Semin Immunopathol (2010) 32:415–430
DOI 10.1007/s00281-010-0221-0

REVIEW

The many roles of NOX2 NADPH oxidase-derived ROS in immunity

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Received: 23 April 2010 / Accepted: 8 August 2010 / Published online: 28 August 2010

Abstract Reactive oxygen species (ROS) have long been studied in the context of their direct toxic effects on cells. As a result, ROS have conventionally been thought of as a necessary nuisance to aerobic living. However, in recent years, much work has been done to examine the contribution of ROS to the field of immunity. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases were identified as one of the key sources of ROS in immune cells. The NOX2 NADPH oxidase in particular has been assigned multiple roles, functioning as a source of antimicrobial ROS, an activator of many signaling pathways, a participant in chemotaxis, an immune modulator, and a critical player in the initiation of antigen cross-presentation. Furthermore, recent studies have revealed a novel role for the NOX2 NADPH oxidase in the activation of autophagy, a cellular degradative pathway. Here, we examine these functions of NOX2 NADPH oxidase in immunity.

Keywords Phagocytes · NADPH oxidase · Reactive oxygen species · Innate immunity · Autophagy

This article is published as part of the Special Issue on Autophagy.

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Introduction

Reactive oxygen species (ROS) are a group of highly reactive free radical and non-radical molecules [1]. ROS have a number of important functions in immunity that have only been recently realized. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) are major sources of ROS in immune cells. The critical role that NOX2 NADPH oxidase-derived ROS play in host immunity was clearly demonstrated by genetic studies done in the late 1980s. These studies showed that patients with mutations in NOX2 NADPH oxidase component genes develop chronic granulomatous disease (CGD) (reviewed by [2]). CGD is characterized by recurrent and severe infections by a particular spectrum of bacterial and fungal infections as a result of the host's inability to mount an effective innate immune response [3]. Since that time, much work has been done to elucidate the contribution of ROS to innate immunity and the defense against invading pathogens. The current opinion on the specific roles that ROS perform has evolved greatly beyond ROS simply as microbicidal compounds, developing into a vastly more complex model of ROS function. The purpose of this review is to discuss the current understanding of all aspects of NOX2 NADPH oxidase-derived ROS contribution to immunity.

The assembly and function of NOX2 NADPH oxidase

There are a number of cellular sources that generate ROS including NOXs, xanthine oxidase, the mitochondrial electron transport chain, peroxisomes, and the endoplasmic reticulum (ER) [4–8]. The NOX family comprises seven members (NOX1-5 and DUOX1-2) with NOX2 NADPH

oxidase being the predominant source of ROS production in humans [9]. The main producers of ROS are phagocytic cells—neutrophils and macrophages. NOX2 NADPH oxidase is composed of functional transmembrane heterodimers, gp91^{phox} and p22^{phox} (also known collectively as the cytochrome *b*558), and four regulatory cytosolic subunits—p40^{phox}, p47^{phox}, p67^{phox}, and the small GTPase, Rac2 (Fig. 1). In the dormant state, cytochrome *b*558 resides in intracellular vesicles [10], while cytosolic Rac2 remains inactive in the guanosine diphosphate (GDP) bound state via interaction with RhoGDI [11, 12]. Upon the initiation of phagocytosis, GDP-Rac2 is converted to GTP-Rac2 through the activity of a Rac guanine nucleotide exchange factor. This allows for Rac2 translocation to the plasma or phagosomal membrane, thereby allowing the subsequent transit of cytochrome *b*558 from the vesicle to the membrane [13]. Concurrently, p47^{phox} is phosphorylated and undergoes a conformational change that now exposes two SRC-homology 3 regions to interact with the proline rich motif on p22^{phox} [14]. Furthermore, Phox homology domains on p47^{phox} allow for binding to phosphatidylinositol 3-phosphate (PI(3)P) and PI(3,4)P_2, transient phosphoinositides that are generated only at the plasma membrane upon phagocytosis, thus further stabilizing p47^{phox} localization to cytochrome *b*558 [9].

Since p47^{phox}, p67^{phox}, and p40^{phox} are trimerized in the cytosol, the translocation of p47^{phox} brings the other two regulatory subunits to the membrane as well [15, 16]. However, there are specific regulatory mechanisms in place that control the activation state of both p67^{phox} and p40^{phox}, which are also required for the proper functioning of the NOX2 NADPH oxidase complex. Phosphorylated p67^{phox} interacts with Rac2 and cytochrome *b*558, inducing a conformational change in the functional subunit that is necessary for ROS production [17]. The role that p40^{phox} plays is less clear, but studies have indicated that phosphorylated p40^{phox}, via its interaction with PI(3)P, is also critical for NOX2 NADPH oxidase activation [18–20]. Recent work suggests that p40^{phox} binds to p67^{phox} via its PB1 domain [21] and assists in p67^{phox} regulation of NOX2 NADPH oxidase activity [22].

There have been many reports about the identity of the kinases responsible for p47^{phox}, p67^{phox}, and p40^{phox} activation, which include several protein kinase C (PKC) isoforms (PKCα [23, 24], PKCδ [24, 25], PKCβ [24, 26], PKCγ [27], and PKCζ [24, 28]), protein kinase A [29], p21 activated kinase [30], ERK1/2 [31, 32], AKT [33], PI3K [34, 35], and possibly others. The complexity of NOX2 NADPH oxidase regulation by such a large number of kinases not only suggests that perhaps a high threshold of activating signals may be required for NOX2 NADPH oxidase activity but also that there may be differing amounts of ROS produced by each individual NOX2 NADPH oxidase complex, depending on the type of local activating signal it receives. In fact, a recent study indicated that NOX2 NADPH oxidase assembly and activity is highly heterogeneous—where only 50% of phagosomes formed upon Fc-γ receptor (FcγR)-mediated phagocytosis have proper p40^{phox} localization and NOX2 NADPH oxidase function [36]. After assembly and activation, NOX2 NADPH oxidase then produces ROS in a reaction on the cytoplasmic region of the gp91^{phox} subunit that converts NADPH to $NADP^+$, resulting in the liberation of two electrons and one H^+. The two electrons are transported

