DOI: 10.1111/imr.13182

INVITED REVIEW

Immunological Reviews WILEY

Inside the phagosome: A bacterial perspective

Mark B. Hampton | Nina Dickerhof

Centre for Free Radical Research, Department of Pathology and Biomedical Science, University of Otago, Christchurch, New Zealand

Correspondence
Mark B. Hampton and Nina Dickerhof, Centre for Free Radical Research, University of Otago, Christchurch PO Box 4345, Christchurch 8140, New Zealand.
Email: mark.hampton@otago.ac.nz and nina.dickerhof@otago.ac.nz

Funding information
Health Research Council of New Zealand

Summary

The neutrophil phagosome is one of the most hostile environments that bacteria must face and overcome if they are to succeed as pathogens. Targeting bacterial defense mechanisms should lead to new therapies that assist neutrophils to kill pathogens, but this has not yet come to fruition. One of the limiting factors in this effort has been our incomplete knowledge of the complex biochemistry that occurs within the rapidly changing environment of the phagosome. The same compartmentalization that protects host tissue also limits our ability to measure events within the phagosome. In this review, we highlight the limitations in our knowledge, and how the contribution of bacteria to the phagosomal environment is often ignored. There appears to be significant heterogeneity among phagosomes, and it is important to determine whether survivors have more efficient defenses or whether they are ingested into less threatening environments than other bacteria. As part of these efforts, we discuss how monitoring or recovering bacteria from phagosomes can provide insight into the conditions they have faced. We also encourage the use of unbiased screening approaches to identify bacterial genes that are essential for survival inside neutrophil phagosomes.

KEYWORDS
bacterial killing, myeloperoxidase, neutrophils, oxidative stress, phagosome, thiols

1 | INTRODUCTION

Bacteria entering a neutrophil phagosome immediately encounter a hostile environment. Upon first contact, the neutrophil plasma membrane wraps over the surface of a microbe until it is completely enclosed (Figure 1). Internalization allows a neutrophil to employ aggressive anti-microbial strategies while limiting collateral damage to neighboring host cells. Cytoplasmic granules quickly fuse with the phagosomal membrane, and anti-microbial peptides and digestive enzymes are emptied onto the bacterium (Figure 1). Assembly of NADPH oxidase complexes enables the transfer of electrons to phagosomal oxygen to produce reactive oxidants directly adjacent to the bacterium. Localization to a phagosome helps to protect the host, but it also enables the neutrophil to regulate and optimize the environment for its microbicidal weaponry. Indeed, neutrophils kill bacteria much better when their prey is inside a fully enclosed phagosome.[1]

Staying out of the phagosome would be the most prudent approach for bacteria. Indeed, some have evolved strategies that prevent recognition or impede neutrophil phagocytosis. In this review, we focus on the experience of bacteria that find themselves inside the phagosomes of human neutrophils. Remarkably, some do survive and it is these bacteria, sheltered from other immune mechanisms, that may pose the greatest threat to the host. We discuss the various protective mechanisms employed by phagosomal bacteria, the impact of viable bacteria on phagosomal biochemistry, and how the

This article introduces a series of reviews covering Neutrophils and Friends appearing in Volume 314 of *Immunological Reviews*.

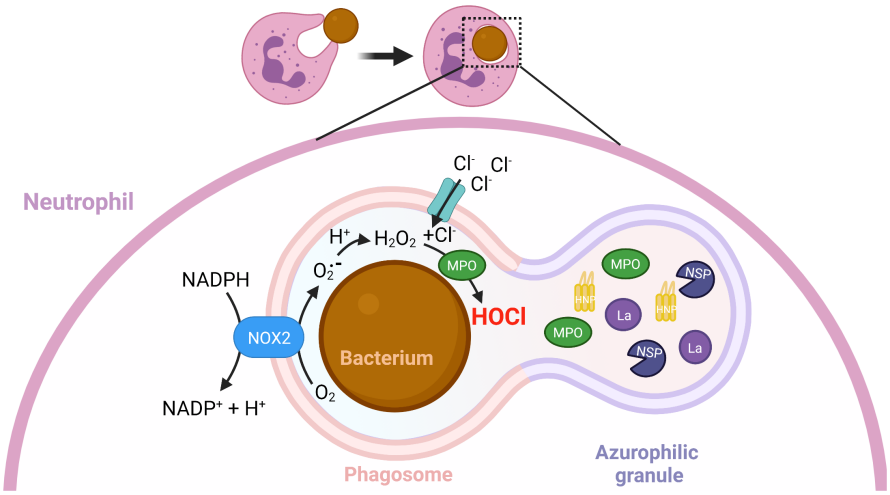

FIGURE 1 Inside the neutrophil phagosome. A phagosome is formed when the neutrophil plasma membrane wraps around a microbe until it is completely enclosed. Cytoplasmic granules quickly fuse with the phagosomal membrane, and anti-microbial peptides (human neutrophil peptides, HNP) and proteins (myeloperoxidase, MPO), digestive enzymes (neutrophil serine proteases, NSP), and the iron-binding protein lactoferrin (La) are emptied into the phagosome. Assembly of NADPH oxidase complexes enables the transfer of electrons to phagosomal oxygen to produce superoxide ($O_2^{\cdot -}$) that is used by myeloperoxidase to oxidize chloride to hypochlorous acid (HOCl). Created with BioRender.com

bacteria themselves are ideally positioned to act as reporters of the dynamic phagosomal environment.

2 | INSIDE THE PHAGOSOME

2.1 | Degranulation

The early phagosome is a very small space that contains only the bacterium and the small amount of extracellular fluid that entered the bacterium at the time of ingestion. The emptying of cytoplasmic granules, termed degranulation, along with the transport of ions across the phagosomal membrane, regulates the environment. Neutrophil granules were originally categorized into two types, azurophilic and specific.[2,3] This grew to four with the identification of gelatinase granules and secretory vesicles, and since then other subtypes have been identified that are either enriched or devoid of different factors (eg, defensin-rich or -poor).[4] As such, there seems to be a continuum of granule types rather than distinct homogenous populations. Indeed, the contents of individual granules are those proteins synthesized at the time of granule formation, with expression profiles varying during the differentiation of myeloid progenitors into mature neutrophils.[5,6]

Azurophilic granules pose the greatest threat for phagosomal bacteria. These granules contain 850 different proteins,[7] including proteases such as elastase, cathepsins, azurocidin, and proteinase-3; cationic defensins/human neutrophil peptides and the bactericidal/permeability-increasing protein that can compromise bacterial membrane integrity; cathelicidins, lactoferrin, and the haem enzyme myeloperoxidase. While other neutrophil granules are mobilized and released during the migration of neutrophils to a site of infection, azurophilic granules are the last to be released.[8] Rab GTPases help co-ordinate the delivery of granules to membranes through the cytoskeletal network of activated neutrophils. A small number of azurophilic granules containing Rab27a fuse with the plasma membrane and release their contents extracellularly upon stimulation with phagocytic stimuli,[9] but the majority of azurophilic granules are delivered to the phagosome.

Processing of azurophilic enzymes occurs prior to packaging into granules, meaning they are ready to function immediately upon release into the phagosome. While in granules, the proteases are restrained from destroying fellow constituents, which is mediated in part by the binding of granule constituents to negatively-charged proteoglycans. Fusion of the granule and phagosomal membranes leads to the dilution of granule constituents and exposure to phagosomal cations, with the detachment of granule proteins visualized by the ability of dyes to access the proteoglycans.[10]

The small volume of the phagosome results in high protein concentrations. Images from electron micrographs, which are consistent with the observed tight contact between the neutrophil plasma membrane and ingested bacteria, result in volume estimates of 0.2–1.2 fL.[11,12] The size of the phagosome increases with time due to osmotic influx of water, but shortly after degranulation the protein concentration is estimated to range from 200 to 500 mg/mL.[11,12] This makes attempts to recapitulate the phagosomal environment in a test tube extremely difficult.

2.2 | Oxidative burst

The phagocytosis of microbes by neutrophils is associated with the activation of a powerful oxidative burst. Increased cytosolic calcium and phosphorylation trigger protein translocation and lipid binding that drive assembly of a multi-protein complex termed the NADPH oxidase at the phagosomal membrane. Two components of the complex, gp91phox (NOX2) and p22phox, transfer electrons from

cytoplasmic NADPH to phagosomal oxygen (Figure 1). Other cells express NADPH oxidases that can be constitutively active or assembled upon stimulation, but the neutrophil stands out as having the greatest capacity to consume oxygen. Phagocytic neutrophils consume up to 5 nmoles of oxygen/min/1×10^6 cells and can quickly deplete oxygen in a solution where gas exchange with air is restricted.

The transfer of electrons one at a time to oxygen results in the production of superoxide. Identification and quantification of superoxide production was achieved by stimulating neutrophils with artificial stimuli that trigger extracellular release and monitoring the superoxide dismutase-inhibitable reduction of cytochrome c.[13] Cytochemical studies with nitroblue tetrazolium (NBT), which is reduced by superoxide to an insoluble formazan, indicate the same process occurs inside the phagosome, but quantification is not possible. However, based on the rate the oxygen consumption by phagocytic neutrophils, there is an estimated flux of 2 mM/s superoxide in the phagosome.[12]

Molecular modelling has also been used to predict the fate of superoxide in the phagosome. Approximately 90% is converted into the potent microbicidal oxidant hypochlorous acid (HOCl),[12] through activity of the azurophilic granule protein myeloperoxidase (Figure 1). Myeloperoxidase dismutates superoxide to hydrogen peroxide and then uses this to oxidize chloride to HOCl. The various redox intermediates of myeloperoxidase, its ability to oxidize a broad range of compounds, and high phagosomal concentrations make it difficult to estimate the actual amount of HOCl produced in phagosomes and its fate. Various methods have been used to confirm HOCl production in phagosomes containing bacteria (Section 2.4).

