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Entry Route of *Salmonella typhimurium* Directs the Type of Induced Immune Response

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SUMMARY

Secretory immunoglobulin A (SIgA) represents a first line of defense against mucosal pathogens by limiting their entrance. By using different strains of *Salmonella typhimurium* that target the two mechanisms of bacterial entry (microfold cell [M cell]- or dendritic cell-mediated), we demonstrated here that the distribution of bacteria after oral infection directed the type of induced immune response. M cell-penetrating invasive, but not noninvasive, *S. typhimurium* was found in large numbers in Peyer's patches (PPs), leading to the activation of immune cells and the release of fecal IgA. In contrast, both strains of bacteria were equally capable of reaching the mesenteric lymph node and the spleen and inducing IgG responses. These data suggest that PPs are absolutely required for the initiation of an IgA response to *Salmonella*, whereas they are dispensable for a systemic response. This compartmentalization could allow the fast generation of both mucosal and systemic acquired immunity to pathogens.

INTRODUCTION

The sites and cells involved in the induction of an immune response to oral invasive pathogens like *Salmonella typhimurium* are still a matter of debate (Mastroeni and Sheppard, 2004; Mastroeni et al., 2000). *Salmonella typhimurium* has the ability to invade host cells via the expression of a type-three secretion system (TTSS) encoded within the *Salmonella* pathogenicity island I (SPI-1) that injects virulence factors into target cells and drives cytoskeleton rearrangements and phagocytosis (Galan, 2001). *Salmonella* is also equipped with a flagellar motor that confers high motility (DeRosier, 1998). These features allow *Salmonella* to efficiently invade microfold cells (M cells) (Baumler et al., 1997; Jones et al., 1994; Marchetti et al., 2004; Penheiter et al., 1997) that are interspersed in the follicle-associated epithelium (FAE) that lines the Peyer's patches (PPs) and isolated lymphoid follicles. In addition, we have recently described that *Salmonella*

can enter the host also through dendritic cells (DCs) that can extend dendrites from the lamina propria (LP) across the mucosal epithelium (Rescigno et al., 2001b). Because DCs are phagocytic cells, they can take up through this route both invasive and noninvasive bacteria. Expression of the chemokine receptor CX3CR1 by the DCs (Niess et al., 2005) and Toll-like receptor (TLR) signaling by epithelial cells (Chieppa et al., 2006) are required for the extension of the DC processes. Entry via these two distinct routes could lead to a different distribution of *Salmonella*. In fact, *Salmonella* entry via M cells delivers bacteria directly into the subepithelial dome of PPs, whereas capture of *Salmonella* by intraepithelial DCs can deliver the *Salmonella* to the LP and the mesenteric lymph nodes (MLNs). Invasiveness of *Salmonella* could influence the immunological outcome, as suggested by Vazquez-Torres et al., who showed that noninvasive *Salmonella* was unable to induce intestinal immunoglobulin A (IgA) (Vazquez-Torres et al., 1999).

In its secreted form, secretory IgA (SIgA) consists of two monomeric IgAs linked by the J chain and the secretory component, which is derived by the proteolytic cleavage of the polymeric Ig receptor (pIgR) that ensures transcytosis of the dimeric IgA across epithelial cells (Mostov, 1994). Intestinal IgAs play a major role in protecting mucosal surfaces against colonization and invasion by pathogenic microorganisms (Brandtzaeg, 2003; Kraehenbuhl and Neutra, 1992; Mestecky and McGhee, 1987) but also in limiting commensal bacteria to the intestinal lumen (Macpherson and Harris, 2004; Macpherson and Uhr, 2004). Mice bearing tumors secreting into the gastrointestinal tract a *Salmonella*-specific monoclonal IgAs are protected against oral challenge with *Salmonella* (Michetti et al., 1992), confirming an important function of SIgA in anti-*Salmonella* immunity.

The site where IgA class switching takes place is still controversial. It was first described that PPs are the major site for the generation of IgA^{+} B cells (Cebra and Shroff, 1994). However, mice lacking or having poorly developed PPs (Neumann et al., 1996; Vajdy et al., 1995) have a large amount of IgA, suggesting that IgA class-switch recombination (CSR) can also occur at sites other than the PP. This hypothesis is supported by data showing that IgA CSR can take place directly in the LP (Fagarasan et al., 2001; Xu et al., 2007). These discrepancies could be due to the different nature of the antigens and/or their route of entry into the host. Two types of B cells have been described in the mouse. B1 cells represent a more ancient type of B

lymphocyte that is dominant in the peritoneal and pleural cavities (Hayakawa and Hardy, 1988). IgAs derived from this cell type have broader specificity and low affinity. B1 cells are found within the LP and are likely to be activated in a T cell-independent fashion (Macpherson et al., 2000). Similarly, in humans, CSR to IgA can occur in a CD40-independent manner by DCs in the presence of interleukin-10 (IL-10) or transforming growth factor-β (TGF-β) via a mechanism that is mediated by the tumor necrosis factor family members B lymphocyte stimulator (BLyS) and a proliferation-inducing ligand (APRIL) (Litinskiy et al., 2002). B2 cells are generated in the bone marrow and are found in the PP, where they require antigenic stimulation and the formation of germinal centers (GCs) for their development and switching to IgA (Cebra et al., 1991; Cebra and Shroff, 1994). Therefore, it is likely that depending on the localization of the antigen (PP versus the LP), one of the two responses are initiated, leading to the production of either antigen-specific or polyreactive IgAs. In this study, we have analyzed whether the ability of *Salmonella* to invade M cells or to enter via the DC-mediated mechanism could affect the type of induced immune response. By using two different strains of *Salmonella*, one that is invasive and another one that is deficient in the *invA* gene and is unable to make a productive SPI-1 TTSS, we showed that localization of *Salmonella* within the host directed the type of induced immune response.

RESULTS

Type-Three Secretion System Is Required for *Salmonella* Localization in Peyer's Patches

Because TTSS encoded within the SPI-1 is needed by *S. typhimurium* to enter M cells (Baumler et al., 1997; Jones et al., 1994; Marchetti et al., 2004; Penheiter et al., 1997), we analyzed whether it is required for PP colonization. We used a mutant of SL1344 strain of *Salmonella* that is deficient in the *invA* gene (*InvA⁻*) and is defective in the expression of the SPI-1 TTSS (Everest et al., 1999). We compared the ability of wild-type (WT) (*InvA⁺*) and *InvA⁻* bacteria to colonize secondary lymphoid organs after oral administration. Six days after infection with replicating bacteria, there was a significant difference in *S. typhimurium* colonization of PPs between mice infected with *InvA⁺* and *InvA⁻* bacteria, whereas both strains were equally present in MLNs and spleens (SPs) (Figure 1A). The bacteria colonization correlated with results from a gross examination of lymphoid organs; PPs were highly enlarged only in mice fed with *InvA⁺* *S. typhimurium*, whereas MLNs and spleens were equally enlarged in mice infected with both strains (data not shown). Consistently, at earlier time points (18 hr after infection), we were able to detect *InvA⁺*, but not *InvA⁻*, *S. typhimurium* in PPs by immunohistochemistry (Figure 1B, bottom row). In contrast, the intestinal villi stained positive for *Salmonella* after both *InvA⁺* and *InvA⁻* infections as compared to untreated samples (Figure 1B, top row), probably because of the ability of DCs to take up

noninvasive *S. typhimurium*. Indeed, mice lacking the chemokine receptor CX3CR1 are unable to extend DC processes, and *Salmonella* is not found in the lamina propria (Niess et al., 2005). We ruled out the possibility that the *InvA⁻* strain was more sensitive to the acidic stomach environment and reached the intestine in limited numbers. Thus, we fed the mice with metabolically defective nonreplicating bacteria (*aroA⁻*) and recovered the feces up to 6 hr after infection for bacterial counts. A similar amount of live bacteria was recovered from feces of mice treated with invasive (*aroA*) or noninvasive (*aroAinvA*) *Salmonella* (Figure 1C). Therefore, both strains have a similar ability to survive in the gastrointestinal tract. These data show that *S. typhimurium* needs a functional TTSS to enter PPs, whereas it can reach other secondary lymphoid tissues even in the absence of TTSS.

