The dual role of splenic mononuclear and polymorphonuclear cells in the outcome of ciprofloxacin treatment of *Salmonella enterica* infections

P. Kanvatirth[1*], O. Rossi[1#], O. Restif[1], B. A. Blacklaws[1], P. Tonks[1], A. J. Grant[1], P. Mastroeni[1]

Author Affiliations

1 Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge, CB3 0ES, UK.

Current affiliation: GSK Vaccine Institute for Global Health, Via Fiorentina 1, Siena, 53100, IT.

* Current affiliation: Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, UK.

Corresponding author: Dr Panchali Kanvatirth, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, UK. Email: pk468@cam.ac.uk

Running title: The dual role of splenic mononuclear and polymorphonuclear cells in the outcome of ciprofloxacin treatment of *Salmonella enterica* infections.

Abstract

Objective: To determine the immune cell populations associated with *Salmonella enterica* serovar Typhimurium before and after ciprofloxacin treatment using a murine model of systemic infection. The effect of depletion of immune cells associating with *Salmonella* on treatment outcome was also determined.

Methods: We infected mice with a *Salmonella enterica* serovar Typhimurium strain expressing GFP and used multicolour flow cytometry to identify splenic immune cell populations associating with GFP-positive *Salmonella* before and after treatment with ciprofloxacin. This was followed by depletion of different immune cell populations using antibodies and liposomes.

Results: Our results identified $CD11b^{+}CD11c^{hi/lo}$ (macrophages/dendritic cells) and $Ly6G^{+}CD11b^{+}$ (neutrophils) leukocytes as the main host cell populations that are associated with *Salmonella* after ciprofloxacin treatment. We therefore proceeded to test the effects of depletion of such populations during treatment. We show that depletion of $Ly6G^{+}CD11b^{+}$

populations resulted in an increase in the number of viable bacterial cells in the spleen at the end of ciprofloxacin treatment. Conversely, treatment with clodronate liposomes during antimicrobial treatment, which depleted the $CD11b^{+}CD11c^{hi/lo}$ populations, resulted in lower numbers of viable bacteria in the tissues.

Conclusion: Our study identified host cells where *Salmonella* bacteria persist during ciprofloxacin treatment and revealed a dual and opposing effect of removal of $Ly6G^{+}CD11b^{+}$ and $CD11b^{+}CD11c^{hi/lo}$ host cells on the efficacy of antimicrobial treatment. This suggests a dichotomy in the role of these populations in clearance/persistence of *Salmonella* during antimicrobial treatment.

Introduction

Salmonella enterica cause severe systemic diseases such as typhoid, paratyphoid fever and invasive Non-Typhoidal Salmonellosis (iNTS). There are currently no licensed vaccines for paratyphoid or iNTS.[1]

Infections including salmonellosis can be difficult to eradicate by antibiotics and persist in the host even when the bacteria retain susceptibility to the drug used for the treatment.[2–4] This can lead to chronic infections, disease reservoirs, prolonged transmission, within-host bacterial evolution and development of antimicrobial resistance (AMR).[3,5,6] This problem is exacerbated in patients with comorbidities that impair the immune system.[3,7]

The role of cells of the immune system in modulating the efficacy of antimicrobial treatment is poorly understood. Immunity can either limit the efficacy of antimicrobials by creating environments hostile to their penetration and efficacy, by reducing bacterial division rates and activating Multi Drug Resistance pumps or synergise with antibiotics in the killing of the microorganisms.[8–11]

Here we show the location of *Salmonella* within different types of murine splenocytes before, during and after ciprofloxacin treatment and illustrate how depletion of host cell populations affects treatment.

Methods

Ethics. Animal experiments were performed under licence issued by the UK Home Office (Animals Act, 1986) approved by the Cambridge Animal Welfare Ethical Review Body.

Bacteria. We used GFP-expressing SL1344 *sifB::gfp* strain *S.* Typhimurium.[12]

Infection and antimicrobial treatment. Female C57BL/6 mice (Charles River), >8 weeks old, were infected intravenously (i.v.) with 10^3 colony forming units (cfu) of SL1344 *sifB::gfp*. Three days post infection (p.i.), treatment with ciprofloxacin hydrochloride (Sigma-Aldrich) was started twice a day for four days after which the infection was allowed to relapse for two days.

