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# Differential timing of antibody-mediated phagocytosis and cell-free killing of invasive African *Salmonella* allows immune evasion

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Nontyphoidal *Salmonellae* commonly cause fatal bacteraemia in African children lacking anti-*Salmonella* antibodies. These are facultative intracellular bacteria capable of cell-free and intracellular survival within macrophages. To better understand the relationship between extracellular and intracellular infection in blood and general mechanisms of Ab-related protection against *Salmonella*, we used human blood and sera to measure kinetics of Ab and complement deposition, serum-mediated bactericidal killing and phagocytosis of invasive African *Salmonella enterica* serovar Typhimurium D23580. Binding of antibodies peaked by 30 s, but C3 deposition lagged behind, peaking after 2–4 min. C5b-9 deposition was undetectable until between 2 and 6 min and peaked after 10 min, after which time an increase in serum-mediated killing occurred. In contrast, intracellular, opsonized *Salmonellae* were readily detectable within 5 min. By 10 min, around half of monocytes and most neutrophils contained bacteria. The same kinetics of serum-mediated killing and phagocytosis were observed with *S. enterica* Typhimurium laboratory strain SL1344, and the *S. enterica* Enteritidis African invasive isolate D24954 and laboratory strain PT4. The differential kinetics between cell-free killing and phagocytosis of invasive nontyphoidal *Salmonella* allows these bacteria to escape the blood and establish intracellular infection before they are killed by the membrane attack complex.

**Keywords:** Antibodies · Complement · Opsonization · Phagocytosis · *Salmonella*



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## Introduction

Immunity against the facultative intracellular bacterium *Salmonella* is complex, involving both cellular [1, 2] and humoral mechanisms [3]. Nontyphoidal *Salmonellae* (NTS), particu-

larly *Salmonella enterica* serovar Typhimurium and Enteritidis (*S. Typhimurium* and *S. Enteritidis*) are a major, yet neglected, cause of fatal bacteraemia in Africa [4, 5]. Among young children in sub-Saharan Africa, the period of susceptibility to invasive NTS infections coincides with the loss of maternally acquired IgG prior to the acquisition of the child's own anti-*Salmonella* IgM and IgG. A lack of specific antibodies in African infants is associated with fatal invasive *Salmonella* disease [6]. In vitro work demonstrates

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potentially important roles for both complement-fixing bactericidal antibodies [6] and opsonizing antibodies [7] in African children, and the majority of invasive NTS isolates in Malawi are sensitive to Ab-mediated killing in serum [6].

Although in healthy adults in developed countries nontyphoidal salmonellosis is usually a self-limiting gastrointestinal infection [8], deficiencies in or immaturity of immunity can lead to severe *Salmonella* disease. Individuals with defects in the IL-12/23-IFN- $\gamma$  axis, which affects killing of *Salmonella* within phagocytes, typically develop severe, though nonfatal, recurrent focal infection in lymph nodes, despite the presence of specific antibodies [1,9]. This suggests that appropriate Ab responses can help prevent the uncontrolled expansion of *Salmonella* numbers even in individuals with marked immunodeficiencies, although antibodies alone are insufficient to resolve infection.

Proliferation, spread, and distribution of *Salmonellae* within the host is heterogeneous and not fully understood. Current models of NTS infection in mice indicate that upon infection, a proportion of *Salmonellae* gain access to the bloodstream where complement-mediated clearance occurs [10], while some gain direct access from the gastrointestinal tract into phagocytes via M cells. Once intracellular infection has been established, spread from infected to uninfected phagocytes is likely to occur through cell necrosis and/or apoptosis. Necrosis of infected cells will release individual bacteria into the extracellular space [11].