In Vivo Development of Ig Responses against *S. typhimurium*

It has been described that PPs are the major site for IgA CSR. Because TTSS seemed to be indispensable to reach PPs (Figure 1A), we analyzed whether the different localization of invasive and noninvasive *Salmonella* could affect the type of induced immune response. We immunized mice with nonreplicating invasive (*aroA*) and noninvasive (*aroAinvA*) *S. typhimurium* and we followed the development of the immune response in terms of anti-*Salmonella* IgG and IgA titers in blood and feces, respectively. Both strains were able to induce secretion of serum IgG (Figure 2A), with the same kinetic, whereas fecal IgA was secreted only after *aroA* infection (Figure 2B). We also measured the secretion of anti-*Salmonella* IgM in the feces, but we could not detect it after vaccination (data not shown). We then examined whether we could evaluate the CSR to IgA by measuring α postswitch transcripts in PPs of mice that were either left untreated or fed with invasive or noninvasive *S. typhimurium*. We found only a slight increase in the postswitch transcripts when mice were treated with invasive bacteria (Figure S1A available online). This small increase was not surprising because we showed that IgA⁺ cells could be detected in both untreated (Figure 2C, left column) and treated mice, even though there was a greater number of cells stained positive for IgA in PPs of mice fed with invasive (Figure 2C, middle column) than with noninvasive *S. typhimurium* (Figure 2C, right column). We also found a higher polymeric Ig receptor (pIgR) expression after infection with invasive bacteria in small intestine isolated epithelial cells (Figure S1B). This, together with the increased amount of IgA⁺ cells in the PP, could be responsible for the augmented secretion of *Salmonella*-specific IgAs in the feces of invasive *S. typhimurium*-infected mice. In agreement, we also found that *Salmonella*-specific IgA antibody-secreting cells (SL-specific IgA ASCs) were generated only after infection with invasive *S. typhimurium*. These cells appeared 2 weeks after the treatment and peaked after 4 weeks (Figure 2D). No SL-specific IgA ASCs were found in PPs of mice that were left untreated or received noninvasive *S. typhimurium* (Figure 2D). Altogether, these data
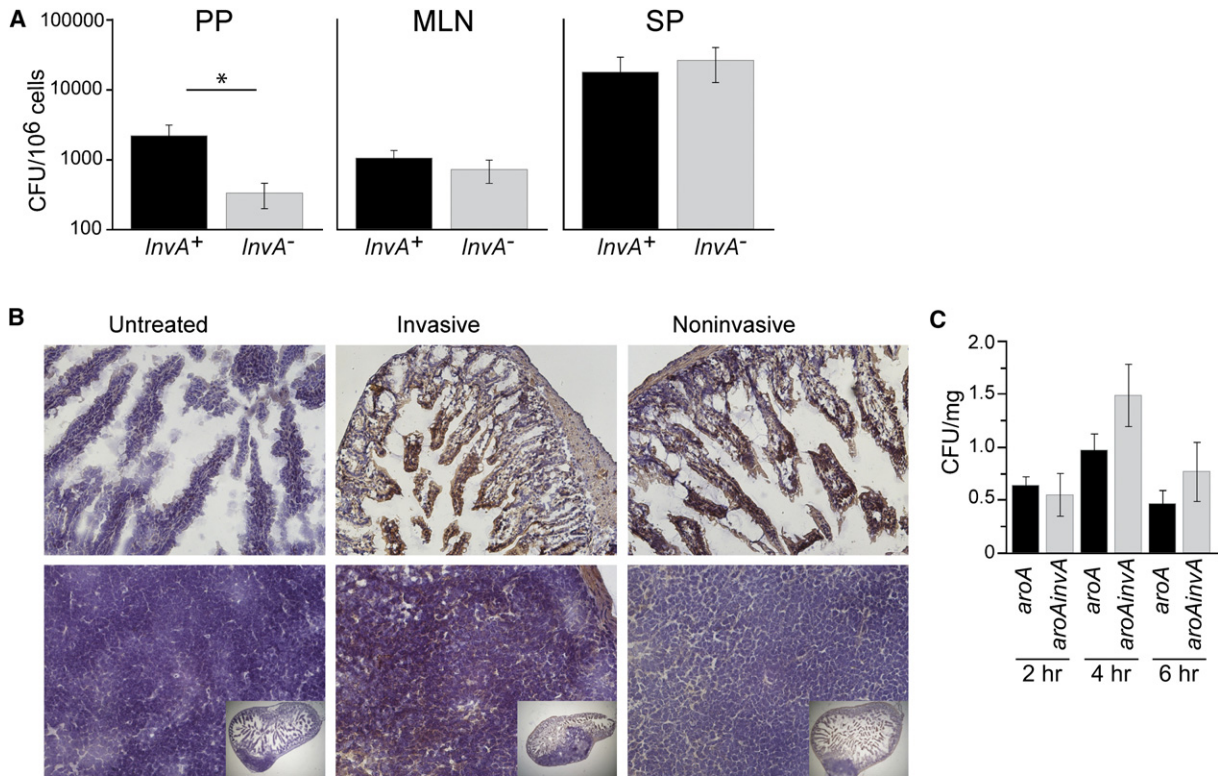
Figure 1. *S. typhimurium* Colonization of Peyer's Patches after Oral Infection Depends on Active Type-Three Secretion System

(A) C57BL/6 mice (n = 12/group) were left untreated (NT) or fed with 10^7 CFUs of invasive (*InvA*$^+$) or 10^8 CFUs of noninvasive (*InvA*$^-$) replicating *S. typhimurium*. Six days after infection, mice were sacrificed. Culturable bacteria in Peyer's patches (PP, left), mesenteric lymph nodes (MLN, middle), and spleens (SP, right) are shown. No bacteria were found in untreated mice. Data are presented as mean ± standard error (SE) and are pooled from five experiments. * indicates p < 0.05.

(B) C57BL/6 mice were left untreated (left column) or fed with 0.5×10^9 CFUs of invasive (middle column) or 10^9 CFUs of noninvasive (right column) *S. typhimurium*. After 18 hr, PPs were collected and embedded in OCT compound. Cryosections were stained with *Salmonella* sp. antiserum (rabbit), HRP-conjugated anti-rabbit and developed with DAB reagent. Sections were counterstained with hematoxilin. The top row shows intestininal ileal villi staining at 20× magnification, the bottom row shows Peyer's patches staining at 40× magnification, and the inset shows a 5× magnification of one representative section.

(C) Invasive and noninvasive *S. typhimurium* have the same capability to survive in the mouse gastrointestinal tract. C57BL/6 mice (n = 4/group) were fed with 10^9 CFUs of recombinant pDsRed *S. typhimurium* (*aroA*, invasive; *aroAinvA*, noninvasive). Total feces were collected from single mice every 2 hr, and serial dilutions were plated onto TB-agar plates supplemented with IPTG for the evaluation of *S. typhimurium* viability. Data are presented as mean ± SE (millions of recovered bacteria) and are pooled from two experiments.

suggest that only invasive *S. typhimurium* that enters the PP can induce specific IgA CSR.

