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MOLECULAR PATHOGENESIS

Synergy between Nutritional Immunity and Independent Host Defenses Contributes to the Importance of the MntABC Manganese Transporter during *Staphylococcus aureus* Infection

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ABSTRACT During infection, the host utilizes a diverse array of processes to combat invaders, including the restriction of availability of essential nutrients such as manganese. Similarly to many other pathogens, *Staphylococcus aureus* possesses two manganese importers, MntH and MntABC. Several infection models have revealed a critical role for MntABC during staphylococcal infection. However, culture-based studies have suggested parity between the two transporters when cells are resisting manganese starvation imposed by the manganese binding immune effector calprotectin. In this investigation, initial elemental analysis revealed that MntABC is the primary transporter responsible for obtaining manganese in culture in the presence of calprotectin. MntABC was also necessary to maintain wild-type levels of manganese-dependent superoxide dismutase activity in the presence of calprotectin. Building on this framework, we investigated if MntABC enabled *S. aureus* to resist the synergistic actions of nutritional immunity and other host defenses. This analysis revealed that MntABC critically contributes to staphylococcal growth when *S. aureus* is subjected to manganese limitations and exposed to oxidative stress. This transporter was also important for growth in manganese-limited environments when *S. aureus* was forced to consume glucose as an energy source, which occurs when it encounters nitric oxide. MntABC also expanded the pH range conducive for *S. aureus* growth under conditions of manganese scarcity. Collectively, the data presented in this work provide a robust molecular basis for the crucial role of MntABC in staphylococcal virulence. Further, this work highlights the importance of synergy between host defenses and the necessity of evaluating the contribution of virulence factors to pathogenesis in the presence of multiple stressors.

KEYWORDS ABC transporters, calprotectin, infection, manganese, MntABC, MntH, nutritional immunity, *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacterium that asymptomatically colonizes the anterior nares and skin of about one-third of the world's population and that, upon breach of the epithelial cell barrier, can infect nearly every organ (1–3). The treatment of *S. aureus* infections is increasingly challenging due to the rise of antibiotic resistance. Both the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) have identified *S. aureus* as a serious threat to human health, stating that there is a critical need to develop novel therapeutics to treat antibiotic-resistant infections (4, 5). One approach to solving this challenge is targeting the mechanisms that pathogens utilize to subvert or evade host defenses.

Citation Radin JN, Zhu J, Brazel EB, McDevitt CA, Kehl-Fie TE. 2019. Synergy between nutritional immunity and independent host defenses contributes to the importance of the MntABC manganese transporter during *Staphylococcus aureus* infection. Infect Immun 87:e00642-18. https://doi.org/10.1128/IAI.00642-18.

Editor Victor J. Torres, New York University School of Medicine

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Received 21 August 2018
Returned for modification 6 September 2018
Accepted 16 October 2018
Accepted manuscript posted online 22 October 2018
Published 19 December 2018

During infection, *S. aureus* and other pathogens must acquire a broad array of essential nutrients such as metal ions from the host. The first-row transition metals iron (Fe), manganese (Mn), and zinc (Zn) are essential for life due to their roles in facilitating the structure and function of proteins (6). Their importance is highlighted by bioinformatic analyses, which suggest that ~30% of all proteins interact with a metal ion (6, 7). The host leverages the essentiality of first-row transition metals to defend itself against invading pathogens by restricting their availability during infection, a strategy known as nutritional immunity (8–10). The prototypic example of Mn and Zn restriction is the staphylococcal abscess, which is rendered devoid of these two essential metals (11, 12). The innate immune effector calprotectin (CP) is a key component of nutritional immunity and contributes to this host defense by withholding Mn and Zn from invading pathogens (9, 11, 13). CP is highly expressed in neutrophils, where it composes ~50% of the total protein in the cytoplasm (14, 15). At sites of infection, CP can be found at levels in excess of 1 mg/ml, and CP-deficient mice, which fail to sequester Mn, are more susceptible to a range of both bacterial and fungal pathogens, including *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Aspergillus fumigatus* (11, 13, 16–20). CP is a heterodimer comprised of S100A8 and S100A9 and features two transition metal-binding sites, which are responsible for sequestration of Mn and Zn (13, 16, 21, 22). The first site (S1) comprises six histidine residues and is able to bind a single Mn or Zn ion with subnanomolar and picomolar affinities, respectively (23). Site 1 also binds Fe(II) and nickel (Ni) (24, 25). However, the physiological relevance and antimicrobial contribution of this activity remain unclear. The second site (S2) consists of an aspartic acid and three histidine residues and binds Zn with subpicomolar affinity (13, 16, 23, 26).

Despite experiencing Mn and Zn starvation during infection, *S. aureus* and other successful pathogens are still capable of causing infection. A common mechanism employed by pathogens to overcome host-mediated metal ion limitation is expression of dedicated high-affinity acquisition systems. *S. aureus* expresses two Mn transporters: MntH, a natural resistance-associated macrophage protein (NRAMP) family member, and MntABC, an ATP-binding cassette (ABC) permease (12, 27, 28). MntH is constitutively expressed, with modest modulation of expression occurring in response to Mn availability, while expression of MntABC is repressed when Mn is available and induced by Mn limitation (12, 27). Expression of both *mntABC* and *mntH* is controlled by the intracellular Mn sensing regulator MntR. In the presence of Mn, MntR represses expression of *mntABC*. While MntR is considered to be a canonical repressor, it is necessary for maximal expression of *mntH* (27). Homologs of both MntABC and MntH are widely distributed throughout prokaryotes and contribute to bacterial virulence (28–39). In the context of *S. aureus*, data from previous studies showed that simultaneous loss of the two Mn transport systems rendered the bacterium more sensitive to CP treatment *in vitro* and profoundly impacted pathogenesis in various models of infection (12, 27, 40, 41). Surprisingly, although loss of MntC, the Mn-recruiting protein of the ABC import pathway, resulted in a modest reduction in superoxide dismutase (SOD) activity, a Δ*mntC* mutant was not more sensitive to CP in the presence or absence of oxidative stress (12). In contrast to those culture-based studies, loss of MntABC resulted in virulence defects in several animal models of infection, implicating this pathway as the dominant Mn import system in the host (12, 42–44). Although *in vitro* growth defects associated with loss of MntABC have been reported, those previous studies investigated conditions that are presumed to represent metal-replete environments (42, 44). As CP has a greater affinity for Mn than MntC (45), this raises the possibility that MntABC may not be the primary transporter responsible for resisting host-imposed Mn starvation. Therefore, the relative contributions of MntABC and MntH to resisting nutritional immunity remain unclear.

