Title: A model of persistent Salmonella infection: *Salmonella* Pullorum modulates the immune response of the chicken from a Th17 towards a Th2-type response

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Abstract

Salmonella enterica infection affects a wide range of animals and human and a small number of serovars cause typhoid-like infections, one characteristic of which is persistent infection in convalescents. The avian specific serovar S. 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 the persistent infection remains unclear. S. Enteritidis is taxonomically closely related but does not show this characteristic. Differences in the immune responses between S. Pullorum and S. Enteritidis were compared using Salmonella-infected chicken monocyte-derived macrophages (chMDMs) and CD4+ T lymphocytes which had been co-cultured 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 of IL-12α and IL-18 and stimulated proliferation of Th2 lymphocytes with reduced expression of IFN-γ and IL-17 and increased expression 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 levels of expression of IL-4 and lower 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 towards a Th2 response, which may promote the 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 (SP); Salmonella enterica serovar Enteritidis (SE); macrophage; CD4+ T cells; Th1; Th2; Th17
1. Introduction

The majority of *Salmonella enterica* serovars that affect human or animal health generally cause gastrointestinal disease of varying severity in a wide range of hosts (1). A small number of serovars, including *Salmonella enterica* serovar Typhi (S. Typhi), S. Gallinarum, S. Pullorum (SP), S. Dublin, S. Choleraesuis and S. Abortusovis/equi, are adapted to a narrow range of host species and generally produce severe, typhoid-like disease sometimes with high mortality (2). S. Typhimurium and S. Enteritidis (SE) 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 the infection produced by the typhoid serovars is asymptomatic persistent infections in a proportion of convalescents in experimental infection involving macrophages in lymphoid tissues (5). This results in localization in the gall bladder, liver and spleen leading to faecal shedding by carriers for long periods and, in some cases, many years (S. Typhi in man and S. Dublin in cattle) (6-8), or localization in the reproductive tract leading either to abortion (S. Dublin, S. Abortusovis in sheep), or vertical transmission through hatching eggs to progeny (S. Pullorum and S. Gallinarum) (9). SP is a good and natural model of the 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 SE from the intestinal tract of infected chickens was also shown to be due to a Th1 dominated response involving increased expression of *IFN-γ* mRNA in the gut and deeper tissues (12-16). SP colonises 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...
serovar *S. Gallinarum* (19). This is attributed to the reduced production of pro-inflammatory chemokines *IL-1* and *IL-6* demonstrated *in vitro* following *S. Gallinarum* infection of avian epithelial cells (20). In the case of SP, a small number of viable bacteria have been shown to persist in macrophages in convalescent birds. These are most easily detectable in the spleen, in a proportion of animals, despite the presence of a high titre antibody response (5, 9, 10). Recrudescence of systemic infection and spread of SP 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, the 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 SP 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 SP and SE, SP-infected birds expressed significantly lower levels of splenic *IL-18* and *IFN-γ* whereas the expression of *IL-4* was increased 14 d after infection (18). This suggested that SP might induce an immune response that more closely resembled the Th2 response in mammals and which could allow SP to establish intracellular carriage evading Th1-mediated clearance.

The nature of the immune response to the other serovars which 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). A significant increase in *IL-17*CD4+ T cells and *in vitro* IFN-γ production was also observed during convalescence from *S. Typhi* (22). These studies suggested that in the majority of individuals *S. Typhi* infection induced a predominant IFN-γ response derived from lymphocytes 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 SP infection using an *in vitro* macrophage-T cell co-culture system and *in vivo* infections. The results indicated that SP is able to drive host immunity towards a Th2-like response.

2. Methods

2.1 Bacterial strains

The *in vivo* behaviour of SP 449/87 (5, 24) and SE P125109 (25, 26) in chickens has been well characterised. SP and SE were cultured in nutrient broth (Oxoid, UK) at 37°C with shaking at 150 rpm prior to use in experimental infection *in vitro* and *in vivo*.

2.2 Isolation and culture of chMDMs, CD4\(^+\) T cells and splenocytes

Chicken peripheral whole blood, obtained from spent Lohmann Lite laying hens, was purchased from the Harlan Laboratories U.K. 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 have been described previously (27). chMDMs enrichment was confirmed using chicken monocytes/macrophages marker antibody (clone KUL01, Santa Cruz Biotechnology, USA) by flow cytometry analysis. Approximately half of the monocytes initially separated from chicken whole blood were KUL01\(^+\)MHCII\(^+\). After 2 d of conversion into macrophages and removal of non-adherent cells this figure increased to more than 95% of adherent cells (Fig. S1). Mouse-anti-chicken CD4 mAb (clone CT-4, Southern
Biotech, USA) and anti-mouse IgG1 microbeads (MiltenyiBiotec, UK) were used to positively select chicken CD4⁺ T cells according to the manufacturer’s instructions. Cell viability was assessed by propidium iodide (20 µg/ml, Life Technologies, UK) uptake detected using flow cytometry analysis. CD4⁺ T cells and chMDMs were isolated from different individual birds. Spleens from newly-hatched Lohmann Lite layers chickens were removed aseptically and homogenised gently using a 70 µm strainer (BD Biosciences, UK) to prepare a suspension of single splenocytes.

