RESEARCH ARTICLE

Salmonella Evades D-Amino Acid Oxidase To Promote Infection in Neutrophils

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ABSTRACT **Neutrophils engulf and kill bacteria using oxidative and nonoxidative mechanisms. Despite robust antimicrobial activity, neutrophils are impaired in directing *Salmonella* clearance and harbor viable intracellular bacteria during early stages of infection that can subsequently escape to more-permissive cell types. The mechanisms accounting for this immune impairment are not understood. We report that *Salmonella* limits exposure to oxidative damage elicited by D-amino acid oxidase (DAO) in neutrophils by expressing an ABC importer specific for D-alanine, a DAO substrate found in peptidoglycan stem peptides. A *Salmonella dalS* mutant defective for D-alanine import was more susceptible to killing by DAO through exposure to greater oxidative stress during infection. This fitness defect was reversed by selective depletion of neutrophils or by inhibition of DAO *in vivo* with a small-molecule inhibitor. DalS-mediated subversion of neutrophil DAO is a novel host-pathogen interaction that enhances *Salmonella* survival during systemic infection.**

IMPORTANCE **Neutrophils engulf *Salmonella* during early stages of infection, but bacterial killing is incomplete. Very little is known about how *Salmonella* survives in neutrophils to gain access to other cell types during infection. In this study, we show that D-amino acid oxidase (DAO) in neutrophils consumes D-alanine and that importing this substrate protects *Salmonella* from oxidative killing by neutrophil DAO. Loss of this importer results in increased bacterial killing *in vitro*, in neutrophils, and in a mouse model of infection, all phenotypes that are lost upon inhibition of DAO. These findings add mechanistic insight into a novel host-pathogen interaction that has consequences on infection outcome.**

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The D-amino acid oxidase (DAO) catalyzes the flavin-dependent deamination of certain D-amino acids to yield an α-ketoacid, ammonium ion, and hydrogen peroxide (1, 2). For example, DAO regulates D-serine levels in the brain, where this amino acid coactivates glutamate-dependent *N*-methyl-D-aspartate receptors on postsynaptic neurons (1). Owing to its ability to generate hydrogen peroxide and its widespread conservation among vertebrates and invertebrates (3), historically, DAO was considered a potential component of the innate host defense system. Work from the 1960s identified DAO activity in the purified granule fraction of human neutrophils (4). This activity was later localized by electron microscopy to the neutrophil phagosome following engulfment of latex beads in the presence of D-alanine (5). Studies of gnotobiotic mice showed DAO in the kidney in response to D-alanine liberated from the microbiota (6), and mice with a spontaneous mutation in DAO were shown to be more susceptible to infection with *Staphylococcus aureus* (7), further linking DAO to an understudied host defense system that is responsive to microbial input. Mammals do not synthesize D-alanine. However, in bacteria, it constitutes the terminal amino acid in peptidoglycan stem peptides, making it a potential discriminator between self and nonself in the context of immunity.

In neutrophil phagosomes, hydrogen peroxide liberated from DAO-catalyzed dehydrogenation of D-alanine would be accessible to myeloperoxidase (MPO), which catalyzes the formation of hypochlorous acid (HOCl) from chloride and hydrogen peroxide. Although debated (8), the latter reaction has for some time been considered the clinically relevant and major microbicidal pathway in neutrophils, with reaction products being directly toxic to bacteria or going on to form secondary chloramines (9–11). Experimental work indicates that hydrogen peroxide is rate limiting for MPO-catalyzed halogenation in neutrophils (12), suggesting that an auxiliary source of peroxide, in addition to dismutation of superoxide formed from the more widely studied NADPH oxidase, would be beneficial toward neutrophil killing activity.

Previously, we identified an ATP-binding cassette transporter in *Salmonella enterica* that imports D-alanine (13). This transporter underwent regulatory evolution for expression in the intracellular environment following host cell invasion (14), providing a clue that its function was related to intracellular survival. Structural work confirmed the specificity and chiral selectivity for D-alanine, showing that DalS, the periplasmic binding component, restricts the beta-carbon of alanine to a D-configuration due to steric hindrance of the L-isomer (13). Interestingly, DalS was dispensable for *Salmonella enterica* serovar Typhimurium growth *in vitro* and for peptidoglycan composition. However, D-alanine

import was required for competitive fitness during infection of mice, and mice infected with DalS-deficient *Salmonella* had a significantly extended survival time than mice infected with wild-type *Salmonella* (13). Our previous results suggested a possible interaction between DalS and the host immune system; however, its function remained enigmatic. Using a murine model of systemic infection with the pathogen *Salmonella enterica* serovar Typhimurium, which is widely used as a model for the host-restricted *Salmonella* serovar Typhi, we show that DalS helps protect *Salmonella* from DAO-dependent killing in neutrophils. *Salmonella* mutants with a deletion of *dalS* were exposed to greater DAO-dependent oxidative stress during host infection, leading to a loss of competitive fitness and increased killing by neutrophils. However, this defect could be repaired upon inhibition of DAO activity or by host neutropenia. Thus, DalS-mediated subversion of neutrophil DAO is an important host-pathogen interaction that enhances bacterial survival during early stages of infection. These data help explain, in part, the incomplete killing of *Salmonella* by neutrophils, allowing secondary dissemination to more-permissive cell types (15).

RESULTS

DalS protects *S.* Typhimurium from the antimicrobial activity of DAO. The flavin-dependent deamination of D-alanine by DAO yields the α-ketoacid, ammonium ion, and hydrogen peroxide (Fig. 1A). To study the susceptibility of *S. enterica* serovar Typhimurium to the activity of DAO, we purified recombinant DAO and confirmed its hydrogen peroxide-generating activity in the presence of 5 mM D-alanine or 50 mM D-serine (Fig. 1B; see Fig. S1 in the supplemental material). Increasing the concentration of D-serine was necessary, as this substrate produces only 10% of the V_{max} activity that D-alanine produces (4). DalS-deficient bacteria were more sensitive to killing by purified DAO in the presence of D-alanine (Fig. 1C). This killing was blocked by the addition of thiourea, a potent hydroxyl radical scavenger that mitigates the toxic effects of hydrogen peroxide on bacterial cells by reducing the formation of hydroxyl radicals from hydrogen peroxide (16, 17). As expected, the addition of myeloperoxidase to the *in vitro* reaction increased the magnitude of killing of both wild-type and *dalS* mutants, yet the enhanced susceptibility of Δ*dalS* bacteria persisted (Fig. 1C). These data established that hydrogen peroxide was the toxic DAO reaction product in these *in vitro* reactions and that *Salmonella* mutants lacking DalS are more susceptible to DAO-dependent killing.