Pathogenic organisms must also contend with other host defenses, such as the oxidative burst of neutrophils (46) and host niches that may impact the function of MntABC and MntH (47–52). Enzymes, such as the superoxide dismutases (SODs), allow bacteria to detoxify damaging reactive oxygen species (53–55). However, this process

is more challenging because of the concomitant restriction of essential metal ion availability, which can inactivate metal-dependent proteins such as Mn-dependent SODs (11, 13). Thus, nutritional immunity and the oxidative burst function synergistically during infection, with Mn sequestration sensitizing *S. aureus* to superoxide generated by immune cells (13). Exposure to host-generated nitric oxide ($NO^{\cdot}$) free radicals forces *S. aureus* to consume glucose (49). Also, recent work revealed that manganese starvation reduces the ability of *S. aureus* to utilize glucose as an energy source (50), suggesting that manganese limitation may function synergistically with host defenses beyond oxidative stress. The contribution of Mn to resisting independent host defenses underscores the need to understand how *S. aureus* obtains this nutrient when Mn availability is limited by the host.

MntC is highly conserved in staphylococcal strains, is expressed on the surface of *S. aureus*, and is currently being investigated as a component of a multiantigen vaccine in clinical trials (56). Hence, how MntABC contributes to infection is crucial to understand. In the current study, the individual contributions of MntABC and MntH to *S. aureus* pathogenicity during Mn limitation were examined. Elemental analyses showed that MntABC is the primary transporter that enables *S. aureus* to compete with CP for Mn acquisition. Our investigations also revealed that loss of MntABC, but not MntH, makes *S. aureus* more vulnerable to Mn limitation and concomitant oxidative stress due to a reduced ability to maintain a robust antioxidant defense. The contribution of MntABC to staphylococcal growth when Mn availability is restricted also becomes more important in environments when the cellular demand for this metal is increased, such as when cells are forced to rely on glucose as an energy source. Similarly to studies of other pathogens that utilize both NRAMP and ABC importers to obtain Mn, in the current study we observed an important role for MntABC in the acquisition of Mn under alkaline conditions. Collectively, these results show that MntABC, not MntH, enables *S. aureus* to compete with the host for Mn during infection and reveal that the crucial role of this transporter is necessitated by the synergistic action of nutritional immunity with other host defenses.

RESULTS

MntABC is the primary transporter responsible for manganese uptake in the presence of calprotectin. To define the respective contributions of MntABC and MntH to resisting host-imposed Mn limitation, wild-type, Δ*mntC*, and Δ*mntH S. aureus* strains were grown in rich medium in the presence and absence of CP, with accumulation of first-row transition metal ions assessed by inductively coupled plasma mass spectrometry (ICP-MS) (Fig. 1). In metal-replete medium, the wild-type and mutant strains accumulated similar levels of Mn (Fig. 1A). Consistent with prior results, CP treatment reduced intracellular Mn levels in all strains (11, 50). However, the Δ*mntC* mutant accumulated significantly less Mn than either the wild type or the Δ*mntH* strain, indicating that MntABC is the primary transporter responsible for resisting host-imposed Mn starvation. Similarly to prior observations with wild-type *S. aureus*, CP did not reduce intracellular Fe or Zn levels in any of the strains examined (Fig. 1B and F). Given the ability of CP to bind other first-row transition metal ions (24, 25), we assessed if loss of MntABC or MntH affected their accumulation. CP did not reduce the accumulation of Co, Ni, or Cu in wild-type bacteria or the Δ*mntC* and Δ*mntH* mutants (Fig. 1C to E). Surprisingly, growth in the presence of CP increased staphylococcal accumulation of Co and Ni (Fig. 1C and D), suggesting that even though CP can bind Co and Ni, it does not starve *S. aureus* for these metals. Collectively, these results indicate that MntABC is the primary system responsible for competing with the host for Mn and that *S. aureus* can successfully compete with CP for other metals.

MntABC facilitates manganese binding by critical enzymes. Although MntABC strongly contributes to Mn acquisition in the presence of CP, it may not be essential to prevent inhibition of Mn-dependent enzymes. In *S. aureus*, the activities of the two SODs, SodA and SodM, strongly correlate with intracellular Mn abundance. In the case of SodA, which is strictly Mn dependent, reduced levels of Mn accumulation result in

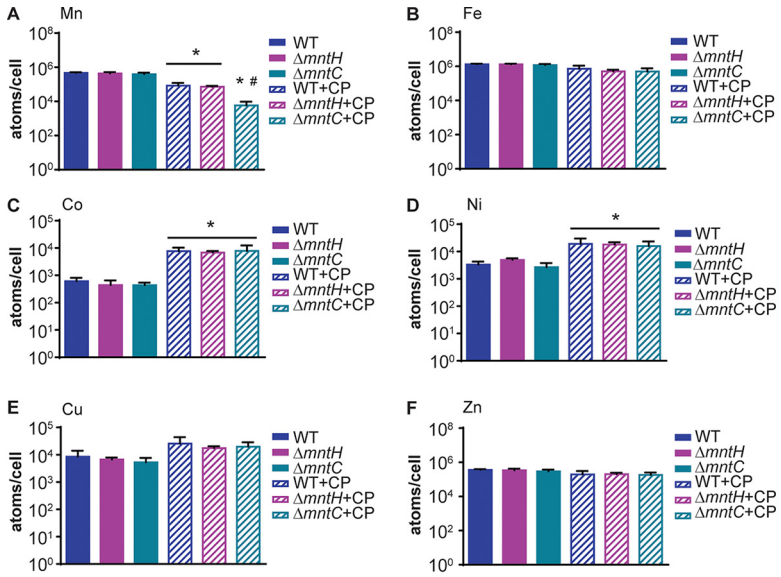

FIG 1 Calprotectin starves *S. aureus* for Mn. *S. aureus* wild-type, Δ*mntH*, and Δ*mntC* strains were precultured in metal-limited medium (NRPMI) and then grown in rich medium (TSB) in the presence or absence of 240 μg/ml of wild-type (WT) CP, and intracellular Mn (A), Fe (B), Co (C), Ni (D), Cu (E), and Zn (F) levels were determined by ICP-MS. * = $P \leq 0.05$ relative to untreated cells and # = $P \leq 0.05$ relative to wild-type *S. aureus* and Δ*mntH* mutant cells grown in the presence of 240 μg/ml of WT CP by one-way analysis of variance (ANOVA) with Tukey's posttests of selected means. $n \geq 3$. Error bars indicate standard errors of the means (SEM).