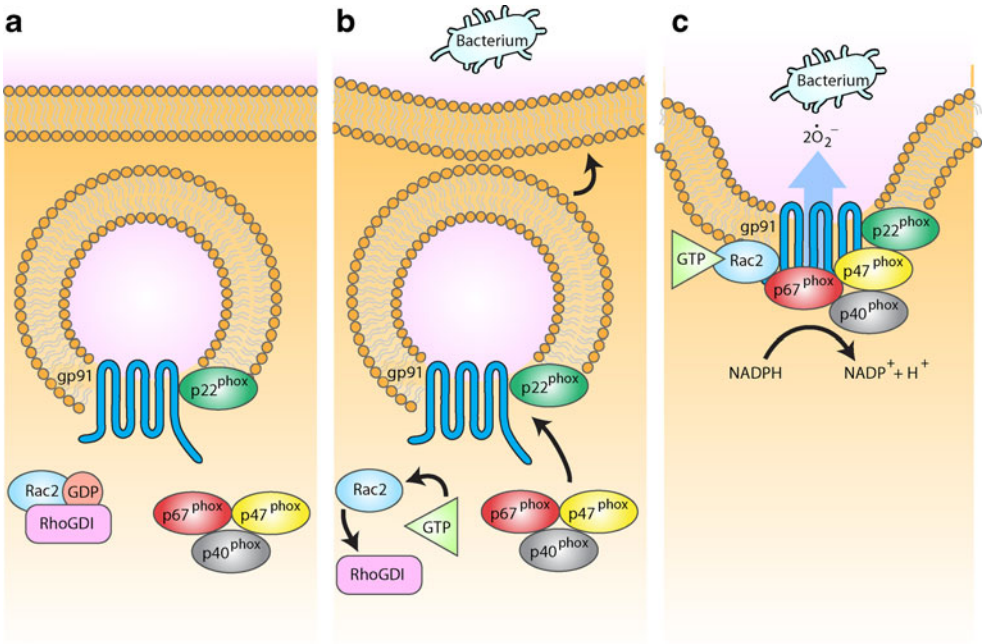

Fig. 1 A schematic of NOX2 NADPH oxidase assembly and activation. **a** In the resting stage, cytochrome *b*558 (gp91^{phox} and p22^{phox}) resides in vesicles. Rac2 in the inactive GDP bound form remains in the cytosol. The regulatory subunits, p47^{phox}, p67^{phox}, and p40^{phox}, are trimerized in the cytosol. **b** Upon receiving signals for activation, cytochrome *b*558 and the trimeric regulatory subunits are recruited to the membrane. RhoGDI inhibition of Rac2 is now released to allow GTP binding. **c** The assembled complex functions at the membrane

through cytochrome *b*558 to the lumen of the phagosome where they react with two oxygen molecules to form two superoxide ions.

While NOX2 NADPH oxidase is the main producer of ROS upon phagocytosis, it must be noted that other NOX family members also generate ROS in macrophages given specific stimulations. In the pathobiology of atherosclerosis, one of the key steps in plaque formation is macrophage to foam cell conversion, mediated by chronic macrophage intake of oxidized low-density lipoproteins (oxLDL) [37]. It was recently shown that lipopolysaccharide (LPS), a toll-like receptor (TLR)-4 agonist, can lead to increased NOX1 NADPH oxidase activity, thereby expediting macrophage conversion to foam cells by increasing the levels of oxLDL [38, 39]. Other groups have found that NOX4 NADPH oxidase also plays a critical role in the formation of oxLDL, leading to macrophage death [40]. Additionally, NOX3 NADPH oxidase expression, albeit low, has been reported in RAW macrophages as well [41]. Thus, while NOX2 NADPH oxidase is the predominant form of NADPH oxidase expressed in macrophages and other phagocytes, the expression and contributions of other NOX members during infection warrants further investigation.

Functions of ROS

Microbicidal activity

Direct inhibitory mechanisms

Upon pathogen infection, neutrophils, macrophages, and dendritic cells (DCs) have a number of defensive strategies that are employed to contain and eliminate pathogens. Such strategies include phagocytosis-mediated lysosomal degradation as well as the production of antimicrobial peptides, defensins, lactoferrins (and other metal chelators), proteases, cathepsins, reactive nitrogen species (RNS), and ROS (reviewed in [42]). Of these strategies, NOX2 NADPH oxidase activity is among the earliest and most robust defenses that phagocytes have against microbes. The importance of NOX2 NADPH oxidase is clearly seen in CGD patients with deficient NOX2 NADPH oxidase activity in which they develop severe innate immune deficiency. Therefore, it is not surprising to find that a number of microbial pathogens have evolved mechanisms to modulate ROS production by NOX2 NADPH oxidase (Table 1).

Despite the predominant production of superoxide by NOX2 NADPH oxidase [9], it remains controversial whether superoxide is the main antimicrobial compound. Some researchers have noted that superoxide is relatively unreactive when compared with the rest of the ROS family and thus may not be a sufficient defensive strategy on its own [43]. Conversely, others have argued that, in the low pH environment of the phagosome, the majority of superoxide is protonated (HO_2•), becoming a much more reactive compound [44].

While the direct antimicrobial effect of superoxides remains contentious, the indirect antimicrobial effect of superoxides, via its reactive products, is well established (Fig. 2). Due to its highly unstable nature, superoxide readily forms a number of other compounds (reviewed in [45, 46]). Superoxide reacts with nitric oxide to produce peroxynitrite, an even stronger oxidizing agent [47]. The combination of two molecules of superoxide ions, catalyzed by the activity of the enzyme superoxide dismutase (SOD), results in the production of hydrogen peroxide (H_2O_2). H_2O_2 acts mainly upon thiol groups in cysteine residues, leading to either oxidation [48, 49] or disulfide bond formation [50, 51]. H_2O_2, in turn, produces hypochloric acid (HOCl) when combined with Cl^- in a reaction catalyzed by myeloperoxidase. Both H_2O_2 and HOCl have been shown to be present in sufficient concentrations in the phagosome to kill microbes [52, 53]. H_2O_2 also interacts with transition metal ions, such as ferrous and ferric ions, to produce hydroxyl radicals (OH•), or with superoxides to generate singlet oxygen ($^1O_2$). OH•, in particular, while short lived, is the most highly oxidizing member of the ROS family, reacting rapidly and non-discriminatorily with DNA, lipids, and proteins.