2.3 In vitro infection of chMDMs and splenocytes with S. enterica

chMDMs and splenocytes were produced at a final concentration of 5×10⁵ cells/ml in RPMI 1640 (Gibco, Life Technologies, UK) supplemented with fetal bovine serum (FBS) (10% v/v) (Gibco, Life Technologies, UK), Hapes (20 Mm) (Sigma-Aldrich, UK), gentamicin sulphate (50 µg/ml) (Sigma-Aldrich, UK), streptomycin/penicillin (10 U/ml) (Gibco, Life Technologies, UK), fungizone (1.25 µg/ml) (Gibco, Life Technologies, UK) and L-glutamine (2 mM) (Gibco, Life Technologies, UK). In vitro invasion was performed 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 ions (NO₂⁻) and cytokine production and phosphate-buffered saline (PBS) only was used as a negative control. After 1 h of incubation, the medium was changed with fresh culture medium supplemented with 100 µg/ml of gentamicin sulphate and incubated for another hour to kill the extracellular S. enterica. Cell preparations were then washed three times with sterile PBS and kept in fresh culture-medium containing 20 µg/ml of gentamicin sulphate prior to use in subsequent studies. Salmonella-infected cells were lysed at 2, 6, 24 and 48 h post-infection (pi) using Triton X-100 (1% v/v) (Thermo Fisher Scientific, UK) to release and determine
intracellular survival of bacteria (Log_{10} CFU/ml). 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 pi, Salmonella-infected cells were collected for cytokine mRNA expression analysis by quantitative real-time PCR (qRT-PCR).

2.4 Avian chMDMs/CD4^+ T cells model in vitro

The chMDMs infected with SP or SE were co-cultured with CD4^+ T cells for 5 d in vitro. The ratio of co-cultured cells was maintained at 1:10 (chMDMs: CD4^+ T cells) throughout the study. In addition, three control groups were set up as follows: (i) co-culture of uninfected (PBS-treated) chMDMs with CD4^+ T cells was used to assess the allogeneic 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 (Con A) (10 μg/ml) (Sigma-Aldrich, UK) as a positive control for proliferation of CD4^+ T cells and (iii) CD4^+ T cells cultured alone were assessed for viability and non-specific proliferation over the 5 d culture period in vitro. All cultures were repeated in triplicate on three separate occasions. After 5 d of co-culture, CD4^+ T cells from each group were collected to measure the proliferation of CD4^+ T cells using the CellTiter96® AQueous 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 SP and SE induction of CD4^+ T cells for proliferation. CD4^+ T cells were also harvested after 5 d of co-culture from each group to measure cytokine mRNA expression analysis by qRT-PCR.
2.5 Phenotypic analysis of infected chMDMs and CD4+ T cells following co-culture with chMDMs

Cells to be analysed for MHCII, CD40, CD80, CD86 or CD28 expression were collected and fixed with PBS/4% formaldehyde (v/v). In each group 10^6 cells were incubated with the antibodies indicated and their isotype controls coupled to PE, FITC, or allophycocyanin. mAbs used were all listed in Table 1. Fluorescence analysis was performed using a FACSCanto II FACS analyser equipped with FACSDivaTM software (BD Biosciences, UK).

2.6 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 SP or SE in 0.1ml 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 d pi, 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 on 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.

2.7 mRNA expression analysis by qRT-PCR
RNA extraction was performed using the RNeasy Plus Mini Kit (Qiagen, UK). 1 µg of total cellular RNA was reverse transcribed to cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, UK) following the manufacturer’s guidelines. The Light Cycler 480 System (Roche, UK) was used to measure the gene expression of selected cytokines and chemokines by qRT-PCR. The sequences of primer and probe are shown in Table 2. Gene expression of CD28 and CTLA-4 was detected by SYBR green based qRT-PCR. Following the methods previously described (20, 29), relative gene expression was normalized against 28S mRNA expression and expressed as fold-difference from levels in uninfected controls.

2.8 Statistical analysis

Data were plotted and analysed using GraphPad Prism 6.0. Comparison between different groups and different groups at different time points p.i. was performed using a two-way ANOVA analysis followed by Tukey’s multiple comparison post-hoc test. Statistical significance was determined at the 5% and 1% confidence limits p<0.05 and p<0.01.

