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Successful treatment of bacterial infection hinders development of acquired immunity[1]

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Abstract

Antibiotics are routinely used to control bacterial infection but the acquisition of acquired immunity following successful treatment has rarely been examined. We developed a model that allows visualization of acquired immunity during and following antibiotic treatment of typhoid. Pathogen-specific humoral and cellular immune responses were activated rapidly in antibiotic-treated mice but were not sustained after successful antibiotic treatment and did not confer protection to secondary infection. In marked contrast, pathogen-specific Th1 and antibody responses matured over several weeks following immunization with a live vaccine strain (LVS). The deficiency in protective immunity following antibiotic treatment could be overcome by administering flagellin during antibiotic therapy. Thus, development of protective immunity is hindered by rapid therapeutic elimination of bacteria but can be overcome by providing additional inflammatory and/or antigenic stimuli.

Keywords

T cells; Bacterial Infections; Memory; Rodent

Introduction

Live attenuated vaccines allow the transient colonization of an immunized host and induce robust humoral and cellular immune responses (1). Successful treatment of an active bacterial infection also results in transient colonization of the host, but it is unclear whether a similar protective response is induced. Induction of acquired immunity following successful antibiotic treatment is particularly important for individuals in endemic areas where re-infection is likely to occur and antibiotic therapy is widely used.

Human typhoid, caused by infection with *Salmonella enterica* serovar typhi (*S*. typhi) is a public health concern in many developing nations (2), and is also recognized as a potential bioterrorism agent in the US (3). *S*. typhi, does not infect other mammals (4), but several *Salmonella* serovars cause a typhoid-like disease in mice, sometimes referred to as murine typhoid (5). It has been demonstrated that immunization with a live vaccine strain (LVS) of *Salmonella* confers robust protective immunity to secondary infection in both murine and human typhoid (6,7), and both CD4 Th1 cells and antibody are required (8,9).

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Much less is known about the induction of acquired immunity during successful treatment of bacterial infections, including typhoid. In theory, antibiotic treatment should liberate bacterial antigens and Pathogen Associated Molecular Patterns (PAMPs) from dead bacteria and therefore allow efficient activation of pathogen-specific T and B cell responses. However, antibiotic treatment of primary typhoid failed to induce significant immunity to secondary infection in a study of human volunteers (10). Other clinical studies indicate that acquired immunity after recovery from primary typhoid is insufficient to prevent re-infection. Relapse of primary infection is reported in 5–10% of typhoid patients and 1–4% can become long-term carriers of disease (11). Indeed, re-infection with a molecularly distinct strain of *Salmonella* has been reported in patients that have previously resolved typhoid (12). Overall, these clinical observations suggest that acquired immunity following clearance of primary typhoid may differ substantially from protective immunity induced by transient colonization with LVS-*Salmonella*.

We decided to examine this issue directly by generating a mouse model that allows visualization of *Salmonella*-specific immunity during and after resolution of typhoid with antibiotic therapy. These experiments demonstrate that antibiotic-mediated resolution of murine typhoid elicits a limited acquired immune response that is insufficient to protect against secondary infection. Although CD4 Th1 cells were efficiently activated during the early stage of antibiotic treatment, they failed to fully develop into an effective Th1 memory pool. In marked contrast, effective Th1 memory matured over a period of several weeks in mice administered LVS-*Salmonella*. However, the weak acquired immunity evident in antibiotic-treated mice could be markedly enhanced by administration of a TLR5 agonist during the period of antibiotic treatment.

Materials and Methods

Mouse strains

RAG-deficient SM1 TCR transgenic mice expressing the CD90.1 allele have been described (13,14). C57BL/6 mice were purchased from NCI (Frederick, MD) and used at 6–12 weeks of age. IFN-γ-deficient, Rag-deficient, B cell-deficient (Igh-6^{tm1Cgn}), and MHC class-II-deficient mice were purchased from The Jackson Laboratory. All mice were housed in specific pathogen-free conditions and cared for in accordance with Research Animal Resources (RAR) practices at the University of Minnesota.

Salmonella infection and antibiotic treatment

S. typhimurium strains BRD509 ($AroA^{-}AroD^{-}$) and SL1344 were grown overnight in Luria-Bertani broth without shaking and diluted in PBS after determining bacterial concentrations using a spectrophotometer. Mice were infected orally by gavage with 5×10^{9} bacteria, immediately following administration of 100ul of a 5% $NaHCO_3$ solution. In all infection experiments, the actual bacterial dose was confirmed by plating serial dilutions onto MacConkey's agar plates and incubating overnight at 37°C. Mice infected with SL1344 were treated with Enrofloxacin (Baytril) at 2mg/ml in their drinking water for five weeks, beginning 2 days post-infection. Five days after antibiotic withdrawal, mice were re-challenged with 5×10^{7} SL1344 and monitored daily for survival. When moribund, mice were euthanized by cervical dislocation as stipulated by our animal care protocol.

Bacterial colonization in vivo

Spleens and mesenteric lymph nodes from infected mice were removed and homogenized in Eagle's Hanks Amino Acids (EHAA, Biofluids, Rockville, MD) containing 2% fetal bovine serum. Serial dilutions were plated on MacConkey's agar plates, incubated overnight at 37°C, and bacterial counts calculated for each organ.

Adoptive transfer of SM1 T cells

Spleen and lymph nodes (cervical, axillary, brachial, inguinal, periaortic, and mesenteric) of RAG-deficient, CD90.1 congenic, SM1 TCR transgenic mice were harvested. After generating a single-cell suspension, the percentage of SM1 cells was determined using a small aliquot of this suspension and antibodies to CD4, CD90.1, and Vβ2 (eBioscience, San Diego, CA). A FACS Canto (BD Biosciences) was used to determine the percentage of $CD4^+$ $V\beta2^+$ SM1 cells, and the total number of SM1 cells calculated. SM1 cells were then incubated with CFSE at 37°C for 8 minutes with shaking every 2–3 minutes. Cells were washed two times in cold HBSS before adjusting the concentration and injecting $1–3\times10^6$ SM1 T cells into the lateral tail vein of recipient C57BL/6 mice.

Flow cytometry

A single-cell suspension was generated from harvested mouse spleens, mesenteric lymph nodes and Peyer's Patches, and samples incubated on ice in the dark for 30 minutes in F_c block (spent culture supernatant from the 24G2 hybridoma, 2% rat serum, 2% mouse serum, and 0.01% sodium azide) containing primary antibodies. FITC-, PE-, PE-Cy5-, or APC-conjugated antibodies specific for CD4, CD11a, CD90.1, Vβ2, TNFα, and IFNγ were purchased from eBioscience and BD Bioscience. After staining, cells were analyzed by flow cytometry using a FACS Canto and data analyzed using FlowJo software (Tree Star, San Carlos, CA).