Impaired Fecal IgA Is Not Dependent on Defects Related to the Lack of SPI-1

We asked whether such different response could be due to the lack of the SPI-1 operon that by itself could affect the overall immune response beyond only affecting the route of infection. To test this possibility, we undertook two strategies. In the first, we used the BCR-HEL VDJ ki mice, in which B cells express a B cell receptor (BCR) that is specific for hen egg lysozyme (HEL) (Figure S2A) but can still undergo CSR. We expressed HEL on the surface of invasive and noninvasive *S. typhimurium* as a fusion protein with the *Pseudomonas Aeruginosa* Opr-I protein (Cornelis et al., 1996) (Figures S2B and S2C) and evaluated the ability of B cells to undergo CSR in vitro in

response to bacteria that expressed or did not express HEL. At day 0 of culture, freshly isolated PP cells were composed primarily of IgM$^+$ cells (>90%). Seven days after exposure to both invasive and noninvasive HEL-expressing *S. typhimurium*, but not control strains, 40%–50% of cells expressed surface IgA (Figure 3A). In addition, when we coincubated total cells from the PP, MLN, and SP of BCR-HEL VDJ ki mice with HEL-surface-expressing *Salmonellae*, we found that, contrary to what was observed in vivo, both strains were equally capable to induce HEL-specific IgG and IgA secretion in vitro (Figures 3B and 3C). In the second strategy, we engineered noninvasive *Salmonella* to express the *invasin* gene of *Yersinia enterocolitica* (*aroAinvA*pInv). The invasin protein of *Yersinia* binds β1 integrins and can selectively target bacteria access to PPs (Grutzkau et al., 1990; Isberg and Leong, 1990). As compared to noninvasive *Salmonella*,
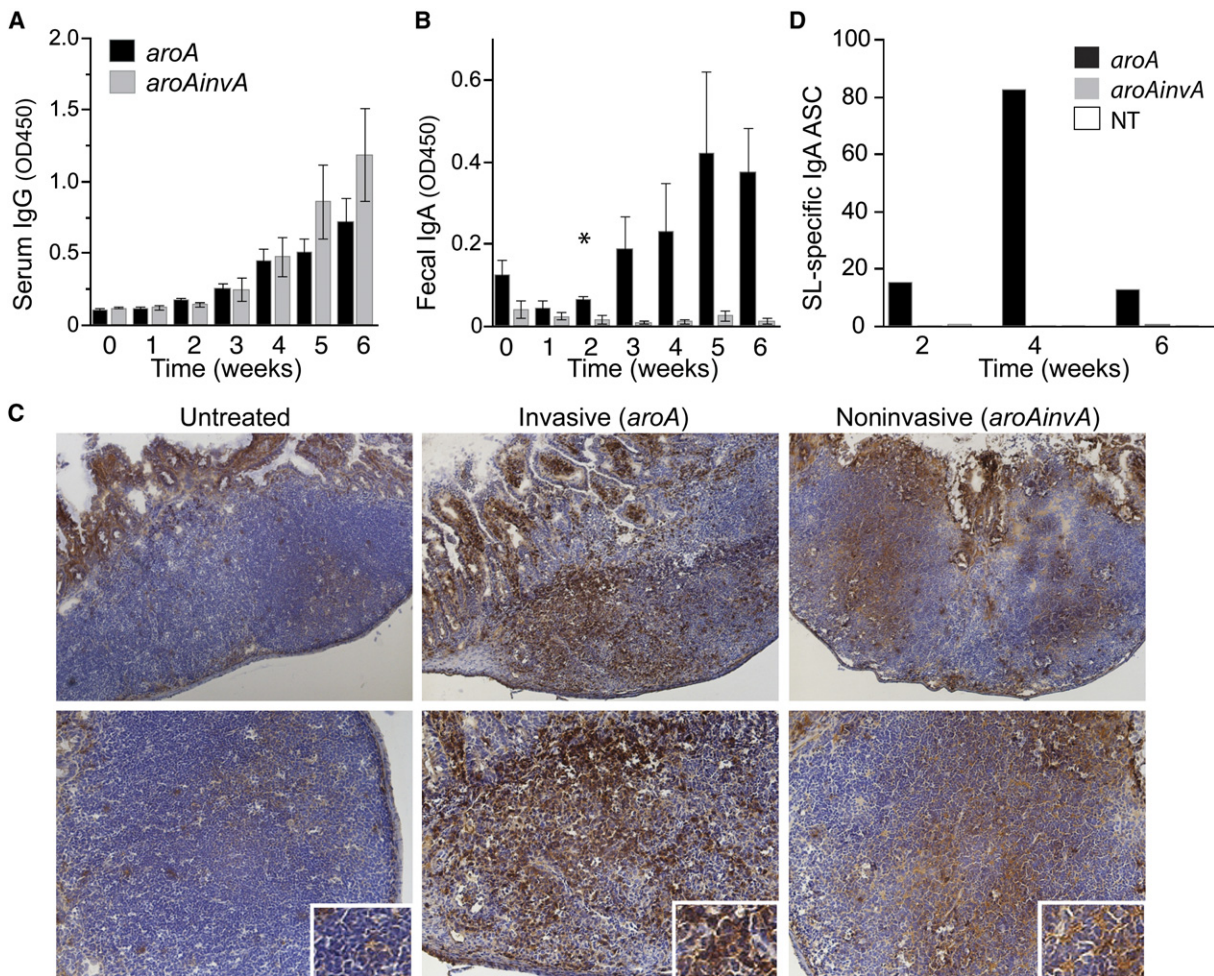
Figure 2. Invasive *S. typhimurium* Induces Mucosal IgA Response

(A and B) C57BL/6 mice (n = 6/group) were fed with 10^9 CFUs of invasive (*aroA*, black bars) or noninvasive attenuated *S. typhimurium* (*aroAinvA*, gray bars) three times on alternate days. Serum and fecal samples were collected before the infection and weekly for 6 weeks after the infection. The presence of *S. typhimurium* specific IgG in serum (A) and IgA in feces (B) was evaluated by ELISA. Data are presented as mean ± SE of OD450 and are representative of six independent experiments. In (B) * indicates that starting from week 2, the content of *Salmonella*-specific IgA in samples from mice that received the two vaccination regimens is statistically significantly different.

(C) C57BL/6 mice were left untreated (left column) or fed with 10^9 CFUs of *aroA* (middle column) or *aroAinvA* (right column) *S. typhimurium* three times on alternate days. Seven days after the last infection, mice were sacrificed, and PPs were collected and embedded in OCT compound. Cryosections were stained with anti-mouse IgA (rabbit), HRP-conjugated anti-rabbit and developed with DAB reagent. Sections were counterstained with hematoxilin. The top row shows 10× magnification; the bottom row shows 20× magnification of one representative section (insets represent digital zooms of selected IgA-enriched areas).

(D) C57BL/6 mice (n = 3/group) were left untreated (NT) or fed with 10^9 CFUs of *aroA* or *aroAinvA S. typhimurium*. PPs were collected 2, 4, and 6 weeks after the infection. The presence of *Salmonella*-specific IgA antibody-secreting cells (ASC) was evaluated by ELIspot.