Generation rates of millimolar per second would suggest that more than enough oxidant is produced to kill phagosomal bacteria. This is misleading, however, because the small phagosomal volume is the main determinant of the high concentration, and it is the number of moles of oxidant per bacterium rather than concentration that is important for killing. Also, most of the HOCl generated in the phagosome was modelled, and confirmed experimentally, to react with neutrophil proteins.[12,14] It is also possible that the superoxide in concert with myeloperoxidase gives rise to oxidants within the phagosome other than HOCl.[15]

As might be expected for a system that can generate large amounts of damaging species, the activation and duration of the oxidative burst are tightly regulated. The duration of the burst, which is difficult to monitor in individual phagosomes using bulk measures of oxygen consumption, differs between stimuli and can be regulated by several different factors. Oxidative damage to NADPH oxidase components is a logical feedback inhibition mechanism, and inhibition of myeloperoxidase extends the oxidative burst.[16] Rapid replenishment of NADPH is critical, with calculations suggesting basal supplies would be exhausted within seconds.[17] The pentose phosphate pathway (PPP), boosted by the inhibition of glycolysis and recycling of its products back to glucose 6-phosphate, is necessary to achieve this goal.[18] Inhibition of this pathway is almost as effective as direct inhibition of the NADPH oxidase in lowering superoxide production.[18] The transfer of electrons across the phagosomal membrane, combined with the generation of protons during NADPH oxidation in the cytoplasm and the consumption of protons in the phagosome during superoxide dismutation, leads to a significant charge imbalance. Voltage-gated proton channels are necessary to rectify this imbalance,[17,19] though a significant amount of proton leakage back into the cytosol has been reported.[20] Myeloperoxidase-mediated lipid peroxidation may contribute to phagosomal membrane damage.[21] Phosphatidylinositol 3-phosphate plays an important role in NADPH oxidase assembly and has been shown to disappear from the phagosomal membrane with time, resulting in disassembly of the complex.[22] Phagosomal chloride is another potential limiting factor for the generation of HOCl. The chloride that enters with the bacterium will be rapidly depleted by myeloperoxidase, and the neutrophil relies on the cystic fibrosis transmembrane regulator (CFTR) and CLC-3 channels for replenishment.[23,24] Neutrophil activation is associated with rapid efflux of chloride, and impaired phagosomal HOCl production and killing has been reported with neutrophils from patients with cystic fibrosis, which carry a deleterious CFTR mutation.[24,25] It is not clear whether the rate of chloride import into phagosomes is effective enough to ensure maximal HOCl generation.

2.3 | Impact of bacteria on the phagosomal environment

Much of the information on conditions within the phagosome has been obtained using experimental models in which neutrophils are fed latex beads or zymosan yeast cell wall preparations. Large inert particles are valuable for microscopy-based applications, including the use of fluorescent dyes, and in delivering proteins to the phagosome. It is important to consider, however, the influence of metabolically active bacteria on phagosomal biochemistry. The impact will vary between species, but many bacteria are constantly secreting metabolites to signal and compete with neighboring microbes. Some of these factors are produced to specifically impede neutrophil function, which we cover in Section 3, but even general metabolic activity may have some impact. Aerobic bacteria consume significant amounts of oxygen, and the use of heat-killed bacteria prevents interference when measuring the oxidative burst of phagocytic neutrophils. It is possible that phagosomal bacteria can lower superoxide production in the phagosome through competing for oxygen. Phagosomal oxygen will be replenished from the external environment, but it has to cross both the plasma and phagosomal membranes, and the oxygen tension at a site of inflammation is already likely to be low.

Phagosomal bacteria present targets for oxidants that are not present in inert particles. Bacteria release proteins and metabolites, including low molecular weight thiols,[26] which will directly scavenge phagosomal oxidants. Also, metabolic waste products such as lactate will influence phagosomal pH, which is important for regulating enzyme activity and the fate of phagosomal oxidants. There has been considerable interest in measuring the pH in early phagosomes and monitoring change with time. An initial slight alkalization to 7.8–8.0 is reported within two minutes of phagosome formation, dependent

on NADPH oxidase activity, followed by slow acidification to around pH 6 over the next two hours.[11,27,28,29] However, these studies are usually undertaken with beads or heat-killed bacteria.

Metabolically active bacteria generate proton gradients across their inner plasma membrane, which is used for energy generation and for the export of proteins and toxins. The impact of bacterial protons on phagosomal pH and the rate of superoxide dismutation has been largely ignored. We speculate that live and dead bacteria will have quite different influences on the phagosomal environment and in so doing could transmit information to neutrophils that they have successfully completed their task. Furthermore, dissipation of proton gradients causes ATP synthases to run in reverse, consuming ATP to maintain the gradient. Proton ionophores such as carbonyl cyanide 3-chlorophenylhydrazone sensitize bacteria to antibiotics by limiting efflux.[30] The consumption of protons during superoxide dismutation may play a similar role in the phagosome.

2.4 | Bacteria as reporters of the phagosomal environment

In addition to making a significant contribution to the phagosomal environment, monitoring or recovering bacteria from a phagosome can provide valuable information, including whether the bacteria have been exposed to lethal doses of oxidants. One of the first pieces of evidence that phagosomal bacteria experience extensive oxidative damage came from a study evaluating the cessation of bacterial DNA synthesis.[31] A close relationship between DNA synthase inhibition and loss of bacterial viability had previously been observed in *Escherichia coli* treated with the purified myeloperoxidase/chloride/H_2O_2 system, with the mechanism proposed to be HOCl-mediated modification of membrane constituents that prevent the chromosomal origin of replication from binding.[32] When extended to co-culture of bacteria with neutrophils, a concomitant decrease in viability and DNA synthesis was also observed in bacteria phagocytosed by neutrophils, and this effect required active NADPH oxidase and myeloperoxidase.[31] This provided strong evidence that *E. coli* are lethally damaged by myeloperoxidase-derived oxidants in the neutrophil phagosome.

Direct evidence for HOCl-mediated damage came with the observation of chlorotyrosine formation on the proteins of ingested *E. coli*, *P. aeruginosa*, and *S. aureus*.[14,24,33] Chlorotyrosine is a stable product that is specific to HOCl. Interestingly, the vast majority of chlorotyrosines were detected on neutrophil proteins, consistent with myeloperoxidase being active in an environment packed with other granule proteins.[14] Tyrosine chlorination was lower in neutrophils from cystic fibrosis patients with defects in the CFTR that imports chloride into the phagosome, confirming that adequate chloride supply is important for optimal HOCl production.[24] However, the level of chlorotyrosine in bacterial protein isolated from neutrophil phagosomes was lower than what was observed when bacteria were exposed to bactericidal levels of HOCl.[14] This suggests that insufficient HOCl is generated in the phagosome to be solely responsible for bacterial killing. This conclusion has to be tempered by the fact that chlorination is a relatively slow reaction of HOCl and only accounts for a small quantum of the HOCl produced in the phagosome.[34]

More accurate quantification of oxidative damage in the neutrophil phagosome became possible when advances in liquid chromatography with mass spectrometry enabled quantitative assessment of the oxidation of sulfur moieties, which is the predominant reaction of HOCl. Up to half of the methionine residues in proteins of *E. coli* and *S. aureus* recovered from neutrophils were found to be oxidized, and this effect was dependent on myeloperoxidase.[35] Oxidation of inner membrane proteins was shown to correlate with the bactericidal activity of the myeloperoxidase/ chloride/H_2O_2 system against *E. coli*.[35] Similarly, two-thirds of bacterial protein cysteines were found to be significantly oxidized in phagocytosed *E. coli*.[36] Quantitative redox proteomics showed the majority of the oxidized proteins were involved in metabolism and stress responses.[36]

Low molecular weight (LMW) thiols are major targets of neutrophil oxidants and quantifying their oxidation during neutrophil phagocytosis using mass spectrometry-based approaches allowed us to gauge the extent of oxidant exposure of phagosomal bacteria. We monitored the oxidation of the bacterial LMW thiols bacillithiol, mycothiol, and glutathione during phagocytosis of *S. aureus*, *Mycobacterium smegmatis*, and *P. aeruginosa*, respectively,[25,37,38] with the latter requiring the use of a heavy isotope to separate it from neutrophil glutathione. A comparison between the LMW thiol oxidation pattern following neutrophil phagocytosis versus exposure to bactericidal doses of HOCl demonstrated that in the case of *P. aeruginosa* enough HOCl reacts with the bacteria in the phagosome to be solely responsible for microbial death.[25] By contrast, killing of phagocytosed *S. aureus* was only partially accounted for by HOCl-mediated damage,[37] consistent with the results obtained using chlorotyrosine as a marker.[14] This study compared the effects between a bolus of HOCl and steady-state generation, which better mimics the phagosome, but the conclusion was not altered. While the loss of bacterial viability and reduced mycothiol were closely associated in HOCl-treated *M. smegmatis*, oxidation of mycothiol was not detected when these bacteria were recovered from neutrophils.[38] This result indicated that the doses of HOCl generated in the phagosome are insufficient to inflict lethal damage to mycobacteria, which was corroborated by the observation that killing of *M. smegmatis* proceeds much slower than for other bacteria and is unaffected by the inhibition of myeloperoxidase.[38] Overall, the investigations on oxidation of bacterial LMW thiols demonstrate that depending on the type of bacteria ingested by neutrophils, oxidant production may be adequate or may be insufficient to be responsible for the eradication of bacteria.