3. Results

3.1 Persistence in SP infection is not the result of increased survival in macrophages

The persistence of SP in comparison with SE is likely to be the result of increased microbial survival within the internal macrophage environment. We therefore assessed this using chMDMs (Fig. S1). We quantified invasiveness and survival of the SP and SE strains, macrophage viability and nitrite ion (NO₂⁻) activity.
SP invaded and/or was taken up by chMDMs in lower numbers than SE at 2 h p.i. \((p<0.01)\) (Fig. 1A). At the later times, there was a significant difference between the viable counts of the two serovars recovered \((p<0.01)\) with SE showing a significantly higher rate of decline \textit{in vitro} than that of SP over 48 h pi (Fig. 1B). Approximately 85% of infected chMDMs cells remained alive until 6 h pi, but this figure was significantly reduced by 24 h and 48 h pi \((p<0.01)\). However, the difference between SP and SE-infected cells was not significant \((p>0.99)\) (Fig. 1C).

NO is a major antibacterial effector during chronic infection (30) so, as expected, \(\text{NO}_2^-\) production was not clearly evident until 24h after infection. SE produced more \(\text{NO}_2^-\) than SP with this difference being significant \((p<0.05)\) at 48 h pi (Fig. 1D). This was mirrored by the difference in the mRNA level of \(iNOS\) measured at 6 h pi with the level induced by SE significantly greater \((p<0.5)\) than that induced by SP (Fig. 1E).

3.2 SP is less effective than SE 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 pro-inflammatory cytokines following infection of cultured epithelial cells (20), we compared the effect of SP and SE infection on induction of mRNA expression of \(IL-1\beta, IL-6, CXCL1\ (K60)\) and \(CXCL2\ (IL-8)\) by chMDMs. With the exception of \(IL-1\beta\) and \(CXCL1\), SP induced significantly lower levels of \(IL-6\) than SE \((p<0.05)\) with \(CXCL2\) levels induced by SP showing a marginally significant reduction compared with that induced by SE \((p=0.0515)\) (Fig. 2A). LPS stimulation enhanced \(IL-6\) expression in chMDMs, which was significantly higher \((p<0.05)\) than that in response to SP infection (Fig. 2A). SP did not completely suppress the
expression of pro-inflammatory cytokines as S. Gallinarum was observed to do in epithelial cells (20). However, they were at lower levels than SE, suggesting that SP may invade splenic macrophages without extensive infiltration of neutrophils during the early stage of infection.

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

3.3 A wider selection of SP and SE strains also displayed a similar pattern of cytokine/chemokine expression

Although the strains used have been shown experimentally to produce infection with characteristics typical of serovars Pullorum (5, 10) and Enteritidis (31), we could not be sure that other strains would behave similarly. We therefore repeated the experiments infecting chMDMs with an additional 5 strains of SP isolated from cases of Pullorum disease and 2 of different phage types (PT) of SE isolated from cases of human food poisoning that were attributed to poultry consumption. The gene expression profiles of IL-12α, IL-18, IL-4, IL-13, IL-10 and TGF-β4 are shown in Fig. 3. Here the patterns of expression for SP 449/87 and SE 125109 were very similar to those observed in the earlier experiment (Fig. 2) with the other strains, within each serovar, behaving in a similar manner with little variation. The patterns of production of the pro-
inflammatory chemokines IL-1β, IL-6, CXCL11, CXCL12 and also iNOS were also similar to those produced by the strains presented in Fig. 1 and 2 (data not shown).

3.4 SP suppressed 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 localised during SP infection (5, 10) as the spleen consists of a variety of cell types. These include dendritic cells (DCs) and lymphocytes, having different immune functions associated with combating infection and initiation of the immune response. We therefore isolated splenocytes, which were infected with the standard strains SP 449/87 and SE 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 SP suppressing Th1-related cytokines and increasing expression of Th2-related cytokines. However, both SP and SE induced lower expression of IL-12α in splenocytes compared with 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 SE when compared with that of SP (p<0.05) or uninfected controls (p<0.01).

3.5 SP suppressed the expression of Th1/Th17 cytokines by CD4+ T cells co-cultured with chMDMs

The pattern of cytokine production by SP compared to SE in chMDMs and splenocytes suggested a response that was anti-inflammatory and which 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 co-cultured these with infected chMDMs.
Initial experiments on the viability of macrophages and the T cells indicated that over 60% of cells were viable after 5 d of *in vitro* culture (Fig. S2).

After 5 d of co-culture, 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, SE-infected chMDMs induced proliferation of CD4$^+$ T cells which expressed high levels of *IFN-γ* (*p*<0.01) and *IL-17F* (*p*<0.05) whereas SP-infected chMDMs induced proliferation of CD4$^+$ T cells but which did not express *IFN-γ* (*p*>0.05) or suppressed the expression of *IL-17F* (*p*<0.05) (Fig. 5). The differences between SP and SE were statistically significant at *p*<0.01. Neither SP nor SE-infected chMDMs induced expression of *IL-17A* in co-cultured CD4$^+$ T cells when compared to the allogeneic control, although there was a significant difference between SP and SE (*p*<0.05). By contrast, SP induced higher levels of expression of *IL-4* than did SE although this difference was not statistically significant (Fig. 5). This suggested that SP was able to switch cytokine production of CD4$^+$ T cells from a dominant *IFN-γ* and *IL-17F*-expression towards *IL-4* expression *in vitro*.