*aroAinvA*pInv displayed a 2-fold increased penetration in epithelial cells (Figure S3A). This correlated with a proportional increase in IgA release in the feces that was significantly higher than in untreated animals (Figure S3B; p = 0.01). Together, these data suggest that the absence of IgA detection after infection with a noninvasive strain of *Salmonella* is not due to the SPI-1 operon and reacquisition of invasive properties restores IgA release.

Spleens are Dispensable for IgG Induction

PP colonization seems not to be necessary for the stimulation of a systemic response because both strains were able to induce anti-*Salmonella* IgG in the serum. Because *Salmonella* could localize to the spleen regardless of the expression of the SPI-1 TTSS, we wondered whether anti-*Salmonella* IgG responses were induced at this site. Interestingly, spleen also seemed to be dispensable to mount an IgG response because we found similar amounts of anti-*Salmonella* IgG in normal or splenectomized mice after vaccination (Figure S4A). *S. typhimurium* could be found only in the MLN and not other nondraining lymph nodes (Figure S4B), suggesting that in the absence of the spleen, there is no major spreading of bacteria to other peripheral lymphoid tissues. Further, we could detect γ1
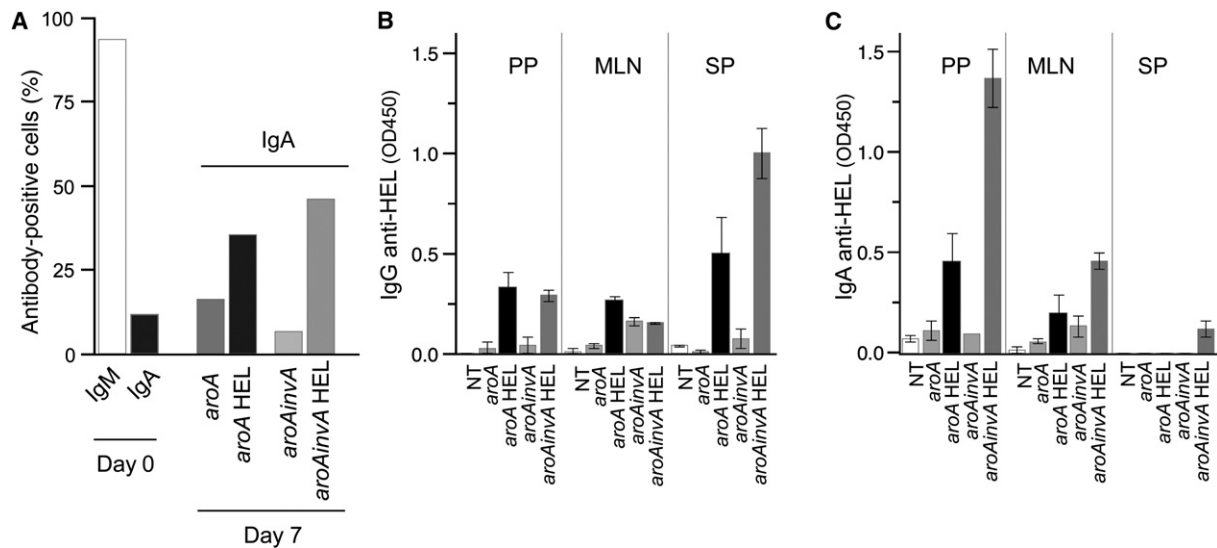
Figure 3. Noninvasive *S. typhimurium* Promotes IgA Production In Vitro

(A) The percentage of IgM- and IgA-positive cells in freshly isolated PP samples was evaluated by flowcytometry (left, day 0). The percentage of total IgA-positive cells 7 days after infection with invasive or noninvasive HEL-expressing bacteria was evaluated by flow cytometry (right, day 7).

(B and C) Total cells (10^6) from the PP, MLN, or SP of BCR-HEL VDJ ki mice were left untreated (NT, white bars) or cocultured for 4 hr with *aroA* (black and dark gray bars) or *aroAinvA* (medium and light gray bars) *S. typhimurium* that expressed or did not express HEL. Seven days after the infection, the presence of HEL-specific IgG (B) or IgA (C) in supernatants was evaluated by ELISA. Data are presented as mean of OD450 ± SE and are representative of three experiments.

circular transcripts that are indicative of ongoing IgG1 class-switch recombination in the MLNs and to a lesser extent also in the spleens of mice infected with both invasive and noninvasive *Salmonella* (Figure S4C). Thus, a systemic anti-*Salmonella* response can be initiated in MLNs.

Only Invasive *S. typhimurium* Activates the Immune System in Peyer's Patches

Because only invasive *S. typhimurium* elicited the secretion of fecal IgAs, we investigated whether the presence of invasive *S. typhimurium* in PPs correlated with the activation of the immune system at this site. *S. typhimurium* infection induces the secretion of proinflammatory cytokines, and we measured the amounts of IL-1β in infected mice. *InvA*+ *S. typhimurium*-infected mice exhibited a statistically significantly higher IL-1β production in the PP when compared with mice fed with *InvA*− bacteria or left untreated (Figure 4A). In contrast, in the MLN and spleen, the production of IL-1β was increased in response to both *InvA*+ and *InvA*− infection, confirming that both bacteria can reach these sites. Because DCs need to be activated in order to mount an adaptive immune response, we analyzed the expression of DC-activation markers (major histocompatibility complex [MHC] class I and CD86) on PP CD11c+ cells. Only *InvA*+ *S. typhimurium*-infected mice upregulated the expression of MHC class I, whereas mice infected with *InvA*− *S. typhimurium* had MHC class I expression similar to that of untreated mice (Figure 4B). No changes in CD86 expression were found in either condition. In contrast, CD86 expression on MLN (Figure 4C) and splenic (Figure 4D) DCs was increased after both treatments, whereas MHC class I seemed to be upregu-

lated by both bacterial treatments only on splenic DCs. Finally, we tested whether PP CD19+ B cells were also activated upon invasive *S. typhimurium* infection. Similar to PP DC activation, only invasive *S. typhimurium*-infected mice showed upregulation of MHC class II and CD86 expression on PP B cells (Figure 4E). Surprisingly, only invasive *S. typhimurium* induced upregulation of CD86 on MLN (Figure 4F) and splenic (Figure 4G) B cells, whereas CD40 expression was not affected at all by bacteria treatment. Whether this indicates that B cells activated in the PP migrate to these sites remains to be elucidated. The downregulation of MHC II on MLN CD19+ cells after invasive *S. typhimurium* treatment remains unclear (Figure 4F). Altogether, these data suggest that the entrance of *S. typhimurium* in PPs correlates with the development of an inflammatory reaction and the activation of immune cells, including dendritic cells and B cells.

