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NOX2-dependent regulation of inflammation

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Abstract

NADPH oxidase (NOX) isoforms together have multiple functions that are important for normal physiology and have been implicated in the pathogenesis of a broad range of diseases, including atherosclerosis, cancer and neurodegenerative diseases. The phagocyte NADPH oxidase (NOX2) is critical for antimicrobial host defence. Chronic granulomatous disease (CGD) is an inherited disorder of NOX2 characterized by severe life-threatening bacterial and fungal infections and by excessive inflammation, including Crohn's-like inflammatory bowel disease (IBD). NOX2 defends against microbes through the direct antimicrobial activity of reactive oxidants and through activation of granular proteases and generation of neutrophil extracellular traps (NETs). NETosis involves the breakdown of cell membranes and extracellular release of chromatin and neutrophil granular constituents that target extracellular pathogens. Although the immediate effects of oxidant generation and NETosis are predicted to be injurious, NOX2, in several contexts, limits inflammation and injury by modulation of key signalling pathways that affect neutrophil accumulation and clearance. NOX2 also plays a role in antigen presentation and regulation of adaptive immunity. Specific NOX2-activated pathways such as nuclear factor erythroid 2-related factor 2 (Nrf2), a transcriptional factor that induces antioxidative and cytoprotective responses, may be important therapeutic targets for CGD and, more broadly, diseases associated with excessive inflammation and injury.

Keywords

host defence; inflammation; NADPH oxidase; NOX2

THE NADPH OXIDASE FAMILY

A large family of NADPH oxidases (NOX) has been identified in plants, fungi, invertebrates and higher animals. As an example, *Drosophila* harbour a dual oxidase (DUOX) homologue in intestinal epithelia that mediates gut antimicrobial host defence [1,2]. Seven members of the NOX family have been characterized (e.g. NOX1–5 and DUOX 1 and 2). NOX homologues have the following characteristics [3]: six transmembrane spanning domains (or seven for DUOX homologues); two haem-binding sites; one cytoplasmic

NADPH-binding site and sequence similarity. These NOX isoforms are expressed in numerous cell types and have diverse physiological functions. NOX2 is the isoform found in professional phagocytes (e.g. eosinophils, macrophages and neutrophils) [4] and dendritic cells [5,6]. Several of the nonphagocyte NOX enzymes, such as those expressed on epithelial surfaces, also probably have host defence functions [7].

The phagocyte NADPH oxidase (NOX2) is a crucial enzyme in antimicrobial host defence and in regulating inflammation. Patients with chronic granulomatous disease (CGD), an inherited disorder of NOX2 in which phagocytes are defective in generation of reactive oxidant intermediates (ROI), suffer from life-threatening bacterial and fungal infections [8]. Among CGD patients, the degree of impairment of NOX2 in neutrophils correlates with mortality [9]. NOX2 is rapidly activated by conditions that, in nature, are associated with infectious threat, such as ligation of specific pathogen recognition receptors (PRR) by microbial products (e.g. formylated peptides and fungal cell wall β -glucans), opsonized particles and integrin-dependent adhesion [6,10,11]. Activation of NOX2 requires translocation of cytoplasmic subunits p40^{phox}, p47^{phox} and p67^{phox} and Rac to a membrane-bound heterodimer cytochrome comprised of gp91^{phox} and p22^{phox} (Figure 1). Molecular oxygen is converted to superoxide anion, which can be converted to downstream metabolites with antimicrobial activity, including H₂O₂ and hydroxyl anion. In neutrophils, myeloperoxidase (MPO) converts H₂O₂ to hypohalous acid, a potent microbicidal metabolite [12–14].

Although the early effects of NOX2 activation – ROI generation, protease activation and neutrophil extracellular trap (NET) generation – are expected to be pro-inflammatory and injurious, observation of CGD patients and experimental models of NOX2 deficiency point to a more complex role for NOX2 in inflammation and injury. In addition to recurrent infections, CGD patients suffer from excessive inflammatory responses. About one-third of CGD patients develop Crohn's-like inflammatory bowel disease (IBD) [15]; other manifestations of excessive inflammation include granulomatous involvement of the genitourinary tract that can lead to obstruction, sarcoid-like lung inflammation and surgical wound dehiscence [8,16,17]. Although this excessive inflammation was thought to result from unresolved infection, work over several decades has shown that NOX2 has a major role in calibrating immune responses in addition to protecting from infection. NOX2 can, in fact, limit inflammation and injury by modulation of key signalling pathways that affect neutrophil accumulation and clearance, as well as adaptive immunity.