Tracking SM1 cells in vivo

After infection with *Salmonella*, spleens, mesenteric lymph nodes, and Peyer's Patches were harvested on days 3, 5, 10, and 20 into EHAA medium containing 2% fetal bovine serum. Cells were stained as described above. The percentage of SM1 cells per organ was determined, as well as the activation and expansion of SM1 cells using the cell-surface marker CD11a and CFSE dye dilution, respectively, using flow cytometry.

Salmonella-specific antibody

Mice were infected with *Salmonella* as described above. Each week post-infection, for five weeks, mice were bled retro-orbitally and serum was prepared by centrifugation and collection of supernatant. In addition, stool was collected, weighed, and suspended at 10% weight/volume in a fecal diluent (10mM Tris, 100mM NaCl, 1mM $CaCl_2$, 0.05% Tween 20, 5mM sodium azide, 1ug of aprotinin/ml, 1mM benzamidine, 10ug of leupeptin/ml, 10ug of pepstatin A/ml [pH 7.4]) (15) before centrifugation to remove fecal solids. Serum and processed stool samples were stored at −20°C before direct use in antibody ELISA. High protein binding plates were coated with heat-killed *S. typhimurium* diluted in 0.1M $NaHCO_3$ and incubated overnight at 4°C. After incubation in F_c block for one hour at 37°C, plates were washed twice in PBS/0.05% Tween 20. Samples were added in serial dilutions, diluted in 10% FCS/PBS, and incubated for two hours at 37°C. Plates were washed four times before biotin-conjugated antibody specific for the desired isotype was added. After incubation for one hour at 37°C, plates were washed six times. Finally, plates were incubated for one hour at 37°C in alkaline phosphatase diluted in 10% FCS/PBS. Plates were washed eight times and a substrate containing sodium phosphate, citric acid, O-phenylenediamene, and H_2O_2 was added. After sufficient color-change was observed, 2N H_2SO_4 was added to stop the reaction before plates were analyzed using a spectrophotometer.

Detection of in vivo cytokine production

Mice were infected with *Salmonella* as described above. Each week post-infection, for six weeks, mice were injected i.v. with bacterial antigens to activate *Salmonella*-specific T cells (10^8 heat-killed S. typhimurium). Six hours later, spleens and mesenteric lymph nodes were harvested into EHAA containing 2% fetal bovine serum and a single-cell suspension generated.

Following rapid surface staining on ice, cells were fixed with formaldehyde, permeabilized using saponin (Sigma-Aldrich), and stained intracellularly using cytokine-specific antibodies.

Flagellin immunization

Mice were infected with virulent *S. typhimurium* and treated with antibiotics as described above. On the first day of antibiotic treatment, mice were injected i.v. with 100ug of highly purified purified *Salmonella* flagellin (16). Injections were repeated each week for the duration of antibiotic treatment. After rechallenge with virulent *S. typhimurium*, as previously described, mice were monitored daily for signs of morbidity and euthanized when moribund.

Results

Effective treatment of murine typhoid with antibiotics

Ciprofloxacin is a fluoroquinolone antibiotic commonly used to treat human typhoid (17). We examined whether the veterinary fluoroquinolone derivative Enrofloxacin would allow resolution of fatal primary typhoid in C57BL/6 mice (18). Indeed, simply adding Enrofloxacin to the drinking water two days after oral infection allowed 100% of mice to survive a fatal infectious dose (Fig. 1A). Although *Salmonella* could not be detected in the spleen 72 hours after Enrofloxacin treatment (Fig. 1B, and data not shown), a much longer period of treatment was required to prevent recurrence of bacterial shedding following antibiotic withdrawal (data not shown). Similar reports of chronic bacterial shedding following apparent resolution of primary typhoid have been reported (19).

Recovery from primary infection confers limited protective immunity to re-infection

Mice that completely resolved primary infection using antibiotic treatment were re-infected to determine whether acquired immunity developed during this period of transient bacterial colonization. Indeed, naïve mice succumbed to infection with virulent *Salmonella* (Fig. 2A), whereas antibiotic-treated mice were protected against secondary infection, as demonstrated by increased survival time (Fig. 2A), and lower bacterial loads in the spleen and liver one week after secondary infection (data not shown). However, despite evidence of acquired immunity, almost all antibiotic-treated mice eventually succumbed to secondary typhoid (Fig. 2A), thus demonstrating that immunity following antibiotic treatment is incomplete. This weak protective immunity was absent in uninfected mice treated with antibiotics and therefore not attributable to residual antibiotics in tissues (Fig. 2B). When compared directly with effective LVS-*Salmonella* immunization, immunity in antibiotic-treated mice was considerably less efficient at protecting against secondary infection (Fig. 2C). Since the protective effect of LVS-*Salmonella* immunization correlated with a longer period of host colonization (Fig. 1C), it was of interest to determine whether antibiotic clearance of LVS-*Salmonella* would also result in incomplete acquired immunity. Indeed, treatment of mice immunized with LVS-*Salmonella* resulted in an inability of these mice to resolve secondary infection with virulent *Salmonella* (Fig. 3).

Acquired immunity following antibiotic therapy is mediated by CD4 T cells and antibody

CD4 Th1 cells and antibody are required for protective immunity conferred by LVS-*Salmonella* immunization (8,20,21). We examined the basis of weak acquired immunity in antibiotic-treated mice by resolving primary infection in several gene-deficient strains and then examining secondary immunity to typhoid. As expected, naïve wild-type (Wt) mice succumbed rapidly to typhoid whereas antibiotic-treated Wt mice survived longer (Fig. 4). Interestingly, antibiotic-treated IFN-γ-deficient mice (Fig. 4A), Rag-2-deficient mice (Fig. 4B), B cell-deficient mice (Fig. 4C), or mice lacking CD4 T cells (Fig. 4D) displayed no evidence of

acquired immunity to secondary infection, demonstrating that both Th1 cells and antibody are required for limited acquired immunity following antibiotic treatment of primary typhoid.

Early activation of pathogen-specific T cells in antibiotic-treated mice

The failure of antibiotic-treated mice to resist secondary infection suggested a deficiency in the initial activation or development of *Salmonella*-specific CD4 T cells. We used *Salmonella*-specific SM1 T cells to compare the initial activation of *Salmonella*-specific CD4 T cells after infection and antibiotic treatment versus immunization with LVS-*Salmonella*. SM1 cells were activated to increase surface expression of CD11a, underwent several rounds of cell division, and accumulated in the Peyer's patch and mesenteric lymph nodes (MLN) of mice administered LVS-*Salmonella* or treated with antibiotics to resolve primary typhoid (Fig. 5). Therefore, the initial activation of *Salmonella*-specific CD4 T cells was similar after oral immunization with LVS-*Salmonella* or antibiotic treatment of typhoid.

