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Superoxide: The enigmatic chemical chameleon in neutrophil biology

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Summary

The burst of superoxide produced when neutrophils phagocytose bacteria is the defining biochemical feature of these abundant immune cells. But 50 years since this discovery, the vital role superoxide plays in host defense has yet to be defined. Superoxide is neither bactericidal nor is it just a source of hydrogen peroxide. This simple free radical does, however, have remarkable chemical dexterity. Depending on its environment and reaction partners, superoxide can act as an oxidant, a reductant, a nucleophile, or an enzyme substrate. We outline the evidence that inside phagosomes where neutrophils trap, kill, and digest bacteria, superoxide will react preferentially with the enzyme myeloperoxidase, not the bacterium. By acting as a cofactor, superoxide will sustain hypochlorous acid production by myeloperoxidase. As a substrate, superoxide may give rise to other forms of reactive oxygen. We contend that these interactions hold the key to understanding the precise role superoxide plays in neutrophil biology. State-of-the-art techniques in mass spectrometry, oxidant-specific fluorescent probes, and microscopy focused on individual phagosomes are needed to identify bactericidal mechanisms driven by superoxide. This work will undoubtably lead to fascinating discoveries in host defense and give a richer understanding of superoxide's varied biology.

KEYWORDS
bacterial killing, hypochlorous acid, myeloperoxidase, phagocytosis, singlet oxygen

1 | INTRODUCTION

Three decades ago, our free radical research group in Ōtautahi Christchurch asked two rather straightforward questions: "What's so super about superoxide?", and "How do neutrophils use superoxide to kill bacteria?"[1–3] Neither question was particularly novel but they were at the heart of understanding the importance of the superoxide radical anion ($O_2^{\bullet-}$) in oxidative stress and host defense. Naively, we thought that an answer to the first question would also satisfy the second question. We hoped that by probing how neutrophils use superoxide to kill ingested bacteria, we would reveal the toxic nature of superoxide. With these answers in hand, we could explain why the enzyme superoxide dismutase is needed to protect cells from oxidative stress. Our naiveté stretched to thinking that these questions would yield quick answers. But, in reality, the central question remains to this day: How does superoxide generation and removal fit into normal physiology?[4]

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

We are still convinced that the remarkable respiratory burst of neutrophils—when these innate immune cells reduce vast amounts of oxygen to superoxide—holds the key to the understanding why superoxide is so super.[5] Superoxide's superiority as an anti-microbial toxin is not due to a highly destructive reactivity, as recently asserted incorrectly.[6] It does not oxidize polypeptides, sugars, or nucleic acids.[7] Nor does superoxide initiate lipid peroxidation.[8] Yet, it is unlikely that superoxide is just a harmless precursor of hydrogen peroxide. This often-assumed possibility can be excluded because when superoxide dismutase was deployed into neutrophil phagosomes, it protected phagocytosed bacteria.[1,9–11] The enzyme would have prevented reactions of superoxide within phagosomes, but hydrogen peroxide would still have been produced through dismutation of superoxide. Thus, superoxide must play a pivotal role in bacterial killing. To date, this role remains an enigma. Imlay concedes that "the surprising bottom line is we still do not know the mechanism by which phagocytic superoxide and/or hydrogen peroxide suppress microbial growth.[12]"

Over the last 30 years, members of our research team have investigated several aspects of the chemistry of superoxide. We have focused on its reactions with other radicals and with myeloperoxidase—the neutrophil's most abundant anti-microbial enzyme.[13–18] On the basis of our work and that of numerous other groups, we will describe how superoxide resembles a chemical chameleon that adopts different reactivities, which depend on the situation and the presence of other reactants. Superoxide can act as a mild reductant and oxidant and as a nucleophile that adds to free radicals to form hydroperoxides. It is also a substrate and a cofactor of myeloperoxidase.[19] We will outline how these reactions of superoxide may explain its vital role in host defense.

2 | THE NEUTROPHIL RESPIRATORY BURST

The serious infections suffered by individuals with neutropenia,[20] as well as defects in neutrophil function, reinforce Metchnikoff's insightful concept that by eating and destroying bacteria, these cells are fundamental in host defense.[21,22] Metchnikoff called neutrophils phagocytes—cells that eat. Neutrophils are the most abundant immune cell and the front-line defenders of innate immunity.[23] In recent years, many discoveries have expanded our understanding of how neutrophils contribute to immunity. We now know that in addition to their classic functions of phagocytosis and bacterial killing (see Figure 1), neutrophils are heterogeneous myeloid cells that communicate with multiple other immune cell types. They are now considered as front-line defenders that train dendritic cells, monocytes, and lymphocytes and are pivotal in directing the initiation and maintenance of an immune response.[23] Neutrophils also present antigens and release nuclear material that contains cytotoxic proteins.[24–26] Whether the latter material—known as neutrophil extracellular traps—is truly a part of host defense remains to be firmly established.[27] By contrast, the evidence that neutrophil oxidants

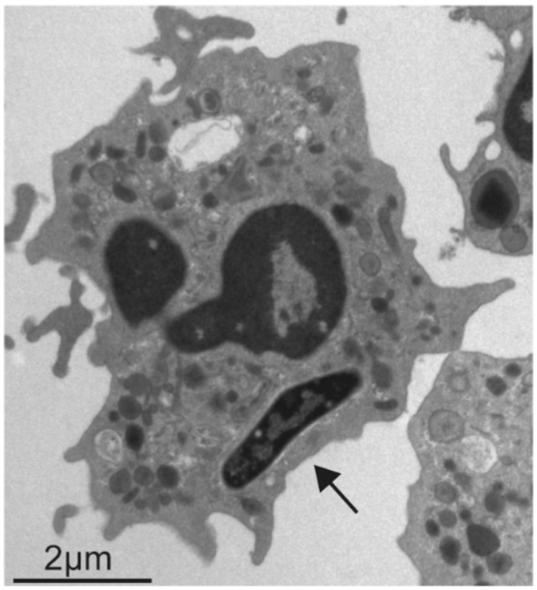

FIGURE 1 Bacterium trapped inside a neutrophil phagosome. A neutrophil has ingested *Mycobacteria smegmatis* (large rod-shaped cell as indicated by the arrow) by encasing it in a tight compartment sealed with neutrophil plasma membrane. Neutrophil granules fuse with the phagosomal membrane to inject an anti-microbial arsenal including myeloperoxidase, proteolytic peptides, and proteins. At the same time, superoxide is generated in the phagocytic space. The electron micrograph was prepared as described previously.[146]

contribute to bacterial killing is overwhelming.[28–32] In light of all these findings, it is surprising that we still do not fully appreciate how neutrophils use superoxide, arguably the most distinctive feature of their biology.

When neutrophils ingest bacteria and other micro-organisms, a series of biochemical steps is initiated that leads to the production of superoxide by an NADPH-dependent oxidase inside phagosomes.[33] Formation of superoxide inside phagosomes can be visualized with dihydroethidium when neutrophils ingest opsonized zymosan particles (see Figure 2). Zymosan is an extract from yeast cell walls that consists of protein and carbohydrate complexes. The chemical probe dihydroethidium is oxidized by superoxide in a slow reaction to form a radical that reacts rapidly with another superoxide molecule to form the specific fluorescent product 2-hydroxyethidium. Dihydroethidium is mainly transformed by other oxidants to ethidium, a similarly fluorescent product.[34] Fluorescence from 2-hydroxyethidium and ethidium can be distinguished by attaching superoxide dismutase to the zymosan. As shown in Figure 2, most of the fluorescence produced inside neutrophils when they ingested zymosan and dihydroethidium was due to superoxide because fluorescence was largely inhibited by active superoxide dismutase but not inactive enzyme.