Fecal IgA Protects Mice against Challenge of Pathogenic *S. typhimurium*

Secretory IgA was shown to play a major role in immune exclusion by impeding pathogens and also commensals to penetrate the epithelium, thus participating in the preservation of the homeostasis of the gut. We found high amounts of fecal IgA specific for *S. typhimurium* only in mice vaccinated with invasive bacteria (Figure 2B). Therefore, we compared resistance to a lethal dose of pathogenic *S. typhimurium* (SL1344) given via the oral route to mice immunized with invasive or noninvasive bacteria (Figure 5). Mice that were pretreated with invasive bacteria were significantly more resistant to infection with very high dose of virulent *Salmonella* (10^7 colony-forming units
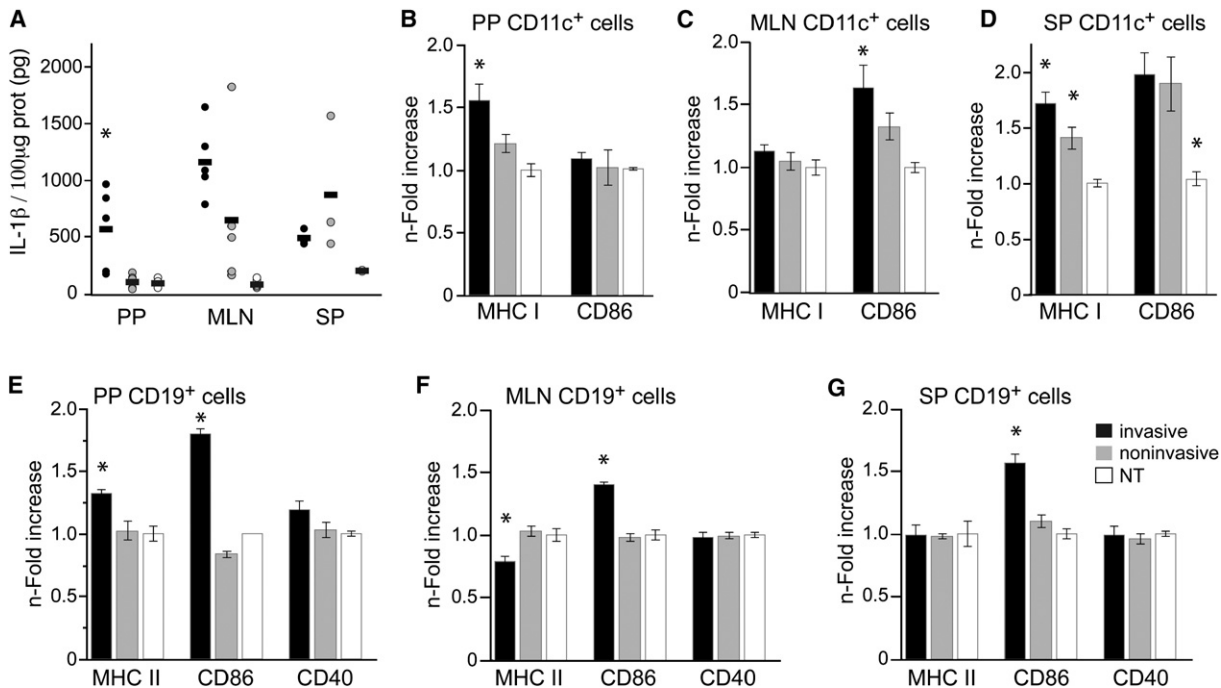
Figure 4. Invasive *S. typhimurium* Activates the Immune System in Peyer's Patches

(A–D) C57BL/6 mice (n = 4–5/group) were left untreated (NT, white) or fed with 10^7 CFUs of *InvA*$^+$ (black) or 10^8 CFUs of *InvA*$^-$ (gray) replicating *S. typhimurium*. Six days after infection, mice were sacrificed, and PPs, MLNs, and SPs were collected. IL-1β in total cell lysates was measured by ELISA (A). Each dot represents an individual mouse. Lines show mean values. Increment in MHC class I and CD86 expression on CD11c$^+$ gated cells in the PP (B), MLN (C), and SP (D) was evaluated in treated versus untreated mice.

(E–G) C57BL/6 mice (n = 4/group) were left untreated (NT, white) or fed with 10^9 CFUs of *aroA* (black) or *aroAinvA* (gray) attenuated *S. typhimurium* three times on alternate days. Four days after the last infection, mice were sacrificed, and PPs, MLNs, and SPs were collected. Increment in MHC class II, CD86 and CD40 expression on CD19$^+$ gated cells in the PP (E), MLN (F), and SP (G) was evaluated in treated versus untreated mice.

(B–G) Data are presented as mean ± SE of the ratio MFI (treated)/MFI (untreated). * indicates $p < 0.05$ when compared to other samples of the same group.

[CFUs]), and 60% of mice survived the lethal dose. Death of mice that were immunized with noninvasive bacteria, which lacked intestinal IgA specific for *Salmonella*, was only delayed when compared to that of control mice, probably due to the systemic response elicited during the vaccination. These data suggest that anti-*Salmonella* IgAs can protect against a lethal dose of virulent *S. typhimurium*.

DISCUSSION

Secretory IgAs represent a first line of defense against mucosal pathogens by limiting the entrance of bacteria, a process named "immune exclusion" (Brandtzaeg, 2003; Kraehenbuhl and Neutra, 1992; Mestecky and McGhee, 1987). By using different strains of *Salmonella typhimurium* that can or cannot invade M cells, we demonstrated here that the distribution of bacteria after oral administration directed the type of induced immune response, the production of SIgAs, and subsequent protective immunity. Invasive *S. typhimurium* that can cross M cells was found in large numbers in PPs, and this correlated with the enlargement of the PP, the activation of immune cells,

and the release of SIgAs in the feces. In contrast, minimal noninvasive *S. typhimurium* was detected in the PP, they did not induce immune cell activation in this site, and they did not lead to SIgA release. Consistent with the finding that PP DCs have the unique ability to drive IgA-secreting B cells (Mora et al., 2006; Sato et al., 2003) and that IgA CSR takes place primarily in the PP and not in the MLN or isolated lymphoid follicles (Bergqvist et al., 2006), our data indicate that PPs are absolutely required for the induction of *Salmonella*-specific SIgA release. We can exclude that noninvasive bacteria were unable to induce IgA CSR for two reasons: (1) In vitro interaction of noninvasive *S. typhimurium* with PP cells resulted in IgA production, and (2) the engineering of noninvasive *Salmonella* with *invasin* gene of *Yersinia*, which targets bacteria to the PP (Grutz-kau et al., 1990; Isberg and Leong, 1990), resulted in IgA release in vivo.

S. typhimurium could be detected in the LP, and this is consistent with recent reports showing that DC extensions are involved in the uptake of luminal bacteria regardless of their invasiveness (Chieppa et al., 2006; Niess et al., 2005; Rescigno et al., 2001a) and that in mice deficient for the chemokine receptor CX3CR1, lacking DC protrusions,
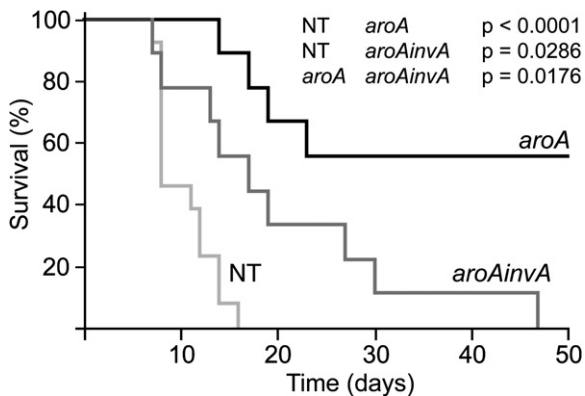
Figure 5. Intestinal IgAs Protect Mice against Oral Challenge with Pathogen *S. typhimurium*
Kaplan-Meier survival curves of C57BL/6 mice (n = 9–13/group) left untreated (light gray line) or fed with 10^9 CFUs of *aroA* (black line) or *aroAinvA* (gray line) *S. typhimurium* three times on alternate days. Six weeks after vaccination, mice received 10^7 CFUs of *InvA*$^+$ pathogenic *S. typhimurium*, and survival was monitored daily.

noninvasive bacteria are unable to cross mucosal surfaces (Niess et al., 2005). Because noninvasive *S. typhimurium* could enter the host and was found in the LP, MLN, and the spleen but still no anti-*Salmonella*-specific SIgAs could be detected in the feces, it is likely that these sites are not required for the production of *Salmonella*-specific SIgAs. This is the first evidence of the compartmentalization of the IgA response in mice that are not immunocompromised, have regularly organized lymphoid tissue, and possess a normal intestinal flora.