Our goals are to discuss the mechanisms by which NOX2 mediates host defence and coordinates the inflammatory response. This knowledge may set the foundation for novel therapeutic approaches in CGD and, more broadly, for diseases associated with excess inflammation and injury.

NOX2 in MACROPHAGES

Alveolar macrophages are the first-line phagocytes that respond to inhaled pathogens (Figure 2). They phagocytose and kill microbes and also coordinate the inflammatory response. Macrophages, neutrophils, and dendritic cells sense microbial motifs through

PRR, such as toll-like receptors, nucleotide-oligomerization domain (NOD)-like receptors and C-type lectin receptors. In addition to microbial products, endogenous products of necrosis, termed damage-associated molecular patterns (DAMPs), activate innate immune responses through pathways that are similar to those engaged by microbes.

Although it is well established that NOX2 is critical for neutrophil-mediated host defence, the importance of NOX2 in macrophages is less established. The strongest evidence for the role of macrophage NOX2 in host defence is from the finding that mutations in *gp91^{phox}* that selectively affect macrophages lead to increased susceptibility to mycobacterial diseases [18]. Prior studies have shown that alveolar macrophages ingest and kill *Aspergillus* spores, whereas neutrophils principally target the hyphal stage [19]. However, there have been conflicting results as to the role of macrophage NOX2 in controlling the growth of *Aspergillus fumigatus* spores [20,21]. Using knockin transgenic mice with NOX2 selectively reconstituted in the monocyte/macrophage and dendritic cell lineages, Grimm et al. [22] showed that macrophage NOX2 was protective against pulmonary challenge with *A. fumigatus*, and limited the germination of phagocytosed spores in isolated alveolar macrophages. These knockin mice have a naturally-acquired disabling mutation of *Ncf1* (which encodes the p47^{phox} protein) and harbour a transgene containing wild-type *Ncf1* under the control of a human CD68 promoter [23]. These knockin mice were also more resistant to *Staphylococcus aureus* and *Burkholderia cepacia* infections compared with NOX2-deficient mice [24]. Together, these results show an important role for macrophage NOX2 in host defence.

NOX2 IN NEUTROPHILS

Following recruitment to sites of infection or damage, activated neutrophils kill microbes through oxidant-dependent and -independent pathways. The rapid activation of NOX2 in neutrophils constitutes an emergency response to invading pathogens. Neutrophil NOX2 activation occurs in response to several stimuli such as opsonized particles, integrin-dependent adhesion [10,25] and ligation of specific PRR (e.g. Dectin-1 [11]). The Syk tyrosine kinase is a critical component of integrin signalling in neutrophils, including NOX2 activation [10,25,26]. NOX2 embedded in the plasmamembrane releases ROI into the extracellular environment whereas NOX2 in neutrophil secondary granules targets phagocytosed pathogens.

Kuhns et al. [9] showed that residual NOX2 activity in neutrophils from CGD patients was associated with less severe illness and a greater likelihood of long-term survival than patients with little or no NOX2 function. This effect on survival was observed in both X-linked and autosomal recessive forms of CGD. These results demonstrate that even low residual levels of NOX2 activity in neutrophils are protective, and support the notion that a small proportion of NOX2-competent neutrophils that may be achievable with gene therapy will reduce infection risk in CGD patients.

CGD patients are at high risk for a limited spectrum of pathogens [27,28], highlighting the fact that pathogens have varying sensitivity to NOX2-dependent host defence. As an example, neutrophils inhibit *A. fumigatus* conidial growth by lactoferrin-mediated iron

depletion, whereas inhibition of growth of hyphae (the tissue-invasive stage of filamentous fungi) required NOX2 [29]. In *ex vivo* studies, NOX2 in neutrophils and peripheral blood mononuclear cells (PBMCs) was required for killing *A. fumigatus*, but not *Aspergillus nidulans* [30]. *A. nidulans* is a major pathogen in CGD patients [31], pointing to the fact that absence of the direct microbicidal effect of ROI observed *ex vivo* does not explain the host defence defect observed in the clinic. Olfactomedin 4 (OLFM4) is a neutrophil granule protein that inhibits host defence against bacterial infection. *Olfm4* deletion augmented defence against *S. aureus*, but not against *A. fumigatus*, in NOX2-deficient mice [32]. Together, these findings demonstrate a complex interaction of NOX2 and ROI-independent pathways in neutrophil-mediated host defence in which NOX2 is required for defence against certain pathogens while being dispensable for others.

