Absent Bactericidal Activity of Mouse Serum against Invasive African Nontyphoidal Salmonella Results from Impaired Complement Function but Not a Lack of Antibody


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Absent Bactericidal Activity of Mouse Serum against Invasive African Nontyphoidal Salmonella Results from Impaired Complement Function but Not a Lack of Antibody


Nontyphoidal strains of Salmonella are a major cause of fatal bacteraemia in Africa. Developing a vaccine requires an improved understanding of the relevant mechanisms of protective immunity, and the mouse model of Salmonella infection is useful for studying immunity to Salmonella in vivo. It is important to appreciate the similarities and differences between immunity to Salmonella in mice and men. Ab is important for protection against nontyphoidal Salmonella in both species, and we have previously found an important role for Ab in cell-free complement-mediated bactericidal activity against Salmonella in Africans. It is unclear whether this modality of immunity is relevant in the mouse model. C57BL/6, BALB/c, and C3H mice immunized with human sera and were unable to kill both wild-type and galE rough mutant of D23580, indicating absent cell-free killing via classical and alternative complement pathways. Supplementing immune mouse sera with human complement enabled killing of Salmonella, whereas addition of human anti-Salmonella Ab to immune mouse sera had no effect. These findings indicate that mouse serum cannot affect cell-free complement-dependent killing of Salmonella, because of the reduced mouse complement ability to kill these bacteria compared with human complement. This difference in Ab-dependent immunity to Salmonella in mice and men must be considered when applying findings from the mouse model of Salmonella disease and vaccination response to man. The Journal of Immunology, 2011, 186: 000–000.

Nontyphoidal Salmonella (NTS) strains, in particular Salmonella enterica serovar Typhimurium, are emerging as a major cause of fatal bacteraemia among African children (1–3) and HIV-infected adults (1, 4, 5). The minimum incidence of NTS bacteraemia is 175 out of 100,000 in Kenyan children under 5 y of age (3), and the case-fatality rate exceeds 20% (2, 6). Partly because of the requirement for blood-culturing facilities in the diagnosis of NTS bacteraemia (2), the scale of the problem caused by this disease has been unappreciated until recently, and currently no vaccine is available. Increasing levels of antibiotic resistance among African strains of NTS (1) and the rapid demise of many children with NTS once they present to hospital indicate that a vaccine is urgently needed. An improved understanding of the relevant protective mechanisms of immunity against Salmonella is required for the development of an effective vaccine against NTS, and this involves complementary studies with human tissues and animal models of salmonellosis. Because S. Typhimurium causes invasive disease in the mouse, this animal provides a useful model for the study of NTS infections in vivo. Salmonellae are adapted to survive within macrophages (7). It has long been appreciated from mouse studies (8, 9) and more recently for studies of patients with rare primary immunodeficiencies (10, 11) that cell-mediated immunity is important for protection against invasive Salmonella disease. From studying NTS bacteremia in Malawi, we have recently shown that NTS bacteremia particularly affects African children between 4 mo and 2 y of age, the period in which Ab levels to NTS are low or absent (12). Ab can potentially protect against Salmonella disease in several ways. First, Ab can protect in a cell-independent manner through complement-dependent bactericidal activity. Second, it can protect through a cell-dependent mechanism involving the oxidative burst by opsonizing bacteria for uptake and killing by phagocytes. In our studies on African children, we have found evidence for an important role for both bactericidal (12) and opsonic Ab (13) in protecting against NTS. Other ways in which Ab can protect against Salmonella disease include Ab-dependent cellular cytotoxicity and blockade of uptake by cells in the gastrointestinal tract.

A second major challenge in developing a vaccine against NTS for Africa is to identify the key molecular targets of the protective immune response against Salmonella. Because much of the assessment of vaccine candidates needs to be performed in vivo, for which animal models of infection are required, it is important to understand the similarities and differences between the immune response to NTS in mice and men.
responses to Salmonella in mice and men. Studies of Salmonella infections in mice have shown that blood Ab titers correlate with protection against Salmonella (14). Ab has been shown to be important for protecting innately susceptible Nramp mouse strains (15, 16), such as C57BL/6 and BALB/c, both in adoptive transfer studies (17, 18) and in studies in T cell-impaired or -deficient mice (19, 20). Recently, studies in C57BL/6 mice have indicated that the outer membrane proteins (19), in particular OmpD (20), are promising targets for vaccine development. Ab has previously been shown to have an important role in opsonizing Salmonella for phagocytosis and cellular killing in C57BL/6 mice (21). However, its ability to kill Salmonella using the cell-independent complement-mediated mechanism described in Africans is uncertain, particularly in view of the recognized limited activity of mouse complement (22, 23).

