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Experimental infection of chickens by a flagellated motile strain of *Salmonella enterica* serovar Gallinarum biovar Gallinarum

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ABSTRACT

Salmonella enterica subsp. *enterica* serovar Gallinarum biovar Gallinarum (SG) causes fowl typhoid (FT), a septicaemic disease which can result in high mortality in poultry flocks. The absence of flagella in SG is thought to favour systemic invasion, since bacterial recognition via Toll-like receptor (TLR)-5 does not take place during the early stages of FT. In the present study, chicks susceptible to FT were inoculated with a wild type SG (SG) or its flagellated motile derivative (SG Fla$^+$). In experiment 1, mortality and clinical signs were assessed, whereas in experiment 2, gross pathology, histopathology, systemic invasion and immune responses were evaluated. SG Fla$^+$ infection resulted in later development of clinical signs, lower mortality, lower bacterial numbers in the liver and spleen, and less severe pathological changes compared to SG. The CD8$^+$ T lymphocyte population was higher in the livers of chicks infected with SG at 4 days post-inoculation (dpi). Chicks infected with SG had increased expression of interleukin (IL)-6 mRNA in the caecal tonsil at 1 dpi and increased expression of IL-18 mRNA in the spleen at 4 dpi. In contrast, the CD4$^+$ T lymphocyte population was higher at 6 dpi in the livers of birds infected with SG Fla$^+$. Therefore, flagella appeared to modulate the chicken immune response towards a CD4$^+$ T profile, resulting in more efficient bacterial clearance from systemic sites and milder infection.

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Introduction

Salmonella enterica subsp. *enterica* serovar Gallinarum biovar Gallinarum (SG) is a naturally aflagellate bacterium which causes fowl typhoid (FT), a severe systemic infection that can affect domestic fowl of all ages, producing high mortality rates (Shivaprasad and Barrow, 2008). The absence of flagella in SG may play a role in the pathogenesis of FT (Iqbal et al., 2005).

Flagellin, the main flagellar protein, is an immunogenic pathogen associated molecular pattern (PAMP) (Medzhitov, 2001; Akira and Takeda, 2004; Miao et al., 2007; Chen et al., 2009). *Salmonella* flagella activate Toll-like receptor (TLR)-5 on enterocyte surfaces (Iqbal et al., 2005; Subramanian and Qadri, 2006) triggering a cascade of cellular events, which lead to the secretion of cytokines and chemokines responsible for stimulating innate and adaptive immunity, mainly through activation of dendritic and T cells (McSorley et al., 2002; Iqbal et al., 2005; Salazar-Gonzalez and McSorley, 2005). Since SG does not express flagella, it would not be expected to stimulate TLR-5 during intestinal invasion (Iqbal et al., 2005). The lack of a strong early innate immune response may be permissive for systemic spread of SG (Chappell et al., 2009; Freitas Neto et al., 2013).

A mutant strain of SG, designated SG Fla$^+$, which expresses flagella, was less pathogenic and stimulated higher mRNA transcription of pro-inflammatory cytokines than the wild type SG in chicken kidney cells (CKCs) (Freitas Neto et al., 2013). This suggested that flagella might alter SG pathogenicity and induce a more efficient immune response. In the present study, this hypothesis was further investigated by infection of 5-day-old chicks from a commercial line of brown egg layers susceptible to FT.

Materials and methods

Bacterial strains

This study used the highly pathogenic strain 287/91 (SG), which produces >90% mortality in susceptible chickens (Thomson et al., 2008), and its derivative, the flagellated strain SG Fla$^+$ (Freitas Neto et al., 2013). SG and SG Fla$^+$ were cultured in lysogeny broth (LB) (Becton Dickinson) at 37 °C for 24 h at 150 revolutions per min (Berchieri et al., 2001). Expression of flagella was confirmed by swimming motility in semisolid agar (SSA), consisting of 0.9% heart infusion broth (Oxoid) and 0.25% LB agar (Becton Dickinson), and by phase contrast microscopy.

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Table 1
Primer sequences and qPCR standard curve data.

