Salmonella Induces a Switched Antibody Response without Germinal Centers That Impedes the Extracellular Spread of Infection[1]

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T-dependent Ab responses are characterized by parallel extrafollicular plasmablast growth and germinal center (GC) formation. This study identifies that, in mice, the Ab response against *Salmonella* is novel in its kinetics and its regulation. It demonstrates that viable, attenuated *Salmonella* induce a massive early T-dependent extrafollicular response, whereas GC formation is delayed until 1 mo after infection. The extrafollicular Ab response with switching to IgG2c, the IgG2a equivalent in C57BL/6 mice, is well established by day 3 and persists through 5 wk. Switching is strongly T dependent, and the outer membrane proteins are shown to be major targets of the early switched IgG2c response, whereas flagellin and LPS are not. GC responses are associated with affinity maturation of IgG2c, and their induction is associated with bacterial burden because GC could be induced earlier by treating with antibiotics. Clearance of these bacteria is not a consequence of high-affinity Ab production, for clearance occurs equally in CD154-deficient mice, which do not develop GC, and wild-type mice. Nevertheless, transferred low- and high-affinity IgG2c and less efficiently IgM were shown to impede *Salmonella* colonization of splenic macrophages. Furthermore, Ab induced during the infection markedly reduces bacteremia. Thus, although Ab does not prevent the progress of established splenic infection, it can prevent primary infection and impedes secondary hemogenous spread of the disease. These results may explain why attenuated *Salmonella*-induced B cell responses are protective in secondary, but not primary infections. ***The Journal of Immunology,* 2007, 178: 6200–6207.**

Salmonella infections are an important health concern worldwide. Typhoid remains a serious public health problem, with 600,000 deaths yearly attributable to this disease. In addition, in sub-Saharan Africa, nontyphoidal *Salmonella* infection resulting in bacteremia is a major cause of death in infants, with 20–30% mortality in hospitalized children (1).

Immunity to *Salmonella* is complex, and effective immunity requires both T and B cells (2, 3). CD4 and CD8 T cells play an important role in curtailing intracellular infection, and impaired IL-12 function is particularly associated with extensive invasive disease (4). An important role for Ab has been demonstrated in *Salmonella* infections. Meta-analysis of vaccine studies has shown that the Ab induced by heat-killed *Salmonella* can be effective, possibly even more so than live vaccines, at protecting humans against typhoid (5). Moreover, Ab induced by conjugate Vi vaccines affords effective immunity (6). Also, despite extensive granuloma formation in individuals with IL-12 or IL-12R deficiency, these patients do not succumb to *Salmonella* bacteremia (7). Lastly, a number of studies have shown varying, but important, roles for Ab in murine models of salmonellosis (8–10). Ab was protective after primary per-oral administration of a virulent strain, but not after i.v. administration. Naive wild-type (WT)[3] and B cell-deficient mice resisted colonization equally after infection with attenuated *Salmonella* strains, but B cell-deficient mice were markedly more susceptible to subsequent per-oral or i.v. infection with a virulent strain of *Salmonella*. Furthermore, some (8, 11), but not all studies (10), showed a degree of protection associated with transfer of immune serum into immunocompromised mice.

In T-dependent responses, plasma cells develop from one of two major pathways, from extrafollicular (EF) growth of activated B cells as plasmablasts (12) or by differentiation of B cells that have proliferated and undergone affinity maturation in germinal centers (GC) (13). EF responses provide first line defense and can be induced by T-dependent and T-independent Ag, and the plasma cells induced during these responses reside primarily in the red pulp of the spleen or the medulla of lymph nodes. The affinity of Ab produced reflects that available after primary Ig V-gene rearrangement and is typically lower than Ab from GC. In mice, T-dependent switching reflects the direction of T cell help; Th1-directed switching is to IgG2a (IgG2c in C57BL/6 mice) and IgG2b, whereas Th2-directed switching is to IgG1 and IgE. In the Th2 response to aggregated proteins, small numbers of IgG1 plasma cells are detectable in EF foci 5 days after primary immunization in lymph node and slightly later than this in the spleen (14). In such responses, functional GC have formed by day 7 and high-affinity

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[3] Abbreviations used in this paper: WT, wild type; EF, extrafollicular; AID, activation-induced cytidine deaminase; Bcl6, B cell lymphoma 6; GC, germinal center; OMP, outer membrane protein of *S. typhimurium*.

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Ab starts to be produced during the second week after immunization along with memory B cells (15). Thus, typically in Th2 responses, the GC and EF responses develop in parallel (16), and in the absence of GC, memory B cell responses are highly curtailed.

The present study analyzes the Ab response in mice to live, attenuated *Salmonella enterica* serovar typhimurium (*S. typhimurium*) and relates this to the course of the infection. It shows the extensive and rapid switched EF response is T dependent, but GC formation and affinity maturation are delayed until the second month of infection. Using mice that cannot develop productive GC, we show that clearance of this attenuated strain of bacteria from the spleen is not dependent on late, high-affinity Ab production. Both transferred high- and low-affinity Ag-specific Ab are shown to impair colonization of the spleen and bacteremia.

