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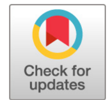
BACTERIAL INFECTIONS



Model of Persistent *Salmonella* Infection: *Salmonella enterica* Serovar Pullorum Modulates the Immune Response of the Chicken from a Th17-Type Response towards a Th2-Type Response

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ABSTRACT *Salmonella enterica* infection affects a wide range of animals and humans, and a small number of serovars cause typhoid-like infections, one characteristic of which is persistent infection in convalescents. Avian-specific *S. enterica* serovar Pullorum produces systemic disease in young chickens, which is followed by a carrier state in convalescent birds, leading to infection of the ovary at sexual maturity and vertical transmission. However, the immunological basis of persistent infection remains unclear. *S. enterica* serovar Enteritidis is taxonomically closely related but does not show this characteristic. Differences in the immune responses between *S.* Pullorum and *S.* Enteritidis were compared by using *Salmonella*-infected chicken monocyte-derived macrophages (chMDMs) and CD4$^+$ T lymphocytes that had been cocultured with infected chMDMs or chicken splenocytes *in vitro* and also in 2-day-old chickens *in vivo*. In comparison with *S.* Enteritidis, *S.* Pullorum-infected chMDMs showed reduced mRNA expression levels of interleukin-12α (*IL-12*α) and *IL-18* and stimulated the proliferation of Th2 lymphocytes, with reduced expression of gamma interferon (*IFN-*γ) and *IL-17* and increased expression levels of *IL-4* and *IL-13*. There was little evidence of clonal anergy or immune suppression induced by *S.* Pullorum *in vitro*. *S.* Pullorum also increased the levels of expression of *IL-4* and decreased the levels of *IFN-*γ in the spleen and cecal tonsil of infected birds. This suggests that *S.* Pullorum is able to modulate host immunity from a dominant IFN-γ-producing Th17 response toward a Th2 response, which may promote persistent infection in chickens. *S.* Pullorum in chickens is presented as a good model of the typhoid group to study persistent infection.

KEYWORDS *Salmonella enterica* serovar Pullorum, *Salmonella enterica* serovar Enteritidis, macrophage, CD4$^+$ T cells, Th1, Th2, Th17, salmonella, T cell immunity, adaptive immunity, chickens, macrophages, persistent infection

The majority of *Salmonella enterica* serovars that affect human or animal health generally cause gastrointestinal disease of various severities in a wide range of hosts (1). A small number of serovars, including *Salmonella enterica* serovar Typhi, *S.* Gallinarum, *S.* Pullorum, *S.* Dublin, *S.* Choleraesuis, and *S.* Abortusovis (*S.* Abortusequi), are adapted to a narrow range of host species and generally produce severe, typhoid-like disease, sometimes with high mortality rates (2). *S. enterica* serovar Typhimurium and *S.* Enteritidis are the serovars most frequently associated with food poisoning, with infection restricted to the lower gastrointestinal tract or transient systemic infection (3), and only produce characteristic typhoid experimentally in mice (4). One of the features of infection produced by the typhoid serovars is asymptomatic persistent infections in a proportion of convalescents in experimental infection involving macrophages in

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lymphoid tissues (5). This results in localization in the gallbladder, liver, and spleen, leading to fecal shedding by carriers for long periods and, in some cases, many years (*S*. Typhi in humans and *S*. Dublin in cattle) (6–8), or localization in the reproductive tract, leading to either abortion (*S*. Dublin and *S*. Abortusovis in sheep) or vertical transmission through hatching eggs to progeny (*S*. Pullorum and *S*. Gallinarum) (9). *S*. Pullorum is a good and natural model of persistent infection shown by these serovars (10).

Studies on murine typhoid with *S*. Typhimurium have indicated the critical role of $CD4^+$ Th1 lymphocytes in controlling salmonellosis (11). Clearance of infection by *S*. Enteritidis from the intestinal tract of infected chickens was also shown to be due to a Th1-dominated response involving increased expression levels of gamma interferon (*IFN-*γ) mRNA in the gut and deeper tissues (12–16). *S*. Pullorum colonizes the gut poorly, with bacteria migrating from the intestine to deeper tissues soon after infection (17), accompanied by relatively little inflammation (18), as does the taxonomically closely related *S*. Gallinarum (19). This is attributed to the reduced production of the proinflammatory chemokines interleukin-1 (*IL-1*) and *IL-6* demonstrated *in vitro* following *S*. Gallinarum infection of avian epithelial cells (20). In the case of *S*. Pullorum, a small number of viable bacteria have been shown to persist in macrophages in convalescent birds. These bacteria are most easily detectable in the spleen, in a proportion of animals, despite the presence of a high-titer antibody response (5, 9, 10). Recrudescence of systemic infection and spread of *S*. Pullorum to the reproductive tissue occur in females at sexual maturity, associated with the reduced T cell responsiveness that occurs at this time (5, 9, 10). In males, infection persists, but bacterial numbers in the spleen and liver gradually decline with time, resulting in very slow tissue clearance by ca. 18 weeks after infection (9). However, the mechanisms by which *S*. Pullorum and other typhoid serovars produce persistent infection in the host and the reasons for the absence of complete clearance are not known. In an initial comparative study using *S*. Pullorum and *S*. Enteritidis, *S*. Pullorum-infected birds expressed significantly lower levels of splenic *IL-18* and *IFN-*γ, whereas the expression of *IL-4* was increased 14 days after infection (18). This suggested that *S*. Pullorum might induce an immune response that more closely resembled the Th2 response in mammals and that could allow *S*. Pullorum to establish intracellular carriage, evading Th1-mediated clearance.

The nature of the immune response to the other serovars that typically produce typhoid-like diseases is poorly understood. In response to *S*. Typhi in humans, IL-17 production was first found in $CD8^+$ T cells, which also produced IFN-γ (21). Significant increases in IL-17^+ $CD4^+$ T cells and *in vitro* IFN-γ production were also observed during convalescence from *S*. Typhi (22). Those studies suggested that in the majority of individuals, *S*. Typhi infection induced a predominant IFN-γ response derived from lymphocyte subsets other than Th1. Persistent infections occur in $<$3% of typhoid patients (23).

However, alternative potential reasons for the absence of a strong Th1 response exist, including immunosuppression, clonal anergy, or reduced lymphocyte proliferation. The aim of the study reported here was to clarify in greater detail the immunological basis for the persistent carrier state observed in *S*. Pullorum infection using an *in vitro* macrophage-T cell coculture system and *in vivo* infections. The results indicated that *S*. Pullorum is able to drive host immunity toward a Th2-like response.

RESULTS

Persistence of *S*. Pullorum infection is not the result of increased survival in macrophages. The persistence of *S*. Pullorum in comparison with *S*. Enteritidis is likely to be the result of increased microbial survival within the internal macrophage environment. We therefore assessed this using chicken monocyte-derived macrophages (chMDMs) (see Fig. S1 in the supplemental material). We quantified the invasiveness and survival of the *S*. Pullorum and *S*. Enteritidis strains, macrophage viability, and nitrite ion (NO_2^-) activity.

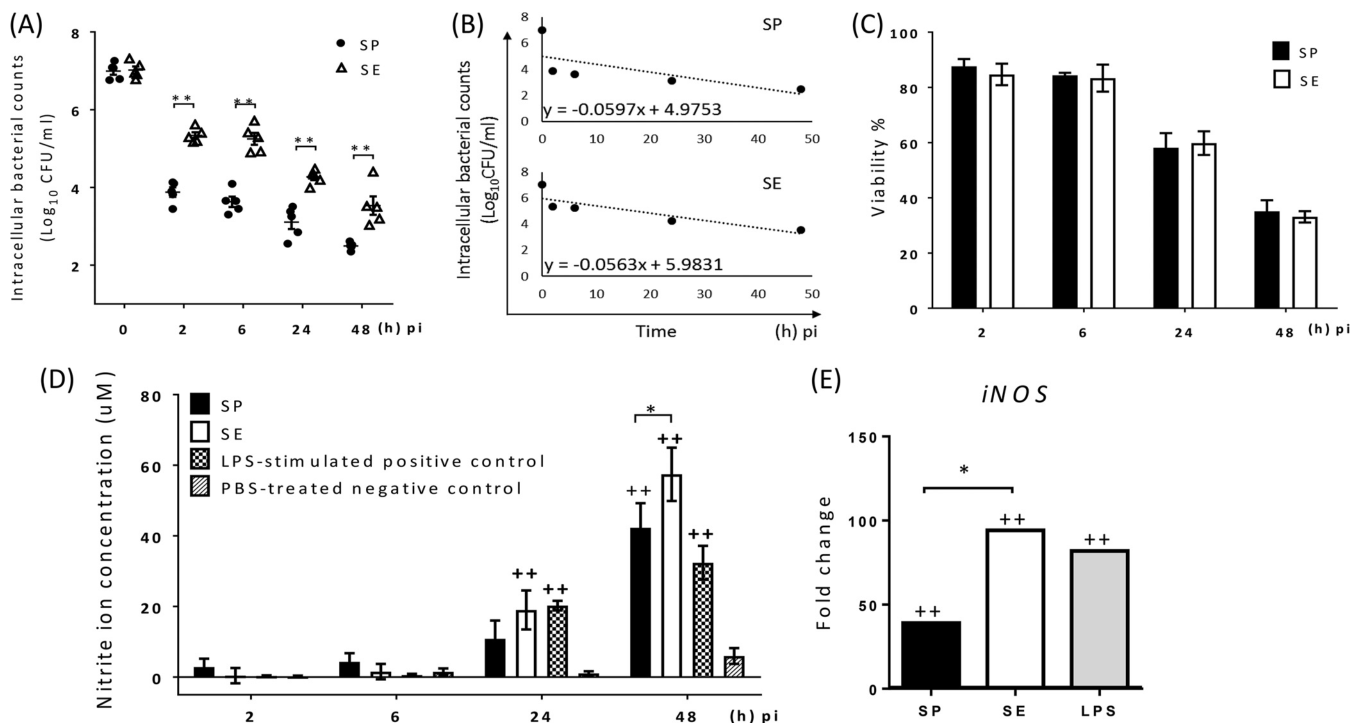


FIG 1 Differences between *S*. Pullorum (SP) and *S*. Enteritidis (SE) in their intracellular survival dynamics (A and B), NO production (D), and *iNOS* expression (E) in infected chMDMs do not correlate with their effect on the viability of infected chMDMs (C). (A and B) Infected chMDMs were lysed to quantify intracellular viable bacteria (A) and the decline rate (B). (D) The supernatant was collected to determine the nitrite ion concentration using a Griess assay. (E) Relative mRNA expression levels of *iNOS* shown as fold changes in comparison to those from uninfected chMDMs (shown as 1) at 6 h p.i. (C) Percentages of viable chMDMs infected with *S*. Pullorum and *S*. Enteritidis were determined by using propidium iodide. Data in panels A, C, and D are presented as means ± standard errors of the means (SEM) ($n = 3$) and are representative of results from at least two independent experiments. For panel B, the decline rate was determined by using averaged counts of intracellular viable bacteria at each time point. For panel E, *iNOS* levels were determined from chMDMs prepared from three birds. + indicates a statistically significant difference from the negative control (+, $P < 0.05$; ++, $P < 0.01$). * indicates statistical differences between different treatment (*, $P < 0.05$; **, $P < 0.01$).

