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Release kinetics and cell trafficking in relation to bacterial growth explain the time course of blood neutrophils and monocytes during primary *Salmonella* infection

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Abstract

Granulocytes and neutrophils are predominantly responding cells during the early phase of infection of rats with *Salmonella*. We propose mathematical and experimental models of the kinetics of neutrophil and monocyte responses in *Salmonella* infection via the oral route. Using the models, we estimate that approximately 1 in 500 inoculated *Salmonella* cells actually infected the rat and multiplied with a doubling time of 5 h in Peyer's patches, reaching a maximum of ~10^6 c.f.u./g. In low-dose infection, neutrophil and monocyte responses are delayed, but further resemble the responses in high-dose infection. Important processes influencing neutrophil and monocyte recruitment are: massive migration into the infected tissue, and non-linear release kinetics of neutrophils and monocytes from the bone marrow. In conclusion, we can predict time series of neutrophil and monocyte responses in low-dose and high-dose experimental infection via the oral route.

Introduction

Salmonella spp. are facultative intracellular pathogens. Infection is often systemic in rodents. Viable *Salmonella* that survived a series of host barriers access deeper tissues via M cells overlaying the Peyer's patches (1) and via dendritic cells (DC) that penetrate gut epithelial monolayers to sample bacteria (2). Some strains of *Salmonella* can persist and multiply inside macrophages (3,4). $CD18^+$ cells, possibly macrophages, disseminate *Salmonella* from the gastrointestinal tract to systemic organs (5). Macrophage depletion increases resistance to *Salmonella* in naive mice (6), underlining the importance of this cell type in systemic *Salmonella* infection. Neutrophil depletion, on the other hand, increases susceptibility to *Salmonella* infection (7), indicating an important role for neutrophils in resistance to *Salmonella*.

Studying *Salmonella* infection in mice, Meynell and Stocker postulated that a single *Salmonella* cell could cause infection (8), albeit with a low probability. To support the idea, they infected mice with a mixture of flagella variants and demonstrated that *Salmonella* isolated from the dying mice following exposure to a low dose were dominated by a monoculture, because the probability of two *Salmonella* cells simultaneously infecting a mouse is vanishingly small. This experiment supported two hypotheses. First, the probability of infection by a single micro-organism is very small but non-zero (single-hit hypothesis). Second, micro-organisms act independently to infect a host. These hypotheses lead to development of a mathematical model relating the probability of infection to ingested doses of a pathogen (9–11). In experimental infection of rats with *Salmonella enteritidis*, we estimated that ~1 per 1000 c.f.u. of ingested micro-organism causes infection (12). Surviving *Salmonella* cells multiply to form a clone in the host body. The doubling time of *Salmonella* in mice ranges between 1 to 37 h, depending on the host–pathogen combination (13,14).

The influx of neutrophils is bacterial-burden dependent (15). Therefore, the kinetics of neutrophil and monocyte responses should be dependent on the dose and the rate of growth of *Salmonella* that determine the bacterial burden in beginning

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infection. To understand quantitatively the dynamics of host–pathogen interactions in the early phase of infection, we performed a series of dose–response experiments and estimated the probability of infection and the rate of growth of *Salmonella* in rodents. Using these estimates, and mathematically modeling neutrophil and monocyte responses, we predict numbers of neutrophils and monocytes in the blood up to a week after inoculation of a low or a high dose of *Salmonella* via the oral route.

Methods

Animals

Specific pathogen-free Wistar Unilever rats were obtained from the breeding colony at the National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands. The animals, 6–9 weeks of age, were housed individually in macrolon cages, 1–2 weeks prior to inoculation. Drinking water and conventional diet (RMH-B, Hope Farms BV, Woerden, The Netherlands) were provided *ad libitum*.

All other husbandry conditions were maintained according to all applicable provisions of the national laws; Experiments on Animals Decree and Experiments on Animals Act. In accordance with Section 14 of this Act, an officer was appointed to supervise the welfare of laboratory animals. All experiments were discussed and approved by an independent ethical committee prior to the study.

Bacterial strains

Salmonella enterica subsp. *enterica* serovar Enteritidis strain 97-198, a patient isolate (origin RIVM); and *Escherichia coli* WG5, a nalidixic acid resistant derivative of *E. coli* C was used as a negative control (16). From all strains, a stock collection was made by pure culturing on brain heart infusion (BHI) agar (18–20 h at 37°C) and inoculating a single colony in BHI, incubated for 18–20 h at 37°C. After incubation, 0.7 ml of the culture was added to cryotubes filled with glass beads and 0.1 ml of glycerol (82% w/v). Directly after adding the cultures the cryotubes were thoroughly mixed and placed in a −70°C freezer.

