Title: The Phagocyte Respiratory Burst: Historical Perspectives and and Recent Advances

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Abstract

When exposed to certain stimuli, phagocytes (including neutrophils, macrophages and eosinophils) undergo marked changes in the way they handle oxygen. Firstly, their rate of oxygen uptake increases greatly. This is accompanied by (i) the production of large amounts of superoxide and hydrogen peroxide and (ii) the metabolism of large quantities of glucose through the hexose monophosphate shunt. We now know that the oxygen used is not for respiration but for the production of powerful microbiocidal agents downstream of the initial production of superoxide. Concomitantly, glucose is oxidised through the hexose monophosphate shunt to re-generate the NADPH that has been consumed through the reduction of molecular oxygen to generate superoxide.

This phagocyte respiratory burst is generated by an NADPH oxidase multi-protein complex that has a catalytic core consisting of membrane-bound gp91*phox* (*CYBB*) and p22*phox* (*CYBA*) sub-units and cytosolic components p47*phox* (*NCF1*), p67*phox* (*NCF2*) and p40*phox* (*NCF4*). Finally, another cytosolic component, the small G-protein Rac (Rac2 in neutrophils and Rac1 in macrophages) is also required for full activation. The importance of the complex in host defence is underlined by chronic granulomatous disease, a severe life-limiting immunodeficiency caused by mutations in the genes encoding the individual subunits.

In this review, I will discuss the experimental evidence that underlies our knowledge of the respiratory burst, outlining how elegant biochemical analysis, coupled with study of patients deficient in the various subunits has helped elucidate the function of this essential part of innate immunity. I will also discuss some exciting recent studies that shed new light on how the abundance of the various components is controlled. Finally, I will explore the emerging role of reactive oxygen species such as superoxide and hydrogen peroxide in the pathogenesis of major human diseases including auto-inflammatory diseases.

Discovery and Characterisation of the Phagocyte Respiratory Burst

The first studies on the oxygen metabolism of phagocytes were carried out in the 1930s by Baldrige and Gerrard who showed that canine neutrophils undergo a marked increase in oxygen consumption while phagocytosing bacteria. No further investigation of this rather startling finding took place until the 1950s when the metabolic properties of neutrophils ingesting foreign material were re-examined. This work demonstrated several unusual features of oxygen consumption by leucocytes and ultimately led to delineation of the workings of the NADPH oxidase. Notably, the increased oxygen consumption first observed by Baldridge and Gerrard was accompanied by the catabolism of glucose through the hexose monophosphate shunt. Crucially, Sbarra and Karnovsky [1] showed that the increase in oxygen consumption was not blocked by inhibitors of mitochondrial oxidative respiration such as cyanide, anti-mycin C and dinitrophenol. Inhibitors of glycolysis did block the process, however. This begged the question of why this pronounced increase in oxygen uptake was necessary if it was not being used for for respiration. Elegant studies by Iyer, Quastel and colleagues in 1961 went some way to answering this question. They showed that, during phagocytosis, formate is effectively converted to $^{14}CO_2$ and concluded that this catalytic oxidation of formate must be due to the production of substantial amounts of hydrogen peroxide. This was formally validated in subsequent work through direct measurement of H_20_2 production [2, 3]. Thus, the augmented

oxygen uptake seen during phagocytosis appeared to be necessary for the production of reactive oxygen species such as hydrogen peroxide but to what end? The hypothesis that the process was related to anti-microbial defence was explored by Klebanoff and colleagues in the late 1960s who showed that the hydrogen peroxide produced could react with halide ions (such as chloride or iodide) to produce the anti-bacterial compound, hypochlorite (HOCL) [4, 5]. Crucially, this process was catalysed by myeloperoxidase, which was abundant in neutrophil granules. Thus a picture emerged where phagocytosis stimulated both MPO release and the production of hydrogen peroxide and then this in turn drove the generation of hypochlorite by the reaction below.

$$Cl^- + H_2O_2 \xrightarrow[\text{myeloperoxidase}]{} OCl^- + H_2O.$$

Hypochlorite, the product of this reaction is itself a potent microbiocidal agent. It is also a precursor of the chloramines, which are oxidised halogens that also exhibit anti-microbial activity and are formed by the reaction of oxidised halogens with ammonia or amines [6]. A major advance in the field was then made by Curnutte and Babior ([7] and reviewed in [8]). They were also working on phagocytosis and had noted that while Klebanoff's work had shown that reactive oxygen species could be microbiocidal via myeloperoxidase, an anomaly remained - namely that myeloperoxidase-deficient individuals were not particularly immunosuppressed except in the setting of other co-morbidities such as diabetes [9]. They reasoned that neutrophils may produce another anion via the phagocyte respiratory burst and that this was most likely to be superoxide. It was already established that mammalian cells could produce superoxide through processes such as the action of the enzyme xanthine oxidase or the oxidation of haemoglobin to methaemoglobin [10] [11]. Furthermore, eukaryotic cells protect themselves from the superoxide free radical by expressing superoxide dismutase (SOD) [12] but an intriguing observation was that many bacteria expressed superoxide dismutase too. Indeed, bacteria that were obligate anaerobes lacked super-oxide dismutase [13] while those that expressed it could survive in conditions where the oxygen tension was higher. Did bacteria express SOD in order to eliminate the superoxide generated by phagocytes? Curnutte and Babior were able to demonstrate that phagocytes certainly did make superoxide. Their seminal paper on the topic showed that human neutrophils reduced cytochrome c upon phagocytosis and that this could be inhibited by superoxide dismutase [7]. The only question that remained was whether these species were truly crucial to host defense.

