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Respiratory protein–generated reactive oxygen species as an antimicrobial strategy

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The evolution of the host-pathogen relationship comprises a series of invasive-defensive tactics elicited by both participants. The stereotype is that the antimicrobial immune response requires multistep processes. Little is known about the primordial immunosurveillance system, which probably has components that directly link sensors and effectors. Here we found that the respiratory proteins of both the horseshoe crab and human were directly activated by microbial proteases and were enhanced by pathogen-associated molecular patterns, resulting in the production of more reactive oxygen species. Hemolytic virulent pathogens, which produce proteases as invasive factors, are more susceptible to this killing mechanism. This 'shortcut' antimicrobial strategy represents a fundamental and universal mode of immunosurveillance, which has been in existence since before the split of protostomes and deuterostomes and still persists today.

Throughout evolution, pathogenic microbes have developed many ways to invade the host and simultaneously evade its immune defense. These tactics include the secretion of virulence factors[1,2], the formation of biofilm[3] and the use of molecular mimicry[4]. Conversely, the host immune system has developed various countermeasures such as the release of antimicrobial peptides[5], the synthesis of highly toxic reactive oxygen species (ROS)[6] and the formation of the complement-mediated membrane-attack complex[7]. It is commonly accepted that the innate immune responses are multistep processes initiated by the recognition of pathogen-associated molecular patterns (PAMPs), followed by successive signal transduction leading to the action of 'downstream' antimicrobial effectors[8]. Over the past two to three decades, researchers have focused their attention on families of host receptors and signaling cascades. However, the primordial forms of immunosurveillance, which might directly link the host's pathogen sensors and the antimicrobial effectors, need to be further examined.

ROS-mediated antimicrobial activity is a powerful host defense mechanism[6]. Studies have shown that hemocyanin (HMC) and hemoglobin, the respiratory proteins of invertebrates and vertebrates, respectively, can produce ROS[9,10]. However, in both cases, how ROS production is regulated by the microbial virulence factors during infection and the importance of ROS-mediated antibacterial activity are not fully understood.

The horseshoe crab, an ancient protostome that has been dubbed a 'living fossil'[11], has an array of frontline defense molecules[12]. The HMC of the horseshoe crab has prophenoloxidase (PPO) potential[9,13] (called 'HMC-PPO' here). Its activation to phenoloxidase (PO) results in the production of highly reactive cytotoxic quinone, which is functionally equivalent to ROS produced by vertebrates[14]; quinone kills the microbial intruder effectively. As the toxicity of ROS poses a dilemma to the host's own survival, its production must be tightly controlled. Several host proteases have been shown to catalyze the conversion of HMC-PPO to PO[15–17]. However, whether the microbial proteases can activate HMC-PPO remains unknown.

The vertebrate respiratory protein hemoglobin binds reversibly to oxygen. When hemoglobin is auto-oxidized to the ferric form, methemoglobin (metHb), it gains a pseudoperoxidase activity capable of catalyzing the production of superoxide ion ($O_2^{\bullet-}$)[10,18]. Although superoxide ion itself is not considered cytotoxic[19], it spawns toxic derivatives such as hydroxyl radicals and hypohalous acid[20]. Therefore, processes that facilitate the generation of $O_2^{\bullet-}$ will perpetuate and potentiate oxygen-dependent microbicidal action[20]. In physiological conditions, hemoglobin is sequestered in erythrocytes, where intracellular reductive mechanisms maintain metHb at a low concentration[21], thereby avoiding host self-destruction[18]. However, research has shown that virulent bacteria produce cytolytic toxins to hemolyse erythrocytes and release hemoglobin[22]. For example, *Staphylococcus aureus* uses hemolysin to convert hemoglobin and selectively retrieves the heme as its 'preferred' iron resource for growth[22]. This raises the issue of how the hemoglobin molecule counters such a microbial assault. We reasoned that when the host is challenged into hemolysis, the released hemoglobin molecule elicits antimicrobial activity through ROS production in the vicinity of the intruding microbe. However, whether such bactericidal activity is regulated by microbial virulence factors after infection remains an enigma.

In addition to cytolytic toxins, microbial intruders have also developed other virulence factors such as extracellular proteases to gain entry into and colonize the host[23–25]. Here we found that the

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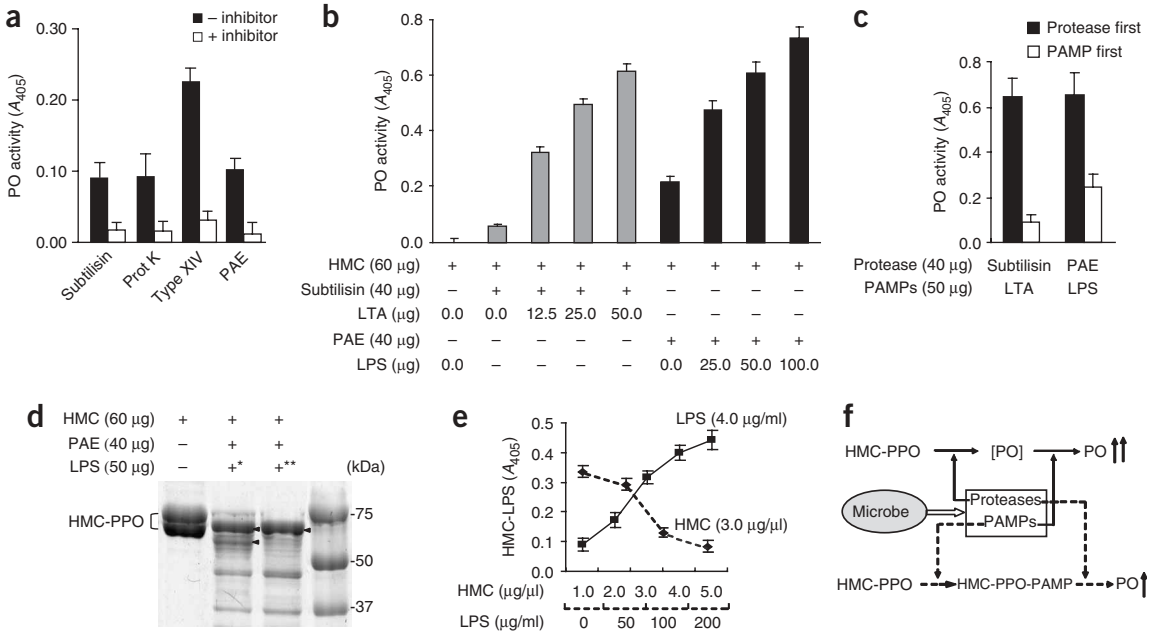