reduced enzymatic activity (41). In contrast, for SodM, which is cambialistic and can utilize either Mn or Fe as a cofactor, Mn limitation results in increased Fe-dependent activity (41). Hence, we sought to determine if reduced Mn accumulation in the Δ*mntC* strain in the presence of CP impacted enzyme function and metalation. The individual activities of SodA and SodM in wild-type bacteria and in the Δ*mntC* and Δ*mntH* single and Δ*mntC* Δ*mntH* double mutants were determined following growth in the presence and absence of CP. In the absence of CP, when Mn was abundant, the predominant SOD activity in all four strains was attributable to SodA (Fig. 2A and B). In the presence of an intermediate concentration of CP (240 μg/ml), SOD activity in both the wild type and the Δ*mntH* strain was mainly from SodA (Fig. 2C and D). In contrast, for both the Δ*mntC* and Δ*mntC* Δ*mntH* mutants in the presence of CP, the activity associated with SodA decreased whereas that of SodM increased. Hydrogen peroxide treatment, which inactivates Fe-containing but not Mn-containing SODs (41, 57), revealed that the activity associated with SodM came from the Fe-loaded form (Fig. 2C). Taken together, these observations indicate that the reduced Mn accumulation of the Δ*mntC* mutant in the presence of CP impacts SOD metalation and function.

Oxidative stress may potentially alter the relative importance of MntABC and MntH pathways in acquiring Mn. To evaluate this possibility, wild-type *S. aureus* and the three transporter mutants were grown in the presence of the superoxide-generating compound paraquat (PQ). In the absence of CP, exposure to PQ did not alter the pattern of SOD activity for any of the strains, with the majority of activity coming from SodA (Fig. 2E and F). In the presence of CP and PQ, SodA was the dominant SOD in the wild type and the Δ*mntH* strain, while the Δ*mntC* and Δ*mntC* Δ*mntH* mutants showed reduced SodA activity and increased Fe-dependent SodM activity (Fig. 2G and H). If anything, the treatment with PQ resulted in a CP-driven transition to SodM as the primary SOD in the Δ*mntC* and Δ*mntC* Δ*mntH* mutants that was more pronounced. These observations highlight the critical role of MntABC as the primary Mn transporter and its contributions to maintaining the activity of Mn-dependent enzymes when Mn starved by the host.

Oxidative stress increases the importance of MntABC. The data reported above indicate that MntABC is the primary Mn transporter and serves to facilitate Mn-SOD

