

Salmonella Virchow infection of the chicken elicits cellular and humoral systemic and mucosal responses, but limited protection to homologous or heterologous re-challenge

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Salmonella enterica serovar Virchow usually causes mild gastroenteritis in humans; however, it is frequently invasive and many isolates are resistant to a broad-range of therapeutic antimicrobials. Poultry meat is considered a major source of human infection. In this study, we characterize the infection biology and immune response to *S. Virchow* in chickens and determine protection against homologous and heterologous re-challenge, with *S. Virchow* or *S. Typhimurium*. Following oral infection of 7-day-old chickens, *S. Virchow* colonized the gastrointestinal tract and the spleen. Infection elicited an increase in specific IgA, IgG, and IgM antibodies and relative quantitative changes in several leukocyte populations, including CD3, CD4, CD8 α , CD8 β , MHC II, KuL01, and $\gamma\delta$ TCR positive cells, both in the gastrointestinal tract and systemically. Increased expression of pro-inflammatory cytokines IL-1 β and IL-6 and the chemokine CXCL12 was also found. Primary infection with *S. Virchow* offered limited systemic protection against re-challenge with *S. Virchow* or *S. Typhimurium*, but no protection against cecal colonization. In conclusion, *S. Virchow* exhibits similar infection biology and immune responses in the chicken to that previously described for *S. Typhimurium*. Unlike *S. Typhimurium*, *S. Virchow* infection is poorly protective to homologous and heterologous re-challenge. These findings suggest that *S. Virchow* is capable of colonizing the chicken well and therefore, presents a risk of entering the food chain in meat production. Furthermore, the development of vaccines that protect effectively against *S. Virchow* and indeed multivalent vaccines that protect across all *Salmonella* serogroups in the chicken would appear to remain a challenging proposition.

Keywords: *Salmonella* infections, animal, vaccines, humoral immune response, cellular immune response, chicken, cytokines

INTRODUCTION

One of the main sources of human non-typhoidal salmonellosis is through the consumption of contaminated poultry meat and eggs (1–3). *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*) and *S. enterica* serovar Typhimurium (*S. Typhimurium*) are the most common serovars causing human foodborne salmonellosis worldwide and are usually associated with mild gastroenteritis (4). Since the 1990s, *S. enterica* serovar Virchow (*S. Virchow*) has increased in prevalence in the UK and has been the third most frequent serovar isolated from human cases in recent years (5, 6). *S. Virchow* infection is most commonly associated with gastroenteritis in humans, but causes bacteremia more frequently than *S. Typhimurium* or *S. Enteritidis*, especially in immuno-compromised patients and in children (5–7). *S. Virchow* is one of the five serovars that have been given priority by the European Union (EU) for control of entry into the food chain, due to their significant risk to public health (8–10).

In Israel, *S. Virchow* has a uniquely high prevalence and association with invasive disease in humans (11, 12). In Switzerland, it has been ranked between the 4th and 8th most frequently

isolated serovar between 2004 and 2009 and is a common cause of human salmonellosis in Australia and other Oceanic countries (13, 14). In other countries, including the United States, *S. Virchow* gastroenteritis in humans is less common, but cases are often associated with invasive infection (15). Increased antimicrobial resistance of *S. Virchow* has been reported in several previous studies (16–20). Recent studies on *Salmonella* prevalence associated with developing poultry industries in Bangladesh have also indicated *S. Virchow* to be a common problem (21, 22). Therefore, *S. Virchow* is a continuing and growing public health problem worldwide, being associated with invasive disease in humans and showing high antimicrobial resistance to therapeutic drugs.

Although *S. Virchow* is commonly isolated from chickens, its mechanisms of invasion and pathogenic behavior is poorly understood, as is the case with other serovars from serogroup C. A previous study has shown that intravenous infection of poultry with *S. Virchow* leads to systemic infection and colonization of organs such as the spleen, to similar levels as *S. Enteritidis* and *S. Typhimurium* (23). However, this study did not address oral

infection, which is the more likely route of *Salmonella* infection in poultry, via fecal–oral transmission in the chicken house. Additionally, previous studies have shown that *S. Virchow* can survive in egg yolk, but has a limited ability to survive in albumen and on egg shells, suggesting the main vehicle of transmission to humans is poultry meat, rather than eggs (23, 24).

The aims of the current study were to characterize the infection biology of *S. Virchow*, following oral infection of chickens. Additionally, we aimed to determine the humoral, cellular, and cytokine response of the immune system following infection, to obtain information for future immunologically based preventative or therapeutic approaches, following further research. Finally, we aimed to get an indication of the protection and cross-protection offered by primary infection with *S. Virchow* against homologous and heterologous re-challenge with *S. Virchow* and *S. Typhimurium*, respectively.

MATERIALS AND METHODS

BACTERIAL STRAINS

Salmonella Virchow 60 was selected from a panel of 12 previously characterized *S. Virchow* isolates (25). *S. Typhimurium* F98 was included in both infection experiments for a comparison, as it is a well characterized strain in chickens (26–28). Bacterial strains were grown from glycerol stocks maintained at -70°C , in 10 ml LB broth, in an orbital shaking incubator overnight, at 37°C and 150 rpm.

EXPERIMENTAL ANIMALS

All work was conducted in accordance with UK legislation governing experimental animals under project license PPL 40/3063 and was approved by the University of Liverpool ethical review process prior to the award of the license. Chicks were reared in the high-biosecurity poultry unit, University of Liverpool, in secure floor pens at a temperature of 30°C until 3 weeks of age, then at 20°C . Birds were allowed *ad libitum* access to water and vegetable protein-based laboratory poultry pelleted diet (SDS, Witham, Essex, UK). All animals were checked a minimum of twice daily to ensure their health and welfare.

EXPERIMENT 1: IMMUNOLOGICAL AND PATHOLOGICAL CHANGES FOLLOWING INFECTION

One-day-old Rhode-Island Red chicks were obtained from the Pirbright Institute, Compton, UK.

Forty-five chicks were housed separately, in 3 groups of 15 animals. At 7 days of age, Group 1 were orally challenged with 10^8 CFU *S. Virchow* 60 in LB broth, Group 2 were orally challenged with 10^8 CFU *S. Typhimurium* F98 in LB broth and Group 3 remained uninfected (controls). The chickens were checked twice daily, for any signs of morbidity and for any mortality. At 5, 11, and 26 days post infection (DPI), five chickens from each group were randomly selected and killed by cervical dislocation for post mortem examination. At post mortem examination, tissue samples were taken aseptically from the spleen and liver into sterile weighed containers. The cecal contents were obtained by removing the caeca aseptically, then by emptying the digesta within each cecum into a sterile container.

EXPERIMENT 2: PROTECTION AND CROSS-PROTECTION TO RE-CHALLENGE

Forty-eight 1-day-old Rhode-Island Red chicks were housed separately in 2 groups of 24 animals. At 7 days of age, the chickens in Group 1 were orally challenged with 10^8 CFU *S. Virchow* 60 in 0.3 ml LB broth. Group 2 remained uninfected as a control. Chickens were checked twice daily, for any signs of morbidity and for any mortality. To determine intestinal clearance of *Salmonella*, cloacal swabs were taken weekly from five randomly selected chickens in each group. Swabs were directly plated onto brilliant green agar (BGA) and then enriched in selenite broth for *Salmonella* detection. BGA plates and enriched swabs were incubated overnight at 37°C and the enriched swabs were re-plated on BGA and incubated overnight at 37°C . Clearance of *Salmonella* was found at 11 weeks post infection (WPI). At 13 weeks post primary infection (WPPI), group 1 and group 2 were each divided into groups of between 10 and 12 birds. Birds were challenged or re-challenged with 10^8 CFU *S. Virchow* (Group 1 = re-challenge, Group 3 = challenge) or *S. Typhimurium* F98 (Group 2 = re-challenge, Group 4 = challenge). At 3 and 5 days post-secondary infection, five or six chickens from each group were randomly selected and killed by cervical dislocation for post mortem examination and samples taken as described above.