Figure 1 Activation of HMC-PPO to PO by microbial proteases and PAMPs. (**a**) PO activity after treatment with various microbial proteases with (+) and without (–) the protease inhibitors phenylmethyl sulfonyl fluoride (1 mM), to inactivate subtilisin A, proteinase K (Prot K) and type XIV protease (Type XIV), or 1,10-phenanthroline monohydrate (1 mM), to inactivate PAE. A_{405}, absorbance at 405 nm. (**b**) PO activity after the addition of HMC with either subtilisin A or PAE (concentration, below graph) with or without increasing concentrations of the PAMPs LTA and LPS. (**c**) PO activity after the addition of microbial proteases (subtilisin A or PAE) and PAMPs (LTA or LPS) in various sequential order (key). (**d**) SDS-PAGE of HMC-PPO incubated with PAE and LPS applied in sequential order: *, PAE before LPS; **, LPS before PAE. Arrowheads indicate proteolytic intermediates and/or proteolytic products of hemocyanine. Each lane is loaded with 12 μg HMC. kDa, kilodaltons (molecular size markers). (**e**) ELISA of HMC-LPS after incubation of HMC-PPO and LPS (concentrations, below graph). (**f**) Synergistic activation of HMC-PPO by microbial proteases and PAMPs. Microbial proteases first lyse HMC-PPO by proteolysis to a partially activated PO intermediate ([PO]), then PAMPs bind the partially activated intermediate to produce fully active PO (PO↑↑; solid arrows) and/or PAMPs interact with HMC-PPO to form an inactive complex that is subsequently cleaved by microbial proteases to PO with only marginal activity (PO↑; dashed arrows). Data are representative of three experiments (mean ± s.d., **a**,**b**,**c**,**e**).

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PPO activity of invertebrate HMC and the pseudoperoxidase activity of vertebrate metHb were both triggered by microbial proteases and were further enhanced by PAMPs. Thus, we have demonstrated a direct antimicrobial strategy by which the host opportunistically exploits the invading microbe's virulence factors to convert its respiratory proteins into potent ROS producers, which effectively kill the intruder. This mechanism has probably been in existence for over 500 million years, before the split of protostomes and deuterostomes, and is particularly potent against virulent bacteria that produce extracellular proteases.

RESULTS

Microbial activation of HMC-PPO to PO

To test the hypothesis that microbial proteases can trigger the conversion of HMC-PPO to PO independently of host proteases, we treated purified HMC-PPO with several microbial proteases and assessed the resulting PO activity. Subtilisin A and type XIV protease from Gram-positive bacteria, pseudomonas elastase (PAE) from Gram-negative bacteria and proteinase K from fungi efficiently activated HMC-PPO (**Fig. 1a**). When we inhibited subtilisin A, proteinase K and type XIV protease with phenylmethyl sulfonyl fluoride and when we inhibited PAE, a metalloprotease, with 1,10-phenanthroline monohydrate, we noted less than 20% PO activity compared with that of their uninhibited counterparts (**Fig. 1a**), indicating that proteolysis was specific and was conditional on PPO activation. However, factor C, a host serine protease that is an effective lipopolysaccharide (LPS)–recognition receptor, did not activate the PPO (data not shown), suggesting that the PPO activation contributes to a non-self type of immune response.

PAMPs are important in triggering the innate immune response[26,27]. To understand their contribution to PPO activation, we tested LPS from Gram-negative bacteria, lipoteichoic acid (LTA) from Gram-positive bacteria and laminarin from fungi, either individually or in combination with the corresponding microbial proteases. None of the PAMPs alone (at concentrations of 0.125–20 μg/ml) activated HMC-PPO substantially. However, when we pretreated HMC-PPO with PAE before adding LPS, the resulting PO activity was enhanced in a dose-dependent way by LPS (**Fig. 1b**). We obtained similar results with the LTA–subtilisin A combination (**Fig. 1b**) but not with the lamarin–type XIV and lamarin–proteinase K combinations. The enhancement of the microbial protease–induced PO activity by PAMPs suggests that HMC-PPO activation is a sensitive non-self recognition mechanism.

Activation of PPO to PO through proteolysis

To delineate the mechanism of PPO activation by microbial proteases and PAMPs, we reconstituted the reactions with the microbial proteases and PAMPs in various sequential orders. When we first lysed HMC-PPO by proteolysis with microbial proteases followed by the addition of PAMPs, higher PO activity was achieved (**Fig. 1c**). Next, we further examined the proteolytic profile of HMC-PPO by PAE with the addition of LPS before or after PAE. There were distinct differences in the proteolytic profiles of HMC-PPO depending on the order of reaction with LPS or PAE (**Fig. 1d**), indicating that LPS

caused a conformational change that exposed an alternate cleavage site for PAE. By enzyme-linked immunosorbent assay (ELISA), we found that HMC-PPO bound LPS specifically (**Fig. 1e**). These observations collectively suggest collaborative activity of the microbial virulence factors, proteases and PAMPs in triggering HMC-PPO and enhancing the activity of PO.

It has been proposed that in the dormant state, entry to the active site of HMC-PPO is blocked by an α-domain Phe49 and thus phenolic substrates cannot be oxidized[28]. Our data indicated two possible PPO-activation mechanisms (**Fig. 1f**). First, after infection, the microbial protease nicks the HMC-PPO, producing only marginal PO activity; when PAMPs bind to the partially activated PO, it causes a conformational change, resulting in a full PO activity (**Fig. 1f**, solid arrows). Second, the HMC-PPO molecule preoccupied by PAMPs undergoes a conformational change and exposes cleavage sites different from those on the free HMC-PPO, after which microbial protease–mediated proteolysis can achieve only marginal PO activity (**Fig. 1f**, dashed arrows). *In vivo*, these two mechanisms of PPO activation may coexist and may constitute a refined regulation of the host immune response: lower PO activity is used to combat strains that produce less extracellular proteases, whereas higher PO activity is unleashed against the strains that produce more extracellular proteases. Given the cytotoxicity of quinone produced by PO[14], such fine-tuning of PPO activation could be crucial for clearing the invading microbes effectively with different combating powers while avoiding host self-destruction.