The road to discovering superoxide production by neutrophils was long. It required seminal discoveries in other fields of research before it became evident that superoxide is the primary product of the NADPH oxidase. Baldridge and Gerard first noticed that there was an associated and dramatic increase in oxygen consumption

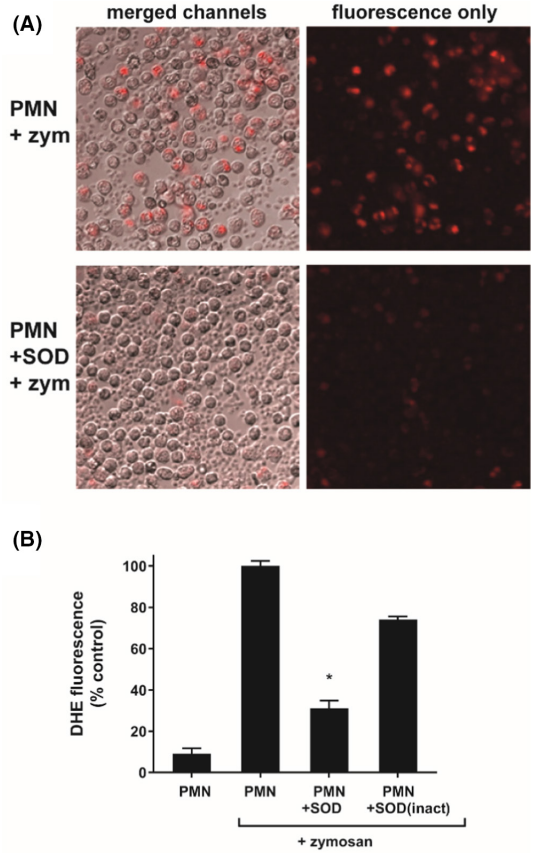

FIGURE 2 Superoxide production inside neutrophils ingesting opsonized zymosan. Neutrophils (5×10^6/mL) were pretreated with the superoxide probe dihydroethidium (10 μM) and then incubated with zymosan preparations (5×10^7/mL) for 30 min at 37°C. (A) Merged red fluorescence and bright-field (Cy3/DIC) images of neutrophils treated for 30 min were recorded by fluorescence microscopy. Neutrophils (PMN) were stimulated either with opsonized zymosan (zym) or opsonized zymosan with covalently attached superoxide dismutase (+SOD). (B) Mean intracellular fluorescence of the neutrophils was measured by flow cytometry and expressed relative to the fluorescence with normal zymosan. *, dihydroethidium fluorescence significantly decreased with active SOD-linked zymosan compared with phagocytosis of normal zymosan or inactive SOD-linked zymosan ($P < 0.01$, paired *t* test). Results are mean ± *SD* from three separate experiments. Full details of these experiments were outlined previously[153]

when neutrophils phagocytose bacteria.[35] Oxygen consumption was not inhibited by cyanide, indicating that it was independent of mitochondrial metabolism. It took another 30 years before it was realized that oxygen consumption resulted in the production of hydrogen peroxide.[36] Klebanoff then reasoned that the hydrogen peroxide formed by neutrophils was most likely used by the neutrophil enzyme myeloperoxidase to aid killing of ingested bacteria.[37] Agner had previously purified myeloperoxidase from neutrophils.[38] He found that it was a typical peroxidase in that it used hydrogen peroxide to oxidize a vast array of substrates. Klebanoff, however, was the first to show that bactericidal activity of myeloperoxidase was related to its ability to oxidize halides (Reaction 1) and the pseudohalide thiocyanate.[39] He demonstrated that oxidation of chloride to hypochlorous acid could be linked to the neutrophil respiratory burst and myeloperoxidase-dependent killing of bacteria.[40] At this time, the conversion of oxygen to hypochlorous acid seemed to neatly explain how the neutrophil respiratory burst was necessary for killing numerous bacteria. Indeed, it had long been known that hypochlorous acid or chlorine bleach was strongly bactericidal to virtually all bacteria. In the 1850s, Semmelweiss was acutely aware of its extreme anti-septic properties. He campaigned hard to have operating theatres sanitized with chlorine bleach.[41] With the discovery of superoxide production by neutrophils as outlined below, it became apparent that there is a deeper complexity to the respiratory burst, and there must be more to explain than just conversion of molecular oxygen to chlorine bleach.

$$X^- + H_2O_2 \xrightarrow{\text{myeloperoxidase}} HOX + HO^- \qquad \text{(Reaction 1)}$$

where $X^- = Cl^-$, Br^-, I^- or SCN^-

3 | A BRIEF HISTORY OF SUPEROXIDE AND SUPEROXIDE DISMUTASE

The discovery of superoxide dismutase in 1969 catapulted superoxide into the front line of biochemical research.[42] In 1968, McCord and Fridovich were investigating how the enzyme xanthine oxidase used molecular oxygen to reduce cytochrome c. This problem had been confounding scientists for decades. Eventually, McCord and Fridovich demonstrated that xanthine oxidase must produce a freely diffusible form of oxygen that reduces cytochrome c.[43] They proposed that this form of oxygen was superoxide. The salient lesson from this early research on xanthine oxidase is that superoxide's biochemistry is a tough nut to crack. Its behavior in cytochrome c reduction was inscrutable because it simply acts as a fleeting relay for a single electron, leaving no chemical footprint of its involvement. Superoxide's chemistry within neutrophil phagosomes may be just as deceptively simple.

McCord and Fridovich also showed that an enzyme was present in several protein preparations they used—including the cytochrome c itself—that could catalytically inhibit the reduction of cytochrome c. This enzyme was purified and named superoxide dismutase. Superoxide dismutases were found to be incredibly efficient enzymes that react with their substrate superoxide faster than for any other bimolecular enzymatic reaction ($k \approx 2 \times 10^9$ M^{-1} s^{-1}).[44] From this work, it became apparent that superoxide could be formed in other biological reactions and that superoxide dismutases are likely to protect cells from the deleterious reactions of superoxide.[45] This hypothesis initially led to a contentious debate about the true function of these enzymes.[46–48] One side, led by Fridovich, was adamant that superoxide dismutases are essential to protect organisms from oxidative stress by defending against superoxide. The opposing camp was led by James Fee who argued strongly that there was justifiable doubt in concluding that dismutation of superoxide is the true function of proteins called superoxide dismutases.[46] The proponents of

superoxide dismutases as essential anti-oxidants swamped the opposition with compelling evidence that these enzymes were needed to quell oxidative stress.[49] Eventually, it became generally accepted that superoxide dismutases defend organisms against the toxicity of superoxide. However, a nagging question remains to this day: How does superoxide cause oxidative stress?

Soon after the discovery of superoxide dismutase, Babior and his research team recognized the potential of the enzyme to confirm or refute the production of superoxide in biological systems. They used cytochrome c and superoxide dismutase to show that stimulated neutrophils produce superoxide.[50] Since then, it has become evident that the vast amount of oxygen consumed in the respiratory burst of neutrophils (see Figure 2) and eosinophils is reduced primarily to superoxide.[5] The initial enthusiasm that superoxide was directly responsible for killing ingested bacteria was swiftly tempered when it was shown that superoxide is not bactericidal.[51]

For a protracted period, it was believed that superoxide acted indirectly in bacterial killing by producing hydroxyl radicals. These extremely reactive radicals were first postulated to form in the Haber-Weiss reaction in which superoxide reduces hydrogen peroxide.[52] This reaction, however, is too slow to be biologically relevant.[53] To account for this kinetic shortcoming, the iron-catalyzed Haber-Weiss reaction, or superoxide-driven Fenton reaction, was invoked to explain the toxicity of superoxide.[54] In this reaction, superoxide reduces ferric iron to ferrous iron, which in turn reduces hydrogen peroxide to hydroxyl radical and hydroxide ions (Reactions 2 and 3). Indeed, in numerous studies using spin traps to detect hydroxyl radical, its production was confirmed in stimulated neutrophils, but only if exogenous iron was added to the cells.[55–57] Normally, there is limited iron in neutrophils to catalyze Fenton chemistry. Any available iron is complexed by lactoferrin, which does not catalyze hydroxyl radical production.[58] Also, myeloperoxidase inhibits iron-dependent hydroxyl radical production by consuming hydrogen peroxide.[59]

$$O_2^{\cdot-} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \qquad \text{(Reaction 2)}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + HO^{-} \qquad \text{(Reaction 3)}$$

