Immune modulation of *Salmonella enterica* serotype Pullorum in the chicken

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Thesis submitted to the University of Nottingham for

the degree of Doctor of Philosophy

July 2016

Abstract

Salmonella enterica infection affects a wide range of animals including humans. The avian specific serotype *S.* Pullorum infection produces systemic disease followed by a persistent carrier state in convalescence birds. Vaccination and other control strategies require an improved understanding of the immunity in response to *S.* Pullorum infection. This study compared the different immune dynamics following infection with (persistent) *S.* Pullorum and related (non-persistent) serovars *S.* Enteritidis and *S.* Gallinarum using co-culture of *Salmonella*-infected macrophages and CD4$^+$ T lymphocytes *in vitro* and 2-day-old chickens *in vivo*. In comparison with *S.* Enteritidis, macrophages infected with *S.* Pullorum had a reduced gene expression of pro-inflammatory cytokines CXCLi2, IL-6, iNOS, IFN-γ, IL-12α and IL-18 and lower level of nitrite production. *S.* Pullorum-infected macrophages were found to be less effective than *S.* Enteritidis in stimulating the CD4$^+$ lymphocytes to proliferate *in vitro*. CD4$^+$ lymphocytes in co-culture with *Salmonella*-infected macrophages also produced lower levels of IFN-γ and IL-17F mRNA in response to *S.* Pullorum compared with *S.* Enteritidis. *S.* Pullorum infection in 2-day-old chickens stimulated proliferation of Th2-like lymphocytes with reduced IFN-γ and IL-17F but increased IL-4, IL-13 and IL-10 in the caecal tonsils and spleens when compared to *S.* Enteritidis. However, the modulation by *S.* Pullorum is not likely to be related to its large virulence plasmid, although the virulence plasmid of *S.* Gallinarum was shown to reduce nitrite production and gene expression of IL-1β and iNOS in infected HD11 cells. Our data showed no

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evidence of clonal anergy or immune suppression induced by *S*. Pullorum *in vitro*.

The experimental work thus shows that the response to *S*. Pullorum infection was characterised by a modulation on host immunity from a dominant IFN-γ-producing Th17 response towards a Th2-like response which may promote persistent infection in chickens.

This study provides insights into mechanisms by which *S*. Pullorum evades host immunity and produces the persistent carrier state. This opens the possibility for therapeutic application of cytokines to restore the host protective immune response to eliminate infection.

Declaration

I declare that the work presented in this thesis is my own work and no part has been submitted for any other degree at the University of Nottingham or elsewhere.

Ying Tang

Conferences

Oral presentation

- Ying Tang, Neil Foster, Michael Jones, Paul Barrow, 2015. *In vitro* immune modulation of *Salmonella enterica* serotype Pullorum. Society for General Microbiology (SGM) annual conference 2015, Birmingham, UK. 30th March-1st April, 2015.

- Ying Tang, Neil Foster, Michael Jones, Paul Barrow, 2015. *In vitro* immune modulation of *Salmonella enterica* serotype Pullorum. 69th The Association for Veterinary Teaching and Research Work (AVTRW) Annual Conference. The Royal Veterinary College, UK. 2nd-3rd September, 2015.

Poster presentation

- Ying Tang, Neil Foster, Michael Jones, Paul Barrow, 2015. In vitro immune modulation of Salmonella enterica serotype Pullorum. Global Alliance for Research on Avian Diseases (GARAD) conference, London, UK, 29thJune – 1st July 2015.

- Ying Tang, Neil Foster, Michael Jones, Paul Barrow, 2015. In vitro immune modulation of Salmonella enterica serotype Pullorum. Third Nottingham Immunology Showcase, University of Nottingham, UK. 4th February, 2016.

- Ying Tang, Neil Foster, Michael Jones, Paul Barrow, 2015. In vitro immune modulation of Salmonella enterica serotype Pullorum. Society for General Microbiology (SGM) annual conference 2016, Liverpool, UK. 21st-24th March, 2016.

Acknowledgement

Firstly my deepest gratitude goes to my supervisor Professor Paul Barrow for stepping in to supervise me and leading me in completion of this PhD project. Without your invaluable guidance and encouragement helping me throughout the venture step by step the work presented in this thesis would not have been possible. Thanks for constant reassurance over the last four years and for every tutorial and every meeting that are special inspiring and always valuable.

Thanks also to my co-supervisors Dr Neil Foster and Dr Michael Jones for your tremendous supports throughout my study. Thank you for every suggestion pushing me further than I thought I could go.

I gratefully acknowledge the financial support from the Chinese Scholarship Council and the University of Nottingham that funded this project.

Finally, I would like to say a huge thank you to my parents and Dr Hongyang Yu for their unwavering support and understanding.

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Abbreviation

°C	Centigrade degrees
β-ME	β-mercaptoethanol
μg	Microgram
μl	Microlitre
μm	Micromitre
μM	Micromole
ANOVA	Analysis of variance
AP-1	Activator protein 1
APC	Antigen-presenting cell
BSA	Bovine serum albumin
C3	Complement 3
cDNA	Complementary DNA
CE	Competitive exclusion
CFU	Colony-forming unit
chMDM	Chicken peripheral blood monocyte-derived macrophages
CKC	Chicken kidney cells
Con A	Concanavalin A
CR3	Complement receptor 3
Ct	Crossing threshold
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
d	Day
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorter
FAM	5-carboxyfluorescein
Fc	Fragment crystallisable
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
FSC	Forward scatter
FT	Fowl typhoid
GALT	Gut-associated lymphoid tissues
gDNA	Genomic DNA
GM-CSF	Granulocyte macrophage colony-stimulating factor

h	Hour
HD11	Avian macrophage-like cell line
IFN	Interferon (e.g., IFN-γ)
Ig	Immunoglobulin
IL	Interleukin (e.g., IL-2)
iNOS	Inducible nitric oxide synthase
LITAF	LPS-induced TNF-α factor
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MALT	Mucosa-associated lymphoid tissues
MCP-1	Monocyte chemotactic protein 1
MHC	Major histocompatibility complex
min	Minute
MIP	Macrophage inflammatory proteins
ml	Millilitre
MLN	Mesenteric lymph nodes
MOI	Multiplicity of infection
mRNA	Messenger RNA
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear factor κB
NO	Nitric oxide
NRAMP1	Natural resistance associated macrophage protein 1
nTreg	Natural regulatory T cells
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PD	Pullorum disease
PD1	Programmed death-1
PE	Phycoerythrin
PI	Propidium iodide
pi	Post infection
PMN	Polymorphonuclear leukocyte
PRR	Pattern recognition receptor
PT	Paratyphoid
PT4	Phage type 4
qRT-PCR	Quantitative real-time polymerase chain reaction

QTL	Quantitative trail loci
RNA	Ribonucleic acid
RNI	Nitrogen intermediates
ROS	Reactive oxygen species
rpm	Revolutions per minute
s	Second
SCV	*Salmonella*-containing vacuole
SEM	Standard error of mean
SPI	*Salmonella* pathogenicity island
spv	*Salmonella* plasmid virulence
SSC	Side scatter
TCR	T cell receptor for antigen
TGF	Transforming growth factor (e.g. TGF-β4)
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor (e.g. TNF-a)
Treg	Regulatory T cell
TTSS	Type Three Secretion Systems
U	Unite
v/v	volume/volume
Vi	Virulence

Chapter 1 Introduction

1. 1   General introduction

1. 1. 1   Classification

Salmonella are Gram-negative bacteria which are members of the family Enterobacteriaceae comprised of a large number of evolutionary-related and biochemically similar pathogenic or potentially pathogenic taxa including the genera *Salmonella, Shigella, Escherichia, Klebsiella, Citrobacter,* and *Proteus*. *Salmonella enterica* (type strain LT2) is the only species of the genus *Salmonella* with new subspecies: *Salmonella enterica* subsp. *arizonae*, *Salmonella enterica* subsp. *bongori*, *Salmonella enterica* subsp*. enterica*, *Salmonella enterica* subsp. *diarizonae*, *Salmonella enterica* subsp. *houtenae, Salmonella enterica* subsp. *indica and Salmonella enterica* subsp. *salamae.* (Le Minor and Popoff, 1987). This has been superseded by a classification involving three species, namely *Salmonella enterica*, *Salmonella bongori* (Brenner and McWhorter-Murlin, 1998) and *Salmonella subterraneae* (Shelobolina *et al.*, 2004). *S. enterica* contains most pathogens that affect human and animal health and is further divided into six subspecies including *enterica, salamae, arizonae, diarizonae, houtenae, and indica* (Brenner *et al.*, 2000). Thus, for example, the former *Salmonella* Pullorum is now designated *Salmonella enterica* subspecies *enterica* serovar Pullorum, which may be shortened to *Salmonella* Pullorum or *S.* Pullorum (Brenner *et al.*, 2000). Biotype and phage type may also be applied to subdivide *Salmonella* serovars

further. Biotypes within a serovar are differentiated by biochemical variation while the differential susceptibilities to lytic bacteriophages can classify microorganisms within the same serovar into the same or different phage type (Ward *et al.*, 1987).

1. 1. 2 *Salmonella* serovars

More than 2600 serovars of *Salmonella* have been described with most of these belonging to the species *S. enterica* (Bugarel *et al.*, 2012). The Kauffmann-White scheme groups *Salmonella* serovars serologically by antigenic variability of the lipopolysaccharides (O antigens) which is the main constituent of outer membrane of Gram-negative bacteria, flagellar proteins (H antigens), and capsular polysaccharides (Vi antigens) (Grimont and Weill, 2007). Alternative methods involving probing the DNA of causative strains at a taxonomic level are now beginning to show an advantage over the traditional serotyping (Wattiau *et al.*, 2011).

From the point of view of infection biology *S. enterica* can be subdivided into two categories (Barrow, 2007). The first group comprises a small number of serovars which are adapted to a narrow range of host species and generally produce severe, typhoid-like disease sometimes with high mortality. These serovars include *S.* Gallinarum and *S.* Pullorum in poultry, *S.* Dublin in cattle and *S.* Typhi in humans. The second group includes the remaining serovars which colonise the intestine of a wide range of unrelated host species resulting in entry into the food chain and causing a self-limiting gastroenteritis

in humans. *S.* Typhimurium and *S.* Enteritidis belong to this group with infection either remaining restricted to the gastrointestinal tract or in some circumstances became systemic. However, *S.* Typhimurium and *S.* Enteritidis can produce typhoid-like infections in mice.

1. 1. 3   *Salmonella* infections in poultry

Salmonellosis is a frequent disease of poultry which affects the poultry industry throughout the world and is a significant source of zoonotic infections through the consumption of contaminated eggs and meat.

1. 1. 3. 1   Paratyphoid infections

Numerous motile *Salmonella* serovars are capable of producing paratyphoid (PT) infections in a wide range of hosts including domestic fowl/chickens. *S.* Typhimurium and *S.* Enteritidis are among the more common serovars isolated which are associated with PT infection in fowl. PT infections are largely confined to the lower gastrointestinal tract with faecal excretion (Gast, 1997). Systemic infection with these serovars is usually more transient and resolved through cellular immunity within 2-3 weeks (Barrow *et al.*, 2004, Beal *et al.*, 2004a) but serious systemic infections can occur in highly susceptible young chickens or when subjected to stressful conditions (Barrow, 2000). For example, infection with *S.* Typhimurium in one-day-old chickens can lead to enteric infection and systemic disease with a high mortality rate whereas infection of older birds generally results in asymptomatic caecal colonisation, with persistent shedding of the organisms in faeces (Barrow *et*

al., 1987a). Newly hatched chickens infected with *S.* Enteritidis can also produce persistent infection with faecal shedding until onset of lay (Berchieri *et al.*, 2001b, Van Immerseel *et al.*, 2004).

Both *S.* Enteritidis and *S.* Typhimurium infections produce an intense pathological consequence that is likely to provoke a strong and rapid immune response to clear the infection. *S.* Enteritidis is able to colonise the reproductive tract of infected chickens more effectively than *S.* Typhimurium (De Buck *et al.*, 2004). *S.* Enteritidis colonises the reproductive tissues of laying hens leading to the production of eggs with contaminated contents and/or shells, depending on strain variation and the route of infection (Barrow and Lovell, 1991, Humphrey *et al.*, 1991). *S.* Enteritidis has replaced *S.* Typhimurium and currently dominates poultry and egg-borne salmonellosis, resulting in an epidemic of human food poisoning outbreaks in the UK but which is now largely under control (Wales and Davies, 2011).

1. 1. 3. 2   Typhoid infections (Fowl typhoid and Pullorum disease)

Fowl typhoid (FT) and Pullorum disease (PD) are two distinct septicaemic diseases of avian species caused by host-adapted serovars *S.* Gallinarum and *S.* Pullorum respectively, which are non-motile serovars of *S. enterica* and have adapted to induce disease exclusively in chickens. Both *S.* Pullorum and *S.* Gallinarum possess the lipopolysaccharide (LPS) O antigens 1, 9 and 12. Both are serologically identical but are thought to have evolved separately from a descendent of *S.* Enteritidis mainly by gene deletion events (Thomson *et al.*,

2008). The three serovars Pullorum, Gallinarum and Enteritidis are phylogenetically closely related (Li *et al.*, 1993).

Both FT and PD cause substantial economic losses to the poultry industry throughout many parts of the world. FT usually affects adult birds, although birds of all ages and breeds may be susceptible. Experimental infection of three weeks old chicken with *S.* Gallinarum resulted in severe fowl typhoid and a mortality of 60% (Jones *et al.*, 2001). PD is an acute systemic disease causing high mortality rates among young birds (Shivaprasad and Barrow, 2008) with those convalescent birds becoming carriers of the disease (Wigley *et al.*, 2001).

PD and FT are reported to be eradicated from commercial poultry in many developed countries, but may not be diagnosed properly such that the incidence of disease is possibly underestimated given the many wild avian species which can be infected by these serovars. Several European countries have witnessed outbreaks of both PD and FT in chickens, either in commercial layer or backyard flocks, up until 2008 (Barrow and Freitas Neto, 2011). The last case of FT in the United States (U.S.) was reported in 1981 but PD was detected in commercial flocks up until 2009 (http://www.aphis.usda.gov/vs/nahss/disease_status.htm#avian). PD remains important in areas of the world where intensive poultry industry is developing and may also become a more important issue due to the ever-increasing popularity of free-range farming.

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Antimicrobials remain in use to reduce mortality associated with PD and FT in many countries, but are not be able to eliminate infection. Uncontrolled or excessive use of antibiotics leads to antimicrobial resistance (AMR) which can then spread into the humans via the food chain (Tollefson and Miller, 2000). For example, 258 Korean isolates of *S.* Gallinarum in 2001 showed resistance to enrofloxacin (6.5%), ofloxacin (82.6%), ampicillin (13.0%), gentamicin (43.4%) and kanamycin (69.6%), compared to a much greater level of susceptibility of all isolates from 1995 (Lee *et al.*, 2003). Multi-resistant strains to three or more antimicrobials accounted for 63.8% out of 105 isolates of *S.* Gallinarum in Korea between 2002 and 2007 (Kang *et al.*, 2010). There was also a high level of resistance to ampicillin, carbenicillin, streptomycin, tetracycline, trimethoprim and sulfafurazole found among 450 isolates of *S.* Pullorum from diseased chicken in China from 1962 to 2007 with 56.2% of them displaying resistance to 4 or more antimicrobials (Pan *et al.*, 2009). In another study, the multi-resistant strains of *S.* Pullorum reached 96.6% of isolates between 1990 and 2010 in eastern China (Gong *et al.*, 2013). Antimicrobial resistance in *S*. Pullorum was most commonly detected to Tetracycline (Lynne *et al.*, 2009).

S. Pullorum typically produces typhoid-like infections in chickens which also generally develops into a disease-free persistent carrier state in convalescent chickens (Wigley *et al.*, 2001). This is a feature shared with some of the other host-specific *Salmonella enterica* serovars including *S.* Typhi in humans, *S.* Dublin in cattle and *S*. Abortusovis in sheep (Wray and Sojka, 1977, House *et*

al., 2001, Uzzau, 2013). Unlike PT infections with *S.* Typhimurium, *S.* Pullorum colonises the gut poorly with bacterial numbers falling during the first 4 days post- challenge due to migration from the intestine to more deeper tissues (Henderson *et al.*, 1999). *S.* Pullorum can be recovered from liver, spleen, caeca, lungs, heart, pancreas, yolk sac, synovial fluid, and reproductive organs of infected chickens (Shivaprasad and Barrow, 2008). In convalescent chickens, a small number of viable *S.* Pullorum persist in the spleen despite the presence of a high titre, specific IgY response and T lymphocyte proliferation (Berchieri *et al.*, 2001a, Wigley *et al.*, 2001, Wigley *et al.*, 2005b), suggesting an intracellular niche. This was demonstrated to be mainly within macrophages of the spleen, which may protect *S.* Pullorum from the antibody response (Wigley *et al.*, 2001). When females come into lay, the increased concentration of female sex hormones reduces T cell responsiveness, resulting in recrudescence of systemic infection and spread of *Salmonella* to the reproductive tissue. This leads to vertical transmission through the developing eggs, from which infected progeny hatch spreading infection horizontally (Wigley *et al.*, 2001). In contrast, the bacteria remain at a low level in male birds during the course of infection and are eventually eliminated after several months (Berchieri *et al.*, 2001a, Wigley *et al.*, 2001, Wigley *et al.*, 2005b). The infected progeny, which carry *S.* Pullorum in the hatchery and intensive poultry units, transmit the infection horizontally (Lister and Barrow, 2008).

In contrast, *S.* Gallinarum reveals a markedly different pathology and infection biology, producing severe systemic disease in mature birds. This results in either high mortality, in susceptible birds, or with bacterial clearance in resistant birds within three to four weeks of the initial infection, although persistent infection in resistant lines can occur occasionally (Berchieri *et al.*, 2001a, Wigley *et al.*, 2002a, Wigley, 2004). *S.* Gallinarum can be found in the gastrointestinal tract either in the early phase after oral infection or in the final stage before the birds die, when the bacteria are shed into the lumen from clusters of lymphoid tissues in the wall of the intestine (Barrow *et al.*, 1994, Wigley *et al.*, 2002a). Moreover, although *S.* Gallinarum has been described as being vertically transmitted in some older reports (Shivaprasad and Barrow, 2008), infection of the oviduct and transmission to eggs rarely occurs with this serovar (Berchieri *et al.*, 2001a). Berchieri *et al.* (2001a) suggested a role for the host genetic background in the inbred lines where this characteristic was expressed.

In humans, typhoid fever is a systemic disease caused primarily by the strictly human-adapted serovar *S.* Typhi. Chronically infected individuals are the reservoirs for the spread of infection with bacterial shedding for periods of time that range from a year to a lifetime (Vogelsang and Boe, 1948). In 2000, there were an estimated 21.7 million cases and 217,000 deaths globally (Crump *et al.*, 2004). In 2010, there were still an estimated total number of 13.5 million typhoid fever episodes worldwide (Buckle *et al.*, 2012). *S*. Dublin, which is closely related to *S*. Gallinarum, *S*. Pullorum and *S*. Enteritidis, is a

major cause of enteritis and systemic diseases in cattle, particularly dairy cattle in which pathology may be exacerbated by parasites such as Liver Fluke (La Ragione *et al.*, 2013). Chronic infection of cattle by *S*. Dublin can lead to persistence in the spleen or gall bladder, while udder infections can lead to infected milk. In sheep, *S*. Abortusovis produces systemic infections with abortion in sheep (Uzzau, 2013). The exact nature of the pathogenesis of these three serovars and how they may result in persistent infection is unclear.

1. 2   Epidemiology and zoonotic infections

Non-typhoid salmonellosis, which causes gastroenteritis, is associated with a massive public health and economic burden globally, resulting in an estimated 93.8 million cases worldwide and 155,000 death each year (Majowicz *et al.*, 2010). In Europe, over 6.2 million cases of non-typhoid *Salmonella* infections in humans are reported each year (Havelaar *et al.*, 2013). *Salmonella* is estimated to cause more than 1.2 million illnesses each year in the U.S., with around 23,000 hospitalizations and 450 deaths (Scallan *et al.*, 2011). It has also been reported that *Salmonella* accounted for the second greatest number (16.73%) of 1082 foodborne disease outbreaks in China between 1994 and 2005, following *Vibrio parahaemolyticus* with 19.50% (Wang *et al.*, 2007).

Most human salmonellosis is typically acquired from contaminated food/water or by contact with a carrier. Consumption of contaminated egg

and egg-products accounted for 44.9% of salmonellosis outbreaks in Europe in 2013 (EFSA, 2015). Besides poultry and eggs, a variety of meats, including pork and beef, can become cross-contaminated with *Salmonella* during slaughter, processing, or distribution (Crum-Cianflone, 2008). Companion animals are also a source of infection with iguanas, turtles and snakes accounting for 3-5% of all *Salmonella* infections in the U.S. (Ackman *et al.*, 1995). Food items not directly derived from animals can also be contaminated with *Salmonella*, such as contaminated peanut butter which caused *S. Tennessee* infection in over 600 patients in Georgia in 2007 (Anonymous, 2007). The risks from manufactured food (Hennessy *et al.*, 1996) and international trade increase the possibility of outbreaks worldwide. In developing regions, non-typhoid *Salmonella* infection is an important cause of neonatal and childhood diarrhoea. Travellers to developing countries can also acquire salmonellosis, which is a cause of 'so-called' traveller's diarrhoea (Boyle *et al.*, 2007).

S. Enteritidis and *S.* Typhimurium are the most commonly isolated serovars from non-typhoid salmonellosis associated with the consumption of contaminated food. These two serovars are responsible for 39.5 % and 20.2 % respectively of all reported serovars in confirmed human cases in the European Union (EU) in 2013 (EFSA, 2015). The prevalence of different *Salmonella* serovars and phage types varies according to country or region. In China, *S.* Enteritidis caused the most outbreaks of foodborne salmonellosis,

which resulted in 28.73% of cases between 1994 and 2005 (Wang *et al.*, 2007) and made up 57.2% of isolates in 2008 (Lu *et al.*, 2011).

In the United Kingdom (U.K.), *S.* Enteritidis was the most commonly reported serovar from human salmonellosis from 2004 to 2013 and *S.* Typhimurium was the second most commonly reported serovar ([https://www.gov.uk/government/publications/*Salmonella*-surveillance-summary-2013](https://www.gov.uk/government/publications/Salmonella-surveillance-summary-2013)), but there are also increasing reports of human outbreaks caused by monophasic variants of *S.* Typhimurium (Martelli *et al.*, 2014). A new *S.* Typhimurium phage type with many distinctive phenotypic and genotypic features, designated DT191a, emerged in England and Wales in 2008 (Harker *et al.*, 2011). *S.* Typhimurium definitive type 104 (DT104), a distinct multidrug-resistant strain, has become widespread since the 1990s (Glynn *et al.*, 1998). In Australia and New Zealand, many other serovars are isolated but *S.* Typhimurium remains predominant. In general, countries with a well-developed intensive poultry industry tend to witness large outbreaks of single strains in contrast to more sporadic cases and various isolated types in countries where the poultry industry is less intensively developed (Barrow, 1993).

1. 3   Pathogenesis of *Salmonella* infection

Salmonella utilise a range of virulence determinants to colonise the intestinal tract after oral infection, invade the intestinal epithelium and persist and multiply within macrophages and other immune cells (Wallis and Galyov,

2000). The outcome of host-pathogen interactions depends on both host susceptibility/resistance and the virulence profiles of the respective serovars of *S. enterica.* The types of infection produced include (i) an invasive typhoid with bacterial multiplication in macrophages in the liver and spleen, (ii) an invasive gastroenteritis and (iii) disease-free intestinal colonisation. *S.* Typhimurium infection in mice usually produces systemic typhoid disease and therefore is only really relevant as a model of typhoid disease.

1. 3. 1   Virulence factors associated with intestinal colonisation

After oral ingestion, efficient adhesion and colonisation to the host cells is required prior to invasion of the mucosal epithelial barrier by *Salmonella*. Fimbriae, found on the bacterial surface, mediate initial attachment and colonisation of the epithelial layer and in murine dendritic cells (DCs) the type-1 fimbrial adhesion FimH protein has been shown to mediate the uptake of *S.* Typhimurium (Humphries *et al.*, 2001, Guo *et al.*, 2007). Mutation in the lipopolysaccharide biosynthesis genes *rfaK, rfaY, rfbK*, and *rfbB* and the genes *dksA, clpB, hupA, and sipC* is associated with reduced intestinal colonisation of *S.* Typhimurium in three-week-old chickens, indicating additional involvement of regulatory and invasion genes (Turner *et al.*, 1998). A wider selection of virulence and metabolic genes have also been associated with colonisation of the intestinal mucosa of chickens and calves (Morgan *et al.*, 2004). Limited studies have been carried out with *S.* Pullorum and *S.* Gallinarum but studies with *S.* Enteritidis indicate that out of the 13 fimbrial

loci, most of which are shared with other serovars, only the *pegA* operon played a significant role in the colonisation of the avian intestine (Clayton *et al.*, 2008). However, *S*. Pullorum and *S*. Gallinarum are not effective colonisers of the avian gut but produce systemic disease and so the roles of fimbrial operons may differ in these two serovars when compared to *S*. Enteritidis or *S*. Typhimurium.

1. 3. 2   Virulence factors associated with gastroenteritis

Large clusters of horizontally acquired virulence-associated genes located within distinct genetic regions, known as *Salmonella* pathogenicity islands (SPIs), have been identified in different serovars (Groisman and Ochman, 1997). *Salmonella* Pathogenicity Island 1 (SPI-1) and *Salmonella* Pathogenicity Island 2 (SPI-2), which encode Type Three Secretion Systems 1 (TTSS-1) and 2 (TTSS-2), are important in both the establishment and persistence of *Salmonella* infections. The TTSSs form needle-like complexes as a delivery system to secrete bacterial effector proteins into host cells. These proteins mediate invasion and associated enteropathogenic response (SPI-1) and intracellular survival (SPI-2), which are required for gastroenteritis and systemic disease respectively.

Invasion is mediated through *Salmonella* directed cytoskeletal rearrangements with TTSS-1 effector proteins. SopE and SopB stimulate the GTPases cdc42 and Rac leading to actin cytoskeleton rearrangement (Wood *et al.*, 1996, Norris *et al.*, 1998). SipA enhances the efficiency of SipC to bind

actin filaments (Hayward and Koronakis, 1999, McGhie *et al.*, 2001). These SPI-1 effector proteins delivered into the host cell work cooperatively to induce rearrangement of the actin cytoskeleton and enable rapid internalization of bacteria into epithelial cells (McGhie *et al.*, 2009). Invasion leads to enteropathogenic responses and neutrophil infiltration (McCormick *et al.*, 1995). However, removal of *S*. Dublin TTSS-1 effector proteins SopA, SopB or SopD resulted in significantly less enteropathogenesis and polymorphonuclear leukocytes (PMN) influx in bovine ligated ileal loops when compared to its wild type (Galyov *et al.*, 1997, Jones *et al.*, 1998, Wood *et al.*, 2000). This indicates the potential for changes in TTSS-1 function to affect inflammatory outcomes. However, there is currently no detailed information on avian-specific serovars.

Flagella is a second factor which is related to the invasiveness of *Salmonella.* Flagellin, the structural component of bacterial flagella, is a virulence factor that is recognized by the Toll-like receptor (TLR) 5 and thus activates pro-inflammatory gene expression (Gewirtz *et al.*, 2001). In non-motile strains the structural genes *fliC* and *fliB* that encode flagellin, are not pseudogenes but the serovars are not able to build up a functional flagellum (Paiva *et al.*, 2009, Kwon *et al.*, 2000). *S. Pullorum* and *S. Gallinarum* have been reported to possess gene *fliC* (Paiva *et al.*, 2009, Kwon *et al.*, 2000, Kilger and Grimont, 1993, Thomson *et al.*, 2008). Non-motility in S. Gallinarum has been partially attributed to mutations in gene *fliC* (Kilger and Grimont, 1993), which would normally express the phase 1 g,m antigens characteristics of *S*. Enteritidis.

Non-flagellated *S.* Pullorum and *S.* Gallinarum typically cause more severe systemic infections in the chicken, compared with flagellated *S.* Typhimurium and *S.* Enteritidis. In a study by Iqbal *et al.* (2005), a non-flagellated *fliM S.* Typhimurium mutant, inducing less IL-6 and IL-1β mRNA in the chicken gut and less heterophil infiltration than did the parent strain, showed increased invasiveness of systemic sites after oral inoculation. These together suggest that the increased invasiveness of non-flagellated serovars may result from a reduced inflammatory response due to the redundancy of TLR 5.

Following invasion, the gastroenteritis-inducing serovars induce and utilize host intestinal inflammation which favours pathogenesis. In an inflamed intestine, the presence of the *iroBCED* operon-encoding salmochelin in *S.* Typhimurium, confers resistance to lipocalin-2, an antimicrobial released from epithelial cells, to prevent bacterial iron acquisition (Hantke *et al.*, 2003, Raffatellu *et al.*, 2009), which contributes to its intestinal colonisation. Furthermore, the *ttrRSBCA* locus was shown to enable *S.* Typhimurium to respire with tetrathionate, $S_4O_6^{2-}$, an electron acceptor that is oxidized from thiosulphate, $S_2O_3^{2-}$ by reactive oxygen species (ROS) generated to produce energy during inflammation (Hensel *et al.*, 1999, Winter *et al.*, 2010a). This promotes bacterial outgrowth in the inflamed intestine.

In poultry, invasion of *S.* Typhimurium and *S.* Enteritidis induce strong inflammatory responses by increasing the production of cytokines IL-1 and IL-6 in the intestine and epithelial cells, which is thought to limit the infection to

the gut in contrast to an absence of inflammation seen with infection of *S. Pullorum* and *S. Gallinarum*, which may facilitate systemic spread (Henderson *et al.*, 1999, Kaiser *et al.*, 2000). Bacterial LPS has multiple effects in modulating immune responses. LPS is a potent inducer of nitric oxide (NO) secretion (Olah, 2008) and expression of splenic IL-6, IL-8, IL-18 and IFN-γ mRNA in chickens early after infection (Sijben *et al.*, 2003). However, stimulation of both chicken macrophage-like HD11 cells and monocyte-derived macrophages by LPS resulted in a release of the anti-inflammatory cytokine IL-10 (Rothwell *et al.*, 2004, Setta *et al.*, 2012a). Production of anti-inflammatory cytokines in the latter study probably represents inflammation accompanied with a later anti-inflammation event during the later stage of infection. However, expression of inflammatory cytokines in the earlier observations *in vivo* may largely result from stimulated epithelial cells.

1. 3. 3   Virulence factors associated with systemic disease

Macrophages were found to be the preferable intracellular niche for the survival and persistence of *S. enterica* (Dunlap *et al.*, 1992, Wigley *et al.*, 2001, Monack *et al.*, 2004). *S.* Pullorum can persist *in vivo* in chicken splenic macrophages for over 40 weeks following experimental infection (Wigley *et al.*, 2001), which is central to the development of the disease-free carrier state in chicken. Several different mechanisms associated with TTSS-2 have been recognised to aid intra-macrophage survival of *S. enterica.*

After internalization by a phagocyte, *S. enterica* occupies a modified phagosome known as the *Salmonella*-containing vacuole (SCV). Several sequential bactericidal cell activities are directed at the SCV. These include the ROS generated through the respiratory burst and reactive nitrogen intermediates (RNI) synthesized by inducible nitric oxide synthase (iNOS) (Vazquez-Torres and Fang, 2001). TTSS-2 enables *S.* Typhimurium to exclude the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase membrane component flavocytochrome *b558* from the membrane of the phagosome in murine macrophages. This prevents the assembly of the NADPH oxidase complex, thereby protecting *S.* Typhimurium from oxidative damage in the SCV (Vazquez-Torres *et al.*, 2000b, Gallois *et al.*, 2001). SPI-2 has also been suggested to abrogate the assembly of the NADPH oxide complex by interfering with the trafficking of oxidase-containing vesicles to the phagosome (Vazquez-Torres *et al.*, 2000b). Efficient localisation of iNOS was observed in murine macrophages infected with *S.* Typhimurium mutants deficient in SPI-2-encoded TTSS, while iNOS was only rarely present in the SCV of macrophages infected with virulent bacteria (Chakravortty *et al.*, 2002). This was later found to be associated with the *Salmonella phoP* regulon which regulates genes located on SPI-2. Hulme *et al.* (2012) reported that the reduced iNOS expression associated with *S.* Typhimurium infection was correlated with inhibition of binding of nuclear factor κB (*NF*-κB) and activator protein 1 (AP-1) to murine J774 macrophage DNA via the *phoP* regulon. The SPI-2-encoded SpiC protein was also found to interfere with intracellular

trafficking and inhibits fusion of *Salmonella*-containing phagosomes with lysosomes and endosomes (Uchiya *et al.*, 1999).

TTSS-2-mediated survival and proliferation within the macrophages has been found to be important for the establishment of systemic infection and gastrointestinal colonisation by *S. enterica* in chickens. *S.* Gallinarum requires TTSS-2 for full virulence, mainly through promoting intra-macrophage survival (Jones *et al.*, 2001). However, *S.* Gallinarum TTSS-1 mutant was still able to effectively infect and persist in avian macrophages, indicating that TTSS-1 had little effect on its virulence (Jones *et al.*, 2001). Similarly, *S.* Pullorum TTSS-2 mutant was fully attenuated and more rapidly cleared from the spleens and livers of infected chickens than the parent strain, whereas TTSS-1 is not essential for its virulence (Wigley *et al.*, 2002b). TTSS-2 was also required for *S.* Typhimurium systemic infection but TTSS-1 is not absolutely required in either systemic infection or gastrointestinal colonisation (Jones *et al.*, 2007). However, the functionality of TTSS-2 used by *S.* Pullorum to produce persistent infection *in vivo* might be different and requires further study.

During the transition of *S.* Typhi through the intestinal epithelium, TviA, a *viaB* locus-encoding regulatory protein that represses expression of flagellin, was induced due to the change in osmolarity, which enabled *S.* Typhi to evade the sentinel functions of TLR5 and weaken flagellin-specific CD4$^+$ cell responses, thus contributing to its ability to disseminate from the intestine to deeper tissues (Winter *et al.*, 2010b, Atif *et al.*, 2014). This is a similar situation to that

observed in chickens where the non-flagellated *S.* Typhimurium mutants had increased invasiveness to systemic sites in chickens (Iqbal *et al.*, 2005). The Vi capsular antigen is another virulence factor used by *S.* Typhi when it transits from the intestinal lumen into the ileal mucosa. *tviB*, a gene necessary for the expression of the virulence (Vi) capsule antigen in *S.* Typhi, was significantly upregulated during invasion of intestinal tissue (Tran *et al.*, 2010). Decreased complement 3 (C3) fixation of Vi-capsulated *S.* Typhi reduced bacterial binding to complement receptor 3 (CR3), resulting in inhibition of CR3-mediated phagocytosis in the livers and spleens of infected mice (Wilson *et al.*, 2011). Another consequence of impairing C3 fixation by the Vi capsule was to reduce the inflammatory response induced by TLR4. For example, CR3-blockade has been shown to attenuate the TLR4-dependent inflammatory response in human neutrophils (van Bruggen *et al.*, 2007) and murine bone-marrow derived macrophages (Wilson *et al.*, 2008) infected with *S.* Typhimurium.

A large virulence-associated plasmid, present in a few serovars, is also required for *Salmonella* virulence during systemic disease caused by *S.* Gallinarum, *S.* Pullorum and *S.* Enteritidis in chickens (Barrow *et al.*, 1987b, Barrow and Lovell, 1988, Halavatkar and Barrow, 1993). The plasmids are 50-90 kb in size with the 7.8 kb *spv* (*Salmonella* plasmid virulence) region essential for virulence (Rotger and Casadesus, 1999). A study by Matsui *et al.* (2001) has suggested that two genes, *spvB* and *spvC*, encode the principle effector factors for plasmid-mediated virulence of *S.* Typhimurium in a mouse

model of systemic infection. T3SS-2 is required for translocation of both SpvB and SpvC proteins into the cytosol of *Salmonella*-infected macrophages (Browne *et al.*, 2008, Mazurkiewicz *et al.*, 2008). Genes homologous to *spv* have not been found in *S.* Typhi but a recently identified large and conjugative antibiotic resistance plasmid (pR$_{ST98}$) in *S.* Typhi (Huang *et al.*, 2005) has been found to modulate the immune response of DCs by inhibiting the expression of co-stimulatory molecules (CD40, CD80, and CD86) (Wei *et al.*, 2012). This plasmid also directed the immune response away from a protective T helper (Th) type 1 (Th1) like response (Wei *et al.*, 2012). This suggested that *S.* Typhi pR$_{ST98}$ may be key in preventing the activation of a protective T-cell mediated immune response to this pathogen. No equivalent study has been performed to investigate the impact of immune response with plasmids of avian-specific serovars Pullorum and Gallinarum.

1. 4   Avian Immunobiology

Among the avian species, the immune system of domestic chicken, *Gallus gallus domesticus*, is the most extensively studied as a result of the availability of inbred lines and their importance in both economy and food security. The immune system of the chicken functions similarly to that of mammals, but there are significant structural and functional differences.

1. 4. 1   The avian immune system

In chicken, the bursa of Fabricius and the thymus are the central (primary) lymphoid organs required for the development of B and T lymphocytes

respectively. The bursa of Fabricius is the primary site for the development of chicken B cells and the antibody repertoire since neonatal surgical removal of the bursa of Fabricius prevents the development of antibodies in response to immunization with *S.* Typhimurium type O antigen (containing LPS) (Glick *et al.*, 1956) while T cells develop from the precursors in the thymus (Olah *et al.*, 2013). The immunologically mature B and T cells then enter the circulation and colonise the peripheral (secondary) lymphoid organs, which comprise the spleen, which directly connects to the blood circulation, and mucosa-associated lymphoid tissues (MALT) (Olah *et al.*, 2013). Many cell types are present in the red pulp of the spleen, including T cells, plasma cells and macrophages. Avian splenic macrophages have been demonstrated to express major histocompatibility complex (MHC) class II, which suggests a role in antigen presentation (Olah *et al.*, 2013). The chicken MHC is an example illustrating a more compact but functionally different immune system in chicken. Chicken MHC is about one-third the size of their mammalian homologues (Kaufman *et al.*, 1999) and strongly associates with resistance and susceptibility to certain infectious pathogens (Kaufman and Wallny, 1996). The gut-associated lymphoid tissues (GALT) that comprises the caecal tonsils and the Peyer's patches is part of the MALT (Olah *et al.*, 2013). Chicken IgM, IgA and IgY have been identified as homologues of their mammalian counterparts whereas IgE is absent in chickens with some of the functions ascribed to IgE in all probability being performed by chicken IgY (Ratcliffe, 2006).