Activated PO has antimicrobial activity

To examine the functional importance of PPO activation, we tested the antimicrobial effect of the PO-catalyzed release of quinone *in vitro*. We tested protease-producing and non-protease-producing strains of the Gram-negative bacterium *Pseudomonas aeruginosa*. The *P. aeruginosa* strain PAO-Iglewski produces PAE[29], the main extracellular protease virulence factor[24], whereas PAO-B1A1 is a 'PAE-knockout' mutant[29]. We incubated each strain of *P. aeruginosa*, at a concentration of 1×10^6 colony-forming units (CFU) per ml, for 1 h with purified HMC-PPO and 4-methylcatehol (4ME), a PO substrate. We counted the residual bacterial population by plating the reaction mixture on nutrient agar plate and incubating the plates overnight at 37 °C. We found that 66% ± 5% of the PAE-producing *P. aeruginosa* were killed within 60 min, whereas only 15% ± 7% of the PAE-deficient *P. aeruginosa* succumbed (**Fig. 2a**). Furthermore, supplementation of the incubation mixture containing PAE-deficient *P. aeruginosa* with exogenous PAE increased the antimicrobial activity in a dose-dependent way (**Fig. 2b**), demonstrating the essential function of PAE in achieving the antimicrobial activity. We confirmed the involvement of PO in this antimicrobial action by our finding that adding the PO-specific inhibitor phenylthiourea (PTU)[30] significantly retarded the killing of PAE-producing strain but did not affect the PAE-deficient strain (**Fig. 2a**). It is known that HMC-PPO contains precursors of antimicrobial peptides[31]; however, in our study, the contribution of the HMC-related antimicrobial peptide seemed to be minor, as HMC-PPO alone did not demonstrate increased antimicrobial activity (**Fig. 2a**). Thus, the PO-induced production of quinone must have been the main contributor of bactericidal activity. In addition to measuring the end-point antimicrobial activity, we visualized real-time bacterial clearance by time-lapse microscopy. We labeled *P. aeruginosa* with green fluorescent protein, incubated the bacteria with purified HMC-PPO and 4ME and examined them for 1 h by fluorescence microscopy. Again we found that more of the PAE-producing strain than the PAE-deficient counterpart succumbed (**Fig. 2c,d** and **Supplementary Movies 1–3** online). These results demonstrate that the HMC-PPO was specifically activated by the microbial protease to PO, which, in the presence of 4ME, produced quinone to kill the bacteria effectively. We obtained similar results with the Gram-positive *S. aureus* laboratory strains PC1839 and AK3 (**Supplementary Fig. 1** online), which produce and do not produce, respectively, active extracellular V8 protease[32].

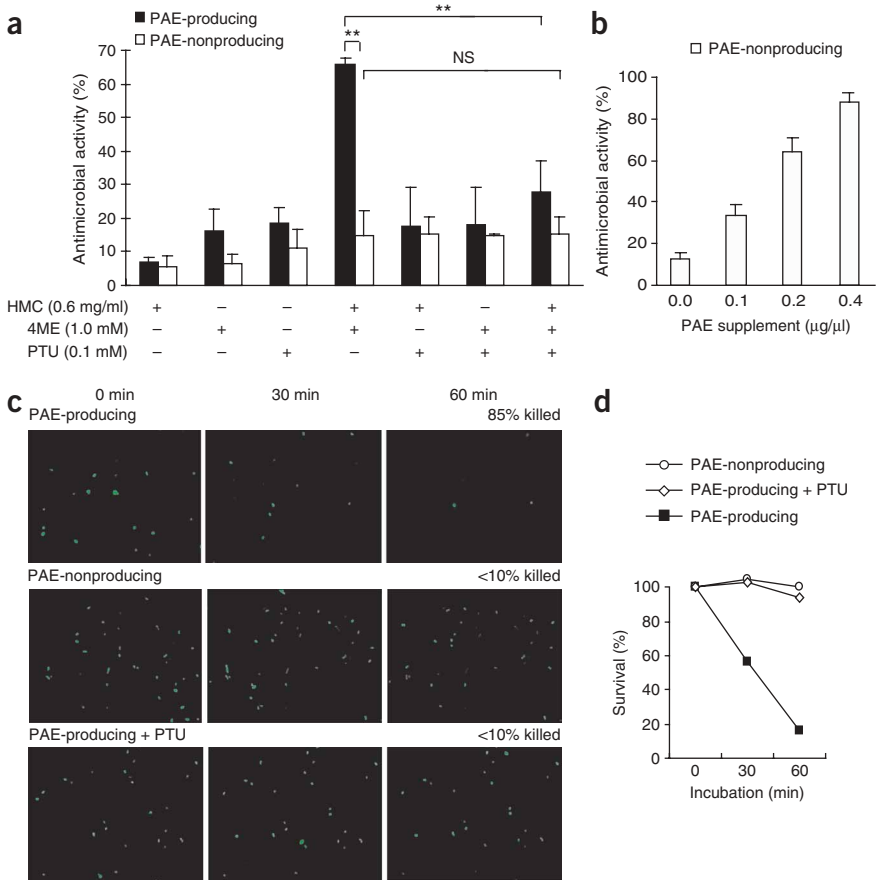

Figure 2 *In vitro* antimicrobial action of activated PO. (**a**,**b**) End-point antimicrobial activity of PAE-producing and PAE-deficient strains of *Pseudomonas aeruginosa*. (**a**) Incubation of each strain (1×10^6 CFU/ml) for 1 h with purified HMC-PPO and 4ME; PTU (0.1 mM), a PO-specific inhibitor, was used to inhibit PO. NS, not significant; **, $P < 0.01$ (Student's *t*-test). Data represent the mean ± s.e.m. of three independent experiments, each done in triplicate. (**b**) PAE-deficient strain supplemented with exogenous PAE. Data represent the mean ± s.d. of three independent experiments. (**c**) Fluorescence microscopy of the samples in **a** (time, above images). Original magnification, 63 × 1.6. (**d**) Survival of bacteria from **c**.

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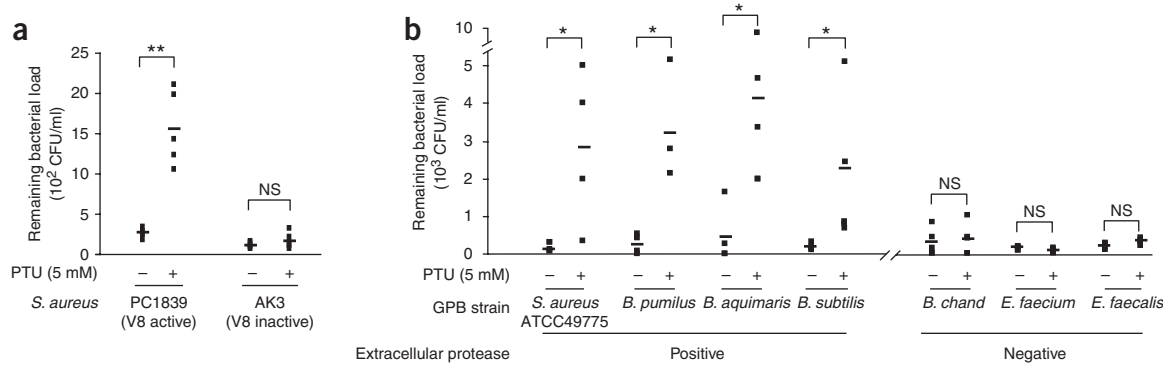