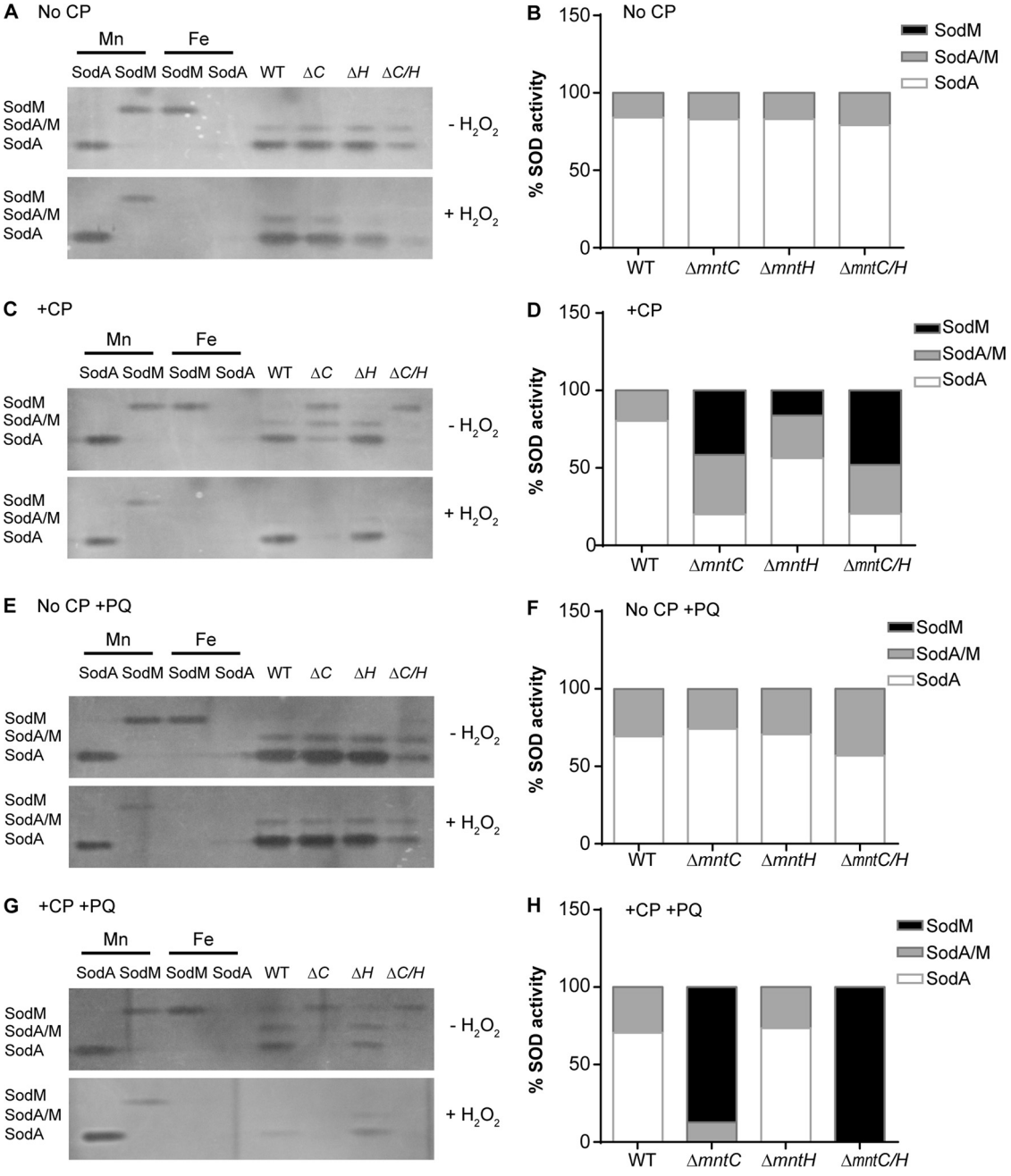

FIG 2 Loss of MntC reduces the ability of *S. aureus* to populate metal-dependent enzymes with manganese. *S. aureus* wild-type, Δ*mntC* (Δ*C*), Δ*mntH* (Δ*H*), and Δ*mntC* Δ*mntH* (Δ*C/H*) strains were prestarved for metals and then grown in rich medium in the absence (A, B, E, and F) or presence (C, D, G, and H) of 240 μg/ml of CP as well as in the absence (A to D) or presence (E to H) of 0.1 mM PQ. The fractional contribution of SodA and SodM to total SOD activity in cell lysates (5.17 μg of total protein) was determined. The lower gel was treated with hydrogen peroxide prior to assessing SOD activity to inactivate Fe-containing SODs. Purified recombinant SodA and SodM (0.3 μg), loaded with either Mn or Fe, were included as controls. Graphs represent averages of results from three independent experiments. Representative gels are shown. "SodA/M" refers to the heterodimer formed between SodA and SodM.

activity in the presence of CP. Despite this, no growth defect has previously been observed for the Δ*mntC* strain in the presence of CP (12). One plausible explanation for the lack of a phenotypic impact could be that the previous study used a rich growth medium (tryptic soy broth [TSB]) that permits sufficient Mn accumulation. To address this inference, we precultured the wild-type and Δ*mntC*, Δ*mntH*, and Δ*mntC* Δ*mntH* strains in Mn-restricted medium (Chelex-treated RPMI medium plus 1% Casamino Acids [NRPMI]) and performed CP growth assays. Differing from those used in prior studies,

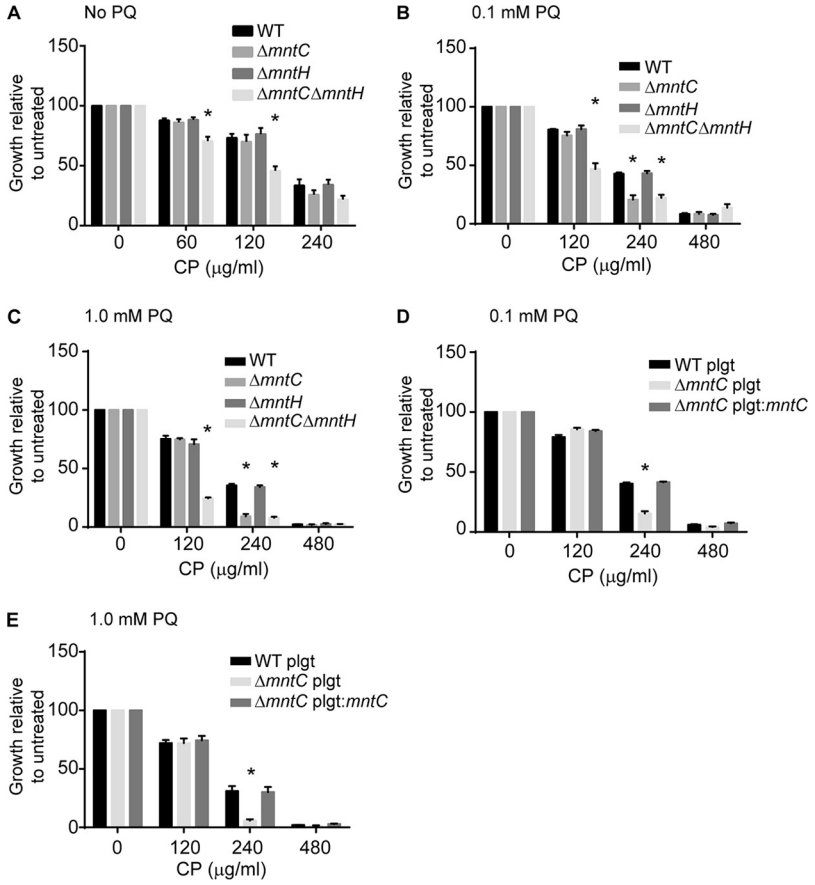

FIG 3 Oxidative stress increases the importance of MntABC. (A to C) *S. aureus* wild-type, Δ*mntC*, Δ*mntH*, and Δ*mntC* Δ*mntH* cells were prestarved for metals, and growth assays were performed in rich medium in the presence of increasing concentrations of CP and in the absence (A) and the presence of 0.1 mM PQ (B) and 1.0 mM PQ (C). (D and E) Growth assays were performed in the presence of increasing concentrations of CP for wild-type and Δ*mntC* strains containing either pOS1 plgt (plgt) or pOS1 plgt:*mntC* (plgt:*mntC*) in the presence of 0.1 mM PQ (D) or 1.0 mM PQ (E). * = $P \leq 0.05$ (two-way ANOVA with Tukey's posttest corrected for repeated measurements). $n \geq 3$. Error bars indicate SEM.

these culture conditions better recapitulate the observed contributions of SodA and SodM to maintaining a defense against oxidative stress during infection (41). When precultured in a Mn-restricted medium, the Δ*mntC* Δ*mntH* mutant showed increased sensitivity to CP, while the Δ*mntC* and Δ*mntH* strains were unaffected (Fig. 3A). In the presence of CP and PQ, both the Δ*mntC* and Δ*mntC* Δ*mntH* strains were more sensitive to CP treatment than the wild-type bacteria (Fig. 3B). By contrast, the Δ*mntH* mutant showed no increase in sensitivity. Increasing the PQ concentration further diminished the growth of the Δ*mntC* strain, but not of the Δ*mntH* strain, compared to the wild-type results (Fig. 3C). Ectopic expression of MntC reversed the phenotype (Fig. 3D and E), confirming that the growth defect was attributable to impaired Mn import by MntABC. These results show that MntABC is crucial for resisting Mn starvation when bacteria are exposed to the synergistic insults of Mn limitation and oxidative stress.