To address this question, we studied the bactericidal capacity of serum, Ab, and complement from C57BL/6, BALBc, and C3H mice immunized with heat-killed S. typhimurium strains D23580 and SL1344 and live-attenuated strain SL3261.

Materials and Methods
Salmonella
S. typhimurium D23580 is an invasive African strain of NTS isolated from a bacteremic girl aged 26 mo who was admitted to Queen Elizabeth Central Hospital in Blantyre, Malawi, in 2004 (12, 24). This strain is sensitive to killing by human adult serum, undergoing a 1–3 log10 kill over a 3-h time course (see Salmonella killing assays below). It is representative of >90% of NTS strains isolated from bacteremic individuals in Malawi since 2002. A rough galE mutant of D23580 that has truncated LPS lacking O-Ag polysaccharide has previously been described (12). SL1344 is a laboratory strain of S. typhimurium that has been used in many experimental studies. Both strains have been sequenced by the Sanger Core Sequencing Facility at the Wellcome Trust Sanger Institute (24, 25). SL3261 is an attenuated mutant of S. typhimurium with a deleted aroA gene and was kindly provided by B.A.D. Stocker. For immunizations, Salmonella were grown in Luria-Bertani media to log phase, washed twice with PBS, and heat-killed as required at 72˚C for 1 h. No exogenous adjuvants were used for immunizations.

Mice
Animal studies had ethical and home office approval. C57BL/6, BALBc, and C3H mice were age and sex matched, housed in pathogen-free conditions, and used between 6 and 12 wk of age. Mice immunized with heat-killed Salmonella were given 5 × 107 bacteria/animal via the i.p. route at day 0, boosted with the same dose of heat-killed bacteria at day 14, and used between 6 and 12 wk of age. Mice immunized with heat-killed African Salmonellae were given 105 bacteria i.p. at day 0 and exsanguinated at 72˚C for 1 h. No exogenous adjuvants were used for immunizations.

Hemolytic classical pathway complement function assay
Washed rabbit erythrocytes were resuspended in complement fixation diluent (CFD) (Oxoid) at 2% (v/v) and sensitized by incubation for 30 min at room temperature with a 1:500 final dilution in CFD of mouse anti-rabbit erythrocyte antiserum. Sensitized erythrocytes were washed, resuspended at 1% in CFD, aliquoted into the wells of a 96-well plate (50 μl/well), and incubated at 37˚C for 30 min with 50 μl dilutions of mouse serum in CFD. Zero and 100% lysis controls were included in all assays. Plates were centrifuged, and complement activity was assessed by hemoglobin release (absorbance at 415 nm). Percentage lysis for individual wells was calculated using the following formula: percent lysis = (A415[max] - A415[min]) / (A415[max] - A415[1%]) × 100.

Results
Immunization of mice with heat-killed African S. Typhimurium strain D23580 induces a switched Ab response
Groups of six C57BL/6, BALBc, and C3H mice were primed and boosted with heat-killed African S. typhimurium D23580 or PBS (negative control groups). Another two groups of six C57BL/6 mice were either primed and boosted with heat-killed laboratory strain S. typhimurium SL1344 or immunized with the live-attenuated vaccine strains SL3261. At 28 and 35 d, mice were exsanguinated and serum assessed for Ab response to Salmonella. Whereas no anti-Salmonella Ab was detected in serum from the control groups, all mice immunized with Salmonella except two produced IgG and IgM Ab to Salmonella (Fig. 1A, 1B). Serum from the two mice in which the immunizations had failed was discarded. Anti-Salmonella Ab titers were similar regardless of whether the Ab assay was performed using S. typhimurium D23580, SL1344, or SL3261 (Supplemental Fig. 1). Sera prepared from the mice were shown to have intact complement function as demonstrated by lytic activity against sensitized rabbit erythrocytes using a hemoglobin release assay to assess classical pathway complement activity (Fig. 1C). This demonstrated that the processing and storage of the mouse sera had not adversely affected the quality of the complement in these sera.

