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 $IL\text{-}17^+CD4^+$ 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 5×10^5 cells/ml in RPMI 1640 (Gibco, Life Technologies, UK) supplemented with fetal bovine serum (FBS) (10% v/v) (Gibco, Life Technologies, UK),Hepes (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 (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 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$^®$AQ$_{ueous}$ 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 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.

147 **2.5 Phenotypic analysis of infected chMDMs and CD4$^+$ T cells following co-culture**
148 **with chMDMs**

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

154

155 **2.6 *In vivo Salmonella* chicken infections**

156 A total of 36 2-day-old Lohmann Lite chickens obtained from the Millennium Hatchery
157 (Birmingham, UK) were divided into three groups with 12 birds each in separate pens and given
158 access to antibiotic-free feed and water *ad libitum* throughout the experiment. Two groups were
159 inoculated orally with 10^8 CFU of SP or SE in 0.1ml of nutrient broth. All animal care and
160 experimentation were carried out under Home Office project license PPL 40/3412 and had local
161 ethical approval. At 1, 2, 4, and 5 d pi, three birds from each group were euthanized. Cecal
162 content and liver were collected aseptically, weighed and homogenized in PBS using Griffiths
163 tubes. Decimal dilutions of the homogenates were then plated on Brilliant Green Agar plates
164 containing sodium nalidixate (20 μg/ml, Sigma-Aldrich, UK) and novobiocin (1 μg/ml, Sigma-
165 Aldrich, UK) to determine bacterial counts. Spleen and cecal tonsil were collected for cytokine
166 mRNA expression analysis.

167

168 **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 (NO_2^-) 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 *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, NO_2^- production was not clearly evident until 24h after infection. SE produced more 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-1β*, *IL-6*, *CXCLi1 (K60)* and *CXCLi2 (IL-8)* by chMDMs. With the exception of *IL-1β* and *CXCLi1*, SP induced significantly lower levels of *IL-6* than SE (p<0.05) with *CXCLi2* 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

213 expression of pro-inflammatory cytokines as *S.* Gallinarum was observed to do in epithelial cells
214 (20). However, they were at lower levels than SE, suggesting that SP may invade splenic
215 macrophages without extensive infiltration of neutrophils during the early stage of infection.

216 Macrophages function as antigen presenting cells and can also shift the direction of
217 differentiation of naïve T cells. Therefore, we investigated the expression levels of cytokines
218 which drive the differentiation of Th1 (*IL-12α* and *IL-18*) and Th2 (*IL-4* and *IL-13*) subsets. SE
219 infection and LPS stimulation (as a positive control) induced strong expression of IL-12α and *IL-*
220 *18*. SE stimulated higher levels of *IL-12α* compared with SP ($p<0.05$) although for *IL-18* this
221 difference was of marginal statistical significance ($p=0.0509$) (Fig. 2B). In contrast, SE induced
222 lower levels of *IL-13* compared with SP ($p<0.05$) (Fig. 2C). This experiment was also repeated
223 using cultured macrophage-like HD11 cells with similar results (data not shown).

224

225 **3.3 A wider selection of SP and SE strains also displayed a similar pattern of**
226 **cytokine/chemokine expression**

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

inflammatory chemokines *IL-1β*, *IL-6*, *CXCLi1*, *CXCLi2* 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 *CXCLi1* and *IL-1β* but did not appear until 2 d pi for *CXCLi2*, *IL-6* and *iNOS* (Fig. 9). The differences were

not as marked in the spleen. Statistically significant differences between SP and SE infection in *CXCLi2* ($p<0.05$), *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 underline 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

393 cells in co-culture, indicating that SP does not initiate an effective IFN-γ-dependent
394 inflammatory response to clear infection.

395 Virulent *S.* Typhimurium can show persistent infection in resistant mice, despite the presence of
396 high levels of circulating anti-*S.* Typhimurium antibody (4). Neutralisation of IFN-γ can
397 reactivate acute infections, probably by interfering with macrophage activation (4), suggesting
398 that functional IFN-γ is probably required to suppress bacterial growth during persistent infection
399 of virulent strains in resistant hosts. It implies an increase in both Th1 and Th2 cytokines in
400 response to *Salmonella* infection. It is rational to consider that the ratio of these cytokine levels
401 will govern the overall direction of the immune response to be mainly Th1 or Th2. It would be
402 interesting to study the kinetics of Th1 and Th2 cytokines during persistent infection because SP
403 persists in the female chickens with gradually reducing bacterial numbers in the spleens,
404 interrupted by the onset of sexual maturity with spread to the reproductive tract, whereas in
405 males elimination eventually occurs at between 10 and 18 weeks of age (9). Thus, although SP
406 appears to suppress the production of IFN-γ in chickens the role of IFN-γ in the *Nramp1*$^{+/+}$mice
407 may be very different since IFN-γ is required to continue to suppress *S.* Typhimurium in an
408 innately resistant mouse line (4).

409 In mice, ablation of Treg early after infection increased the effectiveness of Th1 responses and
410 controlled the tempo of persistent *S.* Typhimurium infection (43). It is unclear whether similar
411 alterations in Treg activities can affect the Th1 responses in susceptible mouse or in chickens.
412 CTLA-4/CD80/86 ligation inhibits T cell proliferation and induces T cell apoptosis (tolerance)
413 (44). In comparison with SE, SP-infected chMDMs did not induce higher levels of *CTLA-4*
414 mRNA expression in co-cultured $CD4^+$ T cells. The suppressive properties of avian Treg cells
415 ($CD4^+CD25^+$) were suggested to be IL-10-dependent (45). In our study, SP infection led to

416 invasion of liver and increased *IL-10* expression in the spleen. It suggests a possible regulatory
417 effect of IL-10 on inhibiting cytokine production during systemic dissemination and possibly
418 persistent infection. Avian $CD4^+CD25^+$ suppressor T cells have been shown to produce high
419 concentrations of IL-10, TGF-β4 and CTLA-4 and suppress T cell proliferation *in vitro* (46). IL-
420 10 inhibits further development of the avian Th1 response and down-regulates the effects of IFN-
421 γ to limit the inflammatory response (47). Increased *TGF-β4* expression in *S.* Typhimurium-
422 infected chickens was also shown to correspond to decreased production of pro-inflammatory
423 mediators (15). The measurement of *IL-10* expression by gene expression rather than the
424 presence of protein opens the possibility that IL-10 protein may conceivably not have been
425 produced. If this is the case it may be the reason that IL-10/TGF-β producing $CD4^+$ T cells did
426 not proliferate. This may be measured in supernatants when a reliable reagent becomes available.