ENUMERATION OF BACTERIA

During both infection experiments, cecal contents and spleens were collected during necropsy for bacteriology and diluted 1:10 in $1 \times$ PBS. The spleen was homogenized using a MicroStomacher 80 (Seward, UK) and cecal contents were mixed using a vortex, to form a suspension. Samples were serial-diluted in $1 \times$ PBS and plated onto BGA. Plates were incubated for 18 h at 37°C and the bacteria were enumerated. Negative samples were enriched overnight at 37°C in selenite broth and then plated onto BGA, to determine if samples contained *Salmonella*.

HISTOLOGICAL EXAMINATION

Samples of ileum and spleen from animals in experiment 1 were fixed in 4% paraformaldehyde for 24–48 h, then trimmed and routinely paraffin wax embedded. Sections ($3\text{--}5\ \mu\text{m}$) were prepared and stained with hematoxylin-eosin (HE) and independently assessed histologically by two veterinary pathologists (Georgios Nikolaou and Anja Kipar), to determine any histological changes in response to infection (29).

PRODUCTION OF SOLUBLE SALMONELLA LYSATE ANTIGEN

Soluble *Salmonella* lysate antigen for each serovar was prepared as described previously (28). Overnight cultures of *S. Virchow* 60 and *S. Typhimurium* F98, as described above, were used to inoculate 100 ml LB broth, which was then incubated overnight at 37°C and 150 rpm. Cultures were aseptically poured into sterile tubes and centrifuged at $4080 \times g$ for 25 min at 4°C to obtain bacterial pellets. The supernatant was poured off and the bacterial pellet was suspended in 20 ml $1 \times$ PBS. Bacterial suspensions were incubated in a waterbath at 65°C for 5 h and a small aliquot was plated onto nutrient agar and incubated at 37°C overnight, to confirm no viable *Salmonella* remained. Following this, bacterial suspensions were sonicated in 10 ml volumes in 20 s bursts on ice at an

amplitude of 15 μm , using a soniprep 150 (MSE Scientific Instruments, UK), for a total of 10 times, allowing the suspension to cool for 1 min between each burst. Suspensions were centrifuged at $4080 \times g$ for 20 min at 4°C and then centrifuged at $30000 \times g$ for 20 min at 4°C . Protein concentrations were measured using the Bradford protein determination kit (Merck, Poole, UK). The soluble antigen preparations were stored in aliquots at -20°C .

ENZYME-LINKED IMMUNOSORBENT ASSAY

Serum was obtained by removing blood from the heart at necropsy, allowing it to clot, then removing the serum after centrifuging at $13000 \times g$ for 5 min for. Serum samples were then stored as aliquots at -20°C until used. Levels of serum specific antibodies against *S. Virchow* and *S. Typhimurium* soluble antigen were determined at each time point, as described previously (28, 30). Each sample was run in triplicate. Flat-bottomed 96-well plates were coated with 100 μl /well of *S. Virchow* or *S. Typhimurium* soluble antigen, diluted in carbonate–bicarbonate buffer (pH 9.6) to a concentration of 16.2 $\mu\text{g}/\text{ml}$ and incubated overnight at 4°C . Subsequently, the plates were washed three times with PBS Tween-20 (0.05%). They were then incubated with 3% blocking buffer (consisting of 0.05% Tween-20 in PBS and 3% skimmed milk powder) for 1 h at 37°C and washed with PBS Tween-20 (0.05%). Serum samples were diluted in blocking buffer for the detection of IgA (1:25), IgM (1:400), and IgG (1:400). Plates were incubated with the diluted chicken serum for 1 h at 37°C and washed three times in PBS Tween-20 (0.05%). Specific antibodies were detected by incubating the samples with alkaline phosphatase conjugated to either goat anti-chicken IgA (1:20000), IgM (1:1000), or IgG (1:2000) (Serotec, Oxford, UK) diluted in blocking buffer, for 1 h at 37°C . Plates were washed with PBS Tween-20 (0.05%) and incubated with 100 μl per well of *p*-nitrophenyl phosphate in the dark for 30 min at room temperature. The reaction was stopped by addition of 100 μl 3N sodium hydroxide to each well. Absorbance was determined using a microplate reader at 405 nm. Negative control serum was obtained from *Salmonella*-free animals and positive controls for *S. Typhimurium* were included using positive serum from previous studies. No positive control was available for *S. Virchow* at the start of the experiment, but a positive sample from the first ELISA run was included on subsequent plates as a control to ensure there was little variation from plate-to-plate.

IMMUNOHISTOCHEMISTRY

Spleen, ileum, and cecal tonsil were collected from all animals of experiment 1 and snap frozen in liquid nitrogen on cork plates in OCT (Tissue-tek, UK). Serial sections (10 μM) were cut from each sample, placed on poly-L-lysine coated slides (VWR International, UK) and fixed in acetone for 10 min. Tissue sections were incubated overnight at 4°C with monoclonal antibodies against chicken CD3, CD4, CD8 α , CD8 β , MHC II (B cells, antigen presenting cells, macrophages, and monocytes) (31), KuL01 (monocytes, macrophages, interdigitating cells, and activated microglia cells) (31), $\gamma\delta$ TCR and Bu1a (B cells and subsets of monocytes and macrophages), antigens (Southern Biotechnology, Cambridge, UK), and diluted 1:100 in $1 \times$ TBS (tris buffered saline). A Vectastain Elite ABC kit (Vector Laboratories, Peterborough, UK) was used for the detection of antibody binding. Sections

were incubated with the secondary antibody (biotinylated horse anti-mouse, diluted 1:100 in $1 \times$ TBS) for 30 min at room temperature. The reaction was visualized by incubating the slides with 3',3'-diaminobenzidine, followed by counterstaining with Papanicolaou's hematoxylin and mounting with DPX mounting media (VWR International).

Immunocytochemically stained sections were analyzed using a Nikon Eclipse 80i microscope and NIS-elements BR 3 software. From each sample, five high power fields (400 \times) of the spleen and cecal tonsil were analyzed. The first field was from the center of the tissue, followed by four fields around the central field. Cells expressing the respective antigen were manually counted for all five fields and then an average cell count was determined for each chicken. A different approach was taken for the Bu1a⁺ cells as these cells comprised the lymphatic follicles in the cecal tonsil. The area of every stained follicle present on the tissue was measured to determine if the follicles changed size over time, after infection. An average follicle area was calculated for each chicken. No differences were found in the intensity of the inflammatory infiltrate between the villous and crypt lamina propria; therefore, cell counts were determined in the villi. Ten medium power (200 \times) fields were analyzed per chicken; fields were selected in which longitudinal sections of villi occupied the whole diameter of the field. An average cell count from 10 fields was determined for each chicken.

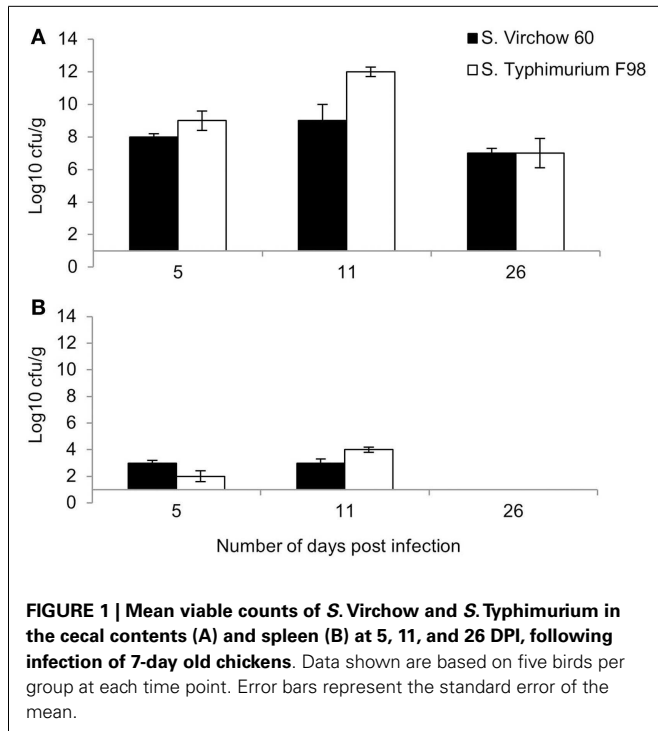
2^{- $\Delta\Delta\text{CT}$} REAL-TIME RT-PCR FOR CYTOKINE EXPRESSION

From animals of experiment 1, spleen and cecal tonsil were collected after euthanasia at 5, 11, and 26 DPI and stored in RNAlater (Sigma-Aldrich, UK) at -20°C . Total RNA was prepared from the tissue samples using an RNeasy kit and following the manufacturer's instructions (Qiagen, UK). The transcription levels of cytokines IL-1 β , IL-6, IL-4, IFN- γ , and the chemokine CXCLi2 were determined using Rotor-Gene Q software v.4.14.2 (Qiagen, UK). Primers and probes for the selected cytokines and chemokine have been described previously (32, 33). One-Step RT-PCR was performed using the Rotor-Gene Probe RT-PCR Master Mix (Qiagen; includes RT stage) in a final concentration of $1 \times 0.25 \mu\text{l}$ Rotor-Gene RT mix, 0.8 μM of both the forward and reverse primers, 0.2 μM of the probe, and 1 μl RNA made up to a total volume of 25 μl with RNase-free water. The following cycling conditions were used for amplification: 50°C for 10 min, 95°C for 5 min, followed by 40 cycles of 95°C for 5 s and 60°C for 10 s.