Selveraj and Sbarra had shown in 1966 that oxygen was necessary for killing of bacteria by phagocytes *in vitro [14]* but the *in vivo* importance of the process was conclusively demonstrated by work on chronic granulomatous disease (CGD). This syndrome had been described as a fatal X-linked disease of childhood characterised by recurrent infections including pneumonia, infectious dermatitis and recurrent abscesses. In a series of papers in 1967-8, Holmes, Page and Good showed that the leucocytes of boys with CGD failed to show increments in respiration, direct oxidation of glucose, and hydrogen peroxide formation during particle uptake [15-17]. A slew of papers from that group and others confirmed and expanded upon these findings including demonstrations by Babior and Curnutte that phagocytes from several males with CGD were also unable to generate superoxide [18]

The hunt was then on to determine the nature of the oxidase enzyme that must be missing in X-linked CGD. The main debate centred on whether NADH or NADPH was oxidised during the reduction of molecular oxygen. A putative NADPH oxidase had been described by Rossi

[19] but Hohn and Lehrer's work [20] is generally deemed to be the first definitive evidence that the enzyme responsible for generating superoxide is an NADPH oxidase. The evidence for this was based on the ability of such an oxidase to produce superoxide at a rate commensurate with that seen in human neutrophils and its absence in patients suffering from CGD.

The exact identity of the oxidase enzyme came in 1978 with A.W. Segal's demonstration that it was the newly described cytochrome b-245 enzyme (more commonly known now as gp91*phox* or Nox2) [21, 22] . It had been suspected for some time that a cytochrome would be involved in the process as these enzymes mediate electron transfer. In a series of papers, Segal and co-workers showed that an unusual b-type cytochrome localised to the plasma membrane of neutrophils and, most importantly, this cytochrome was absent in many patients with CGD. The cloning of the *CYBB* gene that encodes gp91*phox* bore out this hypothesis and is one of the first examples of positional cloning. From chromosome walking experiments , it was known that the gene localised to Xp21. Royer-Pokora [23] and colleagues isolated a cDNA that was present in human leukaemic cells that expressed the NADPH oxidase and used subtractive hydbridisation to show that this cDNA was missing from a cell line with a patient with a large Xp21 deletion. Further validation was obtained by showing that this transcript was expressed in haematopoietic cells but was absent in 4 patients with CGD. This work was beautifully complimented by a follow-up paper in which antibodies were raised to a synthetic peptide derived from the complementary DNA sequence. By western blot, the antisera detected a neutrophil protein of about 90kDa that was absent in X-CGD patients. The antisera also detected gp91*phox* in complex with p22*phox*. [24]

The enzyme has been extensively characterised in the last 30 years and we know now that it is localised to the plasma membrane and specific granules of neutrophils and can also localise to the membrane of the phagocytic vacuole upon ingestion of microbes. At this location, it forms a conduit for electrons to be pumped from NADPH in the cytosol to oxygen in the vacuole. The gp91*phox* molecule contains all the machinery necessary for this process to occur and the molecule comprises two domains specialised for this function. The C-terminal domain contains FAD and NADPH binding sites. The N-terminal domain contains 6 transmembrane spanning helices. Helices III and V contain highly conserved histidines (in fact, they are conserved across the whole family of NOX family enzymes). The function of these residues is to bind and anchor two prosthetic haem groups. Electrons are thus transferred via FAD through these two haem groups across the membrane from the cytosol to the vacuole or extracellular space [25]. The importance of these histidines is supported by subsequent site directed mutagenesis studies [26].

A further step forward in our understanding of the structure of the NADPH oxidase was provided in 1987 when Segal refined the technique for isolation of the gp91*phox* subunit by using more powerful protease inhibitors and a more rapid isolation technique. Isolation using this method showed that an additional 22 kDa protein co-purified with gp91*phox*. The two subunits (gp91*phox* and p22*phox*) were closely linked and remained associated with the heme of the cytochrome through both affinity and gel filtration chromatography and sucrose gradient centrifugation. At around the same time, Parkos and colleagues also demonstrated that gp91phox co-purified with a 22kDa protein [27]. This work marked the discovery of the p22*phox* subunit of the cytochrome b558 heterodimer [28]. In his paper, Segal was able to demonstrate another phenomenon that has been borne out in countless further experiments in mouse and studies in humans, namely that *both* subunits were missing in patients in X-linked