Indirect inhibitory mechanisms

In addition to its direct microbicidal effects on pathogens, ROS also arrests pathogen survival and growth either via the inactivation of critical bacterial products or the modulation of the phagosomal or extracellular environment.

Leukotoxin, a key virulence factor of *Actinobacillus actinomycetemcomitans*, has been shown to induce apoptosis in human macrophages and neutrophils [54, 55]. Myeloperoxidase, through the production of HOCl, has been implicated in the oxidation and subsequent inactivation of leukotoxin [56–58]. Pneumolysin, a vital virulence factor secreted by *Streptococcus pneumophilus* that is required for survival in human neutrophils, is likewise oxidized by HOCl. Pneumolysin is a pore-forming toxin (PFT) whose function is regulated by the ability to form disulfide bonds, without which proper oligomerization for pore formation cannot occur [59, 60]. Myeloperoxidase was found to be effective in inhibiting pneumolysin activity, presumably via the prevention of disulfide bond formation [61]. Another PFT, listeriolysin O (LLO), has been suggested to be inhibited via a similar mechanism [62]. LLO is one of the key virulence factors produced by *Listeria monocytogenes* that mediates bacterial survival and

Table 1 List of pathogens that have been reported to modulate NOX2 NADPH oxidase activity

Organism	NOX activity	Mechanism of NOX2 interaction	Cell type	Reference
Bacteria				
Anaplasma phagocytophilum	↓	AnkA nuclear effector protein interacts with the transcriptional regulatory regions the gp91^{phox} gene	HL-60	[166]
Burkholderia cenocepia	↓	p22^{phox} and p40^{phox} translocation to NOX2 delayed	RAW macrophage	[167]
Chlamydia trachomatis	↓	Mislocalization of Rac2	HeLa	[168]
Coxiella burnetii	↓	p47^{phox} and p67^{phox} translocation to NOX2 inhibited	Human PBMC	[169]
Francisella tulerensis	↓	gp91^{phox}, p22^{phox}, p47^{phox} and p67^{phox} translocation to NOX2 inhibited	Human PBMC	[170, 171]
	↓	migR and fevR mutants unable to prevent ROS production	Human PBMC	[172]
	↓	Acid phosphatases dephosphorylate p47^{phox} and p40^{phox}, preventing translocation to NOX2	Human PBMC	[173]
Helicobacter pylori	↓	Early cessation of p47^{phox} and p67^{phox} translocation to NOX2	Human PBMC	[174]
Legionella pneumophila	↓	p47^{phox} translocation to NOX2 inhibition	U937	[175]
Leishmania donovani	↓	Lipophosphoglycan inhibition of PKC phosphorylation leads to exclusion of p47^{phox} and p67^{phox}	Mouse and RAW macrophage	[176, 177]
Salmonella typhimurium	↑	*Salmonella* pathogenicity island type 2 (SPI-2) dependent exclusion of p22^{phox} and p47^{phox}	Mouse macrophage	[178–180]
Vibrio vulnificus	↓	RtxA1 induces expression of Rac2 to induce ROS	Mouse macrophage in vitro and in vivo model	[181]
Yersinia enterocolitica	↓	Type III secreted effector YopD prevents ROS production via unknown mechanism	BMDM	[182]
Parasite				
Cryptosporidium parvum	↓	Acid phosphatase prevents ROS production via unknown mechanism	Human PBMC	[183]
Virus				
Epstein Barr virus (EBV)	↑	EBNA-1 induces ROS production by increasing gp91^{phox} transcription	DG75 and BJAB (human B cell lines)	[184]
Hepatitis C virus (HCV)	↑	NS3 superoxide production in macrophages and neutrophils, via recruitment of p47^{phox} and p67^{phox}	Human PBMC	[185, 186]
HIV	↑	Nef directly binds to p22^{phox}, increasing ROS production	Human PBMC	[187, 188]
Respiratory syncitial virus (RSV)	↑	Induction of ROS production via unknown mechanism	Human PBMC	[189]

growth in both human and murine macrophages [63, 64]. It has long been known that LLO requires a reducing environment for its activity [65, 66]. Recent work has identified the host protein GILT (γ-interferon inducible lysosomal thiolreductase) to be responsible for the reduction of LLO to mediate disulfide bond formation [67]. It is therefore likely that, similar to pneumolysin, LLO is also inhibited by ROS via the prevention of disulfide bond formation. However, virulence factors are not the only reported microbial target of ROS as quorum sensing by *Staphylococcus aureus* is also affected both in vitro and in vivo [68]. Quorum sensing is the process by which bacteria modulate their behavior once they "sense" that their population has expanded above a certain threshold number. Rothfork and colleagues demonstrated that HOCl and peroxynitrite inhibit the autoinducer peptide activity, a critical player in quorum sensing, leading to an inability to upregulate virulence factor expression once the bacteria has reached sufficient numbers.

In addition to effects on bacterial products, ROS also play a role in the mobilization of other host microbicidal factors. NOX2 NADPH oxidase is in essence an electron transporter, potentially generating a negatively charged environment in the lumen of the phagosome. This buildup requires the concurrent

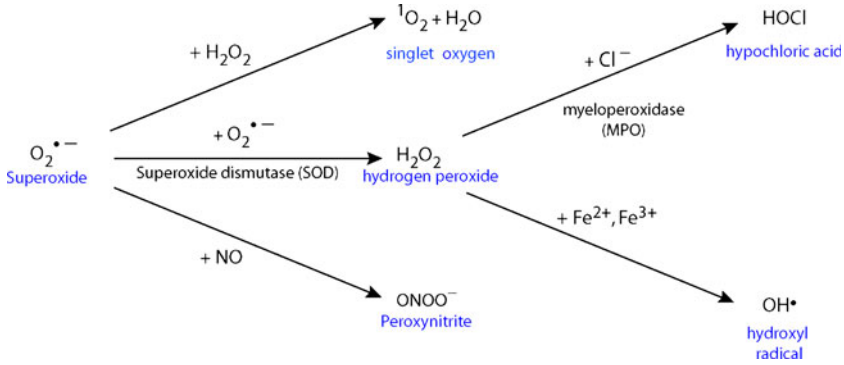

Fig. 2 The generation of common ROS products in phagocytes by NOX2 NADPH oxidase

compensation of positive charges to maintain membrane neutrality. While there has been much speculation on which ions participate in this charge compensation, it has been shown that K^+ contributes, at least in part, to this process [69, 70]. The influx of K^+ increases ionic strength in the phagosome, thereby allowing the release of proteases, such as cathepsin G and elastases, from the negatively charged proteoglycan matrix. This allows the activation and targeting of these proteases to the pathogen for degradation [71].