Inoculum cultures

Both strains were inoculated by placing one glass bead from the stock collection in 10 ml BHI and incubated at 37°C for 6 ± 2 h. Subsequently, 5 μl of the culture was added to 100 ml BHI and further incubated at 37°C for 18 ± 2 h. After incubation, 50 ml of each culture was centrifuged at 5000 *g* for 10 min at room temperature. The supernatant was discarded and the pellet was resuspended in 50 ml physiological saline (PS, 9 g/l NaCl), followed by recentrifugation. Again, the supernatant was discarded and the pellet was resuspended in a volume of 50 ml PS. Then 0.5 ml of the culture was added to 100 ml of PS. The cell suspension and serial dilutions in PS were delivered at the animal department on melting ice. Directly before administration to the animals, 4 ml of each bacterial suspension was mixed with 4 ml of a solution of 6% (w/v) $NaHCO_3$. After administration, the remainder of the inoculum cultures was transported to the microbiological laboratory on melting ice for plate counts on sheep-blood agar (incubated as above) after appropriate dilution in PS plus 1% peptone (PPS).

The plate counts of different dilutions of the inoculum cultures, used as oral doses for different animals, were 1.1×10^2, 9.5×10^2, 8.5×10^3 and 9.8×10^4 per ml for *S.* Enteritidis and 4.9×10^4 per ml for *E. coli* WG5.

Experimental design

On arrival (day –14), the animals were weighed, randomized and housed individually. On day –1, the animals were starved overnight (water *ad libitum*). Five treatment groups ($n = 20$ per group) were evaluated for determination of the kinetics of the *S.* Enteritidis infection and the innate immune response. On day 0, groups 1–4 received 1 ml of different dilutions of *S. enteritidis* per animal. Group 5 received 1 ml of *E. coli* WG5 per animal. The bacterial suspensions were orally administered by gavage. Directly after gavage, food and water was provided *ad libitum*. On days 2–6 after the oral infection, four animals of each group were sacrificed and various parameters were evaluated.

Blood samples were taken via orbita plexus puncture using a capillary under light ether anaesthesia on day –10 to obtain a reference value of each individual animal, and 24 h before autopsy. Part of the blood was used to obtain pre-treatment serum samples.

Daily clinical observations were made with reference to the status of general health of the animals. Special attention was paid to the consistency of the faeces. The animals were weighed each day (early in the morning) starting on day –1 prior to the oral inoculation. Each morning, faeces were obtained from each rat in each group. The faeces were macroscopically evaluated and tested for microbiology the same day.

Animals were sacrificed by bleeding from the abdominal aorta under KRA anaesthesia [intramuscular injection of 100 μl of a cocktail consisting of 7 ml of ketalar (50 mg/ml, Parke Davis, Barcelona, Spain], 3 ml of rompun (20 mg/ml, Bayer, Leverkusen, FRG) and 1 ml of atropin (1mg/ml, OPG, Utrecht, The Netherlands). The following organs were removed aseptically: jejunum, ileum, coecum, colon and the spleen. Peyer's patches were removed from the jejunum and ileum.

Haematology

Haematology for each rat was performed in blood samples, anticoagulated with K_3EDTA, obtained on days –10 and 6. The haematological analyses were performed using the H1-E, a multi-species haematology analyser (Bayer B.V., Mijdrecht, The Netherlands) with multi-species software, version 3.0, as published previously (16).

Microbiology

Internal organs were weighted and homogenized in PPS by using an Ultra Thurrax. For counting *S.* Enteritidis, we used brilliant green agar (Oxoid) after appropriate 10-fold serial dilutions in PPS. *Escherichia coli* was counted on tryptone (10 g/l) yeast extract (1 g/l) NaCl (8 g/l) agar plates containing 100 μg/ml nalidixin acid. For identification, colonies were checked for indole production.

Mathematical model

Probability of infection. The exponential dose response model relates dose D to the probability that the host is infected.

$$\text{Prob}_{\text{infection}} = 1 - e^{-pD}$$

The parameter p is the probability of infection per *Salmonella* cell in inoculum of size D. The model is based on the single-hit hypothesis and the independent action of micro-organisms (8). Derivation of the formula can be found in (12).

Neutrophils. The following set of equations describe two cell-populations, blood neutrophils N_{blood} and tissue neutrophils N_{tissue}. A schematic presentation of neutrophil turnover is shown in Fig. 1(A).

$$\frac{d}{dt}N_{blood} = s_1 + \frac{s_2 B^{n_1}}{h_1 + B^{n_1}} - d_1 N_{blood} - \frac{k_1 N_{blood} B^{n_2}}{h_2 + B^{n_2}}$$

$$\frac{d}{dt}N_{tissue} = \frac{k_1 N_{blood} B^{n_2}}{h_2 + B^{n_2}} - d_2 N_{tissue}$$

$$N_{blood}(0) = \frac{s_1}{d_1}$$

$$N_{tissue}(0) = 0$$

B stands for the number of *Salmonella* cells in the Peyer's patches from jejunum and ileum. N_{blood}, N_{tissue} and B are a function of time, but the symbol for time is suppressed for notational simplicity. d/dt is the differentiation with respect to time. The bone marrow releases s_1 neutrophils into the blood each day. Circulating neutrophils die at the rate d_1 per day. Setting $B = 0$ and solving the equation $d/dt\ N_{blood} = 0$, we obtain the steady state number of blood neutrophils prior to infection, $N_{blood}(0)$. Neutrophils are present in tissue in a very low number, and the number of tissue neutrophils on day 0, $N_{tissue}(0)$, is assumed to be zero.