Large numbers of heterophils and monocytes present in the blood at hatch (Wells *et al.*, 1998). There are further increases in PMN cells in all parts of the intestine during the first two weeks after hatch (Bar-Shira and Friedman, 2005). Antibodies received from the breeder hens (passive immunity) protect the newly hatched chicks from disease for the first few days. The chicken antibody repertoire is generated during the late embryonic stage and for a short period after hatching and a mature repertoire is achieved around 5-7 weeks, when the bursa is fully mature (Davision, 2013). T cells functionally mature in chicks between days 2-4 and by 1 week of age their ability to proliferate and produce cytokines following immune stimulation is equivalent to that of adult chickens (Lowenthal *et al.*, 1994).

1. 4. 2   Innate immune response and recognition in the chicken

Heterophils are primary avian PMNs equivalent to neutrophils in mammals, which can release toxic oxygen species, proteolytic enzymes and various antimicrobial peptides to aid microbial killing (Kogut *et al.*, 2001). Recruitment of heterophils into the intestine was observed in chickens infected with *S.* Enteritidis or *S.* Typhimurium (Withanage *et al.*, 2004, Withanage *et al.*, 2005b, Berndt *et al.*, 2007, Cheeseman *et al.*, 2008). Heterophils from *S.* Enteritidis-resistant chicken had higher levels of pro-inflammatory cytokine mRNA and reduced expression of anti-inflammatory cytokine mRNA when compared to those in susceptible chickens, suggesting that heterophil function contributes to resistance to *Salmonella* infection (Swaggerty *et al.*, 2004). Higher numbers

of circulating heterophils were also seen in chicken lines resistant to intestinal colonisation by *S.* Typhimurium (Barrow *et al.*, 2004). In mammals the equivalent cell type, the neutrophil, also responds to enteric *Salmonella* infection. Massive infiltration of neutrophils into the intestine confers resistance to invasion by subsequently infecting strains (Foster *et al.*, 2003b).

The Toll-like receptors (TLRs) expressed on host cell surfaces are the best characterized membrane-bound pattern recognition receptors (PRRs) which recognise pathogen-associated molecular patterns (PAMPs) conserved on a broad range of invading pathogens (Aderem and Ulevitch, 2000). The chicken TLR repertoire has a pattern of gene duplication/loss and is considered generally less polymorphic when compared with those in mammals (Kaiser, 2010). About ten chicken TLRs have been confirmed with TLR3, 4, 5, 7 having orthologues in mammals whilst duplicated TLR2 gene, termed TLR2A and TLR2B, are both orthologues of a single TLR2 in mammals (Temperley *et al.*, 2008). A broad range of avian tissues and cells types constitutively express TLR4, but a particularly high level of TLR4 is expressed in macrophages and heterophils (Leveque *et al.*, 2003, Kogut *et al.*, 2005a). TLR5 orthologues have been reported in the chicken, which shares 50% amino acid identity with that in humans and induced an up-regulation of IL-6 and IL-1β when exposed to bacterial flagellin (Iqbal *et al.*, 2005). Genomic disruption, generating a pseudogene of avian TLR8 has been identified (Philbin *et al.*, 2005). TLR9 is absent in chickens (Temperley *et al.*, 2008) but is replaced functionally by TLR21 (Keestra *et al.*, 2010). TLR1La, 1Lb and 15 appear to be unique to avian

species (Temperley *et al.*, 2008, Nerren *et al.*, 2009). A recent study indicated that, compared with heterophils isolated from chickens susceptible to *S. Enteritidis* infections, heterophils from resistant chickens had significantly higher levels and stronger up-regulation of TLR15 mRNA expression prior to and after *S. Enteritidis* stimulation respectively (Nerren *et al.*, 2009). The ligand for TLR15 was on a broad-range of bacterial pathogens causing disease in chickens (Nerren *et al.*, 2010), suggesting a potential involvement in the immune responses to bacterial infection in poultry. Another study has shown a significant up-regulation of chicken TLR2, TLR4 and TLR21 expression in gastrointestinal tissues upon infection with *S. Pullorum* in the early stage of infection (Ramasamy *et al.*, 2014) though the involvement of these receptors in avian systemic salmonellosis requires further studies.

1. 4. 3   Adaptive immune response in the chicken

Like mammalian antigen presenting cells (APCs), avian macrophages and DCs capture antigens encountered during scavenging and express the peptides from these antigens through the MHC. MHC class I molecules (MHC I) are expressed on most cells of the body. MHC I-bearing peptides, derived from cytosolic proteins are recognised by $CD8^+$ T cells (cytotoxic T cells) which are specialized to kill infected cells, while MHC class II molecules (MHC II) bind antigenic peptides mainly from endocytosed proteins. MHC II are expressed predominantly by antigen-presenting cells and are recognised by $CD4^+$ T cells (T helper cells, Th) which are specialized to activate other cells (Germain, 1994). Upon recognition and activation, the classical T helper (Th) type 1 (Th1)

and type2 (Th2) (Th1/Th2) paradigm (Mosmann and Coffman, 1989) classifies CD4$^+$ T cell clones into distinct populations on the basis of their patterns of cytokine production. Th1 cells produce IFN-γ to promote cell-mediated immunity against intracellular pathogens whereas Th2 cells produce IL-4 to support humoral immune responses to clear extracellular pathogens. Two functionally different types of macrophages, designated M1 and M2, have been isolated in mammals (Mills *et al.*, 2000). M1- or M2-dominant macrophage responses can influence the occurrence of Th1 or Th2 responses respectively (Mills *et al.*, 2000). It is not yet known whether M1/M2 phenotypes are conserved in avian macrophages but Th1/Th2-polarized immunity has been demonstrated in the chicken (Kaiser, 2010). The conservation of Th1-like pro-inflammatory responses in the chicken was first characterized by IL-18-induced IFN-γ secretion (Gobel *et al.*, 2003). IFN-γ is involved in the activation of macrophages for NO production and promoting intracellular killing of *Salmonella* (Mastroeni and Menager, 2003, Okamura *et al.*, 2005, Babu *et al.*, 2006). Thus it is accepted that the IFN-γ-producing Th1 response plays a vital role in protection against *Salmonella* (Beal *et al.*, 2005, Withanage *et al.*, 2005b, Chappell *et al.*, 2009).

Besides the Th1/Th2 paradigm, Th17 cells were identified as a separate lineage of Th cells in mammals and important regulators of tissue inflammation (Harrington *et al.*, 2006). Th17 cells produce a range of cytokines including IL-17, IL-17F, IFN-γ. They recruit neutrophils and macrophages to infected tissues through production of IL-17 and IL-17F

(Miyamoto *et al.*, 2003, Barin *et al.*, 2012). Upon IL-23 stimulation, induction of both IL-17 and IFN-γ expression was found in CD4⁺ T cells (Oppmann *et al.*, 2000). Th17 cells also displayed considerable plasticity and acquired the capacity to produce IFN-γ *in vitro* (Lee *et al.*, 2009) and *in vivo* (Hirota *et al.*, 2011). There has been little focus on the role of Th17 cells in avian salmonellosis, but IL-17 expression was up-regulated in the caeca of chickens infected with *S*. Enteritidis (Crhanova *et al.*, 2011). It was also found that recombinant chicken IL-17 induced IL-6 production in chicken embryonic fibroblasts (Min and Lillehoj, 2002). These suggest a function of Th17 cells as inflammatory mediators.

Th9 cells were identified by the potent production of IL-9 and appear to be capable of promoting allergic inflammation (Kaplan, 2013). A Th22 subset was recently characterized in epidermal immunity with the cytokine profile of IL-22 and TNF-α (Eyerich *et al.*, 2009). However, the existence and functional significance of any of these CD4⁺ T cell subsets remains to be determined in chickens (Kaiser and Staheli, 2013).

Natural regulatory T (nTreg) cells are specialized in immune suppression to maintain peripheral tolerance and protect the host from autoimmune disease (Vignali *et al.*, 2008). Forkhead box P3 (FoxP3) was defined as a key transcription factor of CD4⁺ CD25⁺ nTregs (Fontenot *et al.*, 2003). A FoxP3 orthologue has yet to be identified in chickens but CD4⁺CD25⁺cells have been described with regulatory T cell properties similar to that of mammalian Treg

(Shanmugasundaram and Selvaraj, 2011). Chicken suppressor CD4$^+$ CD25$^+$ cells suppress naïve T cell proliferation *in vitro* and express higher amounts of IL-10 and TGF-β4 mRNA, compared to naïve CD4$^+$ CD25$^-$ cells (Shanmugasundaram and Selvaraj, 2011).

T cell receptor (TCR) signalling triggered by the antigen-specific recognition of peptide-MHC molecules is not sufficient to initiate an adaptive immune response, which also requires co-signalling molecules to acquire effector function. Cognate antigen recognition by T cells, in the absence of co-stimulation, leads to apoptosis of the T cell clonal population (known as clonal anergy) (Harris and Ronchese, 1999). The interaction between the B7 protein family (CD80 and CD86) found on APCs and their receptors CD28 or CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4, also known as CD152) expressed on T cells, deliver opposing signals for T-cell stimulation. When CD80 or CD86, on the APC surface, binds to CD28 on the surface of T cells it promotes T-cell proliferation, but if CTLA-4 is overexpressed, CD80 and CD86 will preferentially bind to this receptor which prevents proliferation and also induces T cell apoptosis (tolerance) (Perez *et al.*, 1997). In chickens, CD28 and CTLA-4 are both clustered on chromosome 7 (Bernard *et al.*, 2007) and all αβ T cells and a small subset of γδ T cells from peripheral blood have been identified as CD28 positive (Young *et al.*, 1994, Koskela *et al.*, 1998). Besides CTLA-4, inhibitory receptor programmed death-1 (PD1) is also conserved in the chicken (Bernard *et al.*, 2007).

1. 4. 4   Avian cytokines

Cytokines are regulatory proteins or peptides that are produced by various types of cell involved in inflammatory and immune responses while chemokines are a group of cytokines characterized by their chemotactic activity towards various cells (Baggiolini, 1998). Both are key signalling molecules of the host innate and adaptive immune.

Our knowledge of the immunobiology of various avian cytokines has benefited greatly from the availability of chicken genome data (Kaiser *et al.*, 2005). Although there are increasing numbers of monoclonal antibodies commercially available for chicken cytokines and polyclonal antisera have also been raised to several avian cytokines, real-time quantitative RT-PCR assays have been widely applied to measure avian cytokine expression at the mRNA level.

IL-1β belonging to IL-1 superfamily has been characterized in chickens (Weining *et al.*, 1998) and was previously described to induce K60, a chicken IL-8-like pro-inflammatory chemokine now known as CXCLi1, during inflammatory reactions (Weining *et al.*, 1998, Sick *et al.*, 2000). IL-1β appears to play an important role in defining the early stages of enteric versus systemic *Salmonella* infection (Kaiser *et al.*, 2000, Withanage *et al.*, 2004). CXCLi1 and CXCLi2, previously known as K60 and IL-8, respectively share 48% and 50% homology with human CXCL8 (IL-8). These two pro-inflammatory chemokines are different in terms of preferential recruitment of heterophils

(CXCLi1) and monocytes (CXCLi2), respectively (Sick *et al.*, 2000, Kaiser *et al.*, 2005). Enhanced recruitment of granulocytes and macrophages to the site of *S. enterica* invasion in young chickens was accompanied by up-regulated mRNA expression of CXCLi1 and CXCLi2 in the heterophils (Cheeseman *et al.*, 2008). IL-17 (produced by Th17 cells) is a potent pro-inflammatory cytokine in mammals whose function remains to be fully elucidated. Chicken IL-17 is currently best characterized during infection with *Eimeria maxima* and *Eimeria tenella* where IL-17 may play a role in both protection and pathology in infection with *Eimeria tenella* (Kim *et al.*, 2012, Zhang *et al.*, 2013). The potential involvement of chicken IL-17 as a pro-inflammatory mediator in *Salmonella* infection is discussed above.

IFN-γ is produced by many cell types and is an important hallmark of cell-mediated immunity. Upon stimulation by *S.* Enteritidis, up-regulated mRNA expression of IL-18 and IFN-γ was observed in heterophils primed by rChIFN-γ, in comparison with that from heterophils without rChIFN-γ-priming (Kogut *et al.*, 2005b). Recombinant chicken IFN-γ increases the production of NO and the expression of MHC II on macrophages (Weining *et al.*, 1996), and acts synergistically with chicken type I IFN (IFN-α and IFN-β) during anti-viral immune responses (Sekellick *et al.*, 1998). Chicken IL-12 possesses two components, IL-12α and IL-12β, and was shown to induce IFN-γ production and promote the proliferation of chicken splenocytes, which was similar to its activity in driving inflammatory Th1 responses in mammals (Degen *et al.*, 2004). Chicken IL-18 was characterized as a major growth factor of CD4^{+} T

cells and conserved the function to induce IFN-γ secretion from splenocytes (Gobel *et al.*, 2003). IFN-γ induction by IL-18 is IL-12-dependent in mammals whereas IL-12 is not required in mediating the expression of chicken IL-18 receptor (Ahn *et al.*, 1997, Schneider *et al.*, 2000).

IL-6 is a multifunctional cytokine within the IL-6 family. In chicken kidney cells (CKC), infection with *S.* Enteritidis and *S.* Typhimurium up-regulated the expression of IL-6 mRNA by eight to ten fold and induced higher levels of IL-6-like activity compared to uninfected cells but this was not evident during *S.* Gallinarum infection (Kaiser *et al.*, 2000). In another study, however, *in vitro* infection of chicken peripheral blood mononuclear cells (PBMCs) with *S.* Enteritidis down-regulated IL-6 mRNA expression (Kaiser *et al.*, 2006). The down-regulation of IL-6 in the later study may be due *in vitro* invasion of naive PBMCs by *S*. Enteritidis.

Chicken IL-10 has 45% and 42% amino acid identity with human and murine IL-10, respectively. The biological function of IL-10 as an anti-inflammatory cytokine, which inhibits the synthesis of IFN-γ, is conserved in chicken (Rothwell *et al.*, 2004). Expression of IL-10 was seen in *S*. Enteritidis-infected chickens at 4 days after infection, which probably suppressed the inflammatory response to allow *Salmonella* to persist within the gut for a number of weeks (Setta *et al.*, 2012b), although the initial inflammatory response functions to control invasion and clear gastrointestinal infection. Chicken TGF-β4 possesses similar anti-inflammatory properties that suppress

protective inflammatory responses to infection. Heterophils from chickens susceptible to *S*. Enteritidis had a higher levels of TGF-β4 mRNA than that of resistant chickens (Swaggerty *et al.*, 2004).

Mammalian IL-3, IL-4, IL-5, IL-13, and granulocyte macrophage colony stimulating factor (GM-CSF) represent the Th2-associated cytokines in mammals. Production of chicken IL-3, IL-4, IL-13, and GM-CSF was determined by the expression of mRNA and can be expressed as recombinant protein while IL-5 appears to be a pseudogene in chickens (Avery *et al.*, 2004, Kaiser *et al.*, 2005).

The cytokines described in this section are listed in Table 1-1, which is adapted from recent reviews (Kaiser, 2010, Kaiser and Staheli, 2013)

Table 1-1. Chicken cytokine repertoire

Cytokine	Chicken orthologues	Reference
Interferons		
Type I	IFN-α*, IFN-β*, IFN-λ*, IFN-κ, IFN-ω	(Sekellick *et al.*, 1998, Kaiser *et al.*, 2005)
Type II	IFN-γ*	(Digby and Lowenthal, 1995)
Interleukins		
IL-1 family	IL-1β*, IL-1RN*, IL-36RN*, IL-18*	(Weining *et al.*, 1998, Schneider *et al.*, 2000)
IL-10 family	IL-10*, IL-26*, IL-19*, IL-22*	(Rothwell *et al.*, 2004)
IL-12 family	IL-12α*, IL-12β*, IL-23	(Degen *et al.*, 2004, Kaiser *et al.*, 2005)
IL-17 family	IL-17* (A, B, C, D, F)	(Min and Lillehoj, 2002)
Th2 family	IL-3, IL-4*, IL-5*, IL-13*, GM-CSF*, KK34*	(Avery *et al.*, 2004)
T-cell proliferative	IL-2*, IL-15*, IL-21*	(Lillehoj *et al.*, 2001)
Others	IL-6*, IL-7*, IL-9*, IL-16*, IL-34*, IL-11	(Kaiser *et al.*, 2005)
Chemokines		
XCL	XCL1*	(Rossi *et al.*, 1999)
CCL	CCL1*, CCL2*, CCL3*, CCL4*, CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL16, CCL17*, CCL18, CCL19*, CCL20*, CCL21*, CCL23	(Sick *et al.*, 2000, Kaiser *et al.*, 2005, Hughes *et al.*, 2007)
CXCL	CXCL1*, CXCL2*, CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL12, CXCL13, CXCL14	(Sick *et al.*, 2000, Kaiser *et al.*, 2005)
CX3CL	CX3CL1*	(Kaiser *et al.*, 2005)
Transforming growth factors	TGF-β2*, TGF-β3*, TGF-β4*	(Pan and Halper, 2003, Kaiser *et al.*, 2005)
Colony-stimulating factors	GM-CSF*, G-CSF*, M-CSF*	(Avery *et al.*, 2004, Kaiser *et al.*, 2005)
Tumour necrosis factors	TNF-α, OX40L, AITRL, FAST, 4-1BBL, VEGI, CD30L*, CD40L, TRAIL*, RANKL, BAFF*	(Schneider *et al.*, 2004, Kaiser *et al.*, 2005)

* Avian cytokines that have been cloned and characterized in avian species (Kaiser, 2010, Kaiser and Staheli, 2013)

1. 5   Immunobiology to *S. enterica* infection

The nature of host immune response to *S. enterica* are important to consider when investigating its ability to cause disease by overcoming, evading and/or modulating immunity. The avian immune system possesses different properties from that of mammals in various aspects as stated in 1. 4. 1. In addition, much of our understanding of the avian immune response to systemic pathogenesis of *S. enterica* infection has been extrapolated from the typhoid-like infection produced in murine model with *S.* Typhimurium infections. From the point of view of pathogenesis, it more closely resembles *S.* Gallinarum infection in the chickens whilst *S.* Typhimurium usually produces gastrointestinal infection in chickens. It is therefore essential to compare the important aspects in the immunobiology of avian salmonellosis with that observed in mammals.

1. 5. 1   Initial recognition and innate immunity to *S. enterica* infection

Salmonella infection usually occurs via the faecal-oral route than via vertical transmission as a result of infection of infection of the ovary and oviduct. Bacteria enter the small intestine where infection can become established and may disseminate systemically. The epithelial cells lining the intestine are not only a physical barrier against the invading pathogens but also the initiator of the local inflammatory response which can prevent the entry of *Salmonella* into deeper tissues from the gut.

Intestinal epithelial cells express a number of TLRs. While effector proteins secreted via TTSS-1 system can induce cellular changes and inflammation (Hapfelmeier *et al.*, 2004), recognition of flagellin through TLR5 plays a key role in the initial recognition of invading pathogens. These lead to the expression of chemokines such as IL-8 and MCP-1 (monocyte chemotactic protein 1) which attract immune effector cells to the sites of infection and cytokines such as GM-CSF, TNF-α and IL-6 which enhance the effectiveness of local host defence by mediating pro-inflammatory functions of phagocytes (Eckmann and Kagnoff, 2001, Raupach and Kaufmann, 2001, Gewirtz *et al.*, 2001).

Salmonella infection in the chicken intestine results in expression of CXCLi1 and CXCLi2 (Withanage *et al.*, 2004, Withanage *et al.*, 2005b, Setta *et al.*, 2012b), which in turn leads to an influx of heterophils and phagocytes to the gut, resulting in inflammation. The massive inflammatory response in chicken infected with *S.* Typhimurium or *S.* Enteritidis is associated with infiltration of heterophils, macrophages, B cells and CD4^{+} and CD8^{+} lymphocytes subpopulations into the sites of infection (Berndt and Methner, 2001, Berndt and Methner, 2004, Berndt *et al.*, 2006). This is accompanied by an up-regulated expression of various pro-inflammatory cytokines as well as Th1-associated immune mediators (Withanage *et al.*, 2004, Withanage *et al.*, 2005b, Berndt *et al.*, 2007, Fasina *et al.*, 2008, Cheeseman *et al.*, 2008, Setta *et al.*, 2012b, Matulova *et al.*, 2013). Increased resistance to *S.* Enteritidis in neonatal poultry was shown to be related to higher levels of the mRNA

expression of pro-inflammatory (IL-6 and CXCLi2) and Th1-associated (IL-18) cytokine or chemokine and decreased mRNA expression of anti-inflammatory cytokine (TGF-β4) in heterophils (Swaggerty *et al.*, 2006).

In contrast, avian systemic salmonellosis caused by the host-adapted serovars *S.* Pullorum and *S.* Gallinarum is characterized by invasion from the intestine with multiplication in the spleen, liver and other organs. There is little intestinal involvement in the early stage of infection of these two non-flagellated serovars but in the later stage of disease they re-enter the gut, as occurs in *S.* Typhi infection in humans. Infection with *S*. Pullorum and *S*. Gallinarum resulted in little inflammation *in vivo* or *in vitro*. *S*. Pullorum infection induced significantly lower levels of CXC chemokine expression in the ileum than those detected in *S*. Enteritidis-infected chickens (Hughes *et al.*, 2007, Chappell *et al.*, 2009), suggesting a reduced recruitment of heterophils in response to *S*. Pullorum infection. In fact, in an early study, when compared to infections with *S*. Typhimurium, *S.* Pullorum infection resulted in reduced influx of heterophils in the intestine of infected chickens (Henderson *et al.*, 1999). The transcription of IL-6, CXCLi1 and CXCLi2 was also found to be down-regulated in CKC following *S.* Gallinarum infection (Kaiser *et al.*, 2000, Setta *et al.*, 2012a) whereas a motile mutant in *S.* Gallinarum increased its invasive ability for CKC and up-regulated the mRNA expression of CXCLi2, IL-6 and iNOS (Freitas Neto *et al.*, 2013). A reduced inflammatory response was also seen with the non-flagellated *S.* Typhimurium *fli*M mutant (see 1. 3. 2).

Invasion without initiating a strong inflammatory response may reflect the evolutionary adaption of these serovars to the avian host.

Following translocation across the intestinal epithelial barrier, *S.* Typhimurium colonises the lamina propria and Peyer's patches where they are phagocytosed by cells such as macrophages (Vazquez-Torres *et al.*, 1999) and DCs (Rescigno *et al.*, 2001). DCs transport internalised bacteria to the basolateral side of the epithelium (Rescigno *et al.*, 2001). If transported by DC across the gut epithelium, serovar Typhimurium quickly exits DC by inducing cell death to enter macrophages, which is its preferred cell type (van der Velden *et al.*, 2003). Infected macrophages are major producer of anti-microbial factors, such as ROS and reactive nitrogen species (RNS) (Vazquez-Torres *et al.*, 2000a, Vazquez-Torres and Fang, 2001) to kill intracellular bacterial. The reduction of intracellular bacterial numbers correlated with the production of NO and ROS in HD11 or MQ-NCSU cells infected with various serovars of *S. enterica* (Withanage *et al.*, 2005a, Babu *et al.*, 2006, Setta *et al.*, 2012a). Compared with wild-type mice, iNOS$^{-/-}$ mice which is unable to produce NO *in vivo* displayed an increased susceptibility to infection with *S.* Typhimurium (Mastroeni *et al.*, 2000b). Macrophages are also major effector cells eliciting innate immunity. Infected macrophages produce IL-12 and TNF-α during the early acute period of infection in mice (Mastroeni, 2002). IL-12 and IL-18 synergistically promote the differentiation of IFN-γ-producing Th1 cells, which in turn further activates macrophages for induction of NO. It is

likely that DC are also involved in this activity in chickens but to what extent is not known.

Salmonella infection induces apoptosis of mouse macrophages, which is triggered by the activation of caspase-1, resulting in a pro-inflammatory cascade (Monack *et al.*, 2001). During infection, caspase-1 within the resident macrophages is activated by the SipB protein encoded by SPI-1 (Hersh *et al.*, 1999). Activated caspase-1 cleaves and processes the inactive precursors of IL-1β and IL-18 into their biologically active forms that initiate inflammation (Fantuzzi and Dinarello, 1999). In caspase-1-induced pyroptotic macrophages, bacteria released into the extracellular environment were killed by ROS in neutrophils (Miao *et al.*, 2010). The inactive precursors of chicken IL-1β does not contain a caspase-1 cleavage site (Bird *et al.*, 2002) although chickens do express caspase-1 (Johnson *et al.*, 1998). Chicken IL-1β may thus be processed at an alternative cleavage site.

The host genetic background plays a pivotal role in determining the outcome of *Salmonella* infections. *NRAMP1* (natural resistance-associated macrophage proteins, now *SLC11A1:* solute carrier family 11 member 1) codes for an ion transporter and is originally identified in host innate resistance to intracellular pathogens, including infections with *Salmonella* which is reviewed by Blackwell *et al.* (2001). Murine *NRAMP1* expression is restricted to cells of the monocyte/macrophage lineage and NRAMPs required for iron metabolism in macrophages plays an important role in macrophage activation and is

therefore involved in controlling clearance of *Salmonella* infection in mice (Caron *et al.*, 2002).

Macrophage activity also plays an important role in genetic resistance to systemic salmonellosis in the chicken. *NRAMP1* is also known to be a major contributor to the high levels of resistance to systemic disease of *S. Gallinarum* infection in chicken (Bumstead and Barrow, 1993, Hu *et al.*, 1997). In addition, a genetic locus on chromosome 5, designated *SAL1*, has been identified and leads to increased macrophage activity and resistance of chickens to salmonellosis (Wigley *et al.*, 2002a, Wigley, 2004). Macrophages from salmonellosis-resistant birds express pro-inflammatory cytokines and chemokines more effectively and rapidly and have a greater ability to induce Th1 immune responses (Wigley *et al.*, 2006). Macrophages from adult resistant-line birds cleared *S.* Gallinarum from infected macrophages, accompanied by a strong and reproducible respiratory burst response, at least 24 h ahead of that occurs within macrophages from susceptible lines (Wigley *et al.*, 2006). This suggested that increased macrophage antimicrobial activity plays an important role in resistance although heterophils also clearly contribute greatly to host resistance. However, inherently resistant genetic lines of animals may predispose towards a more prolonged/persistent form of infection, as may occur with *S.* Gallinarum in chickens (Berchieri *et al.*, 2001a). This was also suggested by persistent *S.* Typhimurium infection in the mesenteric lymph nodes of *Nramp1$^{+/+}$* mice despite the presence of high titre circulating specific antibody (Monack *et al.*, 2004).

1. 5. 2   Adaptive immune response to *S. enterica* infection

The adaptive immune response is stimulated by the innate immune system through antigen presentation to lymphocytes. Studies with murine models have shown the critical role of CD4^{+} Th1 lymphocytes in controlling salmonellosis. Reduction of systemic bacterial colonisation and increase of mouse survival rates were observed in infected mice treated with IL-12 whereas depletion of IFN-γ and IL-12 by neutralizing antibodies in vaccinated mice resulted in their inability to clear infection from spleen and liver (Mastroeni *et al.*, 1998) and increased levels of bacterial replication and faecal shedding with chronic infection of *S.* Typhimurium (Monack *et al.*, 2004).

Following infection with host non-specific *S. enterica* serovars, such as *S.* Typhimurium and *S.* Enteritidis, in chickens, adaptive immunity is also able to clear infection within 2-3 weeks following infection by a Th1 dominated response involving increased expression of IFN-γ mRNA in the gut and deeper tissues (Beal *et al.*, 2004a, Beal *et al.*, 2004b, Withanage *et al.*, 2005b, Wigley *et al.*, 2005a, Berndt *et al.*, 2007). Increased expression of IFN-γ in turn activates macrophages to produce NO and kill intracellular *Salmonella* (Mastroeni and Menager, 2003, Okamura *et al.*, 2005, Babu *et al.*, 2006), which was correlated with clearance of *S.* Typhimurium from the intestine tract of infected chicken (Beal *et al.*, 2004a). Besides IFN-γ-producing CD4^{+} T cells, a CD8αα^{+} γδ T-cell subset appeared to be another source of IFN-γ in young chickens infected with *S.* Typhimurium or *S.* Enteritidis (Berndt *et al.*, 2006, Pieper *et al.*, 2011), suggesting a potential importance of this T-cell

subset in the development of a protective immune response against *Salmonella* infection.

Macrophages are not only immune effector cells but CD18^{+} phagocytes also transport *Salmonella* to deeper tissues (Vazquez-Torres *et al.*, 1999) and provide an intracellular niche for persistent *Salmonella* infection (Wigley *et al.*, 2001, Monack *et al.*, 2004). Persistent carriage occurs in those birds which recover from infection with *S.* Pullorum. *S.* Pullorum persists in low numbers mainly within macrophages in the spleen for the lifetime of the convalescent hens (Wigley *et al.*, 2001). At the onset of laying the number of bacteria in the spleen increase dramatically, and the infection spreads from the spleen to the reproductive tract (Wigley *et al.*, 2005b), suggesting that some suppression of the chicken immune system may occur at sexual maturity. It is unclear why immune clearance does not occur. Some initial comparative studies using *S.* Pullorum and its closely related serovar *S.* Enteritidis has shown that spleen homogenates from *S.* Pullorum-infected birds expressed significantly lower levels of inflammatory cytokines IL-18 and IFN-γ whereas the expression of IL-4 was increased in the spleen, suggesting that *S.* Pullorum tended to induce an immune response that more closely resembled the Th2 response in mammals and would allow *S.* Pullorum to establish an intracellular carriage evading Th1-mediated clearance (Chappell *et al.*, 2009). In a recent study, Chausse *et al.* (2014) used microarrays to compare the enterocyte gene expression profile during established gastrointestinal tract infections in chickens which are considered either resistant or susceptible to *Salmonella*

colonisation (Barrow *et al.*, 2004). Gene expression linked to a Th1 response was down-regulated in both lines whereas increased expression of genes related to Th2 cytokines, including IL-4, IL-5 and IL-13, were observed in the susceptible line only. This indicated that the Th1 response is essential for immune clearance of avian salmonellosis while Th2 immunity is associated with susceptibility to the carrier-state, at least in the gastrointestinal tract.

Apart from the effective immunity of Th1 cells, there has been recent interest in the role of Tregs and Th17 cells in controlling *Salmonella* infection. In a murine model of persistent *S.* Typhimurium infection, increased potency of Tregs increased bacterial burden in the spleen and liver during the early phase of infection by reducing the effectiveness of Th1 responses (Johanns *et al.*, 2010). Th17 cells are important regulators of tissue inflammation and are potentially involved in intestinal immunity in response to *Salmonella* infection. The absence of IL-17R signalling resulted in an increased systemic dissemination of *S.* Typhimurium probably due to reduced recruitment of neutrophils to the ileal mucosa (Raffatellu *et al.*, 2007). Reduced clearance of *S.* Enteritidis was also found in IL-17-deficient mice which developed increased bacterial loads in spleen and liver along with significantly compromised recruitment of neutrophils when compared with wild type mice (Schulz *et al.*, 2008). In chickens infected with *S.* Enteritidis, an early expression of IL-17 and prolonged high level expression of IFN-γ were detected in the caeca (Crhanova *et al.*, 2011, Matulova *et al.*, 2013). However,

the functional role of Th17 cell and IL-17 in avian salmonellosis is not yet fully defined.

Antigen-specific antibody production (IgA, IgM, IgY) can be detected in primary infection in chicken (Barrow *et al.*, 2004, Beal *et al.*, 2004a, Beal *et al.*, 2004b, Withanage *et al.*, 2005b, Beal *et al.*, 2006). However, neither antibodies nor B cells were demonstrated to be essential to gut clearance of infection (Babu *et al.*, 2004, Beal *et al.*, 2006). Depletion of B cells by surgical bursectomy *in ovo* did not make a difference to intestinal shedding of *S.* Typhimurium when compared to non-bursectomized chickens (Brownell *et al.*, 1970). Similarly in mice infected with *S.* Typhimurium, B cells were revealed to be not required for the clearance of primary infection, although they were involved in immunity to secondary infection and this involved a typhoid-type infection rather than colonisation of the gastrointestinal tract (Mastroeni *et al.*, 2000a).

Table 1-2. Summary of pathogenesis effectors, immune responses and disease outcome of infection with representative serovars of *S. enterica*.

Serovars	Pathogenesis and virulence determinants	Immune response	Outcome of disease
Common features	• TTSS is important for all serovars. SPI-1 and SPI-2 encode TTSS-1 and TTSS-2 are required for invasion and intracellular survival respectively. SPI-3, 4 and 5 are important for colonization, adhesion and enteritis. They may differ in different serovars and has not yet been fully worked out	•Mostly strong Th1 response mediates immune clearance of infection	•*S*. Typhimurium and *S*. Enteritidis infection in poultry and man are usually cleared by Th-1-dominant immune response. •The typhoid group of serovars including Typhi, Gallinarum and Pullorum modulate the host response to enable persistant infection •Uncontrolled replication in macrophages of susceptible individual leading to death
S. Pullorum	• Absence of flagella in avian adapted serovars avoiding TLR5-mediated inflammatory response • TTSS-2 mediate survival and proliferation within the macrophages	•Host adapted serovars that invade causing little or no activation of innate inflammation response •Translocation to spleen and liver and establishment of intracellular infection in macrophages	•Intracellular persistence within macrophages •Recrudescence of infection in hens at sexual maturity
S. Gallinarum		•Initiation of cellular response: key role in IFN-γ-producing T cells •Immunomodulation though up-regulation of Th2 response by *S*. Pullorum •Development of IgM and IgY antibody •Shedding into gut through clusters of lymphoid tissues •Immunological reaction leading to myocarditis	•Animal death or immune clearance •Persistent infection for in resistant birds

Continued

S. Typhi	• Vi-capsule reduces C3 fixation and TLR4-dependent inflammatory response • *viaB* locus represses flagellin expression	• Inflammatory response in gut reduced through down regulation of flagella production • Shedding from gut with infrequent gut perforation	• Systemic clearance in immune competent individual • Persistent carrier in gallbladder, liver and spleen
S. Enteritidis	• Fimbrial adhesion mediate the initial epithelial attachment and invasion • TTSS-1 is required for invasion and gastro-enteritis • Respiration using different carbon sources and with $S_4O_6^{2-}$ as electron acceptor leading to outgrowth in the inflamed intestine • TLR5-dependent inflammatory response	• Activation of innate immune response through action of bacterial effector proteins and host recognition (e.g. TLR4, 5) • Pro-inflammatory CXC chemokines responses leads to influx of heterophils • Secretory IgA response • Salmonella persistence within lower intestinal tract • Mucins and gallinacins limit infection • Initial inflammatory response regulated by regulatory T cells • Role of Th17 response in maintaining gut integrity is not fully known	• Enteritis in chicken and human • Systemic infection in mice • Intestinal clearance 3-12 weeks p.i dependent on Th1 response
S. Typhimurium			

Adapted from (Wigley, 2014)

1. 6   Prevention and control

Control of *Salmonella* infection of poultry is important for both economic and public health reasons.

Preventing contamination of poultry products with *Salmonella* remains a major challenge for poultry industry and public health in terms of safety of food supply. *Salmonella* also remains a major cause of morbidity and mortality in poultry worldwide. Control measures include hygiene and management, the use of antibiotics, competitive exclusion cultures and vaccines.

The most successful measures in controlling *Salmonella* infection include good bio-secure farming and hygienic practice. The WHO has produced effective guidelines on cleaning and disinfection of poultry houses and the surrounding environment to control *Salmonella*-infected poultry flocks (Lister and Barrow, 2008) .

Antibiotics are still used in many parts of the world but their effectiveness is limited and their over use leads to the development of resistance which of increasing global concern (see 1. 1. 3. 2 for more details).

Oral administration of newly hatched chickens with a mixed culture of intestinal bacteria from adult chickens can induce a rapid protection from infection and colonisation by *Salmonella* by conferring on the chick the full mature inhibitory flora normally possessed by the adult (Nurmi and Rantala, 1973). This concept is known as competitive exclusion (CE) and many CE

products are now available for use against *S. enterica* in poultry (Schneitz, 2005). However, most CE products comprise undefined or partially defined cultures and are thereby not accepted in some countries (EFSA, 2004). Probiotics are live microorganisms which are frequently used as feed supplements and which are claimed to confer a health benefit on the host (Fuller, 1992).

Since the development of the first effective live, attenuated vaccine, *S. Gallinarum* 9R (Smith, 1956), vaccination against both avian host-restricted *S. Pullorum* and *S. Gallinarum* has been successful. This vaccine has also been shown to be effective against the serological related *S. Enteritidis* (Barrow *et al.*, 1991). Live attenuated vaccines are considered to be more protective than killed vaccines in eliminating *Salmonella* infection because (i) they stimulate both cellular and humoral immune response (Babu *et al.*, 2004, Van Immerseel *et al.*, 2005) and (ii) the response generated is Th1-like rather than Th2-like, which is normally associated with killed vaccines (see above). Nevertheless, an increased production of IFN-γ and IL-2 by antigen-stimulated splenocytes was observed in chicken vaccinated with a commercial killed *S. Enteritidis* vaccine (Okamura *et al.*, 2004). Killed vaccines may have side effects as a result of their endotoxin (LPS) content (Barrow, 1993).

The development of vaccines against broad host range *Salmonella* strains, especially for vaccines to be effective against food poisoning serovars such as including *S. Typhimurium* and *S. Enteritidis* is a major goal for vaccinologists.

In addition to their effectiveness, through stimulation of the adaptive immune response, live vaccines administrated orally to young chickens can colonise the alimentary tract and themselves induce a more specific form of competitive exclusion. Significant protection against the colonisation of wild strains of *S.* Typhimurium has been achieved in newly-hatched chickens inoculated with a live attenuated *S.* Typhimurium vaccine (Methner *et al.*, 1997), with improved protection derived from combined administration of both *S.* Typhimurium vaccine strain and competitive gut exclusion culture (Methner *et al.*, 1999, Barrow, 2007).