Immune evasion strategies, other than a switch from a resolving Th17/CD4$^+$ profile to a non-resolving Th2/CD4$^+$ profile, may explain the mechanism of carriage of SP 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) failure of APCs to express co-stimulatory signals following engagement of cognate CD4$^+$ T cells, thus inducing clonal anergy.

The results obtained in this current study show that, after 5 days of co-culture, SE-infected chMDMs stimulated significantly increased CD4$^+$ T cell proliferation compared with SP-infected chMDMs (*p*<0.05) (Fig. 6A) indicating that SP did in fact exert a suppressive effect on proliferation.
SP, SE and LPS induced significantly increased \( (p<0.05) \) expression of IL-10 by chMDMs but TGF-β4 was not significantly expressed when compared to control expression by uninfected chMDMs (Fig. 6B). However, these IL-10 expressing chMDMs did not induce 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 allogenic controls (Fig. 6B). We measured levels of expression of MHCII and also co-stimulatory molecules CD40, CD80 and CD86 in infected chMDMs. A significant reduction in the number of MHCII positive chMDMs was measured in response to SP infection at 1 d pi \( (p<0.05) \) (Fig. 7A) compared to uninfected chMDMs. However, the percentage of CD40, CD80 or CD86 positive cells was not lower in SP-infected chMDMs compared with uninfected cells. Compared with SE infection, the number of CD40-expressing SP-infected chMDMs was lower only at 24 h pi (Fig. 7A).

If CTLA-4 is over-expressed on CD4+ T cells, CD80 and CD86 will preferentially bind to this receptor rather than CD28 (which is expressed by activated T lymphocytes). CD28 protein expression by CD4+ T cells over the 5 d pi period was comparable following co-culture with SP or SE-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 towards steady state conditions. We hypothesise that increased CTLA-4 protein (shifting the CD28/CTLA-4 ratio towards CTLA-4) probably also occurred over the 5 d pi period but, due to the lack of commercially available CTLA-4 antibody, we were unable to measure this. Thus, the lower CD4+ proliferation induced by SP in vitro was not a result of the absence of a co-stimulatory signal and therefore not clonal anergy.
3.6 SP showed greater capacity than SE for systemic infection in vivo

Although the evidence so far suggests that SP is able to modulate the immune response of the chicken away from an IFN-γ-producing Th17-type response towards 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 (32). 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 SP or SE and these were compared with uninfected birds. Infection with approximately 10^8 CFU of SP or SE did not induce any clinical signs of disease over the 5 d pi period. Viable SP and SE were detected in the cecal contents of infected chickens in each group after 1 d pi. SE had much higher bacterial counts at all time points examined when compared to SP (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 d pi. At 5 d pi, the mean Log_{10} CFU/g of SP recovered from the liver of infected chickens increased to 5.29, which were significantly higher than that of SE (p<0.01).

3.7 SP infection induced a weaker pro-inflammatory response in vivo

The pattern of induction of pro-inflammatory cytokines in the cecal tonsil was similar to that observed in chMDMs, with higher levels of all cytokines induced by SE compared with SP but with greatest statistically significant differences found in the cecal tonsils compared to chMDMs. The differences between SP and SE (p<0.05) were more apparent earlier (1 d pi) for CXCL1 and IL-1β but did not appear until 2 d pi for CXCL2, IL-6 and iNOS (Fig. 9). The differences were
Statistically significant differences between SP and SE infection in CXCL2 (p<0.05), IL-1β (p<0.01), IL-6 (p<0.05) and iNOS (p<0.01) did not appear until 4 d pi.

3.8  *In vivo* SP infection suppressed the expression of Th1/Th17-related cytokines but up-regulated 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 of Th1 cytokines and lower expression of Th2 cytokines induced by SE infection compared with those induced by SP, but with some key differences (Fig. 10). In the cecal tonsils, gene expression of IFN-γ (p<0.01 at 1 d pi and p<0.05 at 2, 4 and 5 d pi), IL-12α (p<0.05 at 1 d pi and p<0.01 at 2 d pi) and IL-18 (p<0.01 at 2 d pi) were significantly higher in response to SE infection when compared with SP. In the spleen, significant levels of IL-12α and IL-18 (p<0.05) were produced by SE at 1 d pi although no bacteria were isolated from the liver at this time (the lower limit of bacterial enumeration was Log_{10} CFU/tissue(g)=3). In the case of IL-4 (p<0.01 at 2 and 5 d pi and p<0.05 at 4 d pi in the cecal tonsils; p<0.01 at 4 and 5 d pi in the spleens) and IL-13 (p<0.05 at 1 d pi and p<0.01 at 5 d pi in the cecal tonsils; p<0.05 at 4 and 5 d pi in the spleens) this was reversed with higher levels produced by SP infection than those of SE. Expression of IL-17F was slightly different to that observed with CD4⁺ T cells *in vitro*. In the cecal tonsil, SP suppressed the production of IL-17F mRNA at 1 d pi with statistically significant differences to the uninfected controls (p<0.05) and SE infection (p<0.01). SE infection produced higher levels of IL-17F than SP with the difference remaining significant at 4 (p<0.01) and 5 (p<0.05) d pi respectively.