2.5 | Phagosome heterogeneity

The main issue with the bulk measures described previously is that they reflect a population average and do not take phagosomal

heterogeneity into account. Heterogeneity is an important issue because the survival of a small number of bacteria will cause major problems for the host. Bacterial responses to stress can vary depending on their growth phase, and within a population some will be more resilient than others. In the context of this review, it is also important to recognize that there may be significant differences between individual phagosomes and that some pathogens may end up in less destructive environments. Differences between neutrophils will contribute to this phagosomal heterogeneity, as will differences in individual phagosomes within a neutrophil.

Neutrophils are released from the bone marrow into circulation where they have a lifespan of approximately a day before being cleared by apoptosis. At any time some circulating neutrophils will have recently entered circulation and others will be at the end of their lifespan. Increased mobilization of immature neutrophils occurs during infection, increasing the range of phenotypes. Also, molecular profiling has revealed evidence of specific subgroups of neutrophils, independent of maturation or the presence of infection.[39] Neutrophils are primed during their mobilization to and at arrival at a site of infection, primarily driven by pathogen-derived metabolites and host-derived cytokines. Priming pauses the apoptotic program and enhances the functional capacity of neutrophils, including phagocytosis, degranulation and the oxidative burst. Ideally for the host, neutrophils unable to launch a full assault on pathogens would not attempt phagocytosis as they would provide a safe haven for the pathogen, protecting them from more capable neutrophils. Microscopy of samples from in vitro experiments or clinical specimens often reveal a subset of gluttonous neutrophils, while other neighboring neutrophils remain empty. It would be of interest to examine the bactericidal capacity of those neutrophils.

There also appears to be heterogeneity between phagosomes that form within individual neutrophils. Variable distribution of NADPH oxidase components on the phagosomal membrane, which correlated with variable superoxide production as visualized by NBT reduction, was reported in a phagocytic granulocyte cell line.[40] Live cell imaging with an HOCl-sensitive chemical probe revealed differences in the lag period before oxidant generation could be detected, with some phagosomes showing no evidence of HOCl production during the 20 min period of imaging.[41] These did not appear to be phagosomes that formed after the neutrophil had already eaten several particles, so could not be ascribed to the depletion of neutrophil granules. Both studies used artificial phagocytic stimuli, which indicates the results are not explained by bacterial factors disrupting neutrophil pathways. Heterogeneity was also reported in the myeloperoxidase-dependent bleaching of green fluorescent protein being expressed by *S. aureus*,[42] and there was a correlation between the number of bacteria maintaining their fluorescence and bacterial survival. Similarly, GFP-expressing *E. coli* were bleached within neutrophil phagosomes, but some bacteria were protected.[43] Neither of these studies had sufficient visual resolution to determine whether bacteria that ended up in the same phagosome had different fates.

3 | BACTERIAL EVASION STRATEGIES INSIDE THE PHAGOSOME

3.1 | Inhibition of neutrophil function

Considering the challenges of survival in the neutrophil phagosome, it is not unexpected that bacteria have a variety of means to avoid being internalized into this toxic milieu. These include interfering with neutrophil recruitment, evading recognition, and preventing phagocytosis.[44] Once internalized, microbes employ numerous strategies to dampen the anti-microbial potency of the phagosome to increase their chance of survival. Remarkably, some bacteria such as *Yersinia pestis*, the causative agent of pneumonic plague, can survive and even thrive within the phagosome and are transferred to macrophages when the apoptotic neutrophils are cleared from a site of infection.[45] Others, such as various species of mycobacteria, are killed very slowly by human neutrophils,[46] and for some bacteria such as *S. aureus*, neutrophils can quickly kill a significant proportion of the population, but a small number of survivors persist.[37,47,48] *S. aureus* contained within phagosomes ultimately contribute to the destruction of the neutrophil.[48,49] To this end, survivors can be trafficked within neutrophils to tissues, contributing to persistent infection.[50,51] *M. tuberculosis* not only actively replicate within neutrophils but also promote necrosis[52,53] and then continue to grow following internalization by macrophages, thus sustaining the infection.[52]

To avoid destruction by cationic anti-microbial peptides, bacteria remodel their surface charge, proteolytically degrade the peptides or use efflux pumps.[54-58] Protease inhibitors protect against degradation by neutrophil serine proteases.[59] To thwart the production of bactericidal oxidants in the phagosome, many pathogens, including *Y. pestis*, *Neisseria gonorrhoeae*, *Helicobacter pylori*, *Anaplasma phagocytophilum*, *Francisella tularensis*, and *P. aeruginosa* interfere with NADPH oxidase activation and/or assembly.[60-67] *Y. pestis* secretes effector proteins (Yersinia outer proteins, Yop) into the neutrophil cytoplasm that prevent not only NADPH oxidase activation but also granule release.[62,64,68] Furthermore, Yop effectors suppress the synthesis and secretion of the inflammatory mediator CXCL8.[69] *H. pylori* and *F. tularensis* divert assembly to the plasma membrane,[70] while *P. aeruginosa* can inject toxins into cells that block PI3 kinase activation.[67] More is known about the interaction of the intracellular pathogens *M. tuberculosis* and *Salmonella typhimurium* with macrophages, but they express proteins that also disrupt neutrophil NADPH oxidase assembly and activity.[71,72]

Another survival mechanism of *S. aureus* is to thwart the production of bactericidal HOCl by producing an inhibitor of myeloperoxidase (staphylococcal peroxidase inhibitor, SPIN), which prevents H_2O_2 from accessing the enzyme's active site.[73] A knockout strain that lacked this inhibitor showed slightly decreased survival in neutrophils, but the effect was not statistically significant possibly due to a large excess of myeloperoxidase over SPIN in the phagosome.[73]

It is important to note that bacterial strategies that have evolved to damage neutrophils are unlikely to provide protection for the bacteria that first encounter a neutrophil. The neutrophil responses ensure killing begins within seconds of first contact. However, any damage inflicted during this time may help to protect bacteria that are subsequently ingested by the same neutrophil.

3.2 | Detoxification of oxidants and repair of oxidative damage

Bacterial oxidant-degrading enzymes such as superoxide dismutases (SOD), catalases, and peroxidases have the potential to dampen the oxidative insult in the neutrophil phagosome. These enzymes are present in all aerobic cells to protect against reactive oxidants generated during normal metabolism. It is not clear, however, whether these antioxidant systems can cope with the large oxidative burst experienced in the phagosome. Indeed, while deletion of these enzymes is shown to sensitize bacteria to individual oxidants,[74-77] there is only limited evidence to suggest that these enzymes contribute to bacterial survival in the neutrophil phagosome, with the level of protection they afford varying depending on the type of bacteria.

Upregulation of SOD and catalase in *E. coli* prior to phagocytosis failed to protect against neutrophil killing.[78] Similarly, deletion of these two enzymes in *N. gonorrhoeae* did not make the bacteria more susceptible to neutrophil killing.[79] By contrast, *S. aureus* strains producing low levels of catalase (KatA) were killed more rapidly by neutrophils compared with high-expressing strains.[80] Flavoprotein disulfide reductases in *E. coli* (RclA) and *S. aureus* (MerA), although implicated in bacterial HOCl resistance,[81,82] have not shown to be protective against neutrophil killing (Konigstorfer, 2021[107]; Springer et al, unpublished). Recently, these enzymes, along with the *S. pneumoniae* homologue Har, were shown to use NAD(P)H to directly reduce hypothiocyanous acid (HOSCN)[83,84] (Shearer et al, under revision), which is produced when myeloperoxidase and other haem peroxidases use H_2O_2 to oxidizes thiocyanate.[85] Thiocyanate can reach low mM concentrations in respiratory epithelial fluids and like chloride be transported into the neutrophil phagosome by the cystic fibrosis transmembrane regulator (CFTR).[86,87] Being the preferred substrate for myeloperoxidase and able to scavenge HOCl to form HOSCN,[86,88] thiocyanate might contribute to killing by neutrophils. The ability of bacterial HOSCN reductases to promote bacterial survival inside the neutrophil phagosome in the presence of physiological concentrations of thiocyanate needs to be examined.