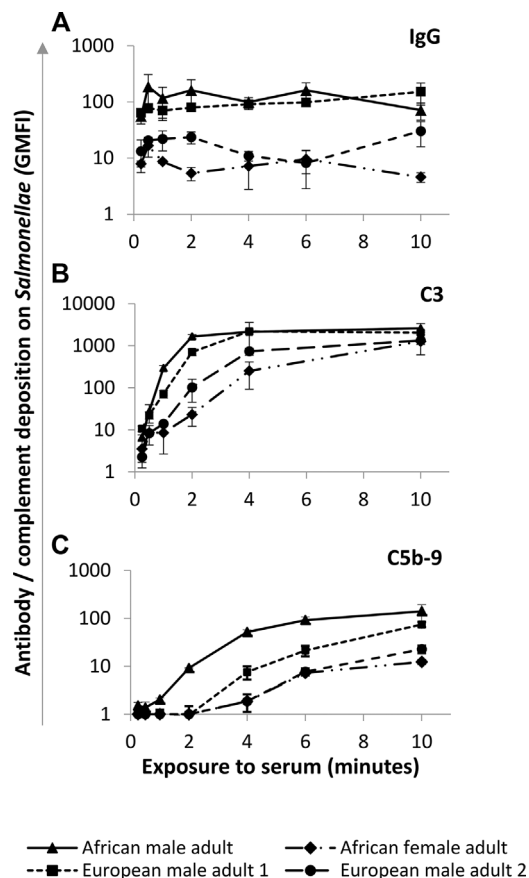
Hence, a proportion of *Salmonellae* are likely to be, at least transiently, present in the extracellular space and blood, where they are exposed to humoral components of the immune system. Ab-dependent complement-mediated killing of extracellular *Salmonellae* appears to be minimal in mice [12], but important in humans [6,13]. As most work on the dissemination of *Salmonellae* within a host has been performed in mouse models, the contribution of cell-free complement killing to the dynamics of infection may be underestimated.

Here we have performed serum bactericidal, phagocytosis, Ab-binding, and complement-deposition time-course assays with human blood and sera in order to better understand the relationship between extracellular killing and uptake into phagocytic cells of invasive African NTS in humans. This demonstrates that uptake of *Salmonella* into the intracellular niche of phagocytic cells can occur before cell-free Ab-mediated killing of bacteria is fully established, allowing a portal through which bacteria can evade extracellular killing.

## Results and discussion

### Deposition of antibodies and complement on *Salmonella* occurs rapidly in serum

In humans, optimal cell-free killing and phagocytosis of *Salmonella* requires deposition of antibodies and complement [6,7]. We investigated the kinetics of binding and deposition of Ab and complement on the bacterial surface. IgG deposition on *S. Typhimurium* D23580 was rapid for all sera and reached peak levels within 30 s (Fig. 1A), although the maximal IgG binding varied between



**Figure 1.** Time course of binding of IgG and deposition of complement components on *S. Typhimurium* D23580 in human serum. *S. Typhimurium* D23580 was incubated in undiluted human serum from four healthy adults for the indicated time points and (A) IgG, (B) C3, and (C) MAC deposition were measured by flow cytometry. Each line style represents the same donor in each panel. Data for each donor were pooled from three independent experiments in which each donor was tested in triplicate and are shown as mean  $\pm$  SD of geometric MFI.

donors. The maximum deposition of the most abundant complement component, C3, occurred at 2–4 min and so lagged behind IgG binding (Fig. 1B).

While the rate of C3 deposition varied between donors, the rank order for the four donors corresponded to the rank order of IgG deposition levels. Nevertheless, by the end of the assay at 10 min, C3 levels on bacteria were comparable between donors. Thus, levels of IgG deposition correspond more closely with the rate of C3 deposition than the final amount of C3 deposited. Percentages of *Salmonellae* labeled with IgG and C3 correlated strongly with the geometric mean IgG and C3 deposited on the bacteria, respectively (Spearman correlation  $r > 0.9$ ,  $p < 0.0001$  for both comparisons) (Supporting Information Fig. 1A–D).

### Formation of bactericidal membrane attack complexes lags behind opsonization of *Salmonellae*

Ab-directed complement-mediated killing of *S. Typhimurium* D23580 is associated with the deposition of terminal complement