Flow cytometry. Single splenocyte suspensions were stained as in Method S1. Cell markers and gating strategies are indicated in Table S1 and Fig. S1 respectively.

Cell Depletions. Polymorphonuclear neutrophils (PMN) were depleted with 0.5 mg of anti-Gr-1 IgG2b (Leinco Technologies)[13] i.p. on day 3 p.i. Macrophage and dendritic cells were depleted with clodronate liposomes (Liposoma) administered i.p. (Method S2). To test the

74 effect of liposomes on the infection during ciprofloxacin treatment, mice were treated with
75 ciprofloxacin and liposomes[14] daily from day 3 to 6 p.i. B-cells were depleted with 0.25 mg of
76 anti-mouse CD20 (Biolegend) i.p. on day 3 p.i.
77 The mice were then killed on day 7 p.i. for enumeration of viable *Salmonella* in the spleen
78 (Method S3) and flow cytometric analysis (Method S1).
79

Results and discussion

Flow cytometric analysis of host cells associated with bacteria.

Mice were infected with 10^3 cfu of *Salmonella*.[12] On day 3 p.i., when bacterial counts in the spleen reached approximately 10^6 cfu, ciprofloxacin treatment was started and continued for 4 days; ciprofloxacin sharply reduced the bacterial load to 10^3 cfu. Treatment was stopped after 4 days (day 7 of the infection) resulting in the relapse of bacterial growth between days 7 and 9.

Before the first antimicrobial dose (day 3 p.i.), $CD19^+$ B-cells were the most abundant type of splenocytes associated with *Salmonella*, (36 to 60%; median 55%) of the total population (Fig.1(a)). GFP^+ bacteria were also associated with cells expressing CD11b and/or CD11c (Fig.1(a), median >5%). $CD3^+$ (T) or $NK1.1^+$ (NK) cells represented <5% each of the GFP^+ cells). $Ly6G^+$ $CD11b^+$ cells (neutrophils) constituted 19 % (range 11-33%) of the GFP^+ cells.

During antibiotic treatment (3-7 days p.i.), despite significant inter-mouse variations within groups, we detected consistent trends in the proportions of specific cell types among GFP^+ infected cells, using generalised linear models with quasi-binomial distributions to account for over-dispersion.

The median proportion of B-cells decreased from 55 to 28%, day 3 *versus* day 6 p.i., p=0.0001 and remained at similar levels (median 33%, p=0.16) for the next 24 h (Fig.1(a)). $Ly6G^+CD11b^+$ cells (neutrophils)[15] followed a similar pattern during treatment, with the median decreasing from 19 to 8%, day 3 *versus* day 6 p.i. (p=0.005), before increasing to 12% on the next day (day 6 *versus* day 7 p.i., p=0.038). These changes were mirrored by increases in the proportions of $CD11b^+CD11c^{hi}$ (dendritic cells) and $CD11b^+CD11c^{lo}$ (macrophages)[15] populations, which cumulatively formed the major proportion of splenocytes associated with GFP^+ *Salmonella* at the end of the treatment. The percentage of $CD3^+$ cells and $NK1.1^+$ cells remained low throughout the experiment (median <5% of GFP^+ cells). Thus, $CD19^+$ B cells and $Ly6G^+CD11b^+$ are the major host cell populations associated with *Salmonella* before the start of ciprofloxacin treatment. $CD11b^+CD11c^{hi}$, $CD11b^+CD11c^{lo}$ and $Ly6G^+CD11b^+$ cells, and a smaller proportion of $CD19^+$ cells are the main *Salmonella*-associated populations after treatment.