chronic granulomatous disease even though p22*phox* is transcribed from a separate, autosomal, locus. As it transpires, the proteins are only stable as a heterodimer and are rapidly degraded in the absence of their partner [29, 30] - in cases of autosomal recessive p22*phox* deficiency, gp91*phox* is also absent. As well as stabilising the expression of gp91*phox*, p22*phox* has an important role in facilitating the binding of the cytosolic components p67*phox* and p47*phox* to the cytochrome b558 heterodimer and allowing full activation of the oxidase [25]. This role is clearly demonstrated in a case of autosomal recessive chronic granulomatous disease caused by a point mutation in the proline rich C-terminus of p22*phox*. This mutation allows normal expression of the protein but recruitment of p67*phox* and p47*phox* to the membrane following activation was virtually abolished [31].

Characterisation and function of the cytoplasmic components of the phagocyte NADPH oxidase

The discovery of cytochrome b558 represented years of incisive work on the basic biochemistry of phagocytes. This combined with shrewd clinical observation of CGD had delineated an entire system of anti-microbial defence. Nevertheless, the recognition that chronic granulomatous disease resulted from deficiency of an NADPH oxidase did not tell the whole story. The *CYBB* gene encoding gp91*phox* is situated on the X-chromosome but work by Borregaard and colleagues [32] demonstrated that some cases of CGD were autosomal recessive, a fact highlighted in one of the first multi-centre descriptions of a large cohort of CGD patients [33]. Moreover, patients with autosomal recessive CGD appeared to have a slightly less severe clinical course and normal levels of gp91*phox* [33]. Nevertheless, such patients had defects in electron transfer suggesting that proteins other than gp91*phox* were necessary to fully "activate" the process. Ultimately, these observations led to the identification of the p47*phox* and p67*phox* cytosolic subunits. The first hint of a cytosolic component to the process came from observations following PMA stimulation of neutrophils. PMA is known to be a potent stimulator of the phagocyte respiratory burst via protein kinase C and work in Segal's laboratory showed that a cytosolic protein of 44-47kDa failed to become phosphorylated in certain cases of autosomal recessive CGD [34].

Further characterisation of p47*phox* and the discovery of p67*phox* were aided by the development of cell-free systems in which the superoxide could be generated by extracts of neutrophil plasma membrane or specific granules mixed with cytosol. This technique allowed fractions of cytosol corresponding to different-sized proteins to be assayed for ability to augment NADPH oxidase activity. The principle of the cell-free assay depends on mixing neutrophil sub-cellular fractions with ionic amphiphiles such as arachidonic acid [35-37] or SDS [38] that stimulate the respiratory burst. Such assays showed that a mixture of crude membrane preparations and cytoplasmic extract were required to re-constitute oxidase activity. Interestingly, in cases of autosomal recessive CGD, the deficiency seemed to lie in the cytoplasmic component whereas in X-linked disease, the problem lay in the membrane fraction.

Volpp et al [39] modified the cell-free technique based on the observation that the oxidase activity of cell free systems was augmented by the addition of GTP, which suggested that GTP-binding proteins might be involved. They purified such proteins by GTP-agarose affinity chromatography and found that all of the super-oxide generating capacity of the cytosol resided in the GTP-bound fractions. Taking the active part of the cytosol, they raised anti-sera against

the proteins within it and found that the antibodies they generated reacted with 2 proteins of approximately 47 and 67kDa. Crucially, either p47*phox* or p67*phox* were absent in some cases of autosomal recessive CGD, confirming the role of these proteins as integral cytosolic components of the NADPH oxidase complex. Another paper also utilising cell free systems, published in the same issue of *Science*, demonstrated very similar results [40]. Cell free systems also demonstrate that recombinant p67*phox* and p47*phox* could rescue the defects in p67*phox* and p47*phox*-deficient cytosol.

Further work has established that both p67*phox* and p47*phox* are rich in motifs required for protein-protein interactions. p67*phox* has two SH3 domains, a TPR (tetratricopeptide repeat) domain and a PB1 domain which is C-terminal to the SH3 domains. p67*phox* interacts with Rac through the TPR domains and p40*phox* through the PB1 domain. p47*phox* also possesses two SH3 domains and a proline rich domain. It has a number of C-terminal serines and is heavily phosphorylated during neutrophil activation. We will see later how p47*phox* is a key organiser and provides a "Go" signal for the activation of the oxidase. Phosphorylation of p47*phox* results in the relief of an auto-inhibitory conformation and exposure of the two SH3 domains to allow its interaction with p22*phox*. The C-terminus of p47*phox* also binds to the C-terminal SH3 domain of p67*phox*, and thus p47*phox* phosphorylation is thought to play a major role in orchestrating assembly and subsequent activation of the oxidase.