Figure 3 PO triggered by microbial protease contributes to *in vivo* bacterial clearance. Killing assay of bacteria (1×10^5 to 1×10^6 CFU bacteria per 100 g horseshoe crab) in the presence (+) or absence (–) of 5 mM PTU, assessed by measurement of the remaining bacterial load in the hemolymph at 30 min after injection. (**a**) Laboratory *S. aureus* strains PC1839 (V8 protease positive) and AK3 (V8 protease negative). (**b**) Naturally occurring *S. aureus* strain ATCC49775 (active V8 protease positive), Gram-positive bacteria *B. pumilus*, *B. aquimaris* and *B. subtilis* (extracellular protease positive), and *B. chandigarhensis* (*B. chand*), *E. faecium* and *E. faecalis* (extracellular protease negative). *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test). Small horizontal lines indicate median CFU/ml recovered. Data represent five independent experiments, unless otherwise stated (**Supplementary Text** online).

In vivo antimicrobial PO is triggered by microbial protease

To demonstrate the ability of PO to clear the invading pathogen *in vivo*, we infected horseshoe crabs in the presence or absence of the PO-specific inhibitors PTU and kojic acid[33]. We compared the remaining bacterial load in those conditions to help clarify the specific contribution of PO to the antimicrobial activity. It has been reported that HMC-PPO is activated by host intracellular factors[15,16] released through LPS-dependent degranulation of hemocytes[34]. To avoid the elicitation of PPO by such cellular components and to unequivocally demonstrate that the microbial factor–activated PPO contributes to the antimicrobial defense, we used Gram-positive bacteria lacking LPS to avoid LPS-induced lysis of hemocytes. We injected the *S. aureus* strains PC1839 and AK3 into the horseshoe crabs. Indeed, at 30 min after injection, the host had cleared over 90% of both strains (**Fig. 3a** and **Supplementary Fig. 2** online). However, injection of PTU or kojic acid together with the bacteria impaired the clearance of the V8 protease–positive strain PC1839, resulting in the recovery of significantly more bacteria from the host ($n = 5$; $P < 0.01$ (Student's *t*-test)). In contrast, clearance of the V8 protease–negative strain AK3 was unaffected by PTU or kojic acid, indicating that the killing of this strain was independent of PO-mediated quinone activity. This also indicated that the killing was possibly mediated by other coexisting extracellular antimicrobial mechanisms, such as the lectin-mediated complement system[26,35], which is not susceptible to inhibition by PTU or kojic acid. Notably, we found that the V8 protease–positive strain PC1839 seemed to be resistant to the coexisting extracellular PO-independent antimicrobial pathways, although the exact mechanism remains to be determined. Furthermore, we obtained similar results with *S. aureus* ATCC49775 as well as six other naturally occurring Gram-positive bacteria species isolated from the habitat of the horseshoe crab (**Fig. 3b**). We obtained further evidence that activation of PO by the Gram-positive bacteria was independent of intracellular components by confirming that collateral hemocyte degranulation and cell lysis were absent (**Supplementary Fig. 3** online). These observations unambiguously support our hypothesis that the PO activity triggered by the invading microbes' proteases contributes to *in vivo* extracellular clearance of the protease-producing bacteria. In the *in vitro* antimicrobial activity assay, we used 4ME as a synthetic substrate for PO on which quinone was produced to kill the microbe. At this juncture, although the physiological substrate for PO remains to be identified[17], the *in vivo* antimicrobial action demonstrates that such a substrate does exist and that the PO activated by microbial proteases forms a powerful defense mechanism.

Synergistic activity of microbial proteases and PAMPs

The functional homology between HMC and hemoglobin prompted us to search for a similar direct antimicrobial mechanism in humans. Given the ROS production by metHb[10,18], we hypothesized that whereas the bacteria seem to usurp hemoglobin through erythrocyte lysis, the hemoglobin molecule elicits antimicrobial action in the vicinity of the microbes by concerted release of ROS. To test that possibility, we monitored the release of ROS by metHb with the chemiluminescence indicator *Cypridina luciferin* analog (CLA)[10] (**Fig. 4a**). The chemiluminescence of CLA was substantially inhibited by superoxide dismutase, indicating that the ROS was $O_2^{\bullet-}$ (**Supplementary Fig. 4** online). Microbial proteases such as proteinase K, type XIV protease, PAE and subtilisin A specifically increased the release of $O_2^{\bullet-}$ by metHb (**Fig. 4b**). Moreover, when subtilisin A was inhibited by recombinant CrSPI-D2 (a 30–amino acid peptidyl serine protease inhibitor specific to subtilisin A; S. Thangamani *et al.*, unpublished data), we noted much less $O_2^{\bullet-}$ production, indicating that the proteolysis of hemoglobin by microbial proteases was specific and was essential for the activation of metHb (**Fig. 4b**). Furthermore, LPS and LTA increased the $O_2^{\bullet-}$ production in a dose-dependent way, whereas the phosphatidyl lipids POPC and POPE, which are common to both the host and the microbe[36], lacked such activity (**Fig. 4c**), indicating that the action of PAMPs on metHb is specific. Notably, consistent with the results obtained with HMC-PPO, when pretreated with PAE or subtilisin A followed by LPS or LTA, respectively, metHb produced tenfold more superoxide ions (**Fig. 4d**). LPS and LTA bound hemoglobin specifically (**Fig. 4e,f**), and such interaction caused a conformational change to metHb (**Supplementary Fig. 5** online), which probably contributed to the increased pseudoperoxidase activity. The consequence of the synergism between these bacterial components on the $O_2^{\bullet-}$ production suggests that hemoglobin functions in non-self recognition by producing deadly ROS in the vicinity of the bacteria. The specific interactions between hemoglobin and PAMPs suggest a mechanism underlying how metHb is recruited into the vicinity of the bacteria, culminating in invader-oriented and localized ROS production.