Each sample was run in triplicate and an average C_T value was taken for each group. The threshold for C_T values was set between 0.20 and 0.23. C_T values were normalized firstly to the endogenous control and then to the uninfected control group (34). Expression levels in the infected groups were represented as the fold-change in expression compared to the uninfected control.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS version 20.0 software. Bacterial counts, immunohistochemical cell counts, and RT-PCR C_T values were compared using one-way ANOVA. Significance between the values was taken if the *P* value was <0.05 .



RESULTS

BACTERIOLOGY

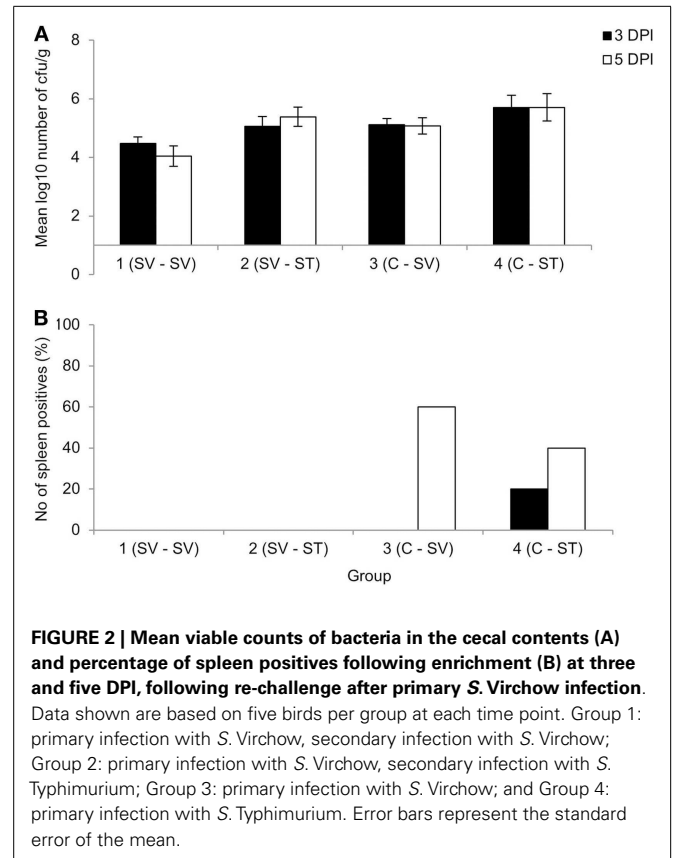
Experiment 1

Following primary infection, *S. Virchow* 60 and *S. Typhimurium* F98 were found in the cecal contents at log₁₀ 8.0 CFU/g and log₁₀ 9.0 CFU/g at 5 DPI (Figure 1A). Bacterial counts in the caeca peaked at 11 DPI, reaching levels of up to log₁₀ 9.0 CFU/g and log₁₀ 12.0 CFU/g for *S. Virchow* and *S. Typhimurium* F98, respectively. By 26 DPI, colony counts had begun to decline in both infected groups.

Both *S. Virchow* 60 and *S. Typhimurium* F98 could be detected in the spleen by 5 DPI, at log₁₀ 3.0 CFU/g and log₁₀ 2.0 CFU/g, respectively (Figure 1B). Counts peaked at 11 DPI; however, no *Salmonella* could be detected in the spleen of either infected group by 26 DPI.

Experiment 2

No animals were lost as a result of infection, though three were euthanized due to unrelated welfare issues. Clearance after primary *S. Virchow* infection occurred by 11 WPPI. At 13 WPPI, Group 1 was divided and given a homologous or heterologous re-challenge and Group 2 was divided and infected with *S. Virchow* 60 or *S. Typhimurium* F98 for age-matched controls. At 3 and 5 days post challenge, the cecal content counts were lower in the re-challenged groups compared to the age-matched control groups (Figure 2A), although the difference in bacterial count was not significant ($P > 0.607$). Viable *Salmonella* could not be directly isolated from the spleen from any of the groups at either time point. Therefore, spleen samples were enriched in selenite broth and the percentage of positive and negative spleens for each group was determined. The re-challenged groups were

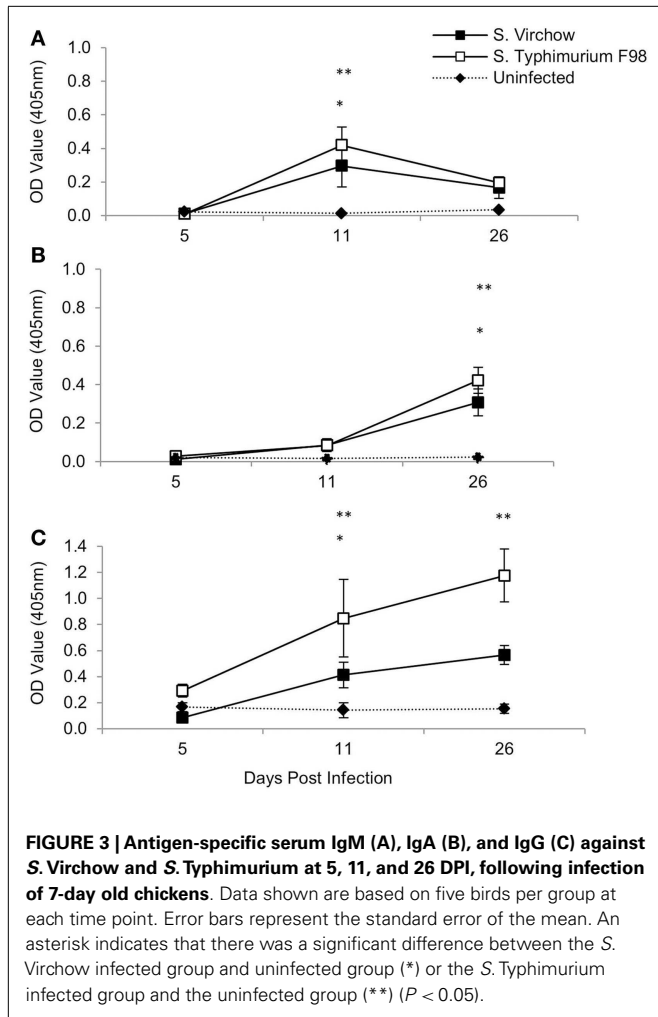


negative following enrichment, whereas at 3 days post challenge 20% of the *S. Typhimurium* age-matched control group were positive and at 5 days post challenge 40 and 60% of the *S. Virchow* and *S. Typhimurium* age-matched control groups were positive, respectively (Figure 2B).

HISTOPATHOLOGY

The histological examination of ileum and spleen from animals in experiment 1, i.e., at 5, 11, and 26 DPI was undertaken to determine the type and degree of pathological changes in response to *S. Virchow* infection. In most control animals, the ileum exhibited scattered lymphocytes in the lamina propria mucosae and occasional lymphocyte exocytosis into the lamina epithelialis mucosae. In some animals, the mucosal infiltration was more prominent (mild to moderate) and comprised not only lymphocytes, but also macrophages and scattered heterophils. At 5 and 11 DPI, chickens infected with either *Salmonella* strain exhibited a slight increase in lymphocytes in the lamina propria, but the lamina epithelialis generally remained unaltered. Animals infected with *S. Virchow* also exhibited occasional loose aggregates of heterophils in the lamina propria mucosae in particular in the villi.