The selection of genetically resistant chickens may in the future be considered as an alternative approach to disease control in the context that the use of vaccine and antibiotics is not sufficient to eradicate salmonellosis, especially the symptom-free carriers of *S. enterica* in poultry. To understand resistance to systemic disease which will have implications for the carrier state, several candidate genes and quantitative trail loci (QTL) have been identified (Kaiser and Stevens, 2013). Developing a breeding regimen to maximise these effects combined with ensuring maximum inheritance of productivity traits remains a major obstacle.

1. 7   Aims and objectives of this project

Pullorum disease remains an important disease of the poultry industry (Barrow and Freitas Neto, 2011). The ever-increasing emergence of antibiotic-resistance, much of which is transferable, requires an alternative approach to

control the problem. Vaccination and other control strategies require an understanding of the immunity in response to *Salmonella* infection.

The aim of this project is to explore the immunobiology of persistent infection and the carrier state of *Salmonella enterica* serovar Pullorum in chickens, and to evaluate the different immune activities of macrophages and T cells in response to the related serovars *S.* Enteritidis and *S.* Gallinarum.

To achieve this aim, the following objectives were proposed:

1. To determine and compare the immune response of PBMC-derived macrophages induced by these three *Salmonella* serovars.

2. To establish co-culture of *Salmonella*-infected macrophages with T cells.

3. To assess the effect of the three serovars on the immune response in the *Salmonella*-infected macrophage/T cell model.

4. To evaluate the role of the virulence-plasmid in modulating the immune response of macrophages in response to different *Salmonella* serovars.

Chapter 2   Materials and Methods

2. 1   Bacterial strains and culture

All the bacterial strains (Table 2-1, Table 2-2 and Table 2-3) used in this study were stored in nutrient broth containing 30% (v/v) glycerol (Fisher Scientific, Leicestershire, UK) at -70°C in the School of Veterinary Medicine and Science (SVMS). Before routine culture on nutrient agar (Oxoid, UK) at 37°C, these strains were checked for purity on MacConkey agar (Oxoid, UK) and resistance to marker antibiotics confirmed. Slide agglutination with anti-serum was used where it was appropriate (see Appendix.1). For experimental infection, these strains were cultured for 18 h in nutrient broth (Oxoid, UK) in an orbital shaking incubator (Forma orbital shaker-Thermo, UK) at 150 rpm and 37°C.

Table 2-1. Strains of *S. enterica* used in this study.

Strain	Description	Reference
SP 449/87	Wt, Nal^r; Poultry-specific strain Produces persistent infection	(Wigley *et al.*, 2001, Wigley *et al.*, 2002b)
SE P125109	Wt, Nal^r; Host non-specific strain, phage type (PT) 4 Virulent in newly hatched chickens, invasive and causing egg contamination in laying hens	(Barrow, 1991, Barrow *et al.*, 1991).
SG 9	Wt, Nal^r; Poultry-specific strain Produce fowl typhoid disease	(Smith, 1955, Barrow *et al.*, 1987b)

SP, *S.* Pullorum; SE, *S.* Enteritidis; SG, *S.* Gallinarum; Wt: wild type strain; Nalr: nalidixic acid resistant

Table 2-2. Extensively selected strains of *S. enterica* used in this study.

Strain	Relevant description
SP 449/87	Detailed in Table 2-1.
SP 3	Detailed in Table 2-3.
SP 5188/86	Isolated in Wilishire Weybridge, UK.
SP 31	Isolated in Copenhagen, Denmark. Poor growth on MacConkey plates.
SP 1002	Isolated from diseased chicken in Yangzhou, China.
SP 380/99	Isolated by Prof. A. Berchieri (University of Sao Paulo) from diseased chickens in Brazil.
SEP125109	PT4, detailed in Table 2-1.
SE PT6	Amp[r]. Isolated from chicken.
SE PT8	Isolated from chicken.
SG 9	Detailed in Table 2-1.
SG 238	Isolated in Greece.
SG 115/80	Antibiotics resistance; isolated in Kenya.
SG 287/91	Isolated by Prof. A. Berchieri from diseased egg-laying hens in Brazil (Jones *et al.*, 2001, Thomson *et al.*, 2008).

SP, *S*. Pullorum; SE, *S*. Enteritidis; SG, *S*. Gallinarum; Amp[r], ampicillin resistant;

Table 2-3. Plasmid-cured and restored strains of *S. enterica* used in this study.

Strain	Plasmid content	Description	Reference
SP 3 parent	pBL001	Parent strain; Wt, Amp[s] Nal[s]	(Barrow and Lovell, 1988)
SP 3 plasmid-cured	None	Virulence plasmid-cured from *S.* Pullorum 3, Amp[s] Nal[s]	(Barrow and Lovell, 1988, Barrow and Lovell, 1989)
SP 3 plasmid restored	pBL001::Tn*3*	Tn3-tagged plasmid reintroduced into *S.* Pullorum 3, Amp[r] Nal[r]	(Barrow and Lovell, 1988, Barrow and Lovell, 1989)
SE P125109 parent	pHH001	Parent strain; Wt, Amp[s] Nal[s]	(Barrow, 1991, Barrow and Lovell, 1991, Thomson *et al.*, 2008)
SE P125109 plasmid-cured	None	Virulence plasmid-cured from *S.* Enteritidis P125109, Amp[s], Nal[r]	(Halavatkar and Barrow, 1993)
SE P125109 plasmid restored	pHH001::Tn*3*	Tn3-tagged plasmid reintroduced into *S.* Enteritidis P125109, Amp[r] Nal[r]	(Halavatkar and Barrow, 1993)
SG 9 parent	pSG090	Field strain; Wt, Amp[s], Nal[r]	(Barrow *et al.*, 1987b)
SG 9 plasmid-cured	None	Virulence plasmid-cured from *S.* Gallinarum 9, Amp[s] Nal[s]	(Barrow and Lovell, 1989)
SG 9 plasmid restored	pSG090::Tn*3*	Tn3-tagged plasmid reintroduced into *S.* Gallinarum 9, Amp[r] Nal[r].	(Barrow and Lovell, 1989)

SP, *S.* Pullorum; SE, *S.* Enteritidis; SG, *S.* Gallinarum; Wt: wild type strain; Amp[s], ampicillin sensitive; Nal[s], nalidixic acid sensitive; Amp[r], ampicillin resistant; Nal[r], nalidixic acid resistant.

2. 2   Tissue culture

2. 2. 1   Determination of cell numbers

A haemocytometer was used to determine cell numbers in all cases. Briefly, 30 µl of cell suspension was mixed with 30 µl of 0.4% Trypan Blue (Sigma-Aldrich, UK) (a restriction dye which can only cross the membrane of dead cells). A 10 µl aliquot of the mixture pipetted onto the edge of the cover slip was allowed to run onto the chamber under the cover slip by capillary action. The haemocytometer was then placed under the microscope (Leica Microsystems, UK) for cell counting.

2. 2. 2   Determination of cell viability

Propidium iodide (PI) (Life Technologies, UK) is a fluorescent restriction dye that interacts with double-stranded nucleic acids. It is excluded by viable cells but can penetrate cell membranes of dying or dead cells. Cell viability was assessed by PI uptake via flow cytometric analysis (see methods in section 2. 4. 2). Briefly, 10 µl of PI staining solution (20 µg/ml) was added and gently mixed with approximately 1×10^6 cells in 100 µl of cell staining buffer (Southern Biotech, Birmingham, AL), which was incubated for 15 min in the dark before being applied for data acquisition using the flow cytometer. Histograms of fluorescence-signal versus counts were then drawn and gated against unlabelled cells to calculate the percentage of dead cells.

2. 2. 3 Chicken peripheral blood monocyte-derived macrophages (chMDM)

2. 2. 3. 1 Chicken peripheral blood collection

Chicken peripheral blood used for the isolation of peripheral blood mononuclear cells (PBMCs) was obtained from two sources. In early experiments blood was purchased from the Harlan Laboratories U.K. Ltd (Leicestershire, UK) (for studies described in Chapter 3 and Chapter 4 except 4. 2. 5). The layers more than 1 year of age were Lohmann Lite and came from the Millennium Hatchery (Studley, Warwickshire, UK). These layers had been vaccinated with live *Salmonella* Vac E+ Vac T Combo Special 2K at ages of 0.4, 6 and 14.4 weeks. There is no reports of protection by a live *Salmonella* vaccine lasting more than 6-9 months. A second source was from unvaccinated breeder blood collected from a spent layer breeder farm containing Lohmann layers (for studies described in section 4. 2. 5).

Chicken whole blood was collected fresh from healthy, adventitious virus-free Harlan barrier-reared layer chickens. Each lithium heparin (200 U/ml Lithium Heparin at 1 in 10 dilution with whole blood)-anti-coagulated blood product was stored in an ice-box during transportation and received within 4 h of bleeding.

2. 2. 3. 2 Preparation of chicken PBMCs

The isolation of chicken PBMCs by Histopaque 1083 (Sigma-Aldrich, UK) density gradient centrifugation has been described previously (Wigley *et al.*, 2002a). The flow diagram (Figure 2-1) below shows the flow of work for this

procedure. In detail, 25 ml of pre-warmed (37 °C) Histopaque 1083 was pipetted into a sterile 50 ml centrifuge tubes. 12.5 ml of lithium heparin anti-coagulated chicken whole blood was mixed with an equal volume of pre-warmed PBS in another sterile 50 ml centrifuge tube and 25 ml of the blood suspension was then slowly overlaid onto the Histopaque 1083 layer. A clear separation between the blood layer and Histopaque 1083 medium was maintained before centrifugation at 400×g for 30 min at 20°C with no brake applied. After the gradient centrifugation, the blood separated into a top layer of plasma and a bottom layer of centrifuged red blood cells. Between the interface of the Histopaque and the plasma, an opaque interface of PBMCs was collected by using a sterile Pasteur pipette (Greiner Bio-one, Gloucestershire, UK) with some red blood cell contamination. The nucleated red blood cells in birds are not lysed by lysing buffer (ammonium chloride (8290.0 mg/L), potassium bicarbonate (1000.0 mg/L) and EDTA (37.0 mg/L), which is used for the lysis of non-nucleated red blood cells in mammals. Thus, the density gradient centrifugation (400×g for 20 min at 20°C) was repeated to eliminate red blood cell contamination from the chicken PBMCs. PBMCs were then collected and washed 3 times in pre-warmed PBS by centrifugation at 300×g for 10min at 20°C to remove excess Histopaque.
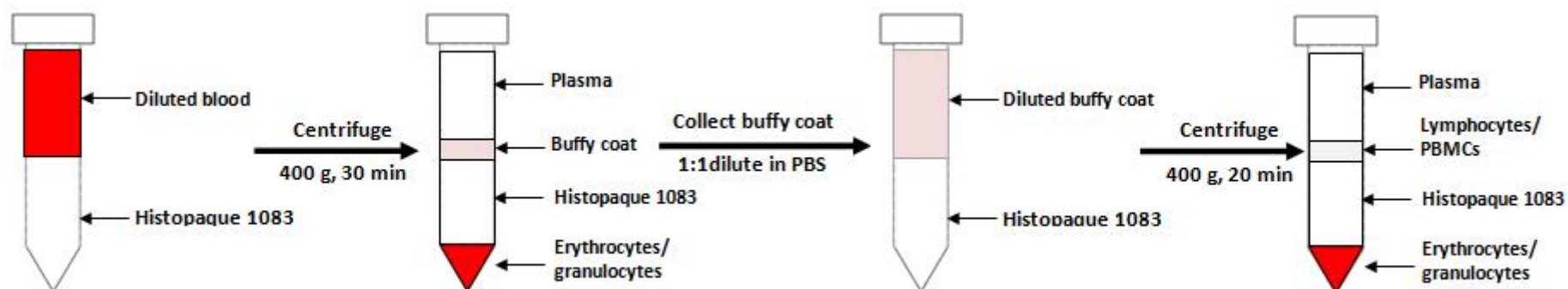
Figure 2-1. The flow of work for the isolation of PBMCs from chicken peripheral blood by density gradient centrifugation

2. 2. 3. 3   Preparation of chMDM

For further culture of chMDM, the cell pellet was resuspended in chMDM culture medium (see Appendix.2) required to make a density of 5×10^6 cells/ ml after performing cell counting. Aliquots of 1 ml of cell preparation were plated into each well of a 24-well plate (Thermo Fisher Scientific, UK). The cells were then incubated at 41°C in a humid 5% CO_2 for 48 h. The medium was changed after 24 h to remove non-adherent thrombocytes and lymphocytes and incubated for a further 24 h at 41°C, 5% CO_2 to allow conversion into macrophages.

2. 2. 4   Chicken CD4$^+$ lymphocytes

Chicken PBMCs were isolated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, UK) by following the technique described in the section 2. 2. 3. 2. Chicken CD4$^+$ lymphocytes (CD4$^+$ T cells) were then selected from PBMCs using magnetic-activated cell sorting (MACS) separation technology (Miltenyi Biotec Ltd., Surrey, UK), which was described previously (Annamalai and Selvaraj, 2010). Based on MACS Microbeads (super-paramagnetic particles conjugated to specific antibody or protein), MACS technology separation was done with a MACS column placed in a MACS separator (a strong magnet with a gradient magnetic field induced on the column matrix). Ice-cold MACS buffer (see Appendix.2) was used throughout the isolation procedure. Briefly, PBMCs collected after density gradient centrifugation were washed in MACS buffer followed by centrifugation at 200×g for 10 min to remove platelets. The cell pellet was resuspended and

labelled with mouse anti-chicken CD4 monoclonal antibody (Table 2-4, #2, 1 µg per 10^6 total cells) in MACS buffer for 30 min at 4°C on a roller. Staining antibody (Table 2-4, #3, 5 µg per 10^6 total cells) was added when necessary to label CD4$^+$ T cells followed by flow cytometric analysis. Unbound antibody was removed by washing cells in 1-2 ml of MACS buffer per 10^7 cells and centrifuging at 300×g for 10 min. The cell pellet was resuspended in 80 µl of MACS buffer per 10^7 cells and incubated with 20 µl of anti-mouse IgG1 Microbeads (Miltenyi Biotec Ltd., Surrey, UK) per 10^7 total cells for 15 min at 4°C on a roller. Unbound Microbeads were removed by washing cells in 1-2 ml of MACS buffer per 10^7 cells and centrifuging at 300×g for 10 min. Up to 10^8 cells were resuspended in 500 µl of MACS buffer before processing by magnetic separation. A LS column (Miltenyi Biotec Ltd., Surrey, UK) was placed in the magnetic field of a MidiMACS™ Separator (Miltenyi Biotec Ltd., Surrey, UK) and prepared by rinsing with 3 ml of MACS buffer. The cell suspension was applied onto the column and the magnetically labelled CD4$^+$ T cells were retained on the column while the unlabelled cell fraction passed through and was separated from cells labelled with primary mouse anti-chicken CD4 antibodies. The column was washed 3 times in 3 ml of MACS buffer before removal from the separator. An appropriate amount of MACS buffer was pipetted onto the column and the fraction of magnetically labelled cells was flushed out by firmly applying the plunger. The number and viability of isolated chicken CD4$^+$ T cells were determined according to the techniques described in the section 2. 2. 1 and 2. 2. 2, respectively.

2. 2. 5   Chicken macrophages-like HD11 cell culture

Chicken macrophage-like cells (HD11) (Leutz *et al.*, 1984) were cultured in 75 cm filtered tissue culture flasks (Thermo Fisher Scientific, UK) in HD11 cell culture medium (see Appendix.2). Before harvesting the cells, old medium was removed and the non-adherent cells were washed 3 times with PBS. 5 ml trypsin/EDTA (Sigma-Aldrich, UK) was then added to the flasks and the cells were incubated at 37°C, 5% CO_2 up to 5 min to release the cells from the flasks. An equal volume of culture medium was added and the contents were transferred to a Falcon tube before being centrifuged at 300×g for 5 min. For the invasion assay, the cells were re-suspended in HD11 cell culture medium and made up to 5×10^5 cells/ml. Aliquots of 1 ml of cell preparation were seeded into 24-well plates and incubated at 37°C, 5% CO_2.

2. 2. 6   Preparation of splenocytes

Spleen from newly-hatched Lohmann Lite layers chickens were removed aseptically and immediately placed in 2 ml PBS containing 20 µg/ml of gentamicin sulphate (Sigma-Aldrich, UK). Spleens were then placed onto a strainer (BD Biosciences, UK) attached to a 50 ml centrifuge tube and pushed through the strainer using the plunger end of a syringe. The strainer was washed with sterile PBS to collect splenic cells. Fresh antibiotic-free chMDM culture medium (see Appendix.2) was added to keep the splenocytes for further use.

2. 3 *Salmonella* infection of avian cells *(in vitro)*

2. 3. 1 Cell invasion

The supernatants of tissue culture were replaced with fresh antibiotic-free medium at least 2 h prior to infection. A 100 µl aliquot of overnight bacterial culture was inoculated into 10 ml nutrient broth and cultured in a shaking incubator (150 rpm/min) at 37°C for 2-6 h to reach their exponential growth phase. Bacteria were pelleted by centrifuge at 1000×g for 10 min and re-suspended in an appropriate volume of PBS calculated by measuring the OD_{600} of bacteria cultures and comparing the values with log counts (see Appendix.3) to produce a suitable density for the correct multiplicity of infection (MOI). The invasion was performed using a MOI of 10:1 (10 bacteria to 1 cell) (Kaiser *et al.*, 2000, Setta *et al.*, 2012a). *S.* Enteritidis LPS (Sigma-Aldrich, UK) (50 µg/ml) was used as a positive control for cytokine production and PBS only was used as a negative controls.

After 1 h of incubation with different strains of *S. enterica* at 37°C, 5% CO_2, the medium of infected cell cultures was changed with fresh culture medium supplemented with 100 µg/ml of gentamicin sulphate and then incubated at 37°C and 5% CO_2 for 1 h to kill the extracellular *S. enterica*. Cell preparations were washed 3 times with sterile PBS and then kept in fresh culture-medium containing 20 µg/ml of gentamicin sulphate thereafter for downstream studies (Kaiser *et al.*, 2000, Jones *et al.*, 2001, Setta *et al.*, 2012a). The invasion assays were performed in triplicate for each strain tested and controls.

Salmonella-infected cells were washed 3 times with pre-warmed sterile PBS and lysed by adding Triton X-100 (1% v/v) (Thermo Fisher Scientific, UK) to release the intracellular bacteria. The lysate was decimal-diluted to 10^{-6} in triplicate for each strain and 100 µl of each dilution were plated on nutrient agar plates to count the number of colonies which were displayed as CFU/ml.

2. 3. 2   Griess assay

NO levels were assessed by measurement of nitrite production using the Griess assay system (Promega, Madison, USA). This assay is based on the chemical reaction between 0.1% sulfanilamide and 1% N-1-napthylethylenediamine dihydrochloride (NED) in 5% phosphoric acid. In this study, at 2, 6, 24, 48 h post-infection (pi), the level of nitrite in the supernatant of infected cell cultures, LPS-stimulated cell culture (positive control) and PBS-treated cell culture (uninfected negative control) were measured. Briefly, 50 µl of the supernatant collected from each tissue cultures were transferred into 96-well flat-bottom plates (Thermo Fisher Scientific, UK) before dispensing 50 µl of the sulfanilamide solution and 50 µl of the NED solution to all wells and incubating at room temperature for 10 min protected from light successively and respectively. The concentration of nitrite was determined by measuring the absorbance at 490 nm using a Micro plate reader LT-4000 (Microplate absorbance reader, Labtech, Sussex, UK). A 7-point double-fold serial dilution (100, 50, 25, 12.5, 6.25, 3.13 and 1.56 µM) of nitrite was performed in triplicate as a standard reference curve of nitrite in each assay.

2. 4   Phenotypic analyses

2. 4. 1   Immunofluorescence labelling

Immunofluorescence labelling of surface antigens on the cells was used to determine the phenotypical characteristics of cells. Cells collected for phenotypic analysis were fixed in 4% paraformaldehyde for 10 min followed by 3 washes in fluorescent-associated cell sorting (FACS) buffer (see Appendix.2). The cell pellet was suspended in staining buffer and the cell number was adjusted to a concentration of 1×10^6 cells/ml.

For direct immunofluorescence staining, cells were incubated with fluorochrome-directly-linked primary antibody for 30 min at 4°C in the dark on a roller followed by 3 washes in FACS buffer to remove unbound antibodies. The cell pellet in each tube was resuspended in FACS buffer and kept on ice, protecting from light prior to flow cytometric analyses (see section 2. 4. 2). An isotype-matched control of each labelled primary antibody was included as negative control and incubated under the same conditions stated above. Isotype controls help to measure the level of non-specific background signal, which is caused by primary antibodies binding non-specifically to Fc (Fragment crystallisable) receptors present on the cell surface.

For indirect immunofluorescence staining, sample preparation and incubation with unlabelled primary antibodies were done under the same conditions as stated above. The cells were washed 3 times in FACS buffer to remove the

unbound primary antibodies. The cell pellet was then re-suspended in staining buffer and incubated with appropriate fluorescein-conjugated secondary antibodies for 25 min at 4°C in the dark on a roller. Cells were washed 3 times in FACS buffer to remove the unbound secondary antibodies and re-suspended FACS buffer and stored on ice, protecting from light prior to flow cytometric analyses (see section 2. 4. 2). Staining with secondary antibody alone using the same conditions described above was included as negative control to eliminate the non-specific binding of each secondary antibody.

2. 4. 2 Flow cytometric analysis

Phenotypic analyses were performed on a FACSCanto II instrument (BD Biosciences, USA) equipped with FACSDiva^{TM} software (BD Biosciences, USA) as standard. The final analyses and graphical output were performed with Flowing software (version 2.5.1, Turku Centre for Biotechnology, University of Turku, Finland).

Non-staining control cells were used to define populations for all samples based on the parameter of forward scatter (FSC) versus side scatter (SSC) in dot-plots. Dot-plots of double fluorescent signals or histograms of fluorescent signals versus cell counts were drawn by gating against the non-staining cells. The percentage of each cell population positive for corresponding fluorescent signals was calculated by the software based on the appropriate gating.

Table 2-4. Monoclonal antibodies used in this study.

No[#]	Antibody: Fluorescein conjugation	Clone	Isotype	Application	†	*
1	Monocytes/macrophages marker Ab (KUL01): PE	KUL01	IgG1κ	Staining mAb, detecting chicken monocytes/macrophages	1	SC
2	Mouse anti-chicken CD4	CT-4	IgG1κ	Labelling mAb, isolating CD4^{+} cells by MACS	1	S
3	Mouse anti-chicken CD4: FITC	2-35	IgG2b	Staining mAb, detecting CD4 expression on CD4^{+} T cells	5	A
4	Mouse anti-chicken CD3	CT-3	IgG1	Primary mAb, detecting CD3 expression on CD4^{+} T cells	2.5	A
5	Mouse-anti-chicken MHC II: FITC	2G11	IgG1	Staining mAb, detecting MHC II expression on chMDM	1	S
6	Mouse-anti-chicken CD40	AV79	IgG2α	Primary mAb, detecting CD40 expression on chMDM	2.5	A
7	Mouse-anti-chicken CD80	IAH: F864:DC7	IgG2α	Primary mAb, detecting CD80 expression on chMDM	2.5	A
8	Mouse-anti-chicken CD86	IAH: F853:AG2	IgG1	Primary mAb, detecting CD86 expression on chMDM	2.5	A
9	Mouse anti-chicken CD28	2-4	IgG2α	Primary mAb, detecting CD28 expression on CD4^{+} T cells	5	A
11	Anti-mouse IgG2α: APC	m2a-15F8		Secondary mAb binding to #6 and #7	2.5	E
12	Anti-mouse IgG1: FITC	M1-14D12		Secondary mAb binding to #4 and #8	2.5	E
13	Mouse IgG1: PE			Isotype control for #1	1	A
14	Mouse IgG1: FITC			Isotype control for #5	1	A
15	Mouse IgG2α: FITC			Isotype control for #9	5	E
16	Mouse IgG2b: FITC			Isotype control for #3	5	E

†working concentration (μg/ml)

*suppliers were referred to by: (A), AbD Serotec (Oxford, UK); (E), eBioscience (Hertfordshire, UK); (SC), Santa Cruz Biotechnology (Texas, US); (S), Southern Biotech (Birmingham, AL). APC, allophycocyanin; FITC, fluorescein; PE, phycoerythrin. mAb, monoclonal antibody.

2. 5   Avian macrophage/ CD4$^+$ T cell model *in vitro*

2. 5. 1   Co-culture infection method

The chicken peripheral blood monocyte-derived macrophages (chMDM) were infected with different serovars of *S. enterica* and co-cultured with peripheral blood CD4$^+$ T cells isolated from peripheral blood to establish an *in vitro* cell model to study the immune modulation of *S. enterica* in adaptive immune response. Three groups of controls were set up: (i) co-culture of uninfected (PBS-treated) chMDM with CD4$^+$ T cells was the control for an allogeneic response resulting from macrophages and lymphocytes isolated from different individual birds; (ii) CD4$^+$ T cells cultured with Concanavalin A (Con A) (10 μg/ml) (Sigma-Aldrich, UK) was the positive control for the proliferation of CD4$^+$ T cells. Con A is a lectin known for its ability to induce mitogenic activity of T-lymphocytes *in vitro*; (iii) culture of CD4$^+$ T cells alone was control for the viability and numbers of CD4$^+$ T cells over a period of 5 days of culture *in vitro*.

The viability and number of chMDM were first determined by following the methods described in the section 2. 2. 2 and 2. 2. 1 at 2 h following infection. Infected or uninfected chMDM were then co-cultured with CD4$^+$ T cells in 1 ml of chMDM culture medium supplemented with 20 μg/ml of gentamicin sulphate in 24-well tissue culture plates at 37°C and 5% CO_2 for 5 days. The ratio of co-culture was kept as 1:10 (chMDM: CD4$^+$ T cells) in each well. At 3 days (d) pi, 500 μl of fresh culture medium (chMDM culture medium supplemented with 20 μg/ml of gentamicin sulphate) was added into each

well. All cultures were repeated in three independent experiments with triplicates on each occasion.

2. 5. 2   Proliferation assay on CD4$^+$ T cells from co-culture

After 5 days of co-culture at 37°C, 5% CO_2, CD4$^+$ T cells from different groups were pipetted off from the tissue culture plates and harvested respectively to measure the proliferation of lymphocytes using the CellTiter® 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, USA). This convenient 'One Solution' assay is a colorimetric method designed to determine the number of viable cells in proliferation assays. The CellTiter 96® AQueous One Solution Reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] combined with an electron coupling reagent (phenazine ethosulfate; PES) of enhanced chemical stability to form a stable solution. The MTS tetrazolium compound is bio-reduced into a coloured formazan product by dehydrogenase enzymes in metabolically active cells. The amount of soluble formazan product in tissue culture medium is directly proportional to the number of living cells in the culture.

All the samples were treated in triplicate as follows: 20 μl of CellTiter® 96 AQueous One Solution Reagent was added into each well of 96-well plates containing 100 μl of cell suspension and the mixture was incubated at 37°C and 5% CO_2 for 4 h in a humidified atmosphere. The absorbance at 490 nm was then recorded on a Micro plate reader and the results are shown as the

number of cells according to the linear regression between the absorbance (490 nm) and the serial dilution of unstimulated CD4$^+$ T cells.

2. 5. 3 Phenotypical analysis of CD4$^+$ T cells from co-cultures

The occurrence of positive (CD28) and negative (CTLA-4) regulation on CD4$^+$ T cells in co-culture with chMDM was determined by following the techniques described in the section 2. 4 and 2. 7.

2. 6 *Salmonella* infection of poultry

2. 6. 1 Experimental animals, infection and sampling

A total of 60 newly-hatched Lohmann Lite chickens were obtained from the Millennium Hatchery (Birmingham, UK). Paper liners were taken randomly from different chick boxes to detect the presence of *Salmonella* prior to infection by incubating in Selenite broth (Sigma-Aldrich, UK) at 37°C for 24 h and then culturing on Brilliant Green agar (BGA) (Oxoid Ltd, UK) plates at 37°C for 24 h. Suspect colonies were examined with *Salmonella*-specific polyclonal antibodies by slide agglutination (see Appendix.1). The day old chickens were divided into four groups with 15 birds each in separate pens and given access to antibiotic-free feed and water throughout the experiment. When 2-days old, three groups were infected with *S.* Pullorum, *S.* Enteritidis or *S.* Gallinarum. Each bird was inoculated orally with 10^8 CFU of the corresponding serovar in 0.1 ml. The uninfected control group was inoculated orally with 0.1 ml of sterile PBS. This work was carried out under Home Office project license PPL 40/3412 and had local ethical approval.

At 1, 2, 4, and 5 d pi, three birds from each group were euthanized to collect tissue samples from each bird for further examination. Post-mortem, (i) spleen and caecal tonsil were collected and stored in RNA*later* (Sigma-Aldrich, UK) at -80 °C before RNA extraction, (ii) caecal content and liver were collected aseptically to determine bacterial loads of *S. enterica* after infection.

2. 6. 2   Bacterial distribution

Tissues from post-mortem, liver and caecal content, were added to a volume of PBS buffer to provide a decimal dilution of tissue. Liver and caecal contents were homogenized by using Griffiths tubes. Further decimal dilutions of the homogenised samples were performed in PBS and 100ul of each dilution were cultured on BGA plates containing sodium nalidixate (20 μg/ml, Sigma-Aldrich, UK) and novobiocin (1 μg/ml, Sigma-Aldrich, UK) at 37°C for 18-24 h. Bacterial colonies at each dilution were enumerated and the tissue count of bacteria was expressed as $Log_{10}CFU/g$.

2. 6. 3   Preparation of tissue samples for RNA extraction

Tissue samples collected from birds were stored in cryovials containing 500 μl RNA*later* to protect RNA with immediate RNase inactivation, which were stored overnight at 4°C before being transferred to -80°C for storage until processing. Prior to extracting the RNA, the RNA*later*-stabilized tissue (less than 30mg) was transferred into a sterile Eppendorf containing 600 μl RLT buffer and a sterile 3mm stainless steel bead. The sample was then disrupted and homogenised in a Retsch MM300 bead mill (Retsch UK Ltd, UK) for 2 min.

The lysate was then centrifuged for 3 min at 16,000×g and the supernatant was carefully removed and transferred to a new tube. RNA extraction from homogenised lysate was carried out by following the methods described in the section 2. 7. 1.

2. 7   Quantification of mRNA gene transcripts

Because cytokine and chemokine production is known to be a descriptive marker of immune clearance or dysfunction in *Salmonella* infection in the chickens (reviewed in the section 1. 4), gene expression of selective chemokines and cytokines and reference gene (28S) *in vitro* or *in vivo* were quantified in the present study using quantitative real-time PCR (qRT-PCR). The primer and probe sequences used in this study are shown in Table 2-5.

2. 7. 1   RNA extraction

RNA extraction was performed using the RNeasy Plus Mini Kit (Qiagen, Crawley, UK). Briefly, cells (typically <5×10^6) were pelleted and lysed using 350 µl of RLT buffer (provided by the kit, containing 10 µl of β-mercaptoethanol (β-ME) per ml RLT buffer). Disruption and homogenization of tissue samples is described in 2. 6. 3.

The homogenized lysate was transferred to a gDNA Eliminator spin column (provide by the kit) and then centrifuged for 30 s at 8,000×g, which was followed by adding 350 µl (600 µl for tissue samples) of 70% ethanol to the flow-through. The mixture was loaded to an RNeasy spin column placed in a 2 ml collection tube (provide by the kit) and centrifuge for 15 s at 8,000×g. This

was followed by the addition of 700 µl Buffer RW1 to the RNeasy Mini spin column and centrifuge for 15 s at 8,000×g to wash the spin column membrane. After discarding the flow-through, 500 µl of Buffer RPE was added to the RNeasy spin column and the tube was centrifuge for 15 s at 8,000×g. The flow through was discarded before adding another 500 µl of Buffer RPE to the column, which was then centrifuged for 2 min at 8,000×g to wash and dry the spin column membrane. The RNeasy spin column was then placed in a new 2 ml collection tube and centrifuged at 8,000×g for 1 min to eliminate any possible carryover of Buffer RPE. This was followed by placing the RNeasy spin column in a new 1.5 ml collection tube, adding 40 µl RNase-free water directly to the spin column membrane and centrifuge for 1 min at 8,000×g to elute the RNA. Purity and quantity were assessed using spectral analysis by NanoDrop spectrophotometer ND-1000 (Labtech International Ltd, UK) and then kept at -80°C until cDNA synthesis.

2. 7. 2   cDNA synthesis

cDNA synthesis was performed with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, UK) using 1 µg RNA per sample in each reaction following the manufacturer's guidelines. Briefly, PCR grade H_2O was added to 1 µg RNA of each sample to make up the volume of 11 µl for each 20 µl transcript reaction in the sterile, nuclease-free, thin-walled PCR tube. The 2 µl kit-included random hexamer primer (600 pmol/µl) was added to each reaction and the template-primer mixture was heated at 65°C for 10 min in a Techne TC-512 thermal cycler (Bibby Scientific Ltd, UK) for denaturation of

RNA secondary structures. The tubes were immediately cooled on ice, followed by addition of these components in order: 4 µl of reverse transcriptase reaction buffer (5× concentration), 0.5 µl of protector RNase inhibitor (40 U/µl), 2 µl of deoxynucleotide mix (10 mM) and 0.5 µl of reverse transcriptase (20 U/µl). cDNA synthesis was conducted with following procedure: 10 min at 25°C for primer annealing, 30 min at 55°C for reverse transcription step and 5 min at 85°C for inactivation of Transcriptor Reverse Transcriptase, then cooled on ice. The cDNA volume amounted to 20 µl of each sample and was tested randomly for quality and quantity on the NanaDrop spectrophotometer ND-1000. The cDNAs were stored at -20°C until used for the qRT-PCR.

2. 7. 3 qRT-PCR

The Light Cycler 480 System (Roche Applied Science, UK) was used for qRT-PCR to measure the gene expression of selected cytokines and chemokines. Primer and probe sequences used in this study have been described previously (Kaiser *et al.*, 2000, Swaggerty *et al.*, 2004, Crhanova *et al.*, 2011, Kim *et al.*, 2012, Setta *et al.*, 2012a) and are listed in Table 2-6. Probes were labelled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5' end and the quencher N,N,N,N'-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end. Primers used to amplify CD28 and CTLA-4 by SYBR green based qRT-PCR are listed in Table 2-7.

Primer concentrations (nM) were optimized by testing various combinations of different concentrations of forward (F) and reverse primer (R) in qRT-PCR. Primer concentrations of 100, 300 and 900 nM, specifically 100F/300R, 100F/900R, 300F/100R, 300F/300R, 300F/900R, 900F/100R, 900F/300R and 900F/900R, were chosen to set up the optimisation. The optimal primer concentration is the lowest concentration that results in the lowest crossing threshold (Ct) and an adequate fluorescence for a given target concentration. Different concentrations of probe (100 nM and 200 nM) were tested to determine the optimal probe concentration using the optimized primer concentrations.

The qPCR mixture for each sample tested consisted of 10 μl Light Cycler probe master mix (2× concentration), the appropriate volume of forward primer, reverse primer and probe at optimized concentrations, 2 μl of template (cDNA) and this was made up to 20 μl with RNase-free water. qPCR was performed following the thermal profile as: one cycle at 95°C for 10 min, 45 cycles at 95°C for 10 s, 60°C for 30 s and 72°C for 1 s and one cycle at 40°C for 30 s. Each qPCR assay contained samples, positive and negative controls and non-template control (NTC) in duplicates. cDNA from LPS-stimulated cells was used for the generation of standard curves for reference gene and target genes, which were performed with 5 point 5-fold serial dilutions in triplicate with replicates on different days. Standard curves were performed for each independent experiment.

Table 2-5. A list of the immune mediators tested in this study and their function.

Immune mediator		Function	Reference
Pro-inflammatory mediators	iNOS	NO production and inflammation Bacterial clearance	(Berndt *et al.*, 2007, Setta *et al.*, 2012a)
	IL-1β	Pro-inflammatory responses	(Weining *et al.*, 1998)
	IL-6	Pro-inflammatory and acute phase responses	(Kaiser *et al.*, 2000, Setta *et al.*, 2012a, Kaiser and Staheli, 2013)
	CXCLi1	Chemo-attraction of particularly heterophils Inflammation	(Setta *et al.*, 2012a, Kaiser and Staheli, 2013)
	CXCLi2	Chemo-attraction of particularly monocytes Inflammation	(Setta *et al.*, 2012a, Kaiser and Staheli, 2013)
Pro-inflammatory (Th1-related) cytokines	IFN-γ	Signature cytokine of Th1-controlled responses Macrophage activating factor	(Kaiser and Staheli, 2013)
	IL-12α	Induce IFN-γ production	(Degen *et al.*, 2004, Kaiser and Staheli, 2013)
	IL-18	Induce IFN-γ production	(Wigley and Kaiser, 2003)
Anti-inflammatory (Th2-related) cytokines	IL-4	Stimulate antibody production	(Degen *et al.*, 2005, Chappell *et al.*, 2009, Kaiser and Staheli, 2013)
	IL-13	Stimulate antibody production	(Degen *et al.*, 2005, Kaiser and Staheli, 2013)
Immune regulator cytokines	TGF-β4	Immuno-regulation Anti-inflammatory	(Kaiser and Staheli, 2013, Kogut *et al.*, 2003)
	IL-10	Immuno-regulation Anti-inflammatory	(Rothwell *et al.*, 2004)
Pro-inflammatory (Th17-related) cytokines	IL-17A	Inflammatory response	(Min and Lillehoj, 2002, Min *et al.*, 2013)
	IL-17F	Inflammatory response	(Kim *et al.*, 2012, Min *et al.*, 2013)

Table 2-6. Sequences of primers and probes used for quantifying gene expression of immune mediators by qRT-PCR.

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

*P, probe; F, forward primer; R, reverse primer

Table 2-7. Sequences of primers tested for quantifying gene expression of CTLA-4 and CD28 in CD4+ T cells by qRT-PCR.

Target RNA	Primers sequence (5' -3')*	Accession number
CTLA-4	F: CAAGGGAAATGGGACGCAAC R: GTCTTCTCTGAATCGCTTTGCC	AM236874.1
CD28	F: GCCAGCCAAACTGACATCTAC R: CTGTAGAAACCAAGAAGTCCCG	NM_205311.1

*F, forward primer; R, reverse primer

2. 8   Statistical analysis

2. 8. 1   General methods for statistical analysis

Statistical analysis was performed using Graphpad Prism 6 software for analysis of variance (ANOVA). Statistical significance was determined at the 5% and 1% confidence limits $P<0.05$ and $P<0.01$.