Another host antimicrobial process activated by ROS is neutrophil extracellular traps (NETs) formation. NETs are fibrous meshworks comprising DNA and protein projected into the extracellular space by mature stimulated neutrophils for the purpose of entrapping and killing pathogens [72, 73]. While the mechanism of NETs formation remains unclear, a recent study suggests that NETs may form as a result of dying neutrophils undergoing a series of cellular reorganization events (i.e., granule disintegration and dissolution of the nuclear envelope) such that, upon the loss of plasma membrane integrity, the toxic intracellular contents are released extracellularly [74]. It has been reported that a number of classical pro-inflammatory signals—interleukin (IL)-8, phorbol 12-myristate 13-acetate (PMA), LPS—serve as activating signals for NET formation [72]. ROS have also been shown to act as signals for induced cell death or "netosis" with the explicit purpose of creating antimicrobial NETs. Interestingly, CGD patients cannot form NETs, further implicating NOX2 NADPH oxidase-derived ROS in this antimicrobial response [72].

Immune modulatory effects of ROS

In addition to its antimicrobial activity, ROS have also been implicated in the modulation of the immune system to help create an environment that allows for an efficient and effective immune response. As such, there has been a wealth of research focused on the interaction between NOX2 NADPH oxidase-derived ROS and various key members of both the innate and adaptive immune systems. The results of these efforts demonstrate a diverse array of ROS functions as they have been reported to participate in both pro- and anti-inflammatory signaling.

Pro-inflammatory effects

The majority of the work linking ROS to the activation of the immune system has focused on the interaction between ROS and DCs. Considered to be the "bridge" between the innate and adaptive immune systems, DCs are professional antigen presenting cells that mediate the activation of different types of adaptive immune responses. Work done by Rutault and colleagues indicate that treatment with H_2O_2 promotes human DC activation and subsequent T-cell engagement and proliferation as a result of enhanced expression of major histocompatibility complex (MHC) II and the co-stimulatory molecules, CD40 and CD86 [75]. Similarly, superoxide produced by xanthine oxidase has also been found to induce dendritic cell maturation [76]. Further examination of the dendritic cell cytokine profile revealed the predominance of two pro-inflammatory cytokines—IL-8 and tumor necrosis factor (TNF)-α—in particular [77]. Thus, while the role of NOX2 NADPH oxidase-derived ROS in DC development has yet to be examined, ROS derived from alternate sources can efficiently induce DC maturation.

Anti-inflammatory effects

While it may seem counterintuitive for ROS to participate in an anti-inflammatory role, a number of studies on the subject suggest that ROS can play a critical role in the prevention of autoimmunity and in the regulation of immune activation. A study of the differences in gene expression between human neutrophils in healthy and CGD patients revealed that a large number of inflammatory genes were upregulated upon phagocytosis in the CGD neutrophils when compared with healthy controls [78]. Further in vitro investigations confirmed that neutrophils from CGD patients produced a more prolonged inflammatory profile accompanied with a delay in apoptosis and clearance.

Interestingly, the chronic granulomas in CGD patients are often found to be sterile, suggesting that the hyperinflammation seen in the absence of ROS production may result in aberrant immune responses to the host itself [79]. An examination of the CGD patient population reveals a high prevalence of concomitant autoimmune diseases such as inflammatory bowel disease (IBD), lupus erythematosus, and chorioretinitis [3, 80]. Experimentally, evidence for the link between ROS and the control of autoimmunity is seen in $p47^{phox}$-deficient mice and rats as they develop a more severe arthritis when challenged with collagen-specific T cells [81]. Transgenic mice that received normal $p47^{phox}$-expressing macrophages via adoptive transfer reduced the number of arthritic animals to the level of wild type (WT) in the T-cell-dependent collagen-induced model but not the T-cell-independent anti-collagen antibody-induced model. Further in vitro investigations revealed that two measures of T-cell activation—proliferation and IL-2 production—are inhibited by macrophage ROS production. $p47^{phox}$ knockout mice also display an increased autoimmune phenotype with more severe arthritis that is reduced with pharmacological activators of NOX2 NADPH oxidase [82]. Taken together, these observations suggest that macrophage-derived ROS are sufficient in inhibiting autoreactive T-cell responses.

Beyond autoimmunity, ROS deficiency has been shown to be a detriment to the host's ability to generate an appropriate immune response against pathogens. Hyperinflammation has also been observed in the context of *Aspergillus fumigatus* extract challenge in murine neutrophils [83], *Helicobacter pylori* mouse infection in vivo [84, 85], and influenza infection in mouse lungs [86].

Cellular signaling effects

ROS, in particular H_2O_2, have a wide range of reported signaling effects that will only be briefly discussed (this topic is extensively reviewed by [87, 88]).

One mechanism by which ROS plays a signaling role is through the modification of cysteine residues. Oxidation of the sulfur molecule to form sulfenic (Cys-SOH), sulfinic (Cys-SO_2H), or sulfonic (Cys-SO_3H) acid can occur [89]. Additionally, oxidation of cysteines can result in reactive thiols, leading to the formation of disulfide bonds [50, 51]. Changes in cysteine oxidation may alter cellular signaling via the inhibition of tyrosine phosphatases, G proteins, and certain ion channels [48, 49], as well as the activation of kinases, such as mitogen-activated protein kinases [90]. Another major effect of ROS on signaling is the modulation of Ca^{2+} signaling. Oxidation of the ryanodine receptor, which contains ROS-sensitive cysteine residues [91], results in the release of intracellular Ca^{2+} stores [92–94]. ROS also activates the inositol triphosphate (IP_3) receptor family Ca^{2+} release channels [95, 96]. Increasing the intracellular concentration of Ca^{2+} in immune cells results in differentiation, proliferation, and/or activation, depending on the cell type [97]. An additional consequence of ROS signaling is the activation of transcription factors. Stimulation of TLR results in the increase of ROS signaling, leading to enhanced expression of nuclear factor-kappa B [98]. ROS signaling also results in the expression of a number of genes involved in antioxidative and tumor suppressive action, such as Nrf2 [99], forkhead box containing transcription factor O (FoxO) [100], and p53 [101].