Spleens exhibited a white pulp composed of small to moderately sized lymphatic follicles without obvious germinal center reaction, and inconspicuous T cell zones in all groups. The red pulp was moderately cellular and contained some heterophils in a few control animals and generally a low to moderate number of heterophils in chickens infected with either



Salmonella strain at 5 and 11 DPI. This difference was not apparent by 26 DPI.

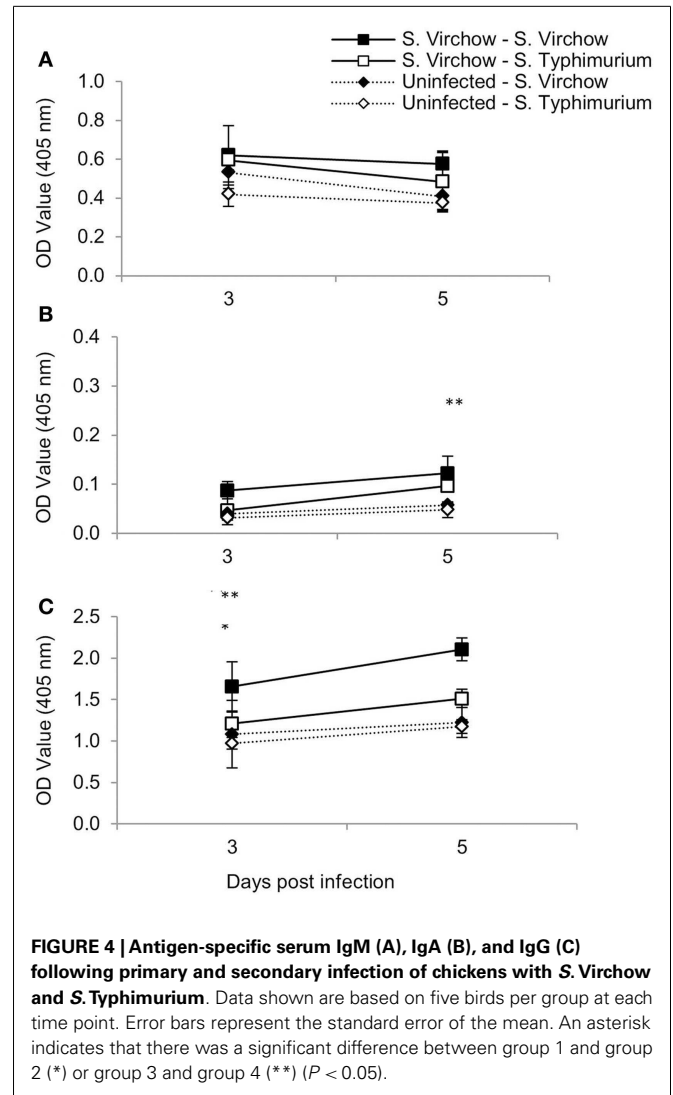
HUMORAL IMMUNE RESPONSE

Experiment 1

Specific IgM, IgG, and IgA antibodies were detected following infection with both *S. Virchow* 60 and *S. Typhimurium* F98 (Figure 3). Between 5 and 11 DPI, serum IgM levels against *S. Virchow* increased rapidly, peaking at 11 DPI. By 26 DPI, anti-*Salmonella* IgM had declined toward levels found in the uninfected group. IgA and IgG increased more slowly, but to greater levels than IgM. Between 5 and 11 DPI, IgA levels against *S. Virchow* increased slowly and were not considerably higher than in the uninfected group. However, between 11 and 26 DPI, there was a sharp increase in IgA and by 26 DPI, levels were considerably higher than in the uninfected group. IgG levels against *S. Virchow* increased steadily throughout the infection period. The IgA, IgM, and IgG response against *S. Virchow* followed a pattern similar to that seen against *S. Typhimurium* F98 throughout the experiment.

Experiment 2

At three and five DPI, serum samples were collected to determine the specific IgA, IgG, and IgM production against *S. Virchow* 60



and *S. Typhimurium* F98 after re-challenge, compared to age-matched controls (Figure 4). Serum IgA antibody levels increased in all four groups between 3 and 5 days post challenge, although they were considerably higher in the re-challenged groups compared to the age-matched controls. Serum IgG antibody levels increased in all four groups between 3 and 5 days post challenge; however, they were highest in the *S. Virchow* re-challenged group at both time points. By 5 days post challenge, IgG levels were higher in the re-challenged groups compared to the age-matched controls. Serum IgM antibody levels decreased between 3 and 5 days post challenge in all four groups. IgM titers were similar between all four groups, although the highest IgM levels were found in the *S. Virchow* re-challenged group, followed by the *S. Typhimurium* re-challenged group.

CELLULAR RESPONSES

To quantify changes in the cellular immune response following *S. Virchow* infection, spleen (Figure 5), ileum (Figure 6), and cecal tonsil (Figure 7) specimens were collected from animals in