The quest to explain how neutrophils use superoxide to kill ingested bacteria seemed to be blocked at every turn. Johnstone and co-worker cleverly demonstrated that when superoxide dismutase and catalase were ingested into phagosomes along with bacteria, fewer bacteria were killed.[9] But they were resigned to conclude that the role of superoxide remains to be clearly defined. Others showed that superoxide dismutases released into the extracellular milieu by bacteria or fungi protected them from neutrophil killing and increased their virulence.[10,60–62] These results also pointed to a direct role for superoxide in the demise of the ingested micro-organisms. Klebanoff, who had focused on myeloperoxidase-dependent production of hypochlorous acid as a major route to bacterial killing by neutrophils,[39] was also stumped when he found superoxide generated by xanthine oxidase had little effect on myeloperoxidase's bactericidal activity.[63]

4 | CHRONIC GRANULOMATOUS DISEASE AND MYELOPEROXIDASE DEFICIENCY

Difficult puzzles in medicine have often been solved by understanding how a mutated gene leads to a loss of protein function and gives rise to a distinct clinical phenotype. There are two of these experiments of nature that affect neutrophil function: chronic granulomatous disease (CGD) and myeloperoxidase deficiency.[64–66] Both arise from genetic mutations and provide compelling evidence that neutrophils generate oxidants to kill phagocytosed bacteria. CGD is a rare disease that results in serious life-threatening infections. The varied mutations in CGD have certainly helped in understanding how oxygen is reduced to superoxide. To date, however, neither CGD nor myeloperoxidase deficiency has shed light on why neutrophils generate superoxide. In fact, they have often made it more difficult to understand what specific role superoxide plays in neutrophil biology. The confusion arises primarily because the multi-layered fabric of host defense is not necessarily weakened when one of its components is removed. In such cases, it is often reasoned that there are layers of redundancy in the immune system. Although at one level this is true, the word redundancy also has negative connotations, implying that particular components of immune defense are not essential. This perspective obscures the role these components play in a fully functional immune system. Myeloperoxidase deficiency is a prime example. Even though many bacteria and fungi are killed poorly by neutrophils that lack myeloperoxidase, this deficiency, unlike CGD, does not predispose individuals to serious infections. Also, neutrophils can kill some bacteria, such as *E. coli*, effectively without the enzyme.[29] However, in normal neutrophils, myeloperoxidase produces sufficient oxidants to be solely responsible for killing *E. coli*.[67] The absence of myeloperoxidase, or its inhibition, simply permits other effective anti-microbial agents to eliminate the bacteria, without any discernible decrease in killing efficiency. Results from myeloperoxidase knock-out mice can also be deceiving when trying to infer how the enzyme contributes to host defense in humans. In mice, myeloperoxidase deficiency gives a relatively mild phenotype compared with CGD.[68,69] Mouse neutrophils, however, normally contain 10-fold less myeloperoxidase than their human counterparts, and their nitric oxide defenses are more developed. Thus, it can be argued that with respect to neutrophil function, mice are not a good model of men.[70,71]

Research into CGD has also provided ambiguous findings in our attempts to understand the critical reactions that superoxide undergoes inside phagosomes. CGD is a rare, mainly X-linked genetic disorder, in which phagocytes including neutrophils, monocytes, macrophages, and eosinophils do not generate superoxide.[64] Individuals afflicted with this disorder are highly susceptible to recurrent life-threatening bacterial and fungal infections from early childhood. Neutrophils from these individuals fail to kill certain

microbes but others are killed effectively. Some of the susceptible bacteria include species that generate hydrogen peroxide during their normal metabolism.[28,72] Intraphagosomal sources of hydrogen peroxide can also compensate for the lack of superoxide production by CGD neutrophils and allow them to kill otherwise resistant bacteria.[73] Compensation by hydrogen peroxide generating systems suggests that superoxide is simply a source of hydrogen peroxide that myeloperoxidase converts to bactericidal hypochlorous acid. However, it is also likely that a functional myeloperoxidase system is an adequate substitute for superoxide-dependent killing. Consequently, these results with CGD do not tell us how superoxide interacts with other phagosomal components in normal neutrophils. Yet, the severe clinical features of CGD indicate that the innate immune system is reliant on the generation of superoxide in phagocytic cells. The absence of superoxide is expected to be more deleterious than myeloperoxidase deficiency because in addition to neutrophils, it affects several other types of innate immune cells.

Given the inherent complexity of the immune system, we believe that the best approach to understanding how neutrophils use superoxide to kill ingested bacteria is to understand its chemistry, identify its potential targets inside phagosomes, and then demonstrate what reactions it affects during phagocytosis and bacterial killing.

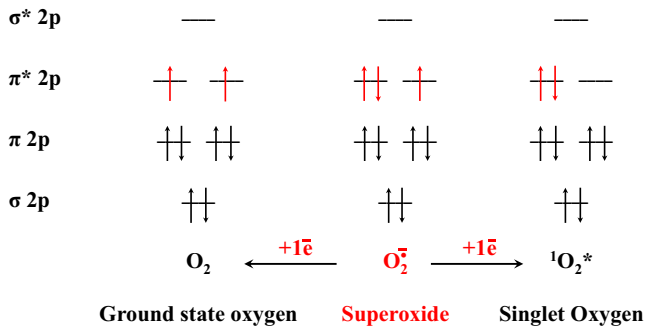

FIGURE 3 Molecular orbital diagrams for ground-state oxygen, superoxide radical anion, and singlet oxygen. Ground-state molecular oxygen is a free radical because it contains two unpaired electrons in its π^* 2p orbitals. When it is reduced with one electron it produces superoxide, another free radical because one of the π^* 2p orbitals still contains an unpaired electron. When superoxide is oxidized and loses one electron, it can form either ground-state oxygen or singlet oxygen. Thermodynamically plausible formation of singlet oxygen from superoxide requires an oxidant with a reduction potential of greater than +0.65 V. Singlet oxygen is not a free radical because it does not contain an unpaired electron. It is more reactive than ground-state oxygen because it has an empty π^* 2p orbital and can readily receive two electrons with opposing spin quantum numbers

5 | THE REACTIVITY OF SUPEROXIDE

Giving a chemical entity a common name, or worse still an acronym, can lead researchers down scientific cul-de-sacs. There have certainly been a few misconceptions and dead-ends for superoxide. Initially, the mystic around its name led biologists to assume that superoxide is super reactive. Superoxide, however, does not derive its name from extreme reactivity. Instead, it was named after the unusual stoichiometry molecular oxygen adopts with alkali metals such as potassium.[74]

Chemists had long known that heating various metals in the presence of molecular oxygen produced metal oxides. This was true for potassium. Based on relative amounts of potassium and oxygen in the molecule, it was claimed that the product was dipotassium tetroxide (K_2O_4). However, the great 20th-century chemist Linus Pauling reasoned that dipotassium tetroxide was too unstable to exist.[74] He proposed that the product formed when burning potassium was in fact KO_2, where there were two possible resonance structures—a species with one single bond containing two electrons and another species with a three-electron bond. In each type of bond, one of the electrons would be unpaired making the molecule paramagnetic. Pauling asked his postdoc Edward Neuman to make the purported dipotassium tetroxide and determine whether it was paramagnetic or diamagnetic, that is, whether it was a free radical with an unpaired electron, or contained paired electrons. Neuman found that dipotassium tetroxide was paramagnetic with a single magnetic moment per two oxygen atoms. Thus, the correct chemical formula was KO_2 and the dioxygen was present as the free radial anion, $O_2^{\cdot-}$. In 1933, Pauling gave a seminar on these findings and asked the audience if they could suggest names for KO_2—the highest possible oxide of potassium. He dismissed potassium dioxide and potassium hydroperoxide as suitable names because they did not suit the radical nature of oxygen. Eventually, Eastman and Bray suggested superoxide. Pauling liked this name because it described the unexpectedly high stoichiometry of oxygen with potassium. Pauling graciously let Neuman publish the work as the sole author with the title "Potassium superoxide and the three-electron bond."[75] The name stuck even though it would soon become apparent that it was a misnomer.