Sera from S. Typhimurium-immunized mice lack bactericidal activity against wild-type S. Typhimurium
We next tested serum from the immunized mice for ability to kill viable S. typhimurium D23580 in the log-growth phase. Our previous work with serum from African children has shown that, in the presence of specific IgG or IgM Ab, human serum is capable of Ab-dependent complement-mediated killing of D23580 (12). Despite the presence of anti-Salmonella Ab, sera from both immunized and unimmunized C57BL/6 (Fig. 2A), BALB/c (Fig. 2B), and C3H (Fig. 2C) mice were unable to kill wild-type D23580 in vitro over
a 3-h time course, regardless of the immunization strategy used. The concentration of viable \textit{Salmonellae} increased by $1 \log_{10}$ in this period. In contrast, African adult serum affected a $2 \log_{10}$ kill over 3 h (Fig. 2D). The lack of killing of \textit{Salmonellae} by sera from all groups of mice compared with the killing of \textit{Salmonellae} by human serum was highly significant (Student $t$ test, $p < 0.0001$).

These findings could result from the Ab induced following immunization of mice with D23580 or SL1344 lacking inherent bactericidal activity. This could occur if the Ab is unable to facilitate the deposition of complement membrane attack complex at the \textit{Salmonella} outer membrane. Alternatively, mouse complement per se may lack the necessary bactericidal activity to kill \textit{Salmonella}, as suggested by early experiments on mouse complement (22, 23).

\textit{Sera from \textit{S}. \textit{Typhimurium}-immunized mice also lack bactericidal activity mediated through the alternative pathway of complement against a galE$^{-}$ rough mutant of \textit{S}. \textit{Typhimurium}}

To explore these possibilities, we repeated the serum killing assays using a galE$^{-}$ knockout mutant of D23580 that is unable to synthesize the O-Ag polysaccharide of LPS (12). Previously, we showed that this rough mutant is susceptible to killing by serum from African children lacking \textit{Salmonella}-specific Ab via alternative pathway complement activity (12). There was no difference between the results from these experiments and those using wild-type D23580. No \textit{Salmonella} killing occurred with sera from C57BL/6, BALB/c, and C3H mice immunized with D23580, SL1344, and SL3261 or from unimmunized mice (Fig. 3A–C). By contrast, African child serum lacking anti-\textit{Salmonella} Ab was able to effect a $3 \log_{10}$ kill of \textit{S. typhimurium} D23580 (Fig. 3D). Again, the lack of killing of \textit{Salmonellae} by sera from all three groups of

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1}
\caption{Ab response and classical pathway complement activity in mouse sera following immunization with African and laboratory strains of \textit{S. typhimurium}. Titers of anti-\textit{Salmonella} Ab at 28 d after i.p. immunization with two doses of heat-killed \textit{Salmonellae} (D23580 and SL1344) and at 35 d after i.p. immunization with live-attenuated \textit{Salmonellae} (SL3261) and unimmunized (Control). Immunizations for heat-killed \textit{Salmonellae} were with $10^{7}$ heat-killed \textit{S. typhimurium} D23580 (African strain) and SL1344 (laboratory strain). Immunizations for live-attenuated SL3261 were with $5 \times 10^{5}$ \textit{Salmonellae}. Groups of BALB/c, C3H, and C57BL/6 mice received D23580 immunizations, with groups of C57BL/6 mice also receiving SL1344 and SL3261. A, IgG. B, IgM. Ab measured by flow cytometric analysis of Ab-binding to fixed \textit{S. typhimurium} D23580. Each point represents serum from one mouse. Horizontal bars indicate median values. C, Lytic activity of mouse sera against rabbit erythrocytes sensitized with mouse anti-rabbit erythrocyte antiserum, measured using a hemoglobin release assay. Fresh C57BL/6 mice sera (diamonds); frozen freshly thawed mice sera: BALB/c (inverted triangles), C3H (triangles), and C57BL/6 (filled circles); and heat-inactivated C57BL/6 sera (empty circles). Data are means of experiments with sera from six mice ± SD.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2}
\caption{Absence of killing of African wild-type \textit{S. typhimurium} D23580 by mouse sera compared with human sera. In vitro serum bactericidal assay with $10^{6}$ \textit{Salmonellae}/ml at 45, 90, and 180 min time points using sera from C57BL/6 (A), BALB/c (B), or C3H mice (C) immunized with D23580 (squares) and unimmunized mice (circles). A also shows data using sera from C57BL/6 mice immunized with SL1344 (triangles) and SL3261 (inverted triangles). Data are means of experiments with sera from three mice ± SD. D, Killing of wild-type D23580 by control serum from an African adult. Lack of killing of \textit{Salmonellae} by sera from all groups of mice compared with the killing of \textit{Salmonellae} by human serum was highly significant (Student $t$ test, $p < 0.0001$).}
\end{figure}
mice compared with the killing by human serum was highly significant (Student t test, p<0.0001). These findings strongly suggest that the lack of bactericidal activity is the result of comparatively limited mouse complement function against Salmonella, regardless of mouse species tested, the deficiency affecting both classical (Ab-dependent) and alternative pathways.