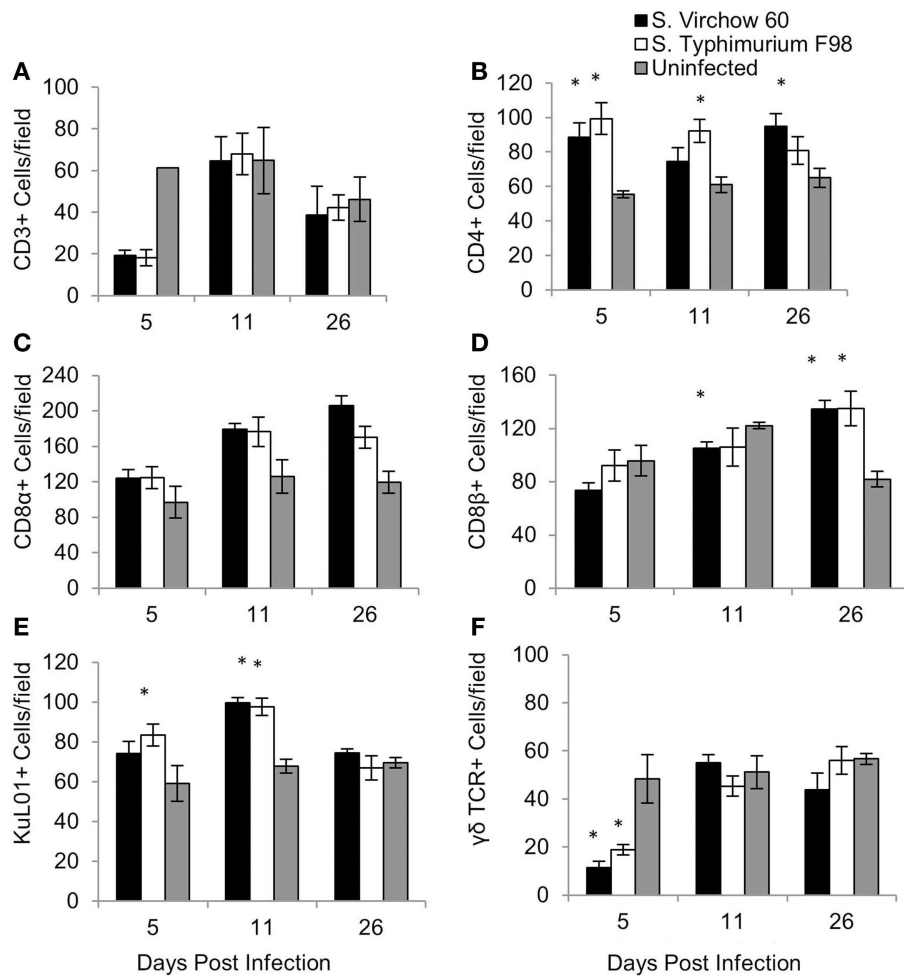


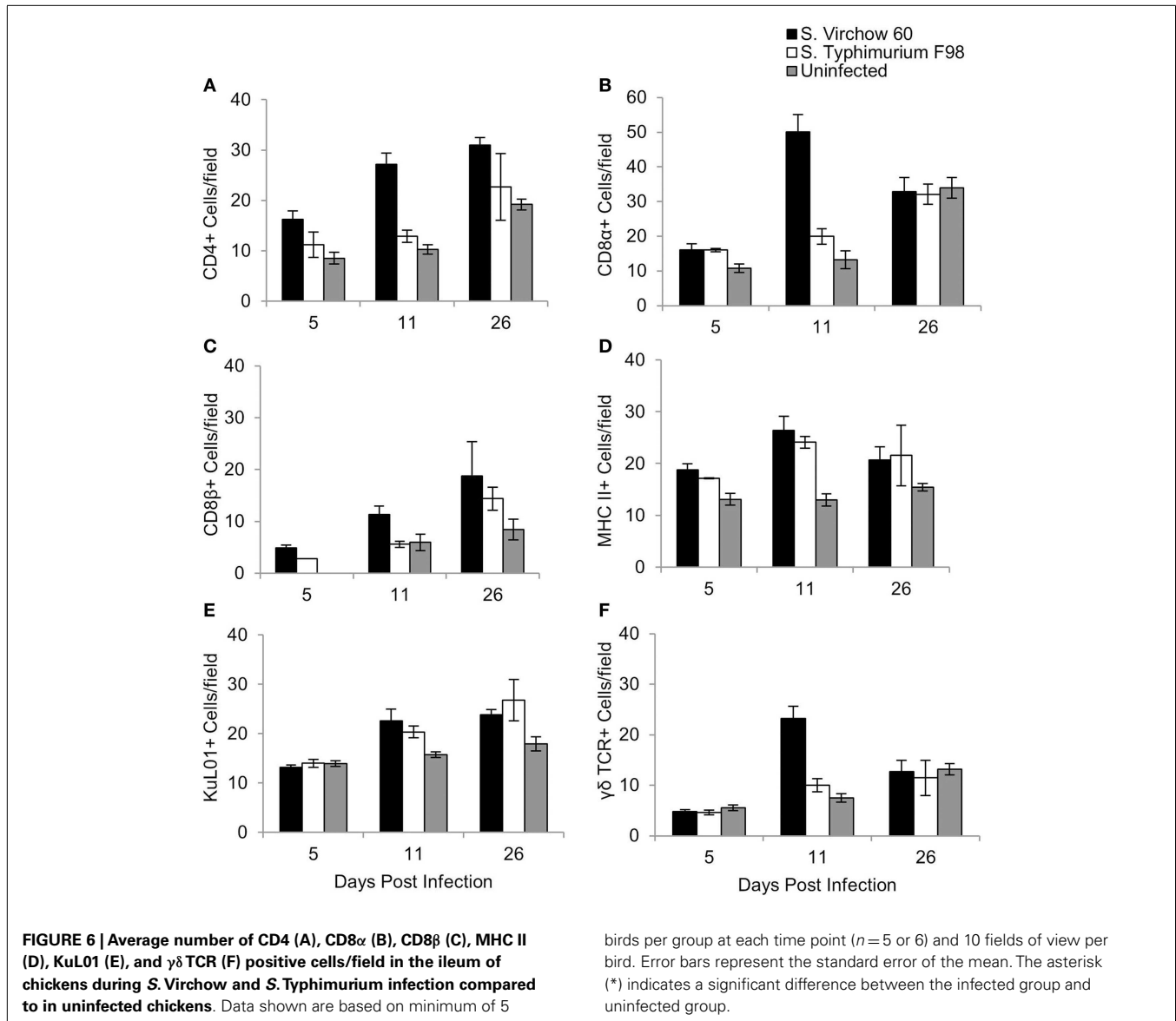
FIGURE 5 | Average number of CD3 (A), CD4 (B), CD8 α (C), CD8 β (D), KuL01 (E), and $\gamma\delta$ TCR (F) positive cells/field in the spleen of chickens during *S. Virchow* and *S. Typhimurium* infection compared to in uninfected chickens. Data shown are based on

minimum of five birds per group at each time point ($n = 5$ or 6) and five fields of view per bird. Error bars represent the standard error of the mean. The asterisk (*) indicates a significant difference between the infected group and uninfected group.

experiment 1, euthanized at 5, 11, and 26 DPI. For the spleen, five high power fields (40X) were selected and the positively stained cells were manually counted. Immunocytochemistry showed that at five DPI, numbers of CD4⁺, CD8 α ⁺, and KuL01⁺ cells were increased in the spleen in chickens infected with *S. Virchow*, compared to those in uninfected chickens, with the increase in CD4⁺ cells being significant ($P < 0.05$). At 11 DPI, CD4⁺, CD8 α ⁺, and KuL01⁺ cells had increased further in the *S. Virchow* infected group, to significantly higher amounts than those found in the uninfected group ($P < 0.003$). By 26 DPI, CD8 β ⁺ cells had significantly increased in the *S. Virchow* infected group above the numbers found in the uninfected group ($P < 0.0025$). In contrast, CD3⁺ and $\gamma\delta$ TCR⁺ cells decreased in the spleen of *S. Virchow* infected chickens, compared to the uninfected chickens at 5 DPI; however, they had returned to levels similar to those found in the uninfected group by 11 DPI.

For the ileum, 10 medium power fields ($\times 200$) were selected and the cells were manually counted. An increase in CD4⁺,

CD8 α ⁺, CD8 β ⁺, and MHC II⁺ cells was observed at five DPI in the *S. Virchow* infected group, in comparison to the uninfected group, with the increase in CD4⁺ and MHC II⁺ cells being significant ($P < 0.028$). CD4⁺, CD8 α ⁺, CD8 β ⁺, and MHC II⁺ cells increased further in the *S. Virchow* infected group at 11 DPI, when a significant increase in KuL01⁺ and $\gamma\delta$ TCR⁺ cells was also found ($P < 0.016$). At 26 DPI, CD4⁺, MHC II⁺, CD8 β ⁺, and KuL01⁺ cells were still elevated in the *S. Virchow* infected group; however, CD8 α ⁺ and $\gamma\delta$ TCR⁺ cells had returned to levels similar to those seen in the uninfected group. In the ileum, the number of several cell populations was higher in the *S. Virchow* infected group compared to the *S. Typhimurium* infected group and the uninfected group. Although the number of CD4⁺ cells were increased in both infected groups at each time point, they were considerably higher in the *S. Virchow* infected group. CD8 α ⁺ and CD8 β ⁺ cells were increased in the infected groups throughout the infection experiment; however, the cell counts were significantly higher, for both subpopulations, in the *S. Virchow*



infected group compared to the *S. Typhimurium* infected group and the uninfected group at 11 DPI ($P < 0.036$). MHC II⁺ cells were significantly more numerous in the infected groups at 5 ($P < 0.028$) and 11 ($P < 0.011$) DPI and numbers remained higher than those of the uninfected group at 26 DPI. At 11 DPI, a significant increase was seen in KuL01⁺ ($P < 0.016$) and $\gamma\delta$ TCR⁺ ($P < 0.001$) cells in both of the infected groups and was still seen at 26 DPI.

For the cecal tonsil, five high power fields ($\times 400$) were selected and the positively stained cells were counted manually. The quantity of CD3⁺, CD8 β ⁺, and KuL01⁺ cells had increased in the *S. Virchow* infected group by five DPI, with the number of CD3⁺ and KuL01⁺ cells being significantly higher than in the uninfected group ($P < 0.016$). At 11 DPI, CD3⁺, KuL01⁺ cell numbers had increased further in the *S. Virchow* infected group. A significant increase was also found in CD4⁺ cells ($P < 0.05$). At 26 DPI, KuL01⁺ cells were still significantly higher in the

S. Virchow infected group compared to the uninfected group. Throughout the infection period MHC II⁺ cells were significantly lower in the *S. Virchow* infected group compared to the uninfected group ($P < 0.045$). The size of the Bu1a⁺ follicles had increased in the *S. Virchow* infected group by 5 DPI and had increased further at 11 DPI ($P < 0.036$). Alterations in the cell numbers in the *S. Virchow* infected group were similar to those in the *S. Typhimurium* F98 infected group, throughout the infection period.