Target gene[a]	Sequence (5′→3′)[b]	Amplicon (bp)[c]	Efficiency[d]	Reference
IL-1β	F: GAAGTGCTTCGTGCTGGAGT R: ACTGGCATCTGCCCAGTTC	144	95.3%	Crhanova et al. (2011)
IL- 6	F: GATCCGGCAGATGGTGATAAA R: CGAAGTAAAGTCTCGGAGGATG	142	94.6%	This study
IL-18	F: ACGTGGCAGCTTTTGAAGAT R: GCGGTGGTTTTGTAACAGTG	88	107%	Rychlik et al. (2009)
CXCLi2 (prior IL-8)	F: ATGAACGGCAAGCTTGGAGCT R: GCAGCTCATTCCCCATCTT	94	91.6%	Rychlik et al. (2009)
iNOS	F: GAATACCCGCCAGAGATTATCC R: CAGAGCATACCACTTCAGATCC	90	91.8%	This study
GAPDH	F: GGCACGCCATCACTATC R: CCTGCATCTGCCCATTT	61	101.8%	De Boever et al. (2008)

[a] IL, interleukin; iNOS, inducible nitric oxide synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
[b] F, forward primer; R, reverse primer.
[c] bp, base pairs.
[d] Primers were decimally diluted (from 10^1 to 10^4) to derive the efficiency curves.

Experimental design

In the present study, two experiments were carried out in which chicks were inoculated with a wild type SG (SG) or its flagellated motile derivative (SG Fla$^+$). In experiment 1, mortality and clinical signs were assessed for 4 weeks. In experiment 2, gross pathology, histopathology, systemic invasion and immune responses were evaluated at different time points. The study used 214 1-day-old chicks from a commercial line of brown egg layers susceptible to FT. Birds were housed in metal cages in a temperature controlled room at Universidade Estadual Paulista, Jaboticabal, Brazil. All chicks received an antibiotic-free, balanced feed, with water provided ad libitum. Prior to inoculation, cloacal swabs were inoculated into selenite broth (Oxoid) containing 4 mg/mL novobiocin (SN^{Nov}), incubated at 37 °C for 24 h and plated onto brilliant green agar (Oxoid) to exclude infection with *Salmonella* spp. (Berchieri et al., 2001). Experiments were approved by the institutional ethical committee (approval number 023482/11; date of approval 18 November 2011).

Experiment 1: Clinical signs and mortality

Chicks were reared until 5 days of age and 60 birds were randomly selected and divided into two groups (A and B), each containing 30 birds, which were moved to a new acclimatised room. An additional 10 chicks were retained as uninfected controls in the clean, acclimatised room. Broth culture (0.2 mL) containing 1×10^6 colony forming units (CFUs) SG (group A) or SG Fla$^+$ (group B) was administered into the crop of each bird using oral gavage needles. Chicks in the uninfected control group were inoculated with 0.2 mL sterile LB. Clinical signs and mortality were recorded daily for 4 weeks. Birds that developed severe clinical signs of FT were killed by manual cervical dislocation.

Experiment 2: Systemic infection and immunity

Chicks (5 days of age; $n = 120$) were selected randomly, transferred to another acclimatised room and distributed into two groups (C and D), each comprising 60 birds. A third, uninfected, control group (E) containing 24 chicks was kept in the clean-acclimatised room. Birds in groups C and D were administered 0.2 mL (1×10^6 CFUs) SG or SG Fla$^+$, respectively, into the crop by oral gavage. Chicks from group E were mock inoculated with 0.2 mL sterile LB.

Pathology and microbiology

In experiment 2, five infected chicks were euthanased by cervical dislocation at 6 and 12 h post-infection (hpi) and at 1, 2, 4, 6 and 12 days post-infection (dpi). Gross lesions in the liver and spleen were recorded. Portions of tissue from the spleen and the right lobe of the liver were collected for bacterial enumeration (Berchieri et al., 2001). In addition, portions of the spleen, bursa of Fabricius and right lobe of the liver from five infected and three uninfected chicks were collected at 1, 2, 4, 6 and 12 dpi, fixed in neutral buffered formalin, embedded in paraffin wax, sectioned at 4 µm and stained with haematoxylin and eosin for histopathology.

Immunohistochemistry

Portions of the right lobe of the liver (4 and 6 dpi) and caecal tonsil (4 dpi) were collected from three chicks in the infected and uninfected groups in experiment 2 and stored at −80 °C for immunohistochemistry (Carvajal et al., 2008). Frozen sections (8 µm thickness) were incubated overnight either with the primary mouse anti-chicken antibody against the CD4 (8210-01, Southern Biotech) or CD8α (8220-01, Southern Biotech). Sections were then incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (DHRR-999, Spring Bioscience) and visualised using 3,3′-diaminobenzidine (Merck), counterstained with Harris-modified haematoxylin. Images (Eclipse Moticam, Nikon) were analysed using Image-Pro Plus (Media Cybernetics).