To determine whether DalS-deficient bacteria allowed a higher concentration of enzymatic hydrogen peroxide product to form from DAO, we constructed an *S.* Typhimurium reporter strain, previously validated to report hydrogen peroxide stress, by fusing the OxyR-dependent *ahpC* promoter (18, 19) to *luxCDABE* from *Photorhabdus luminescens*. We confirmed the activity of this strain in response to hydrogen peroxide as low as 2 μM and showed dose-dependent luminescence in response to peroxide up to 100 μM (Fig. 1D), well below the steady-state peroxide concentration in neutrophil phagosomes estimated to be ~5 μM by kinetic models (10). Importantly, we confirmed that this reporter responded equally in the wild type and *dalS* mutants exposed to nonenzymatically generated hydrogen peroxide at relevant concentrations (Fig. 1E). However, upon exposure to hydrogen peroxide generated enzymatically by DAO in the presence of D-alanine, reporter activity was significantly greater in *dalS* mutants than in the wild type (Fig. 1F). This was consistent with an impaired ability of the *dalS* mutant to reduce an initial 5 mM extracellular concentration of D-alanine to the same extent as the wild type (Fig. 1G), in agreement with our previous data (13). For a control, both wild-type and Δ*dalS* bacteria were killed to equivalent levels following exposure to nonenzymatic sources of hydrogen peroxide or hypochlorous acid (HOCl) (Fig. 1H), indicating that increased killing of the *dalS* mutant was DAO dependent and not due to an inherent sensitivity to hydrogen peroxide or HOCl. Complementing Δ*dalS* with the *dalS* gene under the control of its native promoter restored bacterial survival to wild-type levels in the presence of DAO and D-alanine (Fig. 1I). Together these data indicated that DalS-deficient bacteria allowed a higher concentration of DAO-dependent hydrogen peroxide product to form, resulting in increased killing.

***Salmonella* lacking DalS is more susceptible to DAO killing in neutrophils.** Neutrophils express DAO (4, 5) and are an early host cell target for *Salmonella* (20, 21). To test whether the *S.* Typhimurium *dalS* mutant was more susceptible to the microbicidal activity of neutrophils, we infected purified neutrophils with wild-type *Salmonella* or *dalS* mutants and monitored bacterial survival. After 2 h, 40% of wild-type *Salmonella* had survived, whereas only ~25% of the *dalS* mutants remained viable (Fig. 2A). To confirm that this killing phenotype was dependent on DAO, we used the chemical inhibitor 6-chloro-1,2-benzisoxazol-3(2H)-one (CBIO) that blocks the dehydrogenation reaction catalyzed by DAO (22) (Fig. 2B). We also confirmed that CBIO had no effect on *S.* Typhimurium growth and did not bind to the *S.* Typhimurium DalS protein as measured by fluorescence thermal shift of purified DalS (see Fig. S2 in the supplemental material) (13). In the presence of CBIO, the ability of neutrophils to kill *Salmonella* was impaired, and there was no longer a survival defect of the *dalS* mutant (Fig. 2A). To confirm that this killing phenotype was linked to DAO-dependent hydrogen peroxide stress, we infected neutrophils with the wild type and *dalS* mutants carrying the OxyR-dependent *ahpC* reporter in the presence or absence of CBIO. Consistent with the killing phenotypes observed, peroxide reporter activity in the *dalS* mutant was double that seen in wild-type *Salmonella* during neutrophil infections (Fig. 2C). However, when DAO activity was inhibited with CBIO, the reporter activities from the wild type and *dalS* mutants were reduced by ~35% and ~65%, respectively, and were no longer different (Fig. 2C). Together these data established that DalS enhances *S.* Typhimurium survival in neutrophils in a manner that depends on functional DAO.

***S.* Typhimurium lacking DalS is sensitized to neutrophil DAO *in vivo*.** Neutrophils are the primary source of DAO among polymorphonuclear leukocytes (4, 5), and their numbers increase rapidly in the spleen in response to *S.* Typhimurium infection (19, 21). Given this, we reasoned that the levels of splenic DAO would increase following *S.* Typhimurium infection due to neutrophil influx. We collected spleens from infected and uninfected mice and probed them for DAO using a DAO antibody. Uninfected mice had no detectable DAO in the spleen or cecum by this method (Fig. 3A). In contrast, splenic DAO increased dramatically upon *S.* Typhimurium infection (Fig. 3A). Hepatic DAO has been detected in some studies but not others (23). We detected DAO in livers isolated from uninfected mice, but this did not increase dramatically upon infection when normalized to a control host protein (Fig. 3A). To verify that neutrophils were the