427 In chickens infected with SE, an early expression of *IL-17* and prolonged high-level expression
428 of *IFN-γ* were detected in the caeca (48-49), which suggested a function of Th17 cells as
429 inflammatory mediators in avian immunity. However, the functional role of Th17 cell and IL-17
430 in the mucosal inflammatory response to avian salmonellosis is not yet fully defined. In $17A^{-/-}$
431 mice infected with SE, recruitment of neutrophils was significantly compromised with a reduced
432 clearance of SE from the spleen and liver (50), indicating the potential of Th17 cytokines being
433 involved in intestinal defence against *S. enterica* infection. Our *CXCLi1*/*CXCLi2* data may
434 suggest a difference between SP and SE in heterophil recruitment and avian IL-17 may
435 potentially also function to recruit heterophils to promote inflammatory responses. *IL-17* was
436 elicited rapidly in response to *S.* Typhimurium infection of bovine ligated ileal loops, probably
437 through a non-specific activation of intestinal Th17 cells in response to inflammatory cytokines
438 or recognition of flagellin via the TLR-5 pathway to drive *Salmonella*-specific Th17 cell

development (51). 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 *SAL1* 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

1. Coburn B, Grassl GA, Finlay BB. 2007. *Salmonella*, the host and disease: a brief review. Immunol Cell Biol 85:112-118.

2. Hornick RB, Greisman SE, Woodward TE, DuPont HL, Dawkins AT, Snyder MJ. 1970. Typhoid fever: pathogenesis and immunologic control. N Engl J Med 283:686-691.

3. Rabsch W, Tschape H, Baumler AJ. 2001. Non-typhoidal salmonellosis: emerging problems. Microbes Infect 3:237-247.

4. Monack DM, Bouley DM, Falkow S. 2004. *Salmonella typhimurium* persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1$^{+/+}$ mice and can be reactivated by IFNγ neutralization. J Exp Med 199:231-241.

5. Wigley P, Berchieri A, Jr., Page KL, Smith AL, Barrow PA. 2001. *Salmonella enterica* serovar Pullorum persists in splenic macrophages and in the reproductive tract during persistent, disease-free carriage in chickens. Infect Immun 69:7873-7879.

6. Sojka WJ, Thomson PD, Hudson EB. 1974. Excretion of *Salmonella* dublin by adult bovine carriers. Br Vet J 130:482-488.

7. Wray C, Sojka WJ. 1977. Reviews of the progress of dairy science: bovine salmonellosis. J Dairy Res 44:383-425.

8. House D, Bishop A, Parry C, Dougan G, Wain J. 2001. Typhoid fever: pathogenesis and disease. CurrOpin Infect Dis 14:573-578.

9. Wigley P, Hulme SD, Powers C, Beal RK, Berchieri A, Jr., Smith A, Barrow P. 2005. Infection of the reproductive tract and eggs with *Salmonella enterica* serovar pullorum in the chicken is associated with suppression of cellular immunity at sexual maturity. Infect Immun 73:2986-2990.

10. Berchieri A, Jr., Murphy CK, Marston K, Barrow PA. 2001. Observations on the persistence and vertical transmission of *Salmonella enterica* serovars Pullorum and Gallinarum in chickens: effect of bacterial and host genetic background. Avian Pathol 30:221-231.

11. Mastroeni P, Harrison JA, Robinson JH, Clare S, Khan S, Maskell DJ, Dougan G, Hormaeche CE. 1998. Interleukin-12 is required for control of the growth of attenuated aromatic-compound-dependent salmonellae in BALB/c mice: role of gamma interferon and macrophage activation. Infect Immun 66:4767-4776.

12. Beal RK, Wigley P, Powers C, Hulme SD, Barrow PA, Smith AL. 2004. Age at primary infection with *Salmonella enterica* serovar Typhimurium in the chicken influences persistence of infection and subsequent immunity to re-challenge. Vet Immunol Immunopathol 100:151-164.

13. Beal RK, Powers C, Wigley P, Barrow PA, Smith AL. 2004. Temporal dynamics of the cellular, humoral and cytokine responses in chickens during primary and secondary infection with *Salmonella enterica* serovar Typhimurium. Avian Pathol 33:25-33.

14. Wigley P, Hulme S, Powers C, Beal R, Smith A, Barrow P. 2005. Oral infection with the *Salmonella enterica* serovar Gallinarum 9R attenuated live vaccine as a model to characterise immunity to fowl typhoid in the chicken. BMC Vet Res 1:2.

15. Withanage GS, Wigley P, Kaiser P, Mastroeni P, Brooks H, Powers C, Beal R, Barrow P, Maskell D, McConnell I. 2005. Cytokine and chemokine responses associated with clearance of a primary *Salmonella enterica* serovar Typhimurium infection in the chicken and in protective immunity to rechallenge. Infect Immun 73:5173-5182.

16. Berndt A, Wilhelm A, Jugert C, Pieper J, Sachse K, Methner U. 2007. Chicken cecum immune response to *Salmonella enterica* serovars of different levels of invasiveness. Infect Immun 75:5993-6007.

17. Henderson SC, Bounous DI, Lee MD. 1999. Early events in the pathogenesis of avian salmonellosis. Infect Immun 67:3580-3586.

18. Chappell L, Kaiser P, Barrow P, Jones MA, Johnston C, Wigley P. 2009. The immunobiology of avian systemic salmonellosis. Vet Immunol Immunopathol 128:53-59.

19. Langridge GC, Fookes M, Connor TR, Feltwell T, Feasey N, Parsons BN, Seth-Smith HM, Barquist L, Stedman A, Humphrey T, Wigley P, Peters SE, Maskell DJ, Corander J, Chabalgoity JA, Barrow P, Parkhill J, Dougan G, Thomson NR. 2015. Patterns of genome evolution that have accompanied host adaptation in *Salmonella*. Proc Natl Acad Sci U S A 112:863-868.

20. Kaiser P, Rothwell L, Galyov EE, Barrow PA, Burnside J, Wigley P. 2000. Differential cytokine expression in avian cells in response to invasion by *Salmonella typhimurium*, *Salmonella enteritidis* and *Salmonella gallinarum*. Microbiology 146 Pt 12:3217-3226.

21. McArthur MA, Sztein MB. 2012. Heterogeneity of multifunctional IL-17A producing *S.* Typhi-specific $CD8^+$ T cells in volunteers following Ty21a typhoid immunization. PLoS One 7:e38408.

22. Bhuiyan S, Sayeed A, Khanam F, Leung DT, Rahman Bhuiyan T, Sheikh A, Salma U, LaRocque RC, Harris JB, Pacek M, Calderwood SB, LaBaer J, Ryan ET, Qadri F, Charles RC. 2014. Cellular and cytokine responses to *Salmonella enterica* serotype Typhi proteins in patients with typhoid fever in Bangladesh. Am J Trop Med Hyg 90:1024-1030.

23. Wilson GS, Miles AA, Topley WCC. 1964. Topley and Wilson's Principles of bacteriology and immunity, 5th ed. Edward Arnold, London.

24. Wigley P, Jones MA, Barrow PA. 2002. *Salmonella enterica* serovar Pullorum requires the *Salmonella* pathogenicity island 2 type III secretion system for virulence and carriage in the chicken. Avian Pathol 31:501-506.