Bacteria also produce small antioxidant molecules to defend against oxidant exposure. *S. aureus* produces a carotenoid pigment, which was shown to contribute to surviving the oxidative arm of neutrophil killing.[89] High levels of LMW thiols can be protective by forming disulfides in the presence of oxidants, which are recycled by NAD(P)H-dependent reductases. In addition to forming disulfides with each other, LMW thiols can form disulfides with protein cysteines in a process called S-thiolation[90,91] (Figure 2). This shields protein cysteines from irreversible oxidative damage. While glutathione is used by most Gram-negative bacteria, many Gram-positive bacteria lack glutathione and instead synthesize other LMW thiols such as bacillithiol (BSH), mycothiol (MSH), or ergothioneine.[92] Trypanosomatids employ trypanothione, a bis-glutathionyl conjugate of spermidine.[93] S-glutathionylated, S-mycothiolated, and S-bacillithiolated proteins are reduced by glutaredoxins, mycoredoxins, and bacilliredoxins, respectively.[94,95] Although bacterial strains lacking their major LMW thiol are more sensitive to reagent HOCl,[37,90,96,97] MSH and BSH were shown to provide no or only limited protection in neutrophil killing assays with *M. smegmatis* and *S. aureus*, respectively.[37,38] Deletion of the bacillithiol disulfide reductase in a different *S. aureus* strain did, however, decrease bacterial survival by about half compared with the wildtype, supporting a role for the bacillithiol redox system in coping with oxidative stress in the neutrophil phagosome.[98]

Apart from attempting to modulate exposure to phagosomal oxidants, bacteria may also facilitate their survival by efficiently repairing oxidative damage. Manganese acquisition by *S. aureus*, in addition to playing a role in superoxide detoxification, is crucial for DNA repair carried out by a manganese-dependent ribonucleotide reductase following exposure to phagosomal oxidants.[99] Bacteria employ methionine sulfoxide reductases to cope with the extensive oxidation of protein methionine residues that occurs during neutrophil phagocytosis. Inhibition was shown to sensitize *S. aureus* to neutrophil killing.[100] Protein disulfides can be reduced in many bacteria by thioredoxin (Trx), which itself is recycled by the NADPH-dependent thioredoxin reductase (TrxR).[101] The role of the bacterial Trx system in neutrophil survival has not been widely studied, possibly due to the complications associated with knocking out this important antioxidant system. In *S. aureus*, for example, Trx is essential for bacterial growth,[102] and in *E. coli*, other antioxidant systems are activated in the absence of Trx.[103] Ideally, expression of these defenses should be inhibited to levels that do not affect normal growth, before testing them in a phagosomal model.

3.3 | Upregulation of defenses

Bacteria sense and respond to phagosomal stress by upregulating an array of antioxidant response genes to assist their survival (Figure 2). Rather than examine the contribution of individual antioxidant enzymes through knockout studies, particularly where compensatory mechanisms can come into effect, there may be greater impact in inhibiting transcription factors that co-ordinate the response of bacteria to stress. In Gram-negative *E. coli* and *Granulibacter bethesdensis*, for example, among the most strongly upregulated genes following ingestion by normal, but not NADPH oxidase-deficient neutrophils, are oxidative stress response genes under the control of the transcription factor OxyR.[104,105] Upon oxidation to an intramolecular disulfide,[106] OxyR binds to the promotors and enables the transcription of target genes including katG, trxB, grxA, ahpF, ahpC, encoding catalase, TrxR, glutaredoxin, and the alkyl hydroperoxide reductase system, respectively.[105,106]

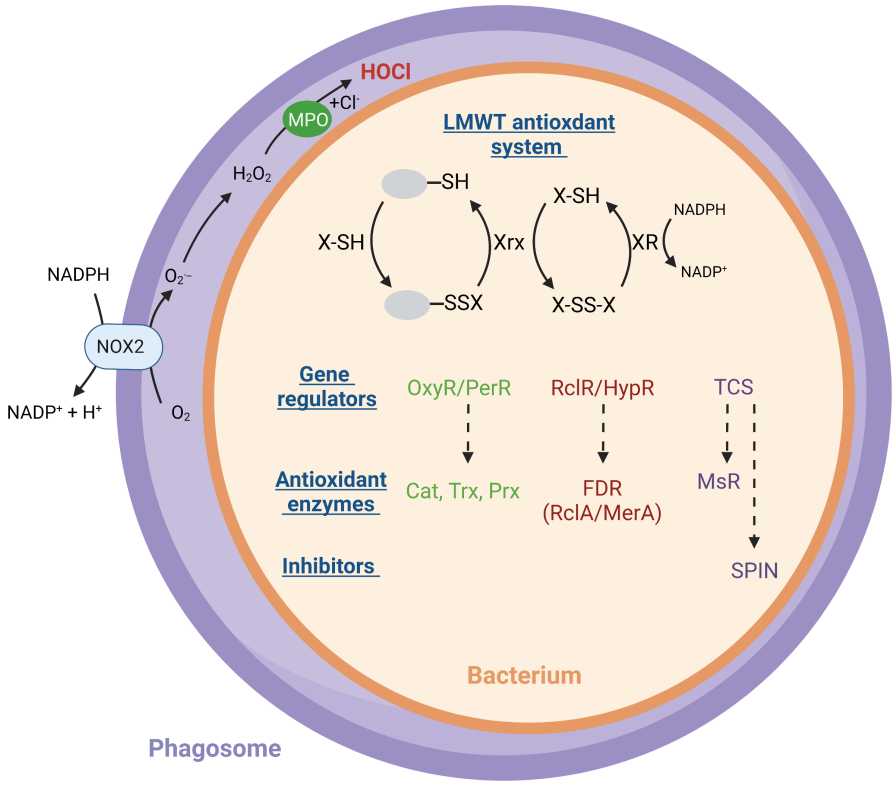

FIGURE 2 Bacterial responses in the neutrophil phagosome. Upon digestion into a neutrophil phagosome, bacteria are exposed to a flux of oxidants derived from the NADPH oxidase (NOX2), including hypochlorous acid (HOCl). Low molecular weight thiols (LWMTs) such as GSH, BSH, and MSH (X-SH) form mixed disulfides with protein thiols, protecting them from irreversible oxidation. Protein thiols are regenerated by thioltransferases such as glutaredoxin (Xrx) using LMWTs as a cofactor, generating oxidized LMWTs (X-SS-X). Oxidized LMWTs are reduced by NADPH-dependent reductases such as glutathione reductase. A variety of antioxidant defense mechanisms are upregulated. OxyR and PerR are H_2O_2-sensitive gene regulators that control the expression of peroxiredoxin (Prx; *ahpCF*), thioredoxin (Trx; *trxC/A*), and catalase (Cat; *katG/A*). RclR and HypR are HOCl-sensitive gene regulators that control the expression of flavoprotein disulfide reductases RclA and MerA in *E. coli* and *S. aureus*, respectively. Two-component systems (TCS) also control the expression of methionine sulfoxide reductases (MsR) and in *S. aureus* the myeloperoxidase inhibitor SPIN. Created with BioRender.com

Deletion of OxyR dramatically decreases *E. coli* survival in normal neutrophils to a greater extent than in NADPH oxidase-deficient neutrophils, demonstrating the role of the OxyR-regulated enzymes in protection against the neutrophil oxidative burst.[105] In addition to OxyR, the reactive chlorine-responsive transcription factor RclR is activated in *E. coli* during neutrophil phagocytosis,[107] resulting in expression of the flavoprotein disulfide reductase RclA, as well as RclB and RclC.[82] Although oxidant-sensing by RclR is important for *E. coli* and *P. aeruginosa* survival of HOCl stress,[82,108] it does not contribute to the survival of *E. coli* in neutrophils.[107] This is possibly due to the rapid bactericidal action of non-oxidative neutrophil killing mechanisms against *E. coli*. Other regulatory factors in *E. coli* that respond to HOCl are HypT and NemR,[109,110] regulating methionine and cysteine biosynthesis and HOCl resistance genes, respectively, as well as the recently discovered HypSR.[111] The role of these regulatory systems in evading neutrophil killing warrants investigation.

S. aureus upregulates the expression of antioxidant genes trxB, katA, ahpF, and ahpC during neutrophil phagocytosis[112] (Figure 2). These genes are controlled by the resistance regulon repressor PerR, which plays an analogous role to OxyR in Gram-positive firmicutes, but senses hydrogen peroxide via metal-catalyzed histidine oxidation rather than via a thiol–disulfide switch.[113,114] Staphylococcal environmental sensing and response is also achieved by two-component gene regulatory systems (TCSs).[115] The VraSR TCS, for example, contributes to the upregulation of methionine sulfoxide reductase during neutrophil phagocytosis,[100] and the SaeR/S TCS controls the expression of the myeloperoxidase inhibitor SPIN[73,116] (Figure 2). While deleting the gene encoding for SPIN had a modest effect on surviving neutrophil killing,[73] the SaeR/S gene regulatory system was shown to be critical.[112] This finding highlights the synergy and/or redundancy of multiple defense mechanisms controlled by the same regulatory system to ensure microbial survival. Hemolysins and regulators of peptidoglycan hydrolase activity, also under the control of SaeR/S,[117] were strongly induced in *S. aureus* following ingestion by neutrophils.[112] Upregulation of these virulence factors may aid in escaping the neutrophil, as demonstrated for α-hemolysin,[118] and simultaneously give rise to a more cytotoxic and antibiotic-resistant phenotype.[119,120]

One of the most upregulated genes in *S. aureus* following ingestion by neutrophils is that of the pyridine nucleotide-disulfide reductase MerA,[112] which is controlled by the hypochlorous acid sensing repressor HypR through a thiol/disulfide switch mechanism.[81] MerA

plays a role in the bacterial response to HOCl and electrophilic stress[81,121] and was recently shown to protect *S. aureus* from HOSCN, which is a predominant oxidant produced from lactoperoxidase and myeloperoxidase in epithelial fluids lining the respiratory tract (Shearer et al, in revision). While merA deletion did not sensitize *S. aureus* to neutrophil killing (Springer et al, unpublished), MerA upregulation may ensure that phagosomal survivors continue to escape killing by the innate immune system once they have escaped the neutrophil.