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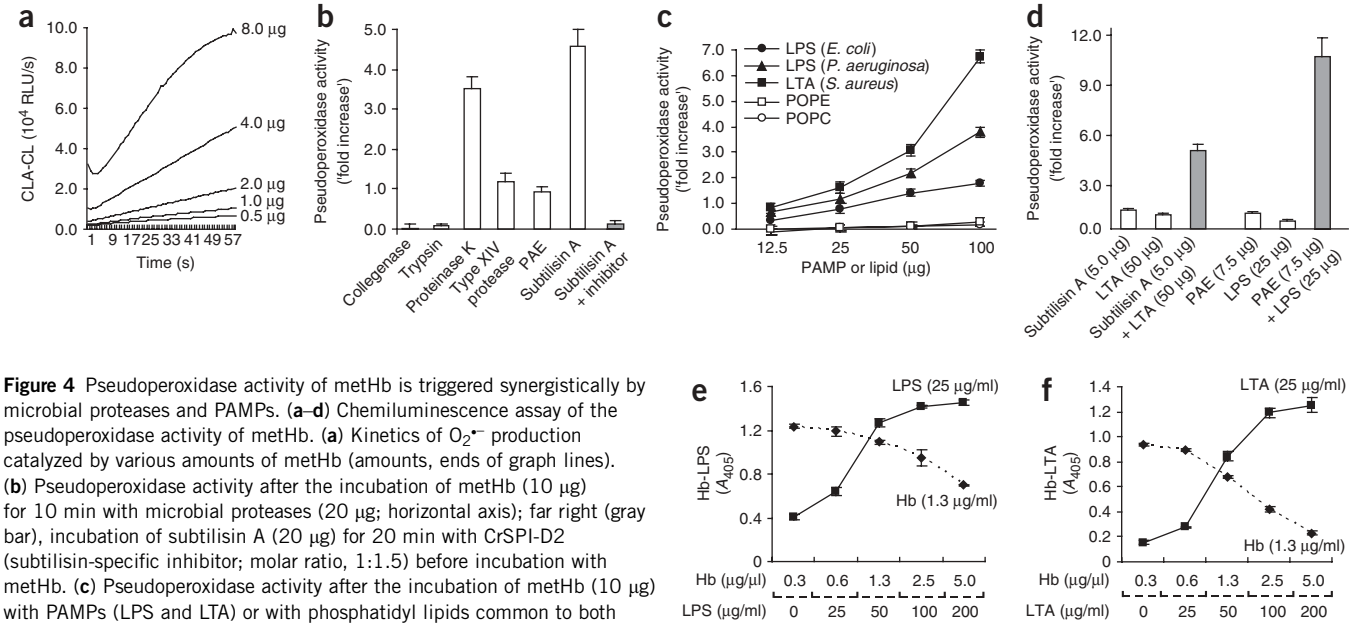

Figure 4 Pseudoperoxidase activity of metHb is triggered synergistically by microbial proteases and PAMPs. (**a**–**d**) Chemiluminescence assay of the pseudoperoxidase activity of metHb. (**a**) Kinetics of $O_2^{\bullet-}$ production catalyzed by various amounts of metHb (amounts, ends of graph lines). (**b**) Pseudoperoxidase activity after the incubation of metHb (10 μg) for 10 min with microbial proteases (20 μg; horizontal axis); far right (gray bar), incubation of subtilisin A (20 μg) for 20 min with CrSPI-D2 (subtilisin-specific inhibitor; molar ratio, 1:1.5) before incubation with metHb. (**c**) Pseudoperoxidase activity after the incubation of metHb (10 μg) with PAMPs (LPS and LTA) or with phosphatidyl lipids common to both host and microbe (POPE and POPC). (**d**) Pseudoperoxidase activity after the incubation of metHb (10 μg) with microbial proteases and/or PAMPs. In **b**–**d**, an aliquot containing 2 μg metHb was used in each assay; 5 μg subtilisin A was used in **d** (in contrast to the 20 μg used in **b**) to demonstrate the further enhancement by LTA due to the synergism between subtilisin A and LTA. (**e**,**f**) ELISA of mixtures of hemoglobin (Hb) and LPS (**e**) or hemoglobin and LTA (**f**). Data represent the mean ± s.d. of three independent experiments.

ROS produced by metHb kills microbes effectively

To investigate the functional importance of the activated metHb, we tested the antimicrobial activity against *S. aureus* PC1839 and AK3 *in vitro* with a biochemically reconstituted system. We incubated each strain with human metHb and a sublethal concentration of H_2O_2 (30 mM). We used the superoxide ion scavenger GSH[37] at a concentration of 10 mM to eliminate superoxide ions, if any were present. More of the V8-producing *S. aureus* than the non-V8-producing *S. aureus* was killed within 60 min (**Supplementary Fig. 6** online), whereas supplementation of the incubation mixture of the V8-negative strain with exogenous subtilisin A increased the antimicrobial activity in a dose-dependent way (**Supplementary Fig. 6b**). Finally, the addition of GSH significantly retarded the killing of V8-positive but not V8-negative *S. aureus* ($P < 0.05$ (Student's *t*-test); **Supplementary Fig. 6a**). These results indicate that the $O_2^{\bullet-}$ production by metHb is enhanced by the microbial protease, thus effectively subverting the pathogen.

Mammalian red blood cells produce bactericidal ROS

In addition to biochemically simulating the bacterial protease–dependent ROS production with metHb, we also examined the function of this mechanism with rabbit and human red blood cells (RBCs). We incubated the bacteria with RBCs and continuously monitored hemolysis, ROS production and the remaining bacterial populations over a period of 30 min. Both rabbit and human RBCs showed similar responses (**Fig. 5**, typical profiles of rabbit RBCs). There was hemolysis within 5 min of the addition of *S. aureus* strains PC1839 and AK3 (**Fig. 5a**). Simultaneously, we noted pseudoperoxidase activity in the chemiluminescence assay (**Fig. 5b**). Within 10 min, over 80% of the protease-producing *S. aureus* was killed, whereas the protease-negative strain remained viable (**Fig. 5c**). The addition of either superoxide dismutase or protease inhibitor (which have been shown to not substantially affect bacterial growth on their own) to the incubation mixture significantly reduced the antibacterial activity against the protease-producing strains ($P < 0.01$, Student's *t*-test), confirming the involvement of superoxide species in the antimicrobial action and the requirement for the bacterial protease in the ROS production.

We obtained similar results when we tested various naturally occurring clinically important bacterial isolates (**Fig. 5d**). *Bacillus subtilis* and *S. aureus* strain ATCC49775, which are hemolytic and positive for extracellular protease, were killed by metHb-mediated ROS production, whereas methicillin-resistant *S. aureus* strains MRSA1 and MRSA2, as well as *Enterococcus faecium* and *Enterococcus fecalis,* which are either nonhemolytic or do not produce protease, were unaffected. Notably, two clinical isolates of *P. aeruginosa*, one producing protease and one not producing protease, were unaffected in this *in vitro* assay. In the conditions of this assay, neither of these *Pseudomonas* strains caused hemolysis, although they appeared hemolytic on blood agar (data not shown), thus supporting the idea that initial hemolysis of the RBC is crucial to the ROS production and subsequent killing event. In conclusion, these observations suggest that in the mammalian system, the hemoglobin released by bacteria-mediated hemolysis of RBCs is activated by the microbial protease to produce ROS, which kills the bacteria effectively (**Supplementary Fig. 7** online).