Roles for ROS in chemotaxis

In addition to the intracellular effects of ROS, recent studies have revealed a critical extracellular role that ROS play in ensuring the proper recruitment of immune cells to the site of infection. Through a small-molecule screen for drugs capable of inhibiting neutrophil chemotaxis, Hattori and colleagues found that the NOX2 NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI), most effectively impaired neutrophil directionality during migration [102]. The DPI effect was found to be ROS specific as the induction of ROS production resulted in an increase in the directed mobility of neutrophils, and this phenotype was reversed when $gp91^{phox}$ or $p22^{phox}$ was silenced by siRNA. Furthermore, neutrophils from CGD patients were found to be slow and disorderly in movement when compared with healthy patients. Finally, a comparison of $gp91^{phox-/-}$ and WT murine neutrophils in an adoptive transfer system revealed that $gp91^{phox-/-}$ but not WT neutrophils were severely stunted in their ability to be properly recruited to the peritoneum in the thioglycolate-induced peritonitis model.

The involvement of ROS in chemotaxis is also seen in other settings. Herpes virus entry mediator binds to host tumor necrosis factor family ligand, LIGHT, leading to the enhanced killing potential of both human macrophages and neutrophils [103]. The mechanism of this increased activation is due to the ROS-mediated increase in migration and expression of chemokine receptors, CCR1 and CCR2 [104]. The 30-kDa antigen produced by *Mycobacterium tuberculosis* elevates both mRNA and protein levels of the chemokine receptors, CXCL8 and CCL2, in primary monocytes via the induction of NOX2 NADPH oxidase-derived ROS [105]. More recently, LPS induction of matrix metalloproteinase (MMP) production and migration of both peritoneal and RAW 264.7 macrophages were found to require ROS [106]. It was reported that the presence of the antioxidant, *N*-acetyl-cysteine (NAC), was sufficient to inhibit both MMP production and migration. Furthermore, it is only the specific silencing of NOX2 NADPH oxidase,

and not NOX1 NADPH oxidase, that results in a decrease in MMPs. Interestingly, NOX1 NADPH oxidase has recently been implicated in a very specific step in chemotaxis—the degradation of extracellular matrix (ECM) [107]. Invadopodia, cellular protrusions found in many metastatic cancers, are thought to proteolytically degrade the ECM. It was shown that NOX1 NADPH oxidase-derived ROS are responsible for this local degradative ability in the invadopodia of human colon cancer cells. In preeclampsia, neutrophil migration and activation at the placental endothelium have been shown to be key pathogenesis events. Recent studies linked neutrophil production of H_2O_2 to the mediation of neutrophil adhesion to the endothelia [108, 109]. It is known that elevated homocysteine levels in the blood correlate with the increased risk of cardiovascular diseases. The mechanism of this relationship appears to hinge upon ROS. It was discovered that homocysteine is a signal for ROS production in macrophages, which results in the production of monocyte chemoattractant protein-1 (MCP-1) [110]. In the model of airway irritation in rats, nicotine induced mRNA expression of the chemokine, macrophage inflammatory protein-1 alpha, an observation that is reversed in the presence of NAC [111]. Together, these studies suggest that, regardless of the model system, ROS appear to be a critical player in phagocyte chemoattraction and migration.

ROS in antigen cross-presentation

Recent studies have demonstrated a pivotal role for ROS in the context of antigen cross-presentation in DCs. Antigen cross-presentation is the process by which antigens taken up via phagocytosis are presented on MHCI molecules, in addition to the conventional MHCII presentation, and vice versa [112]. Upon uptake, antigens are partially degraded in the phagolysosomal pathway, transported into the cytosol, further degraded by the proteasome, and finally transited into the lumen of the ER for loading onto nascent MHCI molecules [113]. However, for proper MHCI or MHCII loading, peptides must, in general, be eight to nine amino acids long. Thus, a degree of control must be exerted over antigen degradation in order for the "right" amount of degradation to occur if antigen cross-presentation is to take place. There are a number of strategies that phagocytes use to degrade engulfed antigens, one of which is the NOX2 NADPH oxidase production of superoxides. The consequence of superoxides in a low pH environment, such as that present in a phagosome, is that the superoxides will readily react with the available H^+ to make H_2O_2 and other ROS. This results in a rapid increase in pH. In neutrophils, the production of ROS is regulated to provide an "oxidative burst," aimed at killing phagocytosed microbes with a high but temporary dose of ROS, thereby leaving the pH largely unchanged. However, DCs express a much lower level of proteolytic enzymes, and engulfed antigen persists longer than in macrophages [114]. A close examination of NOX2 NADPH oxidase activity in human DCs upon phagocytosis revealed that, while there is a tenfold lower level of NOX2 NADPH oxidase activity, it is more prolonged when compared with macrophages [115]. The consequence of the longer-lasting ROS production is a sustained alkalinization of the DC phagosome, as opposed to the rapid acidification of macrophage phagosomes. The increase in pH therefore leads to a consistently lower degree of proteolytic activity, thus attenuating the efficiency of antigen cross-presentation of phagocytosed particles. The evidence for this stems from the comparison of $NOX2^{-/-}$ DCs to WT DCs [116] and $gp91^{phox}$ deficient CGD patient DCs to those of the healthy controls [115]. $NOX2^{-/-}$ DCs of either mouse or human origin showed enhanced phagosomal acidification and antigen degradation, leading to a drastic decrease in antigen cross-presentation. Thus, ROS function as a mode of pH regulation to allow for antigen cross-presentation in DCs, making them important players in innate immunity and in the initiation of adaptive immunity.