The neutrophil influx is non-linearly dependent on the bacterial burden (15). We empirically model the non-linear dependency in the neutrophil influx. It is Michaelis–Menten type when $n_1 = 1$ and sigmoidal when $n_1 > 1$. The maximal release into the blood is s_2 neutrophils per day, and h_1 is the half-maximum constant. A similar term empirically describes non-linear dependency in the neutrophil migration process. The maximal migration rate is k_1 per day, and h_2 is the half-maximum constant.

Monocytes. The following set of equations describe three cell populations, blood monocytes M_{blood}, resting tissue macrophages M_{tissue} and activated tissue macrophages $M_{activated}$. A schematic presentation of monocyte turnover is shown in Fig. 1(B).

$$\frac{d}{dt}M_{blood} = s_3 + \frac{s_4 B^{n_3}}{h_3 + B^{n_3}} - d_3 M_{blood} - k_2 M_{blood} - \frac{k_3 M_{blood} B^{n_4}}{h_4 + B^{n_4}}$$

$$\frac{d}{dt}M_{tissue} = k_2 M_{blood} + \frac{k_3 M_{blood} B^{n_4}}{h_4 + B^{n_4}} - d_4 M_{tissue} - c M_{tissue} B$$

$$\frac{d}{dt}M_{activated} = c M_{tissue} B - d_5 M_{activated}$$

$$M_{blood}(0) = \frac{s_3}{d_3 + k_2}$$

$$M_{tissue}(0) = \frac{k_2 s_3}{d_4 (d_2 + k_2)}$$

$$M_{activated}(0) = 0$$

The baseline release from the bone marrow is equal to s_3 monocytes per day. Circulating monocytes spontaneously enter body tissues at the migration rate k_2 per day. In tissue, monocytes differentiate into macrophages (17,18) or dendritic cells (19,20). In the model, we consider monocytes in tissue as one population and call it 'tissue macrophages'. Resting tissue macrophages die at the rate d_4 per day. Setting $B = 0$ and solving the equations $d/dt\ M_{blood} = 0$ and $d/dt\ M_{tissue} = 0$, we obtain the steady state numbers of blood and tissue macrophages prior to infection, $M_{blood}(0)$ and $M_{tissue}(0)$. On day 0, tissue macrophages are exclusively in a resting state, i.e. $M_{activated}(0) = 0$.

In infection, the bone marrow releases more monocytes into the blood, with the maximum release of s_4 monocytes per day and the half-maximum constant h_3. Circulating monocytes enter the tissue at the maximum migration rate k_3 and the half-maximum constant h_4. The presence of *Salmonella* in the Peyer's patches activates resting tissue macrophages. The activation rate is c per day. Activated tissue macrophages die at the rate d_5.

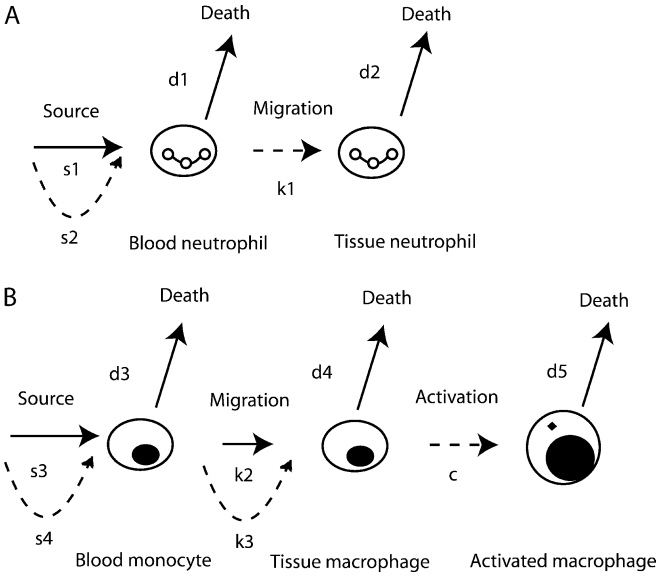

Fig. 1. Schematic presentation of the model. (A) Two populations of neutrophils, blood neutrophils and tissue neutrophils are illustrated. A solid arrow represents a process in a state of health by which a population changes in number. A process that takes place in infection is represented in dashed arrow. Corresponding letter indicates the rate at which the change takes place. (B) Three populations of monocytes are illustrated, blood monocytes, resting tissue macrophages and activated tissue macrophages. In the presence of *Salmonella*, resting tissue macrophages become activated.

Salmonella. The following equations describe the population of *Salmonella B* in the Peyer's patches.

$$\frac{d}{dt}B = rB - z_1 N_{tissue} B - z_2 M_{tissue} B - z_3 M_{activated} B$$

$$B(0) = pD$$

Salmonella cells increase in number at the growth rate *r*. A lag phase and a depletion of essential nutrients due to over-growth are not considered. Elimination of *Salmonella* is modelled by the three mass-action terms. Tissue neutrophils N_{tissue} remove *Salmonella* at the rate z_1. Resting tissue macrophages M_{tissue} remove *Salmonella* at the rate z_2. Activated tissue macrophages $M_{activated}$ remove *Salmonella* at the rate z_3.