3.4 | Metabolic adaptation

In addition to upregulating oxidative stress resistance genes, microbes in the neutrophil phagosome must undergo metabolic reprogramming in order to cope with nutrient privation and to keep up with the demand for NADPH consumed by antioxidant systems. Numerous metabolic pathways were shown to be downregulated in *E. coli* and *G. bethesdensis* ingested by neutrophils, including the tricarboxylic acid cycle, translational machinery, amino acid, and nitrogen metabolism.[104,105] The expression of genes involved in these pathways was similarly altered in normal and NADPH oxidase-deficient neutrophils, indicating that they respond to a non-oxidative trigger in the phagosome environment. Lactoferrin-mediated iron depletion is compensated by upregulating genes involved in iron acquisition.[105]

When cells are under oxidative stress, NADPH is generally known to be replenished by channelling the glycolytic flux into the oxidative PPP, generating CO_2 in the process. This is achieved both by oxidative inhibition of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and through suspension of allosteric inhibition of the first PPP enzyme glucose 6-phosphate dehydrogenase by NADPH[122] (Figure 3). Whether this is happening in bacteria during neutrophil phagocytosis remains to be established; however, glyceraldehyde 3-phosphate dehydrogenase was not found to be among the significantly oxidized thiol proteins in phagocytosed *E. coli*, despite being >90% oxidized under HOCl stress.[36,123] Furthermore, enzymes of the PPP were found to be downregulated in intraphagosomal *G. bethesdensis*.[104] Interestingly, the same study reported that enzymes involved in one and three-carbon metabolism such as pyruvate dehydrogenase were upregulated.[104] Under aerobic growth, pyruvate dehydrogenase converts pyruvate to acetyl-CoA, which enters the tricarboxylic acid cycle. Under anaerobic glucose fermentation, the enzyme's role is to generate endogenous CO_2 required for cell growth.[124] Although this generates slightly fewer reducing equivalents than the oxidative PPP, it is a more energy- and carbon-efficient CO_2-producing route with fewer toxic by-products.[124] CO_2 is known to be growth-limiting, especially in nutrient-poor media and metabolically inactive bacteria,[124] that is, under conditions that are likely to apply in the neutrophil phagosome. It is therefore conceivable that upregulating pyruvate dehydrogenase aids the bacteria in meeting their metabolic demand for CO_2.

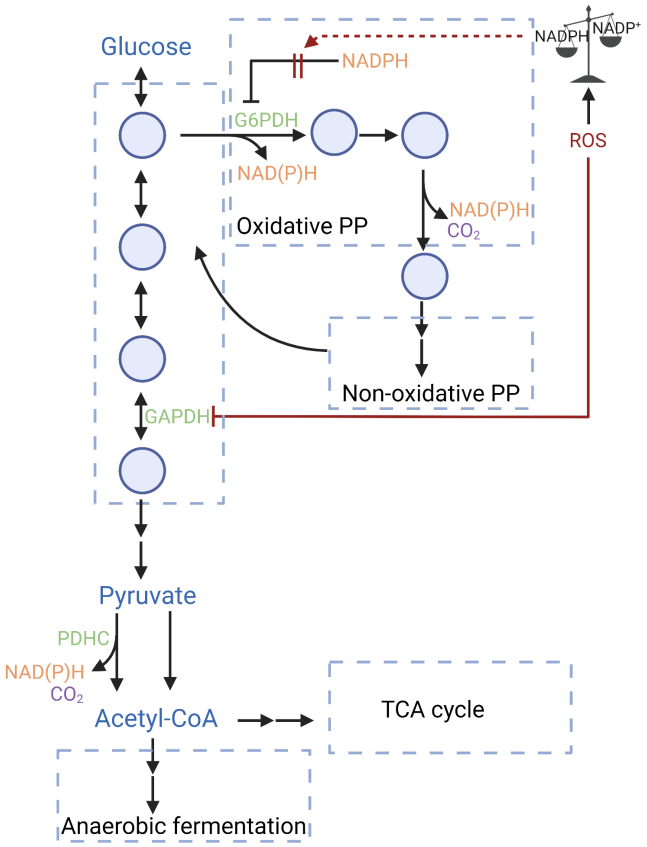

FIGURE 3 Possible metabolic routes within phagocytosed bacteria. Oxidation of glyceraldehyde 3-phosphate dehydrogenase GAPDH and removal of feedback inhibition of glucose 6-phosphate dehydrogenase (G6PDH) by a low NADPH/NAD^+ ratio leads to shunting of glucose and products of the pentose phosphate pathway (PPP) back into this pathway, maximizing NADPH and CO_2 production. The pyruvate dehydrogenase complex (PDHC) either converts pyruvate to acetyl-CoA for the tricarboxylic acid (TCA) cycle under aerobic growth or generates endogenous CO_2 required for cell growth under anaerobic glucose fermentation. The latter produced slightly fewer reducing equivalents than the oxidative PPP, it is a more energy- and carbon-efficient CO_2-producing route with fewer toxic by-products. Created with BioRender.com

Metabolomic studies are warranted to further delineate the extent and nature of bacterial metabolic adaption in the neutrophil phagosome.

4 | FUTURE WORK

Our ability to understand bacterial survival inside the neutrophil requires increased knowledge of phagosomal biochemistry. Chemical probes that can cross membranes and accumulate in the small sealed space are used as oxidant and pH reporters in the phagosome, but the harsh oxidative environment and rapid changes make visualization difficult. High concentrations of proteins and metabolites that prevail in the phagosome are extremely difficult to replicate in a test tube. There is also clear evidence for heterogeneity between

phagosomes. Bacteria themselves can be used as phagosomal probes, indeed their use is encouraged over inert particles, but what is currently missing is the monitoring of individual bacteria. Single-cell transcriptomics and proteomic methods that measure oxidative modifications in individual bacteria would provide valuable information on the variation in insults faced by ingested bacteria. It would also be informative to combine this with microscopy-based methods for monitoring bacterial viability. Colony counts are the traditional method for measuring survival but only provide information at a population level. Experimental systems that report on conditions within individual phagosomes and the response of bacteria enclosed therein would enable us to determine whether survivors were fortunate enough to have arrived in a less threatening phagosome, or whether they have superior defenses.

Bacteria are able to rapidly upregulate their defenses following exposure to environmental stressors. Their major hurdle is they only have seconds to respond when entering a phagosome, and upregulation at that point would seem fruitless. It is possible that bacteria receive signals of impending attack before phagocytosis. Leakage of phagosomal oxidants and even extracellular oxidant generation by neutrophils may provide sufficient warning to those bacteria yet to be phagocytosed. It is also likely that stochastic variation in gene expression means that some bacteria in a population are already better positioned to survive in the phagosome. It is important to recognize that only a few survivors are necessary to cause significant problems for the host.

It is important to consider the conditions under which neutrophil and bacteria co-culture experiments are performed. First, it is important to recognize that there are significant differences between human and murine neutrophils.[125] Also, in vitro studies typically use atmospheric oxygen levels with excess glucose and a neutral buffered pH, and other myeloperoxidase substrates such as thiocyanate and ascorbate are absent. All of these factors can vary in vivo, and it would be valuable to know their impacts on bacterial survival. Neutrophils isolated from peripheral blood do not receive the same priming as neutrophils arriving at a site of infection. Also, to overcome the limitations of working with short-lived neutrophils, cell lines with neutrophil-like properties, such as HL-60, PLB-985, and NB4, have been used in a laboratory setting. These have the advantage of enabling conventional molecular biology approaches to manipulate the expression of genes and therefore test their functional significance, and also enable the use of genetically encoded pH and oxidant reporters. The disadvantage is that they are leukemia cells with significant genetic disruptions. Ongoing improvements in the culture of human hematopoietic stem cells will enable genetic modification prior to differentiation into neutrophils for use in experimental settings.

The emergence of anti-microbial resistance is limiting the tools we have available to directly kill pathogens. Strategies that work in combination with innate defense mechanisms seem to be a logical approach, but appear to have attracted limited research to date. It is therefore important that new strategies are developed to target phagosomal survivors. While specific genes have been shown to protect bacteria from an oxidative bolus in vitro, single deletions of bacterial defense genes have only led to minor increases in susceptibility to neutrophil killing. As with other organisms, bacteria can compensate by upregulating other pathways, and it is likely that multiple hits are required for sensitization. Deletion of gene regulators has been more successful. Unbiased screening tools are now available that do not limit investigators to studying known genes of interest. We recommend the use of bacterial mutant libraries to identify genes that are important for enabling survival in the phagosome. These may identify targets for the development of new classes of antibiotics that assist the neutrophil to eliminate pathogens. Drug libraries should also be exploited to search for compounds that promote the killing of phagosomal bacteria, with the screening performed during and after the co-culture of bacteria with human neutrophils.