Metabolic demands during infection increase the importance of MntABC. Building on the observation that oxidative stress increases the importance of MntABC, we examined the impact of other stresses associated with host resistance to bacterial infection. Previously, we observed that CP treatment shifts *S. aureus* metabolism from sugar to amino acid catabolism due to the increased cellular requirement for Mn imposed by using glucose as a carbon source (50, 58, 59). This shift appears to occur in response to glycolysis being disrupted by Mn limitation, a phenotype that has also been observed in *Streptococcus pneumoniae* and *Bradyrhizobium japonicum* (58, 59). At

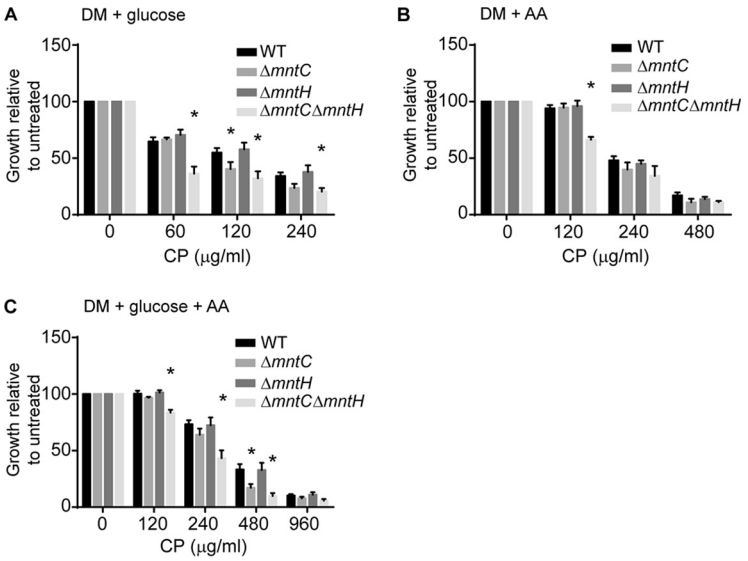

FIG 4 MntABC facilitates glucose consumption when manganese starved. Growth assays were performed with *S. aureus* wild-type, Δ*mntC*, Δ*mntH*, and Δ*mntC* Δ*mntH* strains in defined medium (DM) containing glucose only (A), Casamino Acids (AA) only (B), or glucose and Casamino Acids (AA) (C) as a carbon source in the presence of increasing concentrations of CP. * = $P \leq 0.05$ (two-way ANOVA with Tukey's posttest corrected for repeated measurements). $n \geq 3$. Error bars indicate SEM.

the same time, it is also well established that *S. aureus* resistance to $NO^{\cdot}$ requires glucose consumption (48, 49, 60). Given these divergent pressures, we investigated whether factors beyond oxidative stress, such as the need to consume glucose, contribute to the importance of MntABC during infection. Using a defined medium supplemented with glucose alone, Casamino Acids alone, or glucose and Casamino Acids as carbon sources, the sensitivity of wild-type and mutant variant strains to CP was evaluated. When glucose was the sole carbon source, the wild-type and Δ*mntH* strains grew similarly in the presence of CP. By contrast, the Δ*mntC* and Δ*mntC* Δ*mntH* mutants showed increased sensitivity to metal starvation (Fig. 4A). The diminished ability of the Δ*mntC* and Δ*mntC* Δ*mntH* mutants to resist Mn starvation relative to wild-type bacteria was largely abolished when Casamino Acids were provided as the sole carbon source, with the only exception occurring in the double mutant at 120 μg/ml of CP (Fig. 4B). Surprisingly, in the presence of both glucose and Casamino Acids, the Δ*mntC* and Δ*mntC* Δ*mntH* mutants were still more sensitive to CP than either the wild type or the Δ*mntH* mutant (Fig. 4C). Collectively, these results show that loss of MntABC impairs *S. aureus* growth in Mn-limited environments when glucose is present. Consequently, our findings demonstrate that MntABC provides a crucial mechanism that enhances the capacity of *S. aureus* to retain glycolytic function in Mn-limited environments.

MntABC facilitates growth in alkaline metal-restricted environments. During human infection, bacteria are exposed to pH stress, such as the acidic conditions of the phagosome and the slightly alkaline nature of the blood (51, 61). Notably, alkalization precedes infection by *S. aureus* in burn wounds (52). In *Salmonella enterica* serovar Typhimurium, and in other pathogenic bacteria that contain homologs of both MntH and MntABC transporters, MntH has been shown to be a proton-driven transporter that functions more effectively under acidic conditions, whereas MntABC is more active under alkaline conditions (62, 63). To determine if the same were true for *S. aureus* MntH and MntABC, the sensitivity of the wild-type strain and the Δ*mntC*, Δ*mntH*, and Δ*mntC* Δ*mntH* mutants to CP was evaluated in defined medium supplemented with glucose and buffered to pH 6.4 (acidic), pH 7.4 (neutral), and pH 8.4 (alkaline). In metal-replete medium, all three of the mutants grew similarly to wild-type *S. aureus*. In the presence of CP, however, the Δ*mntC* Δ*mntH* strain had a pronounced growth defect

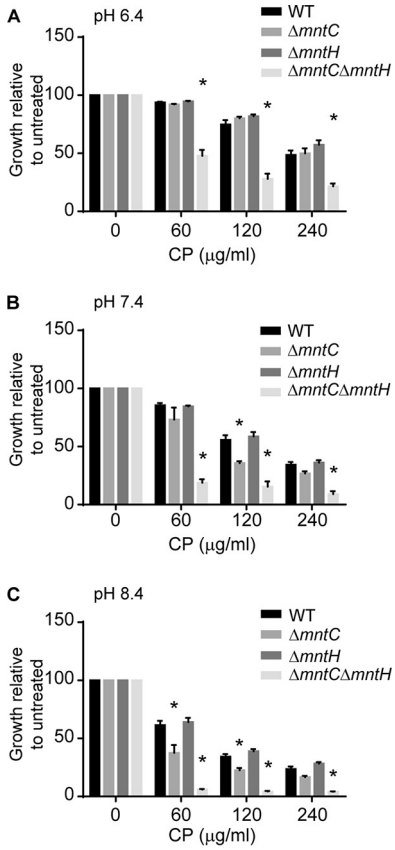

FIG 5 Alkaline environments increase the importance of MntABC to staphylococcal growth. Growth assays were performed in the presence of increasing concentrations of CP with *S. aureus* wild-type, Δ*mntC*, Δ*mntH*, and Δ*mntC* Δ*mntH* strains in defined medium containing glucose as a carbon source and adjusted to pH 6.4 (A), pH 7.4 (B), and pH 8.4 (C). * = $P \leq 0.05$ (two-way ANOVA with Tukey's posttest corrected for repeated measurements). $n \geq 3$. Error bars indicate SEM.