DISCUSSION

ROS production is a major host defense mechanism. However, to avoid self-destruction due to general cytotoxicity, ROS production must be well regulated in the host[17]. Here we have shown that ROS production by horseshoe crab HMC-PPO and human hemoglobin was effectively enhanced by microbial proteases but not by the host proteases (such as horseshoe crab factor C or the mammalian digestive protease-like bovine trypsin). Furthermore, the PAMPs from microbial cell wall but not phosphatidyl lipids such as POPC or POPE, which are common to both the hosts and microbes[36], enhanced the production of ROS. The selectivity and/or specificity of the activating

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factors ensure(s) that ROS are only produced in the vicinity of the invading microbes, in particular against the virulent strains that generate extracellular proteases. Thus, the host avoids self-destruction.

For invertebrates, work on the horseshoe crab has shown that this ancient species has several parallel immunosurveillance mechanisms in its hymolymph[9], including an opsonic complement system[26], which is mediated by a repertoire of plasma lectins[35]. Gram-negative bacteria are effectively eliminated through LPS-induced hemocyte degranulation[9,34], whereas Gram-positive bacteria, which lack LPS, are cleared mainly by extracellular antimicrobial mechanisms. Here we have demonstrated a previously unknown activation mechanism of horseshoe crab HMC-PPO in the extracellular milieu, different from the mechanisms reported before[15,16]. Instead of intracellular serine proteases and/or antimicrobial peptides (which are accessible only after LPS-dependent lysis of the hemocytes and degranulation) to activate the extracellular PPO, we have provided evidence here of a direct and prompt frontline antimicrobial action that immediately responds to the microbial invader as it gains entry to the host. Furthermore, the respiratory protein is triggered by proteases and PAMPs from both the Gram-positive and Gram-negative bacteria, orchestrating equally effective clearance of both types of bacterial invader. Analysis of the *in vivo* physiological importance of this mechanism suggested that PO-mediated quinone production is necessary and sufficient for killing protease-producing bacteria but is not necessarily the only defense against protease-negative bacteria. Other coexisting PO-independent antimicrobial pathways should also be considered, although in this study we found that protease-positive bacteria seemed to be resistant to the PO-independent antimicrobial pathways. It has been noted before that as virulence factors, microbial proteases enable the bacteria to invade and break through the host tissues and suppress the host innate immune mechanism. For example, LasA (staphylolysin or LasA protease) and LasB (elastase), which are both extracellular zinc metalloendopeptidases from *P. aeruginosa*, act synergistically to degrade elastin and a variety of host proteins, including components of the immune system, such as complement factors, immunoglobulins and antimicrobial peptides[23,24]. It is conceivable that the V8 protease from *S. aureus* has similar functions in the PO-independent pathways, although the exact mechanism for this resistance remains to be determined. The observation that the host exploits different defense mechanisms against different species of pathogens recapitulates the idea that the hosts have astutely coevolved mechanisms against the microbes.

In vertebrates, the relative importance of adaptive versus innate immunity depends on the type of infectious agent. However, reports on the catalytic activity of antibodies[20] have apparently shown an inherent link between the innate and adaptive immune mechanisms as well as the importance of innate immunity to the adaptive background. Here we have demonstrated that hemoglobin from both rabbit and human RBCs, when released by hemolytic bacteria, was triggered synergistically by PAMPs and microbial proteases to produce antimicrobial ROS, forming a strong innate immune response. At this juncture, whether this defense mechanism operates in genetic disorders, such as chronic granulomatous disease, remains uncertain. Patients with chronic granulomatous disease, whose phagocytes are defective in producing ROS because of the lack of the NADPH oxidase, are prone to infection by peroxidase-positive bacteria (such as staphylococcus, salmonella and pseudomonas) and fungi (such as aspergillus)[38]. Notably, the infection of chronic granulomatous disease is limited to the organs and septicemia is rare, suggesting that the pathogens may be effectively cleared by the systemic route, possibly through metHb-mediated ROS production. Nevertheless, further investigation on the contribution of this defense mechanism *in vivo* may open new avenues into the development of antibacterial strategies.

In conclusion, we have shown here that host respiratory proteins can be activated to produce ROS, thus forming a strong antimicrobial defense. Such a mechanism, found in both invertebrates and vertebrates, circumvents the need for a chain of events, including PAMP recognition and signal transduction, before antimicrobial action. Instead, the host directly exploits the invading microbe's virulence factors to convert its own respiratory proteins from oxygen carriers to potent ROS producers, thus resulting in a localized surge in cytotoxic free radicals (quinone in invertebrates and superoxide in vertebrates), which rapidly kill the pathogen in their immediate vicinity while sparing the host from

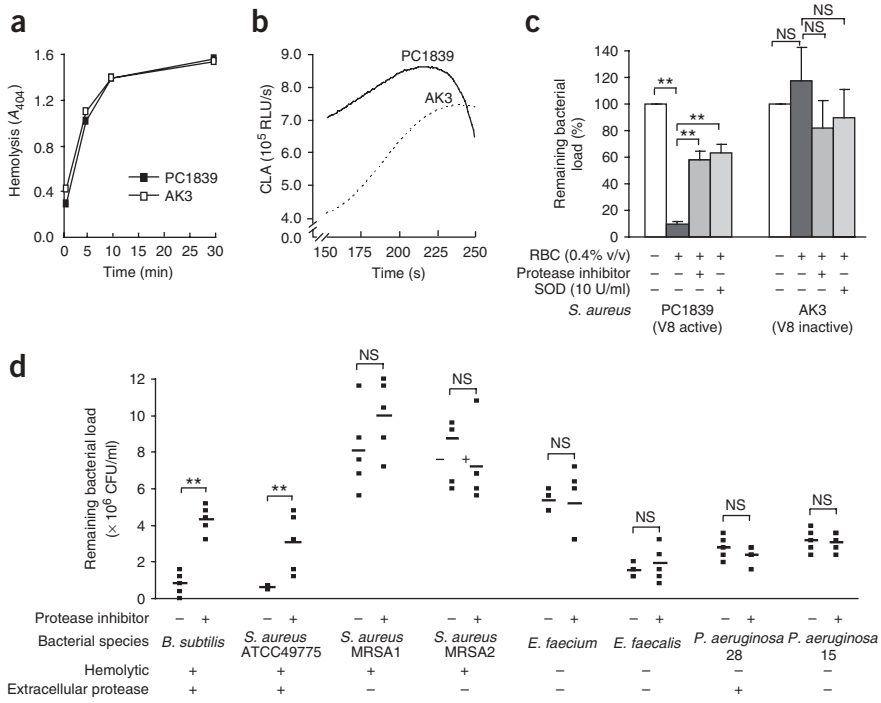

Figure 5 Functional evaluation of the metHb-mediated ROS production in RBC by various bacteria. (**a**) Hemolysis assay of RBCs incubated with *S. aureus* laboratory strains PC1839 (V8 producing) and AK3 (V8 nonproducing). (**b**) Chemiluminescence assay of ROS production after the incubation of RBCs with *S. aureus* strains PC1839 and AK3. (**c**) Killing assay of *S. aureus* strains PC1839 and AK3 after the addition of RBCs plus superoxide dismutase (SOD) or protease inhibitor. (**d**) Killing assay of naturally occurring and/or clinical bacterial isolates with (+) or without (–) hemolytic activity and extracellular protease activity. Small horizontal lines indicate median remaining CFU/ml. *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test). Data represent the mean ± s.d. of three independent experiments.