S. Pullorum invaded and/or was taken up by chMDMs in lower numbers than *S*. Enteritidis at 2 h postinfection (p.i.) ($P < 0.01$) (Fig. 1A). At later times, there was a significant difference between the viable counts of the two serovars recovered ($P < 0.01$), with *S*. Enteritidis showing a significantly higher rate of decline *in vitro* than that of *S*. Pullorum over 48 h p.i. (Fig. 1B). Approximately 85% of infected chMDMs remained alive until 6 h p.i., but this figure was significantly reduced by 24 h and 48 h p.i. ($P < 0.01$). However, the difference between *S*. Pullorum- and *S*. Enteritidis-infected cells was not significant ($P > 0.99$) (Fig. 1C).

NO is a major antibacterial effector during chronic infection (24), so, as expected, NO_2^- production was not clearly evident until 24 h after infection. *S*. Enteritidis produced more NO_2^- than did *S*. Pullorum, with this difference being significant ($P < 0.05$) at 48 h p.i. (Fig. 1D). This was mirrored by the difference in the mRNA level of inducible nitric oxide synthase (*iNOS*) measured at 6 h p.i., with the level induced by *S*. Enteritidis being significantly higher ($P < 0.5$) than that induced by *S*. Pullorum (Fig. 1E).

***S*. Pullorum is less effective than *S*. Enteritidis in inducing strong inflammatory responses by infected chMDMs.** Initiation of macrophage killing of invading bacteria also requires the activity of different chemokines and cytokines. Because the related avian serovar, *S*. Gallinarum, induces lower levels of proinflammatory cytokines following infection of cultured epithelial cells (20), we compared the effects of *S*. Pullorum and *S*. Enteritidis infection on the induction of the mRNA expression of *IL-1β*, *IL-6*, *CXCLi1* (*K60*), and *CXCLi2* (*IL-8*) by chMDMs. With the exception of *IL-1β* and *CXCLi1*, *S*. Pullorum induced significantly lower levels of *IL-6* than did *S*. Enteritidis ($P < 0.05$), with *CXCLi2* levels induced by *S*. Pullorum showing a marginally significant reduction compared with those induced by *S*. Enteritidis ($P = 0.0515$) (Fig. 2A). Lipopolysaccharide (LPS)

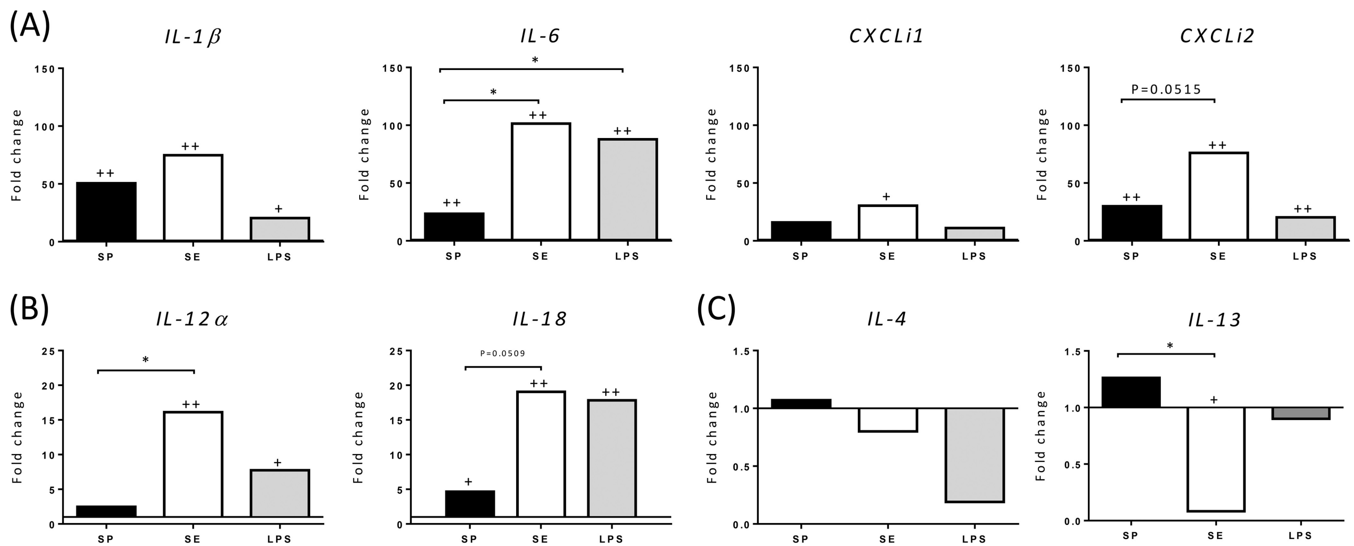


FIG 2 *S*. Pullorum infection induces inflammatory responses that are not as strong as those induced by *S*. Enteritidis in chMDMs. At 6 h p.i., mRNA expression of proinflammatory cytokines (*IL-1β* and *IL-6*) and chemokines (*CXCLi1* and *CXCLi2*) (A), *IL-12α* and *IL-18* (driving the Th1 response) (B), and *IL-4* and *IL-13* (driving the Th2 response) (C) was detected in chMDMs from 3 chickens. The data are shown as fold changes in the mRNA levels of cytokines in comparison to those from uninfected controls (shown as 1) and are representative of results from three independent experiments. + indicates differences between levels of cytokines induced by each serovar compared to PBS-treated uninfected controls (+, $P < 0.05$; ++, $P < 0.01$); * indicates differences between levels of cytokines induced by different serovars (*, $P < 0.05$; **, $P < 0.01$).

stimulation enhanced *IL-6* expression levels in chMDMs, which were significantly higher ($P < 0.05$) than those in response to *S*. Pullorum infection (Fig. 2A). *S*. Pullorum did not completely suppress the expression of proinflammatory cytokines as *S*. Gallinarum was observed to do in epithelial cells (20). However, they were detected at lower levels than with *S*. Enteritidis, suggesting that *S*. Pullorum may invade splenic macrophages without an extensive infiltration of neutrophils during the early stage of infection.

Macrophages function as antigen-presenting cells (APCs) and can also shift the direction of differentiation of naive T cells. Therefore, we investigated the expression levels of cytokines that drive the differentiation of Th1 (*IL-12α* and *IL-18*) and Th2 (*IL-4* and *IL-13*) subsets. *S*. Enteritidis infection and LPS stimulation (as a positive control) induced strong expression of *IL-12α* and *IL-18*. *S*. Enteritidis stimulated higher levels of *IL-12α* than did *S*. Pullorum ($P < 0.05$), although for *IL-18*, this difference was of marginal statistical significance ($P = 0.0509$) (Fig. 2B). In contrast, *S*. Enteritidis induced lower levels of *IL-13* than did *S*. Pullorum ($P < 0.05$) (Fig. 2C). This experiment was also repeated by using cultured macrophage-like HD11 cells, with similar results (data not shown).

A wider selection of *S*. Pullorum and *S*. Enteritidis strains also displays a similar pattern of cytokine/chemokine expression. Although the strains used have been shown experimentally to produce infection with characteristics typical of *S. enterica* serovars Pullorum (5, 10) and Enteritidis (25), we could not be sure that other strains would behave similarly. We therefore repeated the experiments infecting chMDMs with an additional 5 strains of *S*. Pullorum isolated from cases of pullorum disease and 2 of different phage types (PTs) of *S*. Enteritidis isolated from cases of human food poisoning that were attributed to poultry consumption. The gene expression profiles of the *IL-12α*, *IL-18*, *IL-4*, *IL-13*, *IL-10*, and transforming growth factor β4 (*TGF-β4*) genes are shown in Fig. 3. Here the patterns of expression for *S*. Pullorum 449/87 and *S*. Enteritidis 125109 were very similar to those observed in the above-described experiment (Fig. 2), with the other strains of each serovar behaving in a similar manner, with little variation. The patterns of production of the proinflammatory chemokines *IL-1β*, *IL-6*, *CXCLi1*, and *CXCLi2* and also *iNOS* were also similar to those produced by the strains presented in Fig. 1 and 2 (data not shown).

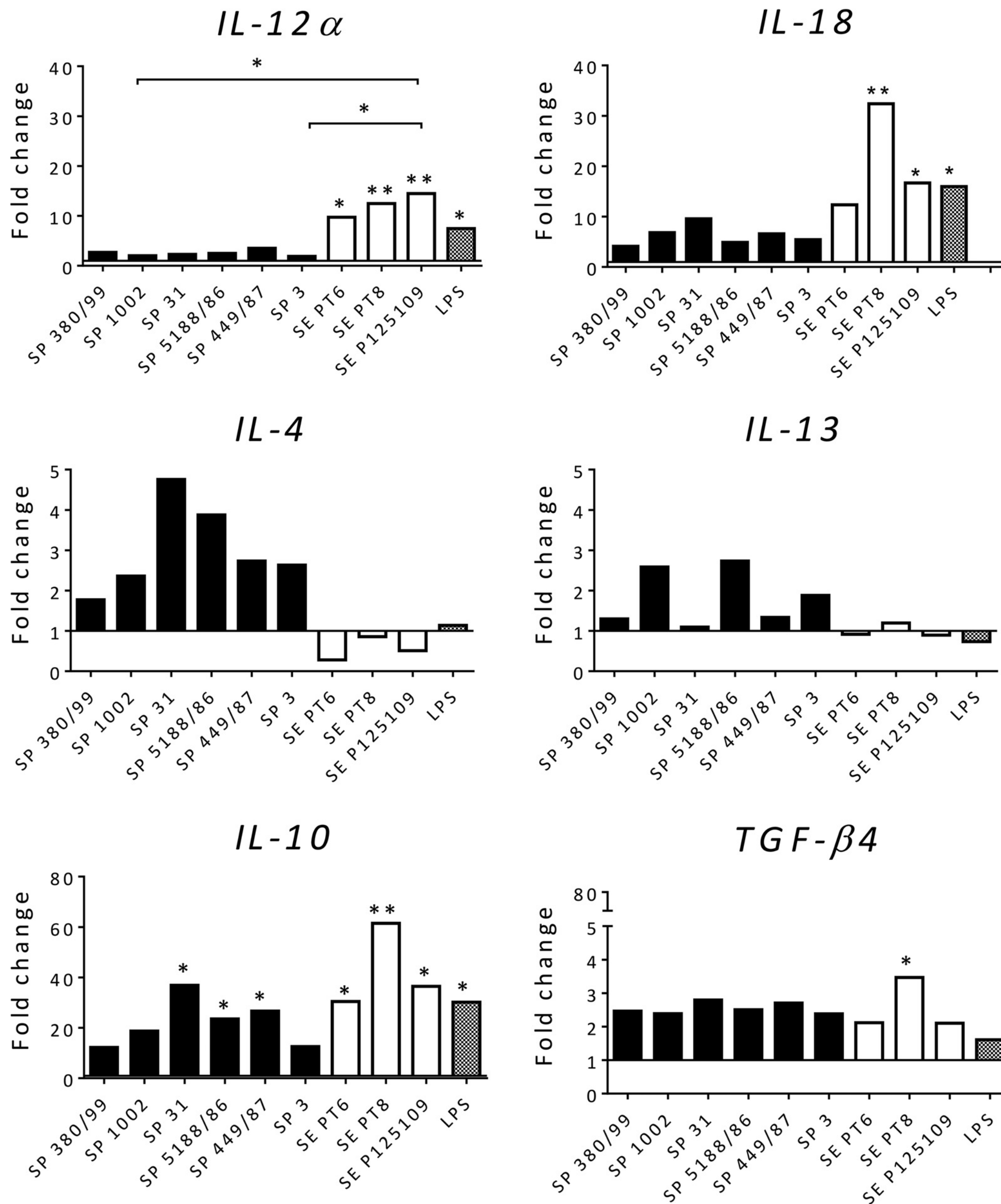


FIG 3 Gene expression profiles of immune mediators in chMDMs in response to infection with a wider selection of *S.* Pullorum and *S.* Enteritidis strains maintain the patterns of the representative strains used. At 6 h p.i., mRNA expression of *IL-12α*, *IL-18*, *IL-4*, *IL-13*, *IL-10*, and *TGF-β4* was detected in chMDMs from 3 chickens. The data are shown as fold changes in the mRNA levels of cytokines in comparison to those from uninfected controls (shown as 1) and are representative of results from three independent experiments. + indicates differences between levels of cytokines induced by each serovar compared to PBS-treated uninfected controls (+, $P < 0.05$; ++, $P < 0.01$); * indicates differences between levels of cytokines induced by different serovars (*, $P < 0.05$; **, $P < 0.01$).