Materials and Methods

Bacteria, mice, outer membrane protein (OMP) preparation, infections, immunizations, and antibiotic regimen

The attenuated *S. typhimurium* SL3261 strain was used throughout these studies (17). C57BL/6 mice were obtained from HO Harlan OLAC. CD154-deficient mice have been fully described elsewhere (18) and were obtained from in-house colonies. Mice were age and sex matched and used between the ages of 6 and 12 wk. B cell-deficient mice are generated from breeding out the QM IgH transgene from QM mice, which have the other IgH locus inactivated (19). Outer membrane fractions were prepared, as previously described (20). Briefly, bacterial cells were harvested by centrifugation and washed and resuspended in 10 mM Tris-HCl (pH 8.0) containing protease inhibitors and RNase. The cells were lysed using a French pressure cell at 20,000 pounds per square inch, and cell envelopes were harvested by centrifugation. The cell envelope fraction was extracted with 2% (v/v) Triton X-100, and the outer membrane fraction was harvested by centrifugation and washed extensively in 10 mM Tris-HCl (pH 8.0) to remove the detergent. Flagellin was prepared, as described previously, as a his-tagged recombinant protein purified by metal affinity chromatography and by immunoprecipitation using a FliC-specific mAb (21). All infections were induced by infecting mice i.p. with 10^5 live organisms. Mice receiving this dose initially exhibit mild clinical signs, such as transient weight loss, but these resolve after 24–48 h. Bacterial numbers were assessed by plating out spleen homogenates and counting the numbers of colonies. Blood bacterial levels were determined by plating 200 μl of blood immediately after cardiac puncture before the blood clotted.

Some mice were given unlimited access to 2.5% (w/v) oral enrofloxacin (Baytril; Bayer HealthCare) between the 7th and 14th day of infection in drinking water, as per the manufacturer's directions.

Immunohistology

Preparation of spleens for immunohistology and staining of spleens was performed, as previously described (21–23). In brief, 5-μm (and 25-μm for PCR; see below) sections from frozen spleens were cut and fixed in acetone. Sections were stained for Ki67; either IgM, IgG2c, or IgD; and either biotinylated peanut agglutinin (Vector Laboratories), rabbit anti-B cell lymphoma 6 (Bcl6) (Santa Cruz Biotechnology), or rat anti-CD138 (BD Pharmingen). Binding of rat anti-IgM, IgG2c, and CD138 was detected with biotinylated sheep anti-rat Abs (The Binding Site). Binding of rabbit anti-Bcl6 was detected using biotinylated swine anti-rabbit Abs (DakoCytomation). These were followed by streptavidinABComplex-alkaline phosphatase (DakoCytomation). The bound alkaline phosphatase activity was then detected using naphthol AS-MX phosphate and Fast Blue salt with levamisole. Sheep anti-mouse IgD (The Binding Site) was labeled with peroxidase-labeled donkey anti-sheep (The Binding Site). Rabbit anti-Ki67 was identified using swine anti-rabbit Ig, followed by rabbit peroxidase/anti-peroxidase (DakoCytomation). HRP was detected using diaminobenzidine tetrahydrochloride solution. Cell numbers and densities were estimated using point counting, and counts were adjusted for the different sizes of the spleens seen throughout the study by multiplying the cells/mm^3 by the mass of the spleen. GCs were evaluated as a percentage of follicle occupied by peanut agglutinin staining.

Reverse transcription of mRNA and its relative quantification by PCR

RNA and cDNA were prepared, as previously described (21–23). Briefly, RNA was purified from tissue sections using the RNeasy Midi kit (Qiagen), according to protocol. The RNA pellet was reverse transcribed by standard methods using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies).

Relative quantification of activation-induced cytidine deaminase (AID) to β-actin message using multiplex PCR, primer, and probe sequences, and adjustment for section size was performed, as described previously (23). Reaction conditions were the standard conditions for the TaqMan PCR, but with 45 PCR cycles.

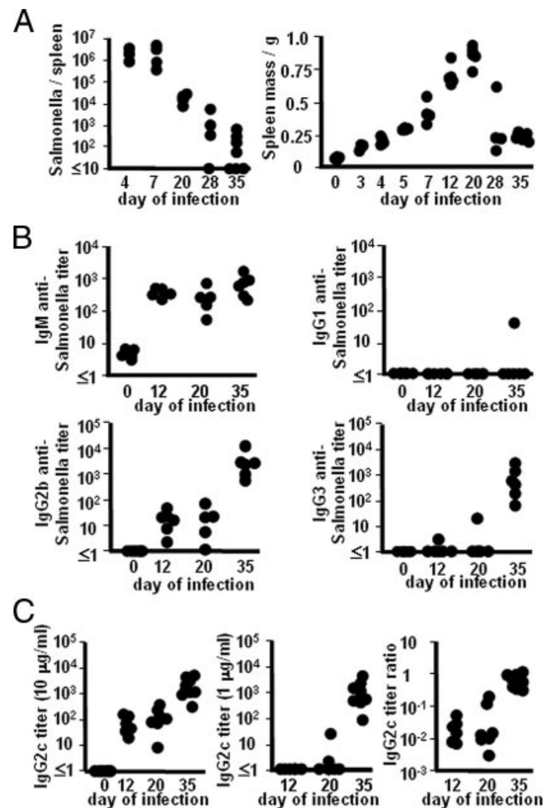

FIGURE 1. High-affinity Ab against *S. typhimurium* does not develop until the bacterial load is reduced to low levels. Mice were infected with 10^5 live *S. typhimurium* i.p. *A*, Splenic bacterial counts (*left*) and spleen size at intervals after i.p. infection. *B*, *Salmonella*-specific Ab titers other than IgG2c assessed using ELISA plates coated with 10 μg/ml *S. typhimurium* homogenate. *C*, IgG2c Ab titers in which Ag availability in the assay is not limiting (10 μg/ml-coated plates, *left*) or limited (1 μg/ml-coated plates, *center*). Affinity is represented (*right*) by the ratio of Ab titers using the nonlimiting plates to those using the Ag-limited plates. The higher the ratio, the higher the affinity of Ab. Each graph represents one experiment, and each point represents values for one mouse. Experiments are representative of three repeats.