self-destruction. Such a seminal 'shortcut' immunosurveillance mechanism, which was entrenched over 500 million year ago, probably represents another ancient form of innate immunity functionally conserved since before the split of protostomes and deuterostomes.

METHODS

Hemoglobin. Human hemoglobin obtained used in a lyophilized form (Sigma). The purity of the protein was analyzed by SDS-PAGE to confirm its purity of about 98% (**Supplementary Fig. 8** online). The PyroGene Endotoxin Diagnostic Kit (Lonza), which detects LPS to a concentration of as low as 0.05 EU/ml (equivalent to about 10 pg/ml) showed no detectable LPS in the hemoglobin.

Purified horseshoe crab HMC-PPO. Horseshoe crabs (*Carcinoscorpius rotundicauda*) were collected from the Kranji estuary in Singapore. Hemolymph was collected in pyrogen-free tubes by cardiac puncture. Only plasma samples from crabs with sterile hemolymph were used for further HMC-PPO purification. Hemocytes were removed by centrifugation for 10 min at 150*g*. Supernatants were further clarified at for 10 min at 9,000*g*, and the resultant second supernatants (called 'plasma' here) were 'quick-frozen' in liquid nitrogen and were stored at –80 °C until further use.

All HMC-PPO purification procedures were done at 4 °C as described[42,43] with modifications. Plasma was centrifuged twice (3 h each) at 40,000*g*. Pellets containing enriched HMC-PPO were resuspended in 0.5 M pyrogen-free NaCl and were further dialyzed at 4 °C against a buffer of 0.1 M Tris-HCl, pH 8.0, 5 mM $CaCl_2$ and 5 mM $MgCl_2$. Then these were further purified by gel filtration through Superdex S200 with elution buffer containing 0.1 M Tris-HCl, pH 8.0, 5 mM $CaCl_2$ and 5 mM $MgCl_2$. Three fractions were collected; the large leading peak contained purified HMC (**Supplementary Fig. 9a** online). The concentration of the HMC-PPO was determined by the Bradford method[39] with bovine serum albumin (fraction V; Sigma) as the standard. The purity of the protein was analyzed by SDS-PAGE to be over 98% (**Supplementary Fig. 9b**).

Proteases. PAE of *P. aeruginosa*, chromatographically purified to over 90% purity from a pathogenic strain, with a specific activity of 261 U per mg protein, was from Elastin Product Company. Proteinase K of *Tritirachium album*, type XIV protease of *Streptomyces griseus*, subtilisin A of *Bacillus licheniformis*, trypsin of bovine pancrease and collagenase of *Clostridium histolyticum* were from Sigma-Aldrich. Factor C was expressed recombinantly and was further activated by LPS[40,41].

PAMPs and phosphatidyl lipids. LPS of *P. aeruginosa* and of *Escherichia coli* 055:B5, both of gel-filtration chromatography grade, LTA of *S. aureus* and laminarin of *Laminaria digitata* were from Sigma. The synthetic phosphatidyl lipids POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) and POPE (1-palmitoyl-2-oleoyl-phosphatidyl-ethanolamine) were from Avanti Polar Lipids. To rule out the possibility of LPS contamination of the non-LPS PAMP products such as LTA, we assayed for LPS with PyroGene Kit and used only LPS-free PAMPs (**Supplementary Fig. 10** online). Other reagents are described in the **Supplementary Methods** online.

Bacterial strains. For determination of microbial protease specificity in driving PPO activation, the wild-type strain of *P. aeruginosa* (PAO-Iglewski), which produces extracellular PAE, and the mutant strain PAO-B1A1 (mutations of *LasA* and *LasB*), which does not produce PAE, were used[29]. A Gram-positive *S. aureus* bacterium lacking LPS was used to preclude the possibility of any LPS-mediated hemocyte degranulation and consequential contribution of intracellular components to PPO activation. *S. aureus* strain PC1839 (a *SarA* mutant of wild-type 8325-4), which produces active V8 protease, and *S. aureus* strain AK3 (an *Aur* mutant of PC1839), which does not produce active V8 protease[32], were used to confirm the specificity of bacterial protease action on HMC-PPO activation and to examine the specific contribution of the extracellular PPO activation to *in vivo* antibacterial action. Kanamycin (50 μg/ml) was used for maintenance of *S. aureus* strains PC1839 and AK3, and erythromycin (10 μg/ml) was used for culture of *S. aureus* strain AK3. Six naturally occurring Gram-positive bacterial species, *Bacillus pumilus*, *Bacillus aquimaris*, *Bacillus subtilis*, *Bacillus chandigarhensis*, *E. faecium* and *E. faecalis*, isolated from the horseshoe crab habitat, as well as *S. aureus* ATCC49775, were also used in the *in vivo* antimicrobial test with horseshoe crabs. For analysis of the antimicrobial activity of metHb-mediated ROS production in RBCs, methicillin-resistant *S. aureus* strains from the National University Hospital (Singapore) were also tested, as well as the naturally occurring bacillus and enterococcus species. The isolation and identification of naturally occurring Gram-positive bacteria from the habitat of the horseshoe crab is described in the **Supplementary Methods**.

PO activity. PO activity was measured as described[44] with modifications. In a reaction volume of 125 μl, purified HMC-PPO was incubated for 10 min at 20 °C with proteases in 50 mM Tris-HCl, pH 8.3, containing 0.05 M NaCl, followed by the addition of the PAMP dissolved in the same buffer. Then, 1 mM 4ME in 0.1 M potassium phosphate, pH 6.0, was added as substrate. The absorbance at 405 nm was monitored continuously with a microplate reader (Molecular Devices). PO activity is presented as the absorbance at 405 nm at 5 min after the addition of substrate (**Supplementary Fig. 11a,b** online).