By day 9 p.i. (the relapse phase), the proportion of GFP^+ bacteria associated with $CD19^+$ cells decreased further, to a median of 15 % (day 9 *versus* day 7, p<0.0001), and $CD11b^+$ cells

111 constituted the majority of host cells associated with *Salmonella* (70-80%). The proportion of
112 neutrophils associated with *Salmonella* reverted to that seen before the commencement of the
113 antimicrobial treatment (median: 19% on day 3 and 22% on day 9 p.i., p=0.08). Thus, during
114 the relapse phase the bacteria did not re-populate individual splenocyte populations in the same
115 proportions as in the pre-antimicrobial phase.
116
117 **Proportions of overall host spleen cell populations in comparison to *Salmonella*-**
118 **associated cells.**
119 We next compared the overall populations of splenocytes in relation to the changes in host cells
120 associated with GFP^+ *Salmonella*. This was to determine whether the shifts in bacterial location
121 described above were a mere consequence of overall changes in the proportions of individual
122 populations of splenocytes.
123 Throughout the experiment, $CD19^+$ cells remained the dominant cell type in infected mouse
124 spleens, decreasing from a median value of 73 to 54% on day 3 p.i. to day 6 p.i. ($p<0.0001$),
125 with no further substantial changes by day 9 p.i. (day 6 *versus* day 7 p.i., p=0.4; day 7 *versus*
126 day 9 p.i., p=0.28) (Fig. 1b). This reduction was matched by initial increases in the proportions
127 of $CD11b^+$ and $CD11c^+$ cells (Fig. 1b), while the proportion of $Ly6G^+CD11b^+$ remained low,
128 increasing from 6 to 10% from day 3 to 7 p.i. ($p<0.0001$). During the relapse phase (days 7-9
129 p.i.), there were only small variations in the overall cell populations, in contrast to the major
130 changes observed in *Salmonella*-associated cells described above.
131
132 **Role of $CD11b^+CD11c^{hi}$ (dendritic cells) and $CD11b^+CD11c^{lo}$ (macrophages) cells on the**
133 **efficacy of antimicrobial treatment.**
134 Since $CD11b^+CD11c^{hi}$ and $CD11b^+CD11c^{lo}$ are the major cell types associated with
135 *Salmonella* at the end of the treatment we explored the effects of clodronate liposomes (CL)
136 on antimicrobial treatment of the infection. CL deplete macrophage and dendritic cells *in vivo*.
137 CL induced a decrease from 5.1 to 0.8% in the proportion of $CD11b^+CD11c^{hi}$ cells, and from
138 4.4 to 1.8% in the proportion of $CD11b^+CD11c^{lo}$ cells by day 6 p.i. (Fig. S2). This led to a 32
139 and 72% reduction in average cell numbers respectively (Table S2). All the other cell types
140 were unaffected by CL (data not shown).

The bacterial load in the spleen of mice receiving PBS liposomes (used as control) and CL alongside ciprofloxacin had a median value of $3.2x10^4$ cfu/spleen and $3.4x10^3$ cfu/spleen ($p=0.0047$) (Fig.2a) respectively.

The data show that, paradoxically, ablation of clodronate-susceptible macrophages, normally involved in host resistance to *Salmonella*[8], had a synergistic effect with the antimicrobial treatment. A plausible explanation could be the fact that these cells can contain persisting bacteria with low replication rates known to be less susceptible to the effect of antibiotics.[16] The reported low/absent replication of *Salmonella* within dendritic cells ($CD11b^+CD11c^{hi}$) could also underpin their role in hindering ciprofloxacin treatment *in vivo*.[17]

Effect of depletion of $Ly6G^+CD11b^+$ neutrophils on the antimicrobial treatment of a *Salmonella* infection.

$Ly6G^+CD11b^+$ neutrophils are key cells in host resistance to *Salmonella.*[18] *In vivo* administration of anti-Gr-1 antibody reduced the proportion of $Ly6G^+CD11b^+$ cells in the spleen from 3.7 to 0.4% (Fig. S3) which led to an 89% reduction in average cell numbers (Table S3). The bacterial loads in the spleen on day 7 p.i. were higher in mice that received anti-Gr-1 antibody compared to mice receiving control IgG with median values for the control and anti-Gr-1 group being $2.5x10^4$ cfu/spleen and $4x10^5$ cfu/spleen ($p=0.0007$) (Fig.2(b)) respectively. Our data indicate that ablation of neutrophils had a detrimental effect on the antimicrobial treatment of the infection. This is consistent with the antimicrobial role of neutrophils in innate immunity to *Salmonella.*[8,18]

Effect of B-cell depletion on the efficacy of antimicrobial treatment of a *Salmonella* infection.