in all three media. In alkaline and neutral media, the Δ*mntC* strain showed a phenotype similar to that seen with the Δ*mntC* Δ*mntH* strain. In contrast, the Δ*mntH* mutant grew the same as the wild type (Fig. 5A and B). In acidic medium, however, both the Δ*mntC* and Δ*mntH* mutant strains grew similarly to wild-type *S. aureus* (Fig. 5C). Taken together, these observations indicate that extracellular pH influences the contribution of MntABC and MntH to competing with CP for Mn. Thus, the environment to which the pathogen is exposed during infection may also impact the relative contributions of the two staphylococcal Mn transporters to resisting host-imposed metal starvation.

DISCUSSION

Mn and other transition metals are essential for the survival of pathogens during infection. In the case of Mn, it is an important cofactor for bacterial enzymes, including enzymes involved in carbon metabolism, the stringent response, and detoxification of reactive oxygen species (62). Additionally, Mn can prevent oxidative damage of mononuclear enzymes by replacing Fe in the active site of metalloenzymes (64). Therefore, it is unsurprising that the ability of pathogens to successfully infect a host depends on their ability to efficiently acquire Mn and that the host uses the restriction of Mn as a defense (9, 10, 65). However, successful pathogens, including *S. aureus*, have evolved mechanisms that allow them to circumvent this host defense, with the most common mechanism being the use of dedicated high-affinity acquisition systems (12, 27–35). *S. aureus* possesses two Mn transporters, MntABC and MntH (12, 27, 28), and loss of MntABC has been shown to reduce staphylococcal virulence during infection (27, 42, 43). However, knowledge of the molecular basis for this defect has remained elusive,

and recent work has suggested that MntABC may not be the primary transporter responsible for competing with the host for Mn (45). The current investigation revealed that MntABC is the primary transporter responsible for resisting host-imposed Mn limitation and that additive insults of nutritional immunity and other host defenses drive the importance of this transporter during infection.

Mn import by MntH- or MntABC-like Mn importers is required for the full virulence of numerous pathogens, including *Salmonella enterica* serovar Typhimurium, *Brucella abortus*, *Shigella flexneri*, multiple *Yersinia* and *Streptococcus* species, and others (28, 29, 31–33, 35, 66–68). Mn acquisition has crucial importance in the pathogenesis of numerous bacterial species, and yet there remains significant heterogeneity in the types of Mn transporters employed. The most common Mn importers in pathogenic bacteria are NRAMP systems, such as MntH, and the ABC permeases, such as MntABC. Many species encode one of these transporters, while others, including *S. aureus*, express both (28–35). *In vitro* studies have suggested near-parity between MntABC and MntH with respect to their contributions to growth in Mn-limited environments. However, binding studies have shown that CP can outcompete MntC for Mn (45), suggesting that MntABC might not be the primary staphylococcal Mn transporter during infection. Despite this, multiple animal models have shown that MntABC has a critical role in staphylococcal pathogenesis (27, 42, 43). Our current work unequivocally shows that while expression of *mntABC* does not enable the bacteria to accumulate levels of Mn that are equivalent to those accumulated by bacteria grown in the absence of CP, loss of MntC, but not of MntH, diminished the ability of *S. aureus* to compete with CP. Further, this function was critical for population of *S. aureus* superoxide dismutases with Mn. Notably, loss of MntABC did not reduce the ability of *S. aureus* to obtain any of the other assayed metals. Collectively, these results indicate that, despite CP having a greater affinity for Mn *in vitro* (45), MntABC is necessary, as it enables *S. aureus* to obtain sufficient (but not optimal) quantities of Mn during infection. Further, our work also suggested that pathogens that express MntABC homologs compete more effectively with the host for Mn than those that express only homologs of MntH. Examples include *Salmonella enterica* serovar Typhimurium (69, 70) and avian *Escherichia coli* (34), where the MntABC-like Mn transporter is more important than the MntH-like transporter.

During infection, *S. aureus* and other pathogens must simultaneously overcome nutritional immunity and a plethora of other host defenses. In the current study, loss of MntC alone was sufficient to sensitize *S. aureus* to oxidative stresses in the presence of CP. This observation differs from those reported from previous studies, where loss of both MntABC and MntH was necessary to sensitize *S. aureus* to oxidative stress in the presence of CP (12). The current study differed from the prior work in that we used a medium that limits intracellular Mn levels to a greater extent than the media used in previous investigations. Glucose is the preferred carbon source for *S. aureus* and many other bacteria (71–74). However, the consumption of glucose in *S. aureus* increases the cellular demand for Mn (50). In response to Mn limitation, both *S. aureus* and *S. pneumoniae* reroute metabolism toward the consumption of amino acids (50, 59). Similarly, in response to Zn limitation, *A. baumannii* increases the consumption of histidine (75). In the case of *S. aureus*, these observations are in tension with the observation that people with diabetes, especially those with hyperglycemia, are more susceptible to *S. aureus* infection than healthy individuals (76–80). Those prior observations suggest that *S. aureus* prefers to consume glucose during infection. Additionally, glycolysis enables *S. aureus* to grow when challenged by $NO^{\cdot}$ produced by activated phagocytes (47, 49). In the presence of CP, loss of MntC impaired the ability to grow when glucose was provided as the sole carbon source. Highlighting the preference of *S. aureus* for consuming glucose, inactivation of MntC also impaired growth in defined medium when both glucose and amino acids were present. These observations suggest that an increased ability to compete with the host for Mn enabled by MntC allows *S. aureus* to consume its preferred carbon source and thereby resist $NO^{\cdot}$ stress during infection. This inference is supported by results from a screen that showed

that MntABC contributes to $NO^{\cdot}$ resistance (48). These observations highlight the necessity of maintaining cellular Mn levels during infection and hence the importance of MntABC in staphylococcal infection.