Quantitative reverse transcription PCR

Fragments of spleen (4 and 6 dpi) and the remaining caecal tonsil (1 dpi) were collected from three chicks in the infected and uninfected groups in experiment 2 and stored at −80 °C. Total RNA was purified using the RNeasy Mini Kit (Qiagen), quantified using the Nanodrop 2000 Spectrophotometer (Thermo Scientific) and reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). Interleukin (IL)-1β, CXCLi2 (previously known as IL-8) and IL-6 mRNA levels were measured in caecal tonsils, whereas inducible nitric oxide synthase (iNOS) and IL-18 mRNA were assessed in spleens, using oligonucleotide primers shown in Table 1. Reverse transcription quantitative PCR (RT-qPCR) was performed using a Real-CFX96 Touch Thermocycler (Bio-Rad) with 6.25 µL SYBR Green Jump Start *Taq* Ready Mix (Sigma-Aldrich), 0.6 pmol each oligonucleotide primer (Sigma-Aldrich), 50 ng template (cDNA) and ultra-pure water (Sigma-Aldrich) to a final volume of 12.5 µL. The cycling profile was 94 °C for 2 min, followed by 40 cycles at 94 °C for 15 s and 58 °C for 1 min. Each reaction was performed in triplicate and non-template controls were run in duplicate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as the reference gene for normalisation of Cq values, since 28S ribosomal RNA and β-actin were less stable or had lower efficiency.

Statistical analysis

Mortality rates were compared between infected groups using the Log-rank (Mantel–Cox) test and the accumulated deaths in experiment 1 were used to construct mortality curves. Statistical differences amongst viable bacteria numbers recovered from livers and spleens were determined using Tukey's test (Daniel, 1991). Differences between the proportions of CD4$^+$ or CD8$^+$ immunostained cells were evaluated using Fisher's exact test. $P < 0.05$ was considered to be statistically significant. Normalised RT-qPCR data were subjected to analysis of variance (ANOVA), followed by comparison amongst groups using Tukey's test. Differences were considered to be significant at $P < 0.05$ or $P < 0.01$. All statistical analyses were performed using the GraphPad Prism software 5.

Results

Clinical signs

In experiment 1, mortality was significantly lower in chicks infected with SG Fla$^+$ (7/30, 23%) than chicks infected with SG (12/30, 40%; $P < 0.05$; Fig. 1). Clinical manifestations, including greenish-yellow diarrhoea, anorexia, prostration, ruffled feathers and decreased feed and water consumption, were first observed at 4 dpi in SG-infected chicks (group A) and persisted to 14 dpi. Milder clinical signs appeared at 6 dpi in the SG Fla$^+$-infected animals (group B); these also persisted to 14 dpi. Chicks of the uninfected group remained healthy throughout the experiment.

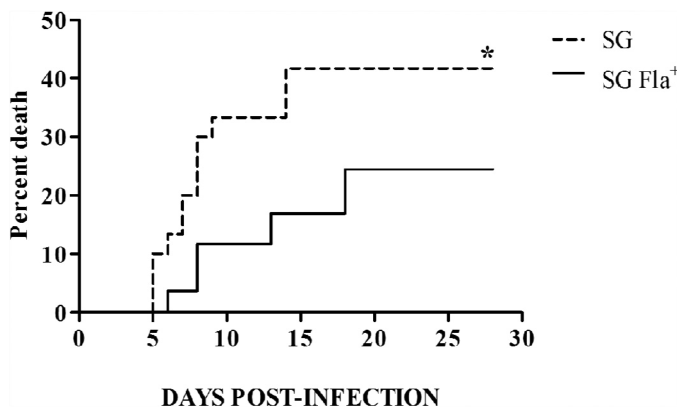

Fig. 1. Mortality curves from total deaths of 5-day-old chickens infected with the wild type *Salmonella enterica* serovar Gallinarum biovar Gallinarum (SG) or the flagellated SG mutant strain (SG Fla⁺). Asterisk indicates significant differences between mortality curves of infected birds by log-rank test (*$P < 0.05$).