Lack of sustained effector Th1 and antibody response following antibiotic treatment

Next we examined the effector Th1 response at the time of secondary challenge. In mice immunized with LVS-*Salmonella*, a large proportion of spleen and mesenteric lymph node $CD11a^{Hi}$ CD4 T cells produced IFN-γ in response to re-stimulation (Fig. 6A). In contrast, a lower percentage of Th1 cells was detected in antibiotic-treated mice (Fig. 6A). Furthermore, Th1 cells in antibiotic-treated mice produced less IFN-γ on a per cell basis than Th1 cells recovered from LVS-*Salmonella* immunized mice (Fig. 6A). Therefore, weak protective immunity in antibiotic treated mice correlates with a deficiency in the number and activity of effector/memory Th1 cells.

Recent reports have suggested that CD4 T cells require sustained antigen presentation for maximal proliferation and development of effector function (22,23). We therefore examined the maturation of effector/memory Th1 responses over several weeks in mice immunized with LVS-*Salmonella* and mice resolving primary typhoid with antibiotic therapy. There was no difference in CD4 IFN-γ production between antibiotic-treated and LVS-*Salmonella* mice at 1 week (Fig. 6B), in broad agreement with the detection of efficient early activation of SM1 cells in antibiotic-treated mice (Fig. 5), and suggest that initial priming conditions are similar in both models. However, at later time points, Th1 cell responses increased dramatically in LVS-immunized mice but actually declined in mice administered antibiotics (Fig. 6B). Together, these data demonstrate a remarkable difference in the development of Th1 cells in LVS-vaccinated and antibiotic-treated mice.

A similar deficiency was noted in the *Salmonella*-specific antibody response. Rising titers of *Salmonella*-specific IgG2c were detected in the serum of LVS-*Salmonella* immunized mice (Fig. 7A). A smaller increase in *Salmonella*-specific IgM (Fig. 7B) and fecal IgA (Fig. 7C) titers was detected, but there was no increase in IgG1 (data not shown). In antibiotic-treated mice, lower titers of *Salmonella*-specific IgG2c were detected and a significant IgM response was absent (Fig. 7A, B). However, *Salmonella*-specific fecal IgA responses developed normally in antibiotic treated mice (Fig. 7C), perhaps because intestinal IgA class switching can occur in the absence of CD4 T cell help (24). Together, these data demonstrate that resolution of primary typhoid with antibiotic therapy causes a deficiency in the maturation of *Salmonella*-specific CD4 Th1 and serum antibody responses.

Recovery of protective immunity by co-administration of flagellin

Incomplete development of Th1 and antibody responses following antibiotic treatment might be due to very rapid elimination of bacterial antigen and PAMPs, causing CD4 T cell to mature in the absence of sustained inflammation. Therefore, we examined whether acquired immunity could be enhanced by administration of the TLR5 agonist, flagellin, which has been shown to

function as an effective adjuvant and is also an immunodominant antigen recognized by CD4 T cells (25). As expected, naïve wild-type (Wt) mice succumbed rapidly to infection but mice that had previously resolved typhoid using antibiotics survived longer (Fig. 8). Weekly administration of bacterial flagellin during the period of antibiotic treatment allowed mice that resolve primary infection to resist re-infection with typhoid (Fig. 8). This protective effect was not simply due to the immunizing effect of flagellin itself as flagellin administration failed to induce significant protective immunity in uninfected mice (Fig. 8). Thus, administration of a simple TLR5 agonist and antigen during the period of antibiotic therapy allowed recovery of robust protective immunity to typhoid.

Discussion

Immunization with live attenuated organisms can induce cellular and humoral immunity responses and effective protection against numerous vial and bacterial pathogens. One unique aspect of these live vaccines is that they involve a short period of host colonization with a weakened microbe. Much less is known about the development acquired immunity following antibiotic treatment of an infection with virulent bacteria, despite the fact that this is a common occurrence in endemic areas.

Unlike several other antibiotics (Griffin et al, unpublished), we found that Enrofloxacin is highly effective at clearing primary typhoid in the mouse model. Indeed, other investigators have recently used Enrofloxacin to resolve *Salmonella* infection in mice (26). Although Enrofloxacin rapidly eliminated bacteria from the spleen and liver, mice recommenced the shedding live bacteria in fecal pellets if treatment was withdrawn before 30 days of treatment. Under these circumstances spleens and livers were re-colonized by rapidly growing bacteria and all mice succumbed to recurrent infection (data not shown). The anatomical site where persistent *Salmonella* survive during treatment is not yet clear, but this issue is under investigation in our laboratory. If mice were treated with Enrofloxacin in drinking water for at least 30 days, no recurrent infection developed, no bacteria were shed in fecal pellets, and these mice survived in our animal facility for over 6 months. Prolonged Enrofloxacin treatment is therefore effective at resolving murine typhoid in highly susceptible mice.

Mice that fully resolved primary infection with antibiotic treatment displayed evidence of acquired immunity to re-infection in that they survived longer and had lower bacterial burdens. This protective immunity required production of IFN-γ and the presence of class-II-restricted T cells indicating an important protective role for Th1 cells, similar to the protective immunity induced by LVS-*Salmonella* (27–30). Our experiments also demonstrate a prominent role for B cells in resistance following antibiotic treatment, consistent with previous studies of immunity mediated by live attenuated *Salmonella* (31–33). However, it should be noted that B cell deficient mice have also been reposted to have a deficiency in the development of Salmonella-specific Th1 cells (32,34), complicating interpretation of this particular experiment. The presence of specific antibody may simply reduce the initial challenge dose before intracellular infection takes place or prevent cell-to-cell transmission after macrophage apoptosis. However, a role for B cells and/or antibody in the enhancement of antigen presentation to CD4 T cells by dendritic cells has also been suggested (32,34,35).

Surprisingly, the adaptive immune response induced following antibiotic therapy was considerably less robust than LVS-*Salmonella* immunization and was insufficient to protect against re-infection. The weak response was not due to an intrinsic difference in virulent versus LVS strains since antibiotic treatment of LVS-immunization severely hindered the development of protective immunity. Failure to develop robust acquired immunity after antibiotic treatment is consistent with clinical reports suggesting weak protective immunity in typhoid endemic areas and the lack of protective immunity in studies using human volunteers

(10–12). It is unlikely that incomplete immunity was due to a lack of antigen, since at the time of antibiotic administration bacterial loads are very high in the spleen and liver of infected mice. Furthermore, *Salmonella*-specific CD4 T cells were effectively activated and initially developed effector Th1 responses that were similar to mice immunized with LVS-Salmonella. These data suggest that the initial priming of bacterial-specific responses occurs normally in antibiotic treated-mice.