CYTOKINE AND CHEMOKINE EXPRESSION

The change in expression of key cytokines and chemokines was determined in the spleen (Figure 8) and cecal tonsil (Figure 9), at each time point, throughout Experiment 1. The magnitude of the response sometimes varied within groups, which can be seen by the standard error bars. These findings have been reported previously (28).

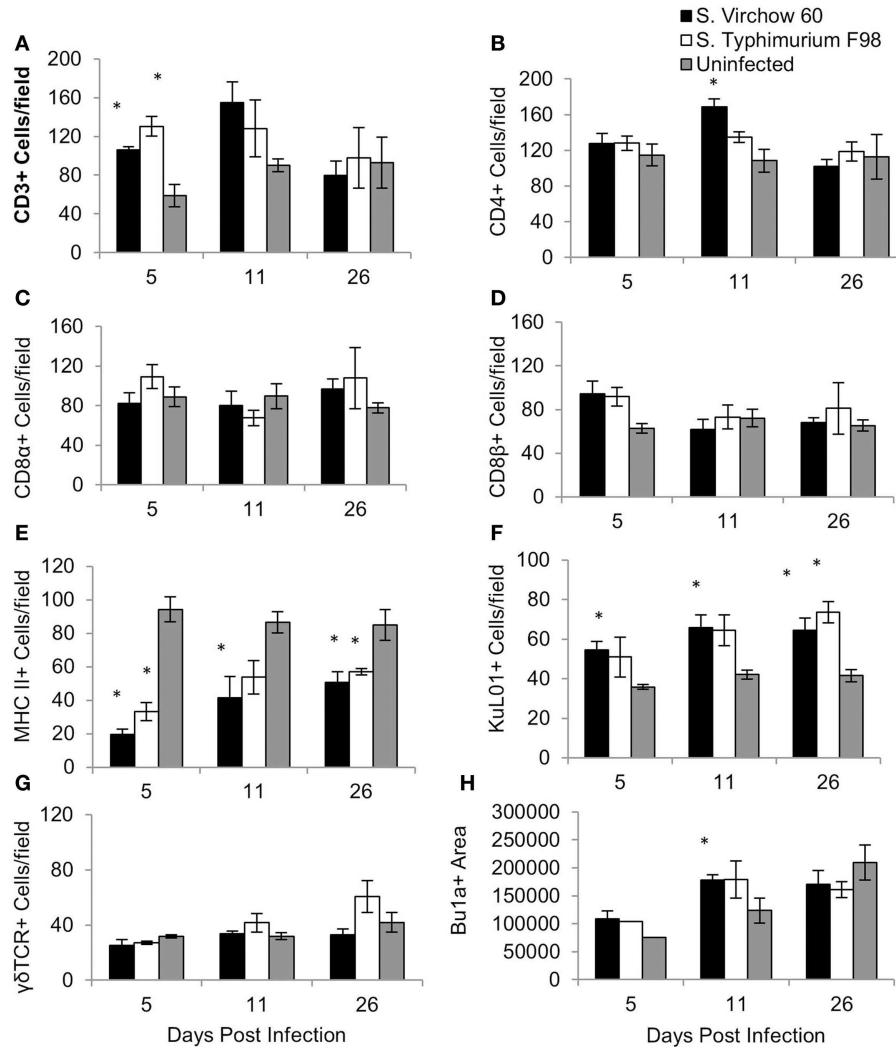


FIGURE 7 | Average number of CD3 (A), CD4 (B), CD8α (C), CD8β (D), MHC II (E), KuL01 (F), γδTCR (G), and Bu1a (H) positive cells/field in the cecal tonsil of chickens during *S. Virchow* and *S. Typhimurium* infection compared to in uninfected chickens. Data shown are based on minimum of

five birds per group at each time point ($n = 5$ or 6) and five fields of view per bird. Error bars represent the standard error of the mean. The asterisk (*) indicates a significant difference between the infected group and uninfected group.

In the spleen, IL-1 β , IL-6, and CXCLi2 mRNA levels were increased in the *S. Virchow* infected group above those of the uninfected group, by 4.23, 5.90, and 2.99-fold, respectively, at five DPI. They were also increased in the *S. Typhimurium* F98 infected group above those of the uninfected group, by 7.58, 7.62, and 3.46-fold, respectively, with the increase in IL-1 β and IL-6 being significant. At 11 DPI, IL-1 β , IL-6, and CXCLi2 transcription in the infected groups had returned to levels similar to those seen in the uninfected group. IFN- γ levels were increased in spleens of the *S. Virchow* infected group at 11 DPI by 2.19-fold. IL-4 was not expressed in the spleen at any time point during the infection period.

In the cecal tonsil, the IL-1 β mRNA level was increased by 1.46-fold in the *S. Virchow* infected group at five DPI. IL-1 β transcription was variable in the *S. Typhimurium* infected group,

with two chickens showing a 6.19-fold increase and three chickens showing no change at five DPI. A 1.25-fold increase in the IL-6 mRNA level was found in 3/5 *S. Virchow* infected chickens at five DPI; however, IL-6 expression in the *S. Typhimurium* infected group did not change in comparison to the uninfected group. CXCLi2 expression was higher in the *S. Virchow* infected group than the uninfected group at each time point and had increased 7.73, 1.65, and 1.80-fold at 5, 11, and 26 DPI, respectively. The increase in CXCLi2 in the *S. Virchow* infected group compared to the uninfected group was significant at five DPI. CXCLi2 transcription had increased by an average of 5.39-fold in two birds from the *S. Typhimurium* F98 infected group at five DPI. IFN- γ levels increased slightly in the *S. Virchow* infected group by 1.29, 1.34, and 1.56-fold at 5, 11, and 26 DPI, respectively. In the *S. Typhimurium* infected group, IFN- γ expression was similar to the

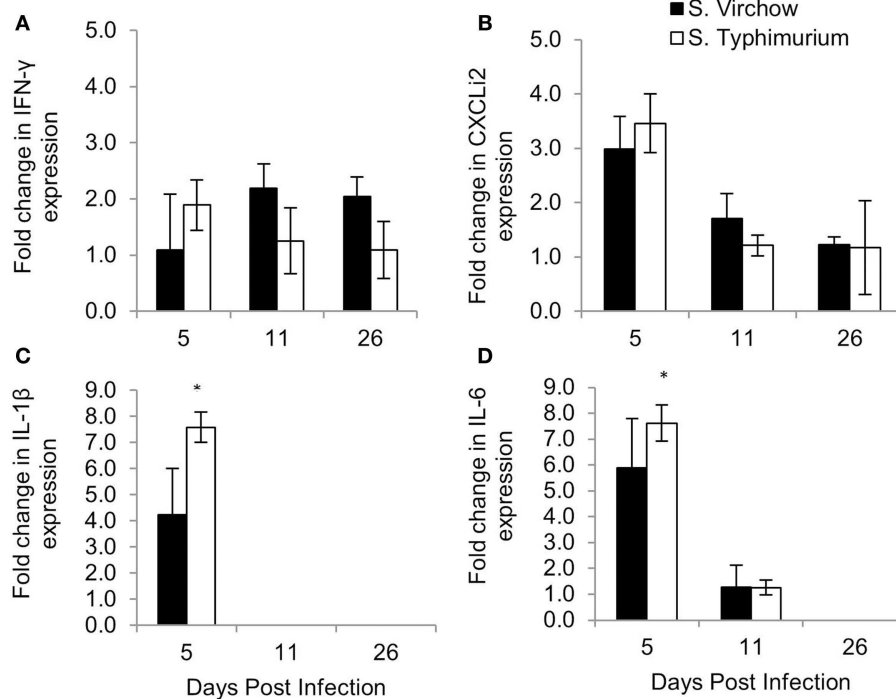


FIGURE 8 | Relative expression of IFN- γ (A), CXCLi2 (B), IL-1 β (C), and IL-6 (D) in the spleen of chickens infected with *S. Virchow* and *S. Typhimurium* F98 compared to uninfected chickens, at 5, 11, and 26 DPI. Data shown are based on minimum of five birds per

group at each time point ($n = 5$ or 6). Error bars represent standard error of the mean. An asterisk (*) indicates that there was a significant fold-change between the infected group and the uninfected group.

uninfected group at five DPI; however, it had increased by three-fold at 11 DPI. At 26 DPI, IFN- γ expression had decreased and was 1.33-fold higher than in the uninfected group. IL-4 expression was not detected in the cecal tonsil for the duration of the experiment.