The number of *Salmonella* cells surviving follows a binomial process with probability of infection *p*. When inoculum of the dose D is given to an animal, the mean *pD* of the binomial process is assumed to be the number of *Salmonella* cells in Peyer's patches of the animal on day 0 immediately after exposure.

Parameter estimates. The present experiments were designed to estimate the probability of infection and the rate of growth of *Salmonella*. Estimates for many of the rate parameters of neutrophil and monocyte response were based on published experimental studies. How we incorporated the results of these studies into our mathematical model is outlined below. References to the experimental studies are listed in Table 2. For other parameters of neutrophil and monocyte response, no independent studies could be found. These were treated as free parameters of the mathematical model.

Probability of infection (p). The exponential dose-response model was fitted to the fractions of infected animals on day 5 and 6 (Table 1). The best fitting parameter value maximizes the log-likelihood function for the binomial process with the probability of infection $\text{Prob}_{\text{infection}}$ (12).

$$-\log \prod_{i=1}^{j} (1 - e^{-pD_i})^{m_i} (e^{-pD_i})^{n_i - m_i}$$

where *j* is the number of dose groups, n_i is the number of animals per dose group, and m_i is the number of infected animals.

Growth rate (r). To the natural logarithms of the number *B*(0) of *Salmonella* cells on day 0 and the number of *Salmonella* cells in the Peyer's patches 2 days after the highest dose challenge, a straight line is fitted by the method of least squares to obtain the exponential growth rate of *Salmonella*.

Release of neutrophils and monocytes into the blood (s_1, s_2, s_3, s_4). In mice, 1.575×10^6 neutrophils per ml per day enter the bloodstream (21). Injection of G-CSF increases the release to 13.8×10^6 neutrophils per ml per day (21).

In mice, 13.6×10^5 monocytes enter the bloodstream in 48 h (18). Assuming 2 ml blood per mouse weighing 30 g (in proportion to 5 litres blood per 75 kg man), we obtain ~3×10^5 monocytes per ml per day. In an acute inflammation reaction in mice provoked by injecting new born calf serum, 27.2×10^5 monocytes enter the bloodstream in 48 h (18). This is equivalent to 8×10^5 monocytes per ml per day.

Table 1. The number of infected animals

Dose (c.f.u.)	Day 2	Day 3	Day 4	Day 5	Day 6
110	0	0	0	1	1
950	0	1	3	4	3
8500	1	3	3	4	4
98 000	2	4	4	4	4

An animal is infected if the Peyer's patches from jejunum, the Peyer's patches from ileum, or the spleen is *Salmonella enteritides* positive. A total of four animals were tested per group.

Migration from the blood to tissues (k_2). In mice in a steady state, circulating monocytes spontaneously enter the body tissue at a rate of 0.03974 per hour, an equivalent of 0.95 per day for the rate of spontaneous migration (18).

Death of neutrophils and monocytes (d_1, d_2, d_3, d_4, d_5). Neutrophils are short-lived cells with $t_{1/2}$ of 6–10 h in the circulation, after which they undergo apoptosis (22,23). Assuming $t_{1/2}$ of 8 h (0.33 day), the rate of death is equal to $\ln(2)/0.33 \approx 2.1$ per day. Lifespans potentially differ in tissue compartments, but we assume them to be the same because relevant *in vivo* data are unavailable ($d_1 = d_2$). Tissue macrophages are generally considered to be long lived cells. In a normal mouse, there are 10^6 blood monocytes and 2.4×10^6 peritoneal-cavity macrophages (18). At least 7.6% of all monocytes that leave the bloodstream arrive in the peritoneal cavity (18). These data give ~3×10^7 tissue macrophages per mouse. To maintain the total body count in a steady state while new monocytes continuously enter the body tissue, macrophages must die at the rate of 0.03 per day (the total monocyte release into the bloodstream divided by the number of tissue macrophages = $10^6/3 \times 10^7$). The equivalent $t_{1/2}$ is about 30 days. Lifespans potentially differ in resting and activated states, but we assume them to be the same because relevant *in vivo* data are unavailable ($d_3 = d_4 = d_5$).

Removal rates (z_1, z_3). Intracellular killing of *Salmonella* by blood granulocytes was estimated using C57/BL and CBA mice. The rates of intracellular killing were 0.007 (C57/BL) and 0.017 (CBA) per min per (5×10^6 granulocytes/ml) (24). A similar study reported the rates of intracellular killing of *Salmonella* by resident peritoneal macrophages (likely to be in an activated state) as being 0.03 (C57/BL) and 0.05 (CBA) per min per (5×10^6 macrophages/ml) (25). Judged by the fit of the mathematical model to our data, the estimates for C57/BL were just as good as the estimates for CBA mice. The estimates for C57/BL mice expressed in ml per blood cell per day were used in this study.