Sera from S. Typhimurium-immunized mice deposit reduced levels of C3 complement on Salmonella compared with human sera

Because cleavage of C3 and deposition of C3b is the central event in all complement activating pathways, we next looked at deposition of C3 on the surface of wild-type S. typhimurium D23580 by sera from mice immunized with Salmonella using flow cytometry. Whereas sera from unimmunized mice were unable to deposit C3 on Salmonella, sera from C57BL/6, BALB/c, and C3H mice immunized with D23580, SL1344, and SL3261 were able to deposit C3 on the surface of D23580, though ~1 log10 less than the amount of C3 deposited by immune African serum (Student t test, p<0.0001) (Fig. 4A). Using confocal microscopy, qualitatively less C3 deposition was visualized on Salmonella incubated with mouse serum compared with human serum (Fig. 4B). These data therefore indicate deficiency in C3 deposition on Salmonella by mouse serum via the classical pathway relative to human immune serum, but do not exclude the possibility of poor activity in the terminal pathway of complement.

Mouse sera supplemented with human bactericidal anti-Salmonella Ab do not kill S. Typhimurium D23580

To check whether the absence of killing of Salmonella by mouse serum was due to a lack of bactericidal Ab, we supplemented sera from unimmunized C57BL/6, BALB/c, and C3H mice with heat-inactivated serum from African adults containing Ab to Salmonella. We had previously shown that sera from African children that lack Ab to Salmonella become bactericidal when exogenous anti-Salmonella Ab is added (12). Addition of human Ab did not correct the inability of mouse sera to kill D23580, the absence of killing of Salmonella being highly significant compared with the
killing by immune human serum (t test, p < 0.0001) (Fig. 5). This finding suggests that lack of killing of *Salmonellae* by immune mouse serum is not due to a lack of Ab function, further implicating limited mouse complement function against *Salmonella* as the reason for absent bactericidal activity.

**Immune mouse sera supplemented with human complement can kill S. Typhimurium D23580**

To confirm that impaired mouse complement function rather than impaired Ab function was the reason for absent killing of *Salmonellae*, we supplemented C57BL/6, BALB/c, and C3H mouse serum with human complement by mixing mouse serum with human serum lacking Ab to *Salmonella*. Mouse sera could now kill and/or prevent the growth of *Salmonella* at 10- or 100-fold dilutions with PBS (Fig. 6). This was statistically significant compared with both the lack of killing by the anti-*Salmonella* Ab-deficient human serum alone and immunized mouse serum alone (Student t test, p < 0.0001 for serum from mice immunized with heat-killed bacteria, and p < 0.05 for serum from mice immunized with live-attenuated bacteria). This experiment confirms that absent killing of *Salmonellae* by mouse sera is the result of limited mouse complement function against *Salmonella* and that the mouse Ab against *Salmonella* has potential bactericidal activity leading to complement-mediated killing of *Salmonella*, provided a suitable source of exogenous complement is available.