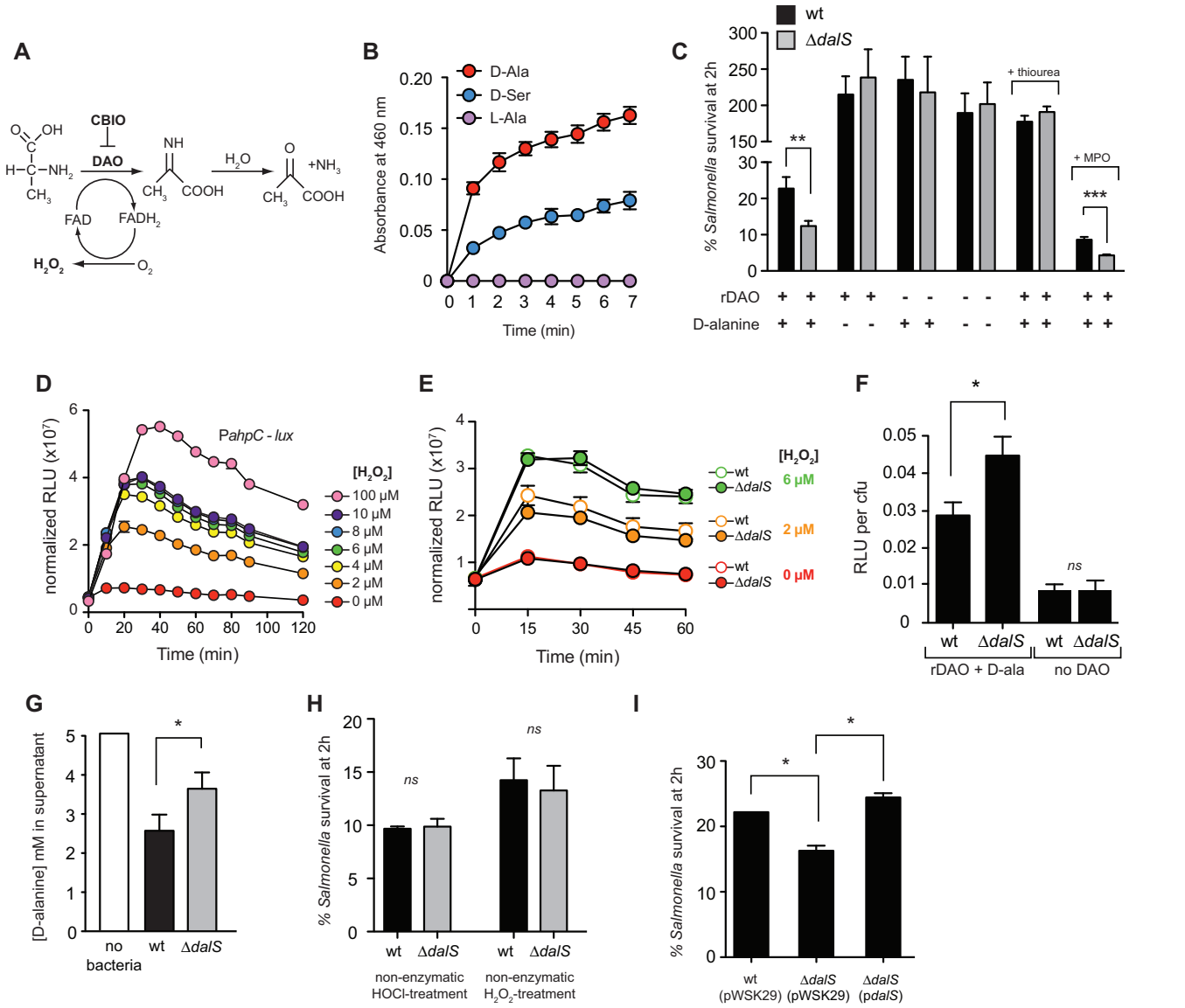

FIG 1 DalS protects *S.* Typhimurium from the antimicrobial activity of DAO. (A) DAO catalyzes the flavin-dependent deamination of D-alanine to yield the α-ketoacid, NH_4^+, and hydrogen peroxide. (B) Recombinant DAO (rDAO) produces hydrogen peroxide upon the addition of D-alanine and D-serine as measured by peroxidase-coupled oxidation of *o*-dianisidine. Data are the means ± standard errors (error bars) from three experiments. (C) *S.* Typhimurium *dalS* mutants are more susceptible to the reaction products of DAO than the wild type (wt) is. Bacteria were incubated with purified DAO in the presence (+) or absence (−) of exogenous D-alanine. Survival data are the means plus standard errors (error bars) from three independent experiments. (D) The OxyR-dependent P*ahpC-lux* reporter strain is sensitive to hydrogen peroxide. Data are mean relative light units (RLU) normalized to the optical density of the culture. (E) Wild-type (wt) *Salmonella* and *dalS* mutant sense nonenzymatic hydrogen peroxide equally. Data are the means ± standard errors from three experiments. RLU data are normalized to the optical density of the culture. Reporter activities for the wild type and *dalS* mutants for each of the treatment groups are not significantly different. (F) A *dalS* mutant is exposed to greater hydrogen peroxide stress compared to the wild type following exposure to DAO and D-alanine. Data are from strains carrying the P*ahpC-lux* reporter and are the means plus standard errors from three separate experiments. (G) *S.* Typhimurium lacking DalS is defective for D-alanine import. The ability of *Salmonella* to remove D-alanine (5 mM) from the culture medium was estimated by DAO-catalyzed peroxidase-coupled oxidation of *o*-dianisidine. (H) Δ*dalS* mutants have wild-type sensitivity to exogenous hydrogen peroxide (0.1%) and HOCl (0.01%) generated nonenzymatically. (I) Sensitivity of *dalS* mutants to DAO-dependent killing is normalized to wild-type levels upon complementation with a *dalS*-encoding plasmid (p*dalS*). All experiments were performed independently three times. Values that are statistically significantly different by the Mann-Whitney test are indicated by bars and asterisks as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Values that are not statistically significantly different (ns) are indicated.

source of splenic DAO, we rendered mice neutropenic by antibody-based neutrophil depletion and verified this by flow cytometry (see Fig. S3 in the supplemental material). In infected neutropenic mice, splenic DAO became undetectable, and liver DAO was reduced to ~25% of that seen in nonneutropenic controls (Fig. 3B). The residual DAO signal in neutropenic mouse liver is likely from hepatocytes, which are a source of DAO activity in a variety of animals (24).

Given that DalS protected *S.* Typhimurium from neutrophil DAO, we hypothesized that depleting neutrophils would restore virulence to the *dalS* mutant *in vivo*. Indeed, in neutropenic mice, the *S.* Typhimurium *dalS* mutant competed equally with wild-

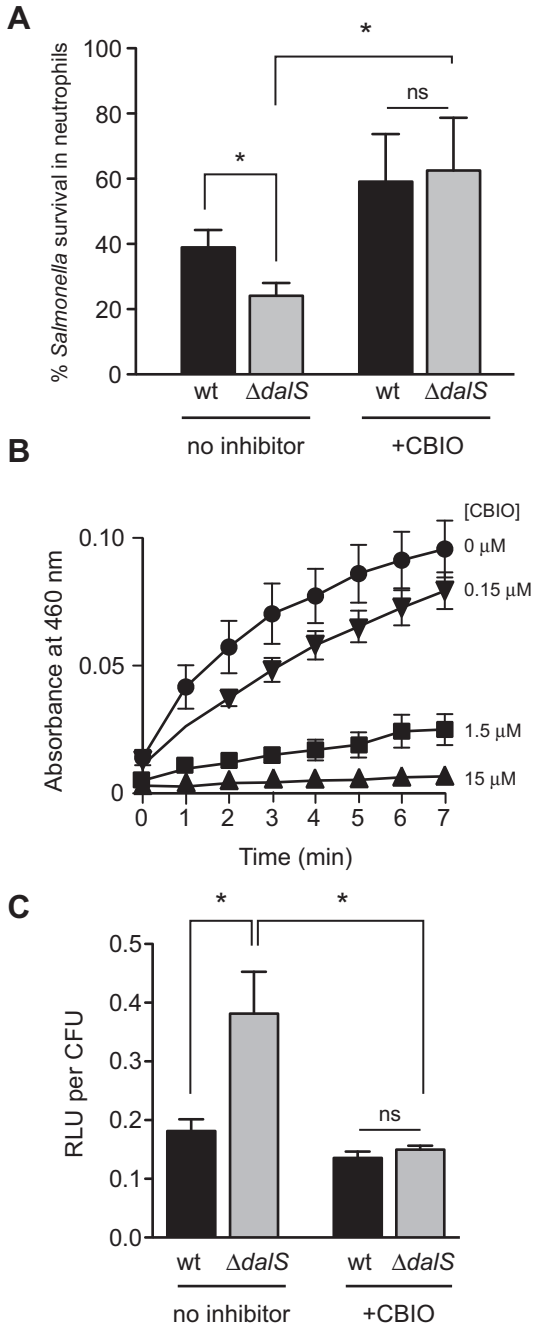

FIG 2 DalS-deficient *Salmonella* bacteria are killed more efficiently in neutrophils in a DAO-dependent manner. (A) *S.* Typhimurium Δ*dalS* bacteria are sensitized to neutrophil killing. Bacterial killing by purified neutrophils was measured after 2 h. Survival was measured as the ratio of the number of viable bacteria at 2 h compared to the initial number of internalized bacteria. The sensitivity of Δ*dalS* mutants to neutrophil killing was inhibited by DAO inhibition with CBIO. Data are from three experiments. (B) CBIO inhibits DAO enzyme activity *in vitro*. Different concentrations of CBIO (0, 0.15, 1.5, and 15 μM) were tested. Data are the means ± standard errors (error bars) from three experiments. (C) *Salmonella* bacteria lacking DalS are exposed to greater hydrogen peroxide stress in neutrophils. Hydrogen peroxide stress levels are restored when DAO is inhibited with CBIO. Data are from strains carrying the P*ahpC-lux* reporter 30 min following infection of purified neutrophils and are the means plus standard error from three separate experiments. Statistical significance is indicated as follows: *, $P < 0.05$ by the Mann-Whitney test; ns, not significantly different.