DISCUSSION

Previously, we demonstrated that *S. Virchow* can colonize the gastrointestinal tract and the spleen of chickens, to levels similar to those seen with *S. Typhimurium* at three DPI, suggesting *S. Virchow* may mirror the infection biology found with broad-range serovars (25). Here, we have further characterized the *S. Virchow* colonization of poultry and confirm that it exhibits an infection biology similar to *S. Typhimurium*, over an infection period of 26 days. *S. Virchow* colonized the gastrointestinal tract and the spleen by 5 DPI, with bacterial counts peaking in the gut and systemically at 11 DPI. By 26 DPI, *S. Virchow* had been cleared from the spleen, showing that it causes transient systemic infection; however, bacterial counts were still high in the cecal contents. As with *S. Typhimurium* F98, chickens infected with *S. Virchow* showed no signs of clinical illness and no significant pathological changes in the ileum during the infection period, suggesting its low pathogenicity in this host. Although, both *S. Virchow* and *S. Typhimurium* have been shown to be invasive, depending on the strain and the host they are infecting (35–39).

The histological analysis of the ileum and spleen did not reveal marked pathological changes at 5, 11, and 26 DPI and no distinct evidence of epithelial damage in the ileum. A slight increase

in lymphocyte infiltration and some heterophil recruitment was seen in the ileum together with evidence of systemic heterophil release, based on the general mild increase in heterophils in the splenic red pulp at the two earlier time points. Our findings differ from those of a previous study, which examined the effect of *S. Typhimurium* in the intestine of 40-day old white leghorn chickens up to four DPI and observed evidence of epithelial damage and a more pronounced mononuclear infiltration (40). The author also found large numbers of heterophils in the intestinal lumen. However, only small numbers of heterophils were seen in the lamina propria that would be more consistent with our findings on day 5. It can also not be excluded that age and breed play a role in the response to infection. The fact that we observed evidence of systemic and local heterophil recruitment after infection is consistent with a more recent study indicating the relevance of heterophils in the innate response to bacterial, including *Salmonella*, infection in chickens (41, 42).

To our knowledge, this is the first detailed study of the humoral, cellular, and cytokine response produced by chickens in response to oral infection with *S. Virchow*. The results show that *S. Virchow* stimulates an immune response in chickens similar to that seen by other broad-range serovars. This suggests that serogroup has limited influence on innate or adaptive immune responses beyond the change in specificity of response to LPS. Indeed recent determination of the innate response to another serogroup C serovar, *S. Infantis*, supports the notion that the immunobiology of infection in the chicken is similar with all invasive broad host range serovars

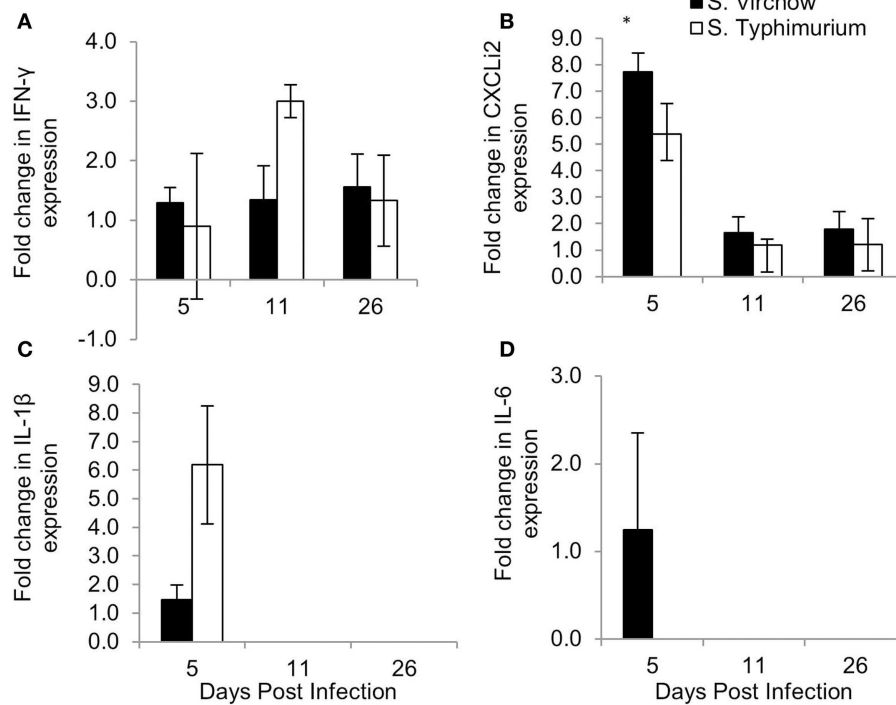


FIGURE 9 | Relative expression of IFN- γ (A), CXCLi2 (B), IL-1 β (C), and IL-6 (D) in the cecal tonsil of chickens infected with *S. Virchow* and *S. Typhimurium* F98 compared to uninfected chickens, at 5, 11, and 26 DPI. Data shown are based on minimum

of five birds per group at each time point ($n=5$ or 6). Error bars represent standard error of the mean. An asterisk (*) indicates that there was a significant fold-change between the infected group and the uninfected group.

(43, 44). After *S. Virchow* infection, numbers of CD4⁺, CD8 α ⁺, and CD8 β ⁺ cells had increased in the ileum by five DPI, indicating a T helper and a cytotoxic T cell response had occurred. Previous studies have found variation in T cell influx into the gut, depending on location in the gastrointestinal tract, infection dose, age of the host at the time of infection, and the genetic background of the host (45–48).

MHC II⁺ cells increased in number in the ileum throughout the experiment and correlated with an increase in KuL01⁺ cells, suggesting local recruitment of macrophages and the likely role of antigen presenting cells in the control of *S. Virchow* infection. At 11 DPI, the quantity of $\gamma\delta$ TCR⁺ cells increased in the ileum in both infected groups; however, the number of cells in the *S. Virchow* infected group was significantly higher than in the *S. Typhimurium* infected group and the uninfected group ($P = < 0.001$). An early increase in $\gamma\delta$ TCR⁺ cells in the cecum has been shown previously, following infection of chicks with *S. Typhimurium*, *S. Enteritidis*, and *S. enterica* serovar Hadar (*S. Hadar*) (47, 49). The difference between time points during this study and previous studies may be due to different areas of the gut being sampled.

The changes in the proportion of leukocyte subpopulations were less prolonged in the cecal tonsil, compared to the ileum. CD3⁺ and CD8 β ⁺ cell numbers increased early in the infected groups, by 5 DPI, followed by an increase in CD4⁺ cells at 11 DPI and CD8 α ⁺ cells by 26 DPI. An increase of CD4⁺ cells in

the cecal tonsil, following *S. Enteritidis* infection, has been previously associated with immunoglobulin class switching (50). This mechanism could explain why, in this study, CD4⁺ cells were only up-regulated in the cecal tonsil at one time point. The size of the lymphatic follicles which were comprised of Bu1a⁺ B cells in the cecal tonsil was increased in the infected groups compared to the uninfected group at 5 and 11 DPI, indicating the humoral immune response has a role in clearance of *Salmonella* infection. Throughout the infection experiment, MHC II⁺ cells decreased, whereas KuL01⁺ cells increased, in the cecal tonsil. An increase in KuL01⁺ cells in the cecal tonsil following *S. Enteritidis* infection has been shown previously (43) and could indicate antigen presentation to immune cells in the cecal tonsil.

CD4⁺ and CD8 α ⁺ cell numbers increased in the spleen in infected groups and remained elevated for the duration of the experiment. CD8 β ⁺ cells did not increase in the spleen until 26 DPI in the infected groups. Varying results have been found for these subpopulation changes in the spleen and could be a result of age of the chickens when infected, infecting serovar or dose (45, 50, 51). At five DPI, the number of CD3⁺ and $\gamma\delta$ TCR⁺ cells (T cells) was decreased in the spleen in the infected groups. A decrease of lymphocytes in the spleen has been shown to coincide with an increase in the cecum following *S. Enteritidis* infection (45) and could indicate cell-trafficking from the spleen to the gut.