Gross pathology and systemic infection

In chicks infected with SG (group C) in experiment 2, lesions typical of FT, including hepatosplenomegaly, were observed in 2/5 chicks at 2 dpi and in all chicks at 4 and 6 dpi. In chicks infected with SG Fla⁺, gross lesions were less severe and appeared at 4 dpi in 3/5 birds, whilst 3/5 birds exhibited gross lesions at 6 dpi. No gross pathology was observed at 6, 12 and 24 hpi, nor at 12 dpi.

There were no significant differences in bacterial counts between groups ($P > 0.05$; Table 2). At 2 dpi, the inoculated strain was recovered from the livers and spleens of two SG-infected chicks (group C), but not from SG Fla⁺-infected chicks (group D). At 4 and 6 dpi, the highest bacterial counts were reached for the liver and spleen of five chicks from group C and three from group D. At 12 dpi, inoculated strains were isolated from the liver and spleen of two SG-infected chicks and one SG Fla⁺-infected chick. No bacteria were recovered from any organs at 6, 12 and 24 hpi.

Histopathology

In experiment 2, histopathological changes were severe at 4 and 6 dpi in chicks infected with SG (group C), including multifocal necrotising hepatitis (Fig. 2A), necrotising splenitis (Fig. 2D) and lymphoid depletion and heterophilic infiltration in the bursa of Fabricius (Fig. 2G). In chicks infected with SG Fla⁺ (group D), lesions were less severe at 4 and 6 dpi (Figs. 2B, E, H). No histopathological changes were observed in the liver, spleen or bursa of Fabricius of uninfected chicks (Figs. 2C, F, I).

Immunohistochemistry

In experiment 2, changes in the CD4⁺ and CD8⁺ T cell populations were measured in caecal tonsils and livers (Table 3; Figs. 3,4). At 4 dpi, the CD8⁺ T lymphocyte population was higher in the livers of chicks infected with SG (group C) than in chicks infected with SG Fla⁺ (group D) and in uninfected birds (group E) ($P < 0.05$). At 4 dpi, the CD4⁺ T lymphocyte population was higher in the caecal tonsils of birds in group D than birds in group E ($P < 0.05$). At 6 dpi, there was a significantly higher CD4⁺ T population in livers of chicks in group D compared to birds in groups C and E ($P < 0.05$).

Cytokine mRNA transcription in spleen and in caecal tonsil by RT-qPCR

Data on cytokine expression in birds in experiment 2 are shown in Fig. 5. At 1 dpi, there were significantly higher levels of IL-6 mRNA (Fig. 5A) in the caecal tonsils of chicks infected with SG (group C) in comparison with chicks infected with SG Fla⁺ (group D) and uninfected birds (group E) ($P < 0.01$). However, no statistically significant changes were detected in the levels of CXCLi2 mRNA (Fig. 5B) or IL-1β (see Appendix: Supplementary Fig. S1) in caecal tonsils at 1 dpi ($P > 0.05$). At 4 dpi, levels of IL-18 mRNA were higher in the spleens of chicks infected with SG compared to chicks infected with SG Fla⁺ ($P < 0.05$; Fig. 5C). At 6 dpi, there were no significant differences in levels of IL-18 mRNA in spleens of infected and uninfected birds ($P > 0.05$; Fig. 5E). There were no significant differences in iNOS mRNA levels in spleens at 4 and 6 dpi ($P > 0.05$; Figs. 5D, F).

Discussion

The absence of flagella in SG is considered to be one of the factors related to the difference between the pathogenesis of FT and fowl paratyphoid provoked by *S. enterica* serovar Enteritidis (Freitas Neto et al., 2013). It has been postulated that SG does not fully stimulate the innate immune system at the intestinal mucosa and that this facilitates the ability of the bacterium to cross the intestinal barrier, producing severe systemic disease (Kaiser et al., 2000; Iqbal et al., 2005; Chappell et al., 2009). In order to further test this

Table 2
Bacterial counts ($\log_{10}$ colony forming units/g) from livers and spleens of 5-day-old chickens infected with SG and SG Fla⁺ at different time points post-infection.