Infection with SE up-regulated the production of splenic IL-17F mRNA, which was significantly
higher than that of SP at 2 and 5 d pi ($p<0.05$). In both organs, as with chMDMs and co-cultured CD4$^+$ T cells the changes in TGF-β4 after infection were generally small. Increased IL-10 expression induced by SP over and above that produced by SE ($p<0.01$) and uninfected controls ($p<0.05$) was observed at 5 d pi.

### 4. Discussion

In contrast to SE, SP infection did not enhance pro-inflammatory cytokine expression in avian macrophages or Th1 and/or Th17 related cytokine expression in CD4$^+$ T cells co-cultured with infected chMDMs. This was also the case in the cecal tonsil and spleen of infected chickens. Although modulation of adaptive immunity by SP towards a non-protective Th2-like response was most evident in vivo, the results of suppressed Th1/Th17 responses derived from the in vitro co-culture experiments are largely consistent with the observations in infection of 2-day-old chickens. These results support our hypothesis that the mechanisms that underlie persistent infection with SP involve a manipulation of adaptive immune responses away from a protective IFN-γ-producing Th17-type response. This may enable SP to evade immune clearance resulting in persistent carriage.

SP may inhibit proliferation of Th1 lymphocytes by inhibiting IL-12 and IL-18. IL-12-stimulated Th1 differentiation is critical in 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 (33) and is also IFN-γ dependent. A previous study with HD11 cells showed an increase in
oxidative burst after *Salmonella* infection but with no significant difference between SE and SP
(28). In the current study, in comparison with SE, failure of SP to increase *IL-12α* expression in
the spleen at 1 d pi was followed by significantly lower levels of *IFN-γ* mRNA observed at 5 d
pi, which may possibly give rise to the persistent infection in the spleen of infected chickens.

Metabolism of arginine utilized by macrophages involves the enzymes iNOS (M1 macrophages)
or arginase (M2 macrophages) (34, 35). In a murine model of persistent infection, *S.
Typhimurium* infection preferentially associated with M2 macrophages activated by Th2
cytokines (36). It is not yet clear whether M1/M2 macrophage polarization occurs in avian
species. We showed SP is a less robust stimulus for *iNOS* mRNA expression in chMDMs in
comparison with SE which is what might be expected from a more chronic, persistent infection.
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 production of
NO (37). It is possible, therefore, that such differences in arginine metabolism occur in SP or SE-
infected chMDMs, although we have not specifically measured this. However, we also show that
SP-infected chMDMs produce low levels of *IL-12α/IL-18* but much higher levels of *IL-4/IL-13*,
which suggest that SP infection alone may induce an M2 phenotype (38, 39).