***S.* Pullorum suppresses *IL-18* and *IL-17F* expression in *ex vivo*-infected splenocytes.** chMDMs may not reflect accurately the infection biology in the spleen, where the bacteria are mainly localized during *S.* Pullorum infection (5, 10), as the spleen consists of a variety of cell types. These types include dendritic cells (DCs) and lymphocytes, having different immune functions associated with combating infection and the initiation of the immune response. We therefore isolated splenocytes, which were infected with the standard strains *S.* Pullorum 449/87 and *S.* Enteritidis 125109. In this case, the expression of *IL-18* and *IL-4* (Fig. 4) was in accordance with that observed with chMDMs in Fig. 2B, with *S.* Pullorum suppressing Th1-related cytokines and

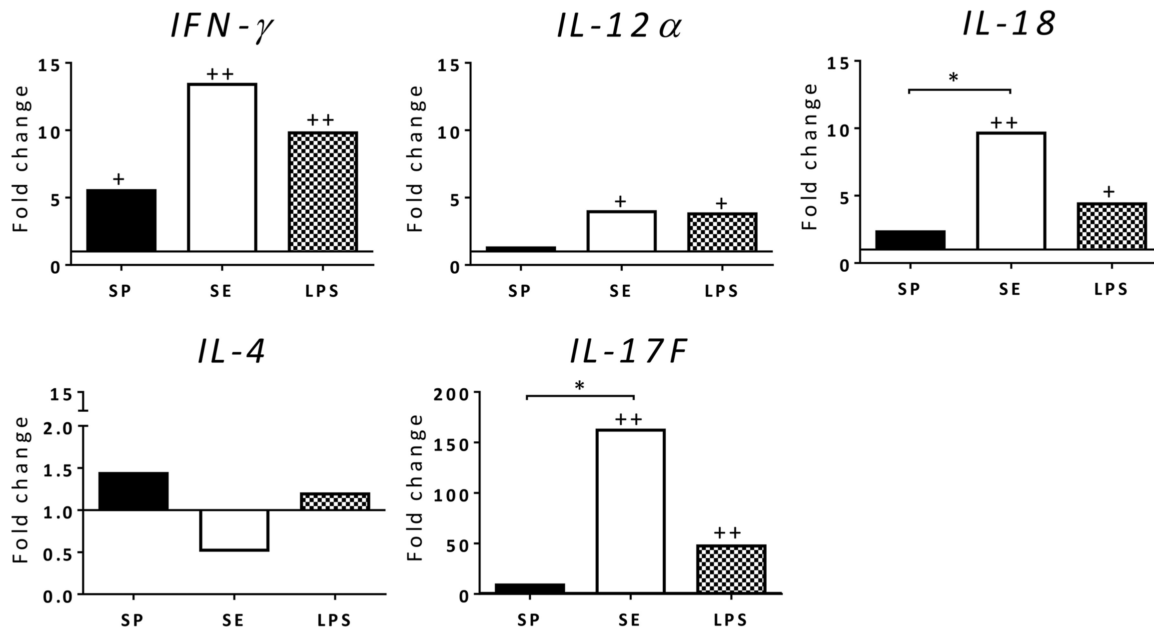


FIG 4 *S*. Pullorum infection induces inflammatory responses that are not as strong as those induced by *S*. Enteritidis in chicken splenocytes *in vitro* at 6 h p.i. Expression of *IFN-γ*, *IL-12α*, *IL-18*, *IL-4*, and *IL-17F* mRNAs was detected in chMDMs from 3 chickens. The data are shown as fold changes in the mRNA levels of cytokines in comparison to those from uninfected controls (shown as 1) and are representative of results from three independent experiments. + indicates differences between levels of cytokines induced by each serovar compared to PBS-treated uninfected controls (+, $P < 0.05$; ++, $P < 0.01$); * indicates differences between levels of cytokines induced by different serovars (*, $P < 0.05$; **, $P < 0.01$).

increasing the expression of Th2-related cytokines. However, both *S*. Pullorum and *S*. Enteritidis induced lower expression levels of *IL-12α* in splenocytes than in chMDMs, which may be regulated by other cell populations other than macrophages in the spleen. We also measured *IL-17F*, which showed very high levels of expression by *S*. Enteritidis compared with those with *S*. Pullorum ($P < 0.05$) or uninfected controls ($P < 0.01$).

***S*. Pullorum suppresses the expression of Th1/Th17 cytokines by CD4$^+$ T cells cocultured with chMDMs.** The pattern of cytokine production by *S*. Pullorum compared to *S*. Enteritidis in chMDMs and splenocytes suggested a response that was anti-inflammatory and that may induce the differentiation of Th2 cells. To test this further, we isolated CD4$^+$ T cells, taken from the blood of different individual birds but from the same genetic line, and cocultured these cells with infected chMDMs. Initial experiments on the viability of macrophages and T cells indicated that over 60% of cells were viable after 5 days of *in vitro* culture (see Fig. S2 in the supplemental material).

After 5 days of coculture, the CD4$^+$ T cells were removed to examine their cytokine profile, which would identify the Th subsets that had proliferated. Compared to those of the control for any allogeneic response, *S*. Enteritidis-infected chMDMs induced the proliferation of CD4$^+$ T cells that expressed high levels of *IFN-γ* ($P < 0.01$) and *IL-17F* ($P < 0.05$), whereas *S*. Pullorum-infected chMDMs induced the proliferation of CD4$^+$ T cells, which did not express *IFN-γ* ($P > 0.05$) or suppressed the expression of *IL-17F* ($P < 0.05$) (Fig. 5). The differences between *S*. Pullorum and *S*. Enteritidis were statistically significant at a P value of <0.01. Neither *S*. Pullorum- nor *S*. Enteritidis-infected chMDMs induced the expression of *IL-17A* in cocultured CD4$^+$ T cells compared to the allogeneic control, although there was a significant difference between *S*. Pullorum and *S*. Enteritidis ($P < 0.05$). In contrast, *S*. Pullorum induced higher levels of expression of *IL-4* than did *S*. Enteritidis, although this difference was not statistically significant (Fig. 5). This suggested that *S*. Pullorum was able to switch cytokine production of CD4$^+$ T cells from dominant IFN-γ and IL-17F expression toward IL-4 expression *in vitro*.

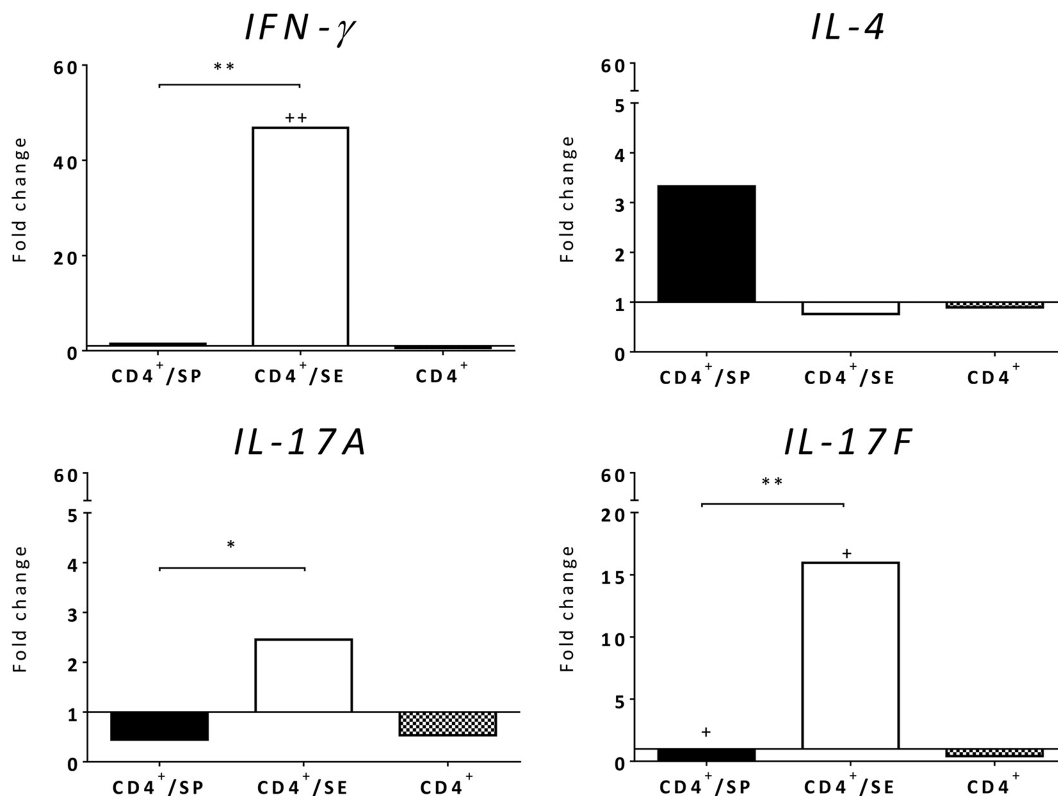


FIG 5 *S.* Pullorum infection suppresses IFN-γ-producing Th17 responses in CD4$^+$ T cells cocultured with infected chMDMs after 5 days of coculture. Expression of *IFN-γ*, *IL-4*, *IL-17A*, and *IL-17F* mRNAs was detected in chMDMs from 3 chickens. The data are shown as fold changes in the mRNA levels of cytokines in comparison to those from uninfected controls (shown as 1) and are representative of results from three independent experiments. + indicates differences between levels of cytokines induced by each serovar compared to PBS-treated uninfected controls (+, $P < 0.05$; ++, $P < 0.01$); * indicates differences between levels of cytokines induced by different serovars (*, $P < 0.05$; **, $P < 0.01$).

Immune evasion strategies, other than a switch from a resolving Th17/CD4$^+$ profile to a nonresolving Th2/CD4$^+$ profile, may explain the mechanism of carriage of *S.* Pullorum in convalescent birds. These include (i) decreased expansion of cognate CD4$^+$ T cell clones, (ii) proliferation of IL-10- and/or TGF-β-producing suppressor T cells, or (iii) a failure of APCs to express costimulatory signals following the engagement of cognate CD4$^+$ T cells, thus inducing clonal anergy.