2. 8. 2   qRT-PCR data analysis method

To account for the variation of cDNA levels among samples within an experiment, the Ct values for the target gene product for each sample were normalized with the Ct value of reference gene product for the same sample, following the methods previously described (Kaiser *et al.*, 2000, Hughes *et al.*, 2007). 28S was used as reference gene to normalize the expression of target genes in this study (Kaiser *et al.*, 2000, Setta *et al.*, 2012a).

The qPCR efficiency of reference gene and each target gene were included into the normalisation of the qPCR data by using linear regression of standard curves with six replicates for each dilution to obtain slopes (regression lines of Ct values against $\log_{10}$ concentration of five point five-fold serial dilution) of either reference gene (S') or each target gene (S).

The experimental mean Ct value for reference gene (Nt) was calculated over all samples in that experiment. The variations between samples in reference gene Ct value (Ct') about the experimental mean were calculated (Nt-Ct'), which was then multiplied by the ratio between the slopes of target gene (S) and reference gene (S'), as follows:

$$\text{Corrected Ct value} = \text{Ct} + (\text{Nt-Ct'}) \times S / S'$$

Where Ct is the mean sample Ct value of target gene, is the mean experimental Ct value of reference gene, Ct' is the mean sample Ct value of reference gene, S is the slope of target gene and S' is the slope of reference gene.

The variation in input total RNA was represented by the different Ct value of reference gene in each sample. Using the slopes of respective target gene and reference gene, the difference with regard to input total RNA was used to correct the corresponding Ct values of target genes. The results were finally expressed as '40-Ct' to allow comparison of gene expression to uninfected controls (Kaiser *et al.*, 2000, Swaggerty *et al.*, 2004). 40-Ct represents 40 cycles of amplification minus the threshold value (Ct). The higher the 40-Ct value the greater the level of gene expression. The fold changes between treatments were calculated as: $F=2^{(T-C)}$, T=corrected 40-Ct for test sample and C= corrected 40-Ct for control sample.

Standard curves were performed on each plate to ensure good consistency of PCR efficiency for each set of primers and probe. The qPCR efficiencies (E) of reference gene and each target gene were determined as:

$$E=(10^{(-1/Slope)}-1) \times 100\%$$

Chapter 3 Immune dynamics of avian macrophages in response to *S.* Pullorum infection and related serovars

3. 1 Introduction

3. 1. 1 General introduction

S. Pullorum is closely related to serovars Gallinarum and Enteritidis at the genomic level (Thomson *et al.*, 2008, Batista *et al.*, 2015). However, they are markedly different in the infection biology. *S.* Enteritidis is generally responsible for a self-limiting enteritis in man or typhoid in mice and intestinal colonisation and vertical transmission to eggs while *S.* Gallinarum usually causes severe systemic infection in chickens with a distinctively different pathology from *S.* Pullorum (Berchieri *et al.*, 2001a).

Following oral infection, phagocytic cells constitute the first line of host defence in response to *Salmonella* infection, of which the important role of macrophages was demonstrated both in chicken (Wigley *et al.*, 2002a, Withanage *et al.*, 2003) and mice (Mastroeni and Sheppard, 2004). Splenic macrophages are the main site of persistent carriage of *S*. Pullorum infection in chicken (Wigley *et al.*, 2001). *S.* Gallinarum may also use macrophage-like cells in the translocation process from gut to deeper tissues of chicken (Jones *et al.*, 2001). It has been found that *S.* Pullorum infection induced much lower levels of splenic IFN-γ with greater levels of IL-4 than occurs during infection by the related serovar *S.* Enteritidis (Chappell *et al.*, 2009). However, the

involvement of *S*. Pullorum-infected avian macrophages in manipulating these changes in cytokine expression and its further causal link to modulation of adaptive immune responses are not clear.

3. 1. 2 Chapter aims and objectives

The ability of *S.* Pullorum to produce persistent infection and avoid immune clearance in chickens might result from an immune response which is clearly different to that induced by the host to related serovars Enteritidis and Gallinarum. We hypothesised that in contrast to serovars such as *S.* Enteritidis, which stimulates a strong Th1 type response, *S.* Pullorum might modulate the host response towards a Th2 immunity by altering macrophage activities (from pro-inflammatory to anti-inflammatory responses) during the innate immune response. The aim of the work described in this chapter was therefore to investigate the innate immune responses of avian macrophages in response to the infection with *S.* Pullorum and related serovars.

3. 2   Results

3. 2. 1   Preparation of chicken peripheral blood monocyte-derived macrophages (chMDM)

Figure 3-1 showed the separation of PBMCs from chicken whole blood by repeated gradient centrifugation as discribed in 2. 2. 3. Chicken monocytes were gated in population (P)-1 (Figure 3-1, f) based on side scatter/forward scatter (SSC/FSC) parameters. The yields of chicken PBMCs from 25 ml of chicken whole blood ranged from 2×10^8 to 5×10^8 cells.
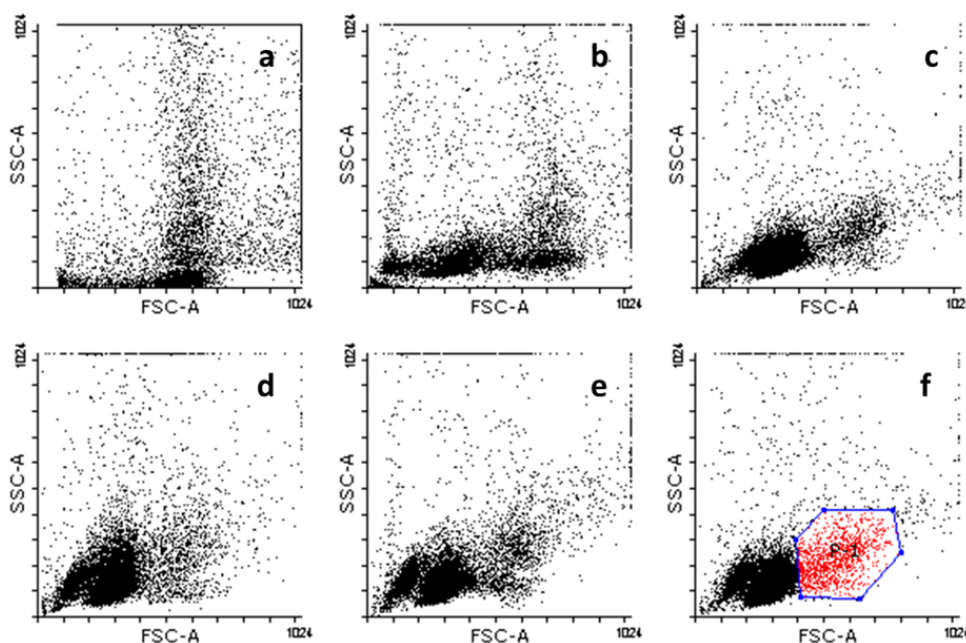
Figure 3-1. Isolation of peripheral blood PBMCs from chicken whole blood by gradient centrifugation. Cells collected at each step of PBMC isolation were analysed using flow cytometer: (a) chicken whole blood (1:1 diluted); (b) buffy coat collected from gradient centrifugation; (c) buffy coat collected from repeated gradient centrifugation; (d-f) PBMCs collected after three washes in PBS followed by centrifugation. The dot-plots represent independent experiments of isolating PBMCs from chicken whole blood**.**

At 24 h and 48 h after incubation, the isolated chicken PBMCs were washed with PBS to remove the non-adherent cells, which are mainly lymphocytes. The proportion of $CD3^+$ cells within adherent and non-adherent populations was determined by flow cytometric analysis, where less than 2 % of adherent cells were found to be CD3-positive at 48 h after incubation, which indicated an effective removal of contaminating lymphocytes by washing off non-adherent cells from the culture plates. During incubation, the morphology of the adherent cells became flatter and more characteristically macrophage-like after 24-48 h of incubation (Figure 3-2) and their viability was found to be in excess of 95 %.
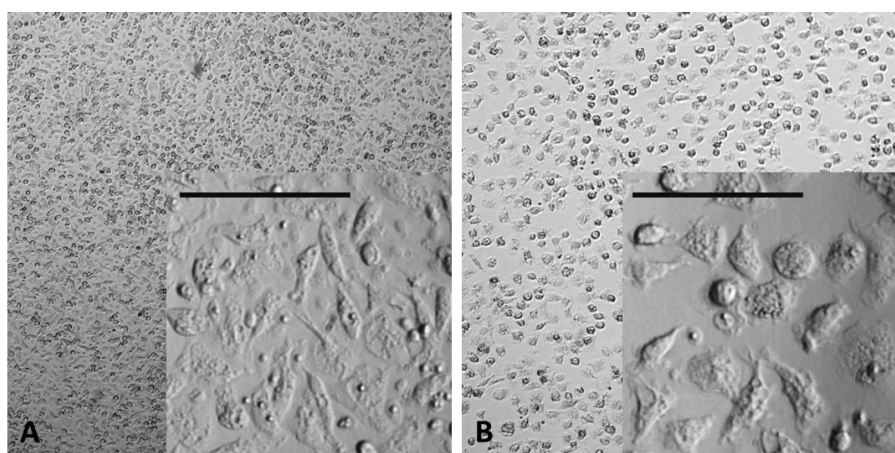
Figure 3-2. Microscopy of chicken peripheral blood monocyte-derived macrophages (chMDM). Adhesive cells become more characteristically macrophage-like after 24-48 h of incubation. PBMCs isolated from chicken whole blood cultured for (A) 24 h and (B) 48 h. The pictures are representative of at least five independent experiments of preparing chMDM from individual batches of chicken whole blood. Scale bar= 50 μm.

The morphological features of adherent cells observed by microscopy coincide with increased granularity and cells size (SSC/FSC) of the adherent cells (Figure 3-3, P2) being assessed in flow cytometric analysis when compared to PBMCs isolated from chicken whole blood (Figure 3-3, P1). Chicken PBMCs and adherent cells after 48 h of incubation were stained selectively with the anti-chicken monocyte/macrophage mAb (clone KUL01, PE), which specifically recognises mononuclear phagocytes in chickens. Approximately 10% of PBMCs isolated from chicken whole blood were KUL01$^+$ cells (Figure 3-3, H1), while the percentage of KUL01$^+$ cells in adherent cells was found in excess of 95% at 48 h after incubation (Figure 3-3, H2), which was phenotypically characterized as chicken peripheral blood monocyte-derived macrophages (chMDM). Prior to the downstream invasion assay and co-culture *in vitro*, the final yield of cells per millilitre was determined by trypan blue exclusion (see section 2. 2. 1).

(a)   (b)

(c)
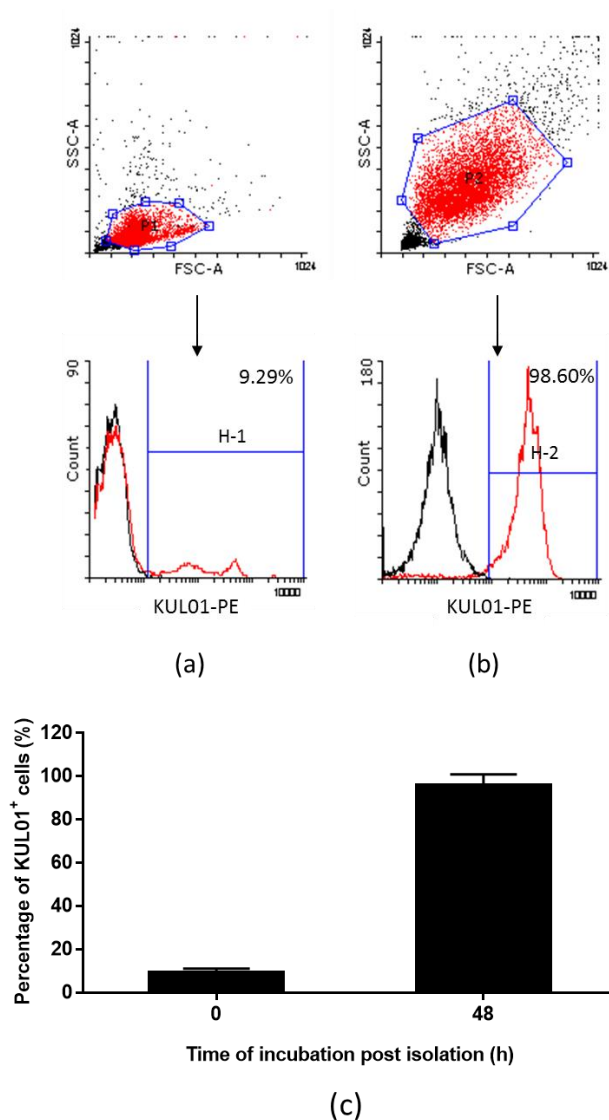
Figure 3-3. Flow cytometric analysis of chicken PBMCs and chMDM. Chicken monocytes and macrophages were specifically recognised by mAb KUL01: PE (a) PBMCs isolated from chicken whole blood are gated within population (P) 1 (P1) in dot-plots. H-1 in histogram represented KUL01+ cells within P1 by IgG1:PE isotype control (black line). (b) PBMCs were cultured for 48 h and non-adherent cells were removed by washing at 24 h and 48 h after incubation. Adherent cells in the culture were analysed and gated within P2 in dot-plots. H-2 in histogram represented KUL01+ cells within P2 by IgG1:PE isotype control (black line). (c) Proportion of KUL01+ cells in *in vitro* culture at different times post-isolation and the data are shown as mean (KUL01+ cells%) ± SEM from three independent experiments.

In addition, the surface expression of Major Histocompatibility Complex (MHC) class II molecules was determined to further characterize chicken PBMCs and chMDM (Figure 3-4). Chicken peripheral blood monocytes were gated in P1 based on their FSC/SSC properties. The cells from within this population were then gated according to the fluorescent signals in the unlabelled control and the percentage of stained cells was indicated by the proportion appearing outside the gate in appropriate treatments. Approximately half of the chicken monocytes were shown to be KUL01$^+$/MHCII$^+$ (Figure 3-4, panel a). After 48 h of incubation, over 95% of adherent cells were found to be KUL01$^+$/MHCII$^+$ cells (Figure 3-4, panel b).
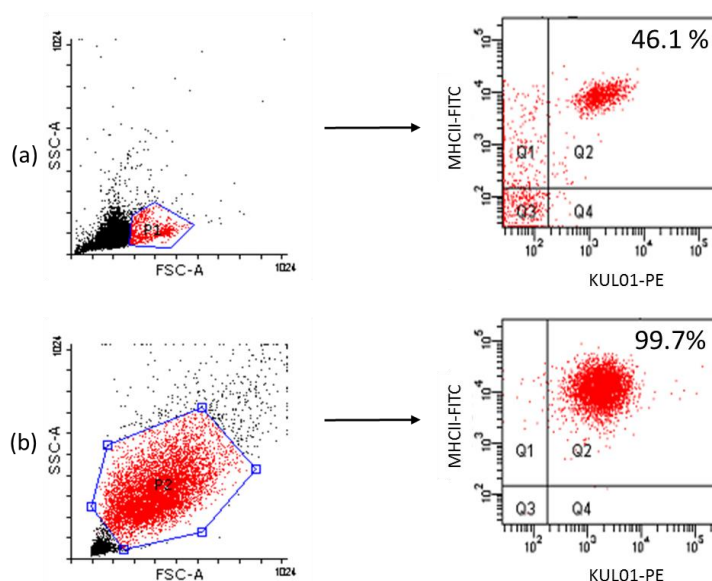
Figure 3-4. Expression of KUL01 and MHCII on chicken PBMCs and chMDM. Chicken PBMCs and chMDM were stained with mAb KUL01 (PE) and mouse-anti-chicken MHC II (FITC). KUL01$^+$/MHCII$^+$ cells are shown in quadrant Q2 accordingly by appropriate isotype control and compensation. (a) PBMCs isolated from chicken whole blood. P1 was gated as monocytes. (b) PBMC-derived macrophages obtained from adherent cells after 48 h of incubation. P2 was gated as macrophages with bigger cell size and more granularity. The dot-plots represent independent experiments of preparing PBMC-derived macrophages from individual batches of chicken whole blood.

3. 2. 2 Invasion and intracellular survival of *Salmonella* in chMDM

The results of invasion by *S*. Pullorum and intracellular survival in chMDM were compared with that of *S*. Enteritidis and *S*. Gallinarum (Figure 3-5). *S*. Enteritidis invaded and/or were taken up by chMDM in greater numbers than *S*. Pullorum and *S*. Gallinarum at all time points post-infection (pi) (*P*<0.05). Bacterial counts in chMDM declined from 6 h pi with bacterial counts of each serovar at 48 h pi being significantly lower than that at 2 h pi (*P*<0.01).
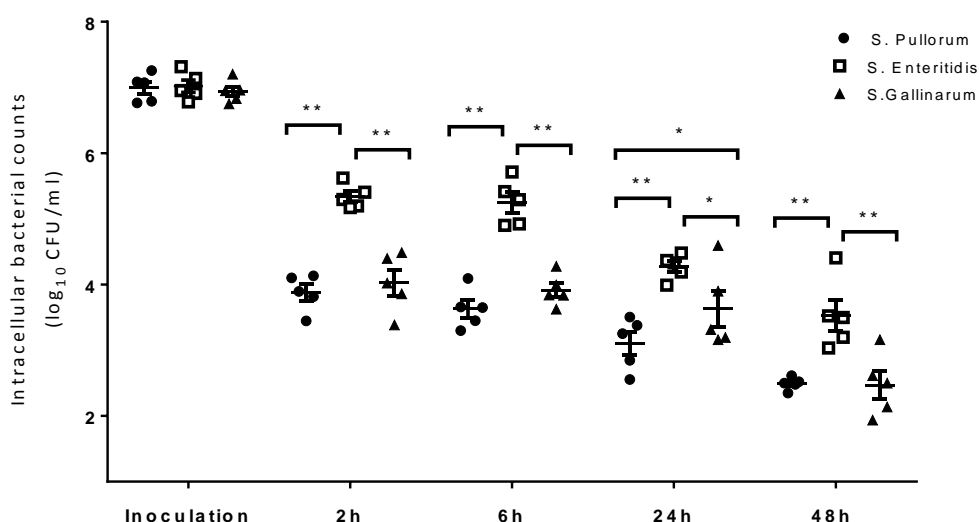
Figure 3-5. Survival dynamics of *Salmonella* serovars in infected chMDM. At 2, 6, 24 and 48 h pi, chMDM infected with different serovars of *Salmonella* were lysed to quantify the intracellular bacterial counts. Viable colony counts were shown as Log_{10} CFU/ml. Results shown are expressed as mean (Log_{10}CFU/ml) ± SEM of independent experiments (n=5). Two-way ANOVA and Tukey's multiple comparisons were performed to determine significant difference between serovars at each time point post infection. (*) shows significant differences between serovars. * *P*<0.05, ***P*<0.01.

The percentage of viable chMDM cells was measured at 2-48 h after infection with different *Salmonella* strains. Following infection, approximately 85% of chMDM cells (84.38%, 83.36% and 82.02% for *S*. Pullorum, *S*. Enteritidis and *S*. Gallinarum, respectively) remained alive until 6 h pi but the percentage of viable cells significantly reduced at 24 h (58.16%, 59.82% and 61.42%) and 48 h (35.1%, 33.12% and 33.87%) pi (*P*<0.01). However, no significance was found between the viability percentages measured from infection with the three serovars (*P*> 0.05).
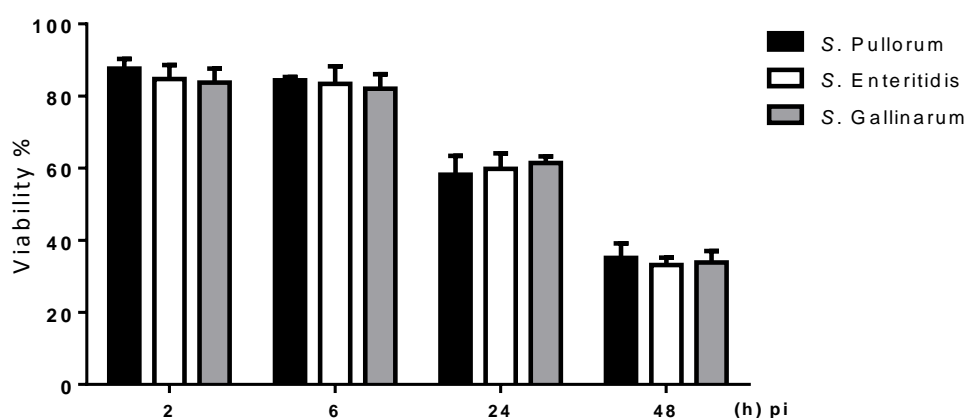
Figure 3-6. Viability of chMDM following infection with different serovars of *Salmonella*. At 2, 6, 24 and 48 h pi, the percentage of viable chMDM infected with *S*. Pullorum, *S*. Enteritidis and *S*. Gallinarum respectively were determined using PI. Data shown are means (viable chMDM %) ± SEM from three independent experiments.

3. 2. 3 NO production

Salmonella infection caused a significant increase in NO production in chMDM at 24 and 48 h pi. At 24 h pi, compared to the uninfected control, increased NO production was only observed from *S.* Enteritidis-infected and LPS-stimulated chMDM (*P*<0.01). The maximal production of NO in chMDM in response to infection with all these serovars was observed at 48 h pi (*P*<0.001), with the level of NO production from *S.* Pullorum-infected chMDM being significantly lower than that produced from *S.* Enteritidis-infected cells (*P*<0.05) (Figure 3-7).
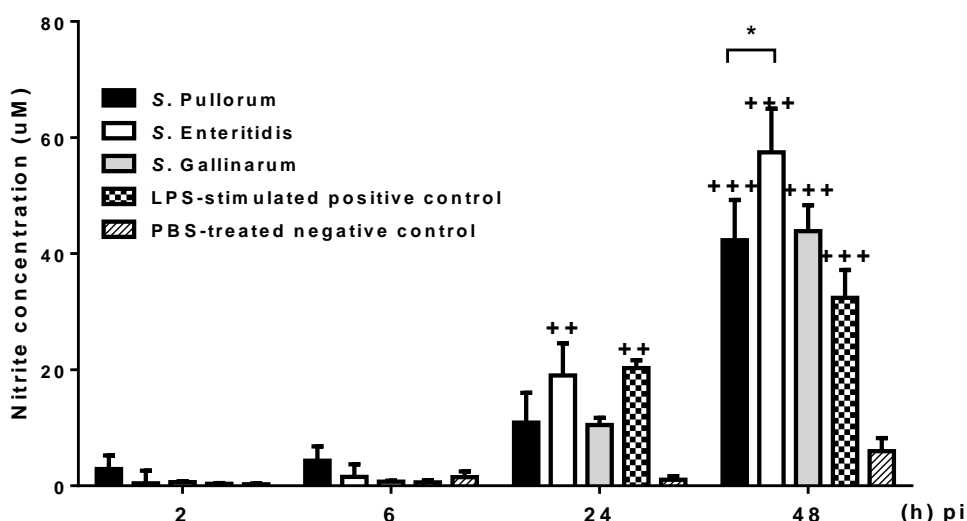
Figure 3-7. NO production by chMDM following infection with different serovars of S. enterica. At 2, 6, 24 and 48 h pi, supernatant was collected from chMDM in different infection or treatment groups to determine the nitrite concentration using Griess assay. The results shown are expressed as means (nitrite concentration, µM) ± SEM of independent experiments (n=3). Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between the groups within each time point. (+) Indicates statistically significant difference from negative control (+*P*<0.05, ++*P*<0.01, +++*P*<0.001). (*) indicates statistical differences between different treatment (**P*<0.05, ***P*<0.01, ****P*<0.001).

3. 2. 4 Quantification of gene expression from chMDM in response to *S.* Pullorum and related serovars

Figure 3-8 showed gene expression of iNOS, the pro-inflammatory chemokines CXCLi1 and CXCLi2 and cytokines IL-6 and IL-1β in avian macrophages in response to infection with different *S. enterica* serovars at 6 h pi. *Salmonella* infection resulted in increased gene expression of these immune mediators in chMDM when compared with the uninfected controls ($P<0.05$ or $P<0.01$). Moreover, the expression levels of CXCLi2, IL-6 and iNOS mRNA from *S.* Enteritidis-infected chMDM were significantly higher than that of *S.* Pullorum- and *S.* Gallinarum-infected cells ($P<0.05$ or $P<0.01$).

(A)

(B)
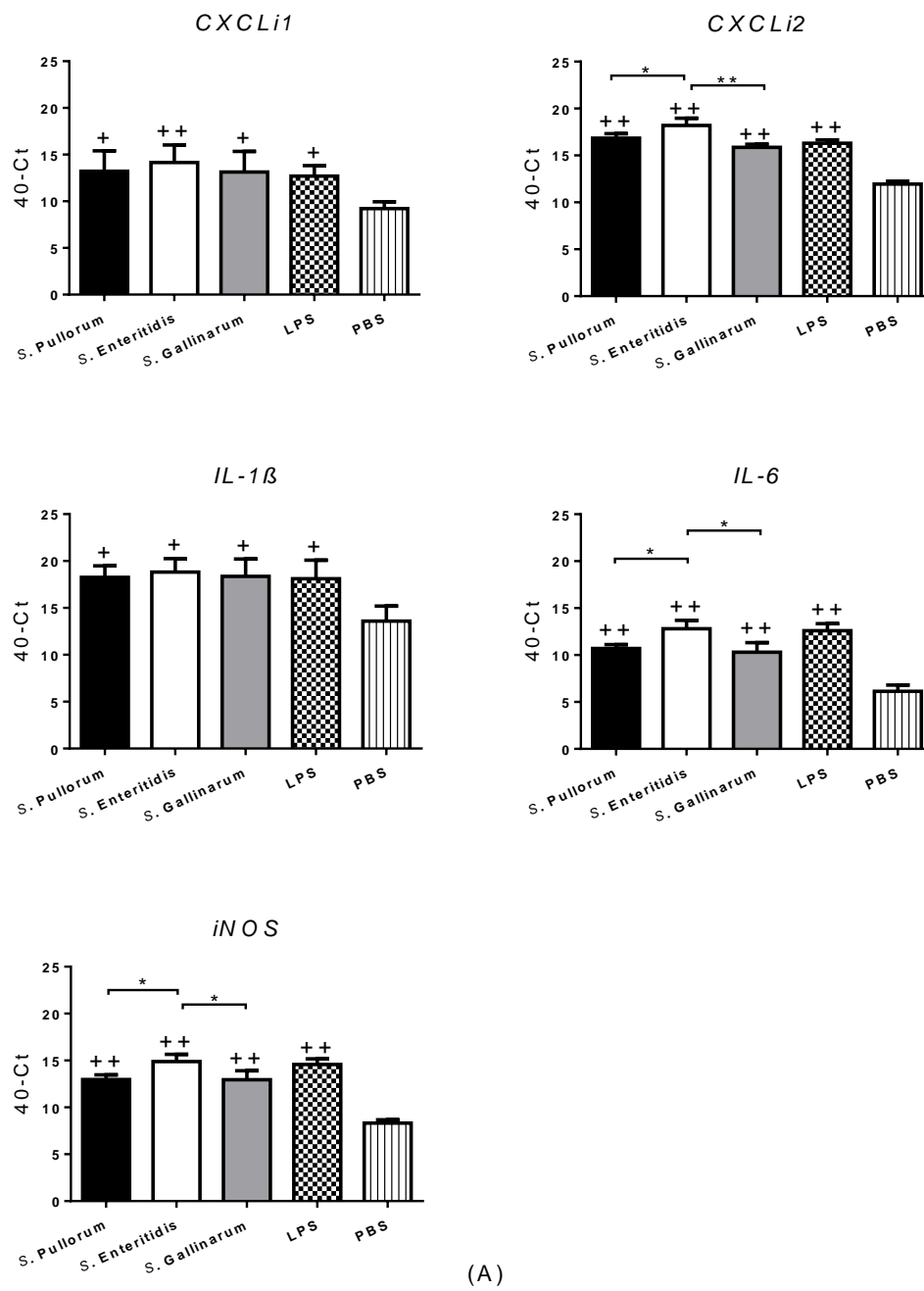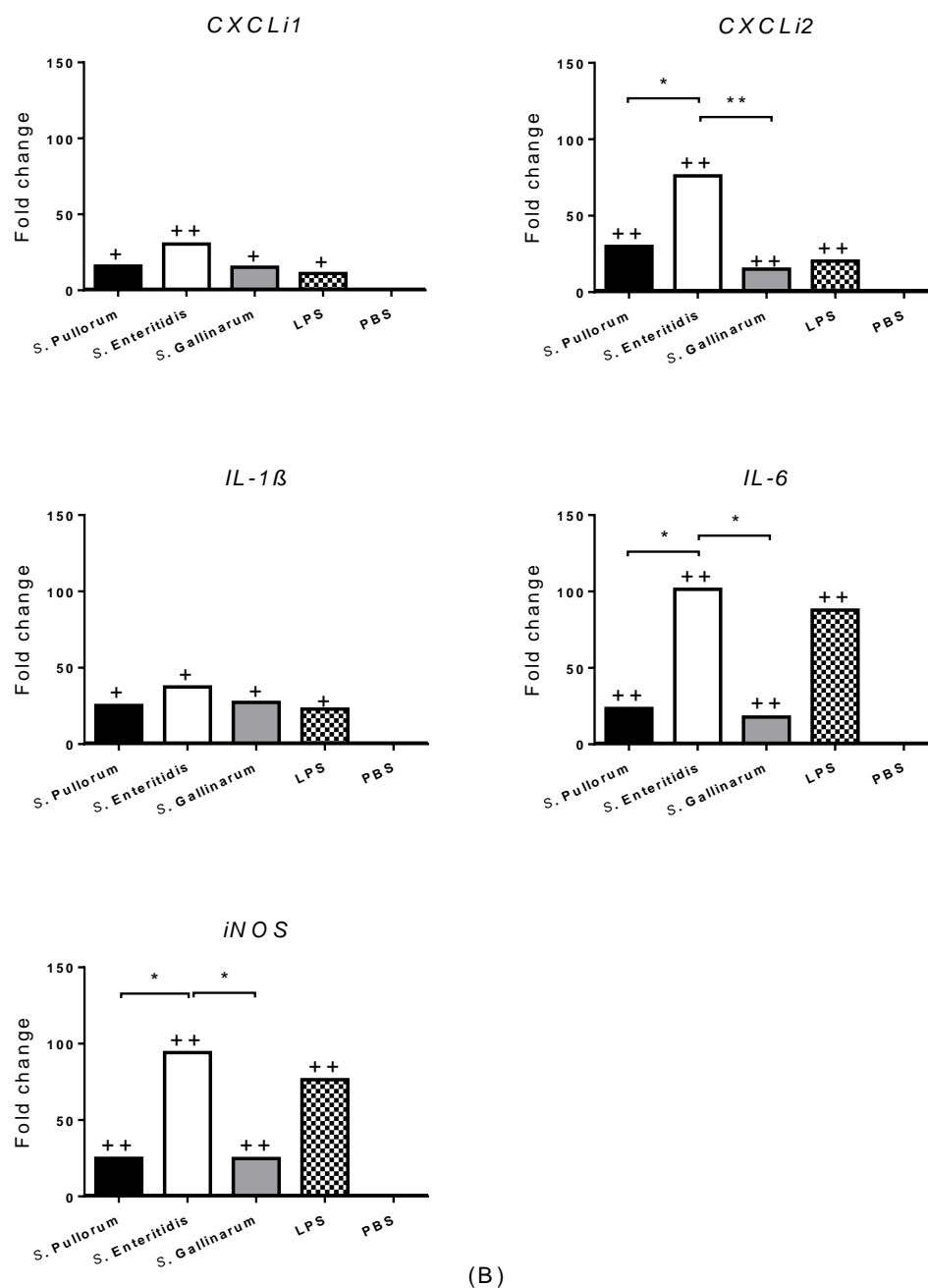
Figure 3-8. CXCLi1, CXCLi2, IL-1β, iNOS and IL-6 mRNA expression from chMDM at 6 h post-infection with different serovars of S*almonella*. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). (+) 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, *: $P<0.05$, **: $P<0.01$.

IFN-γ, IL-12α and IL-18 are pro-inflammatory cytokines related to Th1 immune responses. Infection of chMDM with *S.* Pullorum or *S.* Gallinarum did not up-regulate gene expression of IFN-γ and IL-12α whereas significantly increased expression of IFN-γ, IL-12α and IL-18 was found in *S.* Enteritidis-infected chMDM (*P*<0.01) when compared to uninfected controls. In addition, mRNA levels of IFN-γ, IL-12α and IL-18 from *S.* Enteritidis-infected chMDM were significantly higher than those of *S.* Pullorum-infected cells (*P*<0.05). Moreover, expression of IFN-γ (*P*<0.01), IL-12α (*P*<0.01) and IL-18 (*P*<0.05) were also up-regulated in LPS-stimulated chMDM (Figure 3-9).

(A)

Figure 3-9. IFN-γ, IL-12α and IL-18 mRNA expression from chMDM at 6 h post-infection with different serovars of S*almonella***.** (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). (+) 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, *: $P<0.05$, **: $P<0.01$.

In contrast to the differential expression of IFN-γ, IL-12α and IL-18 mRNA seen above, the overall mRNA expression levels of IL-4 and IL-13, were much lower, without significant differences being observed between experimental groups ($P > 0.05$) (Figure 3-10).
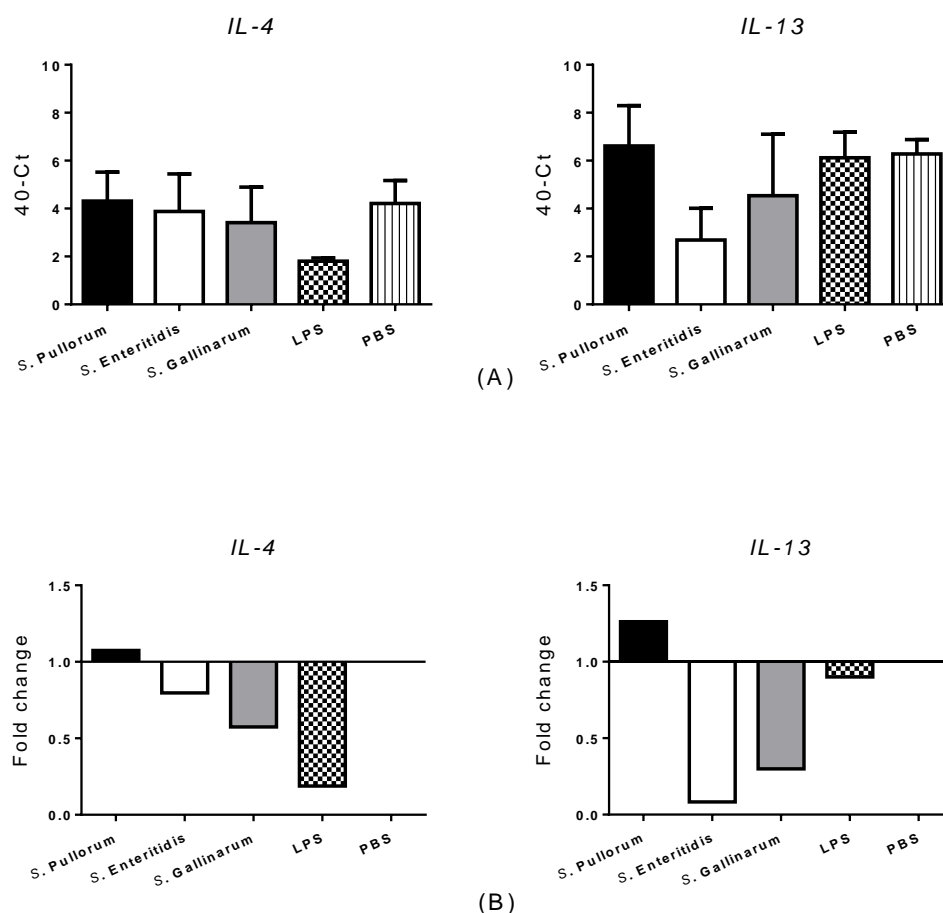
Figure 3-10. IL-4 and IL-13 mRNA expression from chMDM at 6 h post-infection with different serovars of S*almonella*. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). (+) 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, *: $P < 0.05$, **: $P < 0.01$.

Significant levels of IL-10 mRNA was detected in chMDM following infection with *S.* Pullorum (*P*<0.05) or *S.* Enteritidis (*P*<0.01). However, there was no statistical difference between infection groups (*P*>0.05). The mRNA expression of TGF-β4 was not significantly different in *Salmonella*-infected chMDM in comparison with uninfected control cells (Figure 3-11).

Figure 3-11. IL-10 and TGF-β mRNA expression from chMDM at 6 h post-infection with different serovars of S*almonella.** (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). (+) 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.

3. 2. 5 Quantification of gene expression from splenocytes in response to *S.* Pullorum and related serovars

At 6 h pi, infection of chicken splenocytes with *S*. Pullorum did not induce gene expression of IFN-γ, IL-18 and IL-12α while *S.* Enteritidis was again shown to be a robust stimulator of these cytokines, although all the statistical differences between *S*. Pullorum and *S*. Enteritidis seen in infected chMDM were not observed in chicken splenocytes. In addition, the results showed significant up-regulation of IL-17F mRNA following infection with *S*. Enteritidis and *S*. Gallinarum ($P<0.01$). Expression of IL-4 mRNA from *Salmonella*-infected splenocytes was at low levels without significant differences between experimental infection groups (Figure 3-12).