Strain	Chick numbers	Days post-inoculation (dpi)							
		2		4		6		12	
		Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen
SG	1	–	–	6.40	5.66	7.04	6.79	–	–
	2	–	–	5.04	4.79	6.99	6.96	2.00	2.00
	3	3.32	2.00	5.83	4.56	5.65	5.20	2.00	2.00
	4	–	–	6.04	5.32	6.53	6.41	–	–
	5	2.00	2.00	6.34	6.28	7.10	7.00	–	–
Mean ± SD[a]		1.06 ± 1.53[a]	0.80 ± 1.10[a]	5.93 ± 0.55[a]	5.32 ± 0.69[a]	6.66 ± 0.61[a]	6.47 ± 0.75[a]	0.80 ± 1.10[a]	0.80 ± 1.10[a]
SG Fla⁺	1	–	–	5.08	4.66	6.38	6.26	–	–
	2	–	–	–	–	5.11	5.04	–	–
	3	–	–	5.81	5.18	6.30	6.13	–	–
	4	–	–	–	–	–	–	6.23	6.32
	5	–	–	5.89	5.04	–	–	–	–
Mean ± SD[a]		–	–	3.36 ± 3.08[a]	2.98 ± 2.72[a]	3.56 ± 3.29[a]	3.49 ± 3.22[a]	1.25 ± 2.79[a]	1.26 ± 2.83[a]

[a] Mean ± SD, arithmetic mean ± standard deviation of bacterial counts in organs.
Bacterial count averages were compared between the two groups of infection in the same column.
Bacteria were not recovered from organs at 6 and 12 hpi and at 1 dpi.
hpi, hours post-infection; dpi, days post-infection; SG, wild type *Salmonella* serovar Gallinarum biovar Gallinarum; SG Fla⁺, flagellated SG mutant strain.

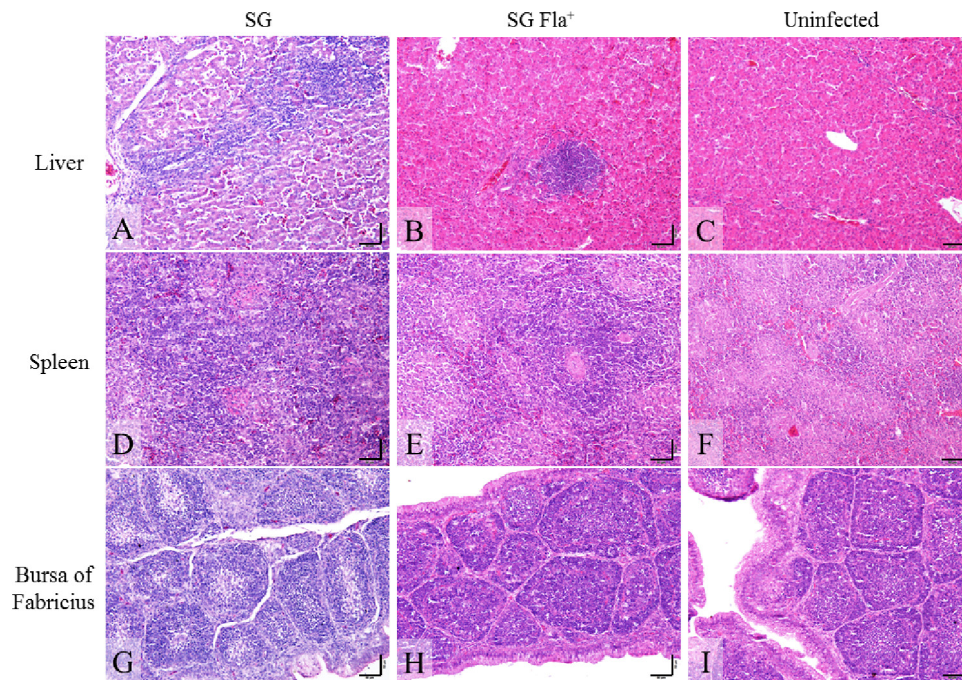

Fig. 2. Photomicrographs of liver, spleen and bursa of Fabricius of *Salmonella* infected and uninfected chicks, showing perivascular inflammation (A), necrosis (D) and lymphoid depletion (G) in chickens infected with wild type *Salmonella* serovar Gallinarum biovar Gallinarum (SG). Chickens infected with the flagellated SG mutant strain (SG Fla^{+}) exhibited lymphoid aggregates (B), hyperplasia of white pulp (E) and discrete lymphocytic depletion (H). No pathological changes were found in uninfected chicken tissues (C, F and I). Haematoxylin and eosin. Scale bar = 50 µm.