The results obtained in this study show that after 5 days of coculture, *S.* Enteritidis-infected chMDMs stimulated significantly increased CD4$^+$ T cell proliferation compared with that in *S.* Pullorum-infected chMDMs ($P < 0.05$) (Fig. 6A), indicating that *S.* Pullorum in fact exerted a suppressive effect on proliferation.

S. Pullorum, *S.* Enteritidis, and LPS induced significantly increased ($P < 0.05$) expression levels of *IL-10* by chMDMs, but *TGF-β4* was not significantly expressed compared to control expression by uninfected chMDMs (Fig. 6B). However, these *IL-10*-expressing chMDMs did not induce the proliferation of *IL-10*/*TGF-β*-producing (tolerogenic) CD4$^+$ T cells, and in this regard, the effect of infected chMDMs on T cells was comparable to the effect of allogeneic controls (Fig. 6B). We measured levels of expression of major histocompatibility complex class II (MHCII) and also the costimulatory molecules CD40, CD80, and CD86 in infected chMDMs. A significant reduction in the number of MHCII-positive chMDMs in response to *S.* Pullorum infection was measured at 1 day p.i. ($P < 0.05$) (Fig. 7A), compared to uninfected chMDMs. However, the percentage of CD40-, CD80-, or CD86-positive cells was not lower in *S.* Pullorum-infected chMDMs than in uninfected cells. Compared with *S.* Enteritidis infection, the number of CD40-expressing *S.* Pullorum-infected chMDMs was low only at 24 h p.i. (Fig. 7A).

If cytotoxic-T lymphocyte-associated antigen 4 (CTLA-4) is overexpressed on CD4$^+$ T cells, CD80 and CD86 will preferentially bind to this receptor rather than CD28 (which

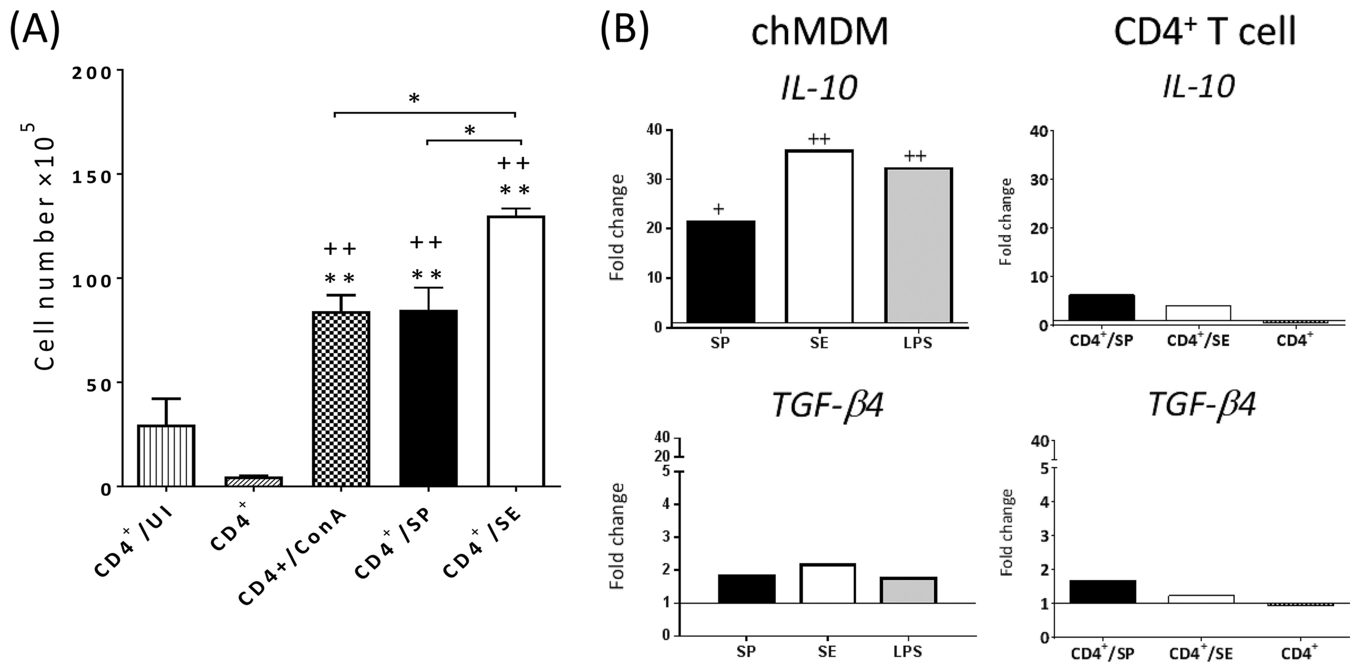
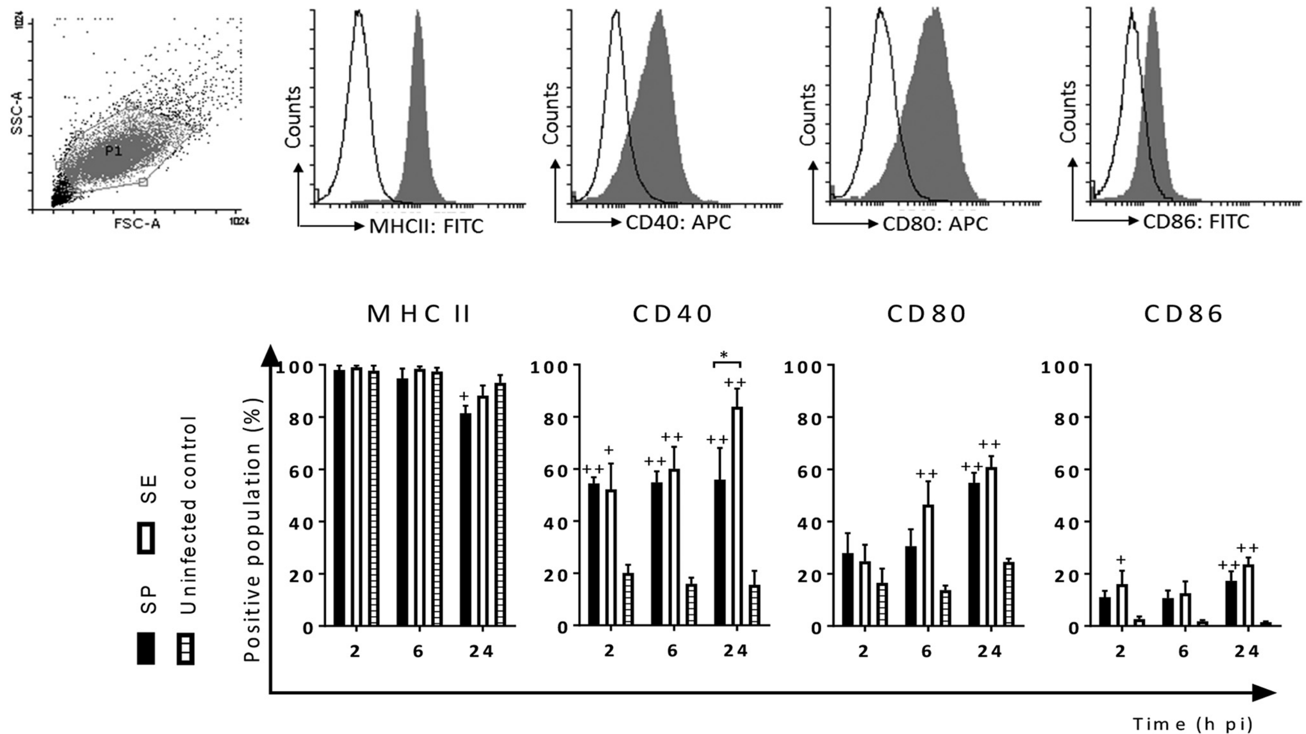
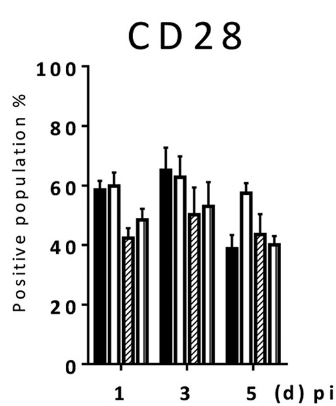


FIG 6 *S.* Pullorum infection neither suppress lymphocyte proliferation nor induces immunosuppression after 5 days of coculture *in vitro*. (A) Numbers of viable proliferating CD4+ T cells are presented as means ± SEM ($n = 3$; chMDMs and CD4+ T cells from 3 chickens each) and are representative of data from two independent experiments. (B) The gene expression levels of *IL-10* and *TGF-β4* (*IL-10* and *TGF-β4* mRNAs in chMDMs are detected at 6 h p.i. without coculture) are shown as fold changes in the mRNA levels of cytokines in comparison to those from uninfected controls (shown as 1) and are representative of results from three independent experiments. CD4+/UI, CD4+ T cells cocultured with uninfected chMDMs (control for allogeneic response); CD4+, CD4+ T cells cultured alone; CD4+/ConA, CD4+ T cells stimulated with ConA (positive control for CD4+ T cell proliferation); CD4+/SP, CD4+ T cells cocultured with *S.* Pullorum-infected chMDMs; CD4+/SE, CD4+ T cells cocultured with *S.* Enteritidis-infected chMDMs. * indicates a statistical difference from the control for the allogeneic response (CD4+/UI) or between different serovar groups (*, $P < 0.05$; **, $P < 0.01$); + indicates a statistical difference from the unstimulated control (CD4+) (+, $P < 0.05$; ++, $P < 0.01$).

is expressed by activated T lymphocytes). Levels of CD28 protein expression by CD4+ T cells over the 5-day-p.i. period were comparable following coculture with *S.* Pullorum- and *S.* Enteritidis-infected chMDMs (Fig. 7B). However, measurement of *CD28* and *CTLA-4* gene expression showed that there was a shift from *CD28* (day 1) to *CTLA-4* (day 5) (Fig. 7C). This would normally be expected as T cells move from an activated state back toward steady-state conditions. We hypothesize that increased CTLA-4 protein levels (shifting the CD28/CTLA-4 ratio toward CTLA-4) probably also occurred over the 5-day period p.i., but due to the lack of a commercially available CTLA-4 antibody, we were unable to measure this. Thus, the lower level of CD4+ proliferation induced by *S.* Pullorum *in vitro* was not a result of the absence of a costimulatory signal and therefore was not clonal anergy.