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 (40,41).
Recombinant chIFN-γ enhanced NO production in PBMC-derived macrophages and reduced
intracellular replication of serovar Typhimurium or Enteritidis (42). However, SP infection
neither induced *IL-12α* expression in chMDMs nor promoted *IFN-γ* expression in the CD4+ T
cells in co-culture, indicating that SP 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). Neutralisation of IFN-γ can reactivate acute infections, probably by interfering with macrophage activation (4), suggesting that functional IFN-γ is probably required to suppress bacterial growth during persistent infection of virulent strains in resistant hosts. It 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 Th1 or Th2. It would be interesting to study the kinetics of Th1 and Th2 cytokines during persistent infection because SP persists in the 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 SP appears to suppress the production of IFN-γ in chickens the role of IFN-γ in the *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, ablation of Treg early after infection increased the effectiveness of Th1 responses and controlled the tempo of persistent *S. Typhimurium* infection (43). It is unclear whether similar alterations in Treg activities can affect the Th1 responses in susceptible mouse or in chickens. CTLA-4/CD80/86 ligation inhibits T cell proliferation and induces T cell apoptosis (tolerance) (44). In comparison with SE, SP-infected chMDMs did not induce higher levels of *CTLA-4* mRNA expression in co-cultured CD4+ T cells. The suppressive properties of avian Treg cells (CD4+CD25+) were suggested to be IL-10-dependent (45). In our study, SP infection led to
invasion of liver and increased *IL-10* expression in the spleen. It 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* (46). IL-10 inhibits further development of the avian Th1 response and down-regulates the effects of IFN-γ to limit the inflammatory response (47). Increased TGF-β4 expression in *S. Typhimurium*-infected chickens was also shown to correspond to decreased production of pro-inflammatory mediators (15). The measurement of *IL-10* expression by gene expression rather than the presence of protein opens the possibility that IL-10 protein may conceivably not have been produced. If this is the case it may be the reason that 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 SE, an early expression of *IL-17* and prolonged high-level expression of *IFN-γ* were detected in the caeca (48-49), which suggested a function of Th17 cells as inflammatory mediators in avian immunity. However, the functional role of Th17 cell and *IL-17* in the mucosal inflammatory response to avian salmonellosis is not yet fully defined. In 17A\(^-\) mice infected with SE, recruitment of neutrophils was significantly compromised with a reduced clearance of SE from the spleen and liver (50), indicating the potential of Th17 cytokines being involved in intestinal defence against *S. enterica* infection. Our *CXCL11/CXCL2* data may suggest a difference between SP and SE 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 non-specific activation of intestinal Th17 cells in response to inflammatory cytokines or recognition of flagellin via the TLR-5 pathway to drive *Salmonella*-specific Th17 cell
SP was able to induce expression of various pro-inflammatory cytokines in chMDMs. The reduced expression of IL-17F in CD4+ T cells in vitro and spleen and cecal tonsils in vivo may thus have resulted from the absence of TLR-5 stimulation by non-flagellated SP. This may also be the case for another non-flagellated serovar S. Gallinarum which is able to show persistent systemic infection in a SALL1 resistant chicken phenotype (27). Murine Th17 cells were reported to produce IFN-γ in vitro (52) and in vivo (53). Although these have not been studied in chickens, SP-infected chMDMs were unable to induce gene expression of IFN-γ and IL-17F from co-cultured CD4+ T cells, indicating a host immunological bias away from IFN-γ-producing Th17 immunity in response to SP infection, which might be associated with the establishment of carriage.

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

S. Typhimurium was shown to reduce T cell proliferation and cytokine production in the absence of DCs (54). 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 (55), although the extent to which this occurs with other host species and serovars is unknown. In chickens at the onset of lay when SP bacteria multiply within splenic
macrophages and spread to the reproductive tract, SP may conceivably utilise a similar strategy to directly inhibit T cells from proliferation.

The bacterial determinants of persistent infection, as opposed to multiplication during acute disease, remain obscure. The Type Three Secretion System 2 (TTSS-2) enables replication and survival of *Salmonella* within macrophages and is essential for inducing systemic infection caused by serovar Pullorum or Typhimurium in chickens (56, 57). However, SPI-2 contributes to, but is not absolutely required for, persistent *S. Typhimurium* infection in mice (58). Further work to identify the bacterial determinants of persistent infection in SP will likely require investigation of all the genes associated with intracellular survival and growth including SPI-2 genes plus a number with metabolic functions. It may be significant that one feature of the serovars which typically produce typhoid-like disease, and which is associated with systemic and persistent infection, is auxotrophy (59). The fact that both serovars Pullorum and Gallinarum are non-flagellate is unlikely to be significant as serovars Dublin, Typhi and Abortusovis/equi are all flagellate. Moreover, the importance of the host genetic background in determining persistent infection has been observed in *S. Gallinarum* infection. In a *SAL1* resistant inbred chicken line, the organism persisted for more than 14 weeks with infection restricted to persistence 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 (60). 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 wild type serovars. How this related to persistence in man or cattle with *S. Typhi* or *S. Dublin* 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 colonisation, in a line of chickens showing long-term faecal shedding, also shows a Th2 bias (61).

It thus seems clear that the true picture in both SP persistence in chickens and *S. Typhimurium* in mice is more complex than first appearances suggest. It is unclear how this compares with the other serovars which typically produce typhoid-like disease and show persistence after convalescence. Chronic infection with *S. Typhi* is associated with shedding via the gall bladder although the spleen, and the liver are known to be infected (62-64). In *S. Dublin* infection in cattle, persistent shedding can occur from the gut and udder but the spleen is also affected and the gall bladder is also sometimes involved (6, 65). Persistent infection within the splenic macrophages may thus also be the key infection site of other serovars producing typhoid-like disease and chronic infections and SP infection in chickens thus may represent a good working model with which to study immune manipulation in greater detail and explore approaches to modifying the host response to adversely affect bacterial persistence.

5. **Acknowledgments**

This work was supported by the China Scholarship Council (CSC) and the University of Nottingham.

6. **References**


29. Hughes S, Poh TY, Bumstead N, Kaiser P. 2007. Re-evaluation of the chicken MIP family of chemokines and their receptors suggests that CCL5 is the prototypic MIP family chemokine, and that different species have developed different repertoires of both the CC chemokines and their receptors. Dev Comp Immunol 31:72-86.


system but not the \textit{Salmonella} pathogenicity island 1 type III secretion system for virulence in chickens. Infect Immun 69:5471-5476.