The discovery and characterisation of p47*phox* and p67*phox* were major milestones in the field but purified extracts of these proteins were not sufficient to re-constitute the "cytoplasmic" element of the oxidase *in vitro*. As mentioned above, it was known that GTP could augment oxidase activity in cell-free systems and that a GTP-bound factor "Sigma 1" was also necessary for NADPH oxidase activity [41]. In 1991, Segal's lab showed, by sequencing "Sigma 1" that it was actually 2 proteins, the small G-protein p21-Rac1 and GDP-dissociation inhibition factor (GDI) [42]. Rac2 was then identified as a component of the neutrophil cytosol that could augment the respiratory burst using very similar techniques. Subsequent work has borne out the early suggestion that Rac2 is the predominant isoform in neutrophil while Rac1 seems more important in macrophages [43, 44].

Cell free systems had a major impact on the field and thus allowed the description of the minimal components of the oxidase but they still did not tell the whole story. For instance, when p67*phox* is purified by gel filtration chromatography, it separates as part of a complex with a mass of 250kDa [45]. One part of this complex is p47*phox* but Segal's lab purified another part of the complex which turned out to be p40*phox* [46]. p40*phox* was, however, not required to reconstitute the oxidase in cell free systems [46]. Early work on p40*phox* offered opposing views of its role, with publications that found that it could have both activating and inhibitory effects on the oxidase [47-49]. Several lines of evidence have shown that this protein contributes substantially to the activation of the NADPH oxidase, albeit in a more context-dependent manner than the other components. In this regard, the two p40*phox*-mutant mouse models have shed light on this mercurial molecule. Mice that are fully deficient in p40*phox* (p40*phox*-/-) [50] or those that carry a point mutation in the PX domain (p40*phox*-/ ^{phoxR105Q}) have been characterised. Both mice show impaired clearance of *Staphylococcus* Aureus and this work has established that the PX domain of p40*phox* is important in binding phosphatidyl-inositol 3-phosphate at the phagosome and consequently, facilitating oxidase activation. However, some differences in the phenotype of the two mice highlight the complex role of p40*phox* in NAPDH oxidase biology. For instance, the fully p40*phox*-deficient mouse exhibits defects in the response to soluble stimuli such as fMLP, whereas the (p40*phox*-/ ^{phoxR105Q})

mouse does not. In this setting, it is important to bear in mind that complete removal of p40*phox* also results in de-stabilisation of p67*phox* expression [50, 51] and an approximate 55% decrease in the abundance of this protein so it can be difficult to differentiate the direct effects of p40*phox*-deficiency from the indirect effect on p67*phox* expression. The *in vivo* importance of p40*phox* in humans, has however, been conclusively demonstrated by the identification of CGD patients with mutations in p40*phox* [52]. The current model of the cytosolic components of the phagocyte NADPH oxidase at rest is that they exist in a complex with 1:1:1 stoichiometry but that p47*phox* may also exist as a free monomer (reviewed in [53]).

Current understanding of oxidase activation

Armed with the information above, it is possible to build a picture of how the oxidase is activated when phagocytes encounter pathogens. Activation of the NADPH oxidase requires the translocation of the cytosolic components either to the plasma membrane (in the case of extracellular ROS) or to the phagosome (in the case of intracellular ROS). This spatial separation of the cytosolic and membrane components prevents the oxidase being activated at rest [54]. After phagocytosis of a particle and closure of the phagocytic vacuole, the oxidase system is activated. Targeting of the components of the oxidase to the phagosome is highly likely to be dependent on the accumulation of phosphoinositol phosphates at the phagosomal membrane [55]. In this context, it is significant that both p40*phox* and p47*phox* contain PX domains which can bind phosphoinositides. Therefore, when the oxidase is activated, the cytosolic components translocate as a heterotrimer to associate with the phagosome. This interaction is also dependent on the presence of the membrane bound components as it is disrupted in their absence [56]. Upon activation, p67*phox* interacts directly with the cytochrome b558 heterodimer and in combination with Rac (in the GTP bound form), it is sufficient to drive electron transport across the phagosome [53]. Rac translocates to the phagosome independently of p67*phox* and interacts both with p67*phox* and the cytochrome b558. Various models of the exact role of Rac1/2 have been proposed. It has been posited to act both as a scaffold that facilitates the interaction between p67*phox* and gp91*phox* and also as a direct participant in electron transfer [57]. The importance of p47*phox* seems to be as an adaptor molecule that forms a bridge between p22*phox* and p67*phox* as well as binding to the cytoplasmic elements in gp91*phox*. It therefore acts to stabilise the interaction between cytochrome b558 and p67*phox*. Indeed, in cell-free systems, micro-molar rather than nano-molar concentrations of p67*phox* are required to induce electron transport if p47*phox* is not present [58, 59]. As well as stabilising the p67*phox*-Rac-cytochrome b558 interaction, there is also evidence that p47*phox* can interact with cytochrome b558 and modify its function directly - most likely through binding of p22*phox*. p47*phox* has two SH3 domains which face each other forming a groove that is filled by a polybasic C-terminal region [53, 60]. In the resting state then, the p47*phox*-SH3 domains interact with the C-terminal region of the non-phosphorylated protein and keep it in an "auto-inhibited" state. Upon activation, this polybasic region is phosphorylated inducing a release from its auto-inhibitory role and allowing the SH3-domain groove to contact the C-terminus of p22*phox* [61]. Thus, in summary, p67*phox* and Rac interact with gp91*phox* and maximise electron transfer while p47*phox* contacts p22*phox* and acts to "organise" the complex into a stable form for maximal activation [53, 54, 62].