In marked contrast, we detected a deficiency in Th1 and antibody responses at later time points after antibiotic treatment. This immune deficiency correlated with the inability of these mice to resist secondary infection. We propose that the rapid elimination of bacterial antigen and PAMPs under antibiotic therapy disrupts the normal development of Th1 immunity that is detected in LVS-*Salmonella* immunized mice. Indeed, it has previously been noted that the full proliferative capacity and effector development of CD4 T cells requires prolonged antigen stimulation (22,23). However, unlike these previous reports, our experiments have been completed in an infectious disease model that requires CD4 T cells for protective immunity. Together, our data indicate that an effective therapy that rapidly eliminates bacteria from the host may actually be detrimental to the subsequent acquisition of CD4-mediated protective immunity to secondary infection. If this is the case, it represents an important challenge to antibiotic therapy in endemic areas since successful treatment may therefore leave the individual susceptible to re-infection.

There are two aspects of bacterial persistence that might be required for the full development of CD4 responses. First, antigen persistence may allow pathogen-specific T cells to gradually acquire full effector function through sequential restimulation of responding clones. Second, the presence of prolonged inflammatory stimuli could provide non-TCR mediated maturation signals that allow Th1 development. Our data show that intervention with flagellin, which unusually happens to be an antigenic target of *Salmonella*-specific T cells and a TLR agonist, can rescue the development of effective protective immunity in antibiotic-treated mice. We are currently examining whether intervention with other adjuvants or *Salmonella* antigens would also have a similar effect. Addition of adjuvant delivery or therapeutic vaccination to antibiotic therapy may therefore be an important requirement for optimal development of CD4 Th1 responses and protection from secondary challenge. Therefore, these studies may aid the design of attenuated vaccines or the development of a novel therapy against bacterial infection.

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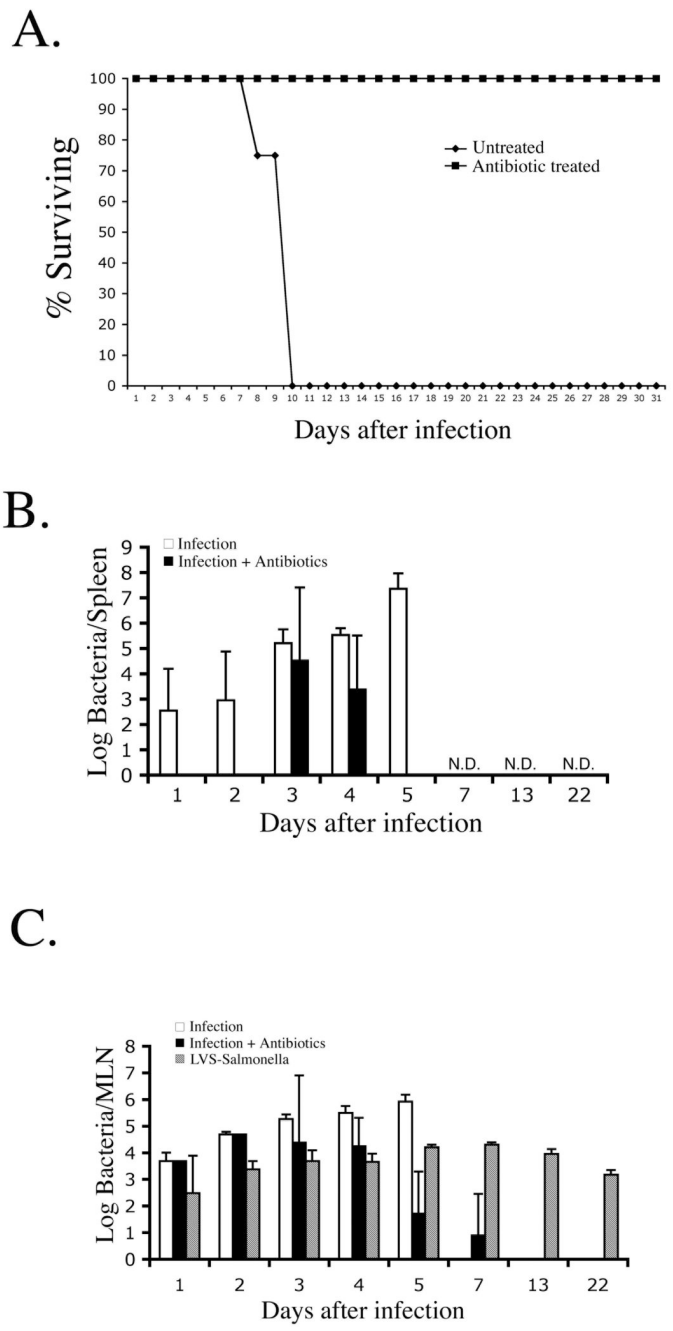

Figure 1. Enrofloxacin effectively resolves active murine typhoid

C57BL/6 mice were orally infected with 5×10^9 virulent *S. typhimurium* (SL1344) and some mice treated with enrofloxacin drinking water. (A) Date show percent survival of antibiotic-treated and untreated mice and is representative of three similar experiments. (B) Spleens were harvested from infected mice at various time-points after infection and bacterial loads were determined by plating organ homogenates on MacConkey's agar. All untreated infected mice were dead by day 7. Data show mean bacterial load +/− SD for 3–5 mice per time point. (N.D. = no bacteria detected in antibiotic-treated mice).