A rodent model of *S. pneumoniae* infection revealed that shortly after infection the pH in the blood increased from neutral to slightly alkaline (51). Burn wound infections have an elevated pH prior to the manifestation of clinical symptoms, and colonization of *S. aureus* or *Staphylococcus epidermidis* was shown previously to be favored by alkaline conditions (52). Therefore, it is highly likely that invading pathogens encounter both alkaline and acidic conditions during infection. Previous studies have investigated the impact that pH has on the relative importance of MntABC and MntH. In *Salmonella enterica* serovar Typhimurium, which encodes both MntH and MntABC homologs, studies have shown that MntABC operates best under slightly alkaline conditions whereas MntH is more active under acidic conditions (62, 63). Similarly to prior work, our findings show that pH impacts the efficacy of the staphylococcal transporters, with MntH functioning optimally in acidic environments. Differing from other pathogens (62, 63), the staphylococcal MntABC appears capable of fully compensating for loss of MntH under the conditions tested. It is possible that MntH may have increased importance in environments that are more acidic than those examined in the current study. While the results of the previous studies have generally been considered from the perspective of transporter function, our current work suggested that altered cellular demand could also contribute to the relative importance of the Mn transporters in acidic and alkaline environments.

If MntABC is important for resisting nutritional immunity and is sufficient under acidic and neutral as well as alkaline conditions, an issue arises as to the necessity of MntH. Our studies failed to reveal physiological conditions that favor MntH, although we did observe that the presence of MntH was sufficient in Mn-replete environments and Mn-depleted acidic environments in the absence of additional stressors. It should be noted that Mn import by the proton-driven MntH is energetically cheaper than that by the ATP-driven MntABC system. Similarly to Mn importers, *S. aureus* expresses multiple inorganic phosphate importers, including a proton-driven importer, PitA, and an ATP-driven ABC transporter, PstSCAB. PitA is constitutively expressed, while PstSCAB is expressed in response to nutrient limitation. Forcing *S. aureus* to rely on PstSCAB in phosphate-replete medium results in a reduced growth rate (81). Hence, it is not unreasonable to speculate that, similarly to PitA, MntH is retained because of its greater energy efficiency for the pathogen.

Since being characterized as a Mn- and Zn-binding protein, CP has been reported to bind nearly all of the first-row transition metals, including Fe and Ni, *in vitro* (24, 25). This has led to suggestions that the antimicrobial activity of CP is driven by its ability to bind a broad array of metal ions. Nevertheless, subsequent studies showed that for *S. aureus*, *A. baumannii*, and *Candida albicans*, the antimicrobial activity of CP in culture is not driven by Fe sequestration (20, 50, 82). Consistent with prior studies (20, 50, 83), our elemental analysis showed that *S. aureus* successfully competes with CP for Fe. Further diminishing the likelihood of a potential role of CP in Fe sequestration is the observation that extracellular Fe exists as Fe(III) during infection and cannot be bound by CP but is bound avidly by transferrin and lactoferrin (16, 84). Hence, the evidence to date does not support the idea of a role for CP as a general metal sequestration protein during infection. The ability of CP to bind Cu and Zn does enable it to limit the ability of *Candida albicans* to obtain both of these metals (82). The current analysis revealed that CP does not reduce the cellular levels of Ni, Co, or Cu in *S. aureus*. Notably, cellular levels of both Ni and Co increased in the presence of CP. This is in contrast to a prior study, which showed reduced cellular Ni concentrations in the presence of CP (25). Differing from the current investigations, the prior study assessed metal accumulation using a metal-depleted defined medium supplemented with excess Ni (25). In the prior study, the observed accumulation of Ni was dependent on the staphylopine-Cnt Zn importer (85). In addition to inducing the expression of Mn transporters, growth of *S. aureus* in the presence of CP also leads to the induction of zinc uptake systems,

including the CntABCDF-staphylopine system (83). While this system functions physiologically as a zinc importer, it is also capable of importing other metals, most notably Co and Ni (85). Induction of this transporter would provide a plausible explanation for the increased accumulation of Co and Ni in the presence of CP. The basis for the difference between the current results and prior results regarding Ni accumulation in the presence of CP is most likely attributable to media formulation. The prior studies used a superphysiological concentration of Ni in the growth medium, facilitating increased accumulation of this metal (85). Thus, despite the ability of CP to bind an array of metals, the antimicrobial activity of this immune effector toward *S. aureus* appears to be driven by Mn limitation, with some contribution from Zn limitation at higher concentrations (16, 50, 83).

The current study identified a diverse collection of host stresses that increase the importance of the MntABC system to infection and provided a molecular rationale for the critical contribution of this system to staphylococcal infection. While diverse, they are unified by increasing the cellular demand for Mn. Thus, nutritional immunity can be viewed as augmenting the activity of well-established host defenses or vice versa. Regardless of perspective, these results highlight the consideration that should be given to potentiation of host defenses in evaluating the contribution of potential virulence factors to the development of disease.

MATERIALS AND METHODS

Bacterial strains. For routine overnight cultures, bacteria were grown in 5 ml of tryptic soy broth (TSB) or Chelex-treated RPMI medium plus 1% Casamino Acids (NRPMI) supplemented with 1 mM $MgCl_2$, 100 μM $CaCl_2$, and 1 μM $FeCl_2$ (12) in 15-ml conical tubes at 37°C on a roller drum. As needed, 10 μg/ml of chloramphenicol was added for plasmid maintenance. *S. aureus* strain Newman and its derivatives were used for all of the experiments. *S. aureus* strains Newman Δ*mntC*, Newman Δ*mntH*, and Newman Δ*mntC* Δ*mntH* and complementation constructs were generated in a previous study (12).