Ab detection

Salmonella-specific serum Ab was detected by ELISA. Whole cell Ag was prepared by homogenizing whole *S. typhimurium*, from a fresh overnight culture, in deionized water containing 0.01% azide, in a Mini Beadbeater. Plates (Maxisorp; Nunc) were then coated with Ag at either 10 or 1 μg/ml. Pilot experiments using a range of coating concentrations show that these Ag concentrations of whole cell lysate added to the plate gave optimal discrimination between high- and low-affinity Ab. OMP or rFliC were coated on plates at 5 μg/ml. *S. typhimurium* LPS was obtained commercially (Quadratech) and used at 5 μg/ml. Primary mouse Abs were added and detected using isotype-specific goat anti-mouse secondary Abs linked to alkaline phosphatase (Southern Biotechnology Associates). Color was developed using the Sigma Fast *p*-nitrophenyl phosphate tablets.

Opsonization of bacteria

Before being used for opsonization, all serum was heated to 56°C for 20 min to inactivate complement. In each experiment, four mice were used to test each serum and at least two different sera were tested for each class of Ab. For anti-*Salmonella*, IgM^+, or $IgG2c^+$ serum, the titer of each serum

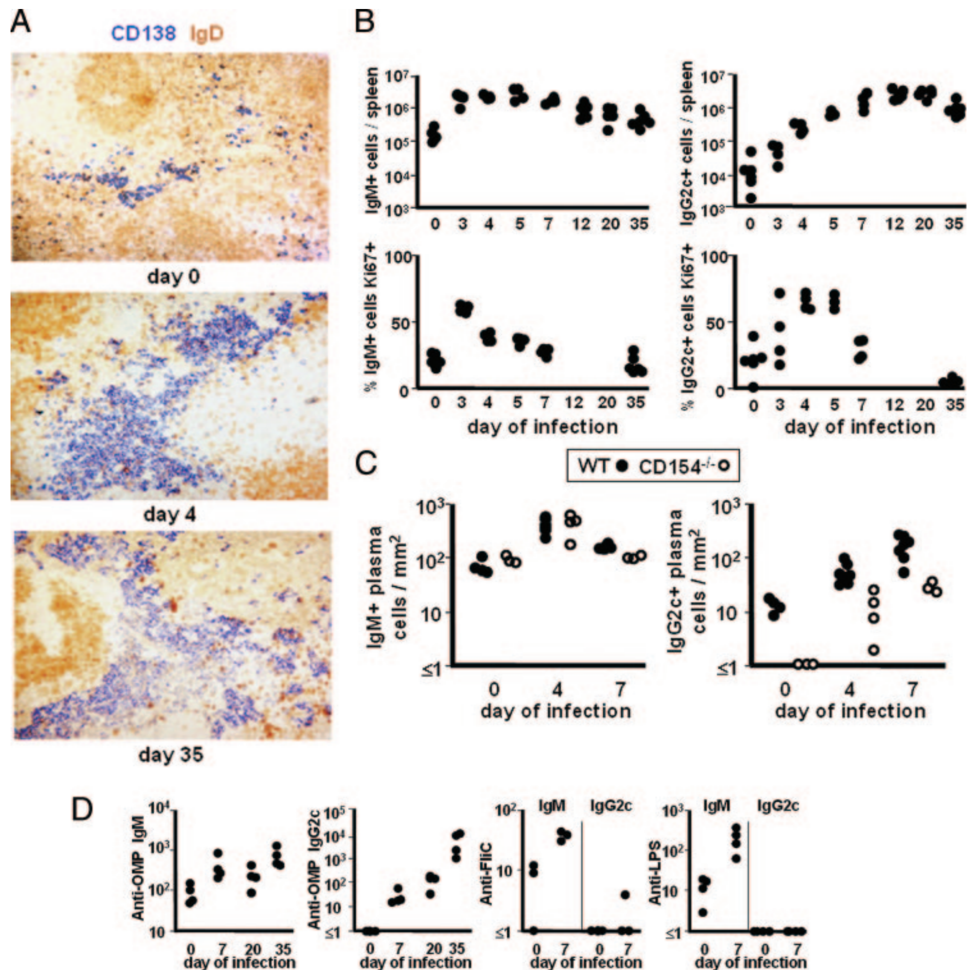

FIGURE 2. *Salmonella* induces a massive, early, and persistent EF Ab response. Mice were infected with 10^5 live *S. typhimurium* i.p. *A*, Representative photomicrographs of spleen sections stained for CD138$^+$ plasmacytoid cells (blue) and IgD (brown) from noninfected WT mice (*top*) and mice infected for 4 days (*center*) or 35 days (*bottom*). *B*, Enumeration of IgM plasmacytoid cells in spleens induced by infection (*top left*) and the proportion of these cells in cell cycle as assessed by expression of the proliferation marker Ki67 (*bottom left*). Equivalent results for IgG2c plasmacytoid cells (*right*). *C*, The influence of CD40 ligation on switching in the EF response. The relative numbers of IgM$^+$ plasmacytoid cells (*left*) and IgG2c$^+$ cells (*right*) in WT (●) and CD154-deficient mice (○) before or 4 or 7 days after infection with *S. typhimurium*. *D*, Serum ELISA using OMP-coated plates, or plates coated with the phase 1 flagellar protein, FliC, or plates coated with LPS. Each graph is representative of one experiment; each point represents values for one mouse; and experiments were repeated three times for WT-only panels and five times for experiments with CD154-deficient mice.

for each isotype was matched as closely as possible. After washing, bacterial concentration was adjusted to 10^5 organisms per 200 μl, the concentration used for infection. Ab was then diluted 1/100, and the Ab and organisms were mixed with gentle agitation for 30 min at room temperature. Afterward, bacteria were cultured to ensure no loss of viability or agglutination, and mice were infected.