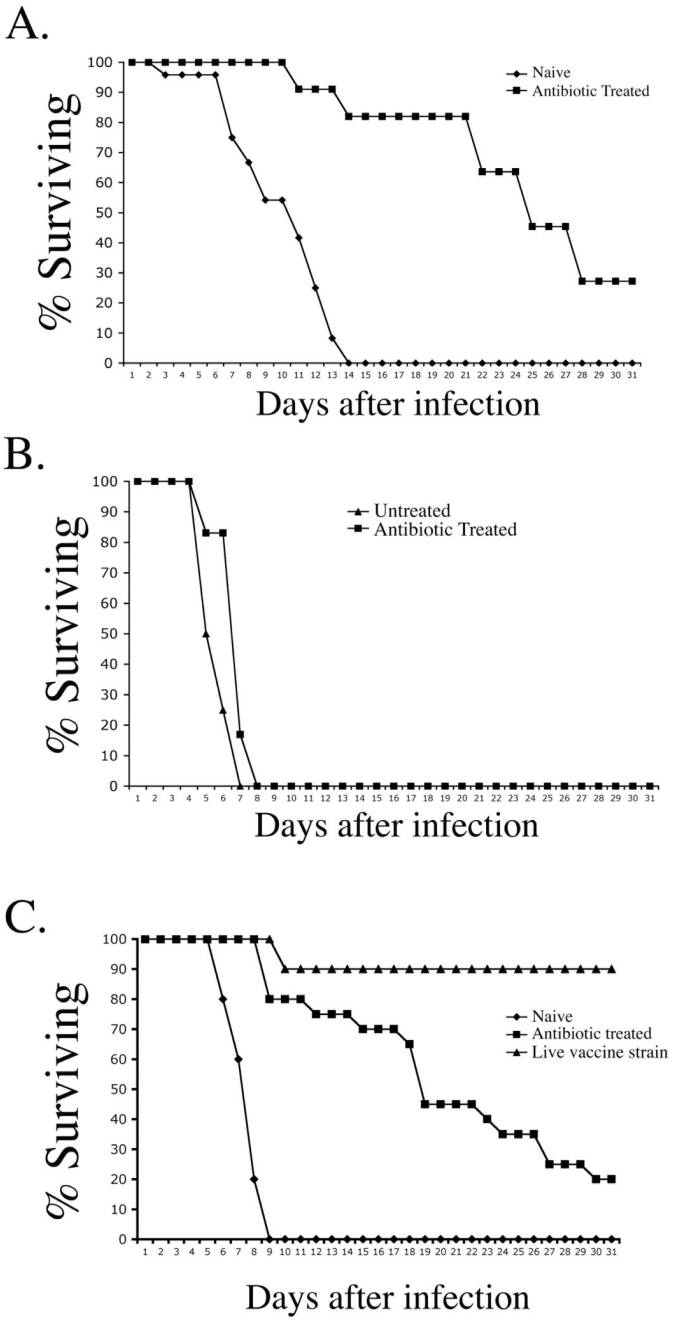

Figure 2. Incomplete protective immunity to typhoid in antibiotic-treated mice

(A) C57BL/6 mice were orally infected with 5×10^9 virulent *S. typhimurium* and treated with enrofloxacin for 35 days to resolve primary infection. Five days after antibiotic withdrawal, these treated mice and a group of naïve C57BL/6 mice were orally infected with 5×10^7 virulent *S. typhimurium*. (B) Naïve C57BL/6 mice were administered enrofloxacin in drinking water for 30 days before oral infection with virulent *S. typhimurium* five days after antibiotic withdrawal (C) Some C57BL/6 mice were immunized orally with 1×10^{10} LVS-*Salmonella* (BRD509) while other mice were treated exactly as in A. Forty two days later, both group of mice were challenged orally with 5×10^7 virulent *S. typhimurium*. Graphs show percent survival of infected mice and are representative of 2–5 similar experiments.

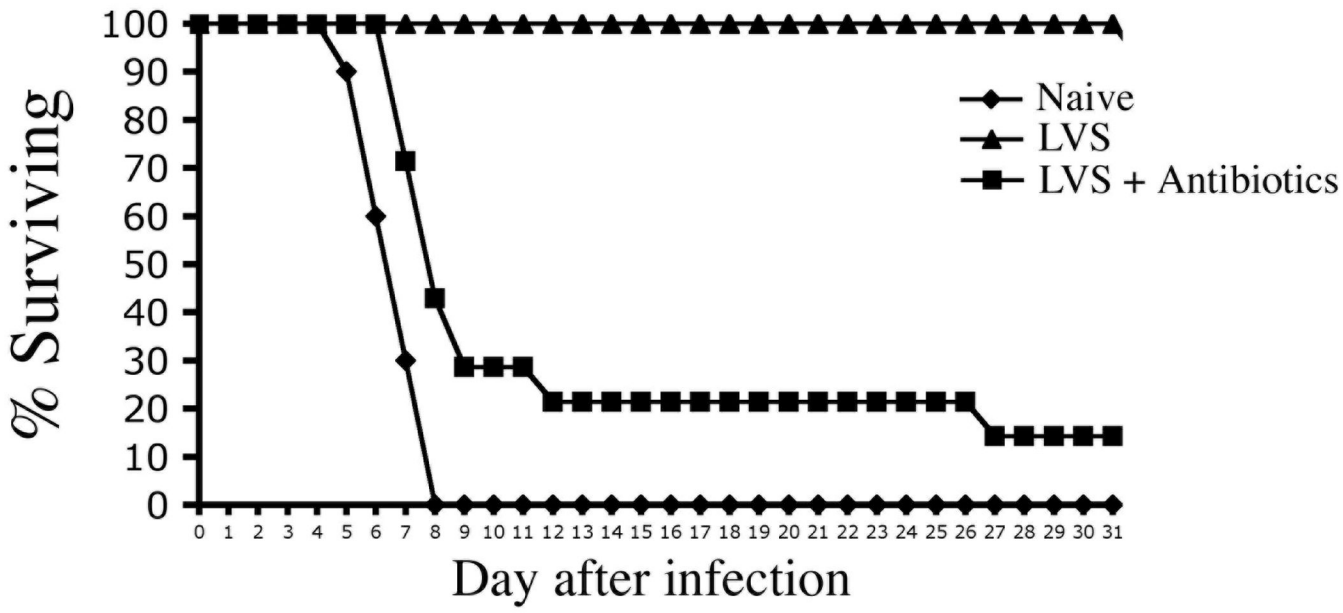

Figure 3. Incomplete protective immunity in antibiotic-treated mice immunized with LVS *Salmonella*

(A) C57BL/6 mice were orally infected with 5×10^9 LVS-Salmonella and some mice were treated with enrofloxacin for 35 days to resolve primary infection. Five days after antibiotic withdrawal, these mice and a group of naïve C57BL/6 mice were orally infected with 5×10^7 virulent *S. typhimurium*.