CP growth assays. CP growth assays were performed as described previously (13, 16), with slight modifications. Briefly, for assays using defined medium, bacteria were grown in TSB overnight. The overnight cultures were then back-diluted 1:50 into fresh TSB and grown for 1 h at 37°C. The cultures were diluted 1:100 into 96-well round-bottom plates containing 100 μl of growth medium in the presence of various concentrations of CP. The growth medium for assays using defined medium consisted of 38% medium and 62% CP buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 1 mM $CaCl_2$, 10 mM β-mercaptoethanol) (50). The defined medium consisted of 1.3 g/liter NaCl, 2.6 g/liter NH_4Cl, 5.2 g/liter KH_2PO_4, 18.2 g/liter Na_2HPO_4, 0.593 μg/liter biotin, 0.593 mg/liter nicotinic acid, 0.593 mg/liter pyridoxine-HCl, 0.593 mg/liter thiamine-HCl, 0.296 mg/liter riboflavin, 1.778 g/liter calcium pantothenate, 0.104 g/liter phenylalanine, 0.078 g/l isoleucine, 0.13 g/l tyrosine, 0.053 g/liter cysteine, 0.26 g/liter glutamic acid 0.026 g/liter lysine, 0.182 g/liter methionine, 0.078 g/liter histidine, 0.026 g/liter tryptophan, 0.234 g/liter leucine, 0.234 g/liter aspartic acid, 0.182 g/liter arginine, 0.078 g/liter serine, 0.15 g/liter alanine, 0.078 g/liter threonine, 0.130 g/liter glycine, 0.208 g/liter valine and 0.026 g/liter proline. The defined medium was supplemented with 6 mM $MgSO_4$, 1 μM $FeCl_2$, 1 μM $MnCl_2$, and 1 μM $ZnSO_4$. Casamino Acids (6.5%) and glucose (1.3%) were provided as carbon sources as indicated. Defined medium supplemented with glucose and adjusted to pH 6.4, pH 7.4, or pH 8.4 was similar to the medium described above and was prepared as described previously (81), with MOPS (morpholinepropanesulfonic acid), HEPES, and Tris buffers being used to adjust the pH. When a metal starvation step was included, the bacteria were grown overnight in NRPMI supplemented with 1 mM $MgCl_2$, 100 μM $CaCl_2$, and 1 μM $FeCl_2$, diluted 1:10 into fresh NRPMI, and then inoculated 1:100 into the assay medium. The assay medium consisted of 38% TSB and 62% CP buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 3 mM $CaCl_2$, 10 mM β-mercaptoethanol). For all assays, the bacteria were incubated with shaking at 37°C and growth was measured by assessing optical density at 600 nm (OD_{600}). In the figures, growth is shown as "growth relative to untreated"; i.e., growth was normalized for each strain to growth in the absence of CP. Calprotectin was purified as previously described (13, 16). Where indicated, 0.1 mM or 1.0 mM paraquat (PQ) was added to the assay medium.

SOD activity. Individual superoxide dismutase activity (SOD) was assessed using a gel-based nitroblue tetrazolium assay, as previously described (41, 86). Bacteria were grown overnight in NRPMI supplemented with 1 mM $MgCl_2$, 100 μM $CaCl_2$ and 1 μM $FeCl_2$, diluted 1:10 into fresh NRPMI, and then inoculated 1:100 into the assay medium (38% TSB and 62% CP buffer supplemented with 1 μM $MnCl_2$ and 1 μM $ZnSO_4$) and grown in the presence and absence of 240 μg/ml of CP and in the presence and absence of 0.1 mM PQ. The bacteria were incubated with shaking at 37°C, and growth was measured by assessing optical density (OD_{600}). The bacteria were harvested in exponential phase (OD_{600} = ~0.1), and cells were collected and resuspended in 0.5 mM KPO_4 at pH 7.8 with 0.1 mM EDTA (41). The bacteria were lysed via mechanical disruption, insoluble material was removed by centrifugation, and protein concentrations were determined using a bicinchoninic acid (BCA) assay kit. In order to evaluate individual SOD activity levels, cell lysates with normalized protein concentrations were resolved on 10% native polyacrylamide gels. The gels were then incubated in buffer containing 0.05 M KPO_4 at pH 7.8 with 1 mM

EDTA, 0.25 mM nitro blue tetrazolium chloride, and 0.05 mM riboflavin and exposed to light, as previously described (86). In order to evaluate if SOD was iron loaded, gels were incubated with 20 mM H_2O_2 or water for 20 min prior to gel staining. Gels were imaged using a Bio-Rad Universal Hood II imager, and the fractional distribution of SOD activity was determined using Bio-Rad Quantity One software.

Elemental analyses. For whole-cell metal accumulation, *S. aureus* strains were grown overnight in NRPMI supplemented with 1 mM $MgCl_2$, 100 μM $CaCl_2$ and 1 μM $FeCl_2$, diluted 1:10 into fresh NRPMI, and then inoculated 1:100 into the assay medium. The assay medium consisted of 38% TSB and 62% CP buffer supplemented with 1 μM $MnCl_2$ and 1 μM $ZnSO_4$. Bacteria were harvested during the exponential phase (OD_{600} = 0.1), washed twice with 100 mM EDTA, and washed twice with water. Bacterial pellets were resuspended in 1 ml of water, and a small aliquot was taken to determine CFU. The bacteria were then pelleted, the supernatant was removed, and pellets were desiccated at 96°C overnight. The dry cell weight was determined, the pellets were resuspended in 35% HNO_3, and samples were boiled at 95°C for 1 h prior to removal of debris by centrifugation. Samples were diluted to a final concentration of 3.5% HNO_3 and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) on an Agilent 8900 ICP mass spectrometer (Adelaide Microscopy, University of Adelaide), as described previously (87, 88).

Statistical analysis. All statistical analyses were performed using GraphPad Prism version 6. For details of the specific statistical tests used, see figure legends.

ACKNOWLEDGMENTS

We thank the members of the Kehl-Fie laboratory for critical reading of the manuscript.

This work was supported by grants from the National Institutes of Health (K22 AI104805 and RO1 AI18880), by a March of Dimes Basil O'Conner award and a Vallee Scholars award to T.E.K.-F., and by grants from the National Health and Medical Research Council (project grants 1122582 and 1140554) and the Australian Research Council (Discovery Project DP170102102 and Future Fellowship FT170100006) to C.A.M. This work does not represent the views of the March of Dimes or the National Institutes of Health.

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