Statistics

Statistics were calculated using the nonparametric Mann-Whitney sum of ranks test. The *p* values were calculated using the Analyze-It program.

Results

Attenuated S. typhimurium *colonizes the spleen and induces progressive splenomegaly that starts to resolve during the fourth week of infection*

Infection of the susceptible C57BL/6 mouse strain with 10^5 viable attenuated *S. typhimurium* bacteria i.p. results in a slowly resolving infection that is characterized by splenomegaly (Fig. 1*A*). Splenic bacterial numbers peak by day 7, and gradually fall to low levels over the next month. Splenomegaly peaks after 3 wk when the spleen is greater than 10 times that of noninfected mice. After this, there is rapid reduction in spleen weight so that by the end of the first month it is only 2–3 times greater than that of noninfected controls.

S. typhimurium*-specific IgG2c is detected soon after infection, but high-affinity Ab only appears in the second month of infection*

Fig. 1, *B* and *C*, *left panel*, shows the time course of *S. typhimurium*-specific Ab production. This occurs in two distinct phases. The first phase lasting through 3 wk is associated with early onset of IgM, IgG2c production with some IgG2b. In the second phase, there is a significant increase in the IgG2c, IgG2b titers ($p < 0.01$), and *S. typhimurium*-specific IgG3 is now detected. Production of IgG1 is rare at any stage of the disease. The sharp rise in Ab titer seen between days 20 and 35 is associated with an increase in Ab affinity (Fig. 1*C*). Thus, whereas low-affinity Ab produced in the first 3 wk only bound to plates coated with 10 μg/ml Ag (Fig. 1*C*, *left panel*), high-affinity Ab seen at day 35 bound to plates coated with 10-fold less Ag (Fig. 1*C*, *central panel*). The affinity of the Ab measured as a ratio of these titers, and Fig. 1*C* (*right panel*) shows that high-affinity Ab was only detected at day 35 when the affinity was nearly 100-fold greater than that on day 20 (*central* and *right panel*; day 20 ratio < day 35 ratio; $p < 0.01$). Thus, high-affinity Ab was only detectable when the infection had largely resolved; the significance of this is considered later. These results differ markedly from classical Th2 responses induced by alum-precipitated proteins in which high-affinity Ab is detectable within 2 wk of immunization (15).

S. typhimurium *induces a massive and persistent EF response*

Immunohistology was used to assess the cellular basis of the splenic Ab response to *S. typhimurium*. Noninfected spleens from specific pathogen-free mice contain small numbers of CD138$^+$ plasmacytoid cells (plasmablasts and/or plasma cells) (Fig. 2*A*, *top panel*). In contrast, by 4 days after infection, there was a massive expansion in plasmacytoid cell numbers, and many of these had switched to IgG2c (Fig. 2, *A* and *B*; day 4 > day 0; $p < 0.01$ for