**Discussion**

The main finding from this study is that although C57BL/6, BALB/c, and C3H mice mount both an IgG and IgM Ab response to heat-inactivated African invasive *S. typhimurium* strain D23580 and laboratory strain SL1344 and to live-attenuated *S. typhimurium* strain SL3261, serum from immunized mice lacks bactericidal activity against *Salmonella*. The lack of killing appears to be due to a lack of specific complement activity against *Salmonella*, because the serum is able to lyse erythrocytes in a standard hemolytic assay. This contrasts with the 1–3 log₁₀ killing of D23580, which is affected by African adult and child serum containing anti-*Salmonella* Ab. Serum from African children does not kill *Salmonella* when it lacks specific Ab, and this was the basis for previously describing bactericidal Ab in Africans as an effector of humoral immunity against NTS (12).

*S. typhimurium* D23580 was chosen for this study because it is a typical invasive strain of NTS from Africa, is sensitive to killing in human serum by bactericidal Ab in the presence of complement, and has been sequenced at the Wellcome Trust Sanger Institute (24, 25). Experiments using *S. typhimurium* laboratory diarrheogenic strains SL1344 and SL3261 were included to demonstrate that the lack of bactericidal activity was not restricted to *Salmonella*. The killing of *Salmonella* by each mouse sera supplemented with human complement compared with the lack of killing of by corresponding mouse serum alone (shown in Fig. 2) was statistically significant (Student t test, p < 0.0001 for immunizations with heat-killed bacteria, p < 0.05 for immunizations with live bacteria).
deficiency in serum bactericidal activity in *Salmonella* is not restricted to one inbred strain of mice. Our findings suggest that the protective effect of mouse anti-*Salmonella* Ab is not mediated by cell-independent bactericidal activity.

The *galE* mutant of *S. typhimurium* D23580 is sensitive to killing via the alternative pathway of complement by human serum lacking anti-*Salmonella* Ab (12). Absent killing of the *galE* mutant by all mouse sera therefore implicates limited complement function against *Salmonella* rather than absence of bactericidal Ab itself as the reason for lack of killing with wild-type D23580. Limited complement function in mouse serum was first described >60 y ago (22). Indeed, our finding that serum from immunized mice supplemented with human complement can affect cell-free killing indicates that the Ab generated following immunization with D23580, SL1344, and SL3261 is capable of direct complement-deposition to the *Salmonella* surface at locations that enable membrane attack complex to kill the bacteria (12).

Our data suggest that complement from C57BL/6, BALB/c, and C3H mice is defective in killing D23580 at multiple points in the complement pathway. Because we have previously shown that deposition of complement and killing of wild-type D23580 is Ab dependent and does not proceed via alternative or mannose-binding lectin pathways (12), the lower titers of C3 deposition on *Salmonella* seen with serum from immunized mice compared with human serum indicate that classical pathway activity is reduced in relation to that in human serum. The lack of killing of the D23580 *galE* mutant also indicates limited alternative pathway activity and/or terminal pathway activity.

Nevertheless, functional classical pathway complement activity was detected against sensitized rabbit erythrocytes in the hemoglobin release assay used. We employed this assay to ensure that the lack of bactericidal complement function against *Salmonella* was not merely an artifact of suboptimal preparation and storage of sera. These findings suggest that differences exist between the susceptibility of erythrocytes and *Salmonella* to the lytic/bactericidal effects of mouse complement, with *Salmonella* being more resistant than erythrocytes. Complement that does not insert into the *Salmonella* outer membrane can be shed from the surface of *S. Minnesota* (26, 27), and various molecules on the *Salmonella* surface, including those encoded by *rck* (28), *traT* (29), and *pgtE* (30), confer some degree of resistance to complement-mediated killing.

Such mechanisms are likely to raise the threshold of complement deposition required for bactericidal activity against *Salmonella* to proceed. Previously, we found that the *rck* gene is encoded in the virulence plasmid of D23580 and that C9 is necessary for serum bactericidal activity against D23580 (12). In contrast, several studies on complement-mediated hemolysis have demonstrated that this can proceed in the absence of C9 by limited perturbation of the membrane by C8 (31–33).