ACTIVATION OF NEUTROPHIL PROTEASES AND NEUTROPHIL EXTRACELLULAR TRAPS

In neutrophils, the NOX2-MPO system exerts direct antimicrobial effects through oxidative damage. In addition, NOX2 activation in neutrophils can further augment host defence by activation of granular proteases and generation of NETs (Figure 2). NOX2 activation leads to alkalization of phagosomes, and normalization of phagocytic pH in CGD phagocytes increased the ability to kill *S. aureus* [33]. NOX2 activation is accompanied by an influx of K⁺ ions and a rise in pH, which, in turn, is linked to activation of primary granular proteases that are predicted to enhance intracellular killing [34]. In support of this model, chloroquine, a lysosomotropic agent that prevents endosomal acidification, increased the antifungal activity of neutrophils from CGD patients [35].

In a landmark study, Brinkmann et al. [36] showed that neutrophils are capable of releasing extracellular traps that target extracellular bacteria. NETosis is triggered as a response to infection and to conditions mimicking sepsis [37,38]. In contrast with necrosis and apoptosis, nuclear decondensation and breakdown of the nuclear and granular membranes are unique features of NETosis [39]. NETs are characterized and visualized by extracellular stretches of chromatin that co-localize with cytosolic and granular proteins [39–41]. NETs can mediate host defence through a number of pathways, including binding to microbes preventing dissemination, degrading pathogen virulence factors and killing of pathogens [36,42,43]. A number of NET constituents (e.g. histones, serine proteases) have antimicrobial activity. MPO, following release with NET formation, can generate hypochlorous acid directed at extracellular microbes [44]. Calprotectin is a NET constituent that mediates nutritional immunity by sequestering divalent metal ions and targets *Candida* and *Aspergillus* species [45,46].

NOX2 activation is linked to NET generation. Administration of H₂O₂ and hypochlorous acid, which are metabolites generated by NOX2 activation, trigger NETosis [39,47,48]. Neutrophils from CGD patients are defective in NETosis and gene therapy resulted in restored NETosis in NOX2-competent neutrophils [43,46]. In pneumococcal lung infection, NETosis of bronchoalveolar lavage fluid (BALF)-recovered neutrophils was reduced, but not eliminated, in NOX2-deficient mice [49]. In this model, NOX2 may trigger NETosis directly

or through stimulation of interferon- γ (IFN- γ), which was shown to augment NETosis. In murine pulmonary aspergillosis, NOX2 was required for NET generation, which is posited to limit hyphal parenchymal invasion [41].

NOX2 can therefore target pathogens through a multi-step process: (i) direct killing from NOX2-MPO-generated ROI; (ii) activation of primary granular proteases that target phagocytosed pathogens and (iii) generation of NETs that target extracellular pathogens. Depending on the stimulus, NETs can also be induced by NOX2-independent pathways [50–52]. Doua et al. [53] showed that NETosis can be activated by mitochondrial ROI independently of NOX2, through a pathway dependent on calcium-induced activation of SK3 (small conductance potassium channels) and Akt.

MACROPHAGE AND NEUTROPHIL NOX2 COORDINATELY REGULATE ACUTE INFLAMMATION

In addition to host defence, there is a growing body of evidence supporting NOX2 in macrophages and neutrophils as critical modulators of inflammation. Neutrophils are rapidly recruited to sites of infection and trauma by activation of specific G-protein coupled receptors that are ligated by formylated peptides produced by bacteria and by host-derived chemoattractants, such as leukotriene B₄, C₅a, and chemokines [e.g. MIP-2 and interleukin (IL)-8]. Activation of specific PRR generally leads to nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation and production of pro-inflammatory cytokines. Termination of neutrophilic inflammation, essential to limit excessive tissue damage, is an active process regulated by several anti-inflammatory pathways that include neutrophil apoptosis and clearance and suppression of cytokine/chemokine responses. Although the immediate effect of NOX2 activation – generation of ROI and activation of proteases – is expected to increase tissue injury, NOX2 activation can also limit inflammation and injury.