type bacteria, whereas they remained significantly attenuated in control treated animals containing neutrophils (Fig. 3C). To verify that this restoration of virulence in the mutant was linked to the attendant loss of DAO following neutrophil depletion, we alternatively inhibited DAO activity in infected mice by *in vivo* administration of CBIO (25). These data showed that the effect of neutropenia on the *dalS* mutant was phenocopied by DAO inhibition (Fig. 3D). The restoration of virulence to *dalS* mutants following DAO inhibition *in vivo* was specific to DalS and not a generalized immune dysfunction, because a *Salmonella* mutant (Δ*SPI2*) containing a lesion in the type III secretion system involved in intracellular replication (26) remained attenuated in mice in the presence of CBIO (Fig. 3D). To confirm the importance of DalS for *Salmonella* during infection and to further connect this virulence factor to host DAO, single infections were performed with the wild type or the *dalS* mutant in mice in which DAO was inhibited or not inhibited. Mice infected with wild-type *S.* Typhimurium carried a significantly higher splenic bacterial burden than mice infected with a *dalS* mutant (Fig. 3E), confirming that DalS contributes to bacterial fitness in the host. Treatment of mice with the DAO inhibitor CBIO significantly increased the bacterial load associated with Δ*dalS* infection (Fig. 3E). Together, these experiments clearly linked the phenotype of the *dalS* mutant to the expression and activity of neutrophil DAO.

DAO contributes to bacterial peroxide stress independent of NOX2. The NADPH oxidase system in neutrophils is a source of superoxide that can spawn an array of reactive oxygen species. Prominent among these is hydrogen peroxide that is consumed by myeloperoxidase to generate hypochlorous acid (HOCl) within the neutrophil phagosome (27). However, our experiments indicated that neutrophils killed *S.* Typhimurium Δ*dalS* mutant bacteria more effectively than wild-type bacteria even in the presence of functional NADPH oxidase, suggesting that DAO is a relevant source of innate immune activity. If this were the case, then the fitness defect in the Δ*dalS* mutant would persist in a host lacking NADPH oxidase. To quantify the contribution of DAO to host protection independent of NADPH oxidase, we infected *CYBB*/gp91/NOX2 mice lacking the flavocytochrome b-245 heavy chain, an essential component of NADPH oxidase. In the liver, *dalS* mutant bacteria remained attenuated and at levels similar to those in wild-type mice. Interestingly, in the spleen where neutrophils accumulate after infection (15, 21), the defect of *dalS* mutants was significantly amplified in *CYBB*$^{-/-}$ mice compared to their defect in wild-type mice (Fig. 4A). The fitness defect of the *dalS* mutant was repaired upon treatment of *CYBB*$^{-/-}$ mice with CBIO (Fig. 4B), implicating DAO as a relevant source of innate immune protection during *S.* Typhimurium infections. These data indicated that DalS increases early *Salmonella* survival, particularly in the spleen, even in the absence of NADPH oxidase.

To confirm that this source of bactericidal peroxide was DAO, we used the OxyR-dependent *ahpC-lux* transcriptional reporter constructed earlier and performed whole-animal *in vivo* imaging of infected mice to quantify hydrogen peroxide stress in *S.* Typhimurium following DAO inhibition. *S.* Typhimurium produced OxyR-dependent luminescence in both wild-type mice and in *CYBB*$^{-/-}$ mice lacking NADPH oxidase, which was reduced in both cases when mice were treated with the DAO inhibitor CBIO (Fig. 4C). Importantly, at these early time points after infection, the bacterial loads in the groups of mice were found to be similar at necropsy (data not shown). OxyR-dependent luminescence in