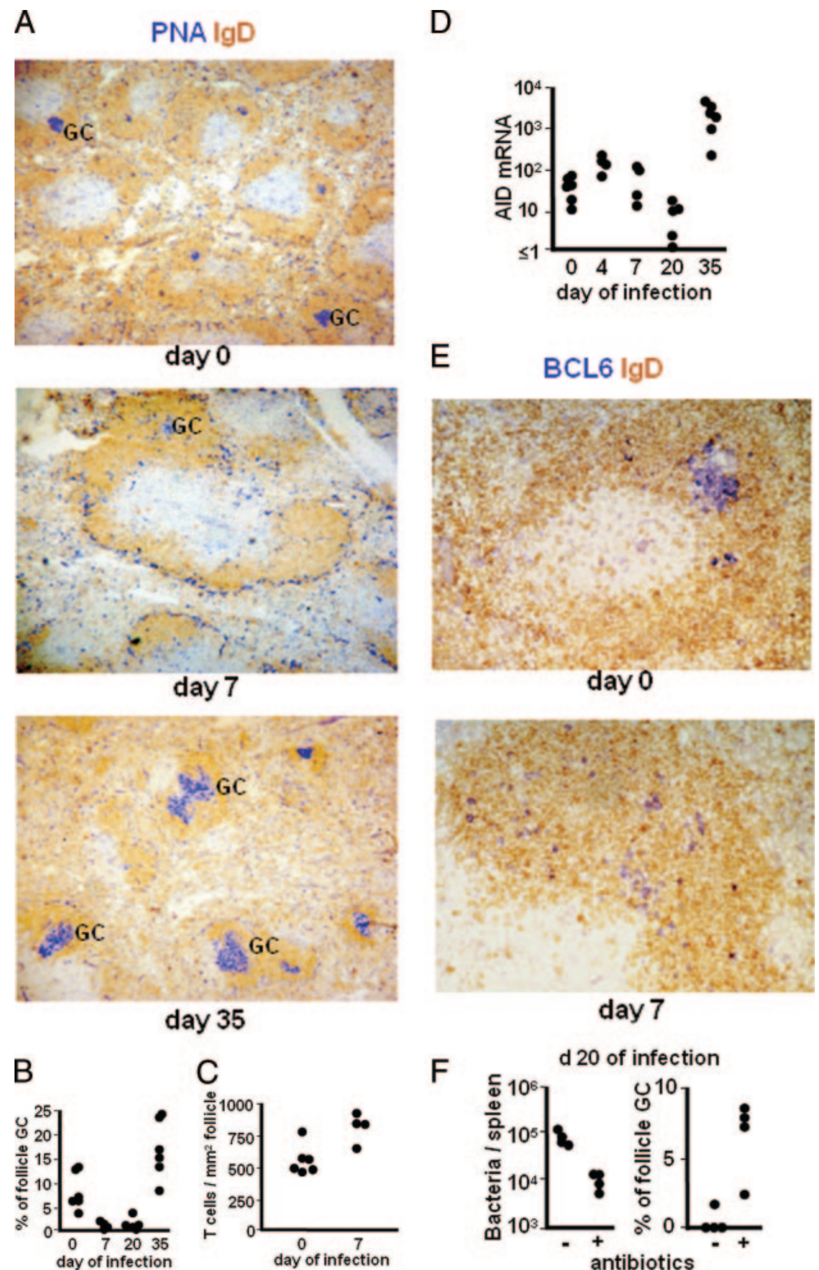

FIGURE 3. GC formation is delayed in the response to S. typhimurium infection. Mice were infected with live *S. typhimurium* i.p. *A*, Representative photomicrographs of spleen sections stained for peanut agglutinin (PNA) (blue) and IgD (brown). GC and the marginal sinus are stained blue. The *central panel* from a mouse infected 7 days previously is selected, as it shows the greatest extent of GC staining found in the spleens from that time. *B*, The proportion of follicles occupied by GC. *C*, The numbers of T cells in follicles. *D*, AID mRNA expression in spleens. *E*, Representative photomicrographs showing $Bcl6^+$ cells in a GC in a noninfected mouse and the small number of isolated $Bcl6^+$ cells seen in follicles in mice 7 days after infection; IgD^+ cells are stained brown. *F*, Bacterial counts (*left panel*) and the proportion of follicles occupied by GC (*right panel*) in nontreated WT mice or WT mice treated with enrofloxacine between days 7 and 14 of infection. Each graph represents one experiment, and each point represents values for one mouse. Experiments were repeated at least four times.

both IgM and IgG2c cells). Even at day 35, when few or no bacteria remain in the spleen, large numbers of plasmacytoid cells persist (Fig. 2*A*, *bottom panel*). Ki67 staining shows IgM and IgG2c plasmablasts from day 3, and that by day 7 a high proportion of the plasmablasts had differentiated to plasma cells. This response is not reflective of activation by commensals because infection with the laboratory-adapted *Escherichia coli* strain JM109 did not induce the same extensive response as *S. typhimurium*, even when given at much higher doses (data not shown).

The T dependence of switching in the EF response was examined using CD154-deficient mice (Fig. 2*C*). This shows that the induction of IgM^+ plasma cells was independent of CD154 signaling, but that the switching of these cells to IgG2c was reduced at least 20-fold in the absence of CD154. The conserved IgM response to *S. typhimurium* in CD154-deficient mice contrasts with the almost total loss of EF responses to alum-precipitated proteins in the absence of CD154 signaling (23). This difference presumably reflects the intrinsic capacity of *S. typhimurium* to stimulate the innate system and initiate some plasma cell responses, even so as shown later (Fig. 4*C*) the small numbers of IgG2c plasma cells induced did not secrete detectable levels of IgG2c Ab.

The T dependence of the switched response indicates that the target of the switched Ab was primarily proteinaceous. To assess the target of the response, we analyzed serum Ab to the two major protein fractions that are surface exposed on the bacterium, OMP, flagella, and another major target of the Ab response, LPS. WT mice gave a clear IgM and IgG2c Ab response against the OMP by day 7 after infection (Fig. 2*D*); CD154-deficient mice also produced IgM anti-OMP, but no switched Ab (data not shown). In contrast, flagellin and LPS induced strong IgM responses at day 7, but either negligible or no switching to IgG2c, respectively (Fig. 2*D*). The IgG2c Ab response against the OMP was maintained throughout the study (Fig. 2*D*). Thus, OMP, but not FliC or LPS, are major targets of this early EF, switched IgG2c Ab response. The capacity of OMP to induce Ab responses that can persist has been noted before (24). In conclusion, the EF plasma cell response

against *S. typhimurium* is atypical in its early appearance, its size, and its longevity when compared with classical responses to Th2 alum-precipitated proteins (25).