The pathway through which neutrophils die probably affects their clearance and cross-signalling to circulating monocytes and macrophages. Apoptosis is the default mode of neutrophil death and occurs spontaneously at sites of inflammation [54]. In addition, macrophages release death receptor ligands [e.g. tumor necrosis factor- α (TNF- α) and Fas ligand], which can induce neutrophil apoptosis [55–57]. Macrophages recognize and ingest apoptotic neutrophils [54], a process known as efferocytosis. To stimulate efferocytosis, phosphatidylserine products are externalized by neutrophils early during apoptosis [58,59]. In contrast, NETotic neutrophils display phosphatidylserine only after plasma membrane rupture [39]. Moreover, release of primary neutrophil granular proteins can recruit circulating monocytes to the site of inflammation, stimulate macrophages to produce cytokines, and enhance the ability of macrophages to phagocytose bacteria [60–62]. Efferocytosis of neutrophils triggers the ‘alternative activation’ or the M2 phenotype of macrophages associated with IL-4 production and resolution of inflammation and repair of injury [63]. Efferocytosis can lead to anti-inflammatory responses, including production of prostaglandin E₂ (PGE₂), IL-10 and transforming growth factor- β (TGF- β) by macrophages

and suppression of the IL-23/IL-17A axis (IL-23 drives the expansion of Th17 cells), that drive neutrophil recruitment [64].

NOX2 in macrophages and neutrophils probably plays an important role in the cross-talk between these cells that promote neutrophil apoptosis and clearance. Accelerating neutrophil death and clearance are likely to be important modes by which NOX2 limits acute inflammation. NOX2 in neutrophils can stimulate neutrophil death in specific settings. NOX2 stimulates neutrophil apoptosis after phagocytosis of opsonized particles [65]. TNF- α and Fas ligand can both induce apoptosis in neutrophils, but through distinct signalling pathways; NOX2 was required for TNF- α -stimulated, but not Fas ligand-stimulated, apoptosis [56,66–68]. Thus, NOX2 can, in certain inflammatory contexts, promote apoptosis (non-inflammatory) whereas in other contexts promote NETosis (pro-inflammatory and injurious). Potentially, the intensity or kinetics of ROI generation or signalling pathways induced by specific mediators modulates the balance between apoptosis compared with NETosis.

Impaired neutrophil apoptosis may also contribute to persistent neutrophil inflammation in CGD. Coxon et al. [65] showed that phagocytosis-induced apoptosis was blocked both in neutrophils treated with the NOX2 inhibitor, DPI, and in neutrophils from CGD patients. Neutrophils from CGD patients are more resistant to spontaneous apoptosis *in vitro* than normal neutrophils and produce less prostaglandin D₂ (PGD₂) [69], an inflammatory mediator with both pro-allergic and anti-inflammatory properties. CGD macrophages are also impaired in both PGD₂ and TGF- β production during phagocytosis of apoptotic targets [69]. Thus, impaired neutrophil apoptosis and increased recruitment may together lead to dysregulated inflammation in CGD.

NOX2 in macrophages may have an important cross-signalling role in limiting neutrophilic inflammation. NOX2-deficient mice developed exuberant neutrophilic lung inflammation and pro-inflammatory cytokine responses to zymosan (fungal cell wall-derived particulate β -glucans), whereas inflammation in transgenic mice with NOX2 reconstituted in the monocyte/macrophage and dendritic cell lineage was self-limited and similar to wild-type mice [22]. NOX2 activation in macrophages can stimulate recognition and removal of apoptotic neutrophils [58,59,63,70]. Fernandez-Boyanapalli et al. [71] showed that impaired efferocytosis in CGD macrophages was associated with defective expression and activation of peroxisome proliferator activated receptor (PPAR)- γ , and that a PPAR- γ agonist limited zymosan-induced peritonitis in CGD mice. Zeng et al. [72] showed that NOX2 was required for efferocytosis-induced IL-4 production and activation of IL-4-producing invariant natural killer T (iNKT) cells by macrophages. These results point to NOX2-regulated cross-talk between neutrophils and macrophages leading to death and clearance of neutrophils, required for resolution of inflammation and tissue repair.