Treatment of infected mice with an anti-CD20 B-cell depleting antibody[19] (Fig. S4) reduced the overall proportion of $CD19^+$ cells in the spleen from 34.5 to 0.1% (Fig. S4), leading to a 99% reduction in B-cell numbers by day 7 p.i. (Table S4). Nevertheless, the bacterial load between B-cell depleted and control mice was similar ($p=0.8248$) (Fig. 2(c)). Our data indicate that depleting B-cells did not impact on the outcome of antimicrobial treatment.

Conclusion

Understanding the location of bacteria can guide treatments that are complementary or alternative to classical antimicrobial regimens. These may include host-cell specific immunological approaches, as shown by the different effects of cell-depletions *in vivo*, or strategies for improved delivery of drugs to infection foci/cells.[20]

Acknowledgments

We thank Prof Dirk Bumann and Dr Beatrice Claudi for providing SL1344 *sifB::gfp* strain, as well as for their invaluable help on implementing FACS-based sorting of *Salmonella* associated splenic cells in house.

Funding

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) grant number BB/M000982/1 (http://www.bbsrc.ac.uk/research/grants/grants/AwardDetails.aspx?FundingReference=BB/M000982/1). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Transparency Declaration

ORo is currently an employee of GSK Vaccines Institute for Global Health (GVGH), part of GSK group of companies; this does not alter the author's adherence to all Journal policies on data and material sharing. All other authors: none to declare.

Authors Contributions

Conceived and designed the experiments: P.K, O.Ro, B.A.B., P.M. Performed the experiments: P.K., O.Ro., B.A.B., P.T., P.M. Analysed the data: P. K., O.Ro., O.Re., B.A.B., P.T., A.J.G., P.M. Contributed to the writing of the manuscript: P. K., O.Ro., O.Re., B.A.B., P.T., A.J.G., P.M. Received the funding: O.Re., A.J.G., P.M.

References

1. Kariuki S, Gordon MA, Feasey N, *et al*. Antimicrobial resistance and management of invasive *Salmonella* disease. *Vaccine* 2015; **33**: C21-C29.

2. Maskell DJ, Horrnaeche CE. Relapse following cessation of antibiotic therapy for mouse typhoid in resistant and susceptible mice infected with salmonellae of differing virulence. *J Infect Dis* 1985; **152**:1044-9.

3. Klemm EJ, Gkrania-Klotsas E, Hadfield J, *et al.* Emergence of host-adapted *Salmonella* Enteritidis through rapid evolution in an immunocompromised host. *Nat Microbiol* 2016; **1**: 15023.

4. Rossi O, Dybowski R, Maskell DJ, *et al*. Within-host spatiotemporal dynamics of systemic *Salmonella* infection during and after antimicrobial treatment. *J Antimicrob Chemother* 2017;

72: 3390-97.

5. Okoro CK, Kingsley RA, Quail MA, *et al.* High-resolution single nucleotide polymorphism analysis distinguishes recrudescence and reinfection in recurrent invasive nontyphoidal *Salmonella* typhimurium disease. *Clin Infect Dis* 2012; **54**: 955-63.

6. Levin-Reisman I, Ronin I, Gefen O, *et al*. Antibiotic tolerance facilitates the evolution of resistance. *Science* 2017; **355**: 826-30.

7. McDermott W. Microbial persistence. *Yale J Biol Med* 1958; **30**: 257-91.

8. Mastroeni P, Vazquez-Torres A, Fang FC, *et al.* Antimicrobial actions of the nadph phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival *in vivo*. *J Exp Med* 2000; **192**: 237-47.

9. Mastroeni P, Grant A, Restif O, *et al*. A dynamic view of the spread and intracellular distribution of *Salmonella enterica*. *Nat Rev Microbiol* 2009; **7**: 73-80.

10. Wu Y, Vulić M, Keren I, Lewis K. Role of oxidative stress in persister tolerance. *Antimicrob Agents Chemother* 2012; **56**: 4922-26.

11. Vazquez-Torres A, Xu Y, Jones-Carson J, *et al. Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. *Science* 2000; **287**: 1655-58

12. Bumann D. Examination of *Salmonella* gene expression in an infected mammalian host using the green fluorescent protein and two-colour flow cytometry. *Mol Microbiol* 2002; **43**: 1269-83.

13. Daley JM, Thomay AA, Connolly MD, *et al*. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol* 2008; **83**: 64-70.