576 25. Barrow PA. 1991. Experimental infection of chickens with *Salmonella enteritidis*. Avian
577 Pathol 20:145-153.
578 26. Barrow PA, Lovell MA, Berchieri A. 1991. The use of two live attenuated vaccines to
579 immunize egg-laying hens against *Salmonella enteritidis* phage type 4. Avian Pathol
580 20:681-692.
581 27. Wigley P, Hulme SD, Bumstead N, Barrow PA. 2002. *In vivo* and *in vitro* studies of
582 genetic resistance to systemic salmonellosis in the chicken encoded by the *SAL1* locus.
583 Microbes Infect 4:1111-1120.
584 28. Setta A, Barrow PA, Kaiser P, Jones MA. 2012. Immune dynamics following infection of
585 avian macrophages and epithelial cells with typhoidal and non-typhoidal *Salmonella*
586 *enterica* serovars; bacterial invasion and persistence, nitric oxide and oxygen production,
587 differential host gene expression, NF-kappaB signalling and cell cytotoxicity. Vet
588 Immunol Immunopathol 146:212-224.
589 29. Hughes S, Poh TY, Bumstead N, Kaiser P. 2007. Re-evaluation of the chicken MIP
590 family of chemokines and their receptors suggests that CCL5 is the prototypic MIP
591 family chemokine, and that different species have developed different repertoires of both
592 the CC chemokines and their receptors. Dev Comp Immunol 31:72-86.
593 30. Mastroeni P, Vazquez-Torres A, Fang FC, Xu Y, Khan S, Hormaeche CE, Dougan G.
594 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide
595 synthase in experimental salmonellosis. II. Effects on microbial proliferation and host
596 survival in vivo. J Exp Med 192:237-248.
597 31. Barrow PA, Lovell MA. 1991. Experimental infection of egg-laying hens with
598 *Salmonella enteritidis* phage type 4. Avian Pathol 20:335-348.

599 32. Berndt A, Pieper J, Methner U. 2006. Circulating γδ T cells in response to *Salmonella*
600 *enterica* serovar enteritidis exposure in chickens. Infect Immun 74:3967-3978.
601 33. Vazquez-Torres A, Jones-Carson J, Mastroeni P, Ischiropoulos H, Fang FC. 2000.
602 Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide
603 synthase in experimental salmonellosis. I. Effects on microbial killing by activated
604 peritoneal macrophages in vitro. J Exp Med 192:227-236.
605 34. Munder M, Eichmann K, Moran JM, Centeno F, Soler G, Modolell M. 1999. Th1/Th2-
606 regulated expression of arginase isoforms in murine macrophages and dendritic cells. J
607 Immunol 163:3771-3777.
608 35. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. 2000. M-1/M-2 macrophages and
609 the Th1/Th2 paradigm. J Immunol 164:6166-6173.
610 36. Eisele NA, Ruby T, Jacobson A, Manzanillo PS, Cox JS, Lam L, Mukundan L, Chawla
611 A, Monack DM. 2013. *Salmonella* require the fatty acid regulator PPARdelta for the
612 establishment of a metabolic environment essential for long-term persistence. Cell Host
613 Microbe 14:171-182.
614 37. Rath M, Muller I, Kropf P, Closs EI, Munder M. 2014. Metabolism via Arginase or Nitric
615 Oxide Synthase: Two Competing Arginine Pathways in Macrophages. Front Immunol
616 5:532.
617 38. Gordon S. 2003. Alternative activation of macrophages. Nat Rev Immunol 3:23-35.
618 39. Martinez FO, Sica A, Mantovani A, Locati M. 2008. Macrophage activation and
619 polarization. Front Biosci 13:453-461.
620 40. Mastroeni P. 2002. Immunity to systemic *Salmonella* infections. CurrMol Med 2:393-
621 406.

622 41. Mastroeni P, Menager N. 2003. Development of acquired immunity to *Salmonella*. J Med
623 Microbiol 52:453-459.
624 42. Okamura M, Lillehoj HS, Raybourne RB, Babu US, Heckert RA, Tani H, Sasai K, Baba
625 E, Lillehoj EP. 2005. Differential responses of macrophages to *Salmonella enterica*
626 serovars Enteritidis and Typhimurium. Vet Immunol Immunopathol 107:327-335.
627 43. Johanns TM, Ertelt JM, Rowe JH, Way SS. 2010. Regulatory T cell suppressive potency
628 dictates the balance between bacterial proliferation and clearance during persistent
629 *Salmonella* infection. PLoSPathog6:e1001043.
630 44. Perez VL, Van Parijs L, Biuckians A, Zheng XX, Strom TB, Abbas AK. 1997. Induction
631 of peripheral T cell tolerance in vivo requires CTLA-4 engagement. Immunity 6:411-417.
632 45. Selvaraj RK. 2013. Avian $CD4^{+}CD25^{+}$ regulatory T cells: properties and therapeutic
633 applications. Dev Comp Immunol 41:397-402.
634 46. Shanmugasundaram R, Selvaraj RK. 2011. Regulatory T cell properties of chicken
635 $CD4^{+}CD25^{+}$ cells. J Immunol 186:1997-2002.
636 47. Rothwell L, Young JR, Zoorob R, Whittaker CA, Hesketh P, Archer A, Smith AL, Kaiser
637 P. 2004. Cloning and characterization of chicken IL-10 and its role in the immune
638 response to *Eimeria maxima*. J Immunol 173:2675-2682.
639 48. Crhanova M, Hradecka H, Faldynova M, Matulova M, Havlickova H, Sisak F, Rychlik I.
640 2011. Immune response of chicken gut to natural colonization by gut microflora and to
641 *Salmonella enterica* serovar enteritidis infection. Infect Immun 79:2755-2763.
642 49. Matulova M, Varmuzova K, Sisak F, Havlickova H, Babak V, Stejskal K, Zdrahal Z,
643 Rychlik I. 2013. Chicken innate immune response to oral infection with *Salmonella*
644 *enterica* serovar Enteritidis. Vet Res 44:37.

50. Schulz SM, Köhler G, Holscher C, Iwakura Y, Alber G. 2008. IL-17A is produced by Th17, γδ T cells and other CD4− lymphocytes during infection with *Salmonella enterica* serovar Enteritidis and has a mild effect in bacterial clearance. International immunology 20:1129-1138.

51. Raffatellu M, Santos RL, Chessa D, Wilson RP, Winter SE, Rossetti CA, Lawhon SD, Chu H, Lau T, Bevins CL, Adams LG, Baumler AJ. 2007. The capsule encoding the viaB locus reduces interleukin-17 expression and mucosal innate responses in the bovine intestinal mucosa during infection with *Salmonella enterica* serotype Typhi. Infect Immun 75:4342-4350.

52. Lee YK, Turner H, Maynard CL, Oliver JR, Chen D, Elson CO, Weaver CT. 2009. Late developmental plasticity in the T helper 17 lineage. Immunity 30:92-107.

53. Hirota K, Duarte JH, Veldhoen M, Hornsby E, Li Y, Cua DJ, Ahlfors H, Wilhelm C, Tolaini M, Menzel U, Garefalaki A, Potocnik AJ, Stockinger B. 2011. Fate mapping of IL-17-producing T cells in inflammatory responses. Nat Immunol 12:255-263.