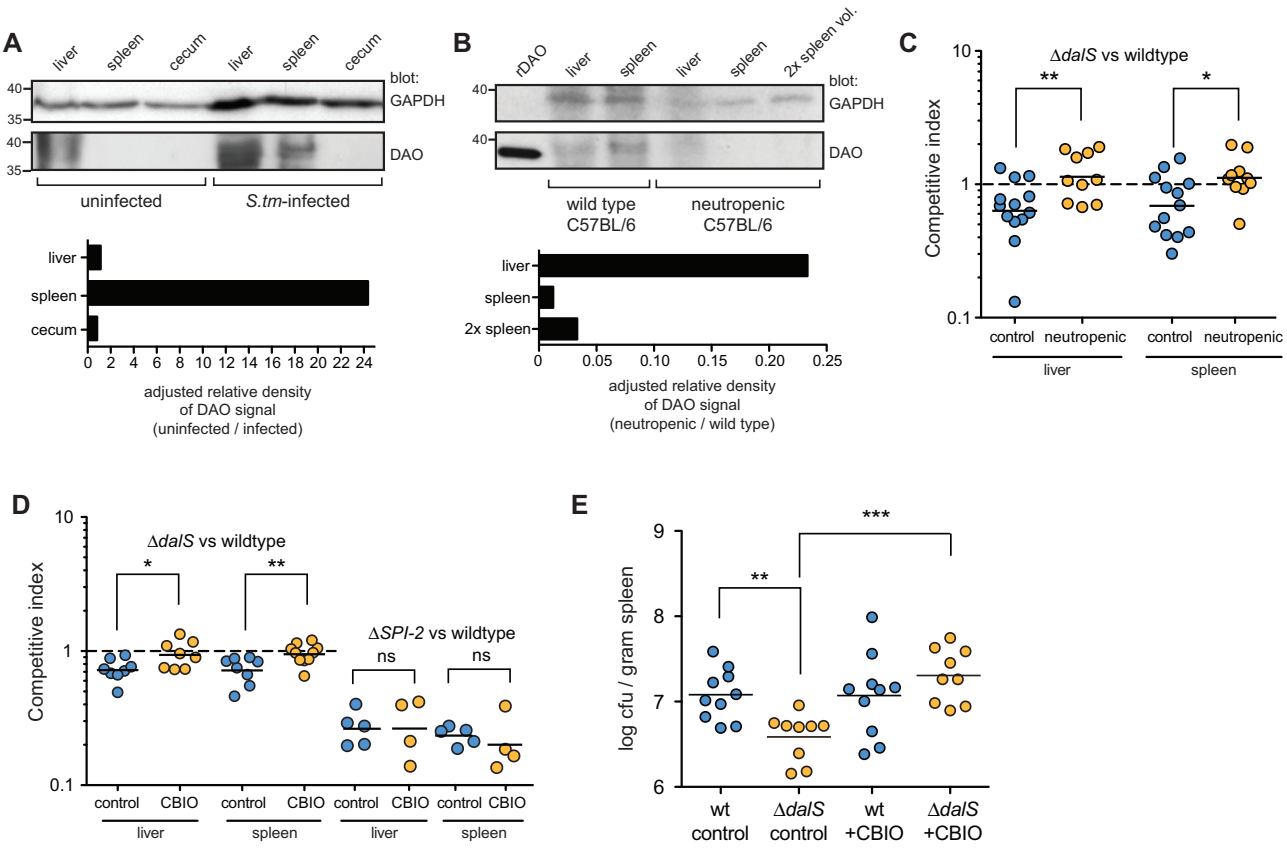

FIG 3 *S.* Typhimurium bacteria lacking DalS are more susceptible to neutrophil DAO killing *in vivo*. (A) Western blot of DAO in liver, spleen, and cecum from uninfected mice or mice infected with *S.* Typhimurium (*S.tm*). The positions of molecular weight markers (in kilodaltons) are shown to the left of the blots. The relative density of the DAO signal adjusted to the GAPDH signal is shown below the Western blot. (B) Neutropenic mice fail to accumulate splenic DAO following *S.* Typhimurium infection. Neutropenic mice were infected with *S.* Typhimurium, and organs were probed for DAO (2× spleen vol., twice the amount of sample loaded). (C) Neutropenia normalizes the fitness defect of the *S.* Typhimurium Δ*dalS* mutant. Control mice and neutropenic mice were infected with an equal mixture of wild-type *S.* Typhimurium and *S.* Typhimurium Δ*dalS* mutant for 2 days. The competitive index data from two experiments are shown. Each symbol represents the value for an individual mouse. The mean value for the group of mice is shown by the short horizontal black line. The broken line shows a competitive index of 1. (D) Inhibition of DAO *in vivo* restores virulence to Δ*dalS* mutants. Mice were infected with an equal mixture of wild-type *S.* Typhimurium and *S.* Typhimurium Δ*dalS* (first set) or an equal mixture of wild-type *S.* Typhimurium and *S.* Typhimurium Δ*SPI-2* (second set) and treated with either carboxymethyl cellulose as a control or the DAO inhibitor CBIO. The competitive index data from two experiments are shown. (E) CBIO restores *dalS* mutant defect in singly infected mice. Mice were infected with either wild-type (wt) *S.* Typhimurium or *S.* Typhimurium *dalS* mutant and treated with CBIO or CMC control for 24 h. The number of CFU was normalized to spleen mass. Values that are statistically significantly different by the Mann-Whitney test are indicated by bars and asterisks as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Values that are not statistically significantly different (ns) are indicated.

wild-type mice was greater than that from *CYBB*$^{-/-}$ mice, a result that was expected because NADPH oxidase activity is broadly conserved among immune cells that interact with *Salmonella* (21, 28), whereas DAO is restricted to neutrophils (4, 5). Luminescence normalized to the bacterial load in the spleen was quantified in both wild-type and *CYBB*$^{-/-}$ mice by *ex vivo* imaging of freshly excised organs (Fig. 4D). This analysis showed that inhibition of DAO activity in both wild-type and *CYBB*$^{-/-}$ mice significantly reduced OxyR-dependent luminescence from splenic *S.* Typhimurium by ~60%, confirming that DAO was a relevant source of hydrogen peroxide during infection in the presence or absence of NOX2.

Our previous results showed that *S.* Typhimurium lacking DalS is exposed to greater DAO-dependent oxidative stress during infection. We quantified this in mice lacking NADPH oxidase by infecting *CYBB*$^{-/-}$ mice with DalS-deficient *Salmonella* carrying the OxyR-dependent luminescence reporter. OxyR-dependent luminescence in mice infected with the Δ*dalS* mutant was significantly greater than from mice infected with wild-type *Salmonella*. Treatment of mice with CBIO to inhibit DAO activity significantly reduced this luminescence signal (Fig. 4E). Together, these experiments revealed that splenic *S.* Typhimurium is exposed to DAO-dependent peroxide stress in mice and that DalS reduces the magnitude of this stress to promote infection.

DISCUSSION

The majority of work on *Salmonella* infection of immune cells has focused on macrophages. However, neutrophils are the major cell type infected by *Salmonella* during the first 2 days of infection. In the gut, 70% of luminal *S.* Typhimurium bacteria are inside neutrophils at day 1 (29), and in the spleen, a neutrophil-enriched population harbored 100% of the viable intracellular *S.* Typhimurium population in the first 24 h (20) and 70% of this bacterial population on day 2 with a concomitant increase in the number of infected macrophages (21). Despite robust antimicrobial activity of neutrophils, which indeed kills many *Salmonella* bacteria dur-