***S.* Pullorum shows a greater capacity than *S.* Enteritidis for systemic infection *in vivo*.** Although the evidence so far suggests that *S.* Pullorum is able to modulate the immune response of the chicken away from an IFN-γ-producing Th17-type response toward a Th2-type response, this is based on the use of chMDMs as representative antigen-presenting cells interacting with CD4+ T cells. However, DCs and CD8+ T cells are also involved in the early response to *S. enterica* infection *in vivo* (26). Thus, it was essential to determine whether the evidence accumulated thus far was mirrored during experimental infections *in vivo*. To examine this effect *in vivo*, 2-day-old chickens were infected orally with *S.* Pullorum or *S.* Enteritidis, and these chickens were compared with uninfected birds. Infection with approximately 10^8 CFU of *S.* Pullorum or *S.* Enteritidis did not induce any clinical signs of disease over the 5-day period p.i. Viable *S.* Pullorum and *S.* Enteritidis bacteria were detected in the cecal contents of infected chickens in each group after 1 day p.i. *S.* Enteritidis had much higher bacterial counts at all time points examined than did *S.* Pullorum ($P < 0.01$), where the counts were also more variable (Fig. 8). Neither serovar was found in the liver of infected chickens at 1 or 2 days





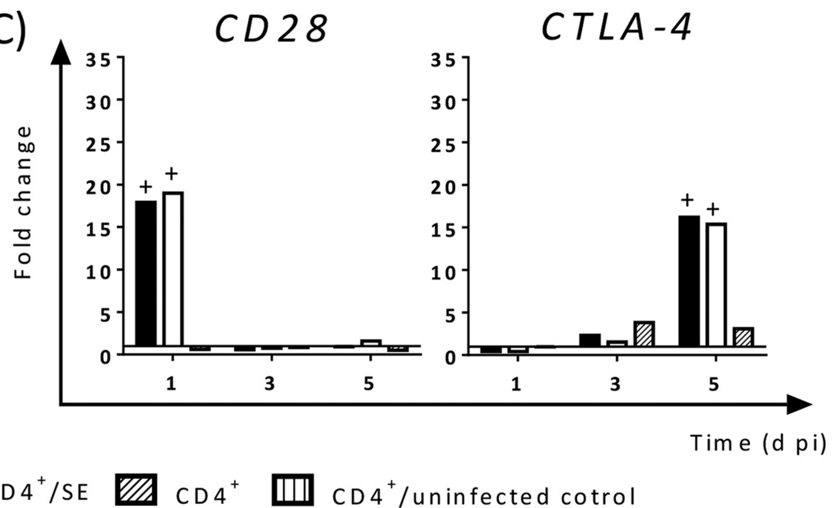


FIG 7 *S.* Pullorum infection does not induce clonal anergy by reducing the number of chMDMs bearing costimulatory molecules. (A) chMDMs (P1) were gated based on side-scatter (SSC)/forward-scatter (FSC) parameters. Shown are representative histograms (top) and average numbers (bottom) of MHCII$^+$, CD40$^+$, CD80$^+$, and CD86$^+$ chMDMs in response to *Salmonella* infection. Black lines, secondary binding or isotype control MAbs; gray shadow, anti-chicken cell surface marker MAbs. (B and C) Numbers of CD28 $^+$ cells (B) and gene expression of CD28 and CTLA-4 (C) in CD4$^+$ T cells from the coculture. For panels A and B, the percentages of MHCII$^+$, CD40$^+$, CD80$^+$, and CD86$^+$ cells from infected chMDMs and CD4$^+$ CD28$^+$ cells out of cocultured CD4$^+$ T cells are shown as means $\pm$ SEM ($n = 3$; chMDMs or CD4$^+$ T cells from 3 chickens). For panel C, the mRNA levels of *CD28* and *CTLA-4* of CD4$^+$ T cells from 3 chickens are shown as fold changes in comparison to those from uninfected controls (shown as 1) and are representative of results from three independent experiments. + indicates statistically significant differences from the uninfected controls (+, $P < 0.05$; ++, $P < 0.01$). * indicates statistical differences between different serovars (*, $P < 0.05$; **, $P < 0.01$).

p.i. At 5 days p.i., the mean counts of *S.* Pullorum recovered from the liver of infected chickens increased to 5.29 $\log_{10}$ CFU/g, which were significantly higher than those of *S.* Enteritidis ($P < 0.01$).

***S.* Pullorum infection induces a weaker proinflammatory response *in vivo*.** The pattern of induction of proinflammatory cytokines in the cecal tonsil was similar to that

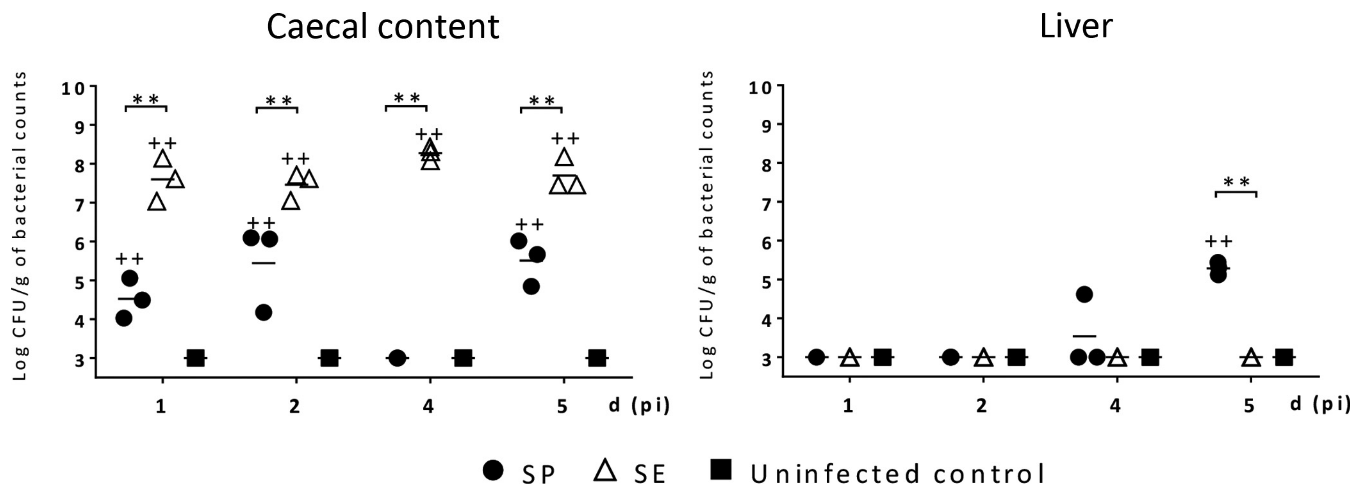


FIG 8 *S.* Pullorum is a poor colonizer of the ceca but an effective invader of the liver. The numbers of viable *S.* Pullorum and *S.* Enteritidis bacteria in the cecal content and the liver of 2-day-old chickens were determined at various times (days) after oral infection. Each symbol represents data for an individual chicken (3 chickens/group) in one independent experiment. When no viable colonies were found at a 10^{-1} dilution after selective enrichment, this suggested a viable count of <3 of log CFU/g, and so log CFU/g=3 was used to represent the bacterial loads in negative animals for statistical analysis. + indicates a statistically significant difference from uninfected controls (+, $P < 0.05$; ++, $P < 0.01$). * indicates statistical differences between different serovars (*, $P < 0.05$; **, $P < 0.01$).

observed in chMDMs, with higher levels of all cytokines being induced by *S.* Enteritidis than by *S.* Pullorum but with the greatest statistically significant differences being found in the cecal tonsils compared to chMDMs. The differences between *S.* Pullorum and *S.* Enteritidis ($P < 0.05$) were more apparent earlier (1 day p.i.) for *CXCLi1* and *IL-1β* but did not appear until 2 days p.i. for *CXCLi2*, *IL-6*, and *iNOS* (Fig. 9). The differences were not as marked in the spleen. Statistically significant differences between *S.* Pullorum and *S.* Enteritidis infection for *CXCLi2* ($P < 0.05$), *IL-1β* ($P < 0.01$), *IL-6* ($P < 0.05$), and *iNOS* ($P < 0.01$) did not appear until 4 days p.i.

***In vivo S.* Pullorum infection suppresses the expression of Th1/Th17-related cytokines but upregulates Th2-related cytokines.** The patterns of production of immune-modulating cytokines measured in the cecal tonsil and spleen were largely similar to each other, with much higher expression levels of Th1 cytokines and lower expression levels of Th2 cytokines being induced by *S.* Enteritidis infection than by *S.* Pullorum, but with some key differences (Fig. 10). In the cecal tonsils, gene expression levels of *IFN-γ* ($P < 0.01$ at 1 day p.i. and $P < 0.05$ at 2, 4, and 5 days p.i.), *IL-12α* ($P < 0.05$ at 1 day p.i. and $P < 0.01$ at 2 days p.i.), and *IL-18* ($P < 0.01$ at 2 days p.i.) were significantly higher in response to *S.* Enteritidis infection than in response to *S.* Pullorum. In the spleen, significant levels of *IL-12α* and *IL-18* ($P < 0.05$) were produced by *S.* Enteritidis at 1 day p.i., although no bacteria were isolated from the liver at this time (the lower limit of bacterial enumeration was 3 $\log_{10}$ CFU/g tissue). In the case of *IL-4* ($P < 0.01$ at 2 and 5 days p.i. and $P < 0.05$ at 4 days p.i. in the cecal tonsils and $P < 0.01$ at 4 and 5 days p.i. in the spleens) and *IL-13* ($P < 0.05$ at 1 day p.i. and $P < 0.01$ at 5 days p.i. in the cecal tonsils and $P < 0.05$ at 4 and 5 days p.i. in the spleens), this was reversed, with higher levels being produced by *S.* Pullorum infection than by *S.* Enteritidis. The expression of *IL-17F* was slightly different from that observed with $CD4^+$ T cells *in vitro*. In the cecal tonsils, *S.* Pullorum suppressed the production of *IL-17F* mRNA at 1 day p.i., with statistically significant differences compared to the uninfected controls ($P < 0.05$) and *S.* Enteritidis infection ($P < 0.01$). *S.* Enteritidis infection produced higher levels of *IL-17F* than did *S.* Pullorum infection, with the difference remaining significant at 4 days p.i. ($P < 0.01$) and 5 days p.i. ($P < 0.05$), respectively. Infection with *S.* Enteritidis upregulated the production of splenic *IL-17F* mRNA, the level of which was significantly higher than that of *S.* Pullorum at 2 and 5 days p.i. ($P < 0.05$). In both organs, as with chMDMs and cocultured $CD4^+$ T cells, the changes in *TGF-β4* after infection were generally small. Increased *IL-10* expression