(A)
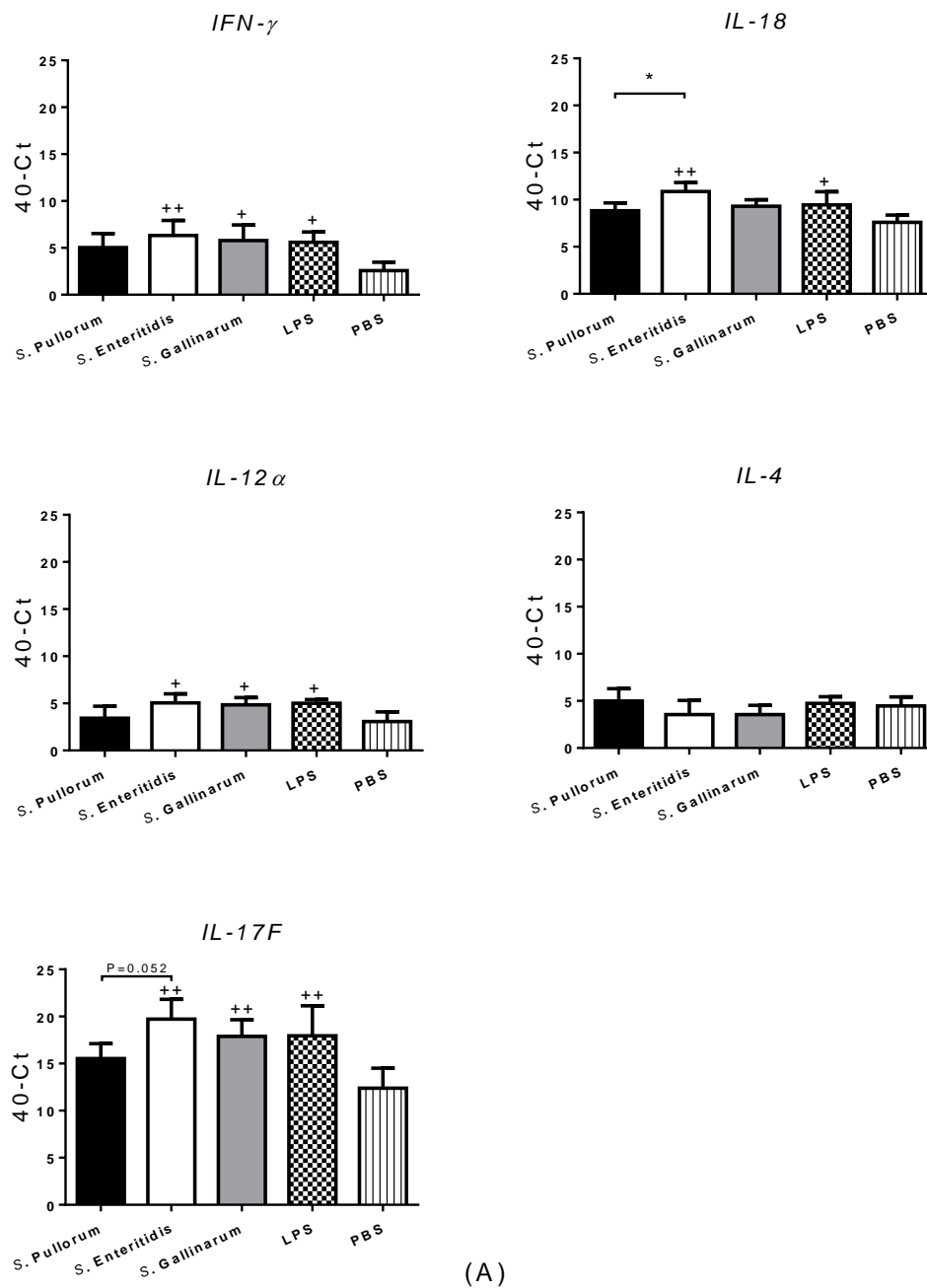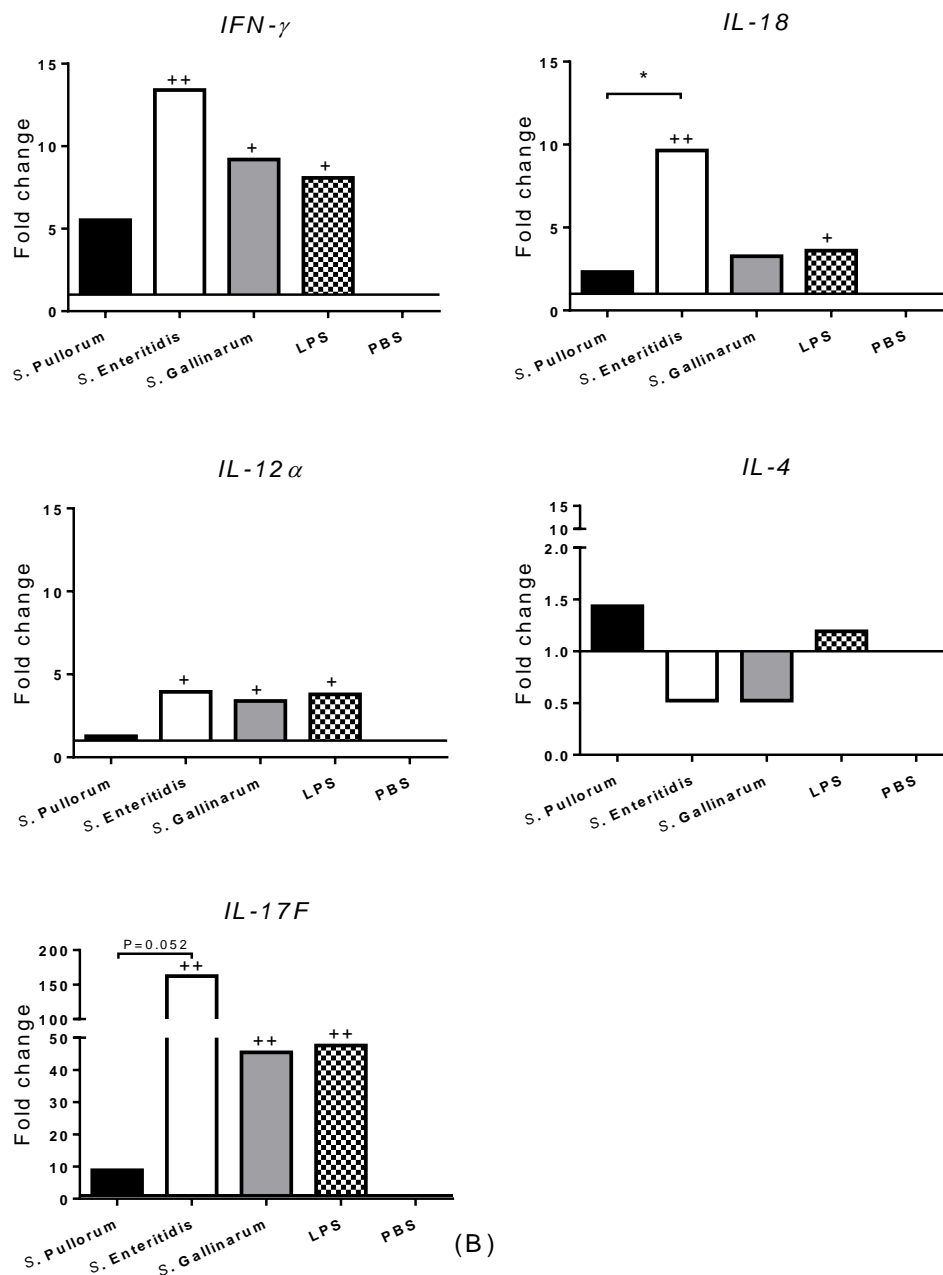
Figure 3-12. IFN-γ, IL-18, IL-12α, IL-4 and IL-17F mRNA expression from chicken splenocytes at 6 h post-infection with different serovars of S*almonella***.** (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=5). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). (+) 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, *: $P<0.05$, **: $P<0.01$.

3. 2. 6 The expression of MHCII and co-stimulatory molecules on *S. enterica* infected macrophages

The expression levels of MHC II and co-stimulatory molecules (CD40, CD80 and CD86) on chMDM in response to *S. enterica* infection was assessed using immunofluorescence labelling followed by flow cytometric analysis as described in section 2. 4. *Salmonella*-infected and uninfected chMDM were gated (P1 in panel A-a, B-a and C-a respectively in Figure 3-13) to exclude cell debris according to cell size and granularity (FSC/SSC parameters). Expression levels of cell surface molecules was then determined by single-colour flow cytometric analysis at 2, 6 and 24 h pi, with results expressed as percentage of the positive population among chMDM collected from each treatment (Figure 3-13, panel D).

Phenotypical analyses indicated that compared with uninfected cells, a significantly increased percentage of CD40$^+$ cells was observed in chMDM infected with *S.* Pullorum or *S.* Enteritidis ($P<0.05$) whereas the proportion of CD80$^+$ and CD86$^+$ cells did not increase in chMDM infected with different serovars of *S. enterica* ($P>0.05$) at 2 h pi. At 6 h after infection, infection with *S. enterica* resulted in a significant increase in the CD40$^+$ population (SP and SE, $P<0.01$; SG, $P<0.05$) while a higher percentage of CD80$^+$ cells was seen with *S.* Enteritidis-infection ($P<0.01$) when compared with uninfected chMDM, However, infection with *S. enterica* did not stimulate the expression of CD86 on chMDM until 6 h pi. In comparison with uninfected chMDM, a significantly increased population of CD40$^+$ (SP and SE, $P<0.01$; SG, $P<0.05$), CD80$^+$ (SP and

SE, $P<0.01$; SG, $P<0.05$) or CD86$^+$ (SP and SG, $P<0.05$; SE, $P<0.01$) cells was found in response to infection with the three serovars of *S. enterica* used at 24 h pi.
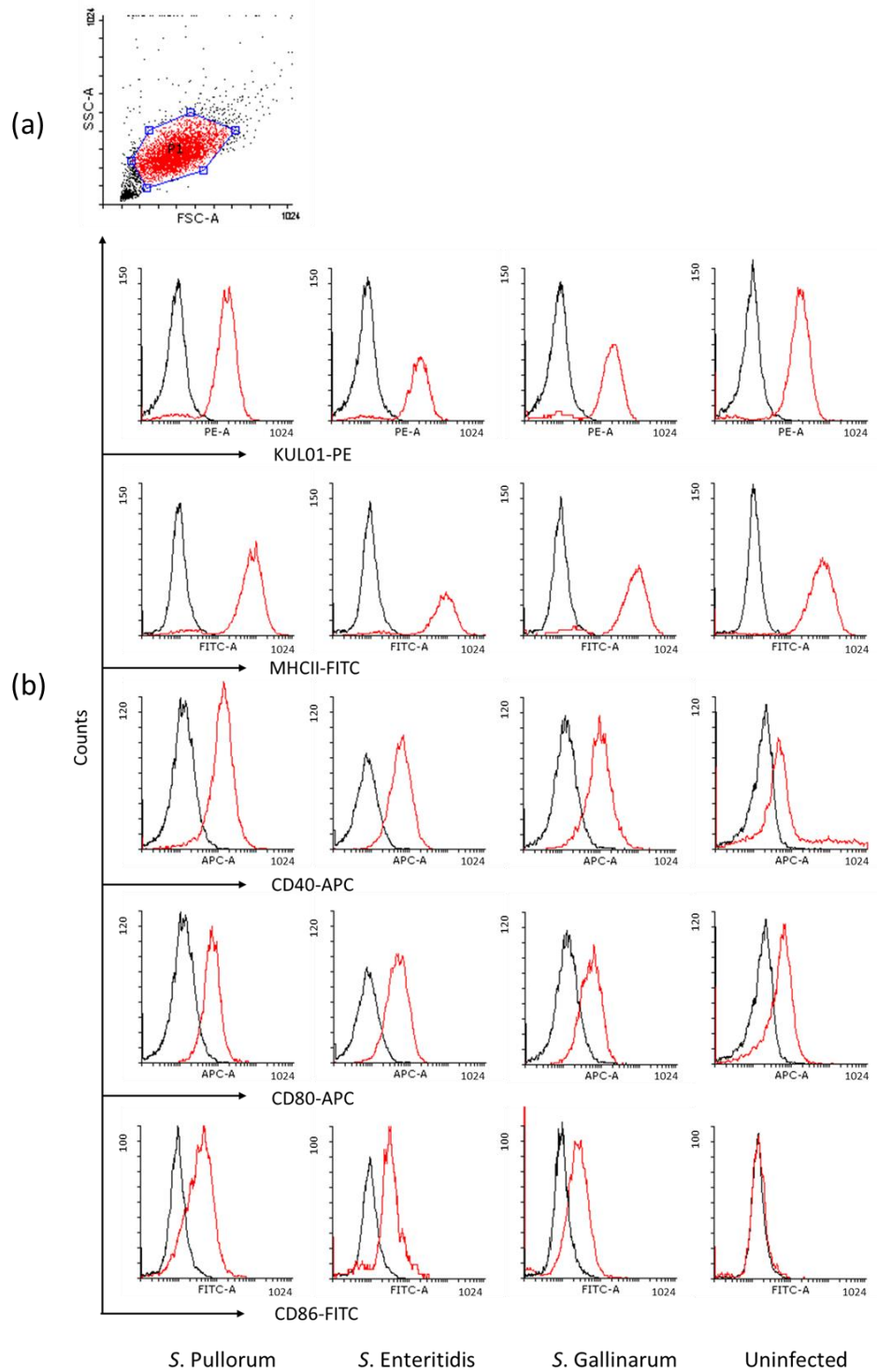
(A) Expression of KUL01, MHCII, CD40, CD80 and CD86 on chMDM infected with different serovars of *Salmonella* at 2 h pi

(B) Expression of KUL01, MHCII, CD40, CD80 and CD86 on chMDM infected with different serovars of *Salmonella* at 6 h pi

(a)

(b)

Counts

KUL01-PE

MHCII-FITC

CD40-APC

CD80-APC

CD86-FITC

S. Pullorum   *S*. Enteritidis   *S*. Gallinarum   Uninfected
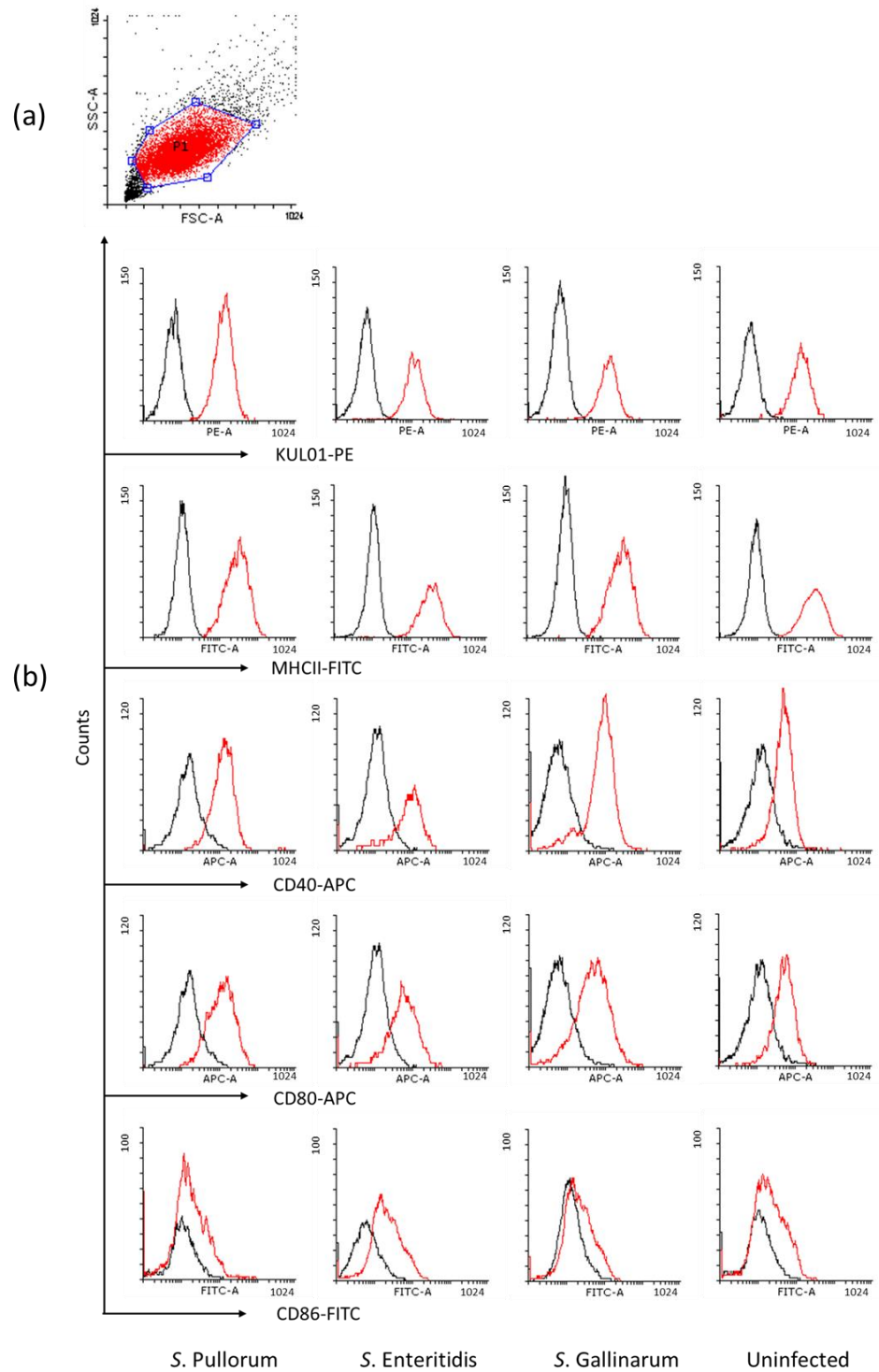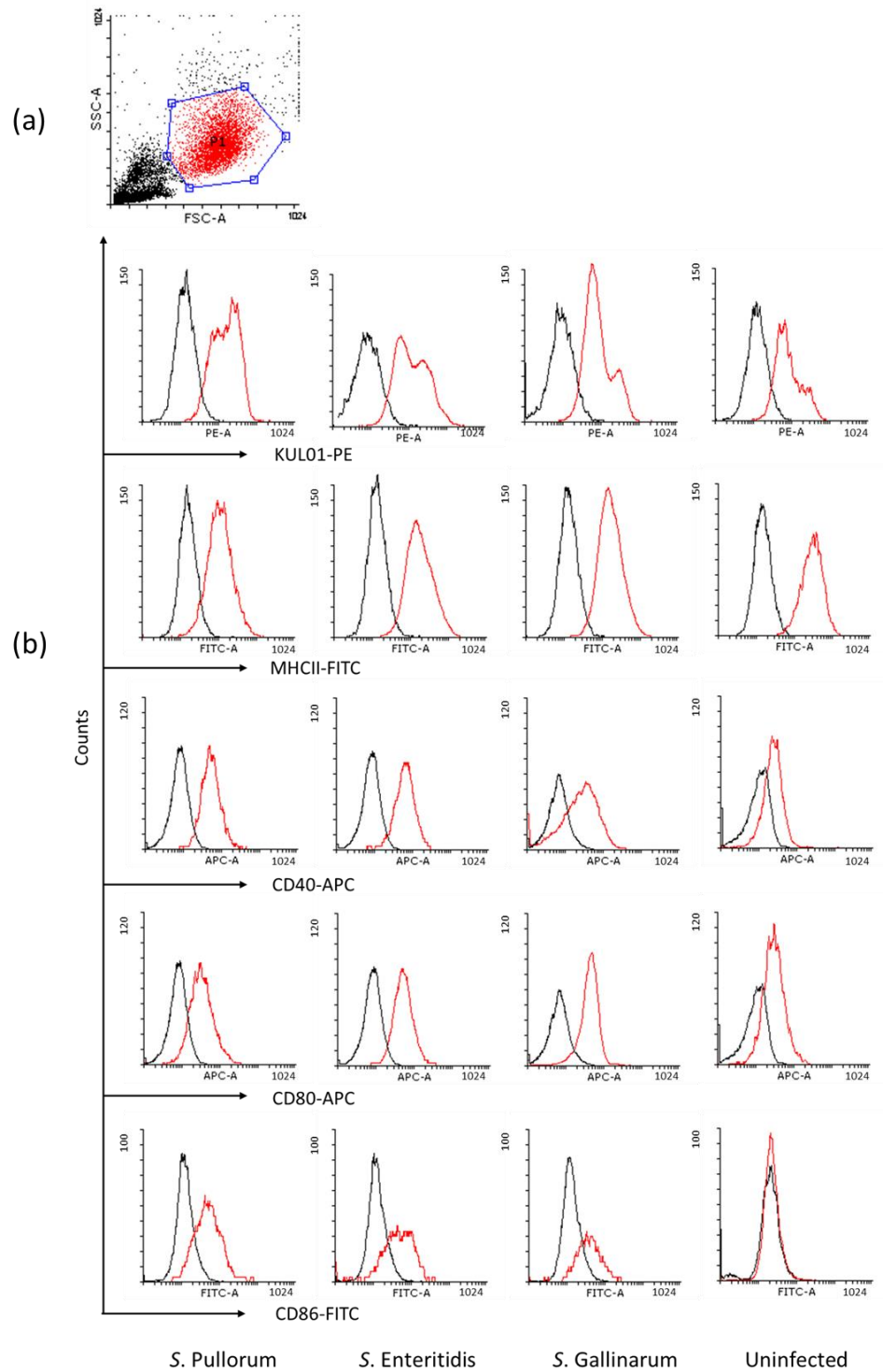
(C) Expression of KUL01, MHCII, CD40, CD80 and CD86 on chMDM infected with different serovars of *Salmonella* at 24 h pi
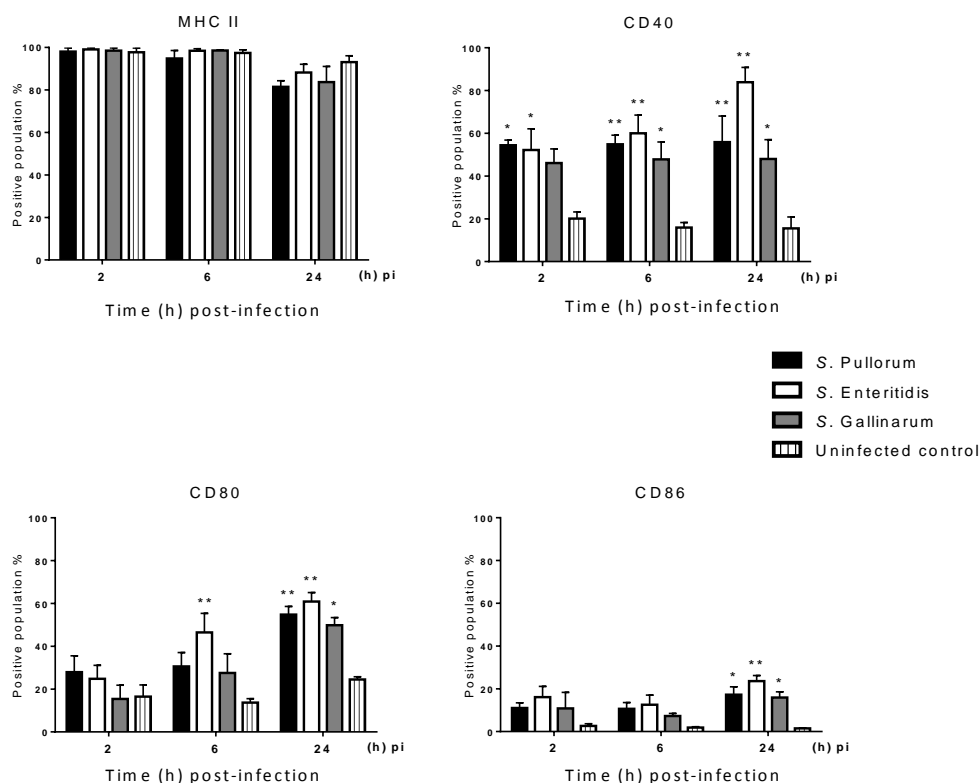
(D)

Figure 3-13. Comparative flow cytometric analysis of surface antigens expression on chMDM in response to _S. enterica_ infection. The chMDM infected with different strains of _S. enterica_ were analysed to determine the positive population in expressing KUL01, MHCII, CD40, CD80 and CD86 respectively at (A) 2, (B) 6 and (C) 24 h pi. KUL01, MHCII, CD40, CD80 and CD86 were recognised by appropriate mAb. At each time point, (a) cells were gated based on side scatter/forward scatter (SSC/FSC) parameters. (b) The histograms shown are representative of three independent experiments. Black lines, secondary binding or isotype control mAbs; red lines, anti-chicken cell surface marker mAbs. (D) The proportion of positive cells from three independent experiments, data shown as mean ± SEM (n=3). Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between experimental groups at each time point. (*) indicates statistically significant differences from uninfected control. *$P<0.05$, **$P<0.01$.

3. 2. 7 Immune responses to infection of chMDM by a wider selection of strains from the three *S. enterica* serovars: NO production and different host gene expression

S. Pullorum 449/87, *S.* Enteritidis P125109 and *S.* Gallinarum 9 are representative strains each of *Salmonella* serovars Pullorum, Enteritidis and Gallinarum, it is essential to demonstrate how representative these strains might be of other strains of the same serovars in terms of their effects in modulating *in vitro* macrophage activities.

3. 2. 7. 1 NO production

NO production from experimental infection of chMDM with these 13 strains of the three different serovars of *S. enterica* maintained the same pattern as shown in Figure 3-7 where infection of chMDM with these strains resulted in significantly higher production of NO at 24 and 48 h pi with the maximal production observed at 48 h pi when compared to the uninfected controls (Figure 3-14, a). Furthermore, there was a trend that NO produced from *S.* Pullorum (6 different strains)-infected cells were significantly lower than that produced from *S.* Enteritidis (3 different strains)-infected chMDM at 24 ($P<0.01$) and 48 h pi ($P<0.05$) (Figure 3-14, b and c).

(a)

(b)

(c)
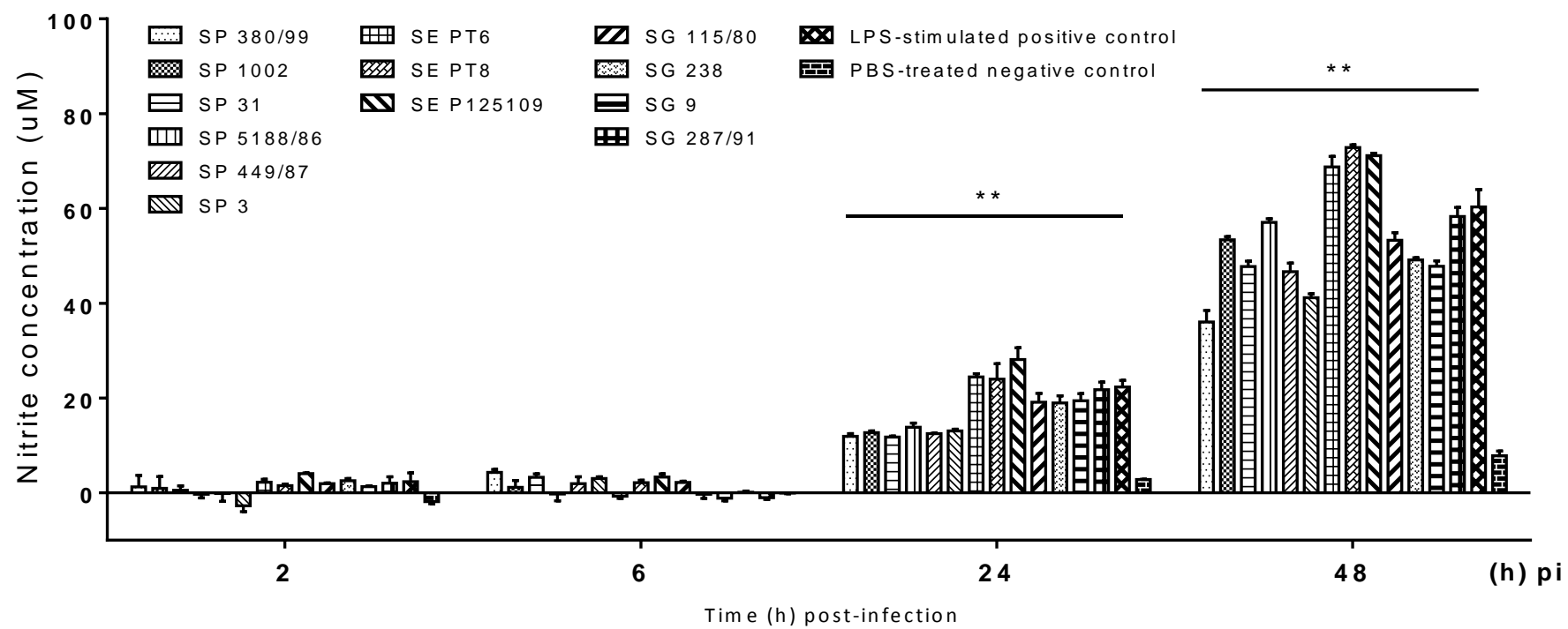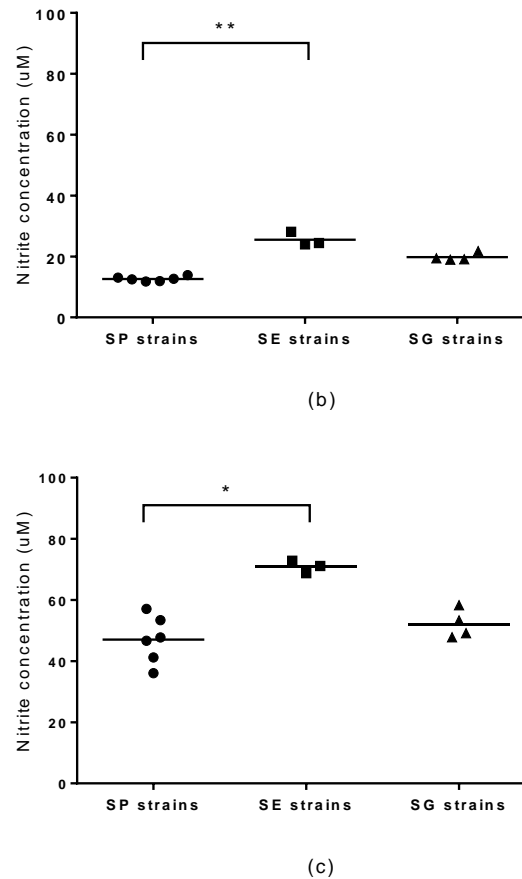
Figure 3-14. NO production by chMDM following infection with different strains of S. Pullorum, S. Enteritidis and S. Gallinarum. (a) NO production from *Salmonella*-infected chMDM at 2, 6, 24 and 48 h pi. Nitrite concentration was analysed by two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between groups at each time point. Each treatment was performed in triplicate and data are shown as mean (nitrite concentration, μM) ± SEM (n=3). (*) indicates statistically significant different from uninfected control. *P<0.05, **P<0.01. Panel (b and c) showed mean of NO production from chMDM infected with *Salmonella* strains grouped within serovars Pullorum, Enteritidis or Gallinarum at (b) 24 and (c) 48 h pi and analysed by Kruskal-Wallis test followed by Dunn's multiple comparisons test. SP, *S.* Pullorum; SE, *S.* Enteritidis; SG, *S.* Gallinarum; c+ve, LPS-stimulated positive control; c-ve, PBS-treated uninfected negative control.

3. 2. 7. 2   Quantification of gene expression of cytokines and chemokines in chMDM infected with 13 different strains of *Salmonella*

Gene expression of pro-inflammatory chemokines and cytokines were determined in avian macrophages infected with 13 different strains of *S. enterica* at 6 h pi. Compared to the uninfected controls, these 13 strains of *Salmonella* up-regulated the mRNA expression of CXCLi1 (except for *S.* Pullorum 31), CXCLi2 (except *S.* Pullorum 1002), IL-1β and IL-6 from infected chMDM to different levels. In addition, expression of CXCLi1 mRNA was significantly higher in chMDM infected with *S.* Enteritidis P125109 than that of two strains of *S.* Pullorum (3 and 31, *P*<0.05). Expression of iNOS mRNA was significantly up-regulated only in chMDM infected with *S.* Enteritidis strains and *S.* Pullorum 31 (Figure 3-15).

CXCLi-1

CXCLi-2

IL-1β

iNOS

IL-6

(A)

(B)

Figure 3-15. CXCLi1, CXCLi2, IL-1β, iNOS and IL-6 mRNA expression from chMDM infected with 13 different strains of *S. enterica* at 6 h post-infection. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). SP, *S.* Pullorum; SE, *S.* Enteritidis; SG, *S.* Gallinarum. (*) indicates statistical difference compared to PBS-treated uninfected control or between groups, *: $P<0.05$, **: $P<0.01$.

Consistent with the results of representative strains, *S*. Pullorum and *S*. Gallinarum strains did not up-regulate gene expression of IFN-γ, IL-12α and IL-18 in chMDM whereas increased expression of these cytokines was observed in chMDM infected with *S*. Enteritidis strains (*P*<0.05 or 0.01 in different strains) or in response to LPS-stimulation (*P*<0.05), when compared with that of uninfected cells (Figure 3-16).

(A)

(B)

Figure 3-16. IFN-γ, IL-12α and IL-18 mRNA expression from chMDM infected with 13 different strains of *S. enterica* at 6 h post-infection. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). SP, *S.* Pullorum; SE, *S.* Enteritidis; SG, *S.* Gallinarum. (*) indicates statistical difference compared to PBS-treated uninfected control or between groups, *: $P<0.05$, **: $P<0.01$.

There was a trend showing slightly higher expression of IL-4 mRNA in chMDM infected with different strains of *S*. Pullorum while S. Enteritidis strains appeared to reduce IL-4 expression when compared with that of uninfected cells. However, the overall expression of IL-4 and IL-13 mRNA from infected chMDM was very low without any difference detected between these 13 strains (*P*>0.05) (Figure 3-17).

(A)

(B)
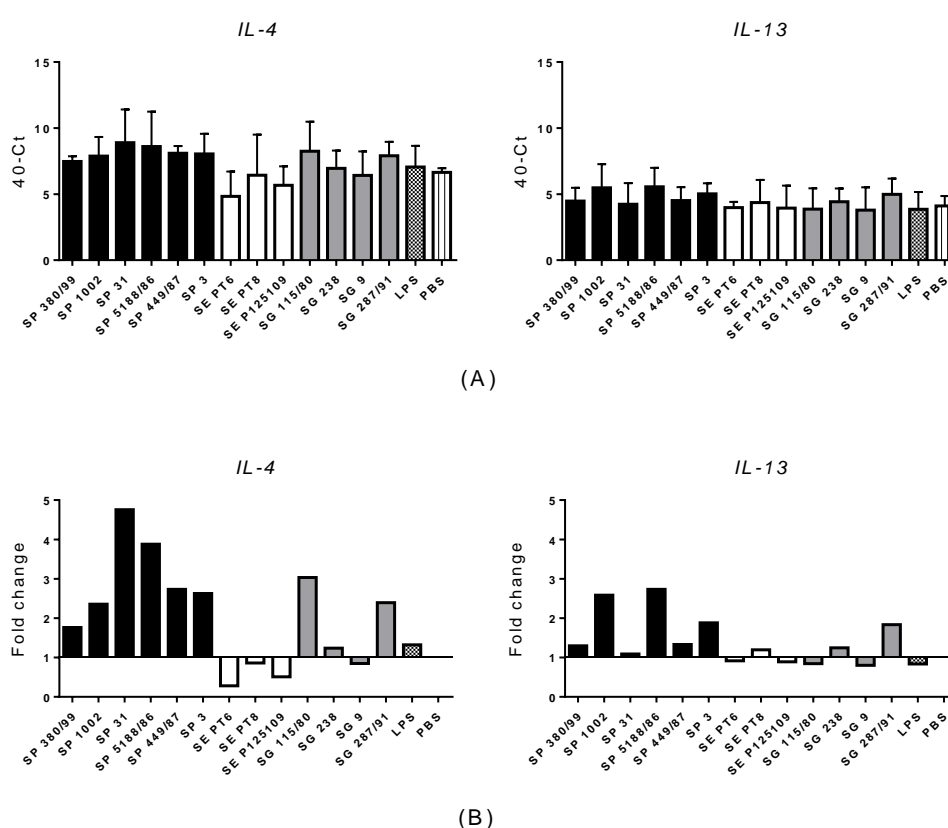
Figure 3-17. IL-4 and IL-13 mRNA expression from chMDM infected with 13 different strains of *S. enterica* at 6 h post-infection. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). SP, *S.* Pullorum; SE, *S.* Enteritidis; SG, *S.* Gallinarum. (*) indicates statistical difference compared to PBS-treated uninfected control or between groups, *: *P*<0.05, **: *P*<0.01.

Increased expression of IL-10 mRNA was observed in chMDM infected with several strains of *S. enterica* (*P*<0.05), but there was no significant difference observed between strains of different serovars. TGF-β4 expression in chMDM infected with *S.* Enteritidis PT8 was found to be higher than that of uninfected cells or of cells infected with strain 238 of *S.* Gallinarum (*P*<0.05).

(A)

(B)
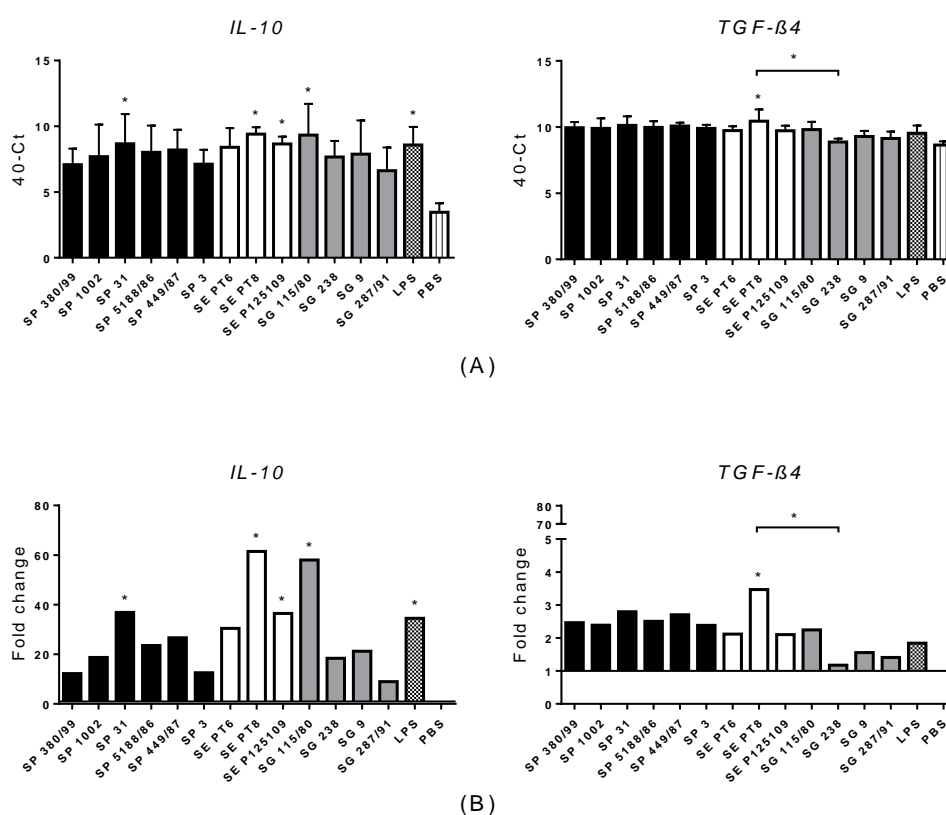
Figure 3-18. IL-10 and TGF-β4 mRNA expression from chMDM infected with 13 different strains of *S. enterica* at 6 h post-infection. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). SP, *S.* Pullorum; SE, *S.* Enteritidis; SG, *S.* Gallinarum. (*) indicates statistical difference compared to PBS-treated uninfected control or between groups, *: *P*<0.05, **: *P*<0.01.