Recent work on control gp91*phox*/p22*phox* expression

Recently, significant advances have been made in understanding the abundance of the gp91*phox*-p22*phox* heterodimer is controlled. It is clear that expression of cytochrome b558 can be controlled at both the transcriptional and post-transcriptional level. gp91*phox* and p22*phox* biosynthesis appears to be a relatively inefficient process as described in a key study by DeLeo and colleagues [63]. Using pulse chase experiments, they found that gp91*phox* was made as a high mannose precursor and glycosylated to gp91*phox* within 4-8 hours of chase. Unassembled monomers are rapidly degraded by the proteasome. Specifically, only around 30% of gp91*phox* translated from the *CYBB* gene product is present at 8h post-pulse chase. This rapid degradation of gp91*phox* is rescued by proteasome inhibitors. They also showed that heme acquisition by gp91*phox* is an absolute requirement for heterodimer formation and that if this process is blocked with succinyl acetone, individual gp65 (the high mannose precursor of gp91*phox*) and p22*phox* monomers get degraded. The glycosylation of gp91*phox*, however, takes place after heterodimer formation. From this data, it follows that gp91*phox* and p22*phox* might interact with proteins in the ER that shorten or prolong its half-life and this is indeed the case. Recently, an important study by Noubade and colleagues identified Nrros (negative regulator of reactive oxygen species) as a protein that associates with gp91*phox* to facilitate its degradation [64]. Deficiency of Nrros in mice causes increased expression of the gp91*phox*-p22*phox* heterodimer with a concomitant increase in the magnitude of the reactive oxygen burst that has beneficial effects for clearance of bacterial infection but detrimental in that it leads to a more severe disease course of experimental allergic encephalomyelitis (EAE). There is precedence for the regulation of gp91*phox* expression by ER chaperones. For instance, the heat shock proteins, hsp70 and hsp90, play reciprocal roles in cytochrome b558 stability [65, 66]. Hsp90 stabilises gp91*phox* expression whereas hsp70 facilitates its degradation via the hsp70-regulated ubiquitin ligase, CHIP [66]. Recently, our laboratory described Eros (Essential for Reactive Oxygen Species), a novel transmembrane protein that has profound effects on the abundance of the cytochrome b558 heterodimer. *Eros*-deficient mice have almost absent levels of gp91phox and p22phox despite expressing normal levels of mRNA for these proteins. Consequently, they have a markedly impaired phagocyte respiratory burst and die very quickly following infection with *Salmonella* or *Listeria [67]*. Control of the phagocyte respiratory burst can also be achieved by varying the stability of the p22*phox* subunit. Yang and colleagues showed that the adaptor protein, Rubicon, better known for its ability to regulate autophagy, could interact specifically with p22*phox* [68]. Rubicon aided p22*phox* localisation to phagosomes and overexpression increased p22*phox* and gp91*phox* levels while Rubicon markedly decreased cytochrome b558 expression. The authors hypothesised that Rubicon might either prevent p22*phox* degradation of facilitate folding of the protein [68]. While the cytochrome b558 can be controlled by the rate of degradation of the protein, its expression is influenced at the level of messenger RNA too [69]. For instance, mouse neutrophils lacking the nuclear envelope protein lamin beta receptor (Lbr) have severely reduced gp91*phox* transcription, lower levels of gp91*phox* protein and a highly impaired phagocyte respiratory burst. Subsequent studies of Lbr also highlighted another important feature of cytochrome b558 biology, which is that the components localise to lipid rafts during activation. The lamin beta receptor has a role in cholesterol biosynthesis and interestingly, restoration of only the sterol reductase domain of the protein to Lbr-deficient neutrophils was able to partially restore the respiratory burst [70]. These results indicated that the lamin beta receptor may act in part by providing sufficient cholesterol for membrane lipid raft formation.

These studies illustrate how it might make sense to regulate gp91*phox* at the level of protein expression so that it can be up-regulated in the setting of infection but tightly controlled to prevent excessive ROS driving auto-inflammatory processes.

Chronic granulomatous disease and the role of ROS in regulating inflammation.

As stated previously, the importance of the phagocyte NADPH oxidase is underlined by chronic granulomatous disease - deficiency of one of the subunits. Several cohorts have now been analysed. In Europe [71-74], the USA [75] and Japan [76], X-linked CGD is the predominant form accounting for around 60% of cases. Of those that remain, around 30% are the result of p47*phox* deficiency and 10% are p67*phox* or p22*phox* deficiency. In cohorts where consanguinous marriage is more prevalent, for instance, in the Turkish [77] or Iranian [78] cohorts, autosomal recessive CGD is the dominant form of the disease.