Detection of $O_2^{\bullet-}$ production by chemiluminescence and pseudoperoxidase activity of metHb. The generation of $O_2^{\bullet-}$ by metHb was monitored by the chemiluminescence of CLA as described[10], with modifications, with the Sirius luminometer (Berthold Detection Systems). The chemiluminescence of CLA, expressed as relative luminescence units per second (RLU/s), specifically indicates the generation of $O_2^{\bullet-}$ but not of ozone, H_2O_2 or hydroxyl radicals[10]. For this analysis, 2.0 μg metHb was added to a substrate mixture containing 5.3 μM CLA and 3.3 mM H_2O_2 in 150 μl PBS, pH 7.4. Chemiluminescence was measured beginning immediately after the metHb was added and was continuously monitored for 1 min at a rate of one 'reading' per second. The pseudoperoxidase activity of the metHb is presented as the maximum speed of $O_2^{\bullet-}$ production in the presence of excess H_2O_2 and CLA. To prove that the ROS indicated by the chemiluminescence of CLA were superoxide anions, superoxide dismutase was applied to specifically quench superoxide anions. Enhancement of pseudoperoxidase activity by LTA alone is described in **Supplementary Figure 11c,d**. Analysis of the auto-oxidation of hemoglobin to metHb is described in the **Supplementary Methods**.

Bacterial culture for antimicrobial assays. Single colonies of *P. aeruginosa* and *S. aureus* were inoculated into 10 ml Luria-Bertani broth and 10 ml tryptone soy broth, respectively, and were shaken at 200 r.p.m for 16 h at 37 °C. For the induction of extracellular protease production (if any occurred), overnight cultures of PAO-Iglewski and PAO-B1A1 were diluted in Luria-Bertani broth to a concentration of 1×10^3 CFU/ml and were incubated for 48 h at 37 °C as standing cultures[29]. For *S. aureus*, overnight cultures of PC1839, AK3, ATCC49775, MRSA1 and MRSA2 were diluted in tryptone soy broth until the absorbance at 600 nm reached 1.0 and then were shaken at 220 r.p.m. for 4 h at 37 °C (ref. 32). For the naturally occurring bacillus species, overnight cultures were diluted with marine broth until the absorbance at 600 nm reached 0.5 and then were shaken at 220 r.p.m. for 2 h at 37 °C. Bacterial cultures were collected by centrifugation for 10 min at 8,000*g*. At this point, extracellular proteases were quantified by the Azocoll protease assay (Sigma) as described in the **Supplementary Methods**.

For exclusion of the possibility of the presence of LPS in Gram-positive bacterial cultures, every precaution was taken to prepare culture media with pyrogen-free water and containers. Bottles made pyrogen-free by being baked for 4 h at 200 °C were used. After sterilization of the media, LPS was assayed with the PyroGene kit, and medium was used only when the LPS was negligible. Gram-positive bacteria were further tested for their inability to cause degranulation of the hemocytes to ensure that the bacteria that were collected lacked LPS. Only pyrogen-free cultures were used for *in vitro* and *in vivo* antimicrobial assays to confirm that the HMC-PPO activation was truly attributable to extracellular factors. Analysis of horseshoe crab hemocyte degranulation and examination of exocytosis are described in the **Supplementary Methods**.

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***In vitro* antimicrobial assay with the chemically reconstituted system.** The end-point measurement of the ROS-mediated antimicrobial activity was done with an initial bacterial population of 1×10^6 CFU/ml. For metHb analysis, bacteria were incubated for 1 h at 37 °C with 36 μg metHb and 1.6 μmol H_2O_2 in 200 μl PBS. The remaining bacterial population in each reaction mixture was counted by plating of 100 μl of serially diluted samples on nutrient agar plates followed by overnight incubation at 37 °C. To further prove that the antibacterial activity was indeed attributable to ROS, superoxide ions were quenched with 2 μmol GSH. Controls comprised incubation of bacteria with metHb, H_2O_2 and GSH separately or in combination. For HMC-PPO analysis, bacteria were incubated for 1 h at 37 °C with 60 μg purified HMC plus 100 nmol 4ME in 100 μl PBS. For analysis of the effect of PO activity, 10 nmol PTU was added to the reactions described above. Controls comprised incubation of bacteria with HMC-PPO or 4ME or PTU separately or in combination. Each condition was tested in triplicate.

Real-time imaging of bacterial clearance elicited by PO-catalyzed production of quinone was done with PAE-producing and PAE-deficient strains of *P. aeruginosa*. Each bacterial strain (1×10^7 CFU) was mixed with 60 μg HMC-PPO and 100 nmol 4ME in 100 μl PBS. An aliquot of 1 μl of the mixture was examined by fluorescence microscopy. Images were obtained at intervals of 30 s for 1 h, at a magnification of 63 (lens objective) × 1.6 (eye piece objective) and movies were made.

***In vitro* antimicrobial assay with mammalian RBCs.** The metHb-mediated antimicrobial activity was also evaluated with mammalian RBCs. Both rabbit and human RBCs were collected into heparinized tubes, were washed with pyrogen-free saline (0.9% (vol/vol) NaCl) and were diluted to 0.4% (vol/vol); then bacteria were incubated with the washed RBCs. Pyrogen-free saline was used as a negative control. The bacterial population was monitored for up to 30 min. Protease Inhibitor Mix G (Serva Chemical) was used to investigate the requirement for microbial protease in triggering the ROS production (if any were required). Superoxide dismutase from bovine erythrocytes (4,733 units/ml; Sigma) was used to determine the specificity of ROS in the antimicrobial action. Measurement of RBC lysis is described in the **Supplementary Methods**.

***In vivo* antimicrobial activity assay.** Gram-positive bacteria at a sublethal dose (1×10^5 to 1×10^6 CFU per 100 g body weight) were injected into horseshoe crabs. At 30 min after injection, cell-free hemolymph was collected from the crabs as described[39]. The residual bacterial load was counted by plating of 100 μl of serial dilutions of the cell-free hemolymph on nutrient agar plates, followed by overnight incubation at 37 °C. To confirm the contribution of PO activity to the bacterial clearance, 5 mM PTU or 5 mM kojic acid was injected along with the bacteria to block *in vivo* PO activity (if any were present).

ELISA-based protein-PAMP interaction and factor C activity. These assays are described in the **Supplementary Methods**.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

N.J. designed and did the experiments and prepared the manuscript; N.S.T. provided some suggestions, helped with real-time fluorescence imaging and manuscript preparation; B.H. provided expertise in microbiology, designed and did some of the experiments and participated in manuscript preparation; J.L.D. conceived the ideas, designed the biochemical, cell and molecular biology experiments, and supervised the studies and preparation of the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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