NOX2 can also modulate acute inflammation and injury by regulation of transcriptional factors. When challenged with either intratracheal zymosan (particulate β -glucan that activates toll-like receptor 2 and Dectin-1) or lipopolysaccharide, NOX2-deficient p47^{phox-/-} mice and gp91^{phox-/-} mice developed exaggerated and progressive lung inflammation, augmented NF- κ B activation and elevated downstream pro-inflammatory

cytokines [TNF- α , IL-17A and granulocyte-colony stimulating factor (G-CSF)] compared with wild-type mice [73]. Replacement of functional NOX2 in bone marrow-derived cells restored the normal lung inflammatory response. Studies *in vivo* and in isolated macrophages demonstrated that NOX2 was required to activate nuclear factor erythroid 2-related factor 2 (Nrf2), a ROI-inducible transcriptional factor that induces anti-inflammatory and cytoprotective responses. Consistent with these findings, zymosan-treated PBMCs from X-linked CGD patients showed impaired Nrf2 activity and increased NF- κ B activation. Additional studies show that NOX2 regulates NF- κ B activation in macrophages by modulation of the intracellular redox status [74]. In addition to regulating inflammation in response to microbial constituents, NOX2 can limit inflammation in response to tissue injury. NOX2 was protective in acute acid aspiration-induced acute lung injury, limiting alveolar neutrophilic inflammation, inducing Nrf2 activation in lungs and reducing alveolar-capillary wall leak [75,76].

These results show that NOX2 can limit lung inflammation and injury in response to pathogens, microbial products and direct tissue injury. Since the current understanding is that ROIs are proinflammatory and injurious, an anti-inflammatory effect of NOX2 – the major source of ROI generation in activated phagocytes – is paradoxical. However, through its modulation of multiple pathways involved in inflammation and injury, NOX2 can serve to resolve inflammation and limit tissue injury.

NOX2 REGULATES ADAPTIVE IMMUNITY

NOX2 can modulate adaptive immunity at multiple levels, including antigen presentation and cross-signalling to T- and B-cells. NOX2 is expressed in dendritic cells and recruited to early phagosomes, leading to sustained production of low levels of ROI and alkalinization of the phagosomal lumen [77,78]. The recruitment of NOX2 can prevent acidification of phagosomes, limiting antigen degradation and enhancing antigen presentation, including antigen cross-presentation [6,78,79]. Rab27a regulates NOX2 recruitment to dendritic cell phagosomes and phagosomal pH [79]. Cross-priming of CD8⁺ T-cells failed to occur in NOX2-deficient mice due to defective endosomal alkalinization and autophagy in dendritic cells. However, vaccination of mice with fungal antigens that activated CD4⁺ T-cells overcame defective cross-presentation, and was protective against *Aspergillus* challenge [80]. Dectin-1 signalling in macrophages and dendritic cells triggers recruitment of light chain 3 (LC3) (a major component of the autophagy pathway) to phagosomes through pathways requiring activation of Syk, NOX2 and autophagy protein 5 (ATG5) [81,82]. This process facilitates recruitment of major histocompatibility complex (MHC) class II to phagosomes and promotes presentation of fungal antigens to CD4⁺ T-cells [82]. In addition to myeloid cells, NOX2 also regulates MHC-II expression on B-cells [83]. Together, these results point to a broad role for NOX2 in antigen presentation in multiple cell types [84], and the potential for vaccination targeted to CD4⁺ T-cell activation to ameliorate impaired host defence in CGD.