XR-CGD patients traditionally have been thought to have a more severe disease course than patients with the AR form of the disease, presumably because they completely lack all oxidase activity. Indeed, the residual oxidase activity of neutrophils is predictive of disease severity and modest production of ROS seems to confer a greater likelihood of long-term survival.

Reactive oxygen species as regulators of immunity

An emerging theme of the last few years has been the role of reactive oxygen species not just as anti-microbial agents but as regulators of the immune system. ROS can have profound influences on redox sensitive cellular pathways. This function in immuno-regulation is not surprising as auto-inflammatory complications, particularly inflammatory bowel disease, have been recognised as a feature of CGD even from the initial characterisation of the syndrome.

There are myriad ways in which reactive oxygen species might regulate immunity but it is thought that the bulk of the effect can be ascribed to the actions of hydrogen peroxide (reviewed in [79]. The superoxide ion has a relatively short half-life but it is readily degraded to hydrogen peroxide, which can pass freely through cell membranes. If produced at the cell surface, it can therefore have both intracellular and intercellular effects. The intracellular targets of reactive oxygen species are generally cysteines on proteins and the proven targets of H_20_2 include major regulators of transcription accounting for their profound effects. These targets include both protein tyrosine kinases and protein tyrosine phosphatases. Several publications have highlighted how reactive oxygen species may influence multiple pathways in mammalian immunity and there is evidence for significant effects on both and adaptive immunity. The body of work on innate immunity, in particular, indicates that reactive oxygen species can regulate a diverse array of key cellular processes including autophagy, inflammasome formation and type 1 interferon signalling. [80-83]. Concomitantly, abnormalities in the generation of reactive oxygen species are associated with a variety of auto-inflammatory manifestations. Some specific examples of immune-regulation by the NADPH oxidase and how this may impact on self-tolerance are discussed below.

ROS regulation of innate immunity

ROS and Type 1 Interferon Signalling

The work of Holmdahl and colleagues on p47*phox* as a regulator of innate immunity provides some key insights into the regulatory role played by ROS in the immune system. This group has investigated p47*phox*-deficiency as a driver of inflammatory arthritis in rat and mouse with

complementary studies in human cells *ex vivo*. They identified *Ncf1* (p47*phox*) as a susceptibility locus in pristine induced arthritis by examining a resistant and susceptible rat strain and making a series of genetically segregated F2 crosses. This work showed that the strongest association with susceptibility or resistance was a quantitative trait locus (QTL) on rat chromosome 12. Surprisingly, the causative mutation was a coding variant in p47*phox* that led to an impaired phagocyte respiratory burst [84]. This was slightly unexpected in that one might expect that *excessive* reactive oxygen species production with concomitant tissue damage might be a more likely route to autoimmunity.

The same group then investigated the mechanisms by which a lack of p47*phox* might influence autoimmunity and focused first on the transcriptional effects of *Ncf1* (p47*phox*)-deficiency in mice and chronic granulomatous disease in children. Using myeloid-derived cells, they showed that a lack of ROS was associated with a large increase in the transcription of genes that are regulated by type 1 interferon. Of note, many of the up-regulated genes, were downstream of STAT1, a transcription factor that was up-regulated both in cells of patients with CGD and *Ncf1*-deficient mice. This finding is of particular interest because genome wide association studies (GWAS) have shown that *NCF2*, which encodes the cytoplasmic p67*phox* component of the phagocyte NADPH oxidase is a susceptibility locus both in childhood and adult SLE [80-83, 85, 86]. Moreover, SLE is known to be characterised by overexpression of the same type one interferon pathway transcripts that are so highly expressed in the CGD patients and p47*phox*-deficient mice. The finding that *NCF2* is a susceptibility locus has been validated in multiple cohorts and some elegant functional work has elucidated the probable role of *NCF2* polymorphisms in disease. Intriguingly, individuals with the risk allele have an impaired phagocyte respiratory burst via reduced Fcγ receptor-elicited NADPH oxidase activity. This is consistent with data suggesting that gp91*phox* deficiency pre-disposes to lupus in both mouse and man [87, 88]. Aside from SLE and IBD [89, 90], genetic variation in p40*phox* (*NCF4*) has also been implicated in atopic dermatitis [91]. A summary of the involvement of the phagocyte NADPH oxidase in autoimmune diseases can be found in **Table 1.**

ROS and autophagy

Autophagy is responsible for degradation and recycling of intracellular proteins and damaged organelles, maintaining the normal cellular function. It is now also clear that autophagy can be used as a form of host defence, the machinery for engulfment and degradation of host proteins is well suited to disposing of microbes. One of the first publications demonstrating that antibacterial autophagy was regulated by ROS was published by Huang and colleagues [92]. This work showed that Nox2 is required to activate anti-bacterial autophagy because it is necessary for LC3 recruitment to phagosomes. Antibacterial autophagy in human epithelial cells was also dependent on ROS generation. Epithelial cells do not express the phagocyte NADPH oxidase so the ROS in this case must come from some other source and therefore this paper makes the important point that the regulation of autophagy by ROS is a general principle not restricted to phagocytes [92].