3. 3   Chapter discussion

S. Pullorum strain 449/87, *S*. Gallinarum strain 9 and *S*. Enteritis strain P125109 were used in this study. It has been previously shown that *S*. Pullorum strain 449/87 and *S*. Gallinarum strain 9 behaved typically of field strains in terms of their virulence and how the disease appears in the field (Berchieri *et al.*, 2001a, Wigley *et al.*, 2001). Infection with the *S*. Enteritis strain P125109 processed typical disease (Barrow, 1991) in broilers and layers (Barrow and Lovell, 1991). This study demonstrated that primary avian macrophages responded differently to infection with these strains in terms of intracellular survival and induction of immune mediators.

Salmonella can use TTSS-1 to actively invade the cells (reviewed in the section 1. 3. 2) or have been phagocytized by the macrophages (Vazquez-Torres *et al.*, 1999, Braukmann *et al.*, 2015). In this study *S*. Pullorum and *S*. Gallinarum were taken up by or invaded chMDM more slowly than *S*. Enteritidis, which was in line with the observations in previous studies (Kaiser *et al.*, 2000, Wigley *et al.*, 2002b, Setta *et al.*, 2012a). This has been attributed to their lack of flagella and motility. Our data also showed that chMDM were able to limit the intracellular replication of *Salmonella* from 6 h pi, which is in agreement with previous studies using chicken macrophages cell line (HD11 cells or MQ-NCSU cells) (Withanage *et al.*, 2005a, Setta *et al.*, 2012a) or PBMC-derived macrophages (Okamura *et al.*, 2005). The reduction of intra-macrophage bacteria can be partly explained by NO-mediated killing mechanism as iNOS expression and NO production are essential for effective host resistance

against *Salmonella* infection, at least in mice (Mastroeni *et al.*, 2000b, Vazquez-Torres *et al.*, 2000a). In this study, the reduction of *Salmonella* within infected chMDM correlated with the up-regulation of iNOS mRNA at 6 h pi and secretion of high amounts of NO at 24 and 48 h pi. In previous studies, significant concentrations of nitrite were detected in MQ-NCSU cells infected with *S.* Typhimurium, *S.* Enteritidis and *S.* Gallinarum (Withanage *et al.*, 2005a). Infection of HD11 cells with *S*. Enteritidis also resulted in significant increase in NO production and iNOS expression (Babu *et al.*, 2006, Setta *et al.*, 2012a). However, infection of chMDM with avian-specific serovars Pullorum and Gallinarum triggered NO production and iNOS expression at lower levels than those produced by *S.* Enteritidis-infected cells in the current study, which may result in a more hospitable environment to improve intracellular survival for *S*. Pullorum and *S*. Gallinarum and further benefit their systemic dissemination. These findings are in line with the data reported by Setta *et al.* (2012a) that *S*. Pullorum and *S*. Gallinarum stimulated iNOS expression in HD11 cells to a lesser extent than other broad-host serovars. Hulme *et al.* (2012) reported that the reduced iNOS expression associated with *S*. Typhimurium infection was correlated with inhibition of binding of *NF*-κB and activator protein 1 (AP-1) to murine J774 macrophage DNA via the *phoP* regulon and suggested that suppression of iNOS is typically associated with typhoid serovars.

Although *S*. Pullorum and *S*. Gallinarum were at lower levels of invasiveness than *S*. Enteritidis, the host gene expression in response to difference

serovars was not likely related to the number of intracellular bacteria. Infection of avian epithelial cells with *S*. Enteritidis or *S*. Typhimurium resulted in greater levels of inflammatory mediators than that produced in *S*. Hadar- or *S*. Infantis-infected cells, though they express comparable levels of invasiveness (Setta *et al.*, 2012a).

A number of publications exist describing expression of pro-inflammatory mediators which play a pivotal role in response to *Salmonella* infection *in vitro* (Swaggerty *et al.*, 2004, Kaiser *et al.*, 2006) and *in vivo* (Beal *et al.*, 2004b, Wigley *et al.*, 2005a, Berndt *et al.*, 2007, Fasina *et al.*, 2008, Chappell *et al.*, 2009, Setta *et al.*, 2012b). In this study, infection of chMDM by all serovars increased gene expression of IL-1β, IL-6, CXCLi1 and CXCLi2, but infection with *S*. Pullorum or *S*. Gallinarum resulted in significantly lower levels of IL-6 and CXCLi2 when compared to *S*. Enteritidis. These findings extend the data on HD11 infected with these serovars (Setta *et al.*, 2012a), which suggested a strong inflammatory response to *in vitro* infection with *S*. Enteritidis. IL-6 produced early after infection plays an important role in both innate immunity and the development of an adaptive immune response (activation of lymphocytes) (Kaiser and Staheli, 2013). Our data was consistent with previous reports of reduced IL-6 production by *S*. Pullorum-infected HD11 when compared to *S*. Enteritidis infection (Setta *et al.*, 2012a). Thus, *S*. Pullorum and *S*. Gallinarum can trigger the expression of pro-inflammatory cytokines in infected avian macrophages, though to a lesser extent than that of *S*. Enteritidis indicating that this is not directly related to the establishment

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of the carrier state. In fact, the down-regulated expression or reduced production of key pro-inflammatory immune mediators, including CXCLi1, CXCLi2, IL-6, IL-1β and iNOS, by infection with systemic serovars Pullorum or Gallinarum is evident in epithelial cells (CKC) (Kaiser *et al.*, 2000, Setta *et al.*, 2012a) and in the ileum of infected chickens (Chappell *et al.*, 2009). In comparison with *S*. Typhimurium, *S*. Enteritidis, *S*. Hadar and *S*. Infantis, which are good colonisers of the gut and effective stimulators of pro-inflammatory mediators (Withanage *et al.*, 2004, Cheeseman *et al.*, 2008, Chappell *et al.*, 2009, Setta *et al.*, 2012b), *S*. Pullorum and *S*. Gallinarum produce typhoid-like infections and invade leading to systemic infection without causing inflammatory responses in the intestinal epithelium. The observed differential effects could be influenced by the presence or absence of flagella-specific effectors. Mutations in the flagellin gene (*fliM*) of *S*. Typhimurium lead to an enhanced ability to establish systemic infection in chickens with decreased expression of IL-6 and IL-1β mRNA and PMN cell infiltration (Iqbal *et al.*, 2005).

The clearance of *Salmonella* infection in chicken has been attributed to the cytokines related to Th1 response (Beal *et al.*, 2004a, Beal *et al.*, 2004b, Wigley *et al.*, 2005a, Withanage *et al.*, 2005b, Berndt *et al.*, 2007). Although rchIFN-γ did not affect phagocytic ability of chicken macrophages, it activated macrophages to enhance NO production and reduced bacterial replication within the infected macrophages (Okamura *et al.*, 2005, Babu *et al.*, 2006). However, our data showed that infection of chMDM and splenocytes with *S*.

Pullorum and *S*. Gallinarum did not induce gene expression of IFN-γ and IL-12α whereas up-regulation of IFN-γ, IL-18 and IL-12α were all detected in chMDM infected with *S.* Enteritidis. It was found that chicken IL-18 stimulated IFN-γ release from chicken CD4^{+} T cells (Gobel *et al.*, 2003). However, mammalian IL-18 can also stimulate Th2 cytokine production in the absence of IL-12 (Nakanishi *et al.*, 2001), which is not yet evident in the chicken. The differential expression of IL-12α and IFN-γ supported our main hypothesis that *S*. Pullorum cannot initiate an effective IFN-γ-dependent inflammatory response to clear infection. We expected to observe evidences indicating manipulation of host immunity towards a Th2 response by *S*. Pullorum. However, the expression of IL-4 or IL-13 mRNA was not significantly changed in *S.* Pullorum-infected chMDM when compared to the infected controls in this study as well as in infected HD11 cells (Setta *et al.*, 2012a). There is so far no information describing the expression of IL-13 in response to *Salmonella* infection in chicken.

IL-10 is a multifunctional cytokine that inhibits further development of the Th1 response and down-regulates the effects of IFN-γ to limit the inflammatory response (Rothwell *et al.*, 2004). A small up-regulation of IL-10 was detected in HD11 cells infected with *S. enterica* serovars Typhimurium, Enteritidis, Pullorum and Gallinarum, suggesting a negative regulation by IL-10 to suppress pro-inflammatory responses (Setta *et al.*, 2012b). In this study, infection of chMDM with *S.* Gallinarum generally did not induce IL-10 expression, although one strain *S.* Gallinarum 115/80 did induce higher levels

of IL-10 mRNA when compared to the uninfected controls. This indicated the potential of less control of inflammatory responses in *S.* Gallinarum-infected avian macrophages which may result in more tissue damage such as hepatosplenomegaly or cardiomyopathy or may be involved in the final stages of intestinal pathology associated with faecal shedding. Th3 cells play a role in suppressing or controlling immune responses in the mucosa. TGF-β enhanced *in vitro* differentiation of Th3 cells from murine Th precursors (Weiner, 2001). Increased TGF-β4 expression at 7 d pi observed in chickens infected with *S.* Typhimurium was shown to correspond to decreased production of pro-inflammatory mediators (Withanage *et al.*, 2005b), but we observed no expression of TGF-β4 from chMDM following infection with *Salmonella* in this *in vitro* study.

Finally, co-stimulation of T cells is required to develop an effective immune response. *S.* Typhimurium was previously reported to up-regulate expression of co-stimulatory molecules (CD40, CD80 and CD86) on murine macrophages and DCs, which would activate cognate T cells (Kalupahana *et al.*, 2005). In this study *S*. Pullorum did not suppress the expression of co-stimulatory molecules on chMDM. Thus, persistent infection of *S*. Pullorum in chicken may not be related to immune evasion by inhibiting the delivery of second signals from infected macrophages.

Chapter 4 Immune modulation of lymphocytes by *Salmonella*-infected avian macrophages

4. 1 Introduction

4. 1. 1 General introduction

In addition to its role as a non-specific barrier to infection the innate immune response makes a crucial contribution in stimulating adaptive antimicrobial immune responses appropriate to combating infection. Macrophages that have phagocytosed bacteria and become activated are capable of activating T lymphocytes. Given the modulation in cytokines observed during infection with different *Salmonella* serovars discussed in Chapter 3 we investigated the potential impact on T-cell activation to determine if there was an impact on the adaptive immune system.

It has been shown that the Th1 response is crucial for protective immunity against primary infection with *S.* Typhimurium in mice, which points to the importance of the IFN-γ-producing CD4$^+$ T cells (Eckmann and Kagnoff, 2001, Raupach and Kaufmann, 2001). Strong Th1 responses are also associated with immunity against a number of other intracellular bacterial pathogens, such as *Mycobacterium leprae* (Garcia *et al.*, 2001) and *Yersinia pestis* (Nakajima and Brubaker, 1993). In poultry, clearance of *S.* Typhimurium from the intestine of infected chickens correlated with IFN-γ levels, which implies a vital role for a strong T-cell response after primary *Salmonella* infection (Beal *et al.*, 2004a, Beal *et al.*, 2005). Splenocytes from *S.* Enteritidis-vaccinated chicken produced

higher levels of IFN-γ and IL-2 in response to antigen stimulation (Okamura *et al.*, 2004). In another study (Chappell *et al.*, 2009), infections with *S*. Pullorum resulted in reduced IFN-γ and elevated IL-4 expression in the spleens when compared with *S*. Enteritidis which induced a strong Th1 type immune response. It was suggested that the carrier-state could be the result of a Th2 bias in the immune responses. A more detailed exploration of the T lymphocyte responses would therefore be helpful to elucidate the interaction of *S*. Pullorum with host immunity.

4. 1. 2 Chapter aims and objectives

A co-culture system, using either macrophages or DCs with T lymphocytes, has been used to explore the host immune responses to *S.* Typhimurium infection *in vitr*o (Niedergang *et al.*, 2000, Kalupahana *et al.*, 2005, Bueno *et al.*, 2008). Here we have used *Salmonella*-infected avian macrophages and blood-derived CD4+ T lymphocytes in co-culture *in vitro* as a model to study the immunomodulation of acquired immunity in response to *S.* Pullorum infection.

The ability of *S.* Pullorum to produce persistent infection and avoid immune clearance in chickens might result from an immune response which is clearly different to that induced by the host to related serovars Enteritidis and Gallinarum. Following the observation in Chapter 3, we hypothesised that, in contrast to serovar *S.* Enteritidis, which stimulates a strong expression of Th1-related cytokines driving the differentiation of Th1 cells, *S.* Pullorum might

modulate the host response towards a Th2 immunity by inducing IL-4-expression Th2 cells. The aim of work described in this chapter was therefore to study the immunomodulation of acquired immunity in response to *S. Pullorum* infection by using *Salmonella*-infected avian macrophages and blood-derived CD4+ T lymphocytes in co-culture *in vitro* as a model.

4. 2 Results

4. 2. 1 Isolation of chicken CD4⁺ T cells by MACS

Chicken CD4⁺ T cells were isolated from PBMCs by MACS separation as described in 2. 2. 4. The magnetically labelled cells were retained within the column and separated from the unlabelled cells which run through (negative separation). The fraction of magnetically labelled cells (positive separation) was then flushed out using plunger. Both the positive and negative separation were then stained for CD4 expression prior to flow cytometric analysis, which indicated good isolation of CD4⁺ T cells by MACS: the positive separation had over 98% (Figure 4-1, panel a, H1) of cells being positive in CD4 expression whereas only less than 5 % (Figure 4-1, panel b, H2) of CD4⁺ T cells present in the negative separation. Anti-mouse IgG1 microbeads did not show non-specific binding to chicken PBMC as only less than 100 out of 2×10^7 cells were directly magnetically labelled with the microbeads and collected from MACS separation (Figure 4-1, panel c).

4. 2. 2 Viability of CD4⁺ T cells

Ensuring the viability of isolated CD4⁺ T cells is an essential component of *in vitro* co-culture. The viability of CD4⁺ T cells was assessed by PI uptake (20 µg/ml) using flow cytometric analysis. There was a > 65 % viability in the first 5 days of culture, followed by an ever-decreasing percentage of viable CD4⁺ T cell from 6 days afterwards (Figure 4-2, a and b). Therefore the co-culture of avian macrophages and CD4⁺ T cells were maintained *in vitro* for 5 days before being processed for further analysis.
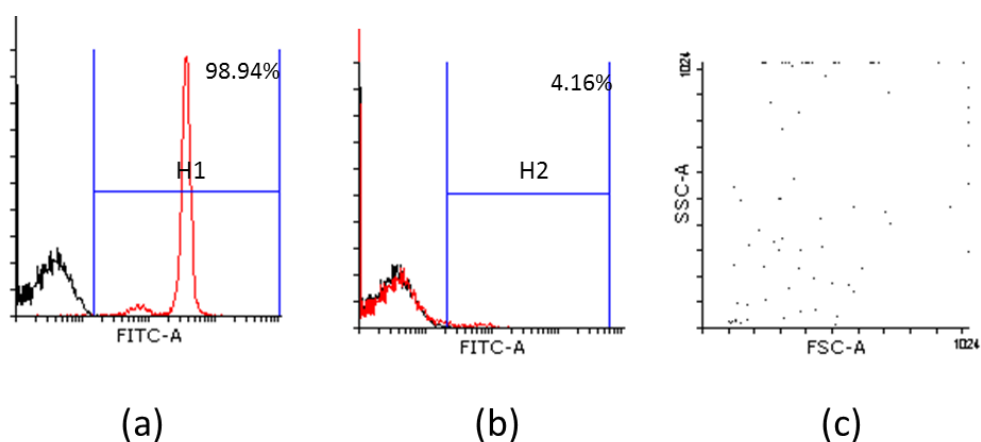
Figure 4-1. Flow cytometric analysis of purity of isolated CD4$^+$ T cells. Chicken CD4$^+$ cells were isolated from PBMCs by MACS (mouse-anti-chicken CD4 mAb and anti-mouse IgG1 microbeads). The (a) positive and (b) negative separation collected after MACS separation were analysed using flow cytometer. The percentage of CD4$^+$ T cells were shown in H1 and H2 by gating against isotype controls respectively (black lines). (c) PBMC stained with microbeads only. The dot-plots and histograms represent independent preparation of CD4$^+$ T cells from individual batches of chicken whole blood. Black lines, isotype control mAbs (Mouse IgG2b: FITC); red lines, stain with mouse anti-chicken CD4 mAb (clone 2-35: FITC).
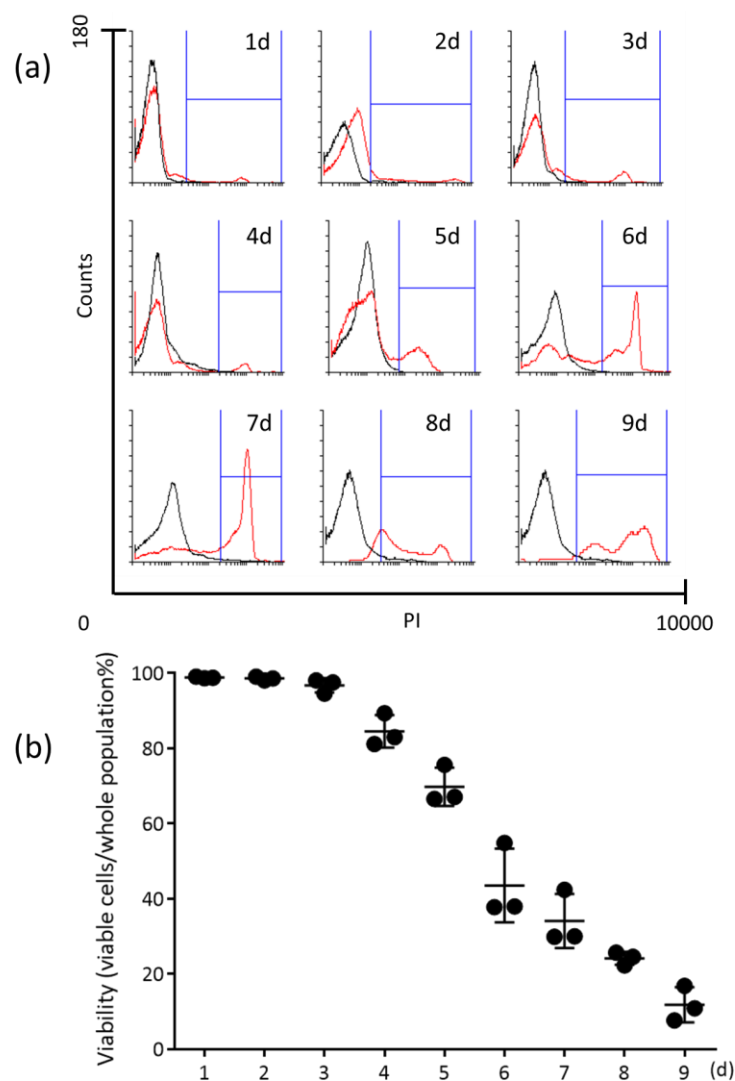
Figure 4-2. Viability of CD4+ T cells cultured *in vitro*. CD4+ T cells were stained with PI (20 µg/Ml) and analysed during 9 d of culture. The PI+ cells were determined as dead cells. (a) Representative overlay histograms on PI+ cells determined by flow cytometric analysis at 9 different days (d) post isolation. Black lines, non-staining negative control; red lines, PI staining. (b) Percentage of viable cells within whole population of CD4+ T cells at each day post isolation and the results were shown as mean± SEM of two independent experiments with duplicates in each experiment.

4. 2. 3 Microscopic pictures of CD4⁺ T cells and *Salmonella*-infected avian chMDM in co-culture

Salmonella-infected avian macrophages in co-culture with CD4⁺ T cells were observed by light microscopy (Figure 4-3). The images in the panel a-1 display chMDM infected with different serovars of *S. enterica* at 1 day pi. The images in the panel a-2 display CD4⁺ T cells in co-culture with *Salmonella*-infected chMDM at 1 day pi. The infected macrophages contained a relatively high number of vacuoles, with more vacuoles being observed in *S.* Enteritidis-infected macrophages, when compared to those in the macrophages infected with *S.* Pullorum and *S.* Gallinarum and the uninfected controls. Lymphocytes are non-adherent mononuclear cells loosely attached to the macrophages at the bottom of the culture plates after one day of co-culture. The images in the panel b-1 display chMDM infected with different strains of *S. enterica* at 5 d pi while CD4⁺ T cells co-cultured with *Salmonella*-infected chMDM after 5 days of co-culture are shown in images in panel b-2. The vacuoles in *S.* Enteritidis-infected macrophages were even bigger and more visible at 5 days pi when compared to that in the macrophages infected with *S.* Pullorum and *S.* Gallinarum and uninfected controls. The majority of CD4⁺ T cells became detached from macrophages and floating in the culture.

(a-1)
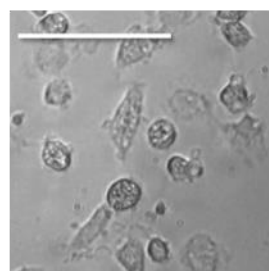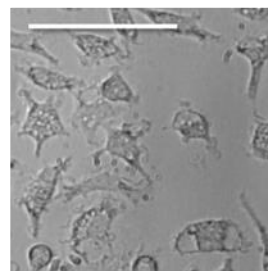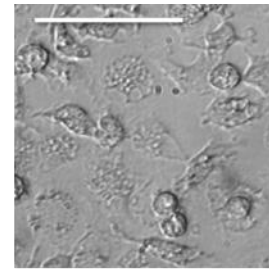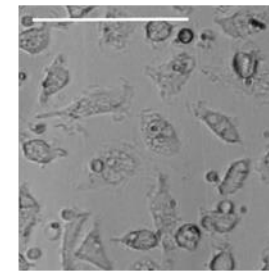
UI SP SE SG

(a-2)
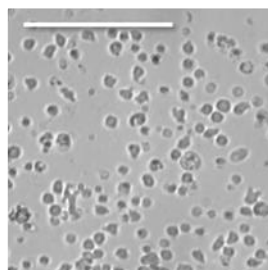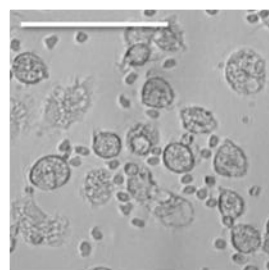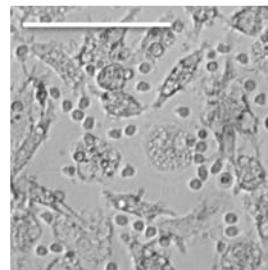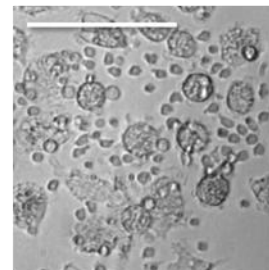
CD4+ UI/CD4+ SP/CD4+ SE/CD4+ SG/CD4+
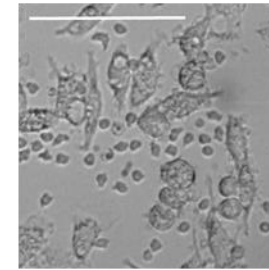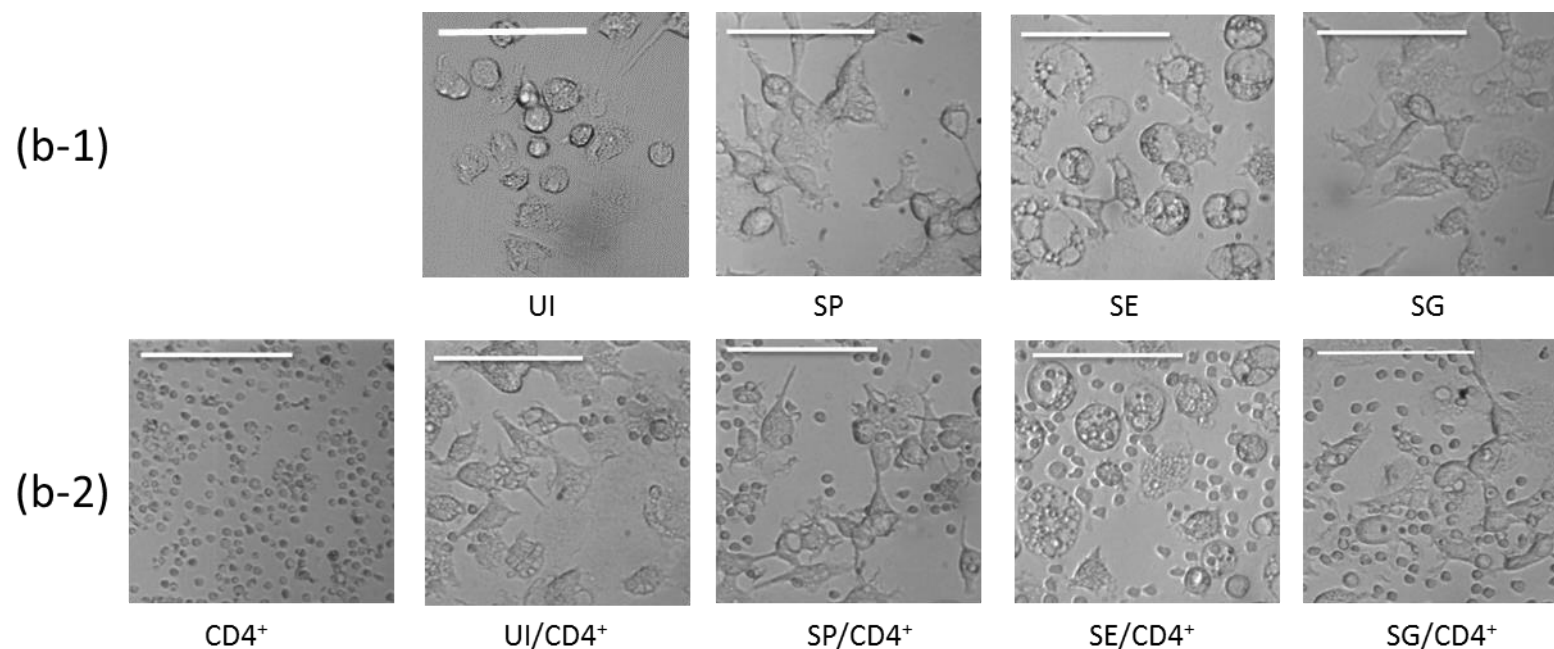
Figure 4-3. Microscopic images of CD4+ T cells in co-culture with *Salmonella*-infected avian macrophages. chMDM were infected with different serovars of *Salmonella*. At 2 h pi, the *Salmonella*-infected and uninfected chMDM were co-cultured with CD4+ T cells (CD4+T cells: chMDM=10:1). The chMDM infected with *Salmonella* (a-1 and b-1) and in co-culture with CD4+ (a-2 and b-2) were observed under the microscope after 1 (a-1 and a-2) 5 (b-1 and b-2) d pi. CD4+, CD4+ T cells cultured alone; UI/CD4+, CD4+ T cells co-cultured with uninfected chMDM as control for allogeneic response; SP/CD4+, CD4+T cells co-cultured with *S.* Pullorum-infected chMDM; SE/CD4+, CD4+T cells co-cultured with *S.* Enteritidis-infected chMDM; SG/CD4+, CD4+T cells co-cultured with *S.* Gallinarum-infected chMDM. Scale bar=50 μm.

4. 2. 4 Proliferation of CD4⁺ T cells in response to *Salmonella* infection

CD4⁺ lymphocytes were cultured for 5 days either in the presence of Con A (10μg/ml, positive control for T cell proliferation), alone (unstimulated CD4⁺ T cells), with uninfected macrophages (control for allogeneic response) or *Salmonella*-infected macrophages. T cell proliferation was determined using the Promega CellTiter 96® Aqueous One Solution Cell Proliferation Assay at 5 days post co-culture and data are shown as the number of cells according to the linear regression between the absorbance (492 nm) and the serial dilution of unstimulated fresh CD4⁺ lymphocytes.

There was only a slightly higher number of viable CD4⁺ T cells co-cultured with uninfected macrophages than CD4⁺ T cells cultured alone ($P > 0.05$), which indicated a moderate allogeneic response occurred due to chMDM and CD4⁺ T cells obtained from different individual birds. CD4⁺ T cell proliferation induced by macrophages infected with different serovars of *Salmonella* was determined by comparison with an uninfected control for the allogeneic response. Our results showed that the avian macrophages infected with each of these three serovars were able to stimulate CD4⁺ T cell proliferation when compared to that of the uninfected controls, but there were no significant differences observed between the different serovars. *S.* Enteritidis-infected macrophages induced a higher level of proliferation of CD4⁺ T cells than that in co-culture with macrophages infected with *S.* Pullorum, but which, however, was of marginal significance ($P = 0.0644$) (Figure 4-4).
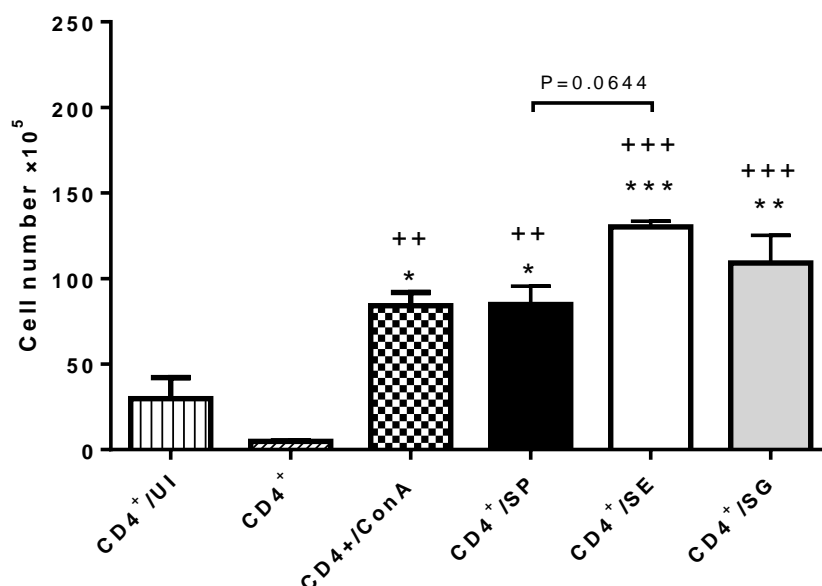
Figure 4-4. CD4+ lymphocyte proliferation induced by *Salmonella*-infected avian macrophages. chMDM were infected with different serovars of *Salmonella*. At 2 h pi, infected chMDM were co-cultured with CD4+ T cells. After 5 days of co-culture, CD4+ T cells were separated to determine the number of viable proliferating CD4+ T cells in each group of co-culture. Results are expressed as mean (cell numbers) ± SEM (n=3). CD4+/UI, CD4+ T cells co-cultured with uninfected chMDM (control for allogeneic response); CD4+, CD4+ T cells cultured alone; CD4+/ConA, CD4+ T cells stimulated with ConA (positive control for lymphocyte proliferation); CD4+/SP, CD4+ T cells co-cultured with *S.* Pullorum-infected chMDM; CD4+/SE, CD4+ T cells co-cultured with *S.* Enteritidis-infected chMDM; CD4+/SG, CD4+ T cells co-cultured with *S.* Gallinarum-infected chMDM;. (*) indicates statistical difference from control of allogeneic response (CD4+/UI), *$P<0.05$, **$P<0.01$, ***$P<0.001$; (+) indicates statistical difference from unstimulated control (CD4+), +$P<0.05$, ++$P<0.01$, +++$P<0.001$.

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4. 2. 5 Unvaccinated control experiment

Chicken whole blood collected from unvaccinated parent breeders (source of blood described in 2. 2. 3. 1) was used to set up the experiment. CD4$^+$ T cells and macrophages were isolated (described in sections 2. 2. 3 and 2. 2. 4) and co-cultured *in vitro* for 5 days (described in section 2. 5. 1). After 5 days T cell proliferation was examined as described in section 2. 5. 2.

Figure 4-5 showed the number of CD4$^+$ T cells after 5 days of co-culture with *Salmonella*-infected and uninfected macrophages. Generally, a similar pattern of results was observed as that shown in Figure 4-4 (using commercial blood obtained from vaccinated birds). Three different serovars of *S. enterica* were all able to stimulate T cell proliferation. In comparison with CD4$^+$ T cells co-cultured with *S*. Enteritidis-infected macrophages, there were lower numbers of proliferating CD4$^+$ T cells in the co-culture with chMDM infected with *S*. Pullorum or *S*. Gallinarum. However, when compared with results shown in Figure 4-4, (i) there were fewer viable CD4$^+$ T cells in each co-culture accordingly, which might result from there being fewer memory T cells in unvaccinated layers; (ii) significantly fewer proliferating CD4$^+$ T cells were observed in co-culture with *S*. Pullorum (*P*<0.01) or *S.* Gallinarum (*P*<0.05) infected-macrophages when compared with *S.* Enteritidis group.
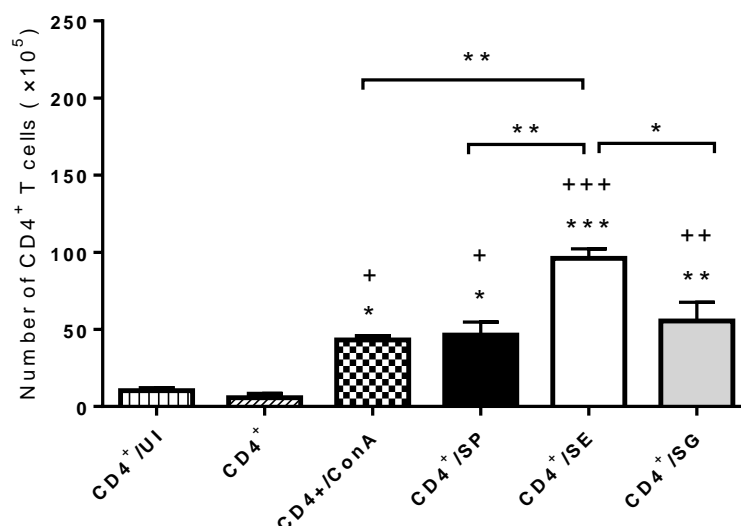
Figure 4-5. Proliferation of CD4+ lymphocytes isolated from unvaccinated chickens induced by *Salmonella*-infected macrophages. chMDM and CD4+ T cells were isolated from unvaccinated chickens. ChMDM infected with different serovars of *Salmonella*. At 2 h pi, infected chMDM were co-cultured with CD4+ T cells. After 5 days of co-culture, CD4+ T cells were separated to determine the number of viable proliferating CD4+ T cells in each group of co-culture. Results are expressed as mean (cell numbers) ± SEM (n=3). CD4+/UI, CD4+ T cells co-cultured with uninfected chMDM (control for allogeneic response); CD4+, CD4+ T cells cultured alone; CD4+/ConA, CD4+ T cells stimulated with ConA (positive control for lymphocyte proliferation); CD4+/SP, CD4+ T cells co-cultured with *S.* Pullorum-infected chMDM; CD4+/SE, CD4+ T cells co-cultured with *S.* Enteritidis-infected chMDM; CD4+/SG, CD4+ T cells co-cultured with *S.* Gallinarum-infected chMDM;. (*) indicates statistical difference from control of allogeneic response (CD4+/UI), *$P<0.05$, **$P<0.01$, ***$P<0.001$; (+) indicates statistical difference from unstimulated control (CD4+), +$P<0.05$, ++$P<0.01$, +++$P<0.001$.

4. 2. 6 Quantification of gene expression from CD4⁺ T cells co-cultured with *S. enterica*-infected macrophages

The mRNA expression of selected immune mediators in CD4⁺ T cells collected from co-cultures was differentially expressed in response to infection with different *S. enterica* serovars as measured by qRT-PCR. Gene expression from CD4⁺ T cells in response to *Salmonella* infection was compared to that from CD4⁺ T cells in co-culture with uninfected macrophages as negative control (for allogeneic response).

In this co-culture system, the level of IFN-γ mRNA of CD4⁺ T cells in co-culture with *S.* Pullorum-infected chMDM was close to that of the uninfected control (*P*>0.05). Increased expression of IFN-γ mRNA was found only in CD4⁺ T cells co-cultured with *S.* Enteritidis-infected chMDM (*P*<0.01), which was also significantly higher than that in response to *S.* Pullorum infection (*P*<0.01).

Although CD4⁺ T cells co-cultured with *S.* Pullorum-infected macrophages appeared to induce higher levels of IL-4 mRNA than that from *S.* Enteritidis (*P*=0.1547) and *S.* Gallinarum (*P*=0.1065) group, the expression of IL-4 mRNA was at low levels in each group with no significant changes being observed from CD4⁺ T cells in response to *Salmonella* infection when compared to the uninfected control (Figure 4-6).

Interestingly, *S.* Pullorum-infected chMDM induced a significantly lower level of IL-17F mRNA in co-cultured CD4⁺ T cells than that detected in CD4⁺ T cells in co-culture with *S.* Enteritidis-infected (*P*<0.01) or uninfected (*P*<0.05)

chMDM. The gene expression of IL-17A was found not to be significantly different between groups in this co-culture experiment, although *S*. Pullorum appeared to reduce the expression of IL-17A from co-cultured CD4$^+$ T cells when compared to *S*. Enteritidis infection (P= 0.0843) (Figure 4-7).