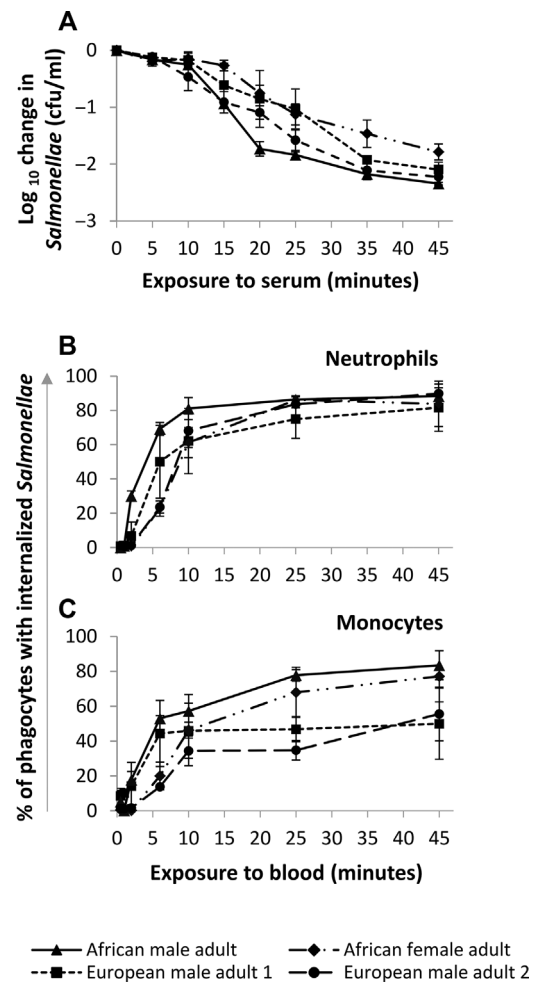
factors to form the bactericidal C5b-9 membrane attack complex (MAC) [6]. Sera lacking specific antibodies are unable to kill *S. Typhimurium* [6] and MAC deposition correlates with IgG binding [14]. In each serum tested, C5b-9 deposition on *S. Typhimurium* developed at a rate that was slower than both IgG binding and C3 deposition (Fig. 1C), with rank order of C5b-9 deposition rate in serum again corresponding to the rank order of IgG binding. Varying between donors, C5b-9 deposition was first detectable at 2–6 min and had not reached a maximum until 10 min. Therefore, although Ab binding is rapid, there can be a lag phase before the machinery that enables cell-free killing is assembled. As for IgG and C3, the percentage of *Salmonellae* labeled with MAC correlated strongly with geometric mean MAC deposition (Spearman correlation  $r > 0.9$ ,  $p < 0.0001$ ) (Supporting Information Fig. 1E and F).

### Cell-free killing of *Salmonellae* by serum follows an initial lag period

Despite the varying rate of deposition of C5b-9 between donors, in all cases serum-mediated killing was limited within the first 5–10 min of the assay (Fig. 2A). After this time there was an increase in the rate of *Salmonella* killing. Such an initial latent period has been observed for other bacterial species and could correspond to the time required for the assembly of the MAC [13]. Our data are consistent with this concept, since maximal serum-mediated killing only occurred after a threshold level of MAC deposition had been exceeded and we have previously reported such a threshold effect [6]. Alternatively, killing could relate to the number of MAC-induced membrane punctures required to cause irreversible damage to the bacterium.

Serum bactericidal assay (SBA) with heat-inactivated serum resulted in no bacterial killing, indicating that killing is complement-mediated and that bacterial agglutination had not led to an artifactual reduction in viable counts. In order to ensure that error was not introduced into the study due to differences in the concentrations of bacteria used in SBA ( $1 \times 10^6$ /mL) and flow cytometric assays to assess Ig and complement difference and phagocytosis ( $2.0$ – $3.3 \times 10^8$ /mL), we repeated the SBA using initial concentrations of  $1 \times 10^6$ ,  $1 \times 10^7$ , and  $1 \times 10^8$ /mL *S. Typhimurium* D23580. There was no significant difference in the killing of *Salmonella* with the three different starting concentrations of bacteria (Supporting Information Fig. 2A). Notably, no net decrease in bacterial numbers was observed during the first 10 min of the assay with any of the three starting concentrations of *Salmonella*.