Not only are ROS required for autophagy but also for LC3-asscociated phagocytosis, a process distinct from autophagy that utilises some of the same molecular machinery [93, 94]. This process results in the lipidation of LC3 on a phagosomal rather than autophagosomal membrane and while dependent on many autophagy proteins, it occurs independently of the pre-initiation complex. A recent detailed description of the process by which LC3-associated phagocytosis proceeds

showed that Rubicon is recruited to phagosomes; this activates class III PI3 kinases which generates PtdIns(3)P. This process, in turn, allows the stabilisation of components of the phagocyte NADPH oxidase at the phagosome. The presence of Nox2-derived ROS is essential for the recruitment of LC3 to the so-called LAPosome and for this structure to fuse with lysosomes [95]. The non-redundant role of the phagocyte NADPH oxidase in this process is especially interesting in light of a recent report from the same group that mice that lack component of LAP, though not canonical autophagy, develop an SLE-like syndrome [96].

While ROS can clearly regulate autophagy, it is important to bear in mind that this is a two-way street, given that autophagy components such as Rubicon also control abundance of cytochrome b558 [68].

ROS and the inflammasome

In addition to regulating type 1 interferon signaling, autophagy and LC3-associated phagocytosis, there is now good evidence that ROS can regulate activation of the inflammasome, the cytosolic multiprotein scaffold that generates IL-1β and IL-18 through the actiavtion of caspase 1. Intriguingly, this regulation seems to take place via autophagy. For instance, De Luca et al [97] showed, consistent with the work described above, that LC3 failed to be recruited to phagosomes of macrophages from p47*phox*-/- mice or gp91*phox*-/- human monocytes and that this led to increased IL-1β production with a concomitant increase in neutrophil recruitment and an exuberant Th17 response. Importantly, treatment with anakinra (anti-IL-1R antibody) improved colitis both in the murine model and the inflammatory bowel disease in patients with CGD. This paper followed an initial report showing that monocytes from patients deficient in gp91*phox*, p22*phox* or p47*phox* all exhibited elevated levels of IL-1β and caspase 1 [98].

Dys-regulated inflammasome activation secondary to ROS-deficiency has also been explored in a mouse model of ANCA-driven crescentic glomerulonephritis. In this model, vasculitis can be transferred to mice by passive transfer of anti-myeloperoxidase (MPO) antibodies to recipient mice. The ANCA-driven glomerulonephritis is much more severe if the host mouse has been irradiated and then re-constituted with either gp91*phox*-/- or p47*phox*-/- bone marrow. Thus, the presence of ROS-deficient haematopoietic cells exacerbates antibody-driven glomerulonephritis and this was associated with increased IL-1β secretion in gp91*phox*-/- and p47*phox*-/- chimaeras. The enhanced IL-1β secretion could be abrogated by caspase1 blockade and indeed, chimaeras that were both gp91*phox* and caspase1-deficient did not show more severe glomerulonephritis than control mice [99].

Interestingly, there is evidence that there is reciprocal interplay between ROS and inflammasome signalling in that activation of the inflammasome via certain specific pathways, such as ATP-driven NLRP3 activation, can lead to rapid ROS induction[100, 101].

ROS regulation of adaptive immunity

It is clear, therefore that reactive oxygen species influence many fundamental processes in the innate immune system. There is also good evidence that reactive oxygen species can also modulate the activity of cells of the adaptive immune system. These effects can be indirect because of the influence of ROS on innate immune signaling and antigen presentation and by

paracrine diffusion of hydrogen peroxide from antigen presenting cells. However, there is also evidence that lymphocytes can express components of the phagocyte NADPH oxidase.

The evidence for indirect effects on T cells mediated via APCs is covered in two excellent reviews of the topic [51, 79]. Three key results, however, emphasise a role for intercellular ROS, however. The first is that wild-type T cells, which cannot transfer arthritis, become arthritogenic when the number of thiol groups on the surface is artificially increased [102] – this emphasises the notion that ROS from other cells diffusing to the T cell surface can influence its phenotype irrespective of any ROS generation by the T cell itself. The second important finding is that a defect in macrophages lacking cytochrome p47*phox* can prime type II collagen-reactive T cells, which can then cause disease [103]. Finally, in the *Ncf1*-deficient collagen induced arthritis model, in which the arthritis is largely T cell driven, the phenotype can be rescued if *Ncf1* expression is restored to the macrophages only [104].

While the actions of intercellular ROS are undoubtedly important, it has long been known that T cells express measurable levels of the cytochrome b558 heterodimer. Stimulation through the T cell receptor drives superoxide production that is dependent on both on the NADPH oxidase and Fas [105] but it is notable that other, non NADPH-oxidase dependent ROS are produced following TCR stimulation, possibly from mitochondria. Nox2-deficiency certainly has a functional effect on T cells in that they are skewed towards Th1 cytokine production [50]. As mentioned above, ROS might influence T cell activation through multiple pathways but the modification of kinases and phosphatases involved in T cell signaling seems the most likely route.