Although not super reactive, superoxide has some interesting and kinetically competent chemistry that lends itself to cytotoxicity. Superoxide is the one-electron reduction product of molecular oxygen (Reaction 4). It is in equilibrium with its protonated form, or conjugate acid, the perhydroxyl radical ($HO_2^{\cdot}$), also known as the hydroperoxyl radical (Reaction 5). At pH 4.8, half of superoxide exists as the anion and half as the perhydroxyl radical. At pH 7.8, close to the pH within neutrophil phagosomes (see below),[76] the concentration of superoxide anion exceeds that of the perhydroxyl radical by a 1000-fold. The distinction between superoxide anion and the perhydroxyl radical is important to appreciate because these two species have different properties and reactivities. They both have the potential to act as an oxidant and a reductant. The perhydroxyl radical is thermodynamically the stronger reactant.[77,78] The duality of superoxide's redox chemistry arises because it has a single unpaired electron in an anti-bonding orbital (Figure 3). Reduction by a single electron converts superoxide to hydrogen peroxide (Reaction 6), whereas its oxidation or removal of an electron produces molecular oxygen (reverse of Reaction 4). The negative

charge on superoxide anion radical restricts its reactivity. It prevents superoxide from diffusing through cell membranes and limits its ability to act as an oxidant by repelling it away from electron-rich centers. By contrast, the perhydroxyl radical freely diffuses into cells and may oxidize lipids on the way through.[79]

Any potentially cytotoxic reaction of superoxide must compete with its spontaneous dismutation, that is, superoxide's reaction with itself.[44] Dismutation occurs most favorably between the superoxide anion and the perhydroxyl radical (Reaction 7). Reaction between two superoxide anion radicals is much less likely because the anions are repelled from each other by their negative charges. Consequently, dismutation is maximal at pH 4.8 where the second order rate constant is $3 \times 10^7\ M^{-1}\ s^{-1}$. Even at pH 7.8, the rate constant is sizeable at approx. $1 \times 10^5\ M^{-1}\ s^{-1}$.

$$O_2 + 1e^- \rightleftarrows O_2^{\cdot-} \quad \text{(Reaction 4)}$$

$$O_2^{\cdot-} + H^+ \rightleftharpoons HO_2^{\cdot} \quad \text{(Reaction 5)}$$

$$O_2^{\cdot-} + 1e^- + 2H^+ \rightarrow H_2O_2 \quad \text{(Reaction 6)}$$

$$O_2^{\cdot-} + HO_2^{\cdot} + H^+ \rightarrow H_2O_2 + O_2 \quad \text{(Reaction 7)}$$

Most rapid reactions of superoxide are with other free radicals. The fastest is with nitric oxide ($NO^{\cdot}$), which is essentially a diffusion-controlled reaction ($2 \times 10^{10}\ M^{-1}\ s^{-1}$).[80] The product peroxynitrite is a strong two-electron oxidant that reacts predominantly with thiols.[80] It also reacts favorably with carbon dioxide ($k = 6 \times 10^4\ M^{-1}\ s^{-1}$) to form a transient intermediate that liberates nitrogen dioxide radical ($NO_2^{\cdot}$) and the carbonate radical ($CO_3^{\cdot-}$).[81] This reaction changes peroxynitrite's chemistry to that of a one-electron oxidant that produces mainly thiyl radicals as well as nitrating tyrosine residues in proteins.[82,83] There is no compelling evidence, however, that $NO^{\cdot}$ is generated in human neutrophils.[29] Furthermore, nitration reactions were not observed when neutrophils phagocytosed either bacteria or beads coated with chemical traps for peroxynitrite.[84]

Within phagosomes, superoxide may react with other radicals. For example, protein-bound tryptophan and tyrosine radicals (P-Tyr) are possible targets for superoxide. These protein radicals are formed when oxidizing radicals, including free tyrosyl radicals, react with proteins.[85,86] The rate constants for reaction of superoxide with tyrosine and tryptophan radicals are high ($k = 1\text{–}2 \times 10^9\ M^{-1}\ s^{-1}$) and the products potentially disruptive.[87–89] Electron transfer from superoxide would repair tryptophan or tyrosine residues (Reaction 8) as well as produce molecular oxygen. Thermodynamically, singlet oxygen, as opposed to ground-state triplet oxygen, could be formed.[77] Although, this route to singlet oxygen has yet to be confirmed by experiment. Repair may be a misleading description for regeneration of tryptophan and tyrosine radicals if these radicals are essential for an enzymatic reaction, as is the case for ribonucleotide reductase.[90]

Alternatively, superoxide can add to these radicals to form hydroperoxides.[88] For tyrosyl radicals, addition (Reaction 9) as opposed to repair is favored by a proximate amine, as is the case with N-terminal tyrosine residues.[18] A mechanism has been invoked whereby hydrogen bonding of superoxide to the amine increases superoxide's oxidizing potential and alters its reactivity.[18,91] Tyrosine hydroperoxides are reactive and form conjugates with thiols.[92] Addition of superoxide is favored for tryptophan radicals and is competitive with oxygen addition.[89] The resulting tryptophan hydroperoxides break down to N-formylkynurenine, kynurenine, alcohols, and diols. These findings suggest that formation of protein hydroperoxides should destabilize or potentially disrupt protein function. So far, hydroperoxide formation in phagosomes has not been explored. Interestingly, similar chemistry is responsible for the chemiluminescent signal produced when neutrophils oxidize luminol.[93,94] A hydroperoxide is also likely to be formed in the superoxide-dependent oxidation of indigo carmine in a reaction that was mistakenly assigned to ozone.[95] Unstable hydroperoxides are formed when superoxide adds to urate and serotonin radicals.[16,96]

$$P - Tyr^{\cdot} + O_2^{\cdot-} \rightarrow P - Tyr + O_2 \quad \text{(Reaction 8)}$$

$$P - Tyr^{\cdot} + O_2^{\cdot-} + H^+ \rightarrow P - Tyr - OOH \quad \text{(Reaction 9)}$$

6 | SUPEROXIDE AS A SUBSTRATE FOR MYELOPEROXIDASE

Superoxide reacts readily with many heme proteins. The classic example is its reduction of cytochrome *c*—the protein that was used to identify superoxide as a biologically important molecule.[43] Bielski and colleagues were quick to follow up this discovery and used pulse radiolysis to investigate reactions of superoxide with horseradish peroxidase.[97] They found that superoxide reacts rapidly with compound I—the enzyme's iron V redox intermediate—with a relatively large rate constant ($2 \times 10^6\ M^{-1}\ s^{-1}$). At this time, it was also shown that superoxide reacts with myeloperoxidase to form oxymyeloperoxidase; the compound III redox intermediate (see Figure 4).[98] Others subsequently showed that formation of compound III by superoxide is a general reaction of peroxidases and catalase.[99,100] There was also good evidence that superoxide reacts with compound I of lactoperoxidase, but no rate data were obtained.[101] We used pulse radiolysis to measure the rates of reaction of superoxide with ferric myeloperoxidase and its compound I redox intermediate (see Figure 4).[102,103] We found that superoxide reacts rapidly with these forms of myeloperoxidase with rate constants of $1\text{–}2 \times 10^6\ M^{-1}\ s^{-1}$. Using steady-state techniques, we also demonstrated that superoxide reduces compound II of myeloperoxidase and estimated its rate constant to be similar to that of the other two reactions.[19] As a consequence of these favorable reactions, we concluded that superoxide is a physiologically relevant substrate for myeloperoxidase. Thus, within neutrophil phagosomes where myeloperoxidase is the major protein, superoxide should be expected