Half saturation constants (h_1, h_2, h_3, h_4), exponents (n_1, n_2, n_3, n_4), migration rates due to infection (k_1, k_3), activation rate (c) and removal rates (z_2). No independent studies could be

Table 2. Parameter estimates

Description	Name	Estimate	Unit	Reference
Probability of infection	p	2.3×10^{-3} (1.0×10^{-3}, 4.3×10^{-3})	/c.f.u.	
Growth rate in Peyer's patches	r	3.1 (2.6, 3.5)	/Day	
Baseline neutrophil release	s_1	1.6×10^6	Cells/ml/day	(21)
Maximum neutrophil release in infection	s_2	1.4×10^7	Cells/ml/day	(21)
Baseline monocyte release	s_3	3.0×10^5	Cells/ml/day	(18)
Maximum monocyte release in infection	s_4	8.0×10^5	Cells/ml/day	(18)
Neutrophil death rate	d_1, d_2	2.1	/Day	(22)
Monocyte/MΦ death rate	d_3, d_4, d_5	0.03	/Day	(18)
Neutrophil migration rate in infection	k_1	1.1 (0.9, 1.4)	/Day	
Baseline monocyte migration rate	k_2	0.95	/Day	(18)
Maximum monocyte migration rate in infection	k_3	1.7 (1.1, 2.4)	/Day	
MΦ activation rate	c	1.4×10^{-9} (0, 3.9×10^{-8})	c.f.u./day	
Rate of removal by neutrophil	z_1	2.0×10^{-6}	ml/neu/day	(24)
Rate of removal by resting MΦ	z_2	1.8×10^{-9} (0, 6.0×10^{-8})	ml/mΦ/day	
Rate of removal by activated MΦ	z_3	8.6×10^{-6}	ml/mΦ/day	(25)
Constant for neutrophil release	h_1	2.1×10^{10} (7.9×10^9, 7.9×10^{10})	None	
Constant for neutrophil migration	h_2	6.0×10^4 (0, 4.8×10^6)	None	
Constant for monocyte release	h_3	3.2×10^9 (3.0×10^8, 1.9×10^{10})	None	
Constant for monocyte migration	h_4	3000 (0, 5×10^4)	None	
Exponent for neutrophil release	n_1	1.9 (1.8, 2.0)	None	
Exponent for neutrophil migration	n_2	1.9 (1.3, ∞)	None	
Exponent for monocyte release	n_3	2.0 (1.8, 2.2)	None	
Exponent for monocyte migration	n_4	1.1 (0.8, ∞)	None	

The minimum value for the log likelihood function was 34.2. Without the parameters c, z_2, h_2, h_4, n_2, n_4 the minimum value for the log likelihood function was 34.8. Therefore, these parameters are statistically insignificant by the likelihood ratio test.

found for these parameters, and they are treated as free parameters of the mathematical model. Estimates were based on the data presented in this study, the number of blood neutrophils, blood monocytes and *Salmonella* cells in the Peyer's patches. The data from all dose groups were used. All data were assumed to be log-normally distributed. We minimize the log likelihood function (l): the sum of squared differences between the data and the model, using a numerical optimization function (NMinimize) in Mathematica version 5 (Wolfram Research). At each optimization step, the system of ODEs was solved numerically using the Mathematica ODE solver (NDSolve). To determine a credible range of values for each parameter, we numerically determined the two roots of the equation, $l - \acute{l} = \chi^2_{1,0.95}$ where $\acute{l}$ is the minimum value for the log likelihood function l and $\chi^2_{1,0.95}$ is the 95th percentile of the Chi square distribution with 1 degree of freedom.

Results

Probability of infection and in vivo growth rate

The number of *Salmonella* infected animals was dose and time dependent. The lower the dose, the longer the time to detect infection (Table 1). By day 5, the number of infected animals in all dose groups appeared to stabilize. To analyse the effect of dose, we fitted the exponential dose–response model to the fraction of infected animals on days 5 and 6 (Fig. 2A). By discarding the results of days 2, 3 and 4, we minimized the risk that the number of infected animals was underestimated. The best-fit dose–response relationship indicates that the probability of infection is 0.0023 per inoculated *Salmonella* cell (= p). Equivalently, only 1 in 500 inoculated *Salmonella* cells actually infected the rat.

The estimated mean number of *Salmonella* cells infecting the rats in the highest dose group is equal to 225 c.f.u. per animal ($\approx 0.0023 \times 98000 = pD$), with the variability described by the binomial distribution (Fig. 2B). Bacterial burden on day 2 post infection was ~10^6 c.f.u. per 0.5 g of Peyer's patches (Fig. 2C). This means that *in vivo* exponential growth rate is 3.1 per day (= r) during the first 2 days. The equivalent doubling time is ~5 h (= ln(2)/3.1 day^{-1}).