ROS and the induction of autophagy

Autophagy is the controlled process of cellular self-digestion where cellular contents are delivered to the lysosome for degradation such that cellular homeostasis may be maintained [117]. Autophagy is characterized by the presence of autophagosomes, double membranous lamellar vesicles bearing the autophagy marker, microtubule-associated protein light chain 3 (LC3) (reviewed in [118]). LC3, or Atg8 in yeast, is a member of a family of over 30 autophagy-related (Atg) proteins that act sequentially to mediate the formation of autophagosomes. While the initial steps of autophagosome formation are currently under much debate, what is known is that the autophagic proteins recruit a crescent-shaped isolation membrane (the source of which is currently unclear) to elongate around and capture cytoplasmic cargo, be it damaged organelles, cytosol, macromolecules, or pathogens. Autophagy is subdivided into three types—chaperone-mediated autophagy, microautophagy, and macroautophagy (reviewed in [119]).

With respect to immunity, there has been a wealth of research demonstrating that macroautophagy, hereafter referred to as autophagy, is a key component of the innate immune defense against many pathogenic microorganisms by removing them from the cytosol, limiting their escape from phagosomes and promoting phagosome maturation (reviewed in [118, 120]). The survival and growth of a number of pathogens have been shown to be restricted by autophagy.

Detection of the presence of streptolysin O, a virulence factor produced by *Streptococcus pyogens*, activates autophagy, resulting in rapid lysosomal degradation and clearance of the bacteria [121]. *Salmonella enterica* serovar Typhimurium resides in vacuoles inside infected host cells. These bacteria can, through the vacuole damaging ability of its type III secretion system, gain access to the cytoplasm. In the presence of the damaged vacuoles, autophagy is activated to limit intracellular spread and replication of the bacteria [122, 123]. Autophagy targeting and clearance of bacteria have likewise been shown to restrict the survival and growth of *L. monocytogenes* [124, 125], *Francisella tulerensis* [126], *M. tuberculosis* [127], *Coxiella burnetii* [128], and others. Furthermore, autophagy has been implicated in the clearance of viruses, such as parvovirus B19 [129], and parasites, such as *Toxoplasma gondii* [130].

A closer examination of the activation and role of autophagy and NOX2 NADPH oxidase in immunity reveals a striking similarity in many regards. Both NOX2 NADPH oxidase and autophagy are early antimicrobial events that occur upon pathogen phagocytosis [131, 132]. There are a number of common activating signals reported for them, including TLR activation [133–135], TNF-α [136, 137], and others. The function of autophagy and NOX2 NADPH oxidase is sensitive to class III PI3K inhibitors [18, 19, 138], and they both can lead to apoptosis [7, 139]. Furthermore, both autophagy and NOX2 NADPH oxidase genes have been implicated in the development of IBD and Crohn's Disease [140–142]. There is also evidence to link ROS to the induction of autophagy. It is known that mounting oxidative stress results in damaged organelles, proteins, and DNA, the clearance of which is one of the main functions of autophagy in a number of cell types [143–145] (reviewed in [146]). If proper clearance cannot take place, increasing ROS levels may then lead to autophagic cell death [147], as seen in the context of TNF-α-induced autophagic cell death in Ewing sarcoma cells [136] or LPS-induced autophagic macrophage cell death [148]. Thus, given the overlap of function and the relationship between ROS and autophagy, it is therefore logical to explore the role that ROS plays in the induction of autophagy in immunity.

The first direct link between ROS and autophagy was provided in the context of starvation-induced autophagy and mitochondrial-derived ROS [149–151]. Starvation-induced autophagy is the induction of autophagy to allow for the autodigestion of the cytoplasm in support of critical cellular processes in times of nutrient deprivation [119]. In response to starvation, it was found that a number of cell lines—HeLa, HEK293, CHO, and mouse embryonic fibroblasts (MEFs)—greatly induced both superoxide and H_2O_2 production in a class III PI3K-dependent manner when compared with controls. Furthermore, in MEFs with and without Atg5, an essential autophagy component, no difference in the ROS levels was observed [151]. These observations combined indicate that PI3K is somehow upstream of ROS production, leading to autophagy. ROS production was proposed to inhibit the redox-sensitive cysteine protease, Atg4—specifically the cysteine residue residing in the active site of the enzyme. Atg4 is one of the autophagy proteins involved in the regulation of LC3 phosphatidylethanolamine (PE) lipidation and delipidation [152], a post-translational event essential for autophagy to occur. When Atg4 encounters H_2O_2, Cys^{81} becomes oxidized in a reversible reaction, rendering the enzymatic activity null [151]. The lack of Atg4 activity is thought to allow LC3 to remain lipidated and localized to the autophagosomal membrane, leading to autophagy.

The link between mitochondrial ROS and starvation-induced autophagy was further confirmed by Chen and colleagues [153]. However, unlike the study done by Scherz-Shouval and colleagues, superoxides but not H_2O_2 were found to be the critical ROS member for the induction of autophagy in HEK293, human glioma cell line U87, and HeLa cells. By pharmacologically inhibiting or siRNA silencing SOD, which converts superoxide to H_2O_2, this study found that there was an increase in autophagy when compared with WT controls. Conversely, the overexpression of SOD resulted in a decrease in superoxide levels and an increase in H_2O_2, leading to decreased autophagosome formation.

More recently, in the context of muscular atrophy, it was found that transgenic mice with SOD deletion exhibit increased numbers of autophagosomes [154]. Since the superoxides are quickly converted to H_2O_2 and other reactive products, there are limitations in ROS assays that make it hard to distinguish one particular type from another. Furthermore, ROS products are linked together in a web of reactions (Fig. 2); thus, it becomes difficult to take away any one enzyme without affecting any other products in a compensatory manner. For example, by knocking out SOD, not only is the level of H_2O_2 affected but also the levels of HOCl, peroxynitrite, and OH•. The limitation of detection techniques available, coupled with the transient nature of ROS members, makes it challenging to pinpoint specific triggers of autophagy. Regardless of the type of ROS, however, it is clear that mitochondrial ROS are indeed involved in the activation of starvation-induced autophagy.