54. van der Velden AW, Copass MK, Starnbach MN. 2005. *Salmonella* inhibit T cell proliferation by a direct, contact-dependent immunosuppressive effect. Proc Natl Acad Sci U S A 102:17769-17774.

55. Pullinger GD, Paulin SM, Charleston B, Watson PR, Bowen AJ, Dziva F, Morgan E, Villarreal-Ramos B, Wallis TS, Stevens MP. 2007. Systemic translocation of *Salmonella enterica* serovar Dublin in cattle occurs predominantly via efferent lymphatics in a cell-free niche and requires type III secretion system 1 (T3SS-1) but not T3SS-2. Infect Immun 75:5191-5199.

56. Jones MA, Wigley P, Page KL, Hulme SD, Barrow PA. 2001. *Salmonella enterica* serovar Gallinarum requires the *Salmonella* pathogenicity island 2 type III secretion

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

57. Jones MA, Hulme SD, Barrow PA, Wigley P. 2007. The *Salmonella* pathogenicity island 1 and *Salmonella* pathogenicity island 2 type III secretion systems play a major role in pathogenesis of systemic disease and gastrointestinal tract colonization of *Salmonella* enterica serovar Typhimurium in the chicken. Avian Pathol 36:199-203.

58. Lawley TD, Bouley DM, Hoy YE, Gerke C, Relman DA, Monack DM. 2008. Host transmission of *Salmonella enterica* serovar Typhimurium is controlled by virulence factors and indigenous intestinal microbiota. Infect Immun 76:403-416.

59. Uzzau S, Brown DJ, Wallis T, Rubino S, Leori G, Bernard S, Casadesus J, Platt DJ, Olsen JE. 2000. Host adapted serotypes of *Salmonella enterica*. Epidemiol Infect 125:229-255.

60. O'Callaghan D, Maskell D, Liew FY, Easmon CS, Dougan G. 1988. Characterization of aromatic- and purine-dependent *Salmonella* typhimurium: attention, persistence, and ability to induce protective immunity in BALB/c mice. Infect Immun 56:419-423.

61. Chausse AM, Grepinet O, Bottreau E, Robert V, Hennequet-Antier C, Lalmanach AC, Lecardonnel J, Beaumont C, Velge P. 2014. Susceptibility to *Salmonella* carrier-state: a possible Th2 response in susceptible chicks. Vet Immunol Immunopathol 159:16-28.

62. Vogelsang TM, Boe J. 1948. Temporary and chronic carriers of *Salmonella* typhi and *Salmonella* paratyphi B. J Hyg (Lond) 46:252-261.

63. Young D, Hussell T, Dougan G. 2002. Chronic bacterial infections: living with unwanted guests. Nat Immunol 3:1026-1032.

691 64. Nath G, Singh YK, Maurya P, Gulati AK, Srivastava RC, Tripathi SK. 2010. Does
692 *Salmonella* Typhi primarily reside in the liver of chronic typhoid carriers? J Infect Dev
693 Ctries 4:259-261.
694 65. Hinton M, Williams BM. 1977. *Salmonella* dublin infection in adult cattle: a slaughter
695 house and knackery survey in South West Wales. J Hyg (Lond) 78:121-127.

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 (*CXCLi1, CXCLi2*), (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.

719 FIGURE 3. The gene expression profiles of immune mediators in chMDMs in response to
720 infection with a wider selection of SP and SE strains maintain the patterns of the representative
721 strains used. At 6 h pi, mRNA expression of *IL-12α, IL-18, IL-4, IL-13, IL-10* and *TGF-β4* was
722 detected in chMDMs from 3 chickens. The data shown as fold change in the mRNA level of
723 cytokines in comparison to those from uninfected controls (shown as 1) and representative of
724 three independent experiments. (+) indicates differences between levels of cytokines induced by
725 each serovar compared to PBS-treated uninfected control, +p<0.05, ++p<0.01; (*) indicates
726 differences between levels of cytokines induced by different serovars, *p<0.05, ** p<0.01.

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

734 FIGURE 5. SP infection suppresses IFN-γ-producing Th17 response in $CD4^+$ T cells co-cultured
735 with infected chMDMs after 5 d of co-culture. Expression of *IFN-γ, IL-4, IL-17A* and *IL-17F*
736 mRNA was detected in chMDMs from 3 chickens. The data shown as fold change in the mRNA
737 level of cytokines in comparison to those from uninfected controls (shown as 1) and
738 representative of three independent experiments. (+) indicates differences between levels of
739 cytokines induced by each serovar compared to PBS-treated uninfected control, +p<0.05,
740 ++p<0.01; (*) indicates differences between levels of cytokines induced by different serovars,
741 *p<0.05, **p<0.01.

742 FIGURE 6. SP infection neither suppress lymphocytes proliferation nor induces
743 immunosuppression after 5 days of co-culture *in vitro*. (A) The number of viable proliferating
744 $CD4^+$ T cells are presented as mean±SEM (n=3, chMDMs and $CD4^+$ T cells from 3 chickens
745 respectively) and representative of two independent experiments. (B) The gene expression of *IL-*
746 *10* and *TGF-β4* (*IL-10* and *TGF-β4* mRNA in chMDMs are detected at 6 h pi without co-culture)
747 are shown as fold change in the mRNA level of cytokines in comparison to those from
748 uninfected controls (shown as 1) and representative of three independent experiments. $CD4^+$/UI
749 (where UI is uninfected), $CD4^+$ T cells co-cultured with uninfected chMDMs (control for
750 allogeneic response); $CD4^+$, $CD4^+$ T cells cultured alone; $CD4^+$/ConA, $CD4^+$ T cells stimulated
751 with ConA (positive control for $CD4^+$ T cells proliferation); $CD4^+$ /SP, $CD4^+$ T cells co-cultured
752 with SP-infected chMDMs; $CD4^+$ /SE, $CD4^+$ T cells co-cultured with SE-infected chMDMs. (*)
753 indicates statistical difference from control of allogeneic response ($CD4^+$/UI) or between
754 different serovar groups, *p<0.05, **p<0.01; (+) indicates statistical difference from
755 unstimulated control ($CD4^+$), +p<0.05, ++p<0.01.

756 FIGURE 7. SP infection did not induce clonal anergy by reducing the number of chMDMs
757 bearing co-stimulatory molecules. (A) chMDMs (P1) were gated based onside scatter/forward
758 scatter (SSC/FSC) parameters. Representative histogram (upper panel) and average number
759 (lower panel) of $MHCII^+$, $CD40^+$, $CD80^+$ and $CD80^+$ chMDMs in response to *Salmonella*
760 infection. Black lines, secondary binding or isotype control mAbs; grey shadow, anti-chicken
761 cell surface marker mAbs. (B) The number of $CD28^+$ cells and (C) gene expression of CD28 and
762 CTLA4 in $CD4^+$ T cells from the co-culture. (A, B) The percentage of $MHCII^+$, $CD40^+$, $CD80^+$
763 and $CD80^+$ cells from infected chMDMs and $CD4^+CD28^+$ cells out of co-cultured $CD4^+$ T cells
764 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 (*CXCLi1* and *CXCLi2*) 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, $*p<0.05$, $**p<0.01$.