Neutrophil and monocyte responses to growing bacteria

In our mathematical model, the number of neutrophils and macrophages in Peyer's patches increases with the bacterial burden. The killing by the phagocytes eventually exceeds the growth of bacteria, resulting in the decrease in bacterial burden on day 3 to day 4 (Fig. 3A). Neutrophil and monocyte responses in high-dose infection are characterized by three time periods (Figs 4A and 5A). In the first period, neutrophils and monocytes in the blood circulation migrate massively into the infected tissue and their numbers drop, reaching the lowest on day 1 after inoculation. Density-dependent migration of neutrophils and monocytes cannot be established based on our data, i.e. both constants h_2 and h_4 can be zero. In the second period, the release of neutrophils and monocytes from the bone marrow exceeds the loss by migration and the pool of blood neutrophils and monocytes increases gradually in number to the highest on day 4. This is when the bacterial burden was the highest and started to decrease (Fig. 3A). The release of neutrophils and monocytes into the blood is a density-dependent response, i.e. both constants h_1 and h_3 are greater than zero. Furthermore, the release-response significantly differs from Michaelis–Menten type, i.e. both exponents n_1 and n_3 are greater than one, indicating a positive feedback mechanism in the responding bone marrow. In the third period,

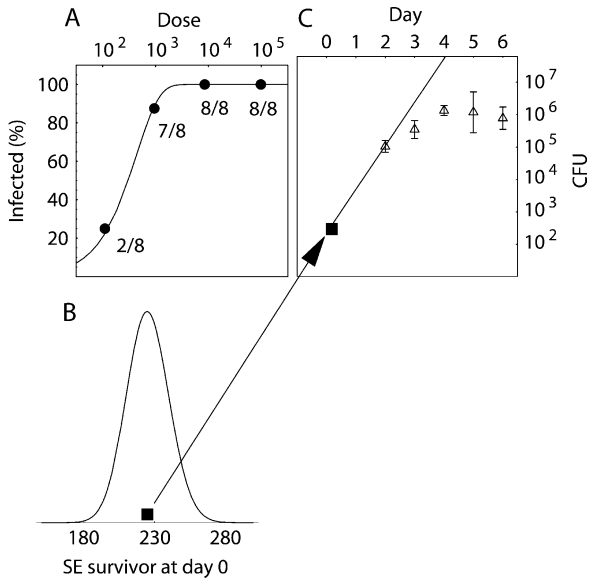

Fig. 2. Estimating the growth rate of *Salmonella*. The exponential dose–response model is fitted to the fractions of infected rats. The ratios indicate the number of infected and total animals on days 5 and 6 (A). Based on the dose response curve, we estimated that on average 225 c.f.u. surviving *Salmonella* infected the animals given the highest dose of ~10^5 cfu (B). The growth rate of *Salmonella* is the slope of the straight line connecting the initial bacterial burden on day 0 and the mean bacterial burden in Peyer's patches of the animals on day 2 (C).

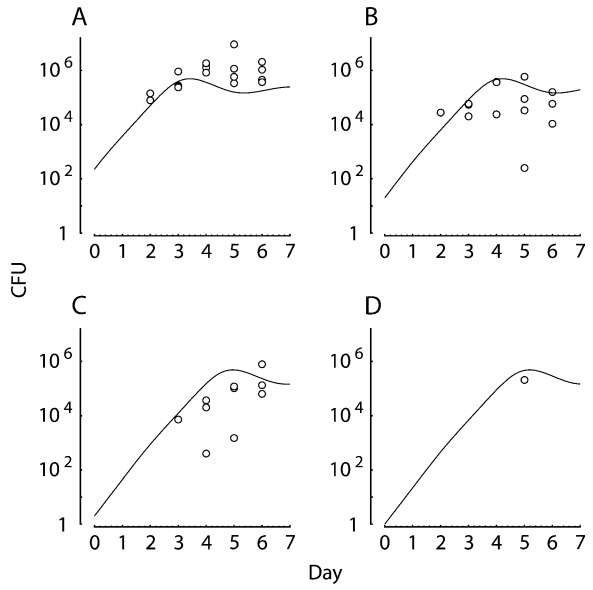

Fig. 3. *Salmonella* in the Peyer's patches in relation to time and dose. Rats were given (A) 98 000, (B) 8500, (C) 950, (D) 110 c.f.u. of *Salmonella* cells on day 0. An open circle represents the number of *Salmonella* per 0.5 g of Peyer's patches. A solid line represents simulated number of *Salmonella*. We calculated the simulated number using the model as described in the Model section and using the parameter values listed in Table 2. We estimated the number of *Salmonella* cells on day 0 for each dose group as illustrated in Fig. 2. (D) Only one of the two infected animals was *Salmonella* positive in Peyer's patches. The other was *Salmonella* positive in the spleen only. Depicted in Figs 3–6 are the results of the same simulation per dose group.

the release from the bone marrow decreases with the bacterial burden. Beyond day 7, the bacterial burden decreases very slowly but not completely. Long term dynamics of bacterial burden exhibited a damped oscillation to the equilibrium ~10^5 c.f.u./g.

In low dose infection, neutrophil/monocyte responses are delayed

The mathematical model predicted that lowering the dose by a factor of 10 would extend the time to reach the highest bacterial burden by 10–20 h, but should reach the same highest bacterial burden. Experimental infection performed with three low doses validated the prediction (Figs 3–5, panels B–D). The model also predicted that activated tissue macrophages would appear 1–2 days later in low dose infection. Although we lack experimental data to validate this prediction, we reasoned that if activated macrophages play a role in developing systemic infection, its time course should correlate with the bacterial burden in the spleen. When we overlaid bacterial burden in the spleen and activated macrophages, their time courses exhibited positive correlation across the four experimental infections (Fig. 6).