GC responses to S. typhimurium *infection are markedly delayed*

In splenic Ab responses to alum-precipitated proteins, GC are usually apparent within 7 days of immunization and reach peak size before 2 wk (16). Strikingly, in the response to *S. typhimurium*, the EF B cell response occurs in the absence of concomitant GC development. Background GC were detected in noninfected mice, but GC were virtually absent between days 7 and 20 of the response, yet by day 35 prominent GC were detected in all mice (Fig. 3, *A* and *B*; median percentage of follicular area for day 0, 6.3%; day 7, 0.7%; day 20, 0.8%; day 35, 15.7%; day 0 > day 7, day 35 > day 0, and day 35 > day 20, $p < 0.01$ for all). This correlates with the onset of affinity maturation of the Ab response described above (Fig. 1*C*). Although GC failed to develop in the early response, T cells retained the capacity to migrate to B cell follicles because by day 7 of the response twice as many T cells were seen in follicles as before infection (Fig. 3*C*; $p < 0.05$). There were two phases of induction of the key enzyme AID. The first was early in the EF response, reflecting the requirement for AID for the switching occurring in this response (Fig. 3*D*; day 0 < day 4, $p < 0.05$). The primary induction of AID was brief, with AID levels returning to the noninfected range or less by day 7 and staying low through day 20. The second phase of AID expression occurred between days 20 and 35 as GC developed (Fig. 3*D*; AID induction day 0 < day 35 and day 20 < day 35, $p < 0.005$ for both). This reflects the known high expression of AID by B cells undergoing Ig V-region hypermutation in the GC (26). Although GC do not develop in *S. typhimurium*-infected WT mice until the second month after infection, small numbers of widely dispersed $Bcl6^+$ cells were seen in the follicles during the first week of infection (Fig. 3*E*). It remains to be assessed whether the phenotype of these cells is that associated with GC founding cells, but clearly at this stage they fail to grow to form GC. The induction of these $Bcl6^+$ cells was T cell dependent, for they did not appear in CD154-deficient mice infected with *S. typhimurium* (data not shown). These scattered $Bcl6^+$ cells do not resemble the abortive GC that can be induced in some responses to TI-2 Ag, which develop fully and then undergo mass involution (27, 28).

The onset of GC formation correlates with the rapid partial resolution of splenomegaly that occurs in the fourth week of infection, and this in turn coincides with bacterial counts in the spleen falling to low or undetectable levels. This raises two questions, as follows. Is *S. typhimurium* actively suppressing GC formation? Is high-affinity Ab production required for resolution of the splenomegaly? To test the first of these questions, the splenic bacterial load was reduced artificially by treating a group of infected WT mice with the fluoroquinolone antibiotic enrofloxacin, a drug similar to ciprofloxacin, during the second week of infection. This reduced the number of bacteria in the spleen by day 20 after infection to approximately one-tenth of that in untreated mice, a level of reduction consistent with other published studies involving *Salmonella* and fluoroquinolones (29, 30) (Fig. 3*F*). All of the spleens in the mice treated with antibiotics contained GC at day 20, but, as before, GC were largely absent from the control group of infected mice not given antibiotics ($p < 0.05$; Fig. 3*F*). This is consistent with repression of GC formation being related, in large part, to the level of splenic *S. typhimurium* burden.

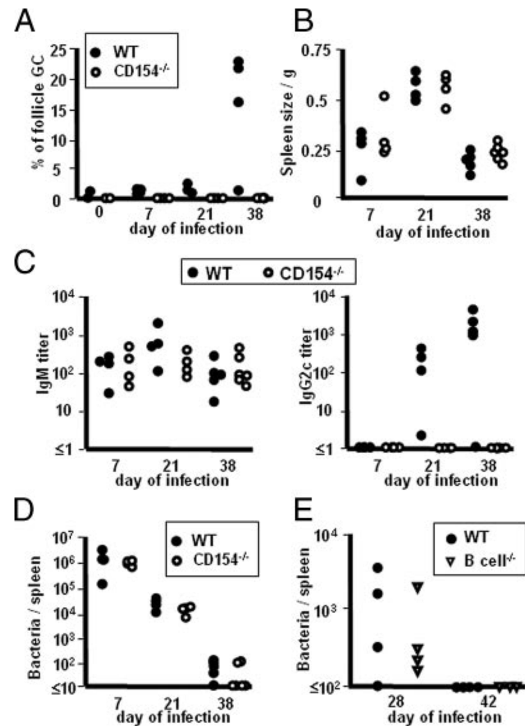

FIGURE 4. Ab does not bring about clearance of *Salmonella* from the spleen. *A*, The GC response of WT (●) and CD154 deficient (○) to infection with 10^5 live *S. typhimurium* i.p. GC size is expressed as a percentage of the follicular area. *B*, Spleen size. *C*, IgM and IgG2c *Salmonella*-specific Ab. *D*, Bacterial colonization of the spleen and responses in WT mice (●) and CD154-deficient mice (○) following infection with 10^5 live *S. typhimurium* i.p. *E*, The relative splenic bacterial load of WT (●) and B cell-deficient mice (inverted triangles) following infection with 10^5 live *S. typhimurium* i.p.; mice were rendered B cell deficient by IgH gene inactivation. Figures are representative of separate experiments. Experiments with CD154-deficient mice were repeated five times; experiments with B cell-deficient mice were repeated twice.

The moderation of splenomegaly and reduction of splenic bacterial load are not dependent on high-affinity Ab production in GC

To assess the contribution, if any, of the B cell responses to the resolution of *S. typhimurium* infection, WT and CD154-deficient mice were infected. As expected, CD154-deficient mice failed to produce GC in response to *S. typhimurium* (Fig. 4*A*). The development and resolution of splenomegaly in infected CD154-deficient mice were comparable to that seen in infected WT mice (Fig. 4*B*), despite the absence of GC and the lack of switched Ab (Fig. 4*C*). Also, the bacterial load in the spleen was similar in the two groups (Fig. 4*D*). A recent study assessed the effect of loss of CD154 on *Salmonella* infection (31), which identified an increased susceptibility of CD154-deficient mice to attenuated *Salmonella* infection. The differences between the current study and that of al-Ramadi et al. (31) may reflect differences in the strains of bacteria or the infection model. The role of natural Ab and EF IgM was then assessed using IgH-deficient mice. In agreement with other studies (10), the total loss of Ab did not modulate the course of this primary infection with attenuated bacteria compared with that in naive WT mice (Fig. 4*E*).