Further evidence has surfaced relating ROS to starvation-induced autophagy. One of the mechanisms of glycolysis downregulation is mediated through TP53-induced glycolysis and apoptosis regulator (TIGAR). An indirect consequence of TIGAR activity on glucose metabolism is the decrease in global intracellular ROS levels [155]. TIGAR activity was found to block autophagosome formation in the human osteosarcoma U2OS cells, even under starvation conditions [156]. This inhibition of

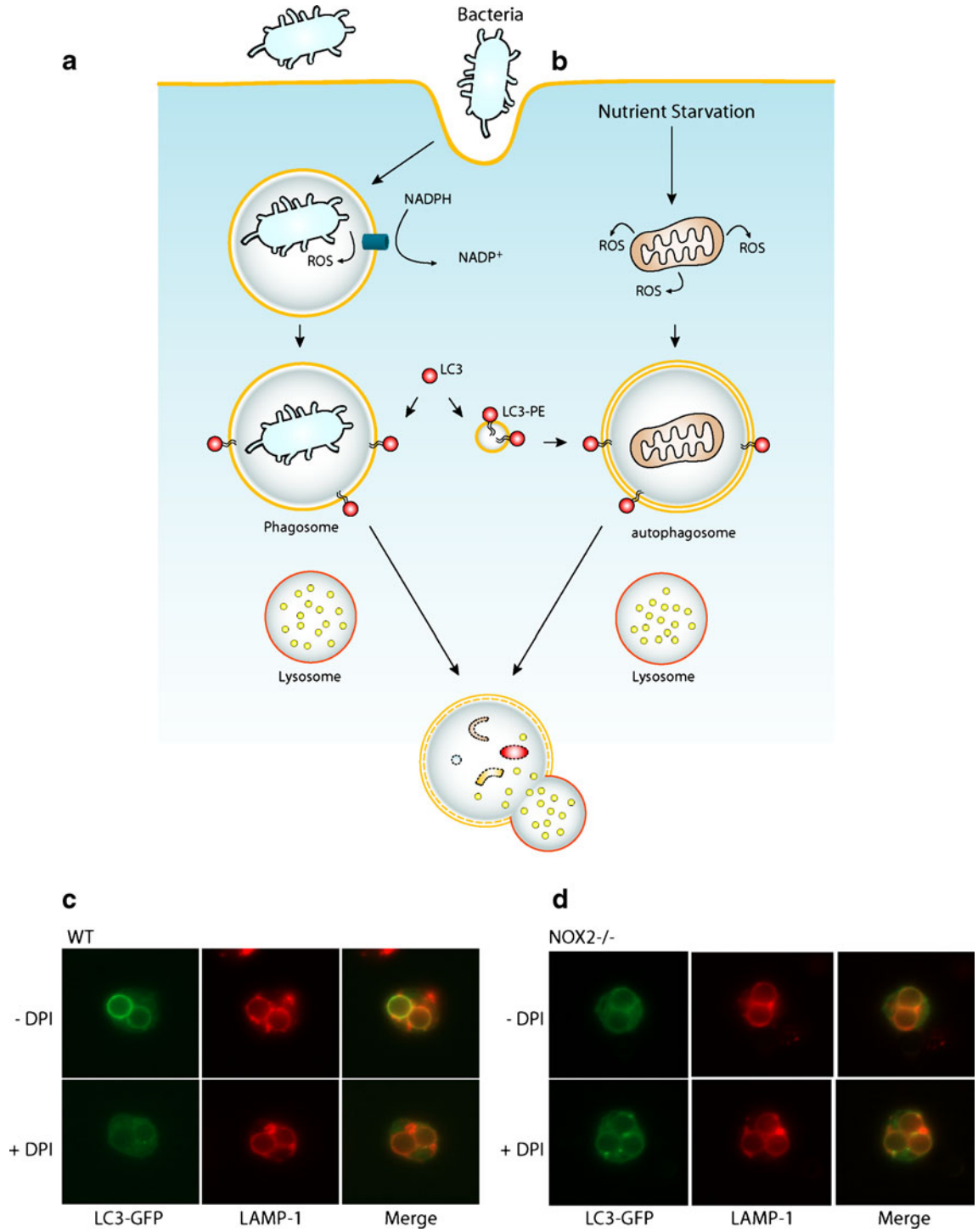

Fig. 3 ROS induction of autophagy. **a** Phagocytosis of microbes results in the recruitment of NOX2 NADPH oxidase complex to the phagosome. The presence of ROS signals the induction of autophagy through an unknown mechanism, resulting in the recruitment of autophagy protein, LC3, to the phagosome, where LC3 is then conjugated to PE. Subsequent fusion with the lysosome allows for microbial degradation in the autophagolysosome. **b** Upon nutrient starvation, ROS are generated in the mitochondria as a result of electron transport chain activity. Loss of mitochondrial integrity results in the detection of ROS, triggering autophagy. The damaged mitochondria and partial cytoplasm are then contained in an autophagosome that is likewise marked for lysosomal fusion and degradation. **c** Epifluorescence images of WT or **d** NOX2 NADPH oxidase deficient bone marrow-derived neutrophils (BMDN) fed with IgG-coated latex beads for 1 h in presence or absence of DPI. Cells were transfected with LC3-GFP and stained with LAMP-1 in red