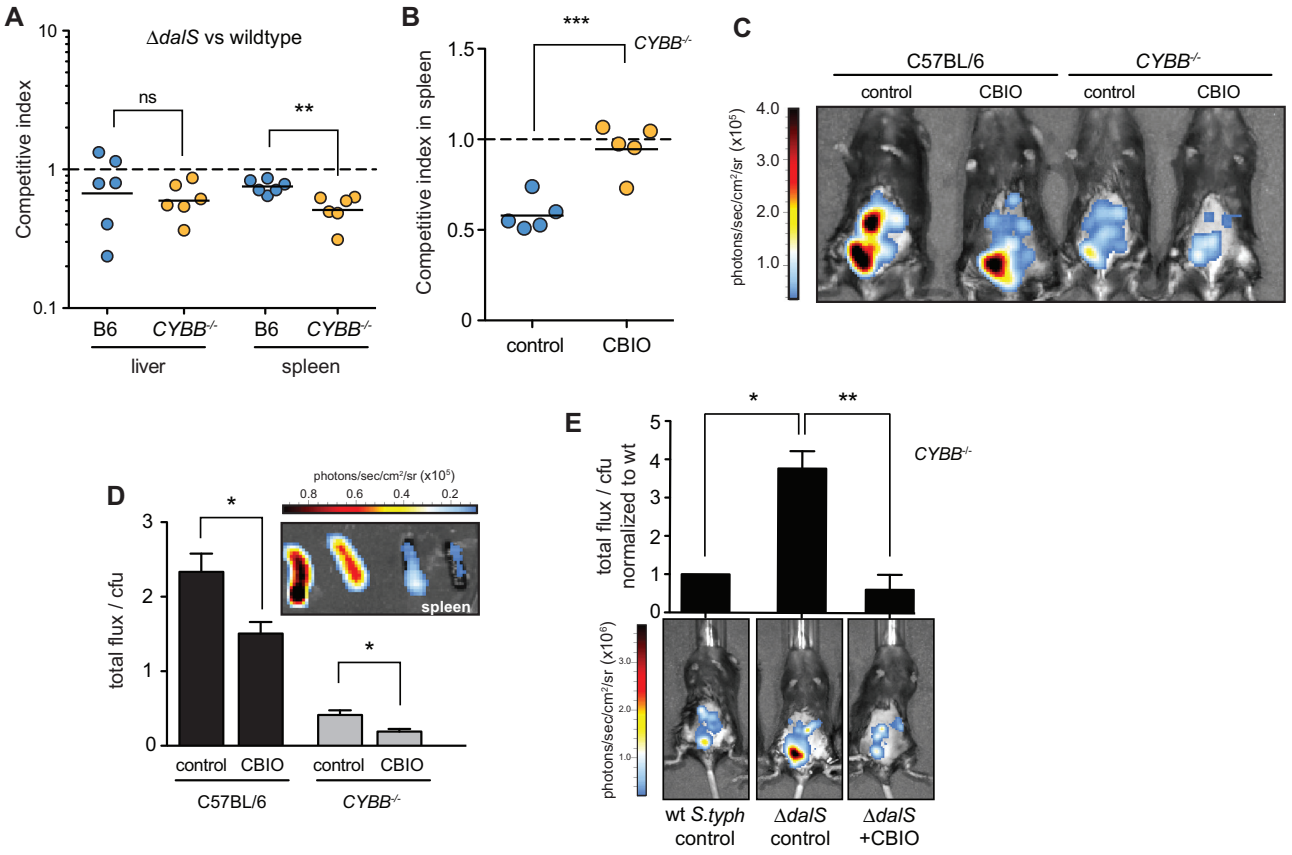

FIG 4 DAO contributes to bacterial peroxide stress independent of NOX2. (A) DalS mutants remain attenuated in mice lacking functional NOX2/NADPH oxidase. Wild-type mice (C57BL/6 [B6]) or *CYBB*$^{-/-}$ mice were infected with an equal mixture of wild-type *S.* Typhimurium and *S.* Typhimurium Δ*dalS* mutant, and the competitive index was calculated after 2 days. (B) DalS mutant defect is restored with CBIO treatment in NADPH oxidase-deficient mice. *CYBB*$^{-/-}$ mice were infected with an equal mixture of wild-type *S.* Typhimurium and *S.* Typhimurium Δ*dalS* mutant and treated with CBIO or carboxymethyl cellulose as a control. The competitive index was calculated after 2 days. (C) DAO elicits peroxide stress in mice. Wild-type and *CYBB*$^{-/-}$ mice with functional (control) or inhibited DAO (CBIO) were infected with the peroxide reporter strain and imaged after 2 h. Images represent 5-s integrations and are representative of the images from three separate experiments. (D) *Ex vivo* imaging of excised spleens from mice in panel B. (Inset) Images were integrated over 5 s and plotted as total photon flux normalized to viable colony counts. (E) DalS mutants are exposed to greater peroxide stress than the wild type *in vivo*. *CYBB*$^{-/-}$ mice were infected with wild-type *S.* Typhimurium (wt *S.typh*) or Δ*dalS* mutants containing the OxyR-dependent luminescent reporter and then treated with the DAO inhibitor CBIO or carboxymethyl cellulose (control). Representative whole-body images from infected mice are shown with normalized luminescence flux plotted above each image. The values plotted in the graph represent the values for three mice per group. Values that are statistically significantly different by the Mann-Whitney test are indicated by bars and asterisks as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

ing infection (15, 30), neutrophils are unable to direct full *S.* Typhimurium clearance (31). This incomplete killing is thought to allow *Salmonella* to spread to a more permissive macrophage population, which has been shown to generate sublethal oxidative bursts (15). The mechanisms that account for this immune subversion of neutrophils are not known but likely involve resistance to the microbicidal effector functions of these cells (32). Interestingly, despite the importance of this host-pathogen interaction, very little is known mechanistically about how *Salmonella* survives in neutrophils. Our data are consistent with neutrophil DAO functioning to exert bactericidal activity on *S.* Typhimurium at early stages of infection. These data provide insight into a host-pathogen interaction in systemic neutrophils that has bearing on the outcome of *Salmonella* infection. It is likely important that this immune subversion mechanism is also conserved in *S.* Typhi, as the DalS transport system is 98% identical in this human pathogen.

Hydrogen peroxide production is rate limiting for MPO-catalyzed halogenation and killing of bacteria in neutrophils (12). These data suggest that an auxiliary source of peroxide, in addition to dismutation of superoxide formed from the more widely studied NADPH oxidase, would be beneficial toward neutrophil killing activity. In mice lacking NADPH oxidase, we showed that *Salmonella* indeed senses an auxiliary source of peroxide stress that can be inhibited by CBIO, a specific small-molecule inhibitor of DAO. This DAO-catalyzed source of oxidative stress was biologically relevant because it elicited a larger response in DalS mutants and was able to better control growth of *Salmonella dalS* mutants that lack the D-alanine import system. In our *in vitro* experiments with purified DAO, exogenous D-alanine was added to elicit *Salmonella* killing by DAO, but D-alanine derived from bacteria was sufficient for killing by DAO in neutrophils. Neutrophils are a rich source of membrane-perturbing antimicrobial peptides, which might sensitize *Salmonella* to the effects of neutrophil DAO due to liberation of D-alanine during membrane damage. This is supported by the fact that neutrophil antimicro-

bial peptides act synergistically with peptidoglycan recognition proteins to kill bacteria (33). In addition, bacterial killing by purified DAO is likely mediated by the terminal hydrogen peroxide product that forms, requiring higher concentrations to reach toxic levels. However, DAO-derived hydrogen peroxide in neutrophils would be converted by MPO to more-toxic secondary products. Indeed, steady-state levels of hydrogen peroxide in the low micromolar range appear sufficient to support killing in neutrophil phagosomes (10).

Salmonella has been reported to actively disrupt the trafficking of NADPH oxidase to the *Salmonella*-containing vacuole (34), although the molecular basis for this observation has not been uncovered. The subversion by *Salmonella* of this additional oxidative stress might provide a more complete inhibition of oxidative killing in neutrophils, allowing *Salmonella* time to access more-permissive splenic macrophages at later stages of infection (15). As predicted by our model, the presence of DalS confers a selective advantage to *Salmonella* in wild-type mice, and it confers a higher selective advantage in NADPH oxidase-deficient mice, but it is dispensable when DAO is inhibited or if neutrophils are depleted. These data highlight a novel interaction between host and microbe that adds to the growing complexity of bacterial pathogenesis. In this context, evasion of neutrophil DAO is a virulence mechanism operating in conjunction with other mechanisms of immune subversion that each confer different but important fitness gains on the infecting pathogen.