The levels of IL-10 and TGF-β4 mRNA from CD4$^+$ T cells in co-culture with *Salmonella*-infected macrophages were found to be close to that of uninfected control (P>0.05) (Figure 4-8).
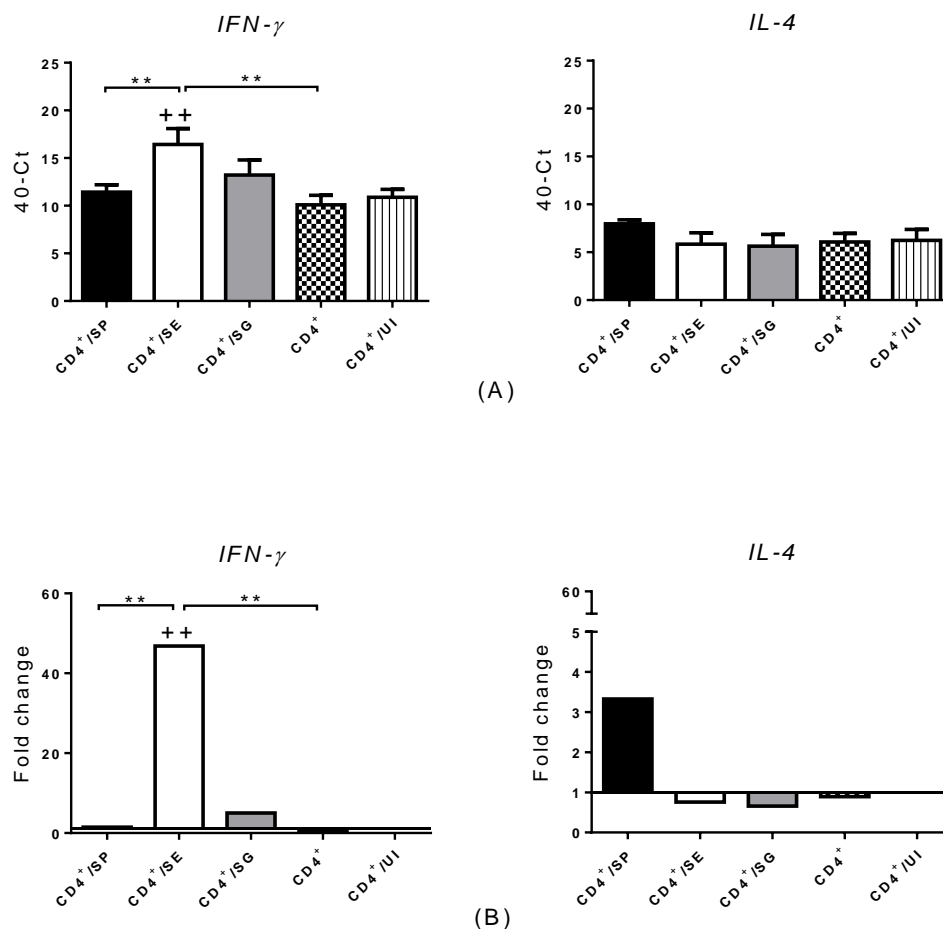
Figure 4-6. Quantification of gene expression of Th1 and Th2 cytokines from CD4+ T cells co-cultured with *Salmonella*-infected chMDM. CD4+ T cells were co-cultured with *Salmonella*-infected or uninfected chMDM for 5 days. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). CD4+/SP, CD4+ T cells co-cultured with *S.* Pullorum-infected chMDM; CD4+/SE, CD4+ T cells co-cultured with *S.* Enteritidis-infected chMDM; CD4+/SG, CD4+ T cells co-cultured with *S.* Gallinarum-infected chMDM; CD4+, CD4+ T cells cultured alone; CD4+/UI, CD4+ T cells co-cultured with uninfected chMDM (control for allogeneic response). (+) indicates differences between levels of cytokines induced by each serovar compared to uninfected control (CD4+/UI), +: $P<0.05$, ++: $P<0.01$; (*) indicates differences between levels of cytokines induced by different serovars, *: $P<0.05$, **: $P<0.01$.

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(A)

(B)

Figure 4-7. Quantification of gene expression of Th17 cytokines from CD4+ T cells co-cultured with *Salmonella*-infected chMDM. CD4+ T cells were co-cultured with *Salmonella*-infected or uninfected chMDM for 5 days. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). CD4+/SP, CD4+ T cells co-cultured with *S.* Pullorum-infected chMDM; CD4+/SE, CD4+ T cells co-cultured with *S.* Enteritidis-infected chMDM; CD4+/SG, CD4+ T cells co-cultured with *S.* Gallinarum-infected chMDM; CD4+, CD4+ T cells cultured alone; CD4+/UI, CD4+ T cells co-cultured with uninfected chMDM (control for allogeneic response). (+) indicates differences between levels of cytokines induced by each serovar compared to uninfected control (CD4+/UI), +: *P*<0.05, ++: *P*<0.01; (*) indicates differences between levels of cytokines induced by different serovars, *: *P*<0.05, **: *P*<0.01.

IL-10

TGF-ß4

(A)

IL-10

TGF-ß4

(B)

Figure 4-8. Quantification of gene expression of regulatory mediators from CD4+ T cells co-cultured with *Salmonella*-infected chMDM. CD4+ T cells were co-cultured with *Salmonella*-infected or uninfected chMDM for 5 days. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). CD4+/SP, CD4+ T cells co-cultured with *S.* Pullorum-infected chMDM; CD4+/SE, CD4+ T cells co-cultured with *S.* Enteritidis-infected chMDM; CD4+/SG, CD4+ T cells co-cultured with *S.* Gallinarum-infected chMDM; CD4+, CD4+ T cells cultured alone; CD4+/UI, CD4+ T cells co-cultured with uninfected chMDM (control for allogeneic response). (+) indicates differences between levels of cytokines induced by each serovar compared to uninfected control (CD4+/UI), +: $P<0.05$, ++: $P<0.01$; (*) indicates differences between levels of cytokines induced by different serovars, *: $P<0.05$, **: $P<0.01$.

4. 2. 7 Expression of CD28/CTLA-4 on CD4+ T cells co-cultured with *S. enterica* infected macrophages

Surface expression of CD28 on CD4+ T cells in the co-culture system was determined by flow cytometric analysis. At 1 day following co-culture, a greater proportion of the CD4+ T cells co-cultured with *S*. Gallinarum-infected chMDM were CD4+CD28+ T cells than that of the uninfected control cells (CD4+/UI) (*P*<0.05). However, the proportion of CD4+ T cells in co-culture with *S*. Pullorum- or *S*. Enteritidis-infected chMDM that were CD4+CD28+ did not increase when compared to the control for an allogeneic response (CD4+/UI) (*P*>0.05). A lower number of CD4+CD28+ T cells in co-culture with *S*. Pullorum-infected chMDM at 5 days of co-culture when compared to that in response to infection with *S*. Enteritidis. However, this apparent difference was not statistically significant (*P*= 0.13).
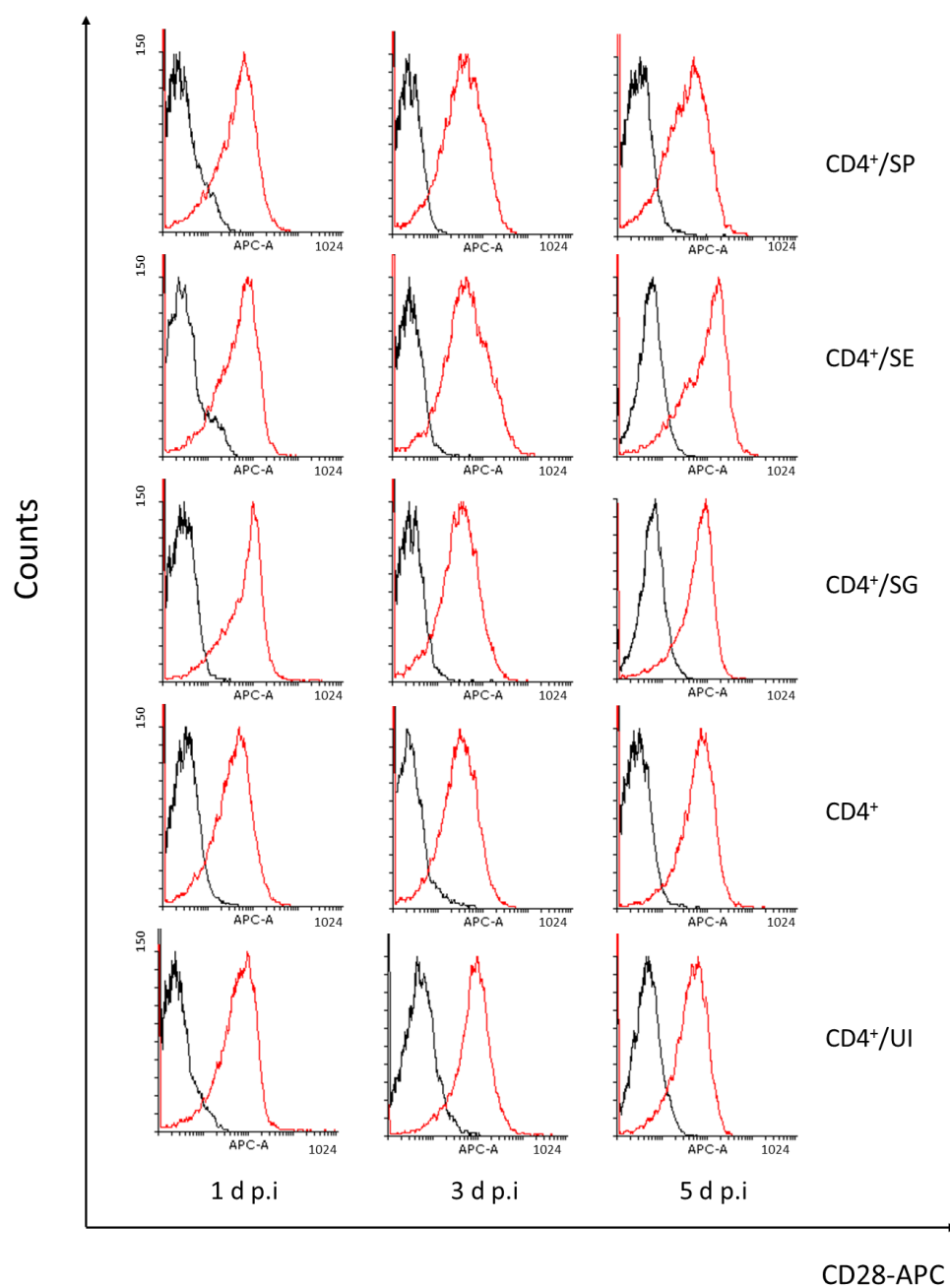
(A)
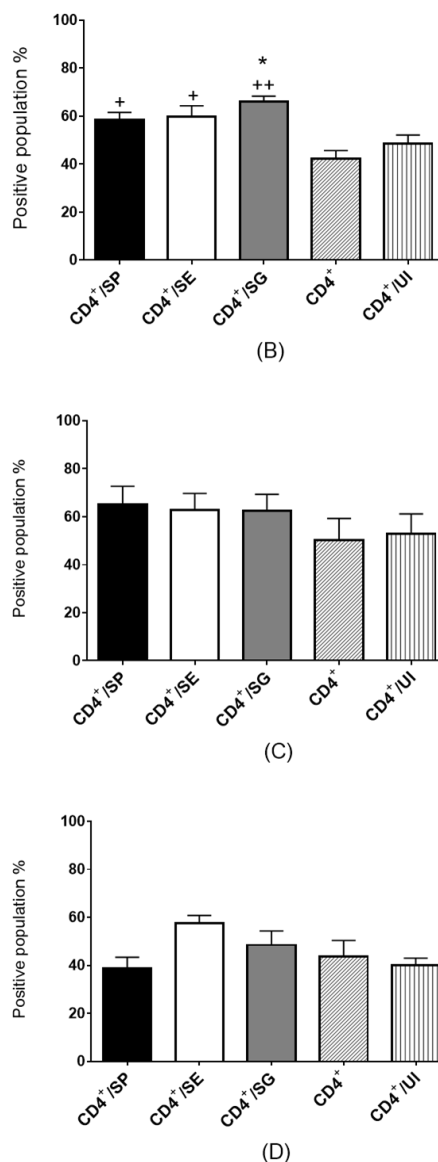
Figure 4-9. Number of CD28+ cells within the CD4+ T cells co-cultured with *S. enterica* infected chMDM. chMDM were infected with different serovars of *Salmonella*. At 2 h pi, infected chMDM were co-cultured with CD4+ T cells. After at 1 (B), 3 (C) and 5 (D) days post co-culture, CD4+ T cells were separated to determine the differential percentage of CD28+/CD4+ T cells within the population of CD4+ T cells. (A) The histograms shown are representative of three independent experiments. Black lines, staining with anti-mouse IgG2α:APC; red lines, staining with mouse-anti-chicken CD28 plus anti-mouse IgG2α:APC. CD4+/SP, CD4+ T cells co-cultured with *S.* Pullorum-infected chMDM; CD4+/SE, CD4+ T cells co-cultured with *S.* Enteritidis-infected chMDM; CD4+/SG, CD4+ T cells co-cultured with *S.* Gallinarum-infected chMDM; CD4+, CD4+ T cells cultured alone; CD4+/UI, CD4+ T cells co-cultured with uninfected chMDM which were controls for allogeneic response. (+) indicates statistical difference from CD4+ T cells (CD4+), +: *P*<0.05, ++: *P*<0.01; (*) indicates statistical difference from uninfected control (CD4+/UI), *: *P*<0.05, **: *P*<0.01.

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Anti-avian CTLA-4 mAb is not commercially available; therefore, qRT-PCR was used to provide an indication of CTLA-4 expression on CD4$^+$ T cells in the co-culture system, according to the methods described in section 2. 7. 3. The single peak displayed of the melting curves for each primer pairs indicated that amplification of these genes by SYBR green qRT-PCR were specific (see Appendix.4). Based on the results from the melting curve and amplifying efficiency, the qRT-PCR amplification was considered to be reliable.

At 1 day after co-culture, the levels of CD28 mRNA were up-regulated in CD4$^+$ T cells co-cultured with chMDM infected with any of the *Salmonella* serovars tested in this study when compared with that in CD4$^+$ T cells co-cultured with uninfected chMDM (control for allogeneic response). There was a subsequent reduction of CD28 expression during the following days of co-culture but this was not significantly different from the uninfected control (*P*>0.05). No changes in the gene expression of CTLA-4 was observed in CD4$^+$ T cells in co-culture with either of the three serovars, indicating that *Salmonella* infection did not induce expression of this inhibitory T cell molecule (Figure 4-10).

(A)

(B)

■ CD4⁺/SP   □ CD4⁺/SE   ▨ CD4⁺/SG   ▨ CD4⁺   ▥ CD4⁺/UI
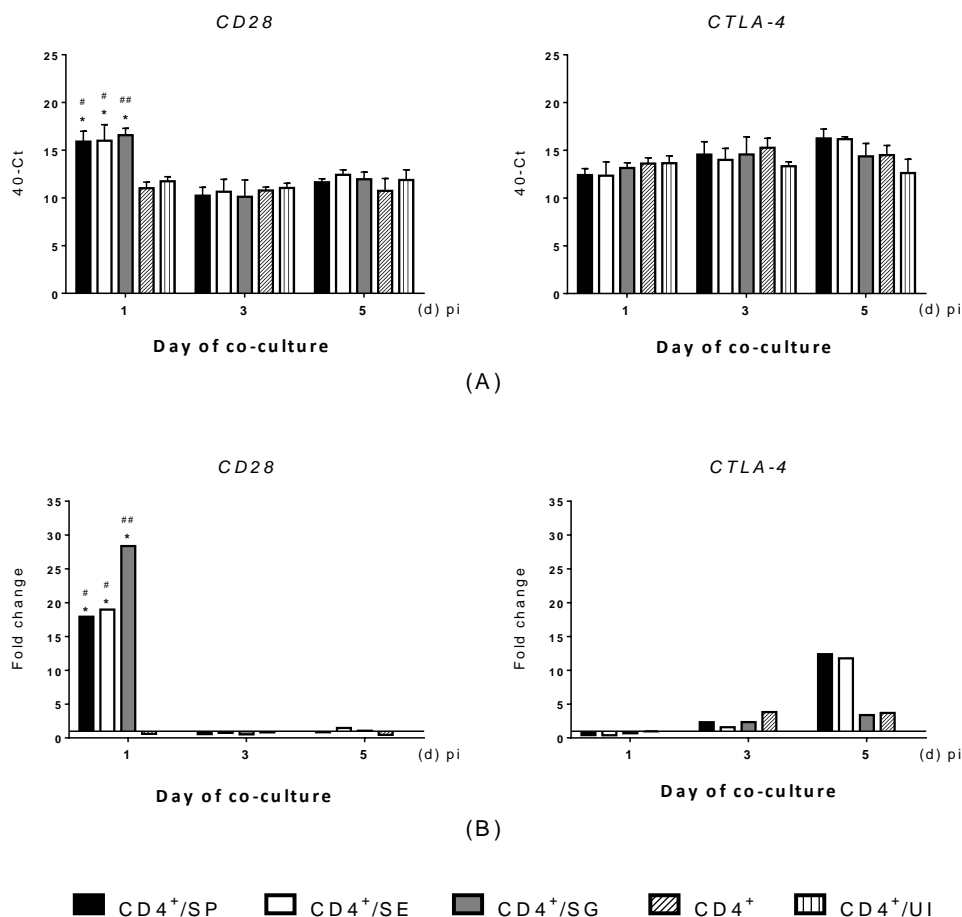
Figure 4-10. Gene expression of CD28 and CTLA-4 from CD4⁺ T cells co-cultured with *S. enterica* infected macrophages. (A) averages (40-Ct) ± SEM and (B) fold changes indicate the changes in mRNA levels of CD28 and CTLA-4 at 1, 3 and 5 days after co-culture (n=3). CD4⁺/SP, CD4⁺ T cells co-cultured with *S.* Pullorum-infected chMDM; CD4⁺/SE, CD4⁺ T cells co-cultured with *S.* Enteritidis-infected chMDM; CD4⁺/SG, CD4⁺ T cells co-cultured with *S.* Gallinarum-infected chMDM; CD4⁺, CD4⁺ T cells cultured alone; CD4⁺/UI, CD4⁺ T cells co-cultured with uninfected chMDM (control for allogeneic response). (#) indicates statistical difference from CD4⁺ T cells (CD4⁺), +: *P*<0.05, ++: *P*<0.01; (*) indicates statistical difference from uninfected control (CD4⁺/UI), *: *P*<0.05, **: *P*<0.01 (no differences were observed).

4. 3   Chapter discussion

A Th1-dominant cellular immune response is essential in controlling and eliminating *Salmonella* infection. However, *S.* Pullorum did not induce the expression of Th1-associated inflammatory cytokines in avian macrophages, as discussed in chapter 3, indicating its inability in initiating a protective Th1 response. Therefore, we used a co-culture system of *Salmonella*-infected macrophages and CD4$^+$ T cells to investigate immunomodulation of the adaptive immunity by *S.* Pullorum in more detail. In the current *in vitro* study, the immune parameters determined in co-cultured CD4$^+$ T cells could be associated with interaction with *Salmonella*-infected avian macrophages, although it has not been definitively proven that cell-cell contact occurs between them. In addition, the frequency of *Salmonella*-specific CD4$^+$ T cells is very low in the endogenous T cell repertoire (McSorley *et al.*, 2002) and also in the current co-culture system. The immune parameters determined in co-cultured CD4$^+$ T cells may largely be a result of non-specific driven by macrophages (e.g. cytokine expression from infected macrophages) as this co-culture system may only represent limited capacity to mount full antigenic signal.

Lymphocyte proliferation is a prerequisite for effective differentiation of T cells into different subsets. Proliferation of CD4$^+$ T cells was observed in co-culture with chMDM infected with each serovar of *Salmonella* tested in this study. *S.* Pullorum stimulated CD4$^+$ T cell proliferation but to a lesser extent than *S.* Enteritidis infection. Infection with *S.* Pullorum did not inhibit the

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expression of co-stimulatory molecules (CD40, CD80 and CD86) on chMDM when compared to those on *S.* Enteritidis-infected cells (Figure 3-13). This suggested that the differential stimulation for CD4$^+$ T cell proliferation was not a result of the absence of a second signal and therefore clonal anergy. CD28 expressed on T cells is an important molecule that recognizes CD80/CD86, providing co-stimulatory signals required for T cell activation and survival. CD28$^{-/-}$ mice are highly impaired in IFN-γ production and are not able to control infection with *S.* Typhimurium *aroA* mutant (Mittrucker *et al.*, 1999). However, neither reduced CD28 nor enhanced CTLA-4 expression was observed with in CD4$^+$ T cells when cultured with *S.* Pullorum-infected chMDM. This would also suggest that the reduced CD4$^+$ proliferation associated with *S.* Pullorum-infected macrophages was not due to clonal anergy. IL-15 derived from *S.* Choleraesuis-infected murine macrophages was suggested to induce γδT cells for proliferation through the β- and γ- chain of IL-2R for signal transduction (Nishimura *et al.*, 1996). Chicken IL-15 was also indicated to be a T-cell growth factor (Lillehoj *et al.*, 2001). It is possible that infection of avian macrophages with *S.* Pullorum induces IL-15 expression at a lower level than that produced from *S.* Enteritidis-infected cells and thus is less effective than *S.* Enteritidis in stimulating CD4$^+$ T cells for proliferation, although this was not measured in this study.

It has been reported that even in the absence of DC, *S.* Typhimurium reduced T cells proliferation and cytokine production (van der Velden *et al.*, 2005). Further study revealed that the inhibitory effect was the result of down-

regulation of TCR β-chain expression that inhibited T cell priming but excluded the involvement of either IL-10 or TGF-β4 in interfering with the expression of the TCR β-chain (van der Velden *et al.*, 2008). It is possible that *S.* Pullorum directly interacts with T cells and utilises a similar strategy to inhibit T cells for proliferation at the onset of laying when bacteria multiply within spleen macrophages and spread to the reproductive tract. It was shown that spread of *S.* Dublin from ligated intestinal loops in calves involved free bacteria that were not present within macrophages (Pullinger *et al.*, 2007), although *Salmonella* resides largely as an intracellular pathogen. We hypothesise that a direct inhibitory effect of *S.* Pullorum on T cell proliferation could impact on the initial stimulation of the adaptive response further avoiding clearance by the adaptive immune system in future studies.

Although antibody production is known to play a role in the immune response against *Salmonella* infection (Mittrücker *et al.*, 2000), IFN-γ production, initiated by IL-12 and IL-18, by Th1 lymphocytes is required for host resolution of *Salmonella* infection in mice (Mastroeni and Menager, 2003) and chicken (Beal *et al.*, 2004a, Beal *et al.*, 2004b, Withanage *et al.*, 2005b, Wigley *et al.*, 2006, Chappell *et al.*, 2009). The results presented in this chapter showed that IFN-γ was not up-regulated in CD4^{+} T cells which had been co-cultured with *S.* Pullorum-infected avian macrophages when compared to the uninfected controls. It suggests that unlike *S.* Enteritidis, *S.* Pullorum inhibits proliferation of Th1 cells. In autologous co-culture of human macrophage and T-cells, IFN-γ- or LPS-activated macrophages preferentially drive Th polarisation towards a

Th1 phenotype whereas IL-4 activated macrophages did not induce T cells to produce IFN-γ or IL-17A (Arnold *et al.*, 2015). This suggests that in our study, *S.* Enteritidis-infected chMDM in co-culture with IFN-γ-producing CD4+ T cells may further drive the development of Th1 cells, although IL-12 has greater importance than IFN-γ in driving Th1 differentiation and IFN-γ produced from Th1 cells functions mainly to mediate macrophage killing. However, there is no evidence to suggest that *S.* Pullorum increased proliferation of Th2 cells, as shown by no difference in IL-4 expression. IL-10 functions to inhibit further development of the Th1 response and down-regulates the effects of IFN-γ (Rothwell *et al.*, 2004). Avian CD4+CD25+ suppressor T cells have been alternatively characterized as nTregs and were shown to produce high amounts of IL-10, TGF-β4 and CTLA-4 and suppress T cell proliferation *in vitro* (Shanmugasundaram and Selvaraj, 2011). Infection of chMDM with both *S.* Pullorum and *S.* Enteritidis induced expression of IL-10 mRNA (Figure 3-11). However, CD4+ T cells in co-culture with *S.* Pullorum-infected chMDM did not produce higher mRNA expression of IL-10 or TGF-β4 when compared to uninfected control (allogeneic responses). This may suggest that *S.* Pullorum does not induce IL-10 or TGF-β4-producing tolerogenic T cells *in vitro*.

In addition, in contrast to *S.* Enteritidis, *S.* Pullorum was shown to suppress Th17 immune responses in this *in vitro* study. In *Salmonella* infected mice, increased bacterial loads were found in spleen and liver of IL-17-deficient individuals (Schulz *et al.*, 2008), indicating the potential of Th17 cytokines being involved in intestinal defence against infection. Increased expression of

IFN-γ and IL-17 was also found in the caeca of chicken infected with *S. Enteritidis* (Crhanova *et al.*, 2011, Matulova *et al.*, 2013). Th17 cytokines were elicited rapidly after *S.* Typhimurium infection of bovine ligated ileal loops, probably through a non-specific activation of intestinal Th17 cells in response to IL-1 or other inflammatory cytokines while recognition of flagellin via the TLR5 pathway, probably driving *Salmonella*-specific Th17 cell development, may also contribute to intestinal mucosal defence against infection (Raffatellu *et al.*, 2007). Our study showed that S. Pullorum induced expression of pro-inflammatory cytokines IL-1β, IL-6 and iNOS in infected chMDM. The reduced expression of IL-17F in CD4^{+} T cells co-cultured with *S.* Pullorum-infected chMDM may thus not the result of down-regulated non-specific activation of Th17 cells in response to inflammatory cytokines. However, suppression of a Th17 response by non-flagellate *S.* Pullorum infection probably resulted from the absence of flagellin in TLR5 stimulation. This may also be the case for the non-flagellated serovar *S.* Gallinarum. In addition, early studies that linked human IL-23 to induction of IL-17 expression by memory CD4^{+} T cells also demonstrated stimulation of IFN-γ production (Oppmann *et al.*, 2000). Th17 cells also displayed considerable plasticity and acquired the capacity to produce IFN-γ *in vitro* (Lee *et al.*, 2009) and *in vivo* (Hirota *et al.*, 2011) where IFN-γ production is a recognized feature of Th17 cells. Although these have not been studied in chickens, in the current study, *S.* Pullorum-infected chMDM 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 *S.* Pullorum infection, which might

be associated with the establishment of carriage.

Chapter 5   Immune response of the chickens to *S.* Pullorum and related serovars

5. 1   Introduction

5. 1. 1   General introduction

S. Pullorum is unable to elicit a protective Th1/Th17 immune response, which was indicated in the *in vitro* infection of avian macrophages alone (chapter 3) and CD4^{+} T cells in co-culture (chapter 4). In contrast, infection with *S. Enteritidis* induced a strong protective Th1/Th17 response *in vitro*, which, however, was not observed in its likely evolutionary descendent, *S. Gallinarum.* In the present study, avian macrophages represent the antigen presenting cells interacting with CD4^{+} T cells, but DCs and CD8^{+} T cells are also involved in response to *Salmonella* infection (Berndt *et al.,* 2006). Thus it is still essential to determine the host immune response during early infection, which, in comparison with the *in vitro* observations addressed above, would inform us whether the immunomodulation of *S.* Pullorum in chicken is mainly due to interaction with macrophages and down-stream presentation to CD4^{+} T cells

5. 1. 2   Chapter aims and objectives

The aim of work described in this chapter was therefore to compare over 5 days the effect of infection by *S*. Pullorum, *S*. Gallinarum and *S*. Enteritidis in 2-day-old chickens, with regard to colonisation of the chicken caeca and liver

and the production of host immune responses during the first week post-hatch.

5. 2   Results

5. 2. 1   Distribution of *S. enterica* in the tissue of chickens following oral infection

Infection of 2 day-old chickens with approximately 10^8 CFU of *S.* Pullorum or *S.* Enteritidis did not induce any clinical signs of disease over the 5 days of infection. However, chickens infected with *S.* Gallinarum began to demonstrate signs of systemic disease from 4 d pi, including ruffled feathers, yellow diarrhoea and reluctance to move or drink. At post-mortem examination at 4 and 5 d pi, the *S.* Gallinarum-infected chickens displayed hepatosplenomegaly, with necrotic liver and spleen lesions and haemorrhaging in the ileum, which are indicative of acute systemic infection of fowl typhoid.

Following infection, viable bacteria of serovar Pullorum, Enteritidis or Gallinarum were detected in the caecal contents of infected chickens in each group at 1 d pi. *S.* Enteritidis had higher levels of bacterial loads in the caecal contents at all time points examined in this study when compared to *S.* Pullorum and *S.* Gallinarum which were poor colonisers of the gut ($P<0.001$). In addition, the mean Log CFU/g of *S.* Pullorum recovered from the caecal contents of infected chickens was higher than that of *S.* Gallinarum at 2 and 5 d pi ($P<0.01$). At 4 d pi, the number of viable *S.* Pullorum and *S.* Gallinarum in the caecal contents of infected chickens dropped to less than 3 of Log CFU/g, which may be a consequence of invasion into deeper tissues from the gut at

this time. The following return to the intestine at 5 d pi may be the later result of systemic dissemination (Figure 5-1).

None of the three serovars was found in the liver of infected chickens at 1 day pi. There were over 10^5 CFU/g of *S*. Gallinarum isolated from the liver at 4 d pi, which was significantly higher than that of *S*. Enteritidis ($P<0.01$). At 5 d pi, the mean Log CFU/g of *S*. Pullorum and *S*. Gallinarum recovered from the liver of infected chickens increased to 5.29 and 7.37, respectively, which were significantly higher than that of *S*. Enteritidis ($P<0.001$). Moreover, the average number of viable bacteria recovered from infected chickens at 5 d pi also indicted that *S*. Gallinarum was more effective than *S*. Pullorum in colonising the livers of infected chickens ($P<0.01$) (Figure 5-2).
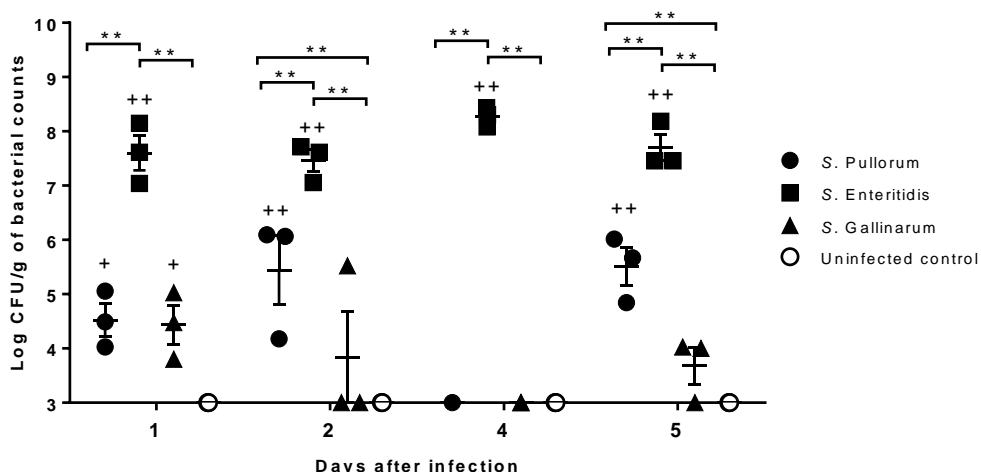
Figure 5-1. The numbers of viable *Salmonella* in the caecal contents following infection with 10^8 CFU in 2-day-old chickens. Viable counts are values from positive animals in each group at each time point in one independent experiment. When no viable colonies were found at 10^{-1} dilution after selective enrichment, it suggested a viable count of <3 of Log CFU/g and Log CFU/g=3 is used to represent the bacterial loads in negative animal for statistical analysis. Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between experimental groups within each time point. (+) 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 5-2. The numbers of viable *Salmonella* in the liver following infection with 10^8 CFU in 2-day-old chickens. Viable counts are values from positive animals in each group at each time point in one independent experiment. When no viable colonies were found at 10^{-1} dilution after selective enrichment, it suggested a viable count of <3 of Log CFU/g and Log CFU/g=3 is used to represent the bacterial loads in negative animal for statistical analysis. Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between experimental groups within each time point. (+) 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).

5. 2. 2 Gene expression profile in the caecal tonsil and spleen of *Salmonella*-infected chickens

In order to characterize the difference in the early host immune response to infection with *S*. Pullorum and related serovars, the gene expression of selected immune mediators in the caecal tonsils and spleens of infected birds was measured by qRT-PCR.

5. 2. 2. 1 Gene expression profile in the caecal tonsils of infected chickens

The mRNA expression of pro-inflammatory cytokines and chemokines in the caecal tonsils of chickens infected with different *Salmonella* serovars are displayed in Figure 5-3. Chickens infected with *S.* Enteritidis expressed higher levels of CXCLi1 ($P<0.05$ at 1 and 4 d pi) and CXCLi2 ($P<0.05$ at 2 d pi), compared to uninfected controls. The levels of CXCLi1 and CXCLi2 expressed in response to *S.* Enteritidis infection were greater than expression in either *S.* Pullorum or *S.* Gallinarum-infected chickens ($P<0.05$ at different time points). Infection with *S*. Pullorum and *S*. Gallinarum did not up-regulate the mRNA expression of any of these immune mediators when compared to that of uninfected controls. Furthermore, *S.* Enteritidis infection induced the highest levels of iNOS mRNA expression from the caecal tonsils of infected chickens among all experimental groups at 2 and 4 d pi ($P<0.05$) while the same scenario was observed with IL-1β expression at 1 d pi ($P<0.05$) and IL-6 at 2 d pi ($P<0.05$).

(A)

(B)
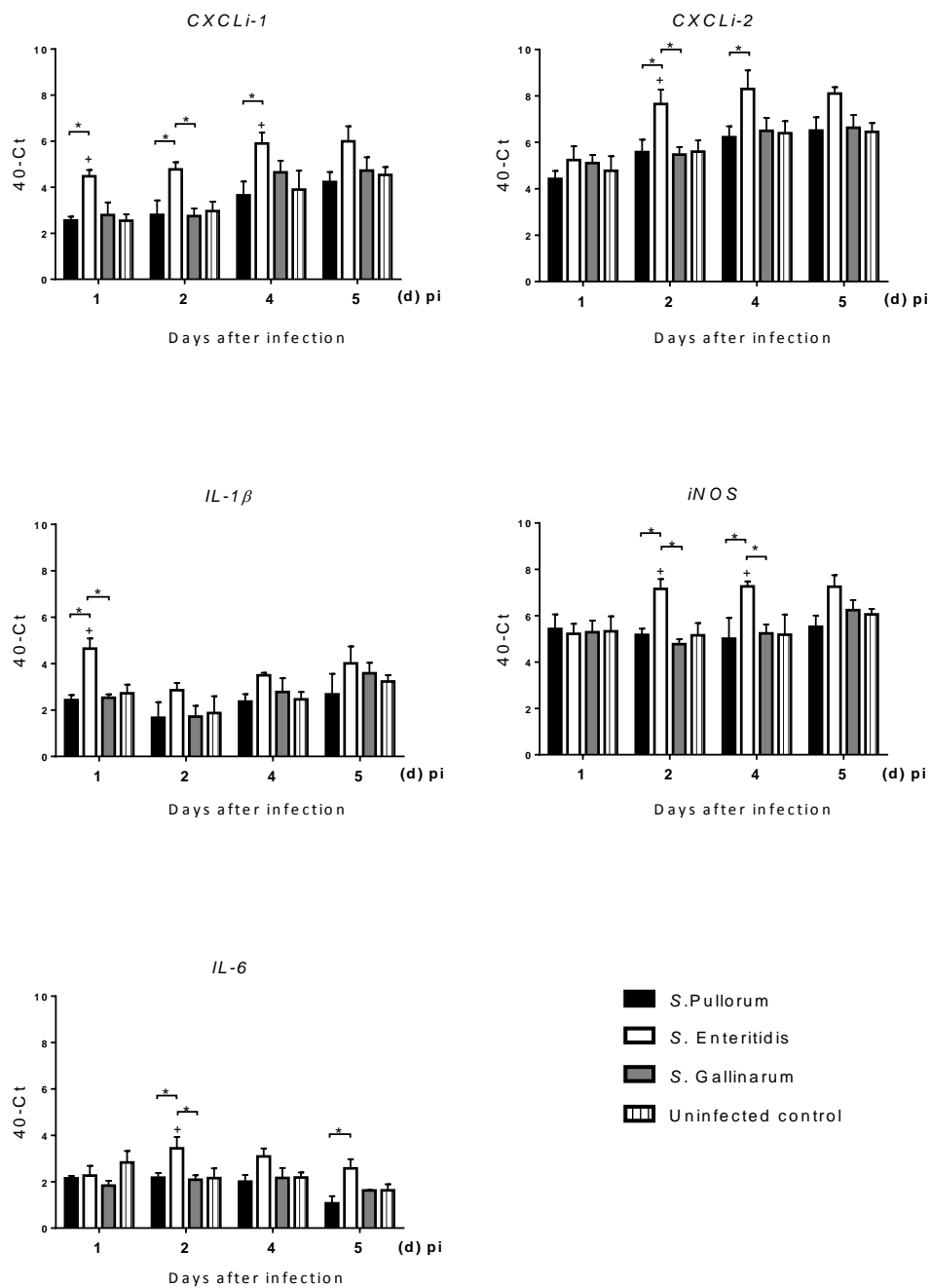
Figure 5-3. Quantification of gene expression of CXCLi1, CXCLi2, IL-1β, iNOS and IL-6 in the caecal tonsils of chickens in response to *Salmonella* infection. Caecal tonsils were sampled from *Salmonella*-infected or uninfected chickens at 1, 2, 4 and 5 d pi. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3, three samples each from three chickens in one independent experiment). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between experimental groups within each time point. (+) 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, *: $P<0.05$, **: $P<0.01$.

In comparison with infection with *S.* Enteritidis, *S.* Pullorum infection was associated with decreased IFN-γ expression in the caecal tonsils at all the time points examined ($P<0.01$ at 1 d pi, $P<0.05$ at 2, 4 and 5 d pi). Lower levels of IL-12α mRNA were found in the caecal tonsils of *S.* Pullorum-infected chickens in the first 2 days after infection when compared to that in birds infected with *S.* Enteritidis ($P<0.05$ at 1 d pi, $P<0.01$ at 2 d pi). Moreover, the significant difference between the infections with *S.* Pullorum and *S.* Enteritidis could also be seen with the gene expression of IL-18 at 2 d pi ($P<0.05$) (Figure 5-4).

The expression pattern of IL-4 and IL-13 following infection with *S.* Pullorum or *S.* Enteritidis was opposite to the trend shown with Th1 cytokines described above. At 2 d pi, significantly increased expression of IL-4 mRNA was found in the caecal tonsils of *S.* Pullorum-infected chickens when compared to that of *S.* Enteritidis-infected ($P<0.01$) and uninfected chickens ($P<0.05$). Chickens infected with *S.* Pullorum also expressed higher levels of IL-13 mRNA when compared with other experimental groups at different time points pi (Figure 5-4).