Our data support the notion that bacteria equipped with a functional TTSS (in our case invasive *S. typhimurium*) induce their own phagocytosis by PP M cells. Once they penetrate in PPs, they promote the activation of B2 cells, the B cell population present in this site. In PP germinal centers, B2 cells undergo CSR and somatic hypermutation (SHM); these events lead to the generation of antigen-specific B cells carrying high-affinity BCR. These cells can then migrate into effector sites (LP) and differentiate into antibody-secreting cells. Consequently, in our system, high-affinity *Salmonella*-specific IgAs were generated and secreted in the intestinal lumen after *S. typhimurium* invasion of PPs. This mechanism does not explain how IgAs are induced toward persistent commensal microorganisms, as described (Macpherson and Uhr, 2004). Two nonmutually exclusive possibilities could be envisaged. (1) Commensals induce the release of broad affinity IgAs from B1 cells directly in the LP via a T-independent mechanism (Macpherson et al., 2000). This is consistent with the finding that IgA CSR can also take place in the intestinal lamina propria (Fagarasan et al., 2001; Xu et al., 2007). Because of their innate and primitive nature, B1 cells might release immunoglobulins with low affinity that are polyreactive. In our system, "natural" or low-affinity IgAs could be induced during noninvasive *S. typhimurium* vaccination but could be undetectable by our enzyme-linked immunosorbent assay (ELISA) sys-

tem. (2) The persistence of commensal bacteria allows the entry in the PP, maybe via receptor-mediated endocytosis after opsonization with low-affinity antibodies (Mantis et al., 2002). Alternatively, DCs scattered in the FAE could participate to commensal bacteria uptake (Rey et al., 2004; Salazar-Gonzalez et al., 2006).

M cell invasion is just the first step of *Salmonella* infection because at later times, *S. typhimurium* is able to move in the mucous layer and to reach and invade enterocytes (Galan, 2001). Our data suggest that the immune system has developed a tool to sense the presence of bacteria that invade M cells and to prepare against their massive entrance by secreting specific SIgAs. Consistently, we found that mice immunized with attenuated *Salmonella* in which we could detect IgA in the feces were also protected against a lethal dose of virulent *S. typhimurium*. Interestingly, although both invasive and noninvasive bacteria induced equal amounts of systemic IgG responses, these were not efficient in protecting against an oral challenge with *Salmonella* in the absence of secreted IgAs. This further confirms an important role of SIgAs in the protection against oral invasive pathogens (Michetti et al., 1992). It is important to mention that natural IgAs are also crucial in an initial protection toward *S. typhimurium* infection because mice lacking pIgR are more susceptible to *S. typhimurium* (Wijburg et al., 2006). This could be a way to contrast the initial attack by pathogens before the initiation of a specific response. In this regard, our finding that *S. typhimurium* infection upregulates the expression of pIgR by intestinal epithelial cells further strengthens this hypothesis.

MacPherson and colleagues have shown that the MLNs limit the spreading of commensal bacteria to mucosal sites (Macpherson and Uhr, 2004). In their system, *E. cloacae* is transported to the MLN by DCs, but it does not induce IgG type of responses. If the MLNs are removed, then the bacteria are found in the spleen and this correlates with the induction of a systemic Ig response. Because *S. typhimurium* could reach the spleen also without cellular transport, we wondered whether the systemic IgG response that we found after both invasive and noninvasive response was due to the ability of *S. typhimurium* to reach the spleen because both strains were found at this site. However, when we splenectomized the mice before *S. typhimurium* administration, we found no bacteria in other lymphoid organs than the draining MLN, and still we found IgG responses, suggesting that, in the absence of the spleen, the MLN can be another site for induction of IgG responses to pathogenic bacteria. This was confirmed by the detection of $\gamma 1$ circular transcripts at this site after infection with noninvasive *Salmonella typhimurium*. Whether this is due to different signals delivered by *S. typhimurium* or by *E. cloacae* to mucosal DCs or whether this is due to the ability of *S. typhimurium* to reach the MLN in the absence of cellular transport and thus be targeted to MLN DCs remains to be established.

In conclusion, we show here that depending on the route of *Salmonella* entry, systemic or mucosal responses are initiated. Further, PPs are absolutely required for the

initiation of a specific IgA response to *Salmonella*, whereas they are dispensable for a systemic response. This com-partmentalization of the immune response could allow the fast and specific generation of anti-*Salmonella* IgAs that are required to protect the host against subsequent challenges with the pathogen.

EXPERIMENTAL PROCEDURES

Mice and Bacterial Strains

C57BL/6 mice (6–8 weeks old) were purchased from Harlan (Udine, Italy). BCR-HEL VDJ ki mice (line VDJ9) were generated by standard procedures with the recombind VDJ region of the HyHEL10 antibody, and they were intercrossed with transgenic mice carrying the HyHEL10 light chain (line k5) and backcrossed to C57Bl/6 for ten generations (a kind gift of Dr. J. Cyster, University of California, San Francisco) and were bred under specific pathogen-free conditions at Charles River Laboratories. All experiments were performed in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC).

The following *S. typhimurium* strains on SL1344 background were provided by G. Dougan (Imperial College, London, UK): wild-type inva-sive strain (*InvA*[+]); noninvasive strain (*InvA*[−]), defective in *invA* gene and therefore unable to form productive type-three secretion system (Everest et al., 1999); metabolically defective invasive strain (*aroA*), with attenuated ability to replicate in vivo in mice; and metabolically defective noninvasive strain (*aroAinvA*), with both mutations (Gots et al., 1974). Recombinant derivatives were generated expressing red fluorescence protein [DsRed, a kind gift of Dr. B. Glick, The University of Chicago (Bevis and Glick, 2002)], invasin from *Yersinia entero-colitica* [pInv vector, (Schulte et al., 1998)], or inactive HEL, under tran-scriptional control of the promoter of LacZ gene. So that HEL protein on the surface of *S. typhimurium* could be exposed, a HEL-encoding gene was cloned in frame with the one encoding for OprI protein from *P. aeruginosa* [pVUB4 vector provided by P. Cornelis, Flanders Institute for Biotechnology, Brussels, Belgium (Cornelis et al., 1996)]. Consistent with other reports (Cote-Sierra et al., 1998), we also de-tected degraded products after overexpression of the OprI-HEL fusion proteins (Figure S2). Bacterial strains were grown at 37°C in Luria broth supplemented with appropriate antibiotics to preserve carried muta-tions and plasmids. Protein expression was induced by the addition of 0.1–1 mmol/L isopropyl-L-thio-B-D-galactopyranoside (IPTG).

In Vivo Infections

For short-term experiments, mice were fed once with 10^7 CFUs of *InvA*[+] or 10^8 CFUs of *InvA*[−] *S. typhimurium* in 200 μl 5% sodium bicar-bonate. For long-term experiments, mice were fed three times on alter-nate days with 10^9 CFUs of *aroA* or *aroAinvA S. typhimurium* in 200 μl 5% sodium bicarbonate. For lethal dose challenges, mice were fed with 10^7 CFUs of *InvA*[+] *S. typhimurium*. Serial dilutions of bacteria preparations were plated onto Terrific Broth (TB)-agar plates so that administered doses could be assessed.