Patients with chronic granulomatous disease (CGD) that lack NADPH oxidase activity are highly susceptible to a variety of bacterial infections. However, these patients are rarely infected with catalase-negative organisms (35), suggesting that a source of hydrogen peroxide stress independent of NADPH oxidase may be selective for certain infections in CGD patients (8). A proposed explanation for this was that the bacteria themselves generate enough hydrogen peroxide to mediate MPO-catalyzed halogenation. However, this idea has been challenged because catalase-negative bacteria are equally virulent in a CGD model system and produce less peroxide (approximately 2 orders of magnitude less) than CGD phagocytes with nonprotective levels of residual NADPH oxidase activity (36). Instead, others have argued that the ability of CGD neutrophils to kill even catalase-positive organisms suggests an incomplete loss of hydrogen peroxide and/or an alternate system of intracellular killing (37). Our results are consistent with both of these suggestions. These findings provide rationale for the targeted augmentation of DAO activity in certain immune deficiencies. For example, polyethylene glycol-conjugated DAO can restore bactericidal activity to congenitally defective CGD neutrophils *in vitro* (38). In this disease, even modest residual oxidative activity offers significant protection from severe illness and greater likelihood of long-term survival (39). Thus, augmenting oxidative killing mechanisms in neutrophils may have clinical benefit in some cases.

MATERIALS AND METHODS

Ethics statement. All animal experiments were conducted according to guidelines set by the Canadian Council on Animal Care using protocols approved by the Animal Review Ethics Board at McMaster University.

Bacteria, cloning, and reagents. All bacterial strains are isogenic derivatives of *Salmonella enterica* serovar Typhimurium strain SL1344 and are listed in Table S1 in the supplemental material. The mRNA encoding porcine D-amino acid oxidase (DAO) was amplified by PCR and cloned with a 6-histidine tag as previously described (7) using primers BRT139 (GGA ATT CCA TAT GCG TGT GGT GGT GAT TGG) and BRT140 (GGA AGA TCT TCA GTG GTG GTG GTG GTG GTG GAG GTG GGA TGG TGG CAT T). DNA was digested with BglII and NdeI, and pET3a was digested with BamHI and NdeI and then ligated. All cloning steps were confirmed by sequencing, and plasmids were transformed into *Escherichia coli* BL21(DE3). The OxyR-dependent *ahpC-lux* transcriptional fusion was generated as described previously with slight modifications (19). Three hundred base pairs upstream of the *S.* Typhimurium *ahpC* gene was amplified using primers BRT169 (CGG GAT CCG TAA TGT AGA GCG CAA CAC TT) and BRT171 (CGT ACG TAT ACT TCC TCC GTG TTT TCG TT) and cloned as a SnaBI/BamHI fragment in pGEN-*luxCDABE*.

Protein purification. Purification of the six-histidine-tagged DalS (DalS-6HIS) was performed as described previously (13). Briefly, *E. coli* BL21(DE3) carrying pDalS-6HIS was grown in LB at 37°C and then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 22°C for an additional 3 h. The cells were lysed by sonication, and soluble supernatant was loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) bead column (Qiagen) and eluted with 80 mM imidazole. Recombinant DAO was purified by growing *E. coli* BL21(DE3) carrying pDAO-6HIS in LB at 37°C to an A_{600} of 0.55, and then the bacteria were induced with 0.1 mM IPTG and grown at 37°C for an additional 20 h. Cultures were centrifuged at 4,000 $\times$ *g* for 13 min at 12°C, resuspended in lysis buffer (20 mM Tris [pH 7.5], 0.5 M NaCl, protease inhibitors), and lysed via sonication (Misonix sonicator, Ultrasonic processor S-400, at 40% amplitude with 6 pulses of 30 s at 1-min intervals). Lysates were pelleted at 10,000 $\times$ *g* for 30 min at 4°C. Supernatant was loaded onto a Ni-NTA bead column and washed with an imidazole gradient (10, 20, and 40 mM) in Tris-buffered saline (TBS) (40 mM Tris [pH 7.5], 0.5 M NaCl). DAO-6HIS was eluted in TBS containing 80 to 320 mM imidazole, and its purity was determined by SDS-PAGE. Purified DAO was dialyzed in TBS, concentrated using 3K Amicon Ultra Centrifugal filters (catalog no. UFC800324; Millipore) and stored at −80°C.

Kinetic assays. (i) DAO production of hydrogen peroxide. Enzymatic activity of DAO was measured by colorimetric assay using peroxidase-coupled oxidation of *o*-dianisidine as previously described (1). Briefly, DAO (5 μg/ml), 5 mM amino acid (D-alanine, D-serine, D-valine, and L-alanine; Sigma), horseradish peroxidase (6.6 μg/ml) (Sigma), and 6-chloro-1,2-benzisoxazol-3(2H)-one (CBIO) (0, 0.15, 1.5, and 15 μM) (Sigma) were incubated at room temperature in phosphate-buffered saline (PBS) containing *o*-dianisidine (75 μg/ml), and A_{460} was measured.

(ii) Estimation of D-alanine concentration. *S.* Typhimurium was grown to mid-log phase in LPM medium (pH 5.8) (40) at 37°C. Bacteria were washed twice in phosphate-buffered saline, and 1 $\times$ 10^7 *S.* Typhimurium bacteria were incubated with 5 mM D-alanine for 3 h at 37°C in LPM medium. Cultures were pelleted at 10,000 *g* for 2 min. The supernatant was filter sterilized and incubated with DAO (5 μg/ml) and horseradish peroxidase (6.6 μg/ml) (Sigma) at room temperature in PBS containing 75 μg/ml *o*-dianisidine. A_{460} was measured after 1 h. The D-alanine concentration was determined by comparison to a standard curve.

(iii) FTS assay. DalS binding was determined by fluorescent thermal shift (FTS) assay as described previously (13). Briefly, the assay was performed with a LightCycler 480 system (Roche) (498-nm excitation, 610-nm emission). Each reaction mixture contained DalS-6HIS (10 mM), SyPro Orange (5 μM) (Invitrogen), and the indicated amino acid or CBIO in a mixture of 100 mM HEPES and 150 mM NaCl (pH 7.5) in a 96-well plate (Roche). A change in the melting temperature (ΔT_m) of 2°C upon the addition of ligand was considered positive binding.

Bactericidal activity assays with purified DAO. *S.* Typhimurium was grown to mid-log phase in LPM medium at 37°C and washed twice in PBS. A total of 1 $\times$ 10^7 *S.* Typhimurium were incubated with 5 μg/ml DAO and 5 mM D-alanine in the presence or absence of 150 mM thiourea, 0.1% hydrogen peroxide, 0.01% HOCl, or 2.5 U/ml myeloperoxidase

(MPO) in LPM medium at 37°C with shaking for 2 h. Cultures were serially diluted in PBS and plated on LB to determine killing activity. The number of CFU were determined and normalized to 0-h growth.