(A)

(B)

Figure 5-4. Quantification of gene expression of IFN-γ, IL-12α, IL-18, IL-4 and IL-13 in the caecal tonsils of chickens in response to *Salmonella* infection. Caecal tonsils were sampled from *Salmonella*-infected or uninfected chickens at 1, 2, 4 and 5 d pi. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3, three samples each from three chickens in one independent experiment). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between experimental groups within each time point. (+) 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, *: $P<0.05$, **: $P<0.01$.

Compared to uninfected control, increased expression of IL-17F mRNA was only found in the caecal tonsil of chickens infected with *S*. Enteritidis at 4 and 5 d pi ($P<0.05$). *S.* Pullorum-infected chickens resulted in significantly lower levels of IL-17F mRNA expression in caecal tonsils when compared to *S.* Enteritidis- ($P<0.01$ at 1 d pi, $P<0.05$ at 5 d pi) and *S.* Gallinarum- ($P<0.05$ at 1 d pi) infected chickens (Figure 5-5).

Gene expression of anti-inflammatory cytokines are shown in Figure 5-6. TGF-β4 mRNA expression was not significantly changed in the caecal tonsils of *Salmonella*-infected chickens when compared with the non-infected controls ($P>0.05$) throughout all the sampling time points. In comparison with chicken infected with *S.* Enteritidis, higher levels of IL-10 mRNA were observed in the caecal tonsils of *S.* Pullorum-infected chickens at 5 d pi ($P<0.01$).

Figure 5-5. Quantification of gene expression of IL-17F in the caecal tonsils of chickens in response to *Salmonella* infection. Caecal tonsils were sampled from *Salmonella*-infected or uninfected chickens at 1, 2, 4 and 5 d pi. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3, three samples each from three chickens in one independent experiment). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between experimental groups within each time point. (+) 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, *: $P<0.05$, **: $P<0.01$.
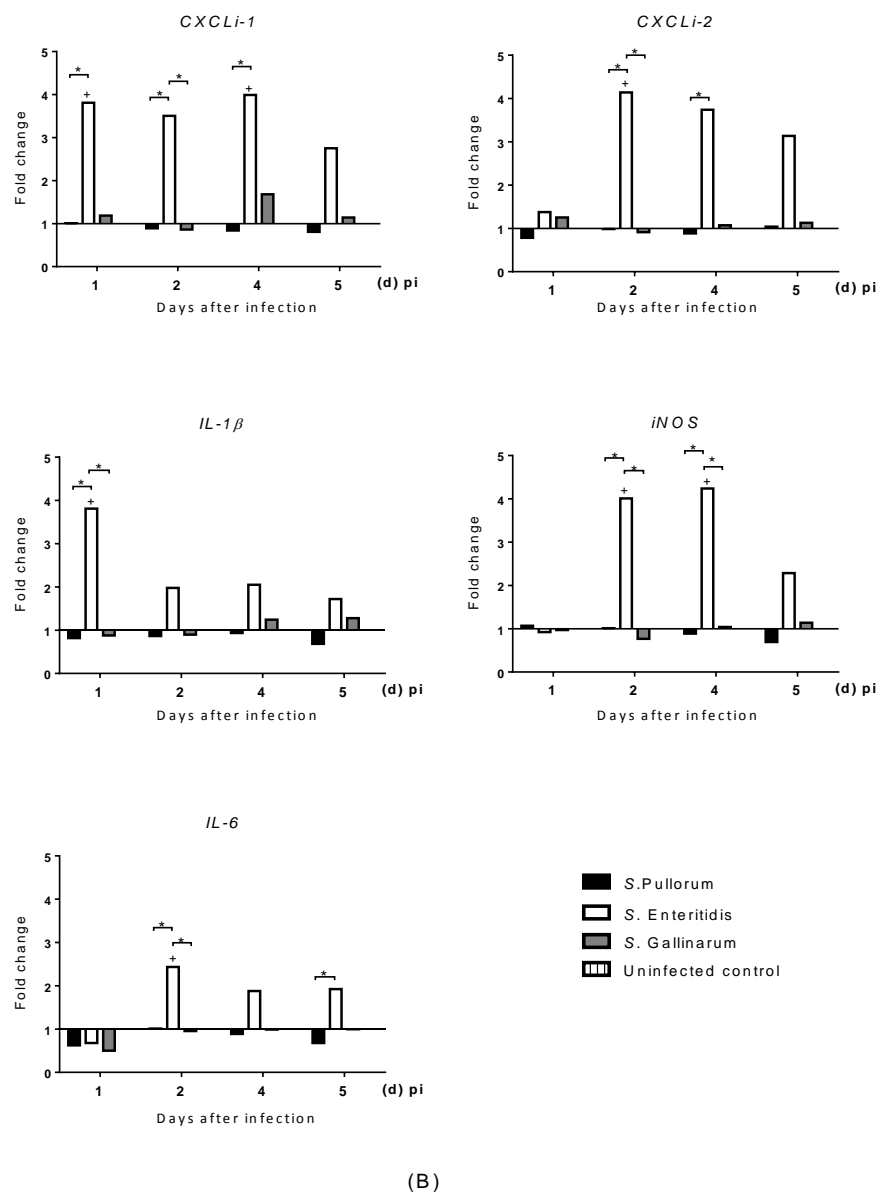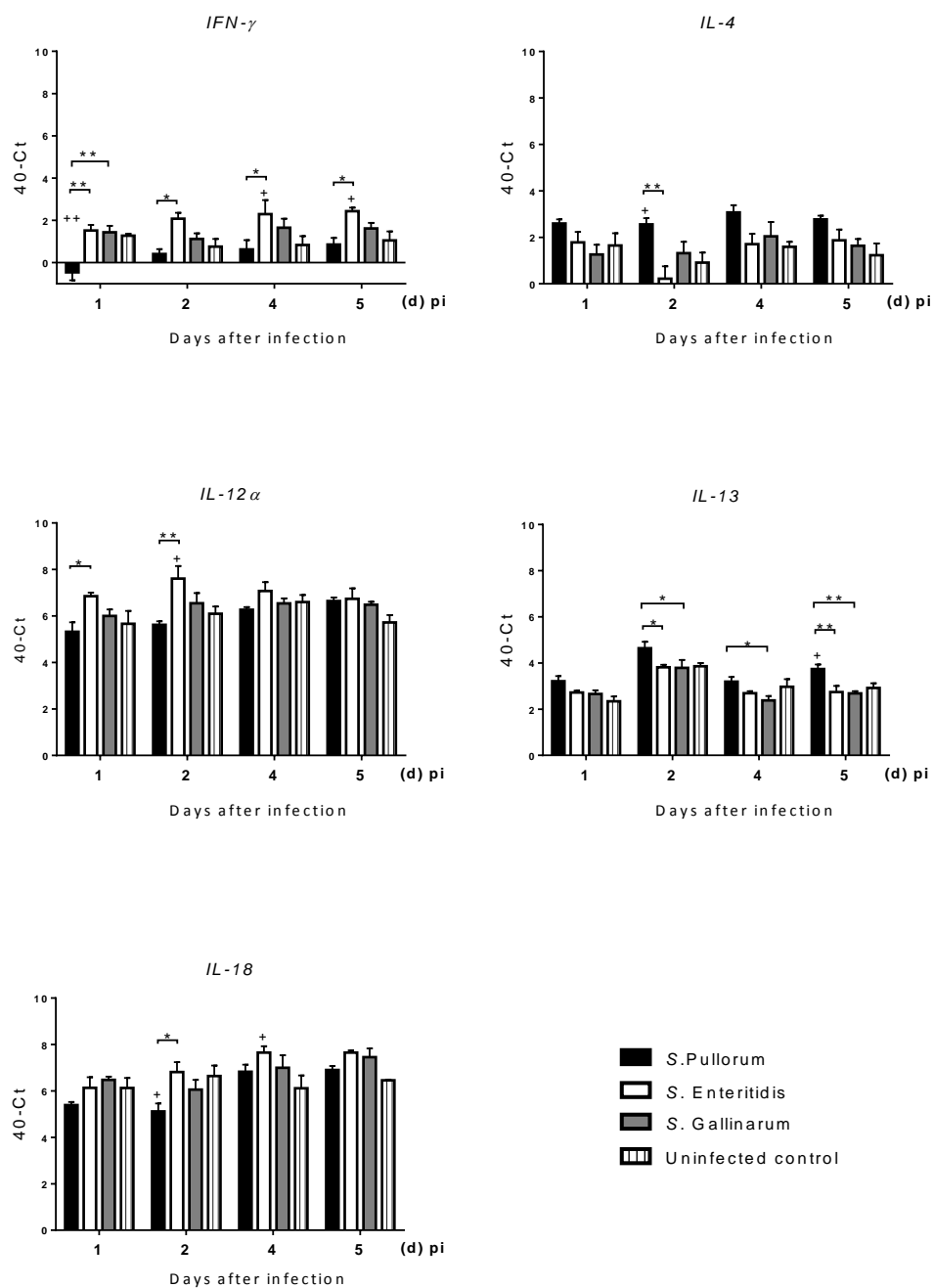
(A)

(B)

S.Pullorum
S. Enteritidis
S. Gallinarum
Uninfected control

Figure 5-6. Quantification of gene expression of IL-10 and TGF-β4 in the caecal tonsils of chickens in response to *Salmonella* infection. Caecal tonsils were sampled from *Salmonella*-infected or uninfected chickens at 1, 2, 4 and 5 d pi. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3, three samples each from three chickens in one independent experiment). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between experimental groups within each time point. (+) 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, *: $P<0.05$, **: $P<0.01$.

5. 2. 2. 2 Gene expression profile in the spleen of infected chickens

The mRNA levels of CXCLi1, CXCLi2 and IL-6 measured in the spleens were not different between infected and uninfected birds at any time points examined in this study. Increased expression of iNOS mRNA was found in response to the infection with *S.* Enteritidis when compared to that in the *S.* Pullorum ($P<0.01$) and *S.* Gallinarum ($P<0.05$)-infected chickens and uninfected controls ($P<0.01$) at 4 d pi. *S.* Pullorum also induced lower levels of IL-1β mRNA in the spleen at 4 d pi compared with *S.* Enteritidis-infected chickens ($P<0.01$). (Figure 5-7).

(A)

(B)
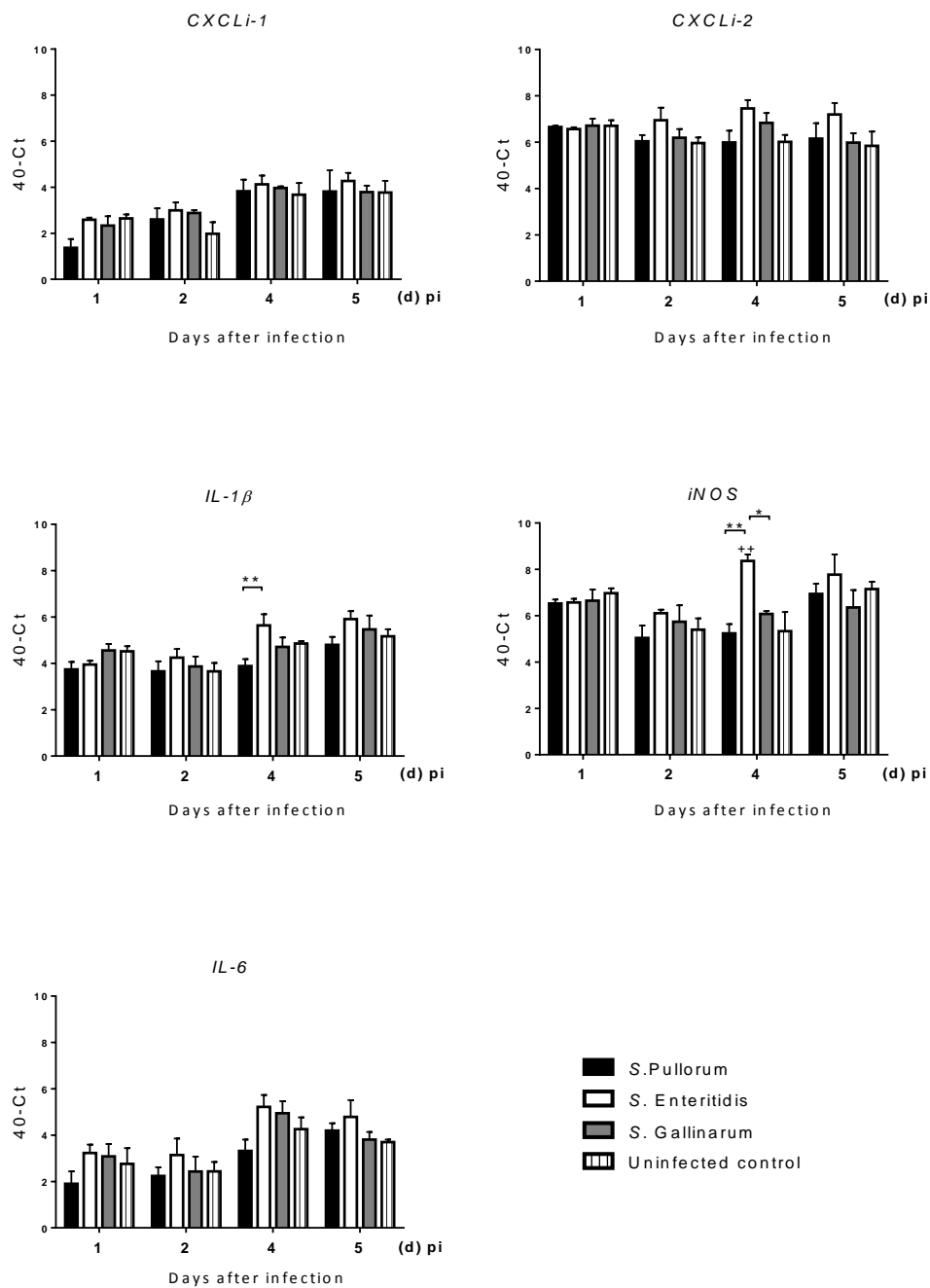
Figure 5-7. Quantification of gene expression of CXCLi1, CXCLi2, IL-1β, iNOS and IL-6 in the spleen of chickens in response to *Salmonella* infection. Spleens were sampled from *Salmonella*-infected or uninfected chickens at 1, 2, 4 and 5 d pi. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3, three samples each from three chickens in one independent experiment). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between experimental groups within each time point. (+) 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, *: $P<0.05$, **: $P<0.01$.

In the spleen, *S.* Pullorum did not stimulate gene expression of Th1-associated cytokines compared to uninfected controls ($P>0.05$). However, *S.* Enteritidis infection was associated with a significant up-regulation in the mRNA expression of IFN-γ ($P<0.05$ at 1 and 5 d pi), IL-12α ($P<0.01$ at 5 d pi) and IL-18 ($P<0.05$ at 1 and 4 d pi) when compared to uninfected controls. Statistically significant differences were also observed between the levels of expression of IFN-γ and IL-12α in chickens infected with *S.* Pullorum and *S.* Enteritidis at 1 and/or 5 d pi (Figure 5-8).

Infection with *S.* Pullorum induced up-regulation of IL-4 mRNA in the spleen of infected birds, which was higher than that in uninfected controls and *S.* Gallinarum-infected chickens at 2 d pi ($P<0.05$) or *S.* Enteritidis-infected chickens at 4 and 5 d pi ($P<0.05$). *S.* Pullorum infection induced higher levels of IL-13 mRNA in the spleen of infected chickens with significantly increased expression being determined at 4 and 5 d pi ($P<0.05$) when compared with that of uninfected controls. At 5 d pi, the level of IL-13 mRNA determined in the spleens of *S.* Pullorum-infected chickens was also found to be higher than that of chickens infected with *S.* Enteritidis ($P<0.05$) or *S.* Gallinarum ($P<0.01$) (Figure 5-8).

(A)
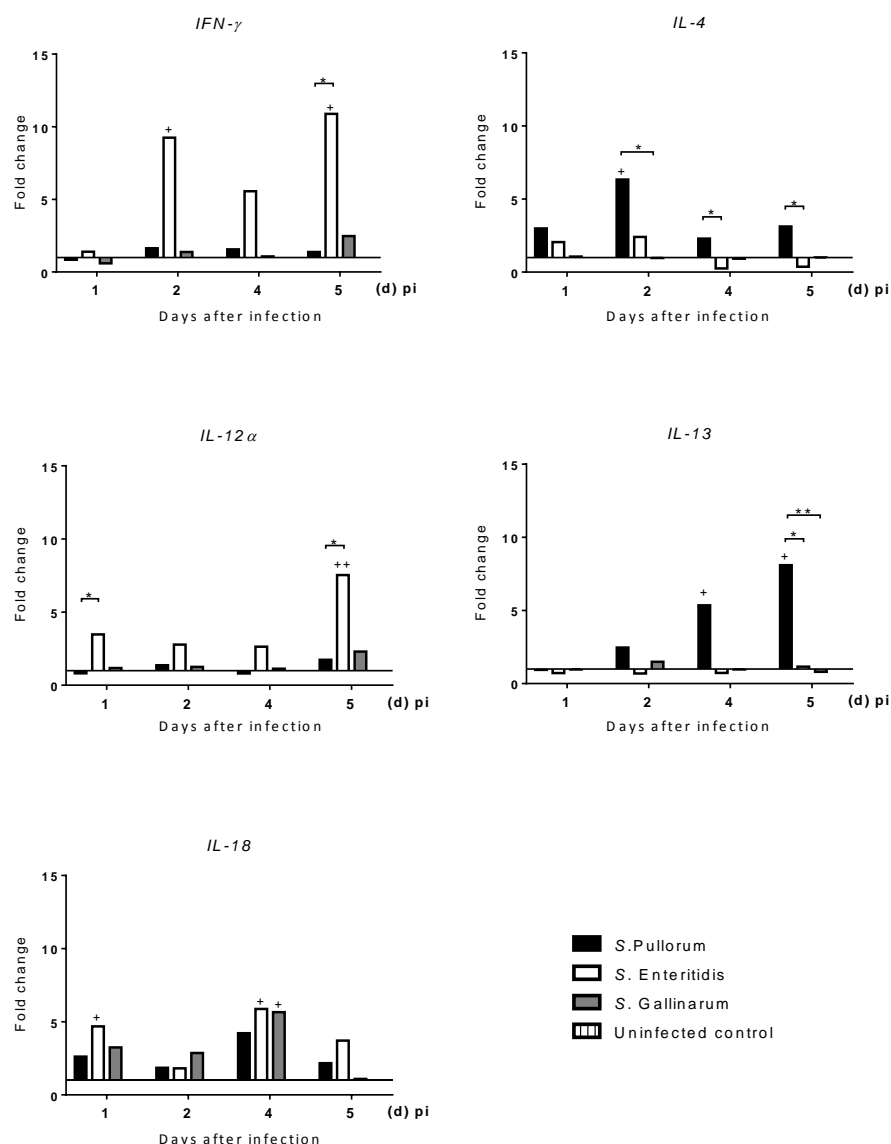
(B)

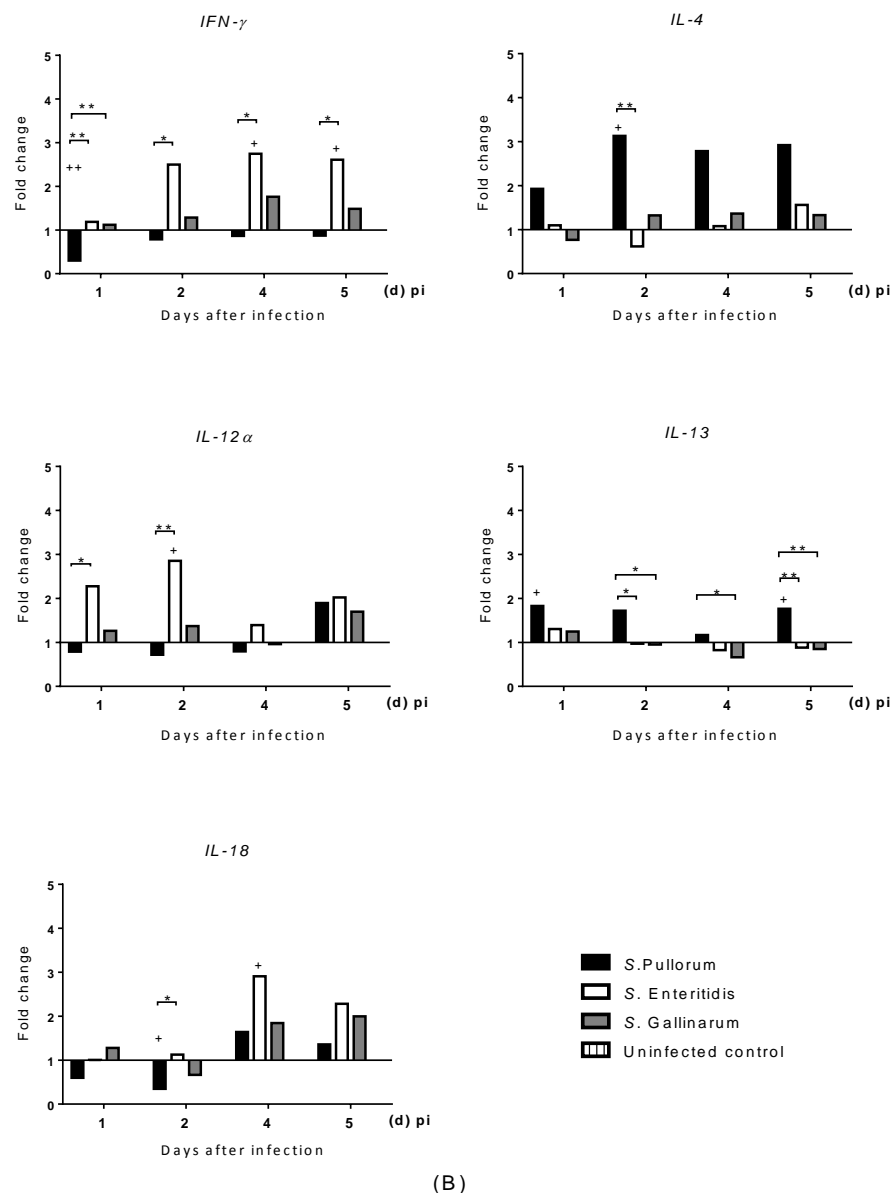**Figure 5-8. Quantification of gene expression of IFN-γ, IL-12α, IL-18, IL-4 and IL-13 in the spleen of chickens in response to *Salmonella* infection.** Spleens were sampled from *Salmonella*-infected or uninfected chickens at 1, 2, 4 and 5 d pi. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3, three samples each from three chickens in one independent experiment). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between experimental groups within each time point. (+) 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, *: $P<0.05$, **: $P<0.01$.

In this *in vivo* study, a significant down-regulation of IL-17F mRNA was observed in the spleens of chickens infected with *S.* Pullorum at 2 or 5 d pi when compared to that of *S.* Enteritidis or *S.* Gallinarum-infected birds ($P<0.05$) (Figure 5-9).
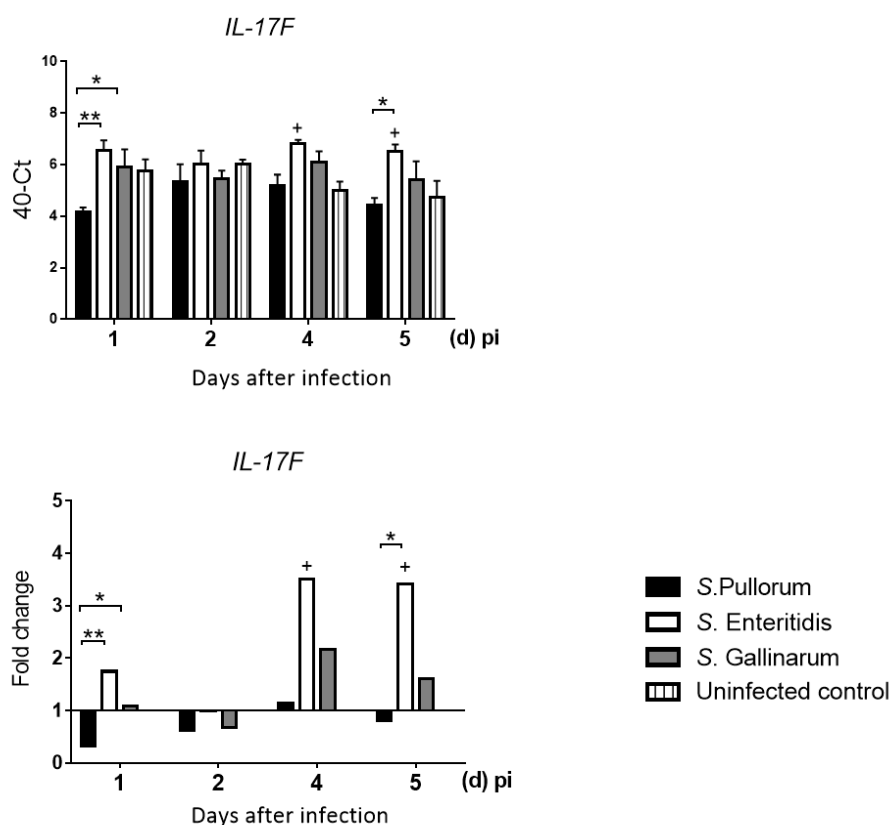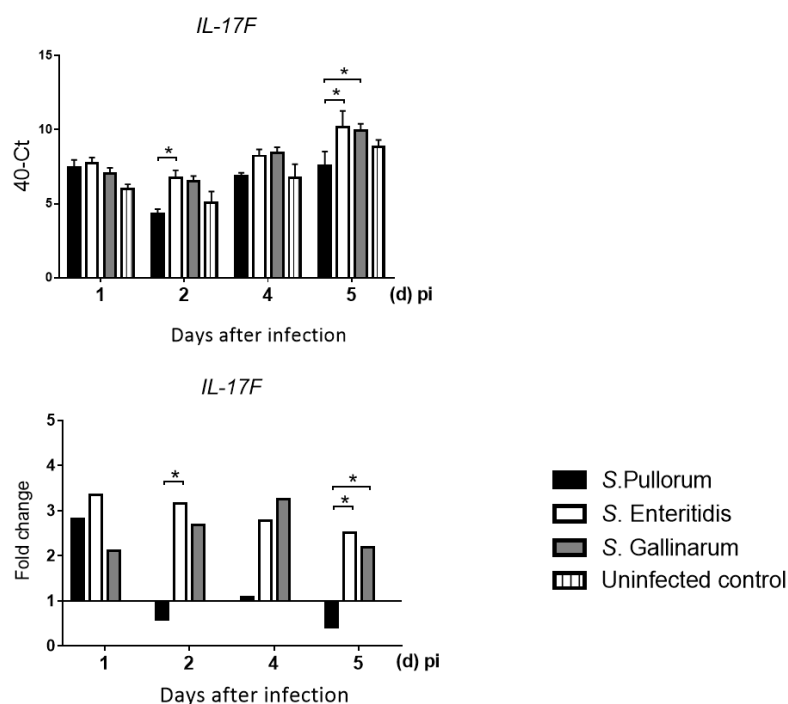

Figure 5-9. Quantification of gene expression of IL-17F cytokine in the spleen of chickens in response to *Salmonella* infection. Spleens were sampled from *Salmonella*-infected or uninfected chickens at 1, 2, 4 and 5 d pi. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3, three samples each from three chickens in one independent experiment). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between experimental groups within each time point. (+) 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, *: $P<0.05$, **: $P<0.01$.
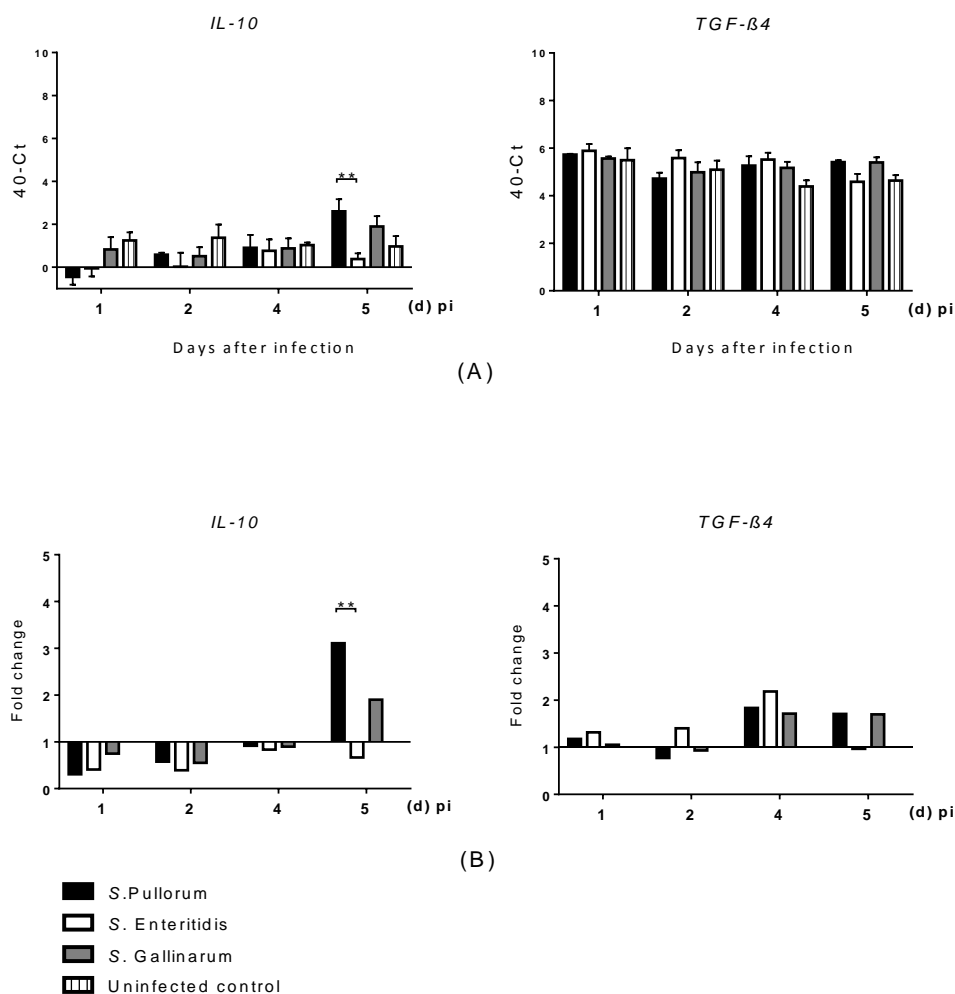
In comparison with uninfected controls, significantly increased expression of IL-10 mRNA in the spleens was observed in chickens infected with *S.* Pullorum ($P<0.05$) or *S.* Gallinarum ($P<0.01$) at 5 d pi. In contrast, IL-10 mRNA expression in the spleen of *S.* Enteritidis-infected chicken remained at levels close to that of uninfected controls, which was found to be significantly lower than that in chickens infected with *S.* Pullorum or *S.* Gallinarum at 4 and 5 d pi. Expression of TGF-β4 in chicken splenic tissues was not affected by infection with different serovars of *Salmonella* at any time point measured in this study ($P>0.05$) (Figure 5-10).
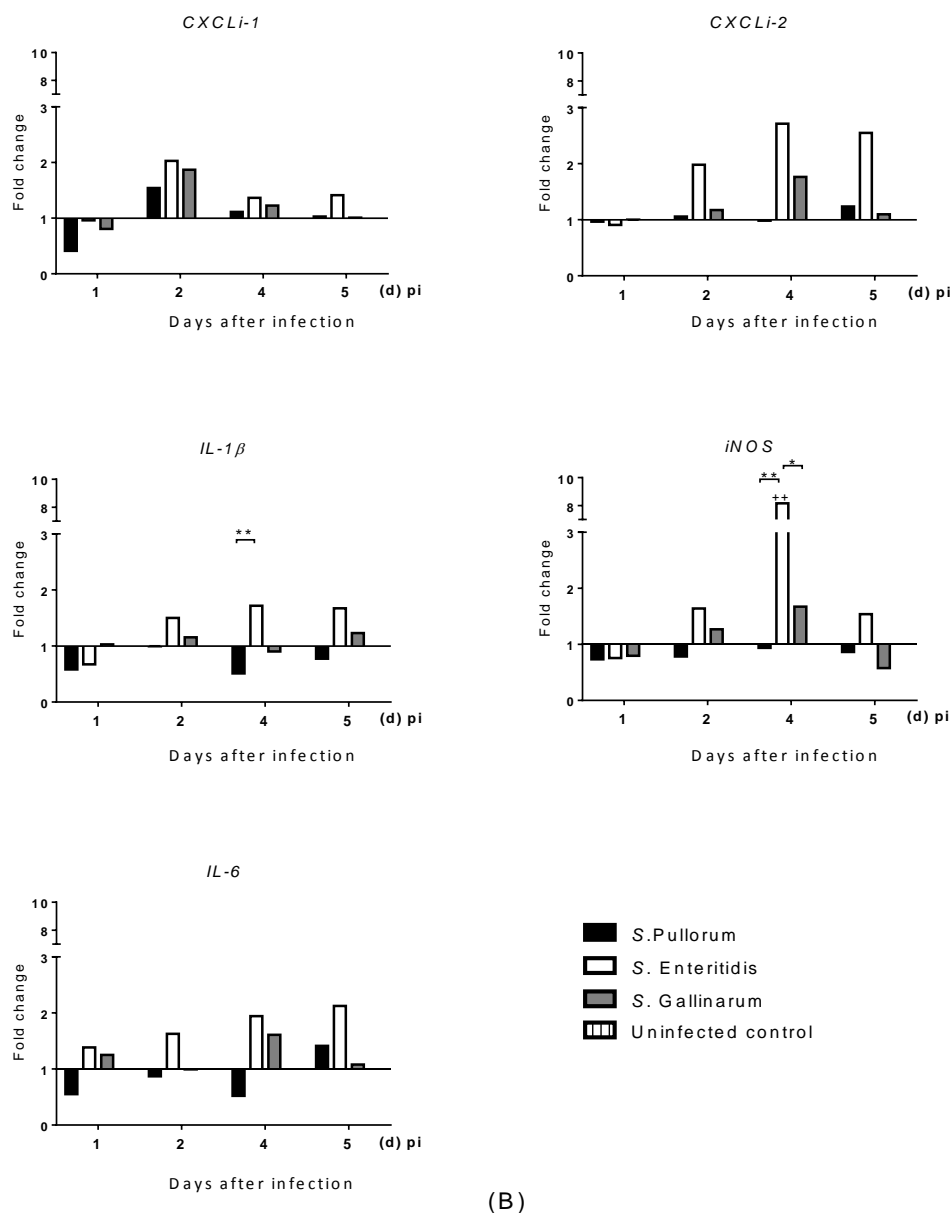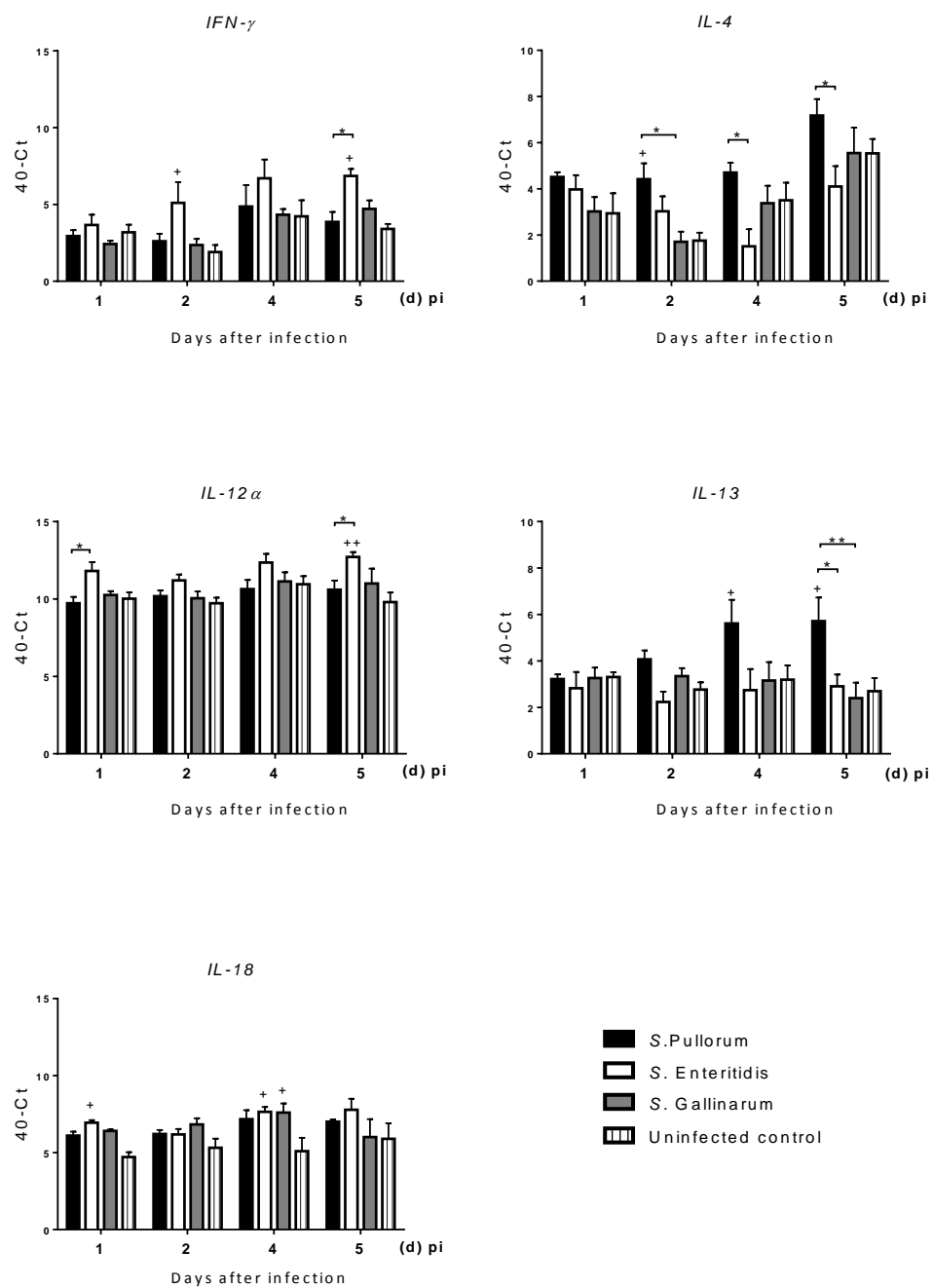
(A)

(B)

S.Pullorum
S. Enteritidis
S. Gallinarum
Uninfected control