ACKNOWLEDGEMENTS

The authors wish to acknowledge funding from the Health Research Council of New Zealand, including a Sir Charles Hercus Health Research Fellowship to N.D. Open access publishing facilitated as part of the Wiley - agreement via the Council of Australian University Librarians.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID

Mark B. Hampton https://orcid.org/0000-0002-7349-3729

REFERENCES

1. Cech P, Lehrer RI. Heterogeneity of human neutrophil phagolysosomes: functional consequences for candidacidal activity. *Blood*. 1984;64(1):147-151.
2. Bainton DF, Farquhar MG. Origin of granules in polymorphonuclear leukocytes. Two types derived from opposite faces of the Golgi complex in developing granulocytes. *J Cell Biol*. 1966;28(2):277-301.
3. Bainton DF, Ullyot JL, Farquhar MG. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. *J Exp Med*. 1971;134(4):907-934.
4. Rice WG, Ganz T, Kinkade JM Jr, Selsted ME, Lehrer RI, Parmley RT. Defensin-rich dense granules of human neutrophils. *Blood*. 1987;70(3):757-765.
5. Le Cabec V, Cowland JB, Calafat J, Borregaard N. Targeting of proteins to granule subsets is determined by timing and not by sorting: the specific granule protein NGAL is localized to azurophil granules when expressed in HL-60 cells. *Proc Natl Acad Sci USA*. 1996;93(13):6454-6457.
6. Cowland JB, Borregaard N. The individual regulation of granule protein mRNA levels during neutrophil maturation explains the heterogeneity of neutrophil granules. *J Leukoc Biol*. 1999;66(6):989-995.
7. Rorvig S, Ostergaard O, Heegaard NH, Borregaard N. Proteome profiling of human neutrophil granule subsets, secretory vesicles,

and cell membrane: correlation with transcriptome profiling of neutrophil precursors. *J Leukoc Biol*. 2013;94(4):711-721.
8. Sengelov H, Kjeldsen L, Borregaard N. Control of exocytosis in early neutrophil activation. *J Immunol*. 1993;150(4):1535-1543.
9. Munafo DB, Johnson JL, Ellis BA, Rutschmann S, Beutler B, Catz SD. Rab27a is a key component of the secretory machinery of azurophilic granules in granulocytes. *Biochem J*. 2007;402(2):229-239.
10. Parmley RT, Doran T, Boyd RL, Gilbert C. Unmasking and redistribution of lysosomal sulfated glycoconjugates in phagocytic polymorphonuclear leukocytes. *J Histochem Cytochem*. 1986;34(12):1701-1707.
11. Reeves EP, Lu H, Jacobs HL, et al. Killing activity of neutrophils is mediated through activation of proteases by K+ flux. *Nature*. 2002;416(6878):291-297.
12. Winterbourn CC, Hampton MB, Livesey JH, Kettle AJ. Modeling the reactions of superoxide and myeloperoxidase in the neutrophil phagosome: implications for microbial killing. *J Biol Chem*. 2006;281(52):39860-39869.
13. Babior BM, Kipnes RS, Curnutte JT. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J Clin Invest*. 1973;52(3):741-744.
14. Chapman AL, Hampton MB, Senthilmohan R, Winterbourn CC, Kettle AJ. Chlorination of bacterial and neutrophil proteins during phagocytosis and killing of *Staphylococcus aureus*. *J Biol Chem*. 2002;277(12):9757-9762.
15. Kettle AJ, Ashby LV, Winterbourn CC, Dickerhof N. Superoxide: the enigmatic chemical chameleon in neutrophil biology *Immunol Rev*. 2023. in press.
16. Nauseef WM, Metcalf JA, Root RK. Role of myeloperoxidase in the respiratory burst of human neutrophils. *Blood*. 1983;61(3):483-492.
17. Decoursey TE, Ligeti E. Regulation and termination of NADPH oxidase activity. *Cell Mol Life Sci*. 2005;62(19–20):2173-2193.
18. Britt EC, Lika J, Giese MA, et al. Switching to the cyclic pentose phosphate pathway powers the oxidative burst in activated neutrophils. *Nat Metab*. 2022;4(3):389-403.
19. Nunes P, Demaurex N, Dinauer MC. Regulation of the NADPH oxidase and associated ion fluxes during phagocytosis. *Traffic*. 2013;14(11):1118-1131.
20. Jankowski A, Scott CC, Grinstein S. Determinants of the phagosomal pH in neutrophils. *J Biol Chem*. 2002;277(8):6059-6066.
21. Wilkie-Grantham RP, Magon NJ, Harwood DT, et al. Myeloperoxidase-dependent lipid peroxidation promotes the oxidative modification of cytosolic proteins in phagocytic neutrophils. *J Biol Chem*. 2015;290(15):9896-9905.
22. Song ZM, Bouchab L, Hudik E, Le Bars R, Nusse O, Dupre-Crochet S. Phosphoinositol 3-phosphate acts as a timer for reactive oxygen species production in the phagosome. *J Leukoc Biol*. 2017;101(5):1155-1168.
23. Moreland JG, Davis AP, Bailey G, Nauseef WM, Lamb FS. Anion channels, including ClC-3, are required for normal neutrophil oxidative function, phagocytosis, and transendothelial migration. *J Biol Chem*. 2006;281(18):12277-12288.
24. Painter RG, Valentine VG, Lanson NA Jr, et al. CFTR expression in human neutrophils and the phagolysosomal chlorination defect in cystic fibrosis. *Biochemistry*. 2006;45(34):10260-10269.
25. Dickerhof N, Isles V, Pattemore P, Hampton MB, Kettle AJ. Exposure of *Pseudomonas aeruginosa* to bactericidal hypochlorous acid during neutrophil phagocytosis is compromised in cystic fibrosis. *J Biol Chem*. 2019;294(36):13502-13514.
26. Sao Emani C, Williams MJ, Wiid IJ, et al. Ergothioneine is a secreted antioxidant in *Mycobacterium smegmatis*. *Antimicrob Agents Chemother*. 2013;57(7):3202-3207.
27. Cech P, Lehrer RI. Phagolysosomal pH of human neutrophils. *Blood*. 1984;63(1):88-95.
28. Jiang Q, Griffin DA, Barofsky DF, Hurst JK. Intraphagosomal chlorination dynamics and yields determined using unique fluorescent bacterial mimics. *Chem Res Toxicol*. 1997;10(10):1080-1089.
29. Dri P, Presani G, Perticarari S, Alberi L, Prodan M, Decleva E. Measurement of phagosomal pH of normal and CGD-like human neutrophils by dual fluorescence flow cytometry. *Cytometry*. 2002;48(3):159-166.
30. Sanchez-Carbonel A, Mondragon B, Lopez-Chegne N, et al. The effect of the efflux pump inhibitor carbonyl cyanide m-chlorophenylhydrazone (CCCP) on the susceptibility to imipenem and cefepime in clinical strains of *Acinetobacter baumannii*. *PLoS One*. 2021;16(12):e0259915.
31. Rosen H, Michel BR. Redundant contribution of myeloperoxidase-dependent systems to neutrophil-mediated killing of *Escherichia coli*. *Infect Immun*. 1997;65(10):4173-4178.
32. Rosen H, Orman J, Rakita RM, Michel BR, VanDevanter DR. Loss of DNA-membrane interactions and cessation of DNA synthesis in myeloperoxidase-treated *Escherichia coli*. *Proc Natl Acad Sci USA*. 1990;87(24):10048-10052.
33. Rosen H, Crowley JR, Heinecke JW. Human neutrophils use the myeloperoxidase-hydrogen peroxide-chloride system to chlorinate but not nitrate bacterial proteins during phagocytosis. *J Biol Chem*. 2002;277(34):30463-30468.
34. Pattison DI, Davies MJ. Absolute rate constants for the reaction of hypochlorous acid with protein side chains and peptide bonds. *Chem Res Toxicol*. 2001;14(10):1453-1464.
35. Rosen H, Klebanoff SJ, Wang Y, Brot N, Heinecke JW, Fu X. Methionine oxidation contributes to bacterial killing by the myeloperoxidase system of neutrophils. *Proc Natl Acad Sci USA*. 2009;106(44):18686-18691.
36. Xie K, Bunse C, Marcus K, Leichert LI. Quantifying changes in the bacterial thiol redox proteome during host-pathogen interaction. *Redox Biol*. 2019;21:101087.
37. Ashby LV, Springer R, Loi VV, et al. Oxidation of bacillithiol during killing of *Staphylococcus aureus* USA300 inside neutrophil phagosomes. *J Leukoc Biol*. 2022;112:591-605.
38. Parker HA, Dickerhof N, Forrester L, et al. *Mycobacterium smegmatis* resists the bactericidal activity of hypochlorous acid produced in neutrophil phagosomes. *J Immunol*. 2021;206(8):1901-1912.
39. Maskarinec SA, McKelvy M, Boyle K, et al. Neutrophil functional heterogeneity is a fixed phenotype and is associated with distinct gene expression profiles. *J Leukoc Biol*. 2022;112:1485-1495.
40. Li XJ, Tian W, Stull ND, Grinstein S, Atkinson S, Dinauer MC. A fluorescently tagged C-terminal fragment of p47phox detects NADPH oxidase dynamics during phagocytosis. *Mol Biol Cell*. 2009;20(5):1520-1532.
41. Albrett AM, Ashby LV, Dickerhof N, Kettle AJ, Winterbourn CC. Heterogeneity of hypochlorous acid production in individual neutrophil phagosomes revealed by a rhodamine-based probe. *J Biol Chem*. 2018;293(40):15715-15724.
42. Schwartz J, Leidal KG, Femling JK, Weiss JP, Nauseef WM. Neutrophil bleaching of GFP-expressing staphylococci: probing the intraphagosomal fate of individual bacteria. *J Immunol*. 2009;183(4):2632-2641.
43. Palazzolo AM, Suquet C, Konkel ME, Hurst JK. Green fluorescent protein-expressing *Escherichia coli* as a selective probe for HOCl generation within neutrophils. *Biochemistry*. 2005;44(18):6910-6919.
44. Kobayashi SD, Malachowa N, DeLeo FR. Neutrophils and bacterial immune evasion. *J Innate Immun*. 2018;10(5–6):432-441.
45. Spinner JL, Winfree S, Starr T, et al. *Yersinia pestis* survival and replication within human neutrophil phagosomes and uptake of infected neutrophils by macrophages. *J Leukoc Biol*. 2014;95(3):389-398.