Changes in IFN- γ , CXCLi2, IL-1 β , and IL-6 transcription were found in the spleen and cecal tonsil during *S. Virchow* and *S.*

Typhimurium infection; although, the magnitude of the response varied within groups. This was particularly evident in the cecal tonsil, as some chickens would exhibit a response, whereas others would not. Variability in cytokine and chemokine response in the cecal tonsil within the same group has been shown in previous studies and may be due to differences in immunological maturation from chicken to chicken (28, 50).

An increase in IL-1 β , IL-6, and CXCLi2 in the spleen and cecal tonsil of chickens occurred by 5 DPI, in both infected groups, showing that a rapid inflammatory response occurs against *S. Virchow*, in a similar manner to *S. Typhimurium*. IL-6 remained elevated in the spleen until 26 DPI, which has previously been associated with lymphocyte and macrophage development, rather than an acute inflammatory response (30). CXCLi2 is a pro-inflammatory chemokine that is highly homologous to human IL-8 and is important for early immune responses in the gut, including an influx of heterophils (52–54). The increase in IL-1 β , IL-6, and CXCLi2 transcription in the spleen and cecal tonsils shows that like *S. Typhimurium* and other broad-range serovars, *S. Virchow* elicits a strong immune response in the chicken, causing a rapid inflammatory response upon infection. The response elicited is unlike that seen with host-restricted serovars, such as *S. enterica* serovar Pullorum (*S. Pullorum*) and *S. enterica* serovar Gallinarum (*S. Gallinarum*), which do not induce inflammatory responses and rely on “stealth” to invade and cause systemic or typhoidal-like disease (55).

IFN- γ expression was modestly increased in both infected groups compared to the uninfected group at every time point, in both the spleen and cecal tonsil. IFN- γ enhances the oxidative burst in macrophages against *Salmonella* infection (56). Elevated levels of IFN- γ support the idea that *S. Virchow* clearance is dependent on IFN- γ T cell mediated responses. The increased level of IFN- γ , in combination with the lack of IL-4 expression (Th2 cytokine) (57), suggests *S. Virchow* clearance is, like *S. Typhimurium*, primarily Th1-mediated.

Serum humoral responses showed a classical pattern of a rapid rise in IgM, followed by a rise in IgG and IgA, against *S. Virchow* infection. The humoral immune response was slightly stronger against *S. Typhimurium* than *S. Virchow* throughout the infection experiment, although it did follow the same pattern in both infected groups. Increased serum antibodies and increased amounts of B cells (Bu1a⁺) in the cecal tonsil suggest the humoral response has a role in *S. Virchow* clearance, although previous studies have shown it is not needed for *Salmonella* clearance (58, 59).

The bacteriology results from Experiment 2 showed primary infection with *S. Virchow* offers some protection against systemic spread, following secondary infection, but no significant reduction in cecal colonization. Additionally, primary infection with *S. Virchow* offered cross-serogroup protection against systemic spread, following secondary infection with *S. Typhimurium*, but no protection against colonization of the gut.

Salmonella-specific IgA, IgG, and IgM were detected following challenge in all four infected groups but levels were always highest in the *S. Virchow* homologous re-challenge group (Group 1). These results are indicative of an antigen-specific secondary immune response in previously challenged birds. Additionally,

the quicker and greater antibody response observed in the *S. Typhimurium* heterologous re-challenge group (Group 2) compared to the age-matched *S. Typhimurium* infected group (Group 4) indicates a degree of immunological cross-reactivity against *S. Virchow* and *S. Typhimurium*, though little protection.

A previous study, investigating the cross-protection and cross-reactivity against *S. Typhimurium* and *S. Enteritidis* infection found reduced cecal content and splenic bacterial counts following re-challenge, compared to the age-matched controls (60). Cross-protection offered by primary infection with *S. Typhimurium* against secondary infection with *S. Enteritidis* was more effective than vice versa (60). In addition to this study, an earlier one looked at the degree of cross-protection in mice, focusing on the protection conferred by the main LPS O antigen. Primary infection with *S. Typhimurium* or *S. Enteritidis* gave high protection against homologous re-challenge; however, no protection against challenge with the heterologous serotype. Here, we show some evidence of cross-protection elicited by *S. Virchow* against systemic infection, following heterologous re-challenge with *S. Typhimurium*, but no cross-protection against cecal colonization. It is possible that *S. Typhimurium* may have protected more effectively against secondary infection with *S. Virchow*, but our findings suggest there is limited cross-protection to intestinal infection between Group B and C serovars of *S. enterica*, suggesting that effective multivalent vaccines for use in controlling *Salmonella* in poultry will be difficult to achieve. However, both the study by Beal et al. (60) and a recent vaccination study have demonstrated a degree of cross-protection between serogroups B and D (60, 61). The use of the live attenuated *S. Enteritidis* vaccine, Gallivac[®] Se, alone or in combination with the *S. Enteritidis*–*S. Typhimurium* inactivated vaccine, Gallimune[®] Se + St, prior to infection with either *S. Typhimurium* or *S. Enteritidis*, resulted in a significant reduction in liver and cecal content colonization in the vaccinated compared to unvaccinated chickens (61).

Overall, the findings in this study have shown that *S. Virchow* colonizes chickens and stimulates an inflammatory immune response, similar to that found with broad-range serovars. *S. Virchow* colonized the ileum of chickens to high levels and caused transient systemic infection. The chickens exhibited no clinical symptoms and this, in combination with the only mild histological changes indicates *S. Virchow* has low pathogenicity in chickens. Although the present study has shown that *S. Virchow* has similar infection biology to broad-range serovars, it is rarely isolated from sources other than humans and chickens (13, 62). In contrast, *S. Typhimurium* is commonly isolated from many species including humans, chickens, pigs, cattle, mice, and domestic animals (63). Although *S. Virchow* could cause disease in these hosts and showed similar infection biology to *S. Typhimurium*, it is therefore, likely to be more host-adapted than *S. Typhimurium*.

Salmonella Virchow stimulated an acute inflammatory response in chickens, including a rapid increase in IL-1 β , IL-6, and CXCLi2 transcription. Evidence of an IFN- γ T cell mediated response was also apparent. An increase in IFN- γ and a lack of IL-4 suggests the immune response against *S. Virchow* is primarily Th1-mediated, although a strong antibody response is elicited by *S. Virchow*. However, unlike *S. Typhimurium* or *S. Enteritidis*, primary infection with *S. Virchow* offers only limited protection

against homologous re-challenge with *S. Virchow* or heterologous challenge with *S. Typhimurium*. This suggests developing effective vaccines to *S. Virchow* or multivalent cross-serogroup vaccines in the chicken may prove problematical. Nevertheless, the findings in this study present valuable information showing the immune responses produced by chickens against *S. Virchow* and could be built on to enable immunological preventative or therapeutic approaches against the serovar.

ACKNOWLEDGMENTS

We would like to thank the technicians in the Histology Laboratories, Veterinary Laboratory Services, School of Veterinary Science, University of Liverpool, with special thanks to Val Tilston, for their help with the histology and immunohistochemistry. We would also like to thank Catherine Hartley for her contribution to the poultry infection experiments and Stuart Carter for his contribution toward supervision of the research. Anne-Marie Salisbury was supported by a Doctoral Training Grant award from the Biotechnology and Biological Sciences Research Council (BB/D526537/1).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 June 2014; accepted: 19 September 2014; published online: 09 October 2014.

Citation: Salisbury A-M, Leeming G, Nikolaou G, Kipar A and Wigley P (2014) *Salmonella* Virchow infection of the chicken elicits cellular and humoral systemic and mucosal responses, but limited protection to homologous or heterologous re-challenge. *Front. Vet. Sci.* 1:6. doi: 10.3389/fvets.2014.00006

This article was submitted to *Veterinary Infectious Diseases*, a section of the journal *Frontiers in Veterinary Science*.

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