14. van Rooijen N, Hendrikx E. Liposomes for specific depletion of macrophages from organs and tissues. *Methods Mol Biol* 2010; **605**: 189-203.

15. Hey YY, Tan JKH, O'Neill HC. Redefining myeloid cell subsets in murine spleen. *Front Immunol* 2016; **6**:652.

16. Helaine S, Cheverton AM, Watson KG, *et al*. Internalization of *Salmonella* by macrophages induces formation of nonreplicating persisters. *Science* 2014; **343**: 204-08.

17. Bueno SM, González PA, Carreño LJ, *et al.* The capacity of *Salmonella* to survive inside dendritic cells and prevent antigen presentation to T cells is host specific. *Immunology* 2008; **124**: 522-33.

18. Conlan JW. Critical roles of neutrophils in host defense against experimental systemic infections of mice by Listeria monocytogenes, *Salmonella* typhimurium, and Yersinia enterocolitica. *Infect Immun* 1997; **65**: 630-35.

19. Naeim F. Principles of Immunophenotyping. In: *Hematopathology*. Elsevier Inc., 2008; 25-

55.

20. Kaufmann SHE, Dorhoi A, Hotchkiss RS, *et al*. Host-directed therapies for bacterial and viral infections. *Nat Rev Drug Discov* 2018; **17**: 35-56.

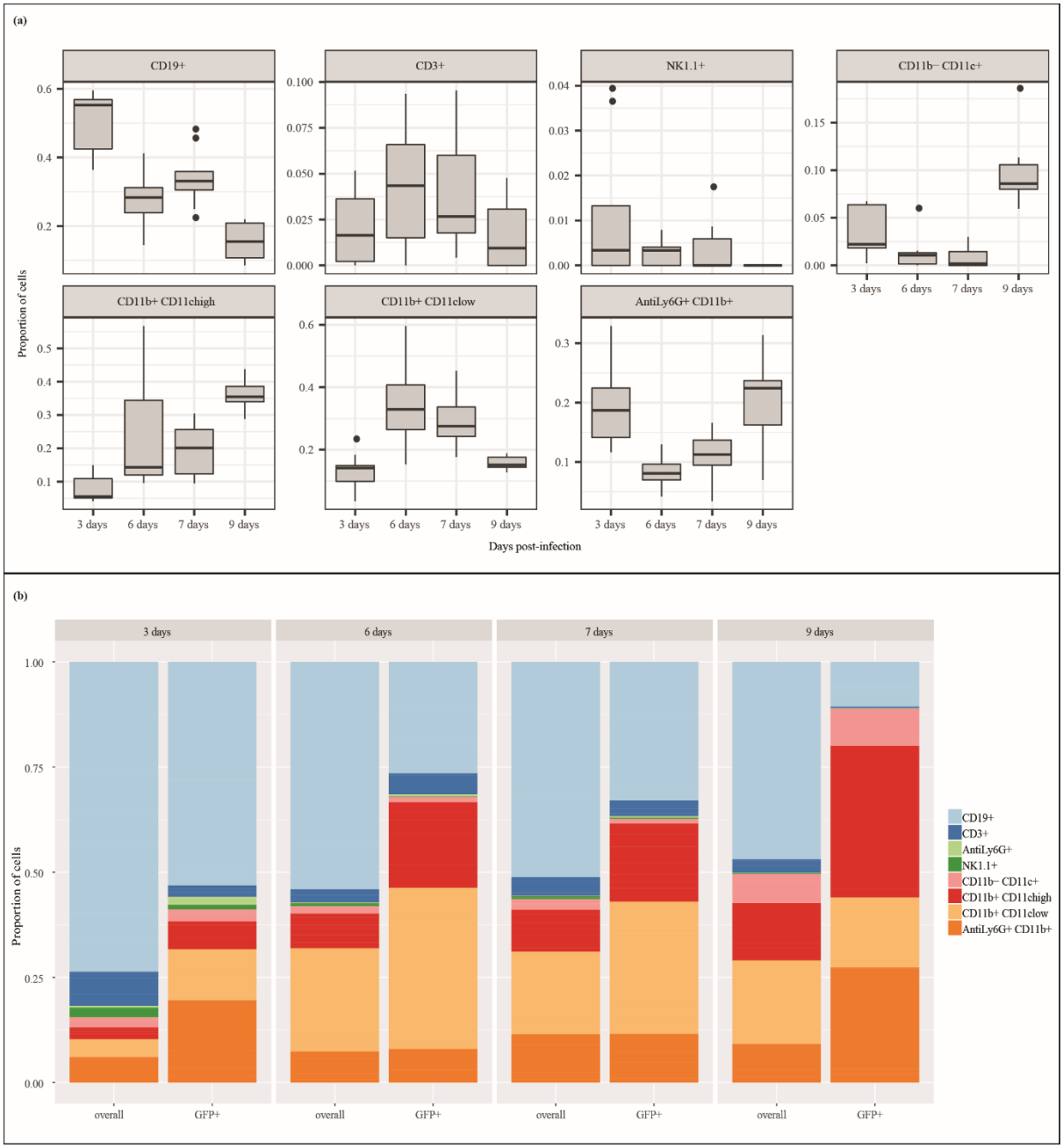
(a)
CD19+
CD3+
NK1.1+
CD11b− CD11c+
CD11b+ CD11chigh
CD11b+ CD11clow
AntiLy6G+ CD11b+
Proportion of cells
3 days
6 days
7 days
9 days
Days post-infection
(b)
Proportion of cells
overall
GFP+
CD19+
CD3+
AntiLy6G+
NK1.1+
CD11b− CD11c+
CD11b+ CD11chigh
CD11b+ CD11clow
AntiLy6G+ CD11b+