Our findings are consistent with IgG increasing the efficiency of complement deposition. Removal of *Salmonella*-specific IgG by adsorption abrogated the binding of C3 and deposition of MAC, preventing serum-mediated killing of *Salmonella* (Supporting Information Fig. 3). Killing could be restored by supplementing with heat-inactivated serum as a source of Ab. However, higher levels of *Salmonella*-specific IgG did not necessarily alter the kinetics of cell-free killing. After this lag time, killing progressed readily and once established there was some relationship between levels



**Figure 2.** In vitro time course of serum killing and peripheral blood cell phagocytosis of *S. Typhimurium*. African *S. Typhimurium* D23580 was exposed to undiluted serum and whole blood from four healthy adult donors for the indicated time points. (A) Serum killing of D23580 was determined by serum bactericidal assay, and phagocytosis of FITC-labeled D23580 by (B) neutrophils and (C) monocytes in whole blood was measured by flow cytometry. Each line style represents the same donor as in Figure 1. Data for each donor were pooled from three independent experiments in which each donor was tested in triplicate and are shown as mean  $\pm$  SD.

of IgG deposition and the speed and extent of bacterial killing (Fig. 2A). Serum-mediated killing continued throughout the entire time course of 45 min. Overall, there was a lack of clear correlation between ranked killing rate and binding and deposition of IgG, C3, and MAC, although the serum with the highest associated Ab and complement deposition affected both the quickest serum-mediated killing and phagocytosis of *Salmonellae*.

### Phagocytosis of *Salmonellae* by neutrophils and monocytes initiates rapidly

Phagocytosis by neutrophils (Fig. 2B) and monocytes (Fig. 2C), determined as the percentage of each cell type with internalized FITC-labeled *S. Typhimurium*, began within the first 5 min of exposure to blood. This intracellular uptake of opsonized bacteria

into phagocytes occurred before cell-free complement-mediated killing was fully established and before maximum levels of C5b-9 deposition were detectable. In contrast, by this time there had been up to a 100-fold increase in levels of C3 deposition following Ab binding. In the absence of Ab and complement for opsonization, no uptake into neutrophils or monocytes was observed.

Although *Salmonellae* possess mechanisms that enable evasion of killing within phagocytes, previous work has indicated that *S. Typhimurium* 14028s opsonized with IgG is targeted to the lysosomal compartment of DCs for degradation through FcγRIII-dependent [15] and FcγR-independent mechanisms [16]. Similarly, engagement of FcR on macrophages by antibodies bound to *Legionella pneumophila* and *Mycobacterium bovis* bacillus Calmette-Guérin targets these pathogens to lysosomes [17]. A study of survival of *S. Typhimurium* SL1344 following internalization within macrophages found that bacteria opsonized with complement survived, while those opsonized with Ab were killed [18].

In order to ensure that *Salmonellae* internalized by blood phagocytes are viable, we conducted gentamicin protection assays using *S. Typhimurium* D23580 that had been opsonized with both Ab and complement, and washed blood cells. After 45 min of exposure to blood phagocytes, we found a fourfold increase in viable *Salmonellae* in the intracellular compartment by comparing numbers of viable bacteria following lysis of blood cells or no lysis (Supporting Information Fig. 2B).

### Applicability of findings to invasive African *S. Enteritidis* and laboratory strains of *S. Typhimurium* and *S. Enteritidis*

In order to gauge the generalizability of these findings to other strains of NTS, we repeated the experiments using the *S. Typhimurium* laboratory strain SL1344, and *S. Enteritidis* African invasive isolate D24954 and laboratory strain PT4. The same kinetics of Ab-dependent complement-mediated killing and phagocytic uptake into neutrophils and monocytes were observed for these three strains as for *S. Typhimurium* D23580 (Supporting Information Fig. 2C–E).

### *Salmonellae* can escape to intracellular locations during the extracellular killing lag period

These findings indicated that there is a period of time that can act as a window of opportunity for opsonized bacteria to evade cell-free complement-dependent killing and escape the extracellular environment into their intracellular niche within phagocytic cells. The results are consistent with *Salmonellae* being rapidly opsonized by specific antibodies and complement upon entering the bloodstream. Opsonization facilitates faster uptake by phagocytes, particularly neutrophils [7], allowing the bacteria to escape potential complement-mediated damage from MAC formation. Although opsonization also facilitates killing of bacteria by oxidative burst [7], *Salmonellae* are adapted to survive inside phagocytes by utilizing virulence genes encoded in their

pathogenicity islands [19]. Therefore, the ability of a proportion of *Salmonellae* to evade the cell-free bactericidal mechanism during an infective episode is likely to be advantageous to the bacteria and clinically important.