Discussion

In a previous work using a similar experimental set-up and mathematical approach, we reported the probability of infection in rats to be 1.2×10^{-3} per ingested *Salmonella* cell (12), which is very close to the current estimate (2.3×10^{-3} per c.f.u.). By exploiting this property and integrating several studies of neutrophil and monocyte turnovers in a state of health and in infection, we tried to go beyond simple statistics and provide explanations for the present observations in the light of *Salmonella* pathology and host response. In the future, we want to extend this quantitative approach. It is possible to block the migration of neutrophils in the mathematical model (by setting the rate of migration to zero) and in the animal model (by suppressing IL-8). This may be one way to assess a role and relative importance of a single cytokine in disease.

In the mathematical model, resting tissue macrophages do not seem to be important to the extent that blocking phagocytosis by setting the parameter z_2 to zero still resulted in a good fit to the experimental data. More surprisingly, blocking the activation of resting macrophages by setting the parameter c to zero did not result in an overgrowth of *Salmonella*. Therefore, the reduction in the number of *Salmonella* as shown in Fig. 3 is due to phagocytosis by tissue neutrophils. However, blocking phagocytosis activity of tissue neutrophils alone (by setting the parameter z_1 to zero) resulted in an initial overgrowth of *Salmonella* that was later controlled by activated tissue macrophages that took over the role of neutrophils. This illustrates a complex interplay between neutrophils and macrophages that could be tested against the animal model.

The estimated doubling time of *Salmonella* (5 h) in the Peyer's patches is based on two assumptions. First, *Salmonella* is predominantly present in the Peyer's patches. In

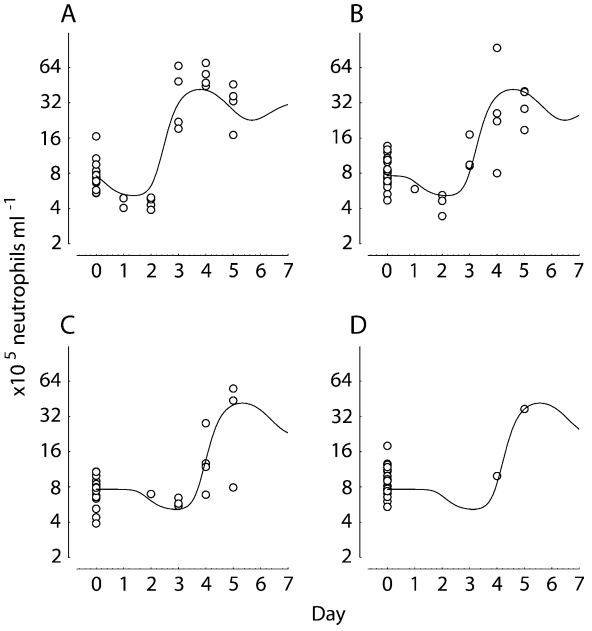

Fig. 4. Blood neutrophils in relation to time and dose. An open circle represents the number of neutrophils per ml of blood. A solid line represents predicted number of neutrophil. Doses: (A) 98 000, (B) 8500, (C) 950, (D) 110 c.f.u. We refer readers to Fig. 3 for details.

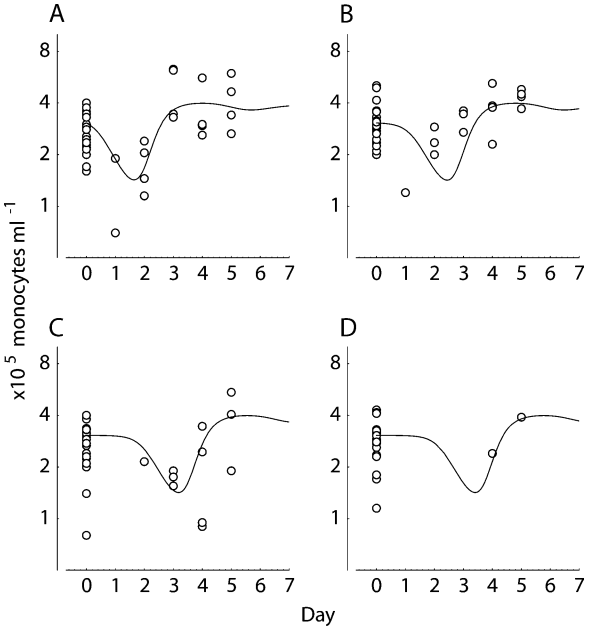

Fig. 5. Blood monocytes in relation to time and dose. An open circle represents the number of monocytes per ml of blood. A solid line represents predicted number of monocyte. Doses: (A) 98 000, (B) 8500, (C) 950, (D) 110 c.f.u. We refer readers to Fig. 3 for details.

jejunum, ileum, coecum, colon and spleen, the number of *Salmonella* cells per gram of tissue are generally 10–100 times lower than the number per gram of Peyer's patch. Only on days 5 and 6 in the highest dose group was *Salmonella* present in ileum, colon and Peyer's patches in a comparable