Ab reduces primary colonization of the spleen by S. typhimurium

Clearly, Ab is not required for clearance of bacteria from the spleen in established *S. typhimurium* infections, but can it provide

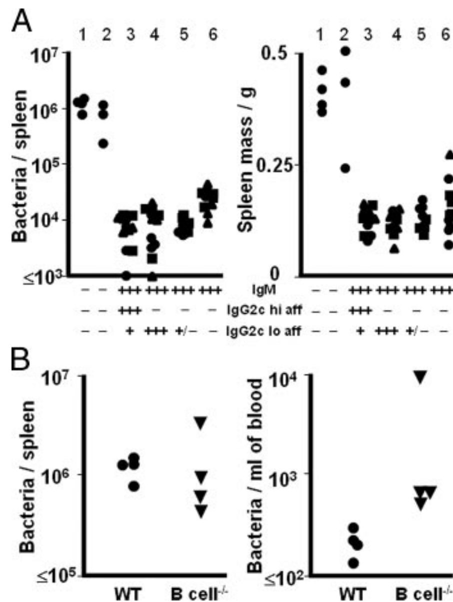

FIGURE 5. Passive Ab reduces splenic colonization, splenomegaly, and bacteremia induced by infection with live *S. typhimurium*. *A*, Splenic bacterial counts (*left*) or spleen weight (*right*) 7 days after infection with 10^5 live bacteria alone (*lane 1*); bacteria preincubated in serum from noninfected mice (*lane 2*); bacteria pretreated with serum that contained IgM and high-titer, high-affinity IgG2c (*lane 3*); bacteria pretreated with serum containing IgM and high-titer, low-affinity IgG2c (*lane 4*); bacteria pretreated with serum that contained IgM and low-titer, low-affinity IgG2c (*lane 5*); or bacteria pretreated with serum that contained only IgM anti-*Salmonella* Ab (*lane 6*). The different symbols represent values in which the bacteria were treated with serum from different donors. *B*, Splenic bacterial load (*left*) and level of bacteremia (*right*) in WT or B cell-deficient mice 7 days after infection with 10^5 live *S. typhimurium*. Each graph represents one experiment, and each point represents values for one mouse. Experiments in *A* were repeated three times, and experiments in *B* were repeated twice.

passive protection of naive mice from primary *S. typhimurium* infection? To test this, mice were infected with bacteria alone, or with bacteria preincubated with heat-inactivated serum from noninfected mice, or with bacteria preincubated with heat-inactivated serum containing *S. typhimurium*-specific Ab. High-affinity and low-affinity *S. typhimurium*-specific switched and nonswitched Ab were compared (Fig. 5*A*). Culture of the bacteria after these preincubations with heat-inactivated serum shows there was no loss of viability. The level of bacterial colonization of the spleen and the degree of splenomegaly were assessed 1 wk after infection (Fig. 5*A*). Preincubating bacteria with nonimmune serum made no significant impact on their capacity to colonize the spleen or to induce splenomegaly (compare *lanes 1* and *2* on the two panels). The effects of preincubating bacteria with qualitatively and quantitatively different immune serum were then assessed. The serum were chosen to have distinct features. Serum in all four groups (*lanes 3–6*) had similar IgM titers. In addition to IgM, the serum used in the mice shown in different lanes were: *lane 3*, high-titer, high-affinity anti-*S. typhimurium* IgG2c from WT mice 5–8 wk after infection; *lane 4*, high-titer, low-affinity anti-*S. typhimurium* IgG2c from WT mice 2–3 wk after infection; *lane 5*, low-titer, low-affinity anti-*S. typhimurium* IgG2c from WT mice 7 days after infection; and *lane 6*, IgM with no switched Ab from CD154-deficient mouse 7 days after infection. Preincubation with any of the anti-*S. typhimurium* serum markedly reduced bacterial colonization in the spleen and splenomegaly (Fig. 5*A*, bacterial count *lane 1* more than that in *lanes 3–6*; $p < 0.0001$ for all). Within the opsonized groups, preincubating with high- or low-affinity IgG2c, respectively, reduced the median number of bacteria in the spleen 4- and 2.5-fold compared with the splenic bacterial load caused by infecting with bacteria incubated in serum with IgM anti-*S. typhimurium* only (Fig. 5*A*, *left panel*, bacterial count *lanes 3* or *4* less than that in *lane 6*; $p < 0.0002$ for both). When the splenic bacterial counts in the high-titer IgG2c groups (*lanes 3* and *4*) and the low-titer IgG2c group (*lane 5*) were compared, no significant difference was observed, suggesting that even small amounts of *S. typhimurium*-specific IgG2c add to the effect of *S. typhimurium*-specific IgM in preventing colonization.

We next assessed whether Ab reduces bacteremia during the course of infection. B cell-deficient mice were infected with *S. typhimurium* to assess the role of *S. typhimurium*-specific Ab induced during infection. As shown in Fig. 5*B* and by others (10), there was no difference in splenic bacterial counts. By contrast, the median blood bacterial burden was more than 3 times greater in B cell-deficient mice than in WT mice. Thus, Ab induced during the course of the primary response restricts subsequent dissemination of bacteria throughout the host after colonization.