The effective MAC deposition and Ab-dependent complement-mediated killing of *Salmonellae* suggests that sustained bacteraemia with high concentrations of bacteria in the presence of specific antibodies and functional complement is unlikely. This is consistent with the relatively low blood-borne bacterial numbers seen in most African adults with NTS bacteraemia [20]. Salmonellosis in patients with IL-12/23-IFN-γ axis deficiencies usually presents as focal extra-intestinal disease, particularly in the cervical lymph nodes. Intracellular densities of *Salmonellae* in challenge studies reach higher levels in IFN-γ<sup>-/-</sup> animals than in WT controls [11]. Yet, despite the impact on cell-mediated immunity and associated increased *Salmonella* growth, patients with IL-12/23-IFN-γ axis deficiencies do not develop fatal *Salmonella* bacteraemia. In contrast, salmonellosis in African infants that lack specific antibodies presents as a bacteraemia, which is often fatal.

Our data demonstrate that the kinetics of Ab and complement binding, serum-mediated killing and phagocytosis are consistent with these clinical observations. The relative delay in maximal serum-mediated killing compared with phagocytosis permits the escape and survival of a proportion of *Salmonellae*, which can enable continued and potentially chronic infection. Such a model could explain how complement-fixing *Salmonella*-specific antibodies are unable to prevent severe and recurrent salmonellosis in patients with defective cellular immunity, despite their ability to mediate bactericidal activity [1]. The model is also consistent with the observation in animals that cell-mediated immunity is required for the effective ultimate clearance of *Salmonellae* following infection [21].

The nearly instantaneous binding of antibodies to *Salmonella*, in combination with an opsonic and bactericidal contribution from complement factors, is likely to prevent persistence of *Salmonellae* in the blood. In the *in vivo* setting, this contribution of antibodies could be lifesaving and thus a vaccine inducing a robust Ab response, even in the absence of a strong cellular response, may prevent mortality caused by invasive NTS disease in Africa. Indeed, this is likely to be the manner that the T-independent Vi capsular polysaccharide vaccine against typhoid contributes its protection. Complementary use of antibiotics with good intracellular penetration, such as the fluoroquinolones, can lead to effective clearance of persistent intracellular infection in immunocompetent individuals. Nevertheless, ideally a vaccine against NTS for Africa, where high numbers of HIV-infected individuals represent a significant proportion of the susceptible population, would also induce effective cell-mediated immunity in order to facilitate clearance of intracellular infection and prevent recurrent disease.

### Concluding remarks

NTS are a major cause of fatal bacteraemia in sub-Saharan Africa. The relationship between intracellular and extracellular survival

of these bacteria in the blood during invasive disease in man is not well understood. Studies in mice are likely to underestimate the importance of Ab-dependent complement-mediated cell-free killing of *Salmonellae* and so we used in vitro assays with human serum and blood to investigate this relationship.

Opsonization with C3 occurred rapidly following Ab binding, but deposition of C5b-9 MAC, required for cell-free killing, was slower. The differential kinetics of deposition of these complement components corresponded with a lag between the rapid uptake of *Salmonellae* into blood phagocytes and cell-free complement-mediated killing. This provides an opportunity for *Salmonella* to escape into its intracellular niche and establish chronic latent infection. These findings need to be considered in the design of optimal vaccine and antibiotic therapies against invasive nontyphoidal *Salmonella* in Africa.

## Materials and methods

### *Salmonellae*

*Salmonella* Typhimurium D23580 and *S. Enteritidis* D24954 are typical invasive African isolate of NTS and were obtained from the Malawi-Liverpool-Wellcome Trust Clinical Research Programme/College of Medicine in Blantyre, Malawi. *Salmonella* Typhimurium SL1344 and *S. Enteritidis* PT4 are commonly used laboratory strains of NTS. They are sensitive to killing by human adult serum, undergoing a one to three  $\log_{10}$  kill over a 3 h time course [6]. D23580 is the index African-specific *S. Typhimurium* isolate of the ST 313 pathovar and is representative of over 90% of NTS isolates from bacteremic individuals in Malawi since 2002 and has been genome sequenced at the Wellcome Trust Sanger Institute [22].

### Materials

Unless otherwise stated, materials were from Sigma. Human sera were from two healthy Malawian and two healthy European adults. Serum was separated within 2 h of venesection and stored in aliquots at  $-80^{\circ}\text{C}$ . Unless otherwise stated, serum was freshly thawed prior to use in assays in order to maintain integrity of endogenous complement function. When required, complement function was inactivated by heating serum at  $56^{\circ}\text{C}$  for 30 min. No external sources of complement were used in the study. Ethical approval for the use of serum samples in this study was granted by the Life and Health Sciences Ethical Review Committee of the University of Birmingham. Informed written consent was obtained from all participants.