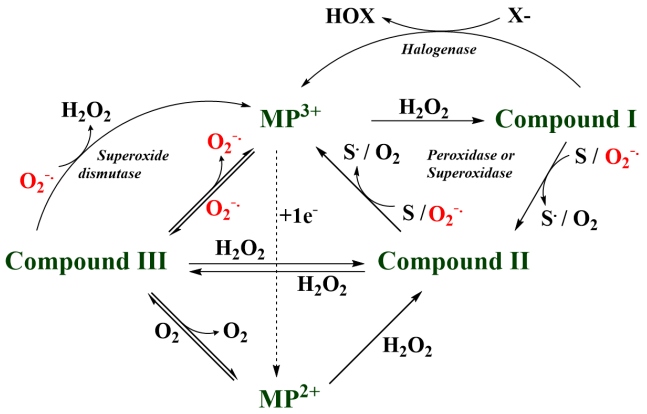

FIGURE 4 Reactions of superoxide with myeloperoxidase. Ferric myeloperoxidase (MP^{3+}) reacts with hydrogen peroxide to form Fe(V) compound I. This redox intermediate has high one and two-electron reduction potentials enabling it to oxidize numerous and varied substrates. It oxidizes halides, thiocyanate, and hydrogen peroxide in two-electron reactions. This is the halogenation cycle for halides (X^-) and thiocyanate, which produces the respective hypohalous acids (HOX). Hydrogen peroxide is oxidized by a catalase activity to molecular oxygen. Chloride and thiocyanate are the major substrates for compound I. Compound I oxidizes numerous substrates (S), including superoxide, by removing a single electron to form compound II, an Fe (IV) redox intermediate, and produce a free radical ($S^{\cdot}$). Compound II also oxidizes these substrates but to a lesser degree. This reaction completes the classic peroxidase cycle. Oxidation of superoxide in this cycle gives myeloperoxidase superoxidase activity. This activity prevents the accumulation of compound II, which would otherwise inhibit the halogenation cycle. Depending on local concentrations, superoxide, serotonin, urate, or ascorbate will be the main substrates for compound II. Compound III or oxymyeloperoxidase ($Fe^{3+}{\cdot}O_2^-$ or $Fe^{2+}{\cdot}O_2$) is formed when superoxide reacts with ferric myeloperoxidase. Compound III reacts with superoxide in a dismutase activity to form the ferric enzyme and hydrogen peroxide. Compound III is also in equilibrium with the ferrous enzyme (MP^{2+}), which can react with hydrogen peroxide to form compound II. Molecular oxygen is formed when superoxide reduces compound I, compound II, and compound III. It could be released as either ground-state or singlet-state oxygen. Compound II and compound III both react with hydrogen peroxide slowly to give the enzyme another, but weaker, catalase activity. We have previously compiled the rate constants for these reactions[164]

to react with this heme enzyme along with its accepted substrates hydrogen peroxide and chloride.

Superoxide's effect on myeloperoxidase's enzyme activity is complex (Figure 4). It inhibits hypochlorous acid production by converting the enzyme to compound III but enhances activity when it prevents the accumulation of compound II. Substrates that react rapidly with compound I but poorly with compound II, such as tryptophan and related indoles, inhibit hypochlorous acid production by trapping the enzyme as compound II.[104–106] Superoxide, however, can relieve this inhibition by reducing compound II to the active ferric enzyme.[13] Superoxide also reacts with compound III of myeloperoxidase to reduce it back to the ferric enzyme.[19] The shuttling of myeloperoxidase between its native and compound III is essentially a dismutase activity that converts superoxide to hydrogen peroxide and oxygen. Compound III has also been implicated in superoxide-dependent hydroxylation reactions of myeloperoxidase, which suggested that the enzyme uses superoxide to produce a reactive species that mimics hydroxyl radical.[107]

TABLE 1 Rate constants for reactions of superoxide and perhydroxyl radical with reactive halogen species[44]

Reaction	k ($M^{-1}\,s^{-1}$)
$HO_2^{\cdot} + Cl_2 \rightarrow Cl^{\cdot} + H^+ + Cl^- + O_2$	1×10^9
$O_2^{\cdot-} + HOCl \rightarrow HO^{\cdot} + Cl^- + O_2$	7.5×10^6
$HO_2^{\cdot} + Br_2 \rightarrow Br^{\cdot} + H^+ + Br^- + O_2$	1.1×10^8
$O_2^{\cdot-} + Br_2 \rightarrow Br_2^- + O_2$	5.6×10^9
$O_2^{\cdot-} + HOBr \rightarrow Br^{\cdot} + HO^- + O_2$	9.5×10^8
$O_2^{\cdot-} + I_2 \rightarrow I_2^- + O_2$	5.5×10^9
$O_2^{\cdot-} + I_3^- \rightarrow I_2^- + I^- + O_2$	8.8×10^8

When superoxide transfers a single electron to the redox intermediates of myeloperoxidase, it will be oxidized to molecular oxygen. For some of these reactions, it is possible that singlet oxygen, rather than ground-state triplet oxygen, is formed (see Figure 3). The redox intermediates of myeloperoxidase would have to have a one-electron reduction potentials greater than +0.65 V. This value is the one-electron reduction potential for the conversion of singlet oxygen to superoxide.[77] Both compound I ($E^{0'}$ = +1.36 V) and compound II ($E^{0'}$ = +.096 V) meet this requirement.[108] Thus, formation of singlet oxygen by the rapid reduction of compound I and compound II by superoxide is thermodynamically plausible. Although there have been many reports of singlet oxygen formation by activated neutrophils,[109] to date the evidence that singlet oxygen is formed inside phagosomes is equivocal.[30] Over 30 years ago, it was reported that about 20% of the oxygen consumed by activated neutrophils was detected as singlet oxygen.[110] Intriguing as it was, this finding has yet to be substantiated. Increased reactivity and specificity of chemical probes used to capture singlet oxygen would help to clarify the involvement of singlet oxygen in the oxidative killing mechanisms of neutrophils.[111] We are currently investigating whether singlet oxygen, or a redox intermediate that has a similar chemical footprint, is formed when superoxide reacts with myeloperoxidase.

In addition to its fast reactions with other radicals, superoxide also reacts at close to diffusion-controlled rates with reactive halogen species (see Table 1).[44] These reactions include reduction of molecular chlorine and bromine as well as hypobromous acid. Superoxide is also likely to react rapidly with hypoiodous acid. Its reaction with hypochlorous acid is fast but about 100-fold slower than with most of these other species.[112] There are no data available for reduction of hypothiocyanous acid by superoxide. In most instances, thermodynamics dictates that the products are halogen radicals, ground-state molecular oxygen, and water.[113] The exception to this is when superoxide reduces hypochlorous acid to give the hydroxyl radical rather than chloride.[114] Extracellular hydroxyl radical formation by neutrophils has been attributed to this reaction.[57] Reactions

of the hypohalite anions with superoxide are less likely due to repulsion by the negative charges on both species.