Discussion

The development and role of the Ab during *Salmonella* infection are complex. During the primary encounter with the pathogen, Ab responses induced during the infection are unlikely to be influential in clearing bacteria from the spleen or other organs because colonization of the spleen and liver is very rapid after the organism enters the blood (32). In addition, because foci of infection expand locally between macrophages of organs, such as the liver, the potential for encounter with Ab is restricted (33). The early, switched, low-affinity Ab induced during the course of infection was effective at restricting bacteremia 1 wk after infection. A complete inhibition of bacteremia may not have been possible by Ab alone because, at least for typhoid, many bacteria in the blood are not free, but intracellular (34). These results share similarities with typhoid infections in which low-grade bacteremia occurs with a few hundred organisms per ml of blood (35). The more severe *Salmonella* bacteremia, in terms of clinical severity, seen in sub-Saharan children under 24 mo old may either reflect an inability to induce rapid, early Ab against certain components of the organism or the absence of pre-existing Ab. Indeed, there is evidence to suggest that *Salmonella* bacteremia in these children only becomes a major problem as levels of IgG passively acquired in the last 3 mo of pregnancy fall (36).

The delay in the GC response induced by infection to *S. typhimurium* (Fig. 3, *A* and *B*) is striking, and such a marked uncoupling between EF and GC responses has not been identified previously. When GC start to appear, it correlates with the bacterial load in the spleen falling to low levels, typically <2% of the peak bacterial burden seen on day 4 of infection or ≤10,000 organisms. Evidence that this association may be causal is provided by the finding that GC formation occurs earlier if the bacterial load is reduced by antibiotic treatment (Fig. 3*F*). It may be that during the early stages of infection, the relatively high levels of Ag present may themselves act to drive GC B cells to apoptose delaying GC development. This phenomenon has been described previously in some model systems as a mechanism of restricting the potential for B cell-mediated autoimmunity to develop (37–39). There is a clear correlation between the onset of high-affinity *S. typhimurium*-specific Ab production with the development of GC, indicating the *S. typhimurium* specificity of the GC. One possibility for the appearance of GC as bacterial numbers fall is that lysis of the bacteria releases sufficient TLR ligands to promote GC formation. Two recent reports (40, 41) have looked at the role of TLRs in Ab

switching at times when GC would contribute the majority of the Ab during Th2 responses to soluble or aggregated proteins, although neither paper assesses GC directly. Whereas Gavin et al. (41) found that mice deficient in TLR signaling could induce good switching to common adjuvants such as alum, CFA, and IFA, the switching pattern resembled the classical Th2 pattern. Jegerlehner et al. (40) found that a Th2 pattern of switching could deviate to a Th1, IgG2a-dominated pattern by linking their proteinaceous virus-like particle Ag to CpG motifs. In the current study, LPS, OMP, and flagellin-specific B cells readily have access to Ag early in the response because there are early increases in Ag-specific IgM titers. Additionally, normal bacterial homeostasis is likely to result in periodic loss of Ag during the infection, although this Ag may not necessarily reach the follicular dendritic cell network. Additional experiments are being undertaken to see whether concomitant *S. typhimurium* infection suppresses GC formation induced by other Ag. The results of these studies are complex and cannot be considered in detail in this work. In brief, GC induced with hapten proteins were suppressed by *S. typhimurium*, whereas those induced by flagellin were not affected. A detailed report on these studies will appear elsewhere. The association of GC with the infection is also shown by the frequency and size of GC at the last experimental time point in infected mice. These were much greater than in noninfected littermates. Chronic low grade bacteremia characterizes this infection, and it may be that this continued load of Ag drives B cells to grow as EF plasma cells rather than forming GC. Evidence for strong BCR ligation causing this polarization was provided in an elegant study in anti-hen egg lysozyme transgenic mice immunized with structurally modified hen egg lysozymes that had altered affinity for the transgenic BCR (42). This is not a universal rule, for the abortive GC induced in response to NP-Ficoll require higher Ag concentration for their induction than is required to induce an EF response (28). Similarly, strong GC and EF responses develop during parasitemia in infections with *Plasmodium chaubadi chaubadi* (43). In contrast, there is a modest delay, of only a few days, in the onset of GC formation during lymph node infection with murine mammary tumor virus (44).

At day 35 of infection, IgG2c plasmacytoid cells numbers were almost as high as at the peak of the EF response, but by this stage few were in cell cycle, indicating they are fully differentiated plasma cells that are not being rapidly renewed. The numbers of nondividing plasma cells found at this stage are greater than in the late stages of responses to alum-precipitated proteins (25), which implies that there has been expansion of stroma that secures plasma cell survival in the spleen. The capacity of stroma that sustains plasma cell survival in the spleen is limited and is the main reason for high death rate among recently produced plasma cells (25).

Major targets for the T-dependent IgG2c-switched EF response to *S. typhimurium* are the OMP. Further work is required to characterize this and test how passive switched Ab impairs colonization of the spleen during primary infection. It is clear, however, that IgG2c-switched Ab to LPS or FliC was not induced during the early EF response, even though good IgM responses were detected against both Ag. The induction of the EF response corresponds to the time when the hemogenous spread of the organism in humans and mice peaks, suggesting Ab contributes to curb the bacteremia (32, 45–47).

In conclusion, the strong EF Ab response to *S. typhimurium* is of early onset and persists through 5 wk of infection. This is effective at moderating bacteremia and protects against further infection. In contrast, this has little or no influence on the intracellular phase of bacterial growth in the spleen and other macrophage beds (10). The reduction in bacteremia afforded by Ab is likely to be of considerably greater clinical importance during infection with virulent strains of bacteria. Thus, whereas inoculation of WT mice with attenuated *S. typhimurium* protects against subsequent challenge with virulent organisms, it does not protect B cell-deficient mice (10). It is plausible that many strains of *Salmonella* that naturally colonize macrophage beds in humans and animals have evolved to be susceptible to moderation by Ab in the extracellular phase. In this way, long-term carriage is achieved without the danger of host death from uncontrolled bacteremia.

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Disclosures

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