### Anti-*Salmonella* Ab assays and complement deposition assays

These were performed by flow cytometry as described previously [6]. Briefly,  $5\ \mu\text{L}$  *S. Typhimurium* D23580 in log-growth phase was

mixed with  $45\ \mu\text{L}$  10% freshly thawed serum for Ab binding, or undiluted freshly thawed serum for complement deposition (final *Salmonella* concentration  $2 \times 10^8/\text{mL}$ ). FITC-conjugated anti-IgG, anti-C3, and anti-C5b-9 (MAC) (DAKO) were used for detection prior to analysis on a FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK). *Salmonellae* were identified by light scatter characteristics and *Salmonella*-specific IgG and complement deposition levels were determined as the FL1-H (FITC) geometric MFI.

### *Salmonella* serum bactericidal assays

These were performed as described previously [6]. Briefly,  $5\ \mu\text{L}$  viable *S. Typhimurium* D23580 in log-growth phase (OD 0.3 at 600 nm) was added to  $45\ \mu\text{L}$  freshly thawed undiluted serum (final *Salmonella* concentration  $1 \times 10^6$ ,  $1 \times 10^7$ , and  $1 \times 10^8/\text{mL}$ ) and incubated at  $37^{\circ}\text{C}$  with numbers of viable *Salmonellae* determined by serial dilution and plating in triplicate on Luria Bertani agar at various time points. SBA were performed with heat-inactivated serum as a control in order to confirm that killing of bacteria was complement-mediated and that bacterial agglutination had not led to an artifactual reduction in viable counts.

### Phagocytosis assays

These were performed as previously described [7]. Briefly,  $10\ \mu\text{L}$  of  $2 \times 10^9/\text{mL}$  FITC-labeled *Salmonellae* were added to  $50\ \mu\text{L}$  of blood (final concentration  $3.3 \times 10^8/\text{mL}$ ) and incubated at  $37^{\circ}\text{C}$ , with a paired sample maintained at  $0^{\circ}\text{C}$ . In order to discriminate internalized and surface-bound bacteria, fluorescence from non-internalized bacteria was quenched using  $50\ \mu\text{L}$  of 0.16% Trypan blue. FACS lysing solution was used to lyse red blood cells and fix leukocytes and propidium iodide was added to allow discrimination of leukocytes from free bacteria and cell fragments. Neutrophils and monocytes were distinguished by light scatter characteristics. The percentages of neutrophils and monocytes that had phagocytosed *Salmonellae* were determined by flow cytometry using the FL1-H (FITC) histogram. To confirm the requirement in the assay of opsonization of *Salmonellae* with Ab and complement for uptake into monocytes and neutrophils, a control was performed using blood cells washed in RPMI to remove Ab and complement.

### Gentamicin protection assays

*Salmonella* Typhimurium D23580 were opsonized with Ab and complement by exposure to immune serum for 20 min. They were then incubated for 10 or 45 min at  $37^{\circ}\text{C}$  (final concentration  $2 \times 10^8/\text{mL}$ ) with peripheral blood cells that had been washed twice with RPMI to remove endogenous Ab and complement. After this, blood cells were washed twice with RPMI and gentamicin was added at a final concentration of 1.25 mg/mL. The cells were incubated for a further 30 min on a rocker plate at 20 rpm to kill extracellular bacteria. Blood cell suspensions were again washed

twice with RPMI and lysed using 1:10 deionized water for 1 min or PBS as a nonlysis control. Numbers of viable bacteria released from blood phagocytes were quantified by serial dilution on agar plates.

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**Conflicts of interest:** C.A.M. is an employee of the Novartis Vaccines Institute for Global Health and the recipient of a Clinical Research Fellowship from GlaxoSmithKline.

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**Abbreviations:** MAC: membrane attack complex, · NTS: nontyphoidal *Salmonella*, · S. Typhimurium: *Salmonella enterica* serovar Typhimurium, · S. Enteritidis: *Salmonella enterica* serovar Enteritidis, · SBA: serum bactericidal assay

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