Superoxide may reduce some reactive halogens inside phagosomes (Table 1). Hypochlorous acid is produced inside neutrophil phagosomes. It could react with superoxide but this reaction would have to compete with more kinetically favored reactions of hypochlorous acid.[84,115–118] Molecular chlorine, which is produced by myeloperoxidase at acidic pH, would be expected to react with superoxide.[119] However, the pH inside phagosomes (see below) overwhelming favors hypochlorite and hypochlorous acid over molecular chlorine in the chemical equilibria between these species (Reaction 10). Bromide, iodide, and thiocyanate are likely to be present at low micromolar concentrations in phagosomes. Consequently, reactions with the HOBr, HOI, and HOSCN would be inconsequential except if the corresponding halides are recycled in catalytic reactions. Higher concentrations of thiocyanate may be present in phagosomes when neutrophils are active in the mouth and respiratory tract, where thiocyanate can reach millimolar concentrations.[120]

$$Cl_2 + H_2O \underset{pKa=3.4}{\rightleftharpoons} HOCl + Cl^- + H^+ \underset{pKa=7.4}{\rightleftharpoons} O-Cl + 2H^+ + Cl^- \quad \text{(Reaction 10)}$$

If the perhydroxyl radical penetrates the outer membranes of bacteria, then along with superoxide it would be damaging. Initial targets within membranes would include unsaturated fatty acids and tocopherols.[44] Within aqueous environments devoid of superoxide dismutase, superoxide would oxidize ascorbate, in a relatively fast reaction ($k = 3 \times 10^5$ M^{-1} s^{-1}).[121] Superoxide rapidly reduces both quinones and hydroquinones, which may disrupt electron transport pathways and energy production.[44] Iron-sulfur proteins, such as aconitase, are vulnerable targets and are crippled by superoxide.[7,122] Enzyme inactivation results from a sequence of increasingly deleterious radical reactions. Initially, superoxide rapidly oxidizes ferrous iron and forms hydrogen peroxide ($k \approx 1 \times 10^7$ M^{-1} s^{-1}), which in turn reacts with more ferrous iron to produce hydroxyl radical—the most reactive of all biological radicals.[123] The hydroxyl radical reacts in its immediate vicinity to irreversibly oxidize the protein. This sequence is the best example of superoxide-driven Fenton reaction in biology and accounts for oxidative stress in cells lacking superoxide dismutase.[12]

From this overview of superoxide's reactivity, it is apparent that superoxide should not be considered simply as a benign source of hydrogen peroxide within phagosomes. It will react rapidly with myeloperoxidase, other radicals, and hypohalous acids. More attention therefore, should be focused on the products of these reactions and whether they are formed during phagocytosis. It is a moot point as to how superoxide itself reacts with and kills bacteria trapped inside phagosomes because, as outlined below, the main target for superoxide will be myeloperoxidase, not the bacteria. Obviously, this premise does not hold for myeloperoxidase-deficient neutrophils. However, in normal neutrophil phagosomes, reactions of superoxide with myeloperoxidase, or its products, are key to understanding superoxide's toxicity (Figure 5A).

7 | SUPEROXIDE PRODUCTION IN PHAGOSOMES

Oxidant production within phagosomes starts with the activation of the NADPH oxidase.[50,124] Activation is tightly controlled by a multistep, but almost synchronous, series of events. Firstly, neutrophils wrap bacteria into individual phagosomes (Figure 5A). A single neutrophil can seal multiple phagosomes simultaneously. During this dynamic rearrangement of the cytoskeleton and cell membrane, there is precise orchestration of other organelles and protein complexes within the neutrophil. In addition, different subgroups of granules and vesicles are mobilized and fused with the phagosome to deliver proteases, cationic peptides, myeloperoxidase, plus many other peptides and proteins. Glucose is fed into the pentose phosphate pathway to ramp up the production of NADPH. At the same time, the multicomponent NADPH oxidase complex assembles at the phagosomal membrane. This is an intricate process in itself, involving modification of the individual components by a series of phosphorylation, lipid binding, conformational changes, and translocation events.[5,125,126] In brief, the NADPH oxidase becomes a functional flavocytochrome for transmembrane electron transfer when the subunits $gp91^{phox}$ and $p22^{phox}$ are correctly bound by FAD, NADPH plus all the cytosolic subunits $p47^{phox}$, $p40^{phox}$, $p67^{phox}$, and rac2. Now the neutrophil starts to rapidly consume oxygen, as the NADPH oxidase uses NADPH in the cytosol to reduce oxygen to superoxide in phagosomes (Reaction 11 and Figure 5A).

$$2O_2 + NADPH \overset{NOX2}{\rightarrow} 2O_2^{\cdot-} + NADP^+ + H^+ \quad \text{(Reaction 11)}$$

One notable feature of superoxide generation in neutrophil phagosomes is that the pH rises to approximately 8.[76,127,128] The pH rises because the NADPH oxidase is electrogenic.[129] As electrons are transferred from the cytosol to the phagosome, the build-up of negative charge is partially maintained by the consumption of protons when superoxide radical anions dismutate (Reaction 7). This charge translocation is compensated by voltage-gated proton channels to prevent self-inhibition of the NADPH oxidase by extreme membrane depolarization. The pH eventually declines to acidic values but not until after bacteria are killed. The early elevated pH has important consequences and should always be considered when assessing oxidative chemistry inside this compartment. For example, alkaline pH slows the dismutation of superoxide by lowering the concentration of the perhydroxyl radical (see above). The reaction of chloride with compound I of myeloperoxidase is also slower at higher pH.[108] Phagosomal pH will also affect the equilibria between hypohalous acids and their conjugate bases. There will be a roughly equal distribution of hypochlorous acid and hypochlorite (pKa = 7.4) in phagosomes.[130] However, the hypothiocyanite anion (pKa = 4.9) will be present in a vast excess over hypothiocyanous acid and consequently limit the bactericidal and bacteriostatic activity of this thiol-specific oxidant.[131]

Once formed in the phagosome, superoxide must react in this compartment because its negative charge restricts it from crossing

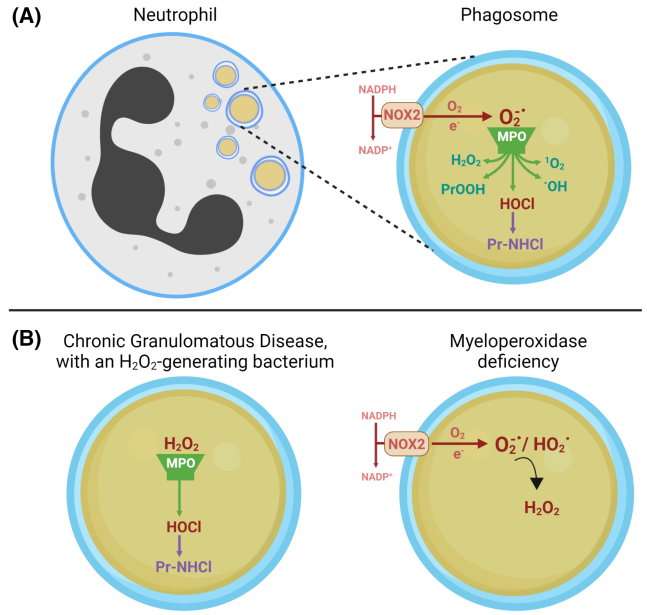

FIGURE 5 Reactions of superoxide inside neutrophil phagosomes produce a variety of oxidants. (A) Neutrophils ingest numerous bacteria into phagosomal compartments into which they release anti-microbial proteins, myeloperoxidase, and superoxide. In normal phagosomes, superoxide is generated when NOX2 transfers electrons from cytosolic NADPH to molecular oxygen in the phagosome. Superoxide reacts preferentially with myeloperoxidase (MPO) to form hydrogen peroxide, which is then converted to hypochlorous acid (HOCl). Some hypochlorous acid reacts with the bacterium (yellow), which may be enough to kill it. Most of the hypochlorous acid reacts with neutrophil proteins to oxidize them and form chloramines (Pr-NHCl). Superoxide may react with myeloperoxidase to form singlet oxygen ($^1O_2$), with hypochlorous acid to form hydroxyl radical ($^{\cdot}OH$), or with protein radicals to form hydroperoxides (PrOOH). The involvement of other halides is at this stage speculative as is a function for protein chloramines. (B) In chronic granulomatous disease (left), oxidants are formed only if bacteria produce hydrogen peroxide. Hypochlorous acid will be produced but is limited by the eventual conversion of myeloperoxidase to compound II. Apart from some protein chloramines, no additional oxidants would be formed. In the absence of myeloperoxidase (right), superoxide would dismutate spontaneously to hydrogen peroxide. High levels of superoxide would accumulate and may allow some perhydroxyl radical to enter bacteria to promote oxidation reactions. This figure was drawn using Biorender