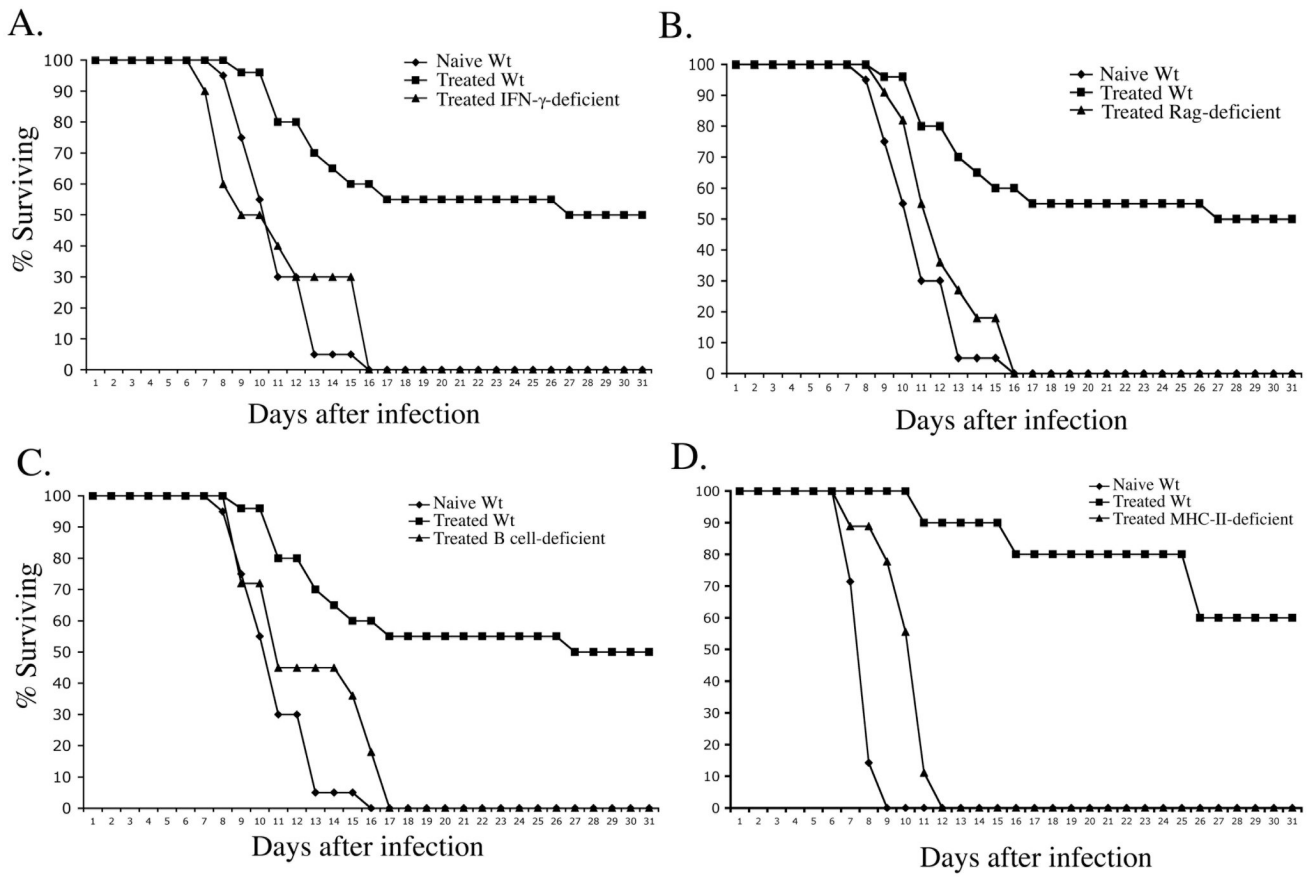

Figure 4. Acquired immunity after antibiotic therapy of typhoid requires class-II restricted T cells and antibody

C57BL/6 (Wt) and, (A) IFN-γ-deficient, (B) Rag-deficient, (C) B cell deficient, or (D) MHC class-II deficient mice, were infected orally with 5×10^9 virulent *S. typhimurium* and treated with antibiotics in drinking water for 35 days. Antibiotic-treated and naïve Wt mice were re-infected orally with 5×10^7 virulent *S. typhimurium*. Graphs show the percent survival of infected mice and are representative of 2–3 similar experiments.