Table 3
Results on CD4^{+} and CD8^{+} T lymphocyte cells in caecal tonsils and in livers of 5-day-old chickens infected with SG or SG Fla^{+}.

dpi	Organ	Immunostained cells (%)					
		CD4+			CD8^{+}		
		SG	SG Fla^{+}	Control	SG	SG Fla^{+}	Control
4	Caecal tonsil	7.8 ± 2.9^{ab}	14.6 ± 6.1^{a}	1.6 ± 2.0^{b}	8.6 ± 4.1^{a}	5.9 ± 4.7^{a}	2.1 ± 1.7^{a}
	Liver	2.8 ± 3.5^{a}	4.5 ± 5.7^{a}	1.1 ± 1.4^{a}	14.2 ± 11.8^{b}	3.2 ± 1.5^{a}	2.0 ± 1.0^{a}
6	Liver	0.9 ± 0.9^{a}	8.9 ± 5.9^{b}	0.0 ± 0.0^{a}	4.4 ± 4.2^{a}	2.7 ± 1.8^{a}	1. ± 1.8^{a}

Results are expressed as the arithmetic mean of different sections followed by standard deviation.
Different letters for each cell population, by day and by organ, represent statistical significance between groups (Fisher's exact test: $P < 0.05$).
dpi, days post-infection; SG, wild type *Salmonella* serovar Gallinarum biovar Gallinarum; SG Fla^{+}, flagellated SG mutant strain.

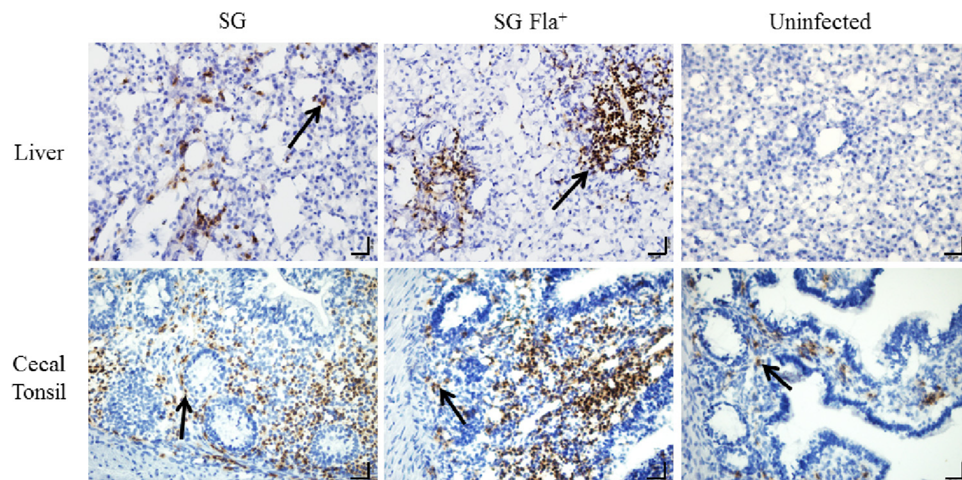

Fig. 3. Immunohistochemical detection of CD4^{+} T lymphocytes in livers (6 dpi) and caecal tonsils (4 dpi) of *Salmonella*-infected and uninfected chicks. Chicks infected with SG Fla^{+} exhibit higher proliferation of CD4^{+} T cells in the liver and caecal tonsil compared to SG-infected and uninfected chicks. Anti-chicken CD4^{+} immunostaining (brown, indicated by black arrow) counterstained with haematoxylin. Scale bar = 20 µm. dpi, days post-infection; SG, wild type *Salmonella enterica* serovar Gallinarum biovar Gallinarum; SG Fla^{+}, flagellated SG mutant strain.