In Vitro Infections

PPs, MLNs, and SPs were collected from naive BCR-HEL VDJ ki mice and processed so that a single-cell suspension could be obtained. One million cells were incubated with HEL-surface-expressing bacteria or the same strains that did not express HEL for 4 hr in B cell medium (Phan et al., 2003) without antibiotics and were then washed and incu-bated in complete medium supplemented with 100 μg/ml gentamicin for 7 days. The percentage of IgM- or IgA-expressing B cells in PPs was evaluated through cytometry. Total cells before and 7 days after infections were stained with PE-conjugated anti-CD19 (PharMingen) and FITC-conjugated anti-IgM (PharMingen) or anti-IgA (Serotec).

Evaluation of Antigen-Specific Antibody Secretion In Vivo and In Vitro

Serum and fecal samples were obtained from nonvaccinated and vac-cinated mice. In brief, blood samples were incubated 1 hr at 37°C, and serum was separated by centrifugation. Fecal pellets were weighted and dissolved in phosphate-buffered saline (PBS) 0.1 mg/ml soybean trypsin inhibitor (1ml/100mg feces); debris were eliminated by centrifu-gation (13000 g, 10'). Serum and fecal samples were then tested for the presence of *Salmonella*-specific antibodies. Supernatants from in vitro-infected cultures were tested for the presence of HEL-specific antibodies. In brief, ELISA plates were coated with lysates of 10^6 CFU/ml of *S. typhimurium* (obtained by repeated cycles of freezing and thaw-ing) or with 10 μg/ml HEL. After the plates were blocked, samples were applied and bound antibodies were detected with horseradish perox-idase (HRP)-conjugated anti-mouse IgA or anti-mouse IgG (Caltag laboratories), thus providing also the characterization of Ig isotype.

Evaluation of Bacteria Survival and Colonization Capabilities

Total fecal samples from single mice infected with DsRed-expressing *aroA* or *aroAinvA S. typhimurium* were collected every 2 hr for 6 hr after infection, weighted, and dissolved in sterile PBS. Serial dilutions of the suspension were plated onto TB-agar plates supplemented with IPTG.

PPs, MLNs, and SPs from mice infected with *InvA*[+] or *InvA*[−] *S. typhi-murium* (day 6) were collected and processed. A fixed number of cells was lysed with 0.5% sodium deoxycholate and plated onto TB-agar plates.

Evaluation of Immune-System Activation

For evaluation of dendritic cell and B cell activation, PPs, MLNs, and SPs were collected, processed, and stained with combinations of the following antibodies (PharMingen): CD11c or CD19 (both APC con-jugated) and H2-Kb, CD86, IA/IE or CD40 (all PE conjugated). For eval-uation of IL-1β production, organs were disrupted in PBS 0.5% Triton-X and analyzed through ELISA (R&D systems).

ELIspot Assay

Ninety-six-well enzyme-linked immunospot (ELIspot) plates (What-man) were coated overnight at 4°C with lysates of 4 × 10^6 CFU/ml of *S. typhimurium*, washed, and blocked with PBS 2% bovine serum albumin (BSA) for 1 hr at 37°C. Total cells from PPs were added in duplicates at two different cell densities (10^6 and 10^5) in Roswell Park Memorial Institute (RPMI) medium supplemented with 2% BSA and 2.5% fetal calf serum (FCS) and incubated 18 hr in humidified at-mosphere at 37°C and 5% CO_2. After extensive washing with PBS and PBS-Tween 20, plates were incubated 4 hr with HRP-conjugated anti-mouse IgA (Caltag laboratories) and washed again. 3-amino, 9 ethyl-carbazole (AEC) (BD ELISPOT) was used as a substrate for per-oxidase, and colored spots were counted with a stereomicroscope.

Immunohistochemistry

PPs were embedded in optimum cutting temperature (OCT) com-pound (Sakura) and snap frozen. Sections 7 μm in thickness were prepared and air dried. After endogenous peroxidases were blocked, sections were incubated with anti-mouse IgA (Open Biosystem) or *Salmonella* sp. antiserum (Virostat), both rabbit. Sections were then incubated with anti-rabbit HRP conjugated (DAKO) and developed with diaminobenzidine (DAB) (DakoCytomation EnVision System). Finally, sections were counterstained with hematoxillin and mounted with Eukitt.

Statistical Analysis

Statistical significance was evaluated by nonparametric Mann-Whit-ney U test (Wilcoxon-Kruskal Wallis) for the analysis of variables that were not normally distributed. Significance was defined at $p < 0.05$ (two-tailed test). Kaplan-Meier plots and long-rank tests were used so that the survival differences of vaccinated mice after virulent *S. typhimurium* infection could be assessed. Statistic calculations were performed by JMP 5.1 software (SAS [Cary, NC]).

REFERENCES

Baumler, A.J., Tsolis, R.M., Valentine, P.J., Ficht, T.A., and Heffron, F. (1997). Synergistic effect of mutations in invA and lpfC on the ability of Salmonella typhimurium to cause murine typhoid. Infect. Immun. *65*, 2254–2259.

Bergqvist, P., Gardby, E., Stensson, A., Bemark, M., and Lycke, N.Y. (2006). Gut IgA class switch recombination in the absence of CD40 does not occur in the lamina propria and is independent of germinal centers. J. Immunol. *177*, 7772–7783.

Bevis, B.J., and Glick, B.S. (2002). Rapidly maturing variants of the Discosoma red fluorescent protein (DsRed). Nat. Biotechnol. *20*, 83–87.

Brandtzaeg, P. (2003). Role of secretory antibodies in the defence against infections. Int. J. Med. Microbiol. *293*, 3–15.

Cebra, J.J., and Shroff, K.E. (1994). Handbook of Mucosal Immunology (San Diego, California: Academic Press).

Cebra, J.J., Logan, A.C., and Weinstein, P.D. (1991). The preference for switching to expression of the IgA isotype of antibody exhibited by B lymphocytes in Peyer's patches is likely due to intrinsic properties of their microenvironment. Immunol. Res. *10*, 393–395.

Chieppa, M., Rescigno, M., Huang, A.Y., and Germain, R.N. (2006). Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. J. Exp. Med. *203*, 2841–2852.

Cornelis, P., Sierra, J.C., Lim, A., Jr., Malur, A., Tungpradabkul, S., Tazka, H., Leitao, A., Martins, C.V., di Perna, C., Brys, L., et al. (1996). Development of new cloning vectors for the production of immunogenic outer membrane fusion proteins in Escherichia coli. Biotechnology (N. Y.) *14*, 203–208.

Cote-Sierra, J., Jongert, E., Bredan, A., Gautam, D.C., Parkhouse, M., Cornelis, P., De Baetselier, P., and Revets, H. (1998). A new membrane-bound OprI lipoprotein expression vector. High production of heterologous fusion proteins in gram (-) bacteria and the implications for oral vaccination. Gene *221*, 25–34.

DeRosier, D.J. (1998). The turn of the screw: The bacterial flagellar motor. Cell *93*, 17–20.

Everest, P., Ketley, J., Hardy, S., Douce, G., Khan, S., Shea, J., Holden, D., Maskell, D., and Dougan, G. (1999). Evaluation of Salmonella typhimurium mutants in a model of experimental gastroenteritis. Infect. Immun. *67*, 2815–2821.

Fagarasan, S., Kinoshita, K., Muramatsu, M., Ikuta, K., and Honjo, T. (2001). In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. Nature *413*, 639–643.

Galan, J.E. (2001). Salmonella interactions with host cells: Type III secretion at work. Annu. Rev. Cell Dev. Biol. *17*, 53–86.