In addition to antigen presentation, NOX2 in myeloid cells can modulate T-cell phenotypes. IL-17A stimulates neutrophilic inflammation by inducing the expression of growth factors and pro-inflammatory cytokines and chemokines. Regulatory T-cells (Tregs;

CD4⁺CD25⁺Foxp3⁺) play an essential role in protection against autoimmunity by suppressing T-cell responses. Romani et al. [85] found that defective tryptophan catabolism led to impaired antifungal host defence and was associated with augmented Th17 and reduced Treg responses in NOX2-deficient mice. However, patients with CGD had normal tryptophan metabolism [86,87]. This difference between species may reflect the fact that superoxide anion is a cofactor for mouse, but not human, indoleamine 2,3-dioxygenase (IDO; which mediates tryptophan oxidation). Additional studies in mice also point to NOX2 in macrophages inducing Tregs and limiting autoimmune responses [23,88]. NOX2-deficient dendritic cells induced responding T-cells to produce higher levels of IFN- γ and IL-17 after antigen-specific or superantigen-induced activation compared with wild-type dendritic cells [89].

Cross-signalling between myeloid cell-derived ROIs and T-cells is not well understood. T-cell surface oxidative stress has been posited to limit T-cell reactivity and sensitivity to autoimmunity [90,91]. However, another potential way NOX2 in myeloid cells can regulate adaptive immunity is by priming the development of myeloid-derived suppressor cells (MDSC). MDSC are immature myeloid cells that are expanded during cancer, sepsis [92,93] and other diseases associated with systemic inflammation. NOX2 has been shown to increase MDSC accumulation in various tumour-bearing mouse models [94,95], and the immunosuppressive capacity of monocytic MDSC in patients with non-small cell lung cancer was ROI-dependent [96]. Granulocytic MDSC have a functional NOX2; however in a mouse model of ovarian cancer, accumulation of MDSCs was not dependent on NOX2 [97]. By definition, MDSC suppress T-cell responses, which may serve to protect the host from excessive inflammation and autoimmunity. However, in the context of cancer, MDSC accumulation can disable T-cell-driven anti-cancer immunity. Although more research is required, these results suggest that NOX2 stimulation of MDSC may be a mode for dampening immune response to avoid injury and autoimmunity, and in the tumour microenvironment may be a target to enhance anti-tumour immunity.

NOX2 CALIBRATES THE INFLAMMATORY RESPONSE TO FUNGI

NOX2 has a dual function; it is essential for antibacterial and antifungal host defence and it regulates both acute neutrophilic responses and adaptive immunity. One of the clearest findings showing a role for NOX2 in limiting fungal inflammation distinct from its host defence function is that NOX2-deficient mice developed excessive and prolonged lung inflammation compared with wild-type mice following intrapulmonary challenge with heat-killed *A. fumigatus* hyphae [98]. Since this study, significant knowledge has been gained about the molecular mechanisms for NOX2 modulating both antifungal host defence and inflammation.

Fungal motifs, including cell wall constituents and DNA, ligate specific PRR. Dectin-1 is the receptor and immunomodulator of particulate β -glucans (ubiquitous cell wall products of fungi and plants) [99,100]. Dectin-1 is expressed on multiple myeloid cells, including monocyte/macrophages, neutrophils and dendritic cells [101]. Dectin-1 signals through the tyrosine kinase Syk and the caspase recruitment domain 9 (CARD9), leading to activation of

NOX2 and NF- κ B-dependent production of IL-17A and other pro-inflammatory cytokine and chemokines that recruit neutrophils [102–105].

Although the specific roles of Dectin-1 in myeloid cell populations has not been fully elucidated, we can speculate that the major effect in neutrophils is to activate NOX2-dependent killing, whereas the major effect in dendritic cells is to prime Th17 responses. In alveolar macrophages, which are the first-line phagocytes that sense inhaled pathogens and microbial motifs, it is likely that the Dectin-1/NOX2 axis plays a dual role in limiting the growth of phagocytosed spores while also calibrating downstream inflammatory responses. *A. fumigatus* spores are coated with a hydrophobic protein that masks pro-inflammatory cell wall products [106]. During germination, which is the developmental transition from spores to hyphae, the cell wall β -glucans become unmasked and able to bind to Dectin-1, which now leads to NOX2 activation and production of pro-inflammatory signals [107–109] (Figure 2). Macrophage Dectin-1 is activated by particulate rather than soluble β -glucans, which is proposed as a mechanism to distinguish cell wall-associated β -glucans associated with live fungi from those that are soluble (probably a fungal degradation product) [110]. The ability of host, lung resident cells to recognize the fungal cell wall products in a manner that is specific to stages of fungal development and, consequently, pathogenic ability probably enables the host to respond to the tissue-invasive forms (germlings and hyphae) while avoiding inflammation to commonly inhaled spores or soluble fungal cell wall products that do not constitute a threat.