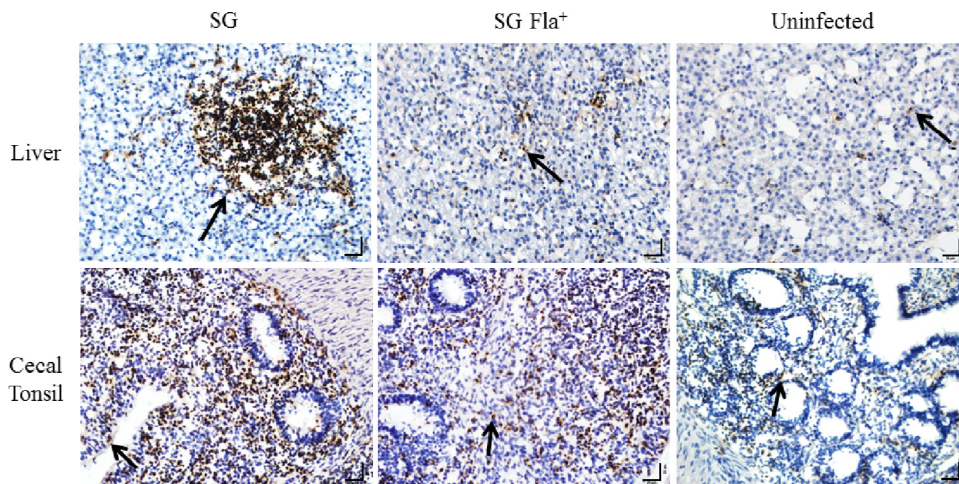

Fig. 4. Immunohistochemistry for CD8+ T lymphocytes in the liver (6 dpi) and caecal tonsils (4 dpi) of *Salmonella*-infected and uninfected chicks. Chicks infected with SG had higher levels of CD8+ T lymphocytes in the liver than SG Fla+ infected and uninfected chicks. Anti-chicken CD8+ immunostaining (brown, indicated by black arrow) counterstained with haematoxylin (blue). Scale bar = 20 µm. dpi, days post-infection; SG, wild type *Salmonella enterica* serovar Gallinarum biovar Gallinarum; SG Fla+, flagellated SG mutant strain.

hypothesis, the present study was conducted by comparing the pathogenicity and immune responses triggered by the flagellated mutant of SG (SG Fla+) and the wild type strain (SG) in a line of chicks susceptible to FT.

Mortality resulting from SG infection was higher than that caused by SG Fla+. Birds from both infected groups demonstrated clinical signs of FT, although the clinical manifestations were milder and later in SG Fla+-infected chicks. Similarly, Freitas Neto et al. (2013) reported that chicks infected with SG Fla+ experienced lower mortality and milder clinical signs than those infected with the parental SG strain. Thus, we speculate that the reduced virulence of SG Fla+ is related to the acquisition of flagella.

In this study, the innate response elicited by SG Fla+ was more efficient in controlling the initial bacterial invasion than that induced by SG. SG Fla+ reached the liver and spleen later (at 4 dpi) and was recovered from a smaller number of birds than SG. In addition, gross pathological changes were milder and observed later (at 6 dpi) in the livers and spleens of three chicks infected with SG Fla+.

IL-6 is involved in acute phase responses, immune regulation and activation of macrophages (Kaiser and Staheli, 2008). Previous studies have reported over-expression of IL-6 gene in caecal tonsils and caeca of *S. enterica* serovar Typhimurium-infected chicks at 1 dpi, when compared to non-flagellated mutant strains (Iqbal et al., 2005; Pan et al., 2012). Conversely, data presented here have shown that the levels of IL-6 mRNA transcription were higher in the caecal tonsils of birds challenged with the non-flagellated SG strain than those challenged with SG Fla+. The host–pathogen interaction between SG and birds is likely to be different from that observed with *S. enterica* serovar Typhimurium, and this may be connected to this higher transcription of IL-6 mRNA in the caecal tonsils of SG-infected birds.

Data from in vitro studies, where epithelial cells were infected, have shown slightly different patterns of pro-inflammatory cytokines and chemokines. Kaiser et al. (2000), using CKCs, reported that invasion of *S. enterica* serovars Typhimurium and Enteritidis caused an 8- to 10-fold increase in IL-6 gene expression, whilst invasion by SG did not result in an increase. Using the same approach, Freitas Neto et al. (2013) reported that SG Fla+ induced higher levels of CXCLi2, IL-6 and iNOS mRNAs than the SG parent strain. However, higher levels of IL-6 mRNA in the caecal tonsils of SG-infected birds suggested that the pro-inflammatory immune response elicited in lymphoid tissue has a different pattern from that observed in vitro with SG-infected epithelial cells and may involve different cell types. A similar picture was observed in epithelial cell lines infected with *S. enterica* serovar Typhi, which triggered antagonistic patterns of IL-8 expression in the early stages of infection (Winter et al., 2008; Fiorentino et al., 2013).

SG induced higher levels of IL-18 mRNA expression in the spleen at 4 dpi in comparison with uninfected and SG Fla+-infected birds. At this time point, chicks infected with SG had clinical signs of FT and more severe histopathological changes than those infected with SG Fla+, suggesting that IL-18 over-expression could be related to the severity of disease. IL-18 promotes inflammation and enhances CD8+ T lymphocyte proliferation and cytotoxic activity (Eckmann and Kagnoff, 2001; Raupach et al., 2006; Ghose et al., 2011).