Isolation of mouse peritoneal neutrophils and bactericidal activity. Female 6- to 10-week-old C57BL/6 mice (Charles River) were injected with 1 ml of 2% Biogel (Bio-Rad) in PBS. Neutrophils were harvested via peritoneal lavage 12 to 16 h later with 6 ml RPMI medium (10% fetal bovine serum [FBS], 1× HEPES, 1× sodium pyruvate, 1× β-mercaptoethanol, 1× essential amino acids). Cells were passed through a 40-μm cell strainer to remove Biogel, and purity was determined by Giemsa staining (Sigma). Approximately 2×10^7 neutrophils were obtained per mouse. Neutrophils were exposed to 3 μM CBIO in PBS or PBS alone at 37°C and 5% CO_2 in RPMI medium for 60 min. Overnight cultures of wild-type *S.* Typhimurium and *S.* Typhimurium Δ*dalS* mutant with or without *ahpC-luxCDABE* were diluted in RPMI medium and added to neutrophils at a multiplicity of infection of 1:100 in tissue culture wells. Infected cells were incubated at 37°C and 5% CO_2 in RPMI medium for 30 min to allow bacterial uptake. Infected cells were washed five times in PBS and incubated for an additional 90 min in RPMI medium or for 30 min for *ahpC-luxCDABE* assays. Luminescence was measured in bacteria containing the *ahpC-lux* reporter. Bacteria were washed five times with PBS and lysed with 250 μl of lysis buffer (1% Triton X-100, 0.1% SDS). Lysates were serially diluted and plated for CFU determination. Bacterial killing was determined as the ratio of CFU at 2 h to the CFU at 0.5 h and normalized to survival of wild-type bacteria.

Animal experiments. For *in vivo* experiments, C57BL/6 (Charles River) or B6.129S6-*CYBB*^{tm1Din}/J (Jackson Laboratory) mice were infected via the peritoneum with 2×10^5 *Salmonella* for competitive experiments as described previously (41). At 24 h (single infections) or 48 to 60 h (competitive infections) after infection, mice were sacrificed by cervical dislocation, and the livers and spleens were harvested. Organs were homogenized in PBS with a mixer mill (10 min, 30 Hz) (Retsch), serially diluted in PBS, and plated on LB containing streptomycin. Colonies were replica plated onto LB agar containing either chloramphenicol or streptomycin to determine the ratio of the wild type to mutant colonies. To induce neutropenia, mice were injected intraperitoneally (i.p.) with 0.15 mg anti-Ly6G clone 1A8 antibody (BioXcell) daily for two consecutive days prior to infection. To confirm neutropenia by flow cytometry, blood was mixed with acid-citrate-dextrose (ACD) anticoagulant and placed on ice. Cells were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C. Red blood cells were eliminated with ACK lysis buffer (150 mM ammoniumchloride, 10 mM potassium hydrogen carbonate, 1 mM EDTA). The cells were first Fc blocked with anti-mouse CD16/CD32 (1:100; eBioscience) antibody and then stained with anti-mouse CD3e-PE-CF594 (anti-mouse CD3e labeled with phycoerythrin and CF594) (1:200; BD Biosciences), anti-mouse CD11b-PE (1:200; eBioscience), and anti-mouse Gr-1 APC-eFluor 780 (anti-mouse Gr-1 labeled with allophycocyanin and eFluor 780) (1:200; eBioscience) antibodies. All samples were run using an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.). For inhibition of DAO *in vivo*, mice were injected i.p. with 25 mg of CBIO/kg of body weight in 0.5% carboxymethyl cellulose (CMC) (Sigma) or 0.5% CMC only every 6 h during the course of infection.

Immunoblotting. Neutropenic or immune replete C57BL/6 mice were infected with 2×10^5 *S.* Typhimurium in 0.1 M HEPES (pH 8.0) and 0.9% NaCl. The spleens, livers, and ceca were removed 48 h after infection and homogenized as described above in a solution of 50 μM Tris, 150 μM NaCl, and protease inhibitor cocktail (Roche), pH 7.5. Samples were centrifuged at 12,000 × *g* for 20 min at 4°C. Supernatants were diluted in an equal volume of SDS-PAGE sample buffer (100 mM Tris-HCl [pH 6.8], 20% [vol/vol] glycerol, 4% [wt/vol] SDS, 0.002% [wt/vol] bromophenol blue, and 100 mM dithiothreitol [DTT]) and boiled for 5 min. Five-microliter portions from the samples, including 5 μg/ml recombinant DAO labeled with histidine (rDAO-HIS) as a control, were separated on 12% polyacrylamide gels and blotted with goat anti-DAO (1:2,000; Sigma) and sheep anti-goat IgG-HRP (sheep anti-goat IgG labeled with horseradish peroxidase) (1:5,000; Abcam). Blots were stripped and reprobed using mouse anti-GAPDH (antibody against glyceraldehyde-3-phosphate dehydrogenase) (1:1,000; Novus Biologicals) and goat anti-mouse IgG-HRP (1:10,000; Cedarlane). Conjugated HRP was detected using chemiluminescence (Western Lightning Plus; PerkinElmer).

Bioluminescent reporter assay. *S.* Typhimurium containing the P*ahpC-luxCDABE* reporter was grown to mid-log phase in 96-well plates. Bacteria were treated with hydrogen peroxide (0, 2, 4, 6, 8, 10, and 100 μM), and A_{600} and luminescence were measured every 10 min. Luminescence was normalized to the A_{600}.

***In vivo* bioluminescent imaging.** C57BL/6 or B6.129S6-*CYBB*^{tm1Din}/J mice were treated with 25 mg/kg CBIO in 0.5% CMC or 0.5% CMC only and infected i.p. with 1×10^7 *S.* Typhimurium containing the *ahpC-luxCDABE* reporter 1 h after treatment. Mice were anesthetized (2% isoflurane carried in 2% oxygen) 2 h postinfection and imaged for 5 s in a Spectrum *in vivo* Imaging System (IVIS) (Caliper Life Sciences). The spleens were isolated and imaged *ex vivo*. Tissues were homogenized as described above, serially diluted, and plated on LB agar to obtain total CFU per organ. Total flux was normalized to tissue CFU.

Statistical analysis. Treatment groups were compared using a nonparametric Mann-Whitney test. All analyses were performed using Graph Prism 4.0 (GraphPad Software Inc. San Diego, CA). A *P* value of 0.05 or less was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01886-14/-/DCSupplemental.

Figure S1, EPS file, 0.4 MB.
Figure S2, EPS file, 0.4 MB.
Figure S3, EPS file, 0.5 MB.
Table S1, DOCX file, 0.1 MB.

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B.R.T. designed and carried out the *in vitro* experiments and performed the *in vivo* experiments. S.A.R.-Y. assisted with neutrophil isolations, provided advice on depletion experiments, and did the fluorescence-activated cell sorting (FACS) analysis. B.K.C. designed experiments, analyzed data, wrote the paper, and acquired funding for the experiments.

We declare that we have no competing financial interests related to this work.

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