Figure Legends:

FIGURE 1. The differences between SP and SE in their (A and B) intracellular survival dynamics, (D) NO production and (E) iNOS expression from infected chMDMs does not correlate with their effect on the (C) viability of infected chMDMs. (A) Infected chMDM were lysed to quantify the intracellular viable bacterial counts and its (B) decline rate. (D) Supernatant was collected to determine the nitrite ion concentration using Griess assay. (E) Relative mRNA expression of iNOS shown as fold change in comparison to those from uninfected chMDMs (shown as 1) at 6 h pi. (C) The percentage of viable chMDM infected with SP and SE were determined using PI. Data in (A), (C) and (D) are presented as mean±SEM (n=3) and representative of at least two independent experiments. (B) Decline rate was determined using averaged intracellular viable bacterial counts at each time points. (E) iNOS levels was determined from chMDMs prepared from three birds respectively. (+) Indicates statistically significant difference from negative control (+p<0.05, ++p<0.01). (*) indicates statistical differences between different treatment (*p<0.05, **p<0.01).

FIGURE 2. SP infection did not induce as strong inflammatory responses as did SE in chMDMs. At 6 h pi, mRNA expression of (A) pro-inflammatory cytokines (IL-1β, IL-6) and chemokines (CXCL1, CXCL2), (B) IL-12α and IL-18 (driving Th1 response), (C) IL-4 and IL-13 (driving Th2 response) was detected in chMDMs from 3 chickens. The data shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1) and representative of three independent experiments. (+) indicates differences between levels of cytokines induced by each serovar compared to PBS-treated uninfected control, +p<0.05, ++p<0.01; (*) indicates differences between levels of cytokines induced by different serovars, *p<0.05, **p<0.01.
FIGURE 3. The gene expression profiles of immune mediators in chMDMs in response to infection with a wider selection of SP and SE strains maintain the patterns of the representative strains used. At 6 h pi, mRNA expression of \textit{IL-12a}, \textit{IL-18}, \textit{IL-4}, \textit{IL-13}, \textit{IL-10} and \textit{TGF-β4} was detected in chMDMs from 3 chickens. The data shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1) and representative of three independent experiments. (+) indicates differences between levels of cytokines induced by each serovar compared to PBS-treated uninfected control, \(+p<0.05, ++p<0.01\); (*) indicates differences between levels of cytokines induced by different serovars, \(*p<0.05, **p<0.01\). 

FIGURE 4. SP infection did not induce as strong inflammatory responses as did SE in chicken splenocytes \textit{in vitro} at 6 h pi. Expression of \textit{IFN-γ}, \textit{IL-12a}, \textit{IL-18}, \textit{IL-4} and \textit{IL-17F} mRNA was detected in chMDMs from 3 chickens. The data shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1) and representative of three independent experiments. (+) indicates differences between levels of cytokines induced by each serovar compared to PBS-treated uninfected control, \(+p<0.05, ++p<0.01\); (*) indicates differences between levels of cytokines induced by different serovars, \(*p<0.05, **p<0.01\). 

FIGURE 5. SP infection suppresses \textit{IFN-γ}-producing Th17 response in CD4\(^+\) T cells co-cultured with infected chMDMs after 5 d of co-culture. Expression of \textit{IFN-γ}, \textit{IL-4}, \textit{IL-17A} and \textit{IL-17F} mRNA was detected in chMDMs from 3 chickens. The data shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1) and representative of three independent experiments. (+) indicates differences between levels of cytokines induced by each serovar compared to PBS-treated uninfected control, \(+p<0.05, ++p<0.01\); (*) indicates differences between levels of cytokines induced by different serovars, \(*p<0.05, **p<0.01\).
FIGURE 6. SP infection neither suppress lymphocytes proliferation nor induces immunosuppression after 5 days of co-culture in vitro. (A) The number of viable proliferating CD4⁺ T cells are presented as mean±SEM (n=3, chMDMs and CD4⁺ T cells from 3 chickens respectively) and representative of two independent experiments. (B) The gene expression of IL-10 and TGF-β4 (IL-10 and TGF-β4 mRNA in chMDMs are detected at 6 h pi without co-culture) are shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1) and representative of three independent experiments. CD4⁺/UI (where UI is uninfected), CD4⁺ T cells co-cultured 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 cells proliferation); CD4⁺/SP, CD4⁺ T cells co-cultured with SP-infected chMDMs; CD4⁺/SE, CD4⁺ T cells co-cultured with SE-infected chMDMs. (*) indicates statistical difference from control of allogeneic response (CD4⁺/UI) or between different serovar groups, *p<0.05, **p<0.01; (+) indicates statistical difference from unstimulated control (CD4⁺), +p<0.05, ++p<0.01.