the phagosomal membrane or entering bacteria. Determining the major reactions of superoxide in phagosomes and what other oxidants it spawns is an exacting challenge, principally because the phagosome occupies a tiny space between the phagosomal membrane and the bacterium (Figure 1). We have estimated this space to have a diameter of 0.25 μm and a volume of 1.5×10^{-15} L.[132] Delineating the chemistry of superoxide in the confines of this microscopically small space has obvious technical hurdles. Segal's group dismissed superoxide's oxidative chemistry within phagosomes as incidental to its function. They posited that superoxide generation has an electrogenic pull, drawing potassium ions into the phagosome to increase ionic strength and solubilize anti-microbial granule proteins.[133] However, this mechanism has been challenged in several convincing publications[129,134,135] and it is clear from numerous publications that the respiratory burst is associated with the generation of oxidants inside the phagosome.[29,31,32]

8 | KINETIC MODELLING OF SUPEROXIDE'S REACTIONS INSIDE PHAGOSOMES

Given the difficulty of interrogating the chemistry that occurs in phagosomes, we developed a kinetic model to evaluate all the likely reactions of superoxide inside this compartment.[132] Our goal was to assess which reactions of superoxide are reasonably expected to occur during phagocytosis, identify the information required to test the model's predictions, and establish what additional data must be gathered to strengthen the model. The model included the spontaneous dismutation of superoxide, all superoxide's reactions with the redox intermediates of myeloperoxidase, and their reactions with other substrates that may be taken up into phagosomes (Figure 4). Reactions of hypochlorous acid with susceptible amino acids on proteins contained in phagosome were also considered as was diffusion of hydrogen peroxide and superoxide. Rate constants for the reactions were obtained either from the literature or our own experimental work. Based on experimental estimates, we assumed the phagosomal concentration of chloride was constant at 100 mM.[136] Two remarkable estimates emerged from constructing the kinetic model. Inside the phagosome, the flux of superoxide will be about 2.5 mM per second and the concentration of myeloperoxidase could be as high as 1 mM. These extraordinary values underscore the impracticality of attempting to recapitulate phagosomal chemistry in experimental systems. The first is physically impossible and the second financially prohibitive.

The model predicts that virtually all the superoxide reacts with ferric myeloperoxidase to form compound III (Figure 4). Spontaneous dismutation of superoxide (Reaction 7) is minimal. This finding is counter to the conventional assumption that hydrogen peroxide inside phagosomes is derived from the uncatalyzed dismutation of superoxide. Instead, superoxide also reacts with compound III in the enzyme's superoxide dismutase-like activity to produce hydrogen peroxide and recycle ferric myeloperoxidase. During the respiratory burst of individual phagosomes, the majority of myeloperoxidase exists as compound III, with the rest mainly ferric enzyme. Superoxide rises to a steady-state concentration of about 25 μM. By contrast, hydrogen peroxide reaches only 2 μM due to its consumption by myeloperoxidase and diffusion out of the phagosome. This concentration is well below bactericidal concentrations of hydrogen peroxide.[28] Incredibly, hydrogen peroxide is converted to hypochlorous acid at a rate approaching 134 mM per minute. This remarkable flux of chlorine bleach creates an extremely oxidizing environment in the phagosome. Importantly, this flux could be maintained only if chloride is recycled in the

oxidation reactions of hypochlorous acid or replenished by a rapidly acting chloride pump. Otherwise, the chloride concentration would decline and enable superoxide to react with compound I and compound II, making these reactions central to the fate of superoxide (Figure 4). A supply of active myeloperoxidase would also need to be maintained as it is known the enzyme is inactivated within phagosomes.[137]

Another notable prediction is that most of the hypochlorous acid will react with neutrophil granule proteins that are packed into the phagosome. A minority of the hypochlorous acid reaches the bacterium. Initially, hypochlorous acid oxidizes methionine and cysteine residues and recycles chloride. After 30 seconds, these resides are exhausted, and hypochlorous acid then reacts with disulfides and amines. Within the following minute, chloramines accumulate on proteins and the chloride concentration declines. At this stage, hydroxyl radicals are produced as hypochlorous acid reacts with superoxide, since other competitive targets are drained.

Other substrates of myeloperoxidase will be taken up into the phagosome during ingestion of bacteria, or released from granules. These substrates could potentially include urate, ascorbate, thiocyanate, bromide, tyrosine and tryptophan, and nitrite.[15] Iodide may also be present as neutrophils contain type 3 deiodinase that removes iodide from thyroid hormones.[138] This enzyme is essential for optimal neutrophil function.[139] These alternative substrates are unlikely to be present at high enough concentrations, either individually or collectively, to compete with chloride for oxidation by compound I (Figure 4). Consequently, their effects are likely to be transient. Some substrates that are good at reducing compound I, but poor at reducing compound II, may promote the accumulation of compound II. Superoxide, however, would rapidly shuttle compound II back to the active enzyme and maintain hypochlorous acid production.

Substrates or inhibitors released by trapped bacteria may also influence the activity of myeloperoxidase. Once again, their concentrations would have to be competitive with chloride. The small protein secreted into phagosomes by *Staphylococcus aureus*, known as staphylococcal peroxidase inhibitor (SPIN), binds myeloperoxidase with high affinity and retards substrate entry to the enzyme's active sites.[140–142] To be maximally effective in neutrophil phagosomes, SPIN would have to be present at a similar concentration to that of myeloperoxidase. A more detailed discussion of how bacteria affect the phagosomal environment is reviewed elsewhere in this issue.[143]

The fate of protein chloramines is intriguing because they account for a large proportion of hypochlorous acid generated in the phagosome. We have shown that protein dichloramines breakdown to release bactericidal monochloramine gas (NH_2Cl).[144,145] However, protein monochloramines on lysine resides are relatively stable. Protein chloramines could react with superoxide to form protein radicals although this reaction is slow.[146,147] Alternatively, they may react with iodide, if present, to liberate hypoiodous acid (HOI).[148]

In summary and as outlined in Figure 5A, superoxide spawns a battery of oxidants inside myeloperoxidase-replete phagosomes. Our kinetic model predicts that hypochlorous acid is the chief oxidant that is derived from superoxide. Superoxide maintains hypochlorous acid production by recycling any compound II that forms during enzyme turnover. Reactions of superoxide with compound I and compound II will become increasingly relevant as the chloride concentration declines. These reactions may generate singlet oxygen. Once protein targets are exhausted, hypochlorous acid should react with superoxide and give rise to hydroxyl radical. Large amounts of protein chloramines will form, which may liberate ammonia monochloramine, and possibly hypoiodous acid. The latter oxidant would react at diffusion-controlled rates with superoxide to form iodide radicals (Table 1).