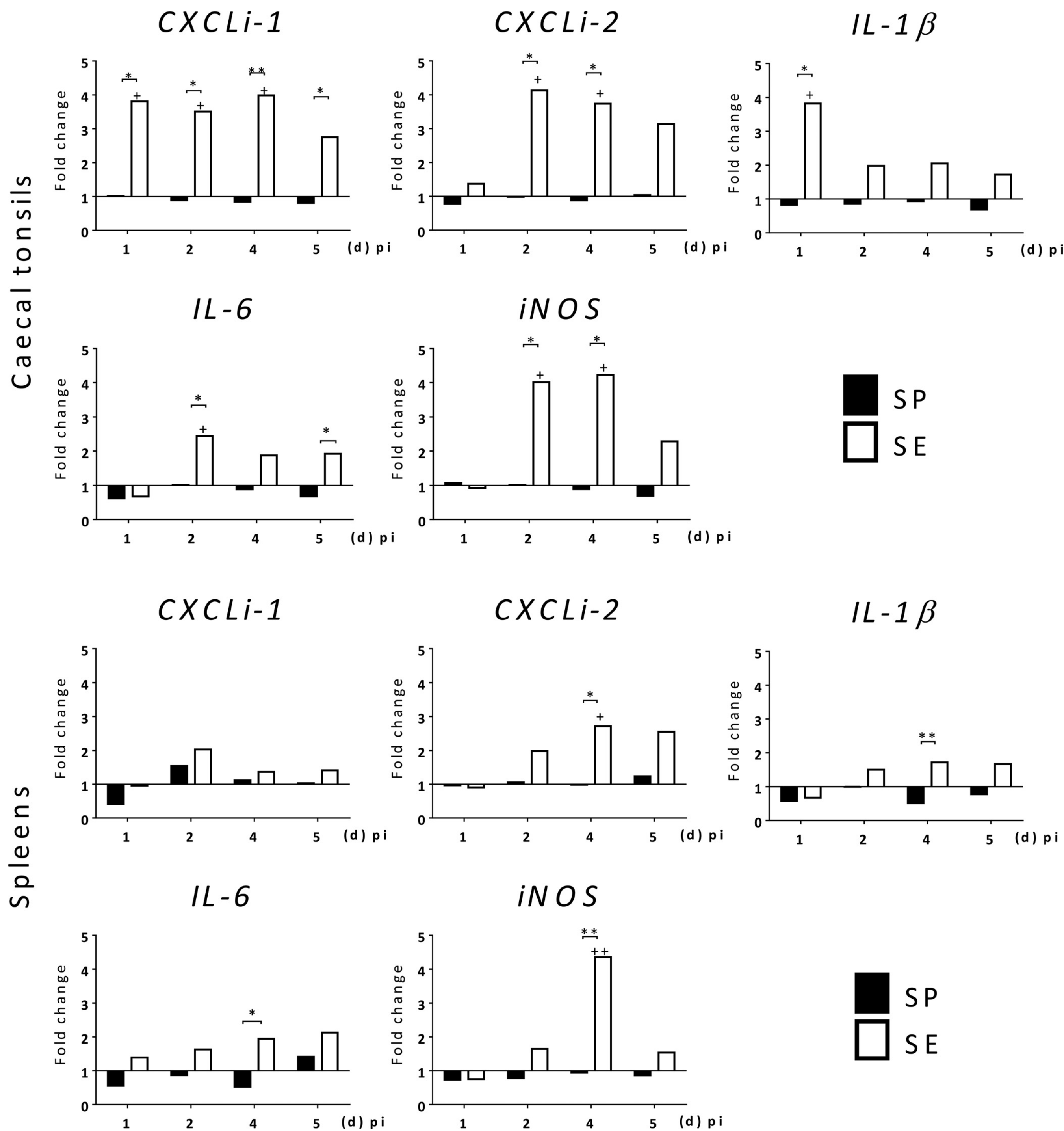
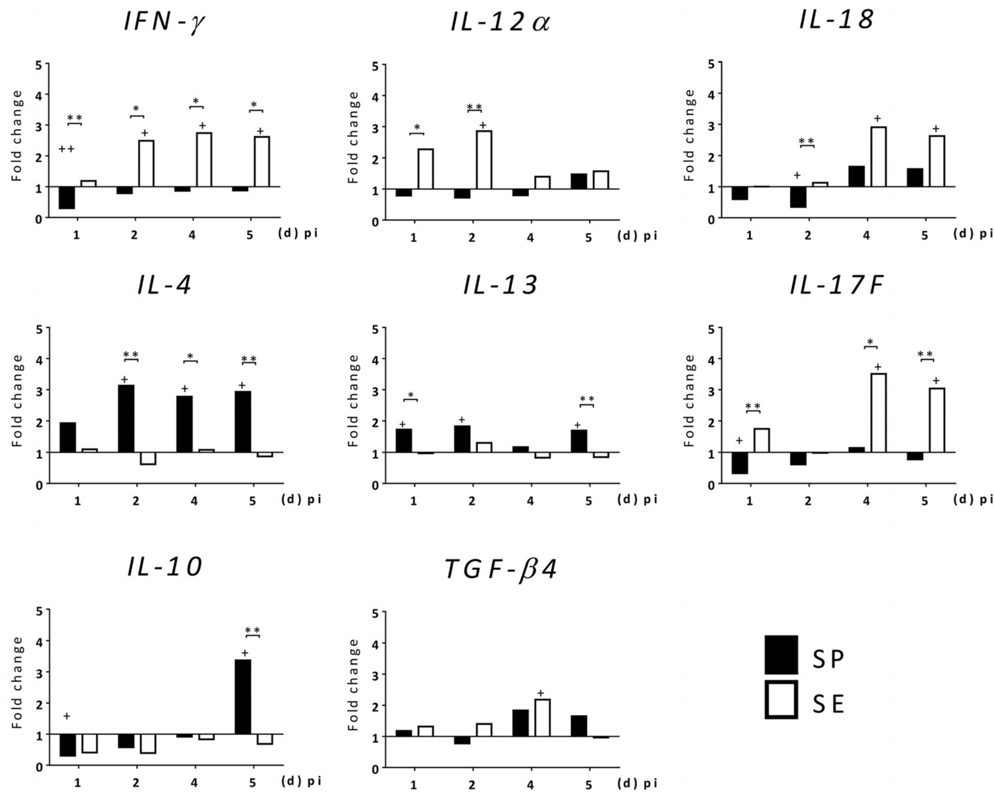


FIG 9 *S.* Pullorum infection suppresses inflammatory responses in the cecal tonsils and spleens of infected chickens at various times (days) after oral infection. The mRNAs of proinflammatory chemokines (*CXCLi1* and *CXCLi2*) and cytokines (*IL-1β*, *IL-6*, and *iNOS*) were detected in 3 chickens from one independent experiment, and the data are shown as fold changes in comparison to values for uninfected controls (shown as 1). + indicates differences in levels of cytokines induced by each serovar compared to uninfected controls (+, $P < 0.05$; ++, $P < 0.01$); * indicates differences between levels of cytokines induced by different serovars (*, $P < 0.05$; **, $P < 0.01$).

induced by *S.* Pullorum over those produced by *S.* Enteritidis ($P < 0.01$) and uninfected controls ($P < 0.05$) was observed at 5 days p.i.

DISCUSSION

In contrast to *S.* Enteritidis, *S.* Pullorum infection did not enhance proinflammatory cytokine expression in avian macrophages or Th1- and/or Th17-related cytokine ex-



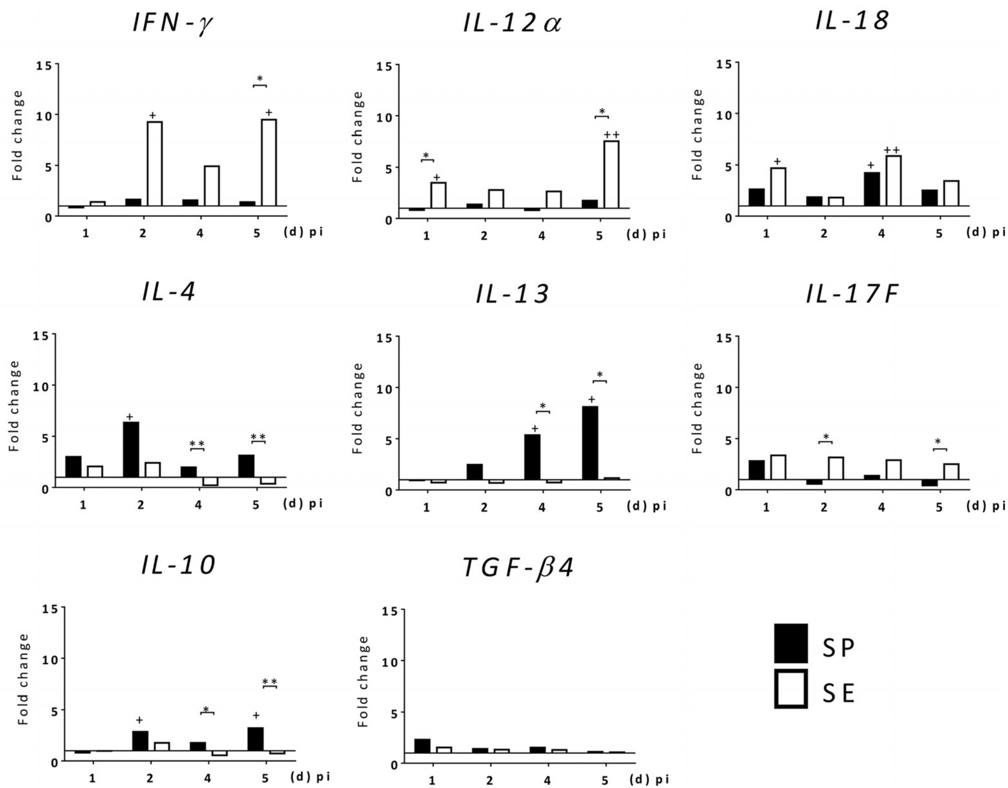


FIG 10 *S.* Pullorum infection modulates an IFN-γ-producing Th17 response toward an anti-inflammatory response in the cecal tonsils and spleens of infected chickens at various times (days) after oral infection. The mRNAs of Th1 (*IFN-γ*, *IL-12α*, and *IL-18*),

(Continued on next page)

pression in CD4$^+$ T cells cocultured with infected chMDMs. This was also the case in the cecal tonsil and spleen of infected chickens. Although modulation of adaptive immunity by *S*. Pullorum toward a nonprotective Th2-like response was most evident *in vivo*, the results of suppressed Th1/Th17 responses derived from the *in vitro* coculture experiments are largely consistent with the observations of infection of 2-day-old chickens. These results support our hypothesis that the mechanisms that underlie persistent infection with *S*. Pullorum involve a manipulation of adaptive immune responses away from a protective IFN-γ-producing Th17-type response. This may enable *S*. Pullorum to evade immune clearance, resulting in persistent carriage.

S. Pullorum may inhibit the proliferation of Th1 lymphocytes by inhibiting IL-12 and IL-18. IL-12-stimulated Th1 differentiation is critical for controlling the early exponential growth of *S*. Typhimurium in the spleen and liver of infected mice by potentiating innate cell-killing pathways (11), while the later control of persistent infection also requires IFN-γ production by Th1 cells (4). The NO pathway is also known to be important for killing of *S*. Typhimurium in murine macrophages. In this case, a biphasic response occurs, such that NO pathways are activated in the later (chronic) phase of infection, whereas reactive oxygen species (ROS) are more important in the earlier stages (27) and are also IFN-γ dependent. A previous study with HD11 cells showed an increase in the oxidative burst after *Salmonella* infection but with no significant difference between *S*. Enteritidis and *S*. Pullorum (28). In the present study, in comparison with *S*. Enteritidis, the failure of *S*. Pullorum to increase *IL-12*α expression in the spleen at 1 day p.i. was followed by significantly lower levels of *IFN-*γ mRNA observed at 5 days p.i., which may possibly give rise to persistent infection in the spleen of infected chickens.

Metabolism of arginine utilized by macrophages involves the enzyme iNOS (M1 macrophages) or arginase (M2 macrophages) (29, 30). In a murine model of persistent infection, *S*. Typhimurium infection was preferentially associated with M2 macrophages activated by Th2 cytokines (31). It is not yet clear whether M1/M2 macrophage polarization occurs in avian species. We showed that *S*. Pullorum is a less robust stimulus for *iNOS* mRNA expression in chMDMs than *S*. Enteritidis, which is what might be expected from a more chronic, persistent infection. The expression of nitric oxide synthase by M1 macrophages metabolizes arginine to NO, whereas arginine is metabolized by M2 macrophages to urea and ornithine, and this limits the production of NO (32). It is possible, therefore, that such differences in arginine metabolism occur in *S*. Pullorum- or *S*. Enteritidis-infected chMDMs, although we have not specifically measured this. However, we also show that *S*. Pullorum-infected chMDMs produce low levels of *IL-12*α/*IL-18* but much higher levels of *IL-4*/*IL-13*, which suggest that *S*. Pullorum infection alone may induce an M2 phenotype (33, 34).