Acute neutrophilic inflammation is an emergency response to infectious threat (or to microbial constituents or products of cellular necrosis that mimic infection). In Dectin-1-deficient mice, production of IL-17A and other pro-inflammatory cytokines and chemokines and neutrophilic inflammation in response to *Aspergillus* infection are blunted. A modest impairment in host defence to *Aspergillus* is also observed, although not as severe as in NOX2-deficient mice [103]. Once neutrophils are engaged, NOX2 is critical for host defence against hyphae. As previously discussed, neutrophil NOX2 can damage hyphae both through a direct effect of ROI generated by the NOX2-MPO pathway, and also amplified by NET generation. It is unclear what the relative requirements for direct ROI injury compared with NET generation are in targeting hyphae during pulmonary *Aspergillus* infection since NOX2 activation promotes NETs. Finally, based largely on murine models, NOX2 probably also limits neutrophilic inflammation by dampening Th17 responses.

NADPH OXIDASE PROTECTS AGAINST INFLAMMATORY BOWEL DISEASE

IBD is thought to result from disordered host–microbe interactions in the bowel [111]. IBD resembling Crohn's disease occurs in about one-third of CGD patients [8,15,112]. Moreover, IBD observed in patients with very early onset IBD (VEOIBD; defined as onset of disease at younger than 6 years of age) often resembles features of CGD. Functional genetic variants in NOX2 components, although not severe enough to result in CGD, have been associated with VEOIBD [113,114]. Muise et al. [115] identified a polymorphism in $p67^{phox}$ gene that led to reduced binding of the protein to RAC2 in a subset of patients with VEOIBD. Mutations in the $p40^{phox}$ gene have also been linked to risk of Crohn's disease [115–118].

These studies suggest shared signalling pathways between NOX2 and pathways that protect from IBD.

Genetic studies have linked IBD to a large number of pathways that shape immune responses, including activation of autophagy, PRR (e.g. NOD2/CARD15, and toll-like receptor 4), cytokines and their receptors and various signalling pathways (e.g. signal transducer and activator of transcription 3 (STAT3) and CARD9). IBD has also been observed in a number of primary immunodeficiencies, such as the Wiskott–Aldrich syndrome and IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), and CGD. These observations raise the question as to whether in patients with primary immune disorders, IBD is primarily the result of disordered inflammation or defective host defence leading to impaired clearance of microbes, pro-inflammatory microbial products and potentially food antigens [119].

NOX2 can affect numerous pathways relevant to IBD, including neutrophilic accumulation, transcriptional factor activation, cytokine responses, processing and clearance of microbial products and antigens, and adaptive immunity. NOX2 deficiency may lead to augmented IL-17A and attenuated Treg responses that, at least in part, account for the extraordinarily high frequency of IBD in CGD. The balance between the IL-23/IL-17A axis and Tregs is likely to be important in responding to intestinal microbial infections while limiting autoimmunity. The importance of Tregs in limiting autoimmunity is demonstrated in IPEX, which results from mutations in the *Foxp3* gene, and characterized by a lack of Treg cells, leading to lymphoproliferation, and multiple autoimmune diseases including IBD. IL-23 and IL-17 are the major mediators of experimental IBD [120,121], whereas Tregs have a protective role [122]. Increased IL-17 and IL-23 expression has been associated with IBD in patients [121,123], and polymorphisms in the IL-23R gene are highly correlated with risk of Crohn's disease [124–127].