B cells can also express components of the phagocyte NADPH oxidase, although, as is the case for T cells, the expression of these is lower than that found in neutrophils and macrophages. B cells deficient in NADPH oxidase components do seem to have altered B cell receptor signaling [106] and patients who have chronic granulomatous disease have abnormal antibody responses, though of course this latter finding might be due to defects in the innate immune system [107].

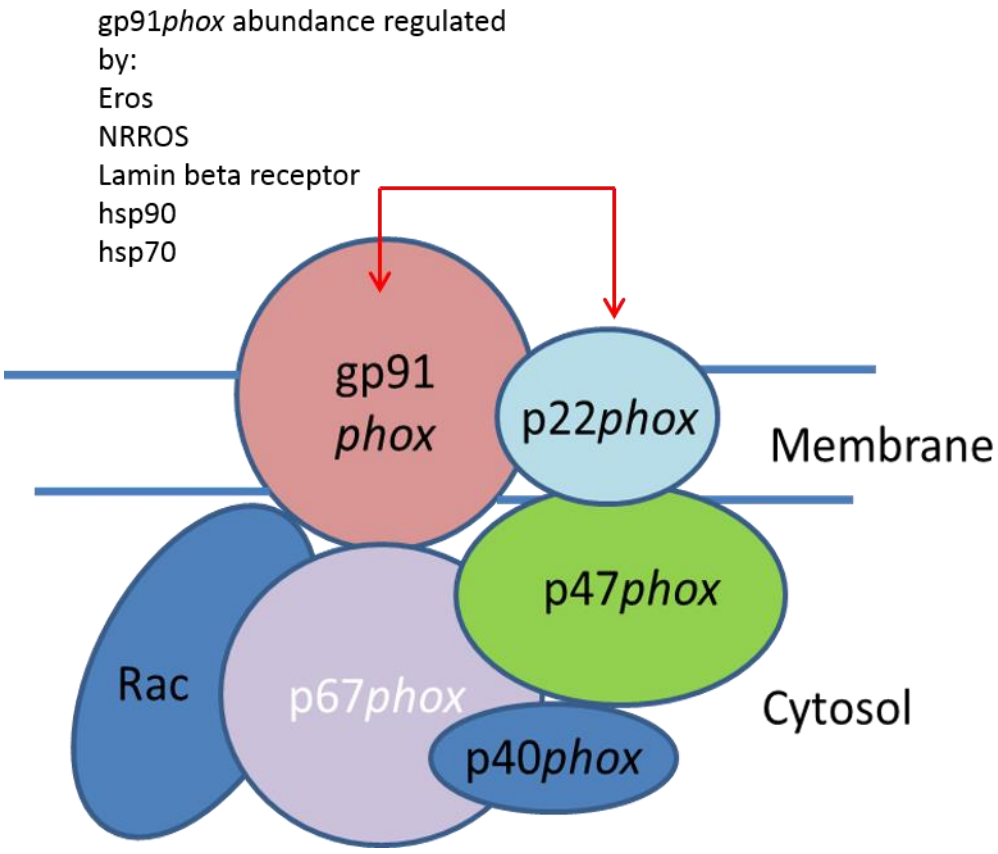
Conclusions

The phagocyte respiratory burst is essential for host defence and the study of chronic granulomatous disease has not only emphasised this point but has illuminated the complex biochemistry of this pathway. Recent work on chaperone proteins that control levels of gp91*phox* and p22*phox* demonstrate that we still have much to learn about the respiratory burst. A particularly exciting development in the last few years has been the recognition that the phagocyte NADPH oxidase regulates not only major pathways in immunity such as type 1 interferon signaling and the inflammasome but basic and ancient cellular processes such as autophagy. Indeed, the finding that genetic variation in NADPH oxidase genes are risk factors in human auto- inflammatory diseases, coupled with abundant evidence from animal studies, emphasises the need to understand the process more fully in order to optimise therapy for such illnesses. Finally, although this review has not dealt with the literature in detail, there is strong evidence that the phagocyte NADPH oxidase influences both cardiovascular disease and melanoma metastasis [108-110]. There is still much work to be done in these areas but the respiratory burst is clearly a fertile area for research that will ultimately lead to improvements in human health.

Table 1 – The involvement of the phagocyte NADPH oxidase in autoimmunity

Subunit	Associated autoimmune disease	Reference
gp91*phox (CYBB)*	S.L.E. (mouse and human) I.B.D	87, 88 71-78
p47*phox (NCF1)*	Arthritis (rat and mouse)	Reviewed in 79
p67*phox (NCF2)*	S.L.E (mouse and human)	80,81,82,83,85,86
p40*phox (NCF4)*	I.B.D., atopic dermatitis (human GWAS studies)	89, 90, 91

Figure 1 – A schematic diagram of the NADPH oxidase and the factors that regulate abundance of gp91*phox*



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