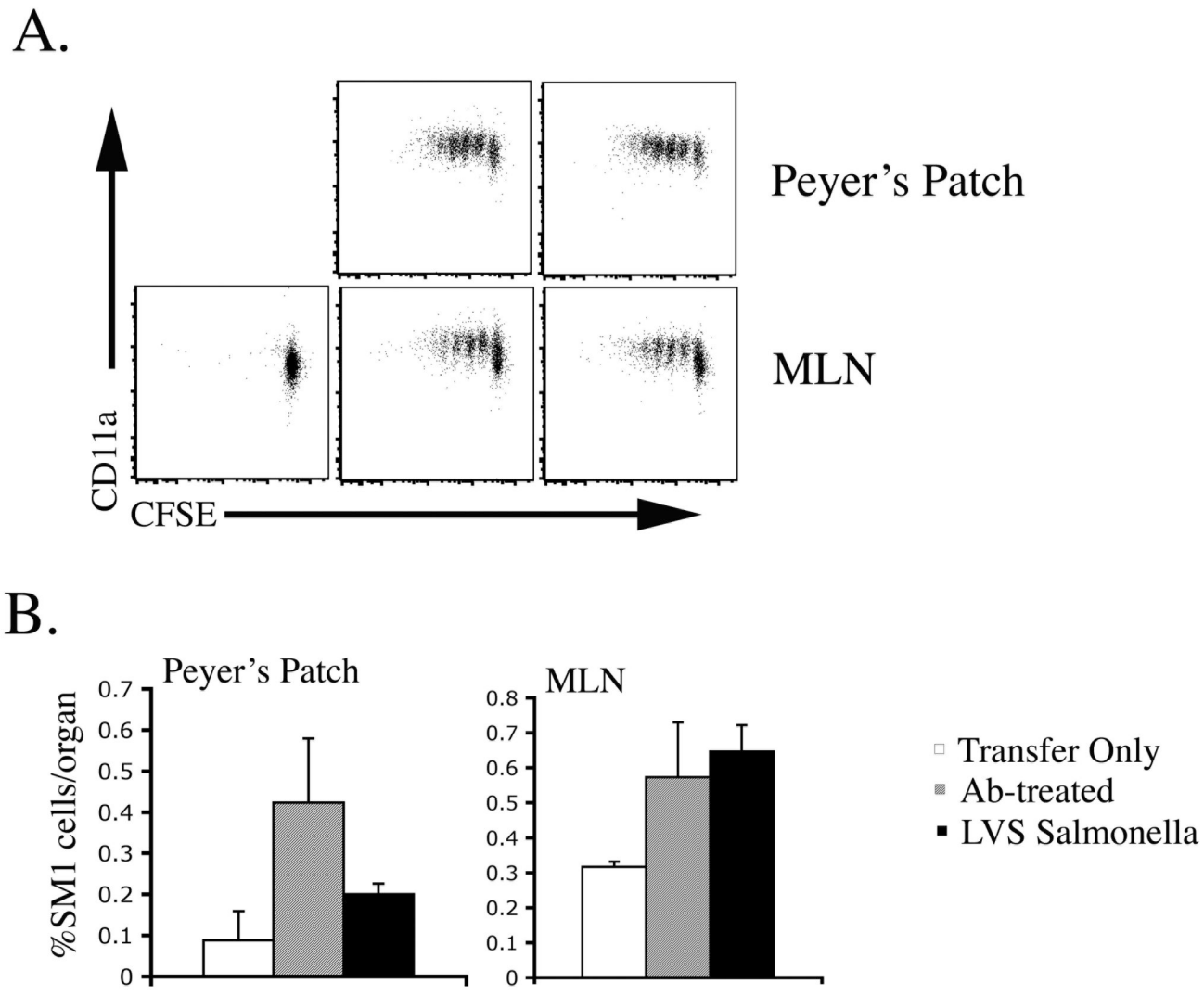

Figure 5. Rapid activation of *Salmonella*-specific CD4 T cells following antibiotic-cure or LVS-*Salmonella* immunization

C57BL/6 mice were adoptively transferred with 2×10^6 CFSE-stained CD90.1+ SM1 T cells before oral infection with 5×10^9 LVS-*Salmonella* (BRD509) or oral infection with 5×10^9 virulent *S. typhimurium* (SL1344) followed by antibiotic treatment 2 days later. Three days after infection Peyer's Patches and mesenteric lymph nodes (MLN) were harvested and SM1 T cells identified by flow cytometry staining for CD4 and CD90.1 (A) CD11a surface staining and CFSE dye dilution, and (B) the percentage of SM1 T cells from antibiotic-treated and LVS-*Salmonella* immunized mice.

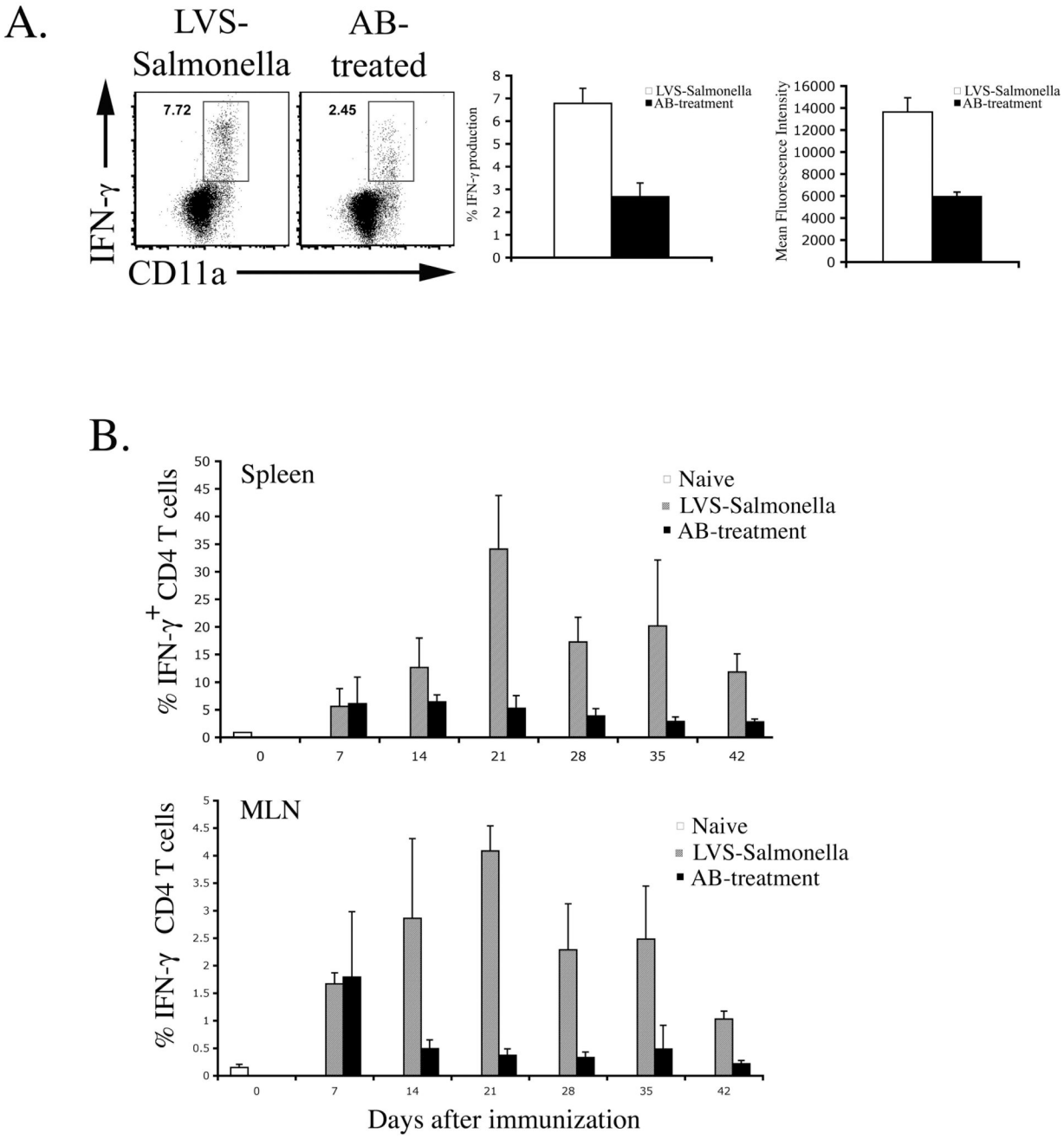

Figure 6. Deficient maturation of Th1 responses in antibiotic-treated mice

C57BL/6 mice were immunized with LVS-*Salmonella* or orally infected with virulent *S. typhimurium* and treated with antibiotics for 35 days. (A) Forty-two days after immunization or infection, CD4 Th1 cell responses in the spleen were examined by re-stimulation with *Salmonella* lysate and direct ex-vivo detection of intracellular cytokine production. FACS plots show IFN-γ production by gated CD4 T cells in response to six hours of stimulation with *Salmonella* lysate. Bar graphs show (middle) mean percentage of IFN-γ^+ CD4 T cells +/− SD, and (right) mean-fluorescence intensity +/− SD of IFN-γ producing cells from 4 mice per group. Data are representative of three similar experiments. (B) At weekly time points, CD4 Th1 responses were examined exactly as described in A. Bar graphs show mean percentage of IFN-

γ^+ CD4 T cells +/− SD in the spleen and MLN of 3–5 mice per group and are representative of 2 similar experiments.