The model also gives insight into how oxidants may be formed in neutrophils from patients with chronic granulomatous disease and myeloperoxidase deficiency (Figure 5B). In chronic granulomatous disease, where the NADPH oxidase is defective, oxidants will be generated only when phagocytosed bacteria such as *Streptococcus pneumoniae* produce hydrogen peroxide as part of their normal metabolism.[149] In this case, myeloperoxidase should be expected to generate hypochlorous acid and form chloramines. Whether toxic amounts of hypochlorous acid reach the bacterium will depend on its sensitivity to the oxidant. For myeloperoxidase deficiency, the model predicts that superoxide accumulates to roughly 100 μM. This is an extraordinary concentration, and consequently, even at a pH of 8.0 in phagosomes, it is probable that a low flux of perhydroxyl radicals enters the bacterium. In contrast to superoxide, hydrogen peroxide accumulates to only about 30 μM because of diffusion out of the phagosome. It will still not be bactericidal at this concentration. However, it may combine with carbon dioxide generated in the pentose phosphate pathway to form peroxymonocarbonate ($HOOCO_2^-$). This formerly disregarded oxidant is now gaining increasing attention because it accentuates the reactivity and toxicity of hydrogen peroxide.[150,151]

9 | CURRENT EVIDENCE FOR REACTIONS OF OXIDANTS INSIDE PHAGOSOMES

Evidence that supports reactions between superoxide and myeloperoxidase in phagosomes includes visible absorption spectra, showing that compound III is formed during phagocytosis by human neutrophils.[152] The interaction of superoxide with myeloperoxidase was also confirmed by our previous results that superoxide dismutase attached to bacteria inhibited their myeloperoxidase-dependent killing by neutrophils.[1] In addition, when superoxide dismutase was linked to zymosan particles, it increased the detection of hypochlorous acid in phagosomes.[153] Much higher levels of 3-chlorotyrosine in neutrophil phagosomal proteins compared with ingested bacteria provided strong evidence that most hypochlorous acid reacts with the neutrophil's own proteins.[115,154]

Although the evidence for oxidation of neutrophil proteins is compelling, it is clear from the high levels of methionine sulfoxide formed in bacterial proteins during phagocytosis that oxidants do reach ingested bacteria.[155] Similarly, 66% of bacterial protein

cysteines were found to be significantly oxidized in *E. coli* phagocytosed by a neutrophil-like cell line.[156] Some of this oxidation is due to hypochlorous acid. We and others used heavy isotopes of bacterial molecules to show that hypochlorous acid definitely reacts with ingested bacteria. Initially, we found that bacterial tyrosine residues are chlorinated when neutrophils kill *Staphylococcus aureus*.[115] Later, 3-chlorotyrosine was detected in proteins from phagocytosed *Pseudomonas aeruginosa*.[157] More recently, we used mass spectrometry to monitor oxidation of the bacterial low molecular weight thiols bacillithiol, mycothiol, and glutathione during phagocytosis of *S. aureus*, *Mycobacterium smegmatis*, and *P. aeruginosa*, respectively.[116,118,158] Our results indicated that enough hypochlorous acid reacted with *P. aeruginosa* in the neutrophil phagosome to be responsible for microbial killing, whereas it only partially contributed to killing of *S. aureus*.[116,118,158] Mycothiol was not oxidized in phagosomal *M. smegmatis*, suggesting that these bacteria are shielded from hypochlorous acid. Our investigations demonstrate that depending on the type of bacteria ingested by neutrophils, hypochlorous acid may be solely responsible for killing, work alongside other toxins, or contribute little to eradication of the bacteria.

To gain a clearer picture of which oxidants superoxide spawns in phagosomes, and their precise contribution to killing, we need to understand chloride dynamics within phagosomes as any decline in its concentration will change oxidant production dramatically. The presence of the cystic fibrosis transmembrane conductance regulator (CFTR) in phagosomal membranes suggests that chloride transport into phagosomes may be important during bacterial killing.[117,136] The demonstration that defective CFTR leads to a decline in bacterial chlorination and poorer killing, suggests that chloride availability is important for effective eradication of bacteria.[116,157] One caveat with these CFTR findings, however, is that this protein complex also transports several other small anions, including iodide, bromide, and thiocyanate.[159] Levels of active myeloperoxidase in phagosomes also need to be established, especially since it is inactivated during phagocytosis.[137]

Recent indications of heterogeneity between phagosomes require deeper investigation. Heterogeneity among phagosomes is evident in relation to their composition, signaling pathways, and the timing of downstream events.[160–162] Even within one neutrophil, neighboring phagosomes can take distinctly different courses. For example, a study using live cell imaging of the ingestion of opsonized zymosan showed that NADPH oxidase assembly, and activity, occurred in only half of the phagosomes.[163] This points to the multiplicity in bactericidal mechanisms employed by neutrophils. A mixture of killing mechanisms, often categorized as oxidative vs non-oxidative, has long been recognized.[29] With new single-cell techniques, we can look closer at the heterogeneity of the host-pathogen interaction. We have observed individuality in phagosomes in terms of the timing and amount of hypochlorous acid produced within them.[153] Hypochlorous acid production was monitored in neutrophils by observing intracellular fluorescence produced when the specific molecular probe R19-S reacted with this oxidant. Populations of neutrophils that were fed opsonized zymosan consistently showed similar increases in R19 fluorescence that were dependent on NAPDH oxidase and myeloperoxidase activities and chloride levels. Real-time monitoring of individual neutrophils revealed that although most fluorescent phagosomes were uniform in their temporal development of fluorescence, there were many phagosomes where the onset of fluorescence was much delayed, and around 2% of phagosomes failed to fluoresce within 20 minutes of ingestion. Many factors may contribute to this variation between phagosomes. Most likely it indicates diversity in the activation of different processes within the phagosome. The generation of different oxidants in different phagosomes may allow neutrophils to hedge their bets with respect to how they kill bacteria in individual phagosomes. This strategy may restrict the ability of bacteria to evolve virulence factors against the anti-microbial mechanisms they defend against inside phagosomes.

10 | CONCLUSIONS AND FUTURE RESEARCH

Based on multiple lines of evidence, superoxide is necessary for timely and efficient killing of bacteria ingested by neutrophils. Kinetic data suggest that superoxide drives oxidant production inside phagosomes rather than simply providing a source of hydrogen peroxide. Hypochlorous acid will be the main oxidant derived from superoxide and will trigger the demise of ingested bacteria. It is still not possible, however, to categorically assign a specific superoxide-dependent bactericidal mechanism that encapsulates the essential reaction or reactions of superoxide. There are, however, several plausible candidate mechanisms that should be investigated in depth. These include reactions of superoxide with myeloperoxidase to maintain efficient production of hypochlorous acid, its oxidation to singlet oxygen and other activated forms of oxygen, and its reduction of reactive halogen species to generate hydroxyl radicals and perhaps halide radicals. Our understanding of superoxide's antimicrobial toxicity will be advanced by addressing the following unresolved questions:

1. What is the concentration of chloride inside phagosomes and how does it change during phagocytosis?
2. What is the concentration of myeloperoxidase inside phagosomes and how long does it remain active?
3. What role do protein chloramines play in bacterial killing and is their potential toxicity released by iodide?
4. Can myeloperoxidase generate singlet oxygen or related oxidants when it oxidizes superoxide?
5. How uniform is oxidant production between phagosomes and what causes it to vary?

Given the fleeting nature of the possible reactive species formed inside phagosomes, sensitive mass spectrometry assays will be needed to identify unique chemical footprints of specific oxidants

on both neutrophil and bacterial proteins. These assays should be used alongside fluorescent chemical probes that can specifically visualize oxidants inside individual phagosomes. In every case, superoxide dismutase should be used to confirm that oxidant production is reliant on superoxide. Tests should also be undertaken to establish that bacterial killing is linked at least partially to oxidant production while acknowledging that other killing mechanisms may compensate for oxidants when they are blocked or removed. These studies will ultimately reveal why superoxide is so super.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data in this article will be made freely available (see Figure 2).

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