794 TABLE 1. mAbs used in this study

Antibody†	Clone	Isotype	concentration (µg/ml)
Monocytes/macrophages marker (KUL01): PE[3]	KUL01	IgG1κ	1
Mouse anti-chicken CD4[4]	CT-4	IgG1κ	1
Mouse anti-chicken CD4: FITC[1]	2-35	IgG2b	5
Mouse anti-chicken CD3[1]	CT-3	IgG1	2.5
Mouse-anti-chicken MHC II: FITC[4]	2G11	IgG1	1
Mouse-anti-chicken CD40[1]	AV79	IgG2α	2.5
Mouse-anti-chicken CD80[1]	IAH: F864:DC7	IgG2α	2.5
Mouse-anti-chicken CD86[1]	IAH: F853:AG2	IgG1	2.5
Mouse anti-chicken CD28[1]	2-4	IgG2α	5
Anti-mouse IgG2α: APC[2]	m2a-15F8		2.5
Anti-mouse IgG1: FITC[2]	M1-14D12		2.5
Mouse IgG1: PE[1]			1
Mouse IgG1: FITC[1]			1
Mouse IgG2α: FITC[2]			5
Mouse IgG2b: FITC[2]			5

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796 †, suppliers: 1, AbDSerotec, UK; 2, eBioscience, UK; 3, Santa Cruz Biotechnology, USA); 4,

797 Southern Biotech, USA.

798 TABLE 2. Sequences of probes and primers used in this study

Target RNA	Probe/ Primers sequence (5′ -3′)*	Accession number
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

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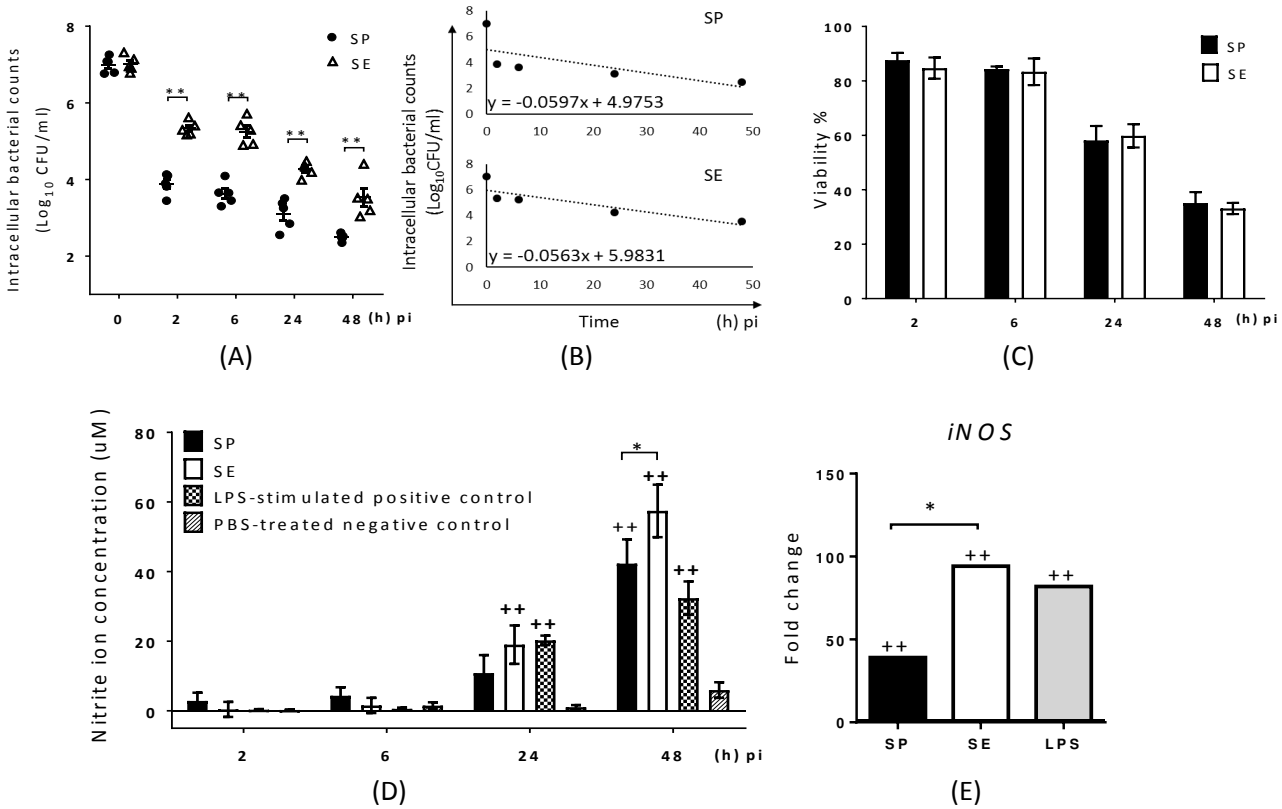


FIGURE 1

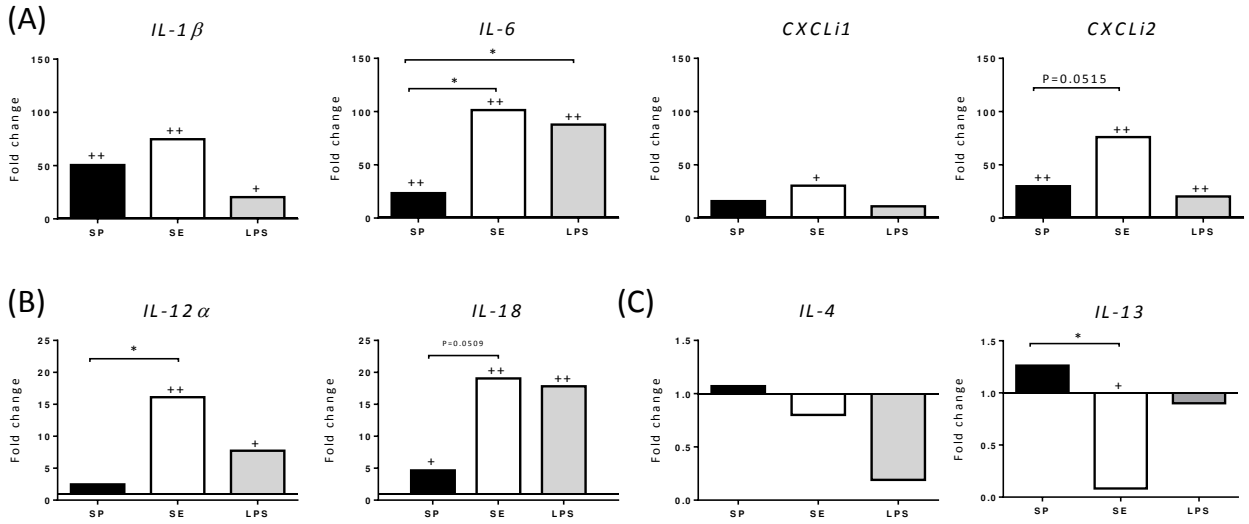
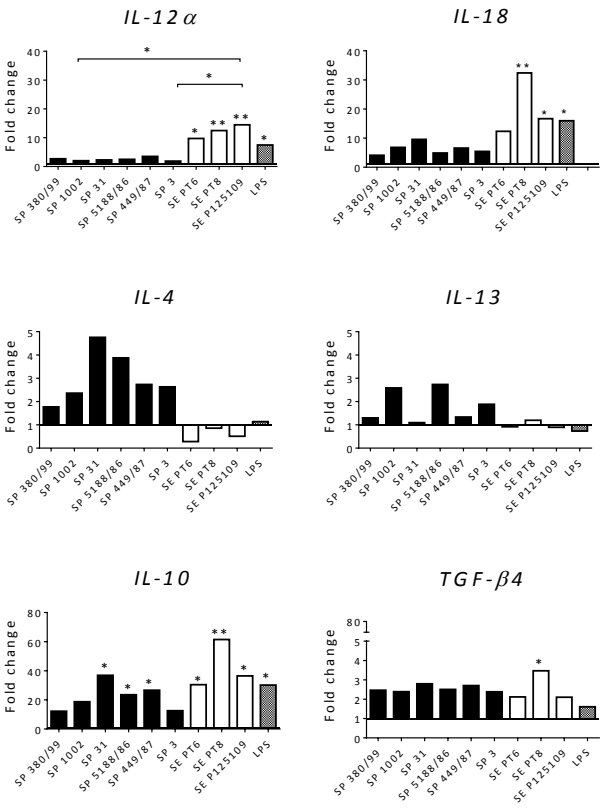