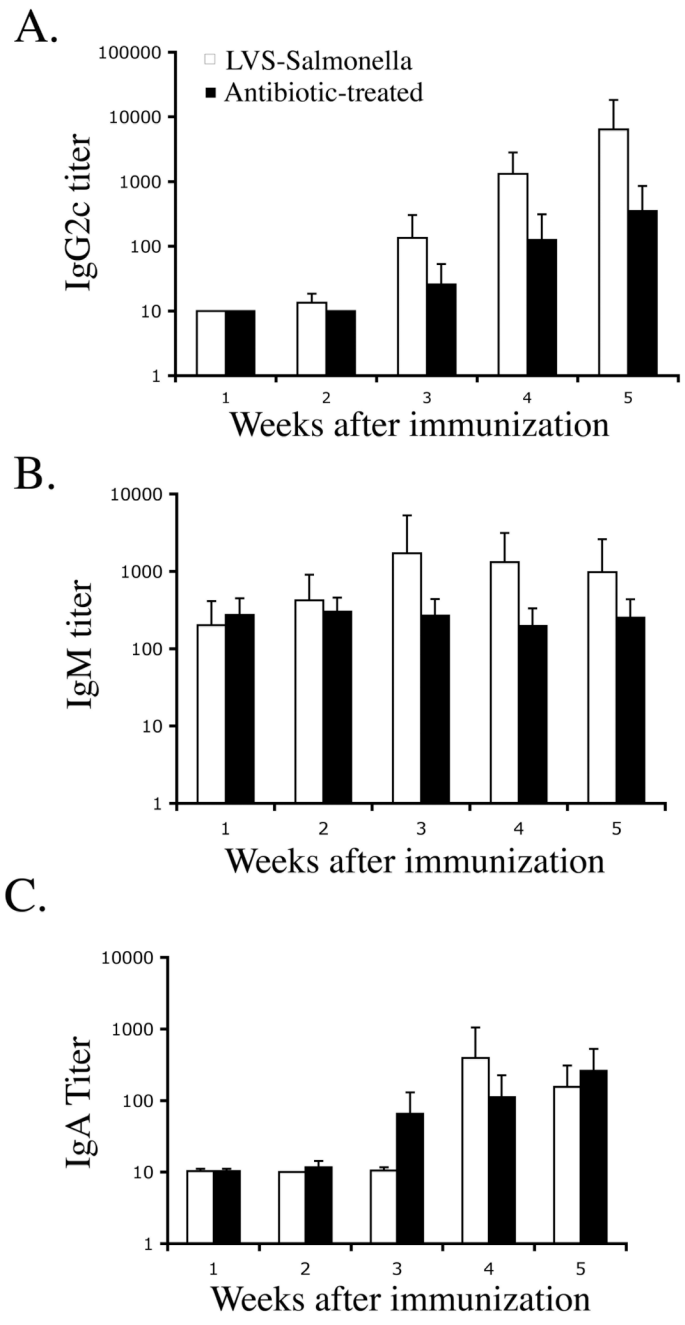

Figure 7. Deficient development of *Salmonella*-specific serum antibody responses in antibiotic-treated mice

C57BL/6 mice were immunized with LVS-*Salmonella* or orally infected with virulent *S. typhimurium* and treated with antibiotics for 35 days. (A–B) Blood was collected at weekly time points and *Salmonella*-specific antibody responses examined using isotype-specific antibody ELISAs (C) Stool was collected, weighed, suspended in a fecal diluent, and the presence of *Salmonella*-specific IgA determined using an antibody ELISA.

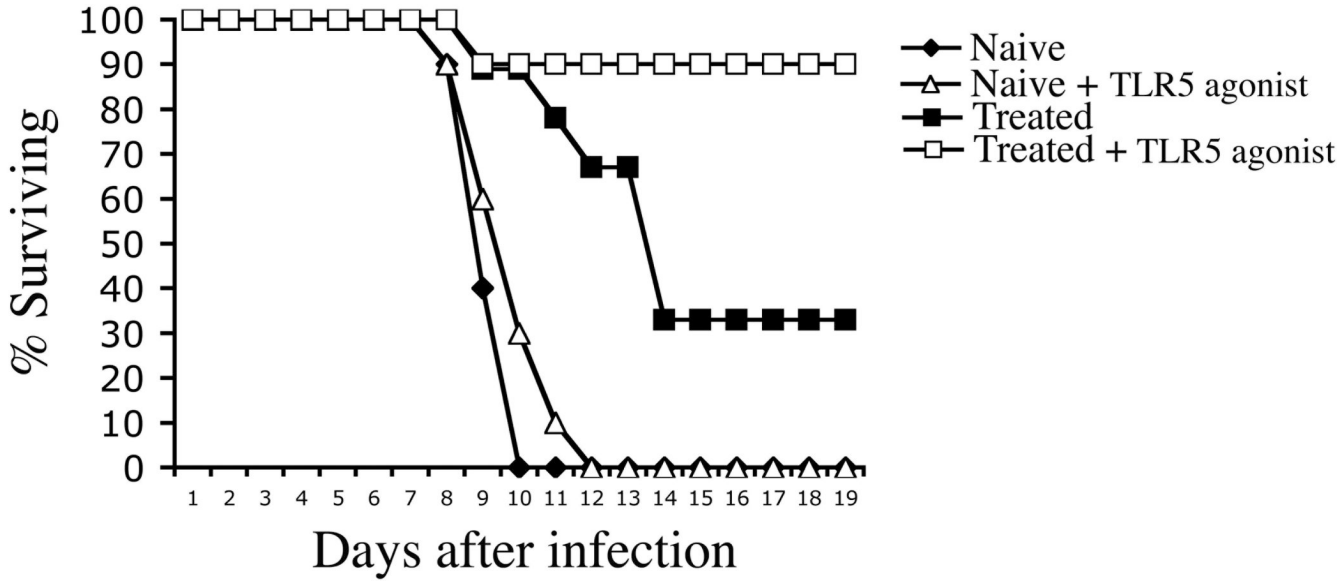

Figure 8. Treatment with flagellin enhances protective immunity in antibiotic-treated mice

C57BL/6 mice were orally infected with virulent *S. typhimurium* and treated with antibiotic drinking water for 35 days. Groups of infected and naïve mice were injected i.v. with 100 μg purified flagellin at weekly intervals during antibiotic treatment. Five days after antibiotic treatment was halted, naïve and antibiotic-treated mice were infected orally with virulent *S. typhimurium*. Data shows the percent survival of infected mice and is representative of 2 similar experiments.