Fig.1

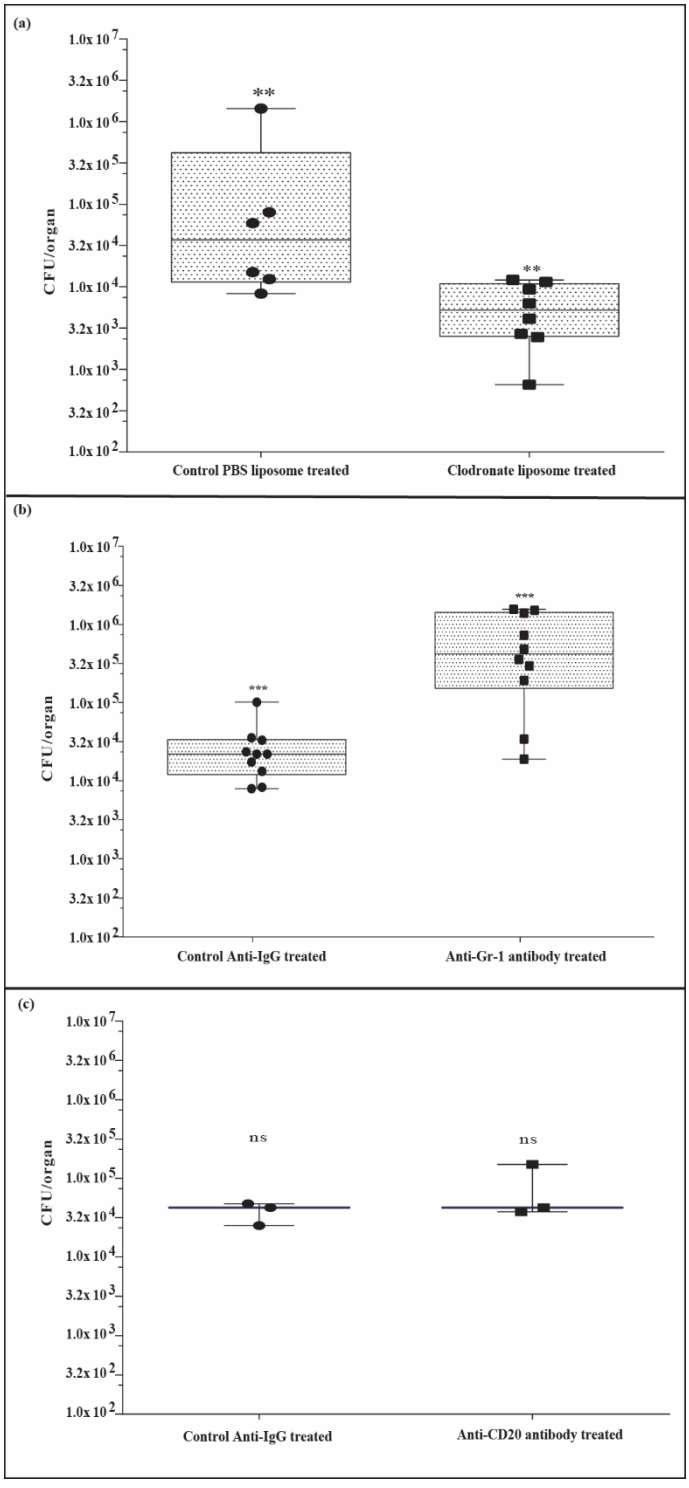
(a)
CFU/organ
1.0x 10 7
3.2x 10 6
1.0x 10 6
3.2x 10 5
1.0x 10 5
3.2x 10 4
1.0x 10 4
3.2x 10 3
1.0x 10 3
3.2x 10 2
1.0x 10 2
**
**
Control PBS liposome treated
Clodronate liposome treated
(b)
CFU/organ
***
***
Control Anti-IgG treated
Anti-Gr-1 antibody treated
(c)
CFU/organ
ns
ns
Control Anti-IgG treated
Anti-CD20 antibody treated