FIGURE 2



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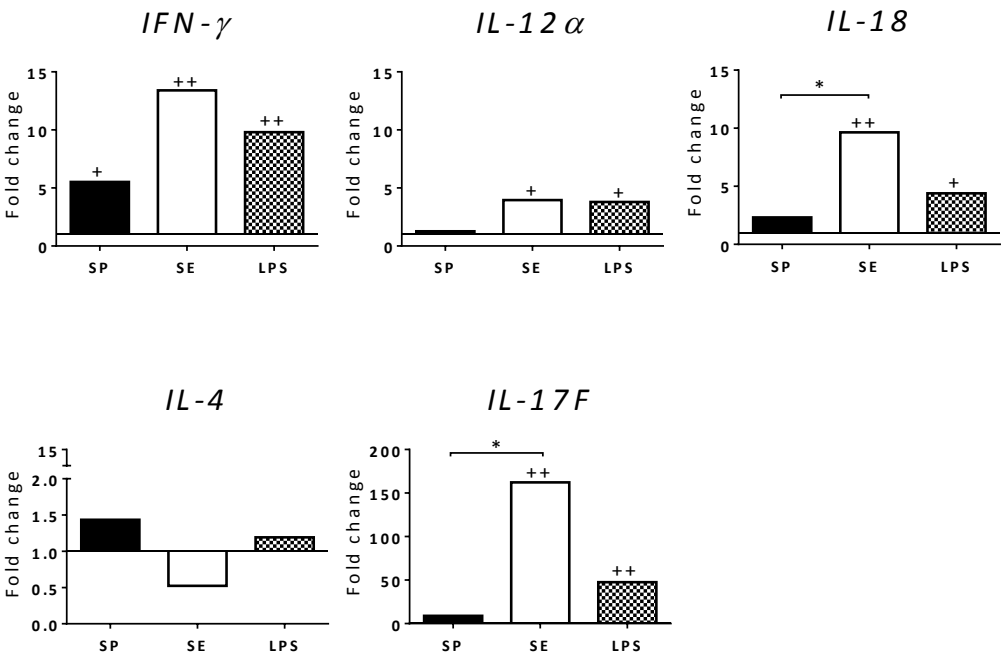

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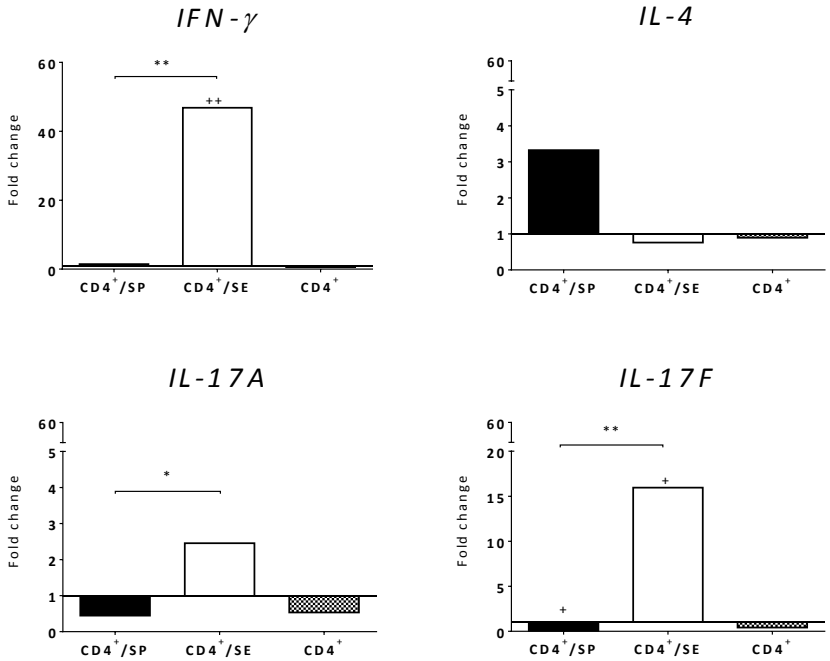

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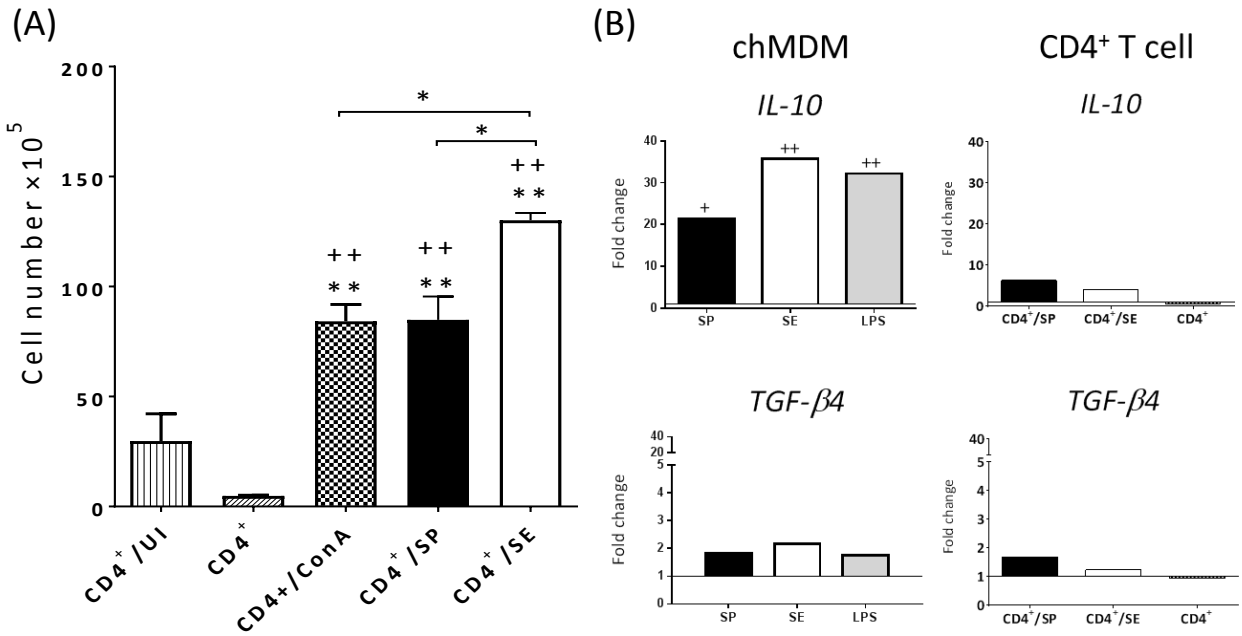