IFN-γ production by a Th1-dominant cellular immune response and initiated by IL-12 and IL-18 is essential for host resolution of *S*. Typhimurium infection in chickens (12–16) and mice (35, 36). Recombinant chicken IFN-γ (chIFN-γ) enhanced NO production in peripheral blood mononuclear cell (PBMC)-derived macrophages and reduced the intracellular replication of *S. enterica* serovar Typhimurium or Enteritidis (37). However, *S*. Pullorum infection neither induced *IL-12*α expression in chMDMs nor promoted *IFN-*γ expression in CD4$^+$ T cells in coculture, indicating that *S*. Pullorum does not initiate an effective IFN-γ-dependent inflammatory response to clear infection.

Virulent *S*. Typhimurium can show persistent infection in resistant mice despite the presence of high levels of circulating anti-*S*. Typhimurium antibody (4). Neutralization of IFN-γ can reactivate acute infections, probably by interfering with macrophage activation (4), suggesting that functional IFN-γ is probably required to suppress bac-

FIG 10 Legend (Continued)

Th2 (*IL-4* and *IL-13*), Th17 (*IL-17F*), and regulatory (*IL-10* and *TGF-*β*4*) cytokines were detected in 3 chickens from one independent experiment, and the data are shown as fold changes in comparison to values for uninfected controls (shown as 1). + indicates differences between levels of cytokines induced by each serovar compared to uninfected controls (+, $P < 0.05$; ++, $P < 0.01$); * indicates differences between levels of cytokines induced by different serovars (*, $P < 0.05$; **, $P < 0.01$).

terial growth during persistent infection by virulent strains in resistant hosts. This implies an increase in both Th1 and Th2 cytokines in response to *Salmonella* infection. It is rational to consider that the ratio of these cytokine levels will govern the overall direction of the immune response to be mainly of the Th1 or Th2 type. It would be interesting to study the kinetics of Th1 and Th2 cytokines during persistent infection because *S*. Pullorum persists in female chickens, with gradually reducing bacterial numbers in the spleens, interrupted by the onset of sexual maturity with spread to the reproductive tract, whereas in males, elimination eventually occurs at between 10 and 18 weeks of age (9). Thus, although *S*. Pullorum appears to suppress the production of IFN-γ in chickens, the role of IFN-γ in *Nramp1*$^{+/+}$mice may be very different, since IFN-γ is required to continue to suppress *S*. Typhimurium in an innately resistant mouse line (4).

In mice, the ablation of Treg early after infection increased the effectiveness of Th1 responses and controlled the tempo of persistent *S*. Typhimurium infection (38). It is unclear whether similar alterations in Treg activities can affect the Th1 responses in susceptible mice or in chickens. CTLA-4/CD80/86 ligation inhibits T cell proliferation and induces T cell apoptosis (tolerance) (39). In comparison with *S*. Enteritidis, *S*. Pullorum-infected chMDMs did not induce high levels of *CTLA-4* mRNA expression in cocultured CD4^{+} T cells. The suppressive properties of avian Treg cells (CD4^{+} CD25^{+}) were suggested to be IL-10 dependent (40). In our study, *S*. Pullorum infection led to the invasion of liver and increased *IL-10* expression in the spleen. This suggests a possible regulatory effect of IL-10 on inhibiting cytokine production during systemic dissemination and, possibly, persistent infection. Avian CD4^{+} CD25^{+} suppressor T cells have been shown to produce high concentrations of IL-10, TGF-β4, and CTLA-4 and suppress T cell proliferation *in vitro* (41). IL-10 inhibits the further development of the avian Th1 response and downregulates the effects of IFN-γ to limit the inflammatory response (42). Increased *TGF-β4* expression in *S*. Typhimurium-infected chickens was also shown to correspond to the decreased production of proinflammatory mediators (15). The measurement of *IL-10* expression by gene expression rather than the presence of protein opens the possibility that the IL-10 protein may conceivably not have been produced. If this is the case, it may be the reason why IL-10/TGF-β-producing CD4^{+} T cells did not proliferate. This may be measured in supernatants when a reliable reagent becomes available.

In chickens infected with *S*. Enteritidis, early expression of *IL-17* and prolonged high-level expression of *IFN-γ* were detected in the ceca (43, 44), which suggested a function of Th17 cells as inflammatory mediators in avian immunity. However, the functional role of Th17 cells and IL-17 in the mucosal inflammatory response to avian salmonellosis is not yet fully defined. In 17A$^{-/-}$ mice infected with *S*. Enteritidis, the recruitment of neutrophils was significantly compromised, with reduced clearance of *S*. Enteritidis from the spleen and liver (45), indicating the potential of Th17 cytokines to be involved in intestinal defense against *S. enterica* infection. Our *CXCLi1*/*CXCLi2* data may suggest a difference between *S*. Pullorum and *S*. Enteritidis in heterophil recruitment, and avian IL-17 may potentially also function to recruit heterophils to promote inflammatory responses. *IL-17* was elicited rapidly in response to *S*. Typhimurium infection of bovine ligated ileal loops, probably through a nonspecific activation of intestinal Th17 cells in response to inflammatory cytokines or the recognition of flagellin via the Toll-like receptor 5 (TLR-5) pathway to drive *Salmonella*-specific Th17 cell development (46). *S*. Pullorum was able to induce the expression of various proinflammatory cytokines in chMDMs. The reduced expression of *IL-17F* in CD4^{+} T cells *in vitro* and in spleen and cecal tonsils *in vivo* may thus have resulted from the absence of TLR-5 stimulation by nonflagellated *S*. Pullorum. This may also be the case for another nonflagellated serovar, *S*. Gallinarum, which is able to show persistent systemic infection in a *SAL1*-resistant chicken phenotype (47). Murine Th17 cells were reported to produce IFN-γ *in vitro* (48) and *in vivo* (49). Although the roles and functions of *IL17* or Th17 cells in regulating immune responses have not been studied in chickens, *S*. Pullorum-infected chMDMs were unable to induce the gene expression of *IFN-γ* and

IL-17F from cocultured CD4$^+$ T cells, indicating a host immunological bias away from IFN-γ-producing Th17 immunity in response to *S*. Pullorum infection, which might be associated with the establishment of carriage.

In this study, *S*. Pullorum was shown to be less effective than *S*. Enteritidis in stimulating the proliferation of CD4$^+$ T cells using commercial blood obtained from spent laying hens, which had been vaccinated more than 1 year previously. Although there are no authenticated reports of immunity against *Salmonella* infection lasting more than 6 to 9 months, we collected blood from unvaccinated layer breeders to repeat the proliferation assay. This produced a pattern of T cell proliferation (see Fig. S3 in the supplemental material) similar to that shown in Fig. 6A, indicating that the vaccination of the birds more than 1 year previously had no effect.

S. Typhimurium was shown to reduce T cell proliferation and cytokine production in the absence of DCs (50). Although *Salmonella* resides largely as an intracellular pathogen, the spread of *S*. Dublin from ligated intestinal loops in calves involves free bacteria that are not present within macrophages (51), although the extent to which this occurs with other host species and serovars is unknown. In chickens at the onset of lay, when *S*. Pullorum bacteria multiply within splenic macrophages and spread to the reproductive tract, *S*. Pullorum may conceivably utilize a similar strategy to directly inhibit the proliferation of T cells.

The bacterial determinants of persistent infection, as opposed to multiplication during acute disease, remain obscure. Type 3 secretion system 2 (TTSS-2) enables the replication and survival of *Salmonella* within macrophages and is essential for inducing systemic infection caused by *S. enterica* serovar Pullorum or Typhimurium in chickens (52, 53). However, *Salmonella* pathogenicity island 2 (SPI-2) contributes to, but is not absolutely required for, persistent *S*. Typhimurium infection in mice (54). Further work to identify the bacterial determinants of persistent infection in *S*. Pullorum will likely require investigation of all the genes associated with intracellular survival and growth, including SPI-2 genes, plus a number of genes with metabolic functions. It may be significant that one feature of the serovars that typically produce typhoid-like disease, which is associated with systemic and persistent infection, is auxotrophy (55). The fact that both *S. enterica* serovars Pullorum and Gallinarum are nonflagellate is unlikely to be significant, as *S. enterica* serovars Dublin, Typhi, and Abortusovis/Equi are all flagellate. Moreover, the importance of the host genetic background in determining persistent infection has been observed for *S*. Gallinarum infection. In a *SAL1*-resistant inbred chicken line, the organism persisted for more than 14 weeks, with infection restricted to persistence and without extensive multiplication in the liver and spleen (10). Similarly, persistent spleen infection involving fully virulent *S*. Typhimurium in mice has also been shown with the *Nramp1*$^{+/+}$ haplotype (4), whereas certain auxotrophic mutants of *S*. Typhimurium are able to persist in the spleens of *Nramp1*$^{-/-}$ mice (56). Persistent infection thus appears to be possible in resistant host phenotypes with fully virulent wild strains or in more susceptible host strains with more attenuated bacterial mutants or with wild-type serovars. How this is related to the persistence of *S*. Typhi or *S*. Dublin in humans or cattle, respectively, also remains to be determined. That the host genetic background is also involved is suggested by the fact that the response to *S*. Enteritidis during intestinal colonization, in a line of chickens showing long-term fecal shedding, also shows a Th2 bias (57).

It thus seems clear that the true picture of persistence of both *S*. Pullorum in chickens and *S*. Typhimurium in mice is more complex than first appearances suggest. It is unclear how this compares with the other serovars that typically produce typhoid-like disease and show persistence after convalescence. Chronic infection with *S*. Typhi is associated with shedding via the gallbladder, although the spleen and the liver are known to be infected (58–60). In *S*. Dublin infection in cattle, persistent shedding can occur from the gut and udder, but the spleen is also affected, and the gallbladder is also sometimes involved (6, 61). Persistent infection within splenic macrophages may thus also be the key infection site for other serovars producing typhoid-like disease and chronic infections, and *S*. Pullorum infection in chickens may thus represent a good

TABLE 1 MAbs used in this study

Antibody	Clone	Isotype	Concn (μg/ml)
Monocyte/macrophage marker (KUL01)-PE[c]	KUL01	IgG1κ	1
Mouse anti-chicken CD4[d]	CT-4	IgG1κ	1
Mouse anti-chicken CD4-FITC[a]	2-35	IgG2b	5
Mouse anti-chicken CD3[a]	CT-3	IgG1	2.5
Mouse anti-chicken MHCII-FITC[d]	2G11	IgG1	1
Mouse anti-chicken CD40[a]	AV79	IgG2a	2.5
Mouse anti-chicken CD80[a]	IAH:F864:DC7	IgG2a	2.5
Mouse anti-chicken CD86[a]	IAH:F853:AG2	IgG1	2.5
Mouse anti-chicken CD28[a]	2-4	IgG2a	5
Anti-mouse IgG2α-allphycocyanin[b]	m2a-15F8		2.5
Anti-mouse IgG1-FITC[b]	M1-14D12		2.5
Mouse IgG1-PE[a]			1
Mouse IgG1-FITC[a]			1
Mouse IgG2α-FITC[b]			5
Mouse IgG2b-FITC[b]			5

[a]AbDSerotec, UK.
[b]eBioscience, UK.
[c]Santa Cruz Biotechnology, USA.
[d]Southern Biotech, USA.

working model with which to study immune manipulation in greater detail and explore approaches to modifying the host response to adversely affect bacterial persistence.