Gots, R.E., Formal, S.B., and Giannella, R.A. (1974). Indomethacin inhibition of Salmonella typhimurium, Shigella flexneri, and cholera-mediated rabbit ileal secretion. J. Infect. Dis. *130*, 280–284.

Grutzkau, A., Hanski, C., Hahn, H., and Riecken, E.O. (1990). Involvement of M cells in the bacterial invasion of Peyer's patches: A common

mechanism shared by Yersinia enterocolitica and other enteroinvasive bacteria. Gut *31*, 1011–1015.

Hayakawa, K., and Hardy, R.R. (1988). Normal, autoimmune, and malignant CD5+ B cells: The Ly-1 B lineage? Annu. Rev. Immunol. *6*, 197–218.

Isberg, R.R., and Leong, J.M. (1990). Multiple beta 1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell *60*, 861–871.

Jones, B.D., Ghori, N., and Falkow, S. (1994). Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. J. Exp. Med. *180*, 15–23.

Kraehenbuhl, J.P., and Neutra, M.R. (1992). Molecular and cellular basis of immune protection of mucosal surfaces. Physiol. Rev. *72*, 853–879.

Litinskiy, M.B., Nardelli, B., Hilbert, D.M., He, B., Schaffer, A., Casali, P., and Cerutti, A. (2002). DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. Nat. Immunol. *3*, 822–829.

Macpherson, A.J., and Harris, N.L. (2004). Interactions between commensal intestinal bacteria and the immune system. Nat. Rev. Immunol. *4*, 478–485.

Macpherson, A.J., and Uhr, T. (2004). Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science *303*, 1662–1665.

Macpherson, A.J., Gatto, D., Sainsbury, E., Harriman, G.R., Hengartner, H., and Zinkernagel, R.M. (2000). A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science *288*, 2222–2226.

Mantis, N.J., Cheung, M.C., Chintalacharuvu, K.R., Rey, J., Corthesy, B., and Neutra, M.R. (2002). Selective adherence of IgA to murine Peyer's patch M cells: Evidence for a novel IgA receptor. J. Immunol. *169*, 1844–1851.

Marchetti, M., Sirard, J.C., Sansonetti, P., Pringault, E., and Kerneis, S. (2004). Interaction of pathogenic bacteria with rabbit appendix M cells: Bacterial motility is a key feature in vivo. Microbes Infect. *6*, 521–528.

Mastroeni, P., and Sheppard, M. (2004). Salmonella infections in the mouse model: Host resistance factors and in vivo dynamics of bacterial spread and distribution in the tissues. Microbes Infect. *6*, 398–405.

Mastroeni, P., Simmons, C., Fowler, R., Hormaeche, C.E., and Dougan, G. (2000). Igh-6(−/−) (B-cell-deficient) mice fail to mount solid acquired resistance to oral challenge with virulent Salmonella enterica serovar typhimurium and show impaired Th1 T-cell responses to Salmonella antigens. Infect. Immun. *68*, 46–53.

Mestecky, J., and McGhee, J.R. (1987). Immunoglobulin A (IgA): Molecular and cellular interactions involved in IgA biosynthesis and immune response. Adv. Immunol. *40*, 153–245.

Michetti, P., Mahan, M.J., Slauch, J.M., Mekalanos, J.J., and Neutra, M.R. (1992). Monoclonal secretory immunoglobulin A protects mice against oral challenge with the invasive pathogen Salmonella typhimurium. Infect. Immun. *60*, 1786–1792.

Mora, J.R., Iwata, M., Eksteen, B., Song, S.Y., Junt, T., Senman, B., Otipoby, K.L., Yokota, A., Takeuchi, H., Ricciardi-Castagnoli, P., et al. (2006). Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science *314*, 1157–1160.

Mostov, K.E. (1994). Transepithelial transport of immunoglobulins. Annu. Rev. Immunol. *12*, 63–84.

Neumann, B., Luz, A., Pfeffer, K., and Holzmann, B. (1996). Defective Peyer's patch organogenesis in mice lacking the 55-kD receptor for tumor necrosis factor. J. Exp. Med. *184*, 259–264.

Niess, J.H., Brand, S., Gu, X., Landsman, L., Jung, S., McCormick, B.A., Vyas, J.M., Boes, M., Ploegh, H.L., Fox, J.G., et al. (2005). CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science *307*, 254–258.

Penheiter, K.L., Mathur, N., Giles, D., Fahlen, T., and Jones, B.D. (1997). Non-invasive Salmonella typhimurium mutants are avirulent because of an inability to enter and destroy M cells of ileal Peyer's patches. Mol. Microbiol. *24*, 697–709.

Phan, T.G., Amesbury, M., Gardam, S., Crosbie, J., Hasbold, J., Hodgkin, P.D., Basten, A., and Brink, R. (2003). B cell receptor-independent stimuli trigger immunoglobulin (Ig) class switch recombination and production of IgG autoantibodies by anergic self-reactive B cells. J. Exp. Med. *197*, 845–860.

Rescigno, M., Rotta, G., Valzasina, B., and Ricciardi-Castagnoli, P. (2001a). Dendritic cells shuttle microbes across gut epithelial monolayers. Immunobiology *204*, 572–581.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.P., and Ricciardi-Castagnoli, P. (2001b). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat. Immunol. *2*, 361–367.

Rey, J., Garin, N., Spertini, F., and Corthesy, B. (2004). Targeting of secretory IgA to Peyer's patch dendritic and T cells after transport by intestinal M cells. J. Immunol. *172*, 3026–3033.

Salazar-Gonzalez, R.M., Niess, J.H., Zammit, D.J., Ravindran, R., Srinivasan, A., Maxwell, J.R., Stoklasek, T., Yadav, R., Williams, I.R., Gu, X., et al. (2006). CCR6-mediated dendritic cell activation of pathogen-specific T cells in Peyer's patches. Immunity *24*, 623–632.

Sato, A., Hashiguchi, M., Toda, E., Iwasaki, A., Hachimura, S., and Kaminogawa, S. (2003). CD11b+ Peyer's patch dendritic cells secrete IL-6 and induce IgA secretion from naive B cells. J. Immunol. *171*, 3684–3690.

Schulte, R., Zumbihl, R., Kampik, D., Fauconnier, A., and Autenrieth, I.B. (1998). Wortmannin blocks Yersinia invasin-triggered internalization, but not interleukin-8 production by epithelial cells. Med. Microbiol. Immunol. (Berl.) *187*, 53–60.

Vajdy, M., Kosco-Vilbois, M.H., Kopf, M., Kohler, G., and Lycke, N. (1995). Impaired mucosal immune responses in interleukin 4-targeted mice. J. Exp. Med. *181*, 41–53.

Vazquez-Torres, A., Jones-Carson, J., Baumler, A.J., Falkow, S., Valdivia, R., Brown, W., Le, M., Berggren, R., Parks, W.T., and Fang, F.C. (1999). Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes. Nature *401*, 804–808.

Wijburg, O.L., Uren, T.K., Simpfendorfer, K., Johansen, F.E., Brandtzaeg, P., and Strugnell, R.A. (2006). Innate secretory antibodies protect against natural Salmonella typhimurium infection. J. Exp. Med. *203*, 21–26.

Xu, W., He, B., Chiu, A., Chadburn, A., Shan, M., Buldys, M., Ding, A., Knowles, D.M., Santini, P.A., and Cerutti, A. (2007). Epithelial cells trigger frontline immunoglobulin class switching through a pathway regulated by the inhibitor SLPI. Nat. Immunol. *8*, 294–303.