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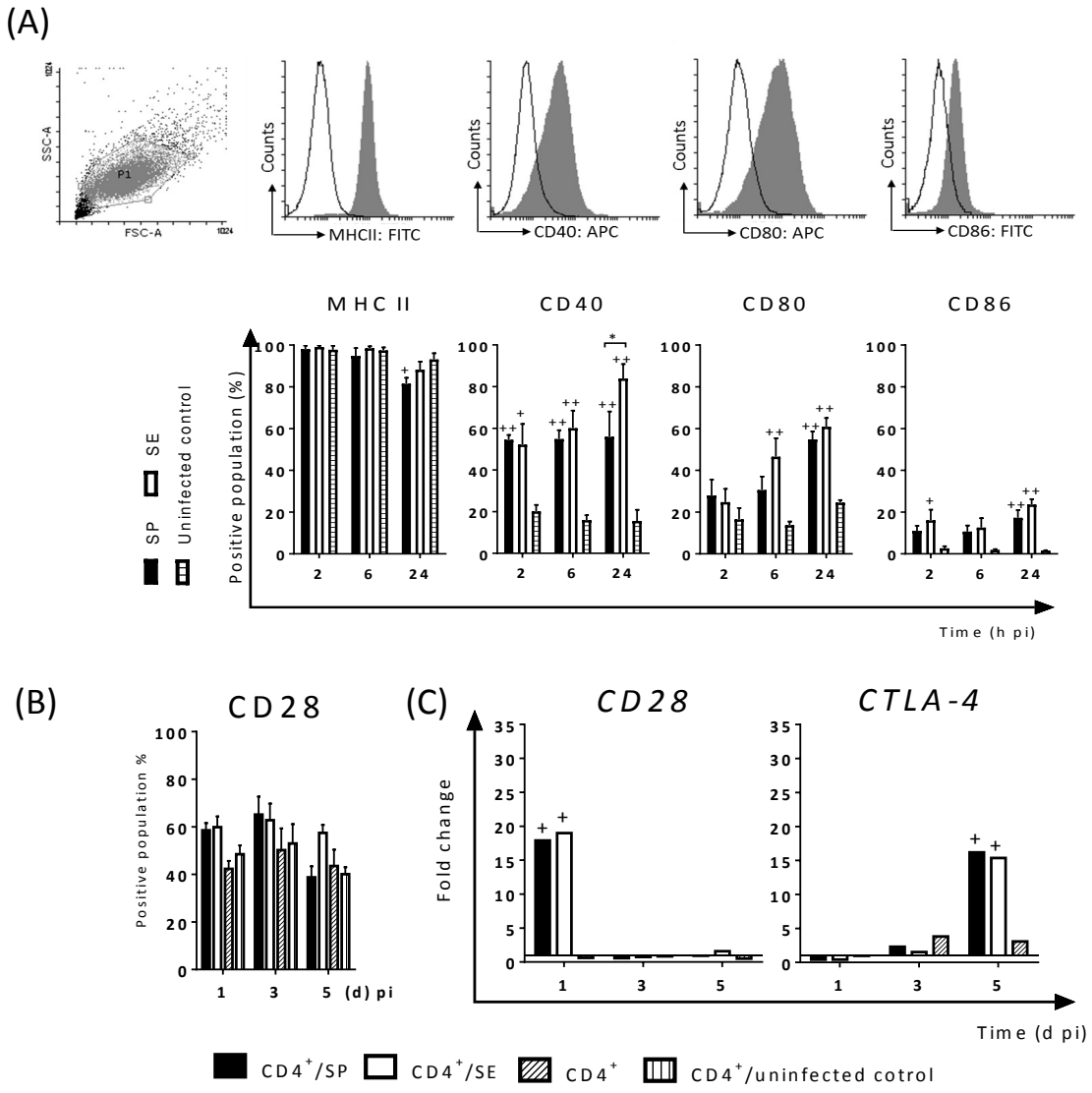