FIGURE 7. SP infection did not induce clonal anergy by reducing the number of chMDMs bearing co-stimulatory molecules. (A) chMDMs (P1) were gated based on side scatter/forward scatter (SSC/FSC) parameters. Representative histogram (upper panel) and average number (lower panel) of MHCII⁺, CD40⁺, CD80⁺ and CD80⁺ chMDMs in response to Salmonella infection. Black lines, secondary binding or isotype control mAbs; grey shadow, anti-chicken cell surface marker mAbs. (B) The number of CD28⁺ cells and (C) gene expression of CD28 and CTLA4 in CD4⁺ T cells from the co-culture. (A, B) The percentage of MHCII⁺, CD40⁺, CD80⁺ and CD80⁺ cells from infected chMDMs and CD4⁺CD28⁺ cells out of co-cultured CD4⁺ T cells are shown as mean ± SEM (n=3, chMDMs or CD4⁺ T cells from 3 chickens). (C) The mRNA
level of CD28 and CTLA-4 of CD4+ T cells from 3 chickens is shown as fold change in comparison to those from uninfected controls (shown as 1) and representative of three independent experiment. (+) indicates statistically significant differences from the uninfected control. +p<0.05, ++p<0.01. (*) indicates statistical differences between different serovars (*p<0.05, **p<0.01).

FIGURE 8. SP is a poor coloniser in the caeca but an effective invader into the liver. The numbers of viable SP and SE in the caecal content and the liver of 2-day-old chickens was determined at various times (d) after oral infection. Each symbol represents an individual chicken (3 chicken/group) in one independent experiment. When no viable colonies were found at 10^-1 dilution after selective enrichment, a viable count of <3 of Log CFU/g and Log CFU/g=3 was used to represent the bacterial loads in negative animal for statistical analysis. (+) Indicates statistically significant difference from uninfected control (+p<0.05, ++p<0.01). (*) indicates statistical differences between different serovars (*p<0.05, **p<0.01).

FIGURE 9. SP infection suppresses inflammatory response in the caecal tonsils and spleens of infected chickens at various times (d) after oral infection (pi). The mRNA levels of pro-inflammatory chemokines (CXCL1 and CXCL2) and cytokines (IL-1β, IL-6 and iNOS) was detected in 3 chickens of one independent experiment and the data was shown as fold change in comparison to those from uninfected control (shown as 1). (+) indicates differences between levels of cytokines induced by each serovar compared to uninfected control, +p<0.05, ++p<0.01; (*) indicates differences between levels of cytokines induced by different serovars, *p<0.05, **p<0.01.

FIGURE 10. SP infection modulates an IFN-γ-producing Th17 response towards an anti-inflammatory response in the caecal tonsils and spleens of infected chickens at various times (d)
after oral infection (pi). The mRNA levels of Th1 (*IFN-γ, IL-12α and IL-18*), Th2 (*IL-4 and IL-13*), Th17 (*IL-17F*) and regulatory (*IL-10 and TGF-β4*) cytokines was detected in 3 chickens of one independent experiment and the data was shown as fold change in comparison to those from uninfected control (shown as 1). (+) indicates differences between levels of cytokines induced by each serovar compared to uninfected control, +p<0.05, ++p<0.01; (*) indicates differences between levels of cytokines induced by different serovars, *p<0.05, **p<0.01.
### TABLE 1. mAbs used in this study

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FIGURE 1

(A) Intraacellular bacterial counts (Log_{10} CFU/ml) over time (h pi).

(B) Intraacellular bacterial counts (Log_{10} CFU/ml) plotted against time (h pi).

(C) Viability % for SP and SE over time (h pi).

(D) Nitrite concentration (μM) for SP and SE over time (h pi).

(E) iNOS expression fold change for SP, SE, and LPS-stimulated positive control compared to PBS-treated negative control.
FIGURE 2

(A) IL-1β

(B) IL-12α

(C) IL-18

IL-6

CXCL1

CXCL12

P = 0.0515

FIGURE 2

(A)

(B)

(C)
FIGURE 3
FIGURE 4

**IFN-γ**

**IL-12α**

**IL-18**

**IL-4**

**IL-17F**
FIGURE 6

(A) [Bar graph showing cell number × 10^5 for CD4/Unl, CD4, CD4/Con A, CD4/SP, CD4/SE.]

(B) [Bar graph showing fold change for TGF-β4, IL-10, TGF-β4, and IL-10 for chMDM and CD4+ T cells.]
FIGURE 7
FIGURE 8
FIGURE 9

CXCLi-1   CXCLi-2   IL-1β

C a e c a l t o n s i l s

( d )  p i

Fold change

IL-6   iNOS

S p l e e n s

( d )  p i

Fold change

CXCLi-1   CXCLi-2   IL-1β

Fold change

IL-6   iNOS

SP

SE

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**Caecal tonsils**

**FIGURE 10**

**Spleens**

**FIGURE 10**