46. Parker HA, Forrester L, Kaldor CD, Dickerhof N, Hampton MB. Antimicrobial activity of neutrophils against mycobacteria. *Front Immunol*. 2021;12:782495.
47. Melly MA, Thomison JB, Rogers DE. Fate of staphylococci within human leukocytes. *J Exp Med*. 1960;112:1121-1130.
48. Rogers DE, Tompsett R. The survival of staphylococci within human leukocytes. *J Exp Med*. 1952;95(2):209-230.
49. Kobayashi SD, Braughton KR, Palazzolo-Ballance AM, et al. Rapid neutrophil destruction following phagocytosis of *Staphylococcus aureus*. *J Innate Immun*. 2010;2(6):560-575.
50. Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, Lindberg FP. Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. *J Immunol*. 2000;164(7):3713-3722.
51. Rogers DE. Studies on bacteriemia. I. Mechanisms relating to the persistence of bacteriemia in rabbits following the intravenous injection of staphylococci. *J Exp Med*. 1956;103(6):713-742.
52. Dallenga T, Repnik U, Corleis B, et al. *M. Tuberculosis*-induced necrosis of infected neutrophils promotes bacterial growth following phagocytosis by macrophages. *Cell Host Microbe*. 2017;22(4):519-530 e513.
53. Eum SY, Kong JH, Hong MS, et al. Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB. *Chest*. 2010;137(1):122-128.
54. Bauer ME, Shafer WM. On the *in vivo* significance of bacterial resistance to antimicrobial peptides. *Biochim Biophys Acta*. 2015;1848(11 Pt B):3101-3111.
55. Galvan EM, Lasaro MA, Schifferli DM. Capsular antigen fraction 1 and Pla modulate the susceptibility of *Yersinia pestis* to pulmonary antimicrobial peptides such as cathelicidin. *Infect Immun*. 2008;76(4):1456-1464.
56. Munch D, Engels I, Muller A, et al. Structural variations of the cell wall precursor lipid II and their influence on binding and activity of the lipoglycopeptide antibiotic oritavancin. *Antimicrob Agents Chemother*. 2015;59(2):772-781.
57. Peschel A, Jack RW, Otto M, et al. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. *J Exp Med*. 2001;193(9):1067-1076.
58. Shafer WM, Qu X, Waring AJ, Lehrer RI. Modulation of *Neisseria gonorrhoeae* susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family. *Proc Natl Acad Sci USA*. 1998;95(4):1829-1833.
59. Stapels DA, Kuipers A, von Kockritz-Blickwede M, et al. *Staphylococcus aureus* protects its immune-evasion proteins against degradation by neutrophil serine proteases. *Cell Microbiol*. 2016;18(4):536-545.
60. Allen LA, Beecher BR, Lynch JT, Rohner OV, Wittine LM. *Helicobacter pylori* disrupts NADPH oxidase targeting in human neutrophils to induce extracellular superoxide release. *J Immunol*. 2005;174(6):3658-3667.
61. Carlyon JA, Abdel-Latif D, Pypaert M, Lacy P, Fikrig E. *Anaplasma phagocytophilum* utilizes multiple host evasion mechanisms to thwart NADPH oxidase-mediated killing during neutrophil infection. *Infect Immun*. 2004;72(8):4772-4783.
62. Eichelberger KR, Jones GS, Goldman WE. Inhibition of neutrophil primary granule release during *Yersinia pestis* pulmonary infection. *MBio*. 2019;10(6):e02759-19.
63. McCaffrey RL, Schwartz JT, Lindemann SR, et al. Multiple mechanisms of NADPH oxidase inhibition by type A and type B *Francisella tularensis*. *J Leukoc Biol*. 2010;88(4):791-805.
64. Pulsifer AR, Vashishta A, Reeves SA, et al. Redundant and cooperative roles for *Yersinia pestis* Yop effectors in the inhibition of human neutrophil exocytic responses revealed by gain-of-function approach. *Infect Immun*. 2020;88(3):e00909-19.
65. Smirnov A, Daily KP, Criss AK. Assembly of NADPH oxidase in human neutrophils is modulated by the opacity-associated protein expression state of *Neisseria gonorrhoeae*. *Infect Immun*. 2014;82(3):1036-1044.
66. Tauber AI, Pavlotsky N, Lin JS, Rice PA. Inhibition of human neutrophil NADPH oxidase by chlamydia serovars E, K, and L2. *Infect Immun*. 1989;57(4):1108-1112.
67. Vareechon C, Zmina SE, Karmakar M, Pearlman E, Rietsch A. *Pseudomonas aeruginosa* effector ExoS inhibits ROS production in human neutrophils. *Cell Host Microbe*. 2017;21(5):611-618 e615.
68. Songsungthong W, Higgins MC, Rolan HG, Murphy JL, Mecsas J. ROS-inhibitory activity of YopE is required for full virulence of Yersinia in mice. *Cell Microbiol*. 2010;12(7):988-1001.
69. Spinner JL, Hasenkrug AM, Shannon JG, Kobayashi SD, Hinnebusch BJ. Role of the Yersinia YopJ protein in suppressing interleukin-8 secretion by human polymorphonuclear leukocytes. *Microbes Infect*. 2016;18(1):21-29.
70. Allen LA, McCaffrey RL. To activate or not to activate: distinct strategies used by *Helicobacter pylori* and *Francisella tularensis* to modulate the NADPH oxidase and survive in human neutrophils. *Immunol Rev*. 2007;219:103-117.
71. Miller JL, Velmurugan K, Cowan MJ, Briken V. The type I NADH dehydrogenase of *mycobacterium tuberculosis* counters phagosomal NOX2 activity to inhibit TNF-alpha-mediated host cell apoptosis. *PLoS Pathog*. 2010;6(4):e1000864.
72. Westerman TL, Bogomolnaya L, Andrews-Polymenis HL, Sheats MK, Elfenbein JR. The Salmonella type-3 secretion system-1 and flagellar motility influence the neutrophil respiratory burst. *PLoS One*. 2018;13(9):e0203698.
73. de Jong NWM, Ramyar KX, Guerra FE, et al. Immune evasion by a staphylococcal inhibitor of myeloperoxidase. *Proc Natl Acad Sci USA*. 2017;114(35):9439-9444.
74. Clements MO, Watson SP, Foster SJ. Characterization of the major superoxide dismutase of *Staphylococcus aureus* and its role in starvation survival, stress resistance, and pathogenicity. *J Bacteriol*. 1999;181(13):3898-3903.
75. Cosgrove K, Coutts G, Jonsson IM, et al. Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in *Staphylococcus aureus*. *J Bacteriol*. 2007;189(3):1025-1035.
76. Hajaj B, Yesilkaya H, Benisty R, David M, Andrew PW, Porat N. Thiol peroxidase is an important component of *Streptococcus pneumoniae* in oxygenated environments. *Infect Immun*. 2012;80(12):4333-4343.
77. Paterson GK, Blue CE, Mitchell TJ. An operon in *Streptococcus pneumoniae* containing a putative alkylhydroperoxidase D homologue contributes to virulence and the response to oxidative stress. *Microb Pathog*. 2006;40(4):152-160.
78. Schwartz CE, Krall J, Norton L, McKay K, Kay D, Lynch RE. Catalase and superoxide dismutase in *Escherichia coli*. *J Biol Chem*. 1983;258(10):6277-6281.
79. Seib KL, Simons MP, Wu HJ, et al. Investigation of oxidative stress defenses of *Neisseria gonorrhoeae* by using a human polymorphonuclear leukocyte survival assay. *Infect Immun*. 2005;73(8):5269-5272.
80. Mandell GL. Catalase, superoxide dismutase, and virulence of *Staphylococcus aureus*. *In vitro* and *in vivo* studies with emphasis on staphylococcal-leukocyte interaction. *J Clin Invest*. 1975;55(3):561-566.
81. Loi VV, Busche T, Tedin K, et al. Redox-sensing under hypochlorite stress and infection conditions by the Rrf2-family repressor HypR in *Staphylococcus aureus*. *Antioxid Redox Signal*. 2018;29(7):615-636.
82. Parker BW, Schwessinger EA, Jakob U, Gray MJ. The RclR protein is a reactive chlorine-specific transcription factor in *Escherichia coli*. *J Biol Chem*. 2013;288(45):32574-32584.