Salmonella spp. flagellin enhances antigen specific CD4+ T cell expansion and memory development in vivo (McSorley et al., 2000, 2002; Salazar-Gonzalez and McSorley, 2005). In the present study, higher numbers of CD4+ T cells were present in the caecal tonsils and liver of chicks challenged with SG Fla+.

Conclusions

In this study, SG Fla+ modulated the immune response of chicks towards a CD4+ T profile, resulting in more efficient bacterial clearance from systemic sites and milder infection. Chickens infected with SG exhibited a CD8+ T profile, which correlated with systemic infection, more severe lesions and higher mortality.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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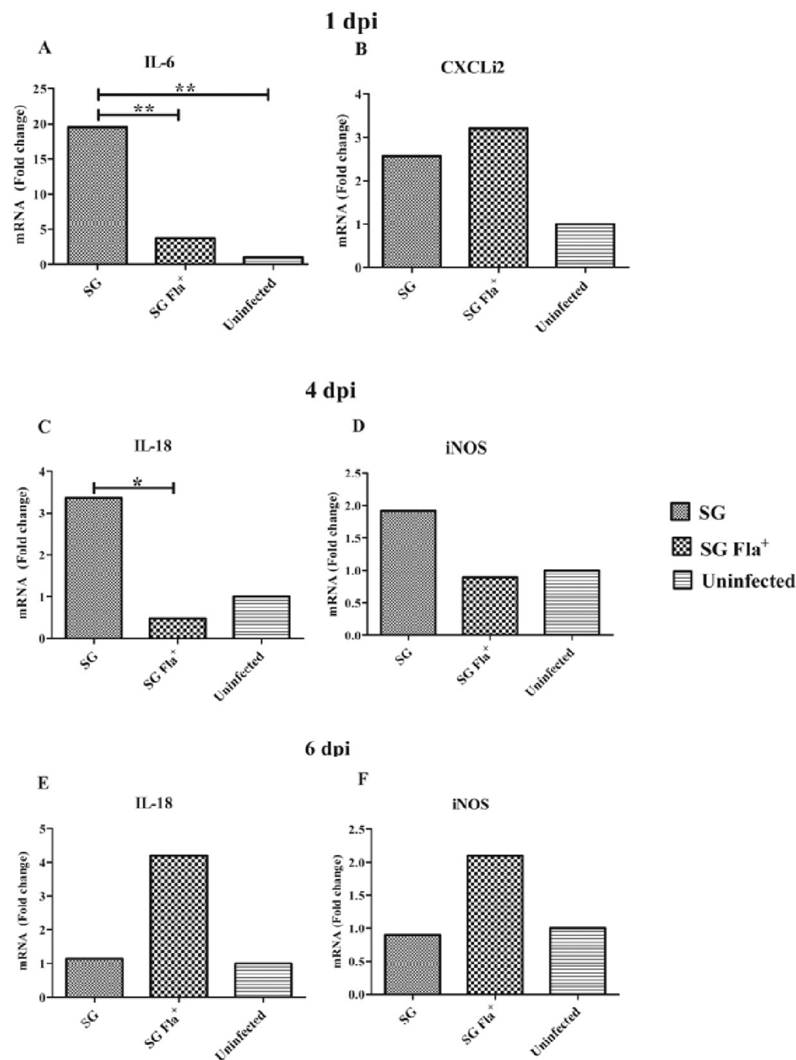

Fig. 5. Expression of CXCLi2 (A) and IL-6 mRNA (B) in caecal tonsils at 1 dpi, and IL-18 and iNOS in the spleens at 4 dpi (C and D, respectively) and at 6 dpi (E and F, respectively) of 5-day-old chicks. SG-infected chicks had higher IL-6 levels in caecal tonsils than chicks infected with SG Fla+ and uninfected chicks. At 4 dpi, IL-18 levels were higher in the spleens of SG infected chicks than SG Fla+ infected chicks. No significant differences were observed for other mRNA levels measured. Cytokine expressions are presented as fold-changes. Statistical significance in relation to the uninfected chicks: $^{*}P < 0.05$, $^{**}P < 0.01$. dpi, days post-infection; SG, wild type *Salmonella enterica* serovar Gallinarum biovar Gallinarum; SG Fla+, flagellated SG mutant strain.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.tvjl.2016.05.006.

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