Fig. 2

Figure legends

Fig. 1. (a) Distribution of *Salmonella*-infected spleen cells at different time points post infection. The data are presented as box and whisker plots with the central line indicating the median of the values. Mice were infected with 10^3 cfu of GFP-expressing *S.* Typhimurium SL1344 *sifB::gfp* and treated with ciprofloxacin for four days (3-7 days p.i.) at 12 h intervals. Splenic cells were analysed using multicolour flow cytometry and gated for $CD45^+$ cells and then for GFP expression before analysis for the presence or absence of other cell markers. Each boxplot represents data from nine mice and each panel shows the proportion of GFP^+ *Salmonella*-associated cells expressing a particular combination of receptors. The X-axes of the graphs represent days post infection and the Y-axes represent the proportion of cells expressing the cell marker. **(b) Comparative proportion of spleen cells ("overall") and GFP^+ *Salmonella*-associated spleen cells ("GFP^+").** Mice were infected and spleen cells were analysed as described in method S1 and Fig. S1. In addition to the distribution of GFP^+ *Salmonella*-associated spleen cells phenotypes, the overall proportions of the different cell types (marker expression) in the $CD45^+$ population are shown, for each time point post infection. On the Y-axis the relative proportion of each spleen cell population at each time point are presented as different colours, side-by-side for overall population and GFP^+ subsets. At each time point (X-axis), the data represent the median value obtained from nine mice.

Fig. 2. (a) Effect of depletion of $CD11b^+CD11c^{lo}$ and $CD11b^+CD11c^{hi}$ populations using clodronate liposomes on bacterial load. A box and whisker representation with the central line depicting the median value of cfu counts from the spleen on day 7 p.i. from *Salmonella* infected, antimicrobial treated mice, either when treated with PBS liposomes or with clodronate liposomes from day 3 to day 6 p.i.. The Y–axis indicates cfu/spleen. The values are cumulative from two independent experiments with n=6 for the control group and n=8 for the depleted group. The statistical analysis was performed using the Mann Whitney test. ** indicates $p<0.01$. **(b) Effect of depletion of $Ly6G^+CD11b^+$ neutrophil populations on bacterial load.** A box and whisker representation with the central line depicting the median value of cfu counts taken from spleens on day 7 p.i. from *Salmonella*-infected, antimicrobial-treated mice, either injected with a control IgG or an anti-Gr-1 antibody. The values are cumulative from two independent experiments with n=10 mice for both the control and the depleted groups. The

statistical analysis was performed using the Mann Whitney test. *** indicates $p<0.001$. **(c) Effect of depletion of B-cells on bacterial load.** A box and whisker representation with the central line depicting the median value of cfu counts taken from spleens on day 7 p.i. from *Salmonella*-infected, antimicrobial-treated mice, injected with either a control IgG or an anti-CD20 antibody. The values are from n=3 mice for the control and the depleted group. The statistical analysis was performed using the Mann Whitney test. Ns: non-significant.