Rivas et al. [128] identified a highly significant association between a splice variant of CARD9 and protection from IBD. Since activation of Dectin-1 can result in CARD9-dependent production of IL-17A and other pro-inflammatory cytokines, this observation raises the notion that immune responses to β -glucan (e.g. from *Candida* species colonizing the bowel or foods containing β -glucan) in bowel mucosa may influence the risk for IBD. Iliev et al. [129] demonstrated that mice lacking Dectin-1 had altered responses to indigenous fungi (referred to as the 'mycobiome') and more severe chemically-induced colitis. In addition, a polymorphism in CLEC7A, the gene encoding Dectin-1, was linked to a more severe form of ulcerative colitis. Seen in this light, NOX2, which is also activated by Dectin-1 ligation, might dampen IL-17A responses and protect from IBD.

de Luca et al. [130] recently linked defective autophagy (associated with impaired phagosomal recruitment of LC3) in CGD to increased IL-1 β -driven inflammation. The IL-1 receptor antagonist, anakinra, restored autophagy in CGD mice. Moreover, anakinra decreased neutrophil recruitment and Th17 responses in CGD mice, and was protective in models of colitis and aspergillosis. These findings show a new mechanism for hyperinflammation in CGD and point to IL-1 blockade as a potential therapeutic approach.

CONCLUSION

CGD is a rare inherited disorder of the phagocyte NOX2 from which we can learn about the function of this enzyme, both as a mediator of host defence and of inflammation. From CGD patients and CGD mouse models, we have learned that NOX2, in addition to defending against a spectrum of bacterial and fungal pathogens, also limits neutrophilic inflammation through several mechanisms. Effective calibration of neutrophilic inflammation is critical in protecting the host from infection, while averting injury associated with excessive or persistent inflammation. Knowledge gained about mechanisms by which NOX2 and its downstream pathways modulate host defence and inflammation may lead to novel therapeutic targets both for CGD patients, and, more broadly, for diseases associated with excessive inflammation (e.g. autoimmunity) and inflammation-induced organ injury.

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Abbreviations

ATG5	autophagy protein 5
BALF	bronchoalveolar lavage fluid
CARD	caspase recruitment domain
CGD	chronic granulomatous disease
DAMPs	damage-associated molecular patterns
DUOX	dual oxidase
G-CSF	granulocyte-colony stimulating factor
IBD	inflammatory bowel disease
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon- γ
IL	interleukin
iNKT	invariant natural killer T cell
LC3	light chain 3
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MPO	myeloperoxidase

NET	neutrophil extracellular trap
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOD	nucleotide-oligomerization domain
Nrf2	nuclear factor erythroid 2-related factor 2
OLFM4	olfactomedin 4
PBMC	peripheral blood mononuclear cell
PGD₂	prostaglandin D ₂
PPAR	peroxisome proliferator-activated receptor
PRR	pathogen recognition receptors
ROI	reactive oxidant intermediates
STAT3	signal transducer and activator of transcription 3
Syk	spleen tyrosine kinase
TGF-β	transforming growth factor- β
TNF-α	tumor necrosis factor- α
Treg	regulatory T-cell
VEOIBD	very early onset IBD

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Figure 1. The phagocyte NADPH oxidase (NOX2) is a crucial enzyme in antimicrobial host defence and in regulating inflammation

Activation of NOX2 requires translocation of the cytoplasmic subunits p40^{phox}, p47^{phox} and p67^{phox} and Rac to the membrane-bound cytochrome comprised of p22^{phox} and gp91^{phox}. Molecular oxygen is converted to superoxide anion, which can be converted to downstream metabolites with antimicrobial activity, including H₂O₂ and hydroxyl anion. In neutrophils, MPO converts H₂O₂ to hypohalous acid.

Figure 2. NOX2-mediated antifungal host defence

NOX2 mediates host defence by both ROI generated by the NOX2-halide system, and downstream signalling, including autophagy in macrophages and the generation of NETs in neutrophils. Alveolar macrophages, which are the first line of phagocyte host defence against inhaled fungi, phagocytose and kill *Aspergillus* spores. Although spores are relatively immunologically inert, exposure of the fungal cell wall component, β -glucan, during the germling stage activates Dectin-1 and other PRR. This results in NF- κ B activation, production of pro-inflammatory cytokines, and activation of NOX2. Macrophage NOX2 limits the growth of phagocytosed spores and germlings by ROI generation and through activation of autophagy. Autophagy, in addition to its host defence function, limits IL-1 β . NOX2 in macrophages, and potentially in dendritic cells, can also limit Th17 expansion. Neutrophils principally target the extracellular hyphal stage. NOX2-mediated hyphal injury can occur via direct ROI injury, as well as through NET generation, in which chromatin and granular constituents are released extracellularly.