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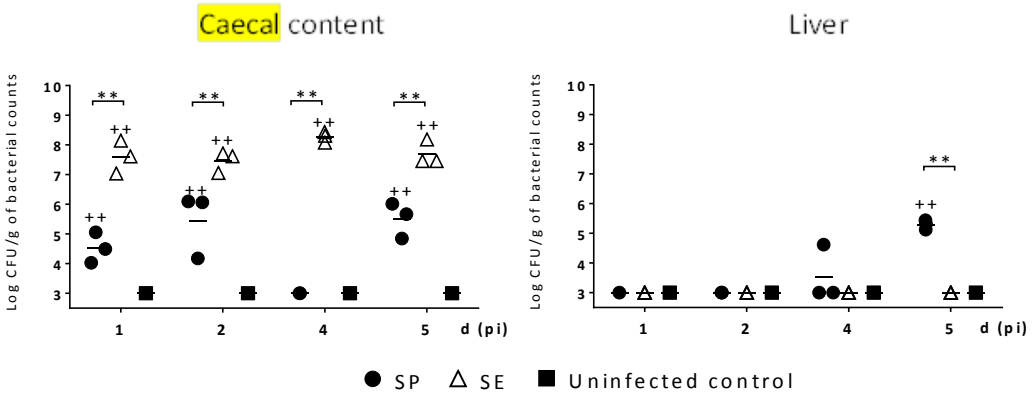
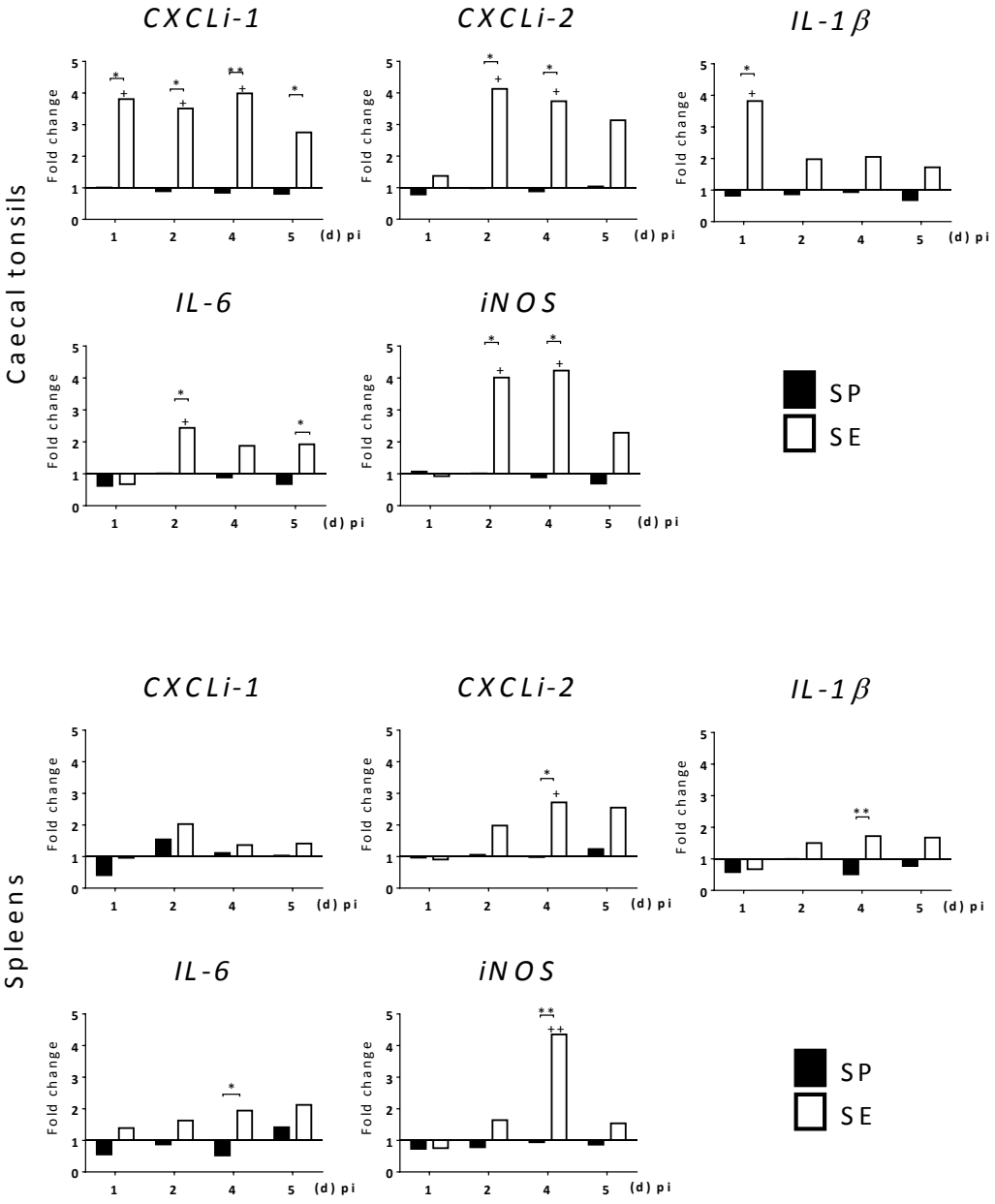


FIGURE 8



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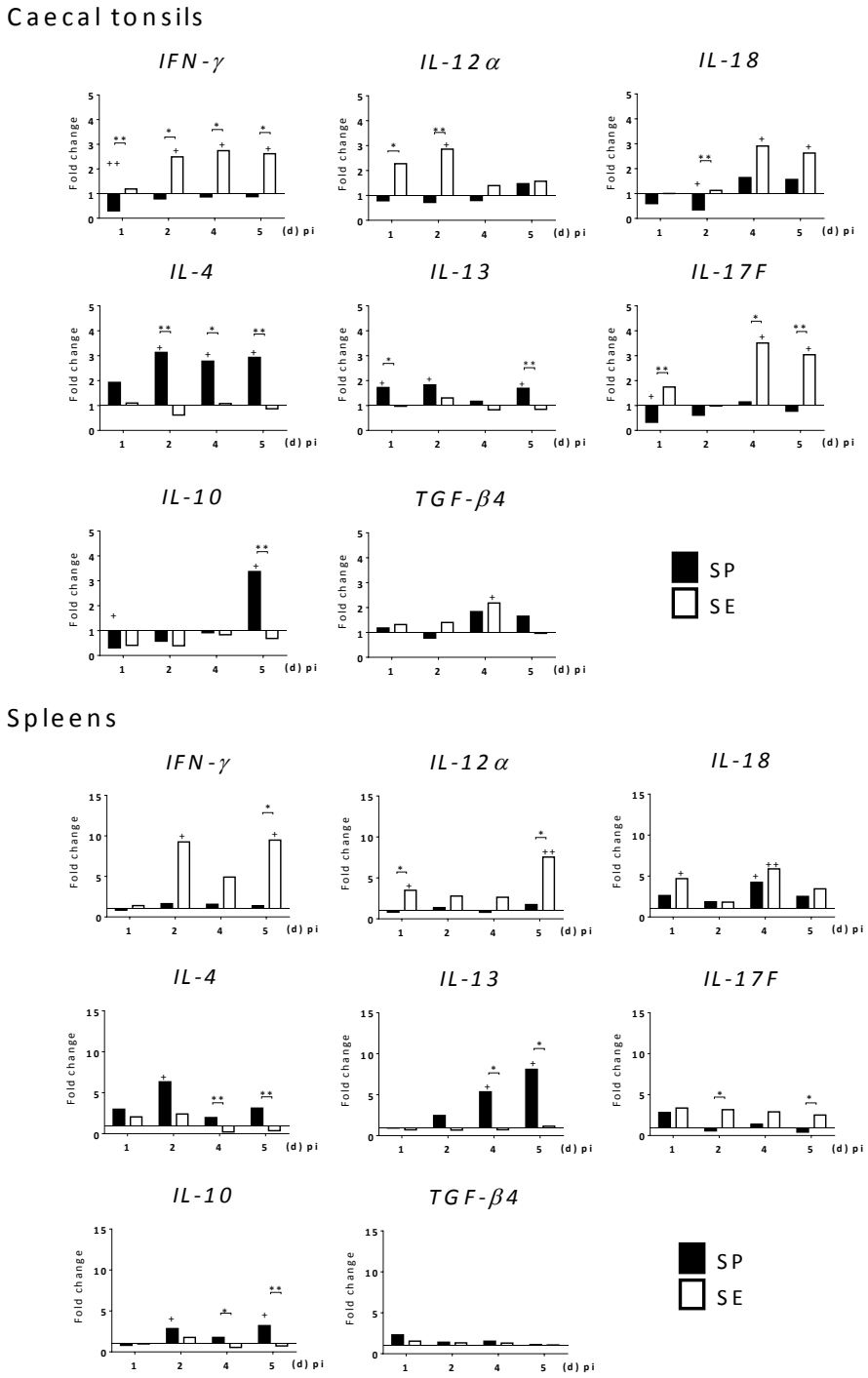

FIGURE 10