With mouse complement lacking bactericidal activity against *Salmonella*, it is likely that the protective effect of anti-*Salmonella* Ab in C57BL/6, BALB/c, and C3H mice is mediated by opsonization facilitating phagocytosis, respiratory burst, and intracellular killing of *Salmonella* by neutrophils, monocytes, and macrophages. The importance of anti-*Salmonella* Ab for these modalities of cell-mediated immunity has been previously demonstrated using serum and cells from C57BL/6 mice (21). Other work in men and mice has indicated that opsonization of *Salmonella* with C3 is required for optimal intracellular killing (13, 34–36), and work with C1q-deficient mice has shown a role for the classical pathway of complement in protecting against *S. typhimurium* (37).

If C3 deposition on *Salmonella* is a requirement for such killing, this suggests that the threshold level of C3 deposition required for opsonization leading to intracellular killing of *Salmonella* is lower than the level required for bactericidal terminal pathway complement activity.

Although *Salmonellae* are facultative intracellular bacteria, we have previously identified that the high case-fatality rate among young African children is associated with extracellular bacterial growth unchecked by bactericidal Ab (12). Because our findings indicate a lack of bactericidal activity in mouse serum against *Salmonella*, this could contribute to the sensitivity of C57BL/6, BALB/c, and C3H mice to infection with *S. typhimurium*. Different mouse strains differ in their ability to handle infection with *Salmonella*, and this may be due to a variety of reasons, including differences in innate Nrp polymorphism-mediated genetic susceptibility of each strain to *Salmonella* and complement function. Variations in complement function between strains can be complex. For example, A/J mice are more effective at depositing C3b on *Salmonella* than C57BL/6 mice but are deficient in the C5 component of complement, hence preventing complement-mediated bactericidal activity against *Salmonella* in such mice (36).

A positive outcome from the current study is the clear demonstration of the bactericidal potential of Ab produced in mice following immunization with *Salmonella* provided suitable exogenous complement is provided. We have been able to use this finding to demonstrate that porins Omp F, C, and D are targets of bactericidal Ab by immunizing mice with these porins and performing bactericidal assays supplemented with human complement (38). Furthermore, this study suggests that the mouse model underestimates the potency of Ab in protecting against *Salmonella* infections. A consequence of this is that if immunizations with an Ag offer Ab-mediated protection in the mouse, they may be more likely to offer protection in humans.

In conclusion, Ab-mediated immunity against NTS in C57BL/6, BALB/c, and C3H mice and African children involves both distinct and shared immunological mechanisms. Bacterial Ab is able to mediate cell-free complement-mediated killing of extracellular bacteria in children, whereas Ab in mice appears incapable of this modality of immunity. In contrast, both human and mouse Ab are able to protect against *Salmonella* via opsonization in conjunction with cell-mediated mechanisms.

**Acknowledgments**

We thank Timothy Hughes for help with mouse complement studies, Robert Kingsley for the *galE* mutant of *S. typhimurium* D23580, the Biomedical Services Unit at the University of Birmingham, and B. Paul Morgan and Pietro Mastroeni for helpful discussions.

**Disclosures**

The authors have no financial conflicts of interest.

**References**

Corrections


In the second to last sentence of the Abstract, the word “affect” should be “effect.” The correct sentence is: “These findings indicate that mouse serum cannot effect cell-free complement-dependent killing of Salmonella, because of the reduced mouse complement ability to kill these bacteria compared with human complement.”

In the Results section, in the second paragraph, line 11, the word “affected” should be “effected”. The correct sentence is: “In contrast, African adult serum effected a 2 log_{10} kill over 3 h (Fig. 2D).”

In the Discussion section, in the first paragraph, line 10, “affected” should be “effected”. The correct sentence is: “This contrasts with the 1–3 log_{10} killing of D23580, which is effected by African adult and child serum containing anti-Salmonella Ab.”

In the Discussion section, in the third paragraph, line 9, “affect” should be “effect.” The correct sentence is: “Indeed, our finding that serum from immunized mice supplemented with human complement can effect cell-free killing indicates that the Ab generated following immunization with D23580, SL1344, and SL3261 is capable of directing complement-deposition to the Salmonella surface at locations that enable membrane attack complex to kill the bacteria (12).”

All instances of “Salmonella typhimurium” and “S. typhimurium” in the article should be changed to “Salmonella Typhimurium” and “S. Typhimurium.” This change was requested by the authors at proof stage and was not made in production, with the exception of the first page of the printed article (page 2365).

All of the above errors have been corrected in the online version, which now differs from the print version as originally published.