83. Meredith JD, Chapman I, Ulrich K, Sebastian C, Stull F, Gray MJ. Escherichia coli RclA is a highly active hypothiocyanite reductase. *Proc Natl Acad Sci USA*. 2022;119:e2119368119.
84. Shearer HL, Pace PE, Paton JC, Hampton MB, Dickerhof N. A newly identified flavoprotein disulfide reductase Har protects *Streptococcus pneumoniae* against hypothiocyanous acid. *J Biol Chem*. 2022;298:102359.
85. van Dalen CJ, Whitehouse MW, Winterbourn CC, Kettle AJ. Thiocyanate and chloride as competing substrates for myeloperoxidase. *Biochem J*. 1997;327(Pt 2):487-492.
86. Day BJ. The science of licking your wounds: function of oxidants in the innate immune system. *Biochem Pharmacol*. 2019;163:451-457.
87. Wang G. Chloride flux in phagocytes. *Immunol Rev*. 2016;273(1):219-231.
88. Ashby MT, Carlson AC, Scott MJ. Redox buffering of hypochlorous acid by thiocyanate in physiologic fluids. *J Am Chem Soc*. 2004;126(49):15976-15977.
89. Liu GY, Essex A, Buchanan JT, et al. *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *J Exp Med*. 2005;202(2):209-215.
90. Hillion M, Bernhardt J, Busche T, et al. Monitoring global protein thiol-oxidation and protein S-mycothiolation in *Mycobacterium smegmatis* under hypochlorite stress. *Sci Rep*. 2017;7(1):1195.
91. Imber M, Huyen NTT, Pietrzyk-Brzezinska AJ, et al. Protein S-bacillithiolation functions in thiol protection and redox regulation of the glyceraldehyde-3-phosphate dehydrogenase gap in *Staphylococcus aureus* under hypochlorite stress. *Antioxid Redox Signal*. 2018;28(6):410-430.
92. Salinas G, Comini MA. Alternative thiol-based redox systems. *Antioxid Redox Signal*. 2018;28(6):407-409.
93. Manta B, Comini M, Medeiros A, Hugo M, Trujillo M, Radi R. Trypanothione: a unique bis-glutathionyl derivative in trypanosomatids. *Biochim Biophys Acta*. 2013;1830(5):3199-3216.
94. Van Laer K, Buts L, Foloppe N, et al. Mycoredoxin-1 is one of the missing links in the oxidative stress defence mechanism of Mycobacteria. *Mol Microbiol*. 2012;86(4):787-804.
95. Linzner N, Loi VV, Fritsch VN, et al. *Staphylococcus aureus* uses the bacilliredoxin (BrxAB)/bacillithiol disulfide reductase (YpdA) redox pathway to defend against oxidative stress under infections. *Front Microbiol*. 2019;10:1355.
96. Loi VV, Harms M, Muller M, et al. Real-time imaging of the bacillithiol redox potential in the human pathogen *Staphylococcus aureus* using a genetically encoded bacilliredoxin-fused redox biosensor. *Antioxid Redox Signal*. 2017;26(15):835-848.
97. Pother DC, Gierok P, Harms M, et al. Distribution and infection-related functions of bacillithiol in *Staphylococcus aureus*. *Int J Med Microbiol*. 2013;303(3):114-123.
98. Mikheyeva IV, Thomas JM, Kolar SL, et al. YpdA, a putative bacillithiol disulfide reductase, contributes to cellular redox homeostasis and virulence in *Staphylococcus aureus*. *Mol Microbiol*. 2019;111(4):1039-1056.
99. Coady A, Xu M, Phung Q, et al. The *Staphylococcus aureus* ABC-type manganese transporter MntABC is critical for reinitiation of bacterial replication following exposure to phagocytic oxidative burst. *PLoS One*. 2015;10(9):e0138350.
100. Pang YY, Schwartz J, Bloomberg S, Boyd JM, Horswill AR, Nauseef WM. Methionine sulfoxide reductases protect against oxidative stress in *Staphylococcus aureus* encountering exogenous oxidants and human neutrophils. *J Innate Immun*. 2014;6(3):353-364.
101. Lu J, Holmgren A. The thioredoxin antioxidant system. *Free Radical Biol Med*. 2014;66:75-87.
102. Uziel O, Borovok I, Schreiber R, Cohen G, Aharonowitz Y. Transcriptional regulation of the *Staphylococcus aureus* thioredoxin and thioredoxin reductase genes in response to oxygen and disulfide stress. *J Bacteriol*. 2004;186(2):326-334.
103. Ritz D, Patel H, Doan B, et al. Thioredoxin 2 is involved in the oxidative stress response in *Escherichia coli*. *J Biol Chem*. 2000;275(4):2505-2512.
104. Greenberg DE, Sturdevant DE, Marshall-Batty KR, et al. Simultaneous host-pathogen transcriptome analysis during *Granulibacter bethesdensis* infection of neutrophils from healthy subjects and patients with chronic granulomatous disease. *Infect Immun*. 2015;83(11):4277-4292.
105. Staudinger BJ, Oberdoerster MA, Lewis PJ, Rosen H. mRNA expression profiles for *Escherichia coli* ingested by normal and phagocyte oxidase-deficient human neutrophils. *J Clin Invest*. 2002;110(8):1151-1163.
106. Zheng M, Aslund F, Storz G. Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science*. 1998;279(5357):1718-1721.
107. Konigstorfer A, Ashby LV, Bollar GE, et al. Induction of the reactive chlorine-responsive transcription factor RclR in *Escherichia coli* following ingestion by neutrophils. *Pathog Dis*. 2021;79(1):ftaa079.
108. Farrant KV, Spiga L, Davies JC, Williams HD. Response of *Pseudomonas aeruginosa* to the innate immune system-derived oxidants hypochlorous ccid and hypothiocyanous acid. *J Bacteriol*. 2021;203(2):e00300-20.
109. Drazic A, Miura H, Peschek J, et al. Methionine oxidation activates a transcription factor in response to oxidative stress. *Proc Natl Acad Sci USA*. 2013;110(23):9493-9498.
110. Gray MJ, Wholey WY, Parker BW, Kim M, Jakob U. NemR is a bleach-sensing transcription factor. *J Biol Chem*. 2013;288(19):13789-13798.
111. El Hajj S, Henry C, Andrieu C, et al. HprSR is a reactive chlorine species-sensing, two-component system in *Escherichia coli*. *J Bacteriol*. 2022;204(2):e0044921.
112. Voyich JM, Braughton KR, Sturdevant DE, et al. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J Immunol*. 2005;175(6):3907-3919.
113. Horsburgh MJ, Clements MO, Crossley H, Ingham E, Foster SJ. PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. *Infect Immun*. 2001;69(6):3744-3754.
114. Ji CJ, Kim JH, Won YB, et al. *Staphylococcus aureus* PerR is a hypersensitive hydrogen peroxide sensor using iron-mediated histidine oxidation. *J Biol Chem*. 2015;290(33):20374-20386.
115. Bleul L, Francois P, Wolz C. Two-component systems of *S. aureus*: signaling and sensing mechanisms. *Genes*. 2022;13(1):34.
116. Guerra FE, Addison CB, de Jong NW, et al. *Staphylococcus aureus* SaeR/S-regulated factors reduce human neutrophil reactive oxygen species production. *J Leukoc Biol*. 2016;100(5):1005-1010.
117. Voyich JM, Vuong C, DeWald M, et al. The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus*. *J Infect Dis*. 2009;199(11):1698-1706.
118. Pang YY, Schwartz J, Thoendel M, Ackermann LW, Horswill AR, Nauseef WM. Agr-dependent interactions of *Staphylococcus aureus* USA300 with human polymorphonuclear neutrophils. *J Innate Immun*. 2010;2(6):546-559.
119. Berube BJ, Bubeck WJ. *Staphylococcus aureus* alpha-toxin: nearly a century of intrigue. *Toxins (Basel)*. 2013;5(6):1140-1166.
120. Groicher KH, Firek BA, Fujimoto DF, Bayles KW. The *Staphylococcus aureus* lrgAB operon modulates murein hydrolase activity and penicillin tolerance. *J Bacteriol*. 2000;182(7):1794-1801.
121. Loi VV, Huyen NTT, Busche T, et al. *Staphylococcus aureus* responds to allicin by global S-thioallylation - role of the Brx/BSH/YpdA pathway and the disulfide reductase MerA to overcome allicin stress. *Free Radic Biol Med*. 2019;139:55-69.
122. Christodoulou D, Link H, Fuhrer T, Kochanowski K, Gerosa L, Sauer U. Reserve flux capacity in the pentose phosphate pathway enables *Escherichia coli's* rapid response to oxidative stress. *Cell Syst*. 2018;6(5):569-578.e567.

123. Leichert LI, Gehrke F, Gudiseva HV, et al. Quantifying changes in the thiol redox proteome upon oxidative stress *in vivo*. *Proc Natl Acad Sci USA*. 2008;105(24):8197-8202.
124. Murarka A, Clomburg JM, Moran S, Shanks JV, Gonzalez R. Metabolic analysis of wild-type *Escherichia coli* and a pyruvate dehydrogenase complex (PDHC)-deficient derivative reveals the role of PDHC in the fermentative metabolism of glucose. *J Biol Chem*. 2010;285(41):31548-31558.
125. Nauseef WM. Human neutrophils not equal murine neutrophils: does it matter? *Immunol Rev*. 2022. in press.

How to cite this article: Hampton MB, Dickerhof N. Inside the phagosome: A bacterial perspective. *Immunol Rev*. 2023;00:1-13. doi:10.1111/imr.13182