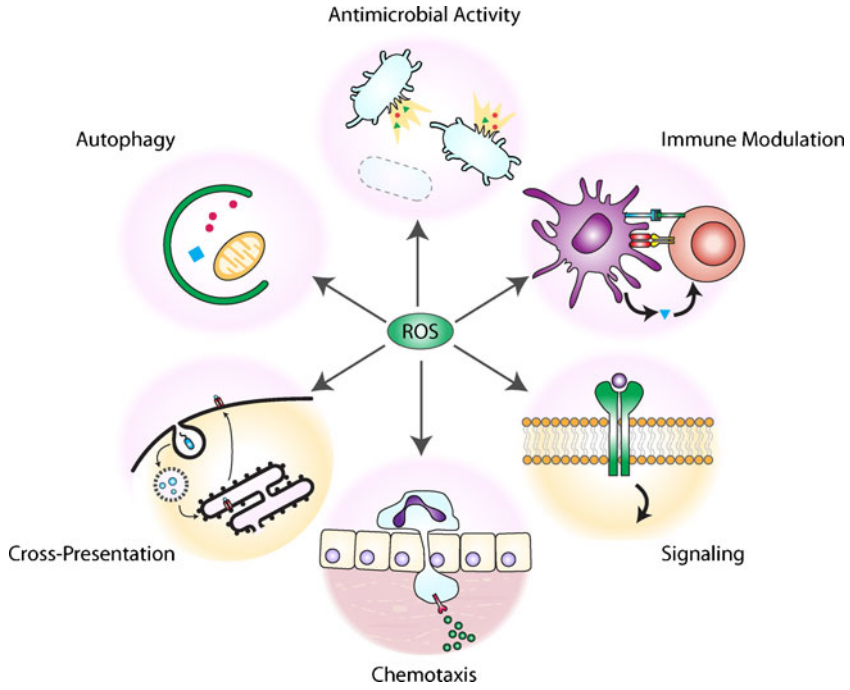

Fig. 4 Summary of the known roles that ROS play in immunity

autophagy was correlated with lowered ROS levels. Adding TIGAR exogenously results in an even greater decrease in ROS and autophagy. Thus, TIGAR is potentially expressed only under nutrient-rich conditions to inhibit autophagy activation via the inhibition of ROS.

NOX2 NADPH oxidase-derived ROS induction of antimicrobial autophagy

Unlike starvation-induced autophagy, the mechanism by which antimicrobial autophagy is activated is less clear. The first detection of invading pathogens occurs via the binding of pattern recognition receptors, such as TLRs, to their pathogen-associated molecular patterns, resulting in the activation of autophagy in both murine RAW 264.7 macrophage cell line and primary bone marrow-derived macrophages [135, 157, 158] (reviewed by [159]). Nucleotide-binding oligomerization domain (NOD) and NOD-like receptor signaling has also been shown to regulate autophagy [160, 161]. Upon the activation of TLR, FcγR, complement receptors, or others, phagocytosis of microbes is initiated, and autophagy is triggered [135, 162]. Seminal work done by Sanjuan and colleagues showed that the activation of TLR upon phagocytosis results in the recruitment of LC3 to the phagosome [135], forming a single membrane $LC3^{+}$ phagosome. The induction of autophagy leads to phagosomal maturation and subsequent degradation of its contents.

Given that TLR activation leads to both NOX2 NADPH oxidase activation and autophagy, we hypothesized that ROS might also play a role in the activation of antimicrobial autophagy. To this end, we first examined if TLR- or FcγR-activated autophagy is mediated through ROS production. Using bone marrow-derived neutrophils (BMDN), we challenged cells with IgG-coated latex beads or dead yeast cells (zymosan particles), and found that LC3 was recruited to the phagosome [163]. This recruitment was further examined using either pharmacological agents to block/neutralize ROS or comparing WT with $NOX2^{-/-}$ BMDN with respect to LC3 recruitment upon stimulation. We found that, in the absence of NOX2 NADPH oxidase activity, LC3 recruitment to the phagosome was impaired (Fig. 3c, d). It is hypothesized that ROS recruits the assembly of the Atg5–Atg12–Atg16L1 complex, a core event in the autophagic pathway to allow for LC3II (the lipidated form of LC3) localization on the phagosomal membrane. An analysis of the purified phagosomes revealed enhanced localization of Atg5–Atg12 conjugate and LC3-II on IgG-coated latex bead containing phagosomes, and this recruitment was inhibited by the addition of DPI. Recently, another independent study confirmed some of our observations in human neutrophils, suggesting that NOX2 NADPH oxidase-derived ROS induction of autophagy is not a murine-only phenomenon [164].

We further established that other cell types (epithelial cells) also employ other member(s) of the NOX family for antimicrobial autophagy, suggesting that the NOX family may be a general mechanism of antimicrobial defense in a range of cell types. Using $p22^{phox}$ siRNA silencing in the human embryonic intestinal epithelial cells, Henle-407, we observed a reduction in autophagy targeting of *Salmonella* Typhimurium. The specific NOX family member(s) that mediate antimicrobial autophagy in non-phagocytic cells remain undetermined. Thus, ROS plays an important role in

the induction of both starvation-induced (Fig. 3b) and antimicrobial autophagy (Fig. 3a).

Conclusion

The current understanding of NOX2 NADPH oxidase-derived ROS has evolved tremendously over the past few years to give a more holistic and complex picture of just how important ROS are to host immunity. Researchers now have a greater appreciation of ROS function beyond simply that of the antimicrobial agent but also as an integral player in immunity—participating in immune modulation, adaptive immune activation, intracellular signaling, chemoattraction, and the induction of autophagy (Fig. 4). Given the plethora of immunological roles that ROS are both directly and indirectly involved in, perhaps a new paradigm of thinking about immunological diseases and microbial pathogenesis in general is in order. Certainly, an analysis of the CGD population reveals that a number of immunological diseases are reported to be comorbidities in CGD patients; chiefly among them is IBD [165]. In a recent genome-wide association study examining the Crohn's disease patient population, *Ncf4*, the human gene encoding $p40^{phox}$, was identified as a significant susceptibility gene [142]. Together, these observations suggest that NOX2 NADPH oxidase deficiency can be thought of as a syndrome of related diseases that are characterized by a distinct panel of immunological disorders resulting from the loss of NOX2 NADPH oxidase function. Therefore, further studies into the role of NOX2 NADPH oxidase in immunity would contribute greatly to the understanding of not only CGD but also a number of other diseases as well.

Acknowledgements The authors would like to thank Michelle Ang and Michal Bohdanowicz for their technical assistance in the generation of the figures. The authors would also like to thank Veronica Canadien for the use of her images in Figure 3c-d. John H. Brumell, PhD, holds an Investigators in Pathogenesis of Infectious Disease Award from the Burroughs Wellcome Fund. Infrastructure for the Brumell Laboratory was provided by a New Opportunities Fund from the Canadian Foundation for Innovation and the Ontario Innovation Trust. G.Y.L. is supported by a M.D/Ph.D Studentship and Canadian Graduate Scholarship Doctoral Research Award from the Canadian Institutes of Health Research. J.H. holds a Canadian Association of Gastroenterology/Canadian Institutes of Health Research/Crohn's and Colitis Foundation of Canada postdoctoral fellowship administered by the Canadian Association of Gastroenterolgy.

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