MATERIALS AND METHODS

Bacterial strains. The *in vivo* behavior of *S.* Pullorum 449/87 (5, 62) and *S.* Enteritidis P125109 (63, 64) in chickens has been well characterized. *S.* Pullorum and *S.* Enteritidis were cultured in nutrient broth (Oxoid, UK) at 37°C with shaking at 150 rpm prior to use in experimental infections *in vitro* and *in vivo*.

Isolation and culture of chMDMs, $CD4^+$ T cells, and splenocytes. Chicken peripheral whole blood, obtained from spent Lohmann Lite laying hens, was purchased from Harlan Laboratories UK Ltd. (Leicestershire, UK). The methods of isolation of chicken peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation using Histopaque 1077 and conversion into macrophages were described previously (47). chMDM enrichment was confirmed by flow cytometry analysis using chicken monocyte/macrophage marker antibody (clone KUL01; Santa Cruz Biotechnology, USA). Approximately half of the monocytes initially separated from chicken whole blood were $KUL01^+$ $MHCII^+$. After 2 days of conversion into macrophages and removal of nonadherent cells, this figure increased to more than 95% of adherent cells (see Fig. S1 in the supplemental material). Mouse anti-chicken CD4 monoclonal antibody (MAb) (clone CT-4; Southern Biotech, USA) and anti-mouse IgG1 microbeads (Miltenyi Biotec, UK) were used to positively select chicken $CD4^+$ T cells according to the manufacturers' instructions. Cell viability was assessed by propidium iodide (20 μg/ml; Life Technologies, UK) uptake detected by flow cytometry analysis. $CD4^+$ T cells and chMDMs were isolated from different individual birds. Spleens from newly hatched Lohmann Lite layer chickens were removed aseptically and homogenized gently by using a 70-μm strainer (BD Biosciences, UK) to prepare a suspension of single splenocytes.

***In vitro* infection of chMDMs and splenocytes with *S. enterica*.** chMDMs and splenocytes were produced at a final concentration of 5×10^5 cells/ml in RPMI 1640 (Gibco, Life Technologies, UK) supplemented with fetal bovine serum (FBS) (10%, vol/vol) (Gibco, Life Technologies, UK), HEPES (20 mM) (Sigma-Aldrich, UK), gentamicin sulfate (50 μg/ml) (Sigma-Aldrich, UK), streptomycin-penicillin (10 U/ml) (Gibco, Life Technologies, UK), amphotericin B (Fungizone) (1.25 μg/ml) (Gibco, Life Technologies, UK), and L-glutamine (2 mM) (Gibco, Life Technologies, UK). *In vitro* invasion was performed by using a multiplicity of infection (MOI) of 10 (20, 28). *S.* Enteritidis LPS (50 μg/ml) (Sigma-Aldrich, UK) was used as a positive control for nitrite ion (NO_2^-) and cytokine production, and phosphate-buffered saline (PBS) only was used as a negative control. After 1 h of incubation, the medium was replaced with fresh culture medium supplemented with 100 μg/ml of gentamicin sulfate, and the mixture was incubated for another hour to kill extracellular *S. enterica* bacteria. Cell preparations were then washed three times with sterile PBS and kept in fresh culture medium containing 20 μg/ml of gentamicin sulfate prior to use in subsequent studies. *Salmonella*-infected cells were lysed at 2, 6, 24, and 48 h postinfection (p.i.) by using Triton X-100 (1%, vol/vol) (Thermo Fisher Scientific, UK) to release and determine the intracellular survival of bacteria (log_{10} CFU per milliliter). The concentration of NO_2^- produced by infected and uninfected chMDMs was assessed by a Griess assay kit (Promega, USA) at the same time points. At 6 h p.i., *Salmonella*-infected cells were collected for cytokine mRNA expression analysis by quantitative real-time PCR (qRT-PCR).

Avian chMDM/$CD4^+$ T cell model *in vitro*. chMDMs infected with *S.* Pullorum or *S.* Enteritidis were cocultured with $CD4^+$ T cells for 5 days *in vitro*. The ratio of cocultured cells was maintained at 1:10 (chMDMs to $CD4^+$ T cells) throughout the study. In addition, three control groups were set up, as follows: (i) coculture of uninfected (PBS-treated) chMDMs with $CD4^+$ T cells was used to assess the allogeneic

TABLE 2 Sequences of probes and primers used in this study

Target RNA	Probe or primer sequence (5′–3′)[a]	GenBank accession no.
28S	P, (FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA) F, GGCGAAGCCAGAGGAAACT R, GACGACCGATTTGCACGTC	X59733
iNOS	P, (FAM)-TCCACAGACATACAGATGCCCTTCCTCTTT-(TAMRA) F, TTGGAAACCAAAGTGTGTAATATCTTG R, CCCTGGCCATGCGTACAT	U46504
IL-1β	P, (FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA) F, GCTCTACATGTCGTGTGTGATGAG R, TGTCGATGTCCCGCATGA	AJ245728
IL-6	P, (FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA) F, GCTCGCCGGCTTCGA R, GGTAGGTCTGAAAGGCGAACAG	AJ250838
CXCLi1	P, (FAM)-CCACATTCTTGCAGTGAGGTCCGCT-(TAMRA) F, CCAGTGCATAGAGACTCATTCCAAA R, TGCCATCTTTCAGAGTAGCTATGACT	AF277660
CXCLi2	P, (FAM)-TCTTTACCAGCGTCCTACCTTGCGACA-(TAMRA) F, GCCCTCCTCCTGGTTTCAG R, TGGCACCGCAGCTCATT	AJ009800
IFN-γ	P, (FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA) F, GTGAAGAAGGTGAAAGATATCATGGA R, GCTTTGCGCTGGATTCTCA	Y07922
IL-12α	P, (FAM)-CCAGCGTCCTCTGCTTCTGCACCTT-(TAMRA) F, TGGCCGCTGCAAACG R, ACCTCTTCAAGGGTGCACTCA	AY262751
IL-18	P, (FAM)-GGAAGGAG-(TAMRA) F, AGAGCATGGGAAAATGGTTG R, CCAGGAATGTCTTTGGGAAC	AJ276026
IL-4	P, (FAM)-AGCAGCACCTCCCTCAAGGCACC-(TAMRA) F, AACATGCGTCAGCTCCTGAAT R, TCTGCTAGGAACTTCTCCATTGAA	AJ621735
IL-13	P, (FAM)-CATTGCAAGGGACCTGCACTCCTCTG-(TAMRA) F, CACCCAGGGCATCCAGAA R, TCCGATCCTTGAAAGCCACTT	AJ621735
TGF-β4	P, (FAM)-ACCCAAAGGTTATATGGCCAACTTCTGCAT-(TAMRA) F, AGGATCTGCAGTGGAAGTGGAT R, CCCCGGGTTGTGTGTTGGT	M31160
IL-10	P, (FAM)-CGACGATGCGGCGCTGTCA-(TAMRA) F, CATGCTGCTGGGCCTGAA R, CGTCTCCTTGATCTGCTTGATG	AJ621614
IL-17A	P, (FAM)-ATCGATGAGGACCACAACCGCTTC-(TAMRA) F, TATCAGCAAACGCTCACTGG R, AGTTCACGCACCTGGAATG	NM_204460.1
IL-17F	P, (FAM)-GTTGACATTCGCATTGGCAGCTCT-(TAMRA) F, TGAAGACTGCCTGAACCA R, AGAGACCGATTCCTGATGT	JQ776598.1
CTLA-4	F, CAAGGGAAATGGGACGCAAC R, GTCTTCTCTGAATCGCTTTGCC	AM236874.1
CD28	F, GCCAGCCAAACTGACATCTAC R, CTGTAGAAACCAAGAAGTCCCG	NM_205311.1

[a]P, probe; F, forward primer; R, reverse primer; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

immune response due to culture of chMDMs and CD4^{+} T cells isolated from different individual birds, (ii) CD4^{+} T cells were cultured with concanavalin A (ConA) (10 μg/ml) (Sigma-Aldrich, UK) as a positive control for the proliferation of CD4^{+} T cells, and (iii) CD4^{+} T cells cultured alone were assessed for viability and nonspecific proliferation over the 5-day culture period *in vitro*. All cultures were repeated in triplicate on three separate occasions. After 5 days of coculture, CD4^{+} T cells from each group were collected to measure the proliferation of CD4^{+} T cells using the CellTiter96AQ$_{ueous}$ One Solution cell proliferation assay (Promega, USA). Supernatants from infected and uninfected chMDMs cultured alone under the same conditions were also tested for cell proliferation to ensure that chMDMs did not affect the difference between *S*. Pullorum and *S*. Enteritidis induction of CD4^{+} T cells for proliferation. CD4^{+} T cells were also harvested from each group after 5 days of coculture to measure cytokine mRNA expression by qRT-PCR.

Phenotypic analysis of infected chMDMs and CD4^{+} T cells following coculture with chMDMs. Cells to be analyzed for MHCII, CD40, CD80, CD86, or CD28 expression were collected and fixed with PBS–4% (vol/vol) formaldehyde. In each group, 10^6 cells were incubated with the antibodies indicated and their isotype controls coupled to phycoerythrin (PE), fluorescein isothiocyanate (FITC), or allophycocyanin. MAbs used are all listed in Table 1. Fluorescence analysis was performed by using a FACSCanto II fluorescence-activated cell sorter (FACS) equipped with FACSDiva software (BD Biosciences, UK).

***In vivo Salmonella* chicken infections.** A total of 36 2-day-old Lohmann Lite chickens obtained from the Millennium Hatchery (Birmingham, UK) were divided into three groups with 12 birds each in separate pens and given access to antibiotic-free feed and water *ad libitum* throughout the experiment. Two groups were inoculated orally with 10^8 CFU of *S*. Pullorum or *S*. Enteritidis in 0.1 ml of nutrient broth. All animal care and experimentation were carried out under Home Office project license PPL 40/3412 and had local ethical approval. At 1, 2, 4, and 5 days p.i., three birds from each group were euthanized. Cecal content and liver were collected aseptically, weighed, and homogenized in PBS using Griffiths tubes. Decimal dilutions of the homogenates were then plated onto Brilliant Green agar plates containing sodium nalidixate (20 μg/ml; Sigma-Aldrich, UK) and novobiocin (1 μg/ml; Sigma-Aldrich, UK) to determine bacterial counts. Spleen and cecal tonsil were collected for cytokine mRNA expression analysis.

mRNA expression analysis by qRT-PCR. RNA extraction was performed by using the RNeasy Plus minikit (Qiagen, UK). One microgram of total cellular RNA was reverse transcribed to cDNA by using a Transcriptor first-strand cDNA synthesis kit (Roche, UK) according to the manufacturer's guidelines. The LightCycler 480 system (Roche, UK) was used to measure the gene expression levels of selected cytokines and chemokines by qRT-PCR. The sequences of primers and probes are shown in Table 2. Gene expression of *CD28* and *CTLA-4* was detected by SYBR green-based qRT-PCR. According to methods described previously (20, 65), relative gene expression was normalized against 28S mRNA expression and expressed as a fold difference from levels in uninfected controls.

Statistical analysis. Data were plotted and analyzed by using GraphPad Prism 6.0. Comparisons between different groups and between different groups at different time points p.i. were performed by using two-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison *post hoc* test. Statistical significance was determined at the 5% and 1% confidence limits as *P* values of <0.05 and <0.01.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00307-18.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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