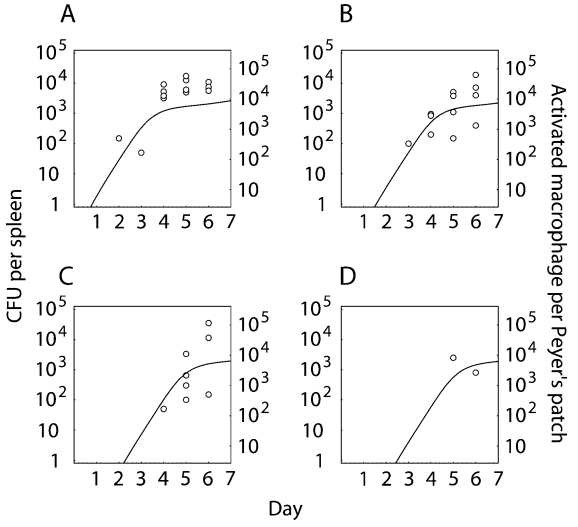

Fig. 6. The predicted time course of activated macrophage in the Peyer's patches coincides with the time course of *Salmonella* in the spleen in infected animals. An open circle represents the number of *Salmonella* cells per spleen of a rat. A solid line represents the predicted number of activated macrophage per 0.5 g of Peyer's patch. Doses: (A) 98 000, (B) 8500, (C) 950, (D) 110 c.f.u. We refer readers to Fig. 3 for details.

density. Second, we assumed that Peyer's patches weigh ~0.5 g per infected rat. We had to make the assumption because the weights per individual rat were not recorded following the measurements. We examined 4–10 of the largest Peyer's patches per infected rat, which weighted roughly between 0.1 and 0.5 g in total. The results should hold unless this assumption is grossly violated, e.g. by a factor 100, which we feel is unlikely. The estimated doubling time based on these assumptions lies within the range previously reported (14).

The numbers of *Salmonella* cells in the Peyer's patches in infected rats in the highest dose group tend to be higher than the simulation results using the mathematical model (Fig. 3A) whereas those in the lower dose groups tend to be lower (Fig. 3B). We regarded experimental noise as the source for the variability. However, alternative possibilities, such as a decision by a group of densely populated *Salmonella* cells in the intestine to turn on additional virulence mechanisms, cannot be excluded.

A protective role of B and T cell mediated immunity against *Salmonella* infection in mice was demonstrated in an adoptive transfer study (26). In the present experiment, Ag-specific T cell immunity was already detected from day 3 and onwards by ear swelling after injecting heat-killed *Salmonella* into the ears (unpublished observations). Although this read-out does indicate the presence of T cell dependent immune response, it does not by itself demonstrate T cell activity in the killing of *Salmonella*. In addition, *Salmonella* infections in nude rats that lack T cells and in which antibody production is diminished were not more severe than in normal rats during the first week of infection (our unpublished observations). So, in the mathematical model, we attributed the diminished growth of *Salmonella* entirely to tissue neutrophils and (activated) macrophages. In an experiment that lasted to 12 days following the challenge

with the dose of 2.0×10^6 c.f.u. of *S.* Enteritidis, the bacterial load per gram of Peyer's patch on day 12 ranged between 4.6×10^4 and 7.4×10^4 c.f.u. (our unpublished observations). The simulated bacterial load on day 12 using the mathematical model was 2×10^5 c.f.u. per gram of Peyer's patches. This number is only marginally higher than the experimental data, despite the fact that T cell immunity is missing from the mathematical model.

In Peyer's patches or proximal gut tissues, *Salmonella* cell may enter the bloodstream and reach the liver and the spleen. Alternatively, cells of monocyte–macrophage lineage may actively transport *Salmonella* cells into these organs. We observed in this study that *Salmonella* appears first in Peyer's patches and then in the spleen about a day later (Figs 3 and 6). Moreover, the appearance of *Salmonella* in the spleens of infected animals coincides with the appearance of a cell population in the model that we termed 'activated tissue macrophages'. Although activated macrophages are not generally considered to be mobile, dendritic cells are. After taking up *Salmonella* in inflamed tissue, DC downregulate antigen uptake and migrate to a draining lymph node, where they activate Ag-specific T cells (19). The DC biology and the population dynamics of activated macrophages in our mathematical model substantiate the idea that *Salmonella* cells are actively transported to the spleen by a cell of monocyte–macrophage lineage.

In conclusion, we estimated the *in vivo* growth rate of *Salmonella* in rats. The estimated doubling time is 5 h. The main strength of this estimate is that *Salmonella* is given via the oral route, which is the better route of infection to model foodborne infection compared to intra-peritoneal or other routes. We can predict the time course of neutrophil and monocyte responses up to a week in low and high dose infection. Predictions can be extended to a longer time period and to other altered situations, such as diminished/disabled neutrophil migration. Importantly, predictions can be tested quantitatively by experiments.

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Abbreviations

DC	dendritic cells
BHI	brain heart infusion
PS	physiological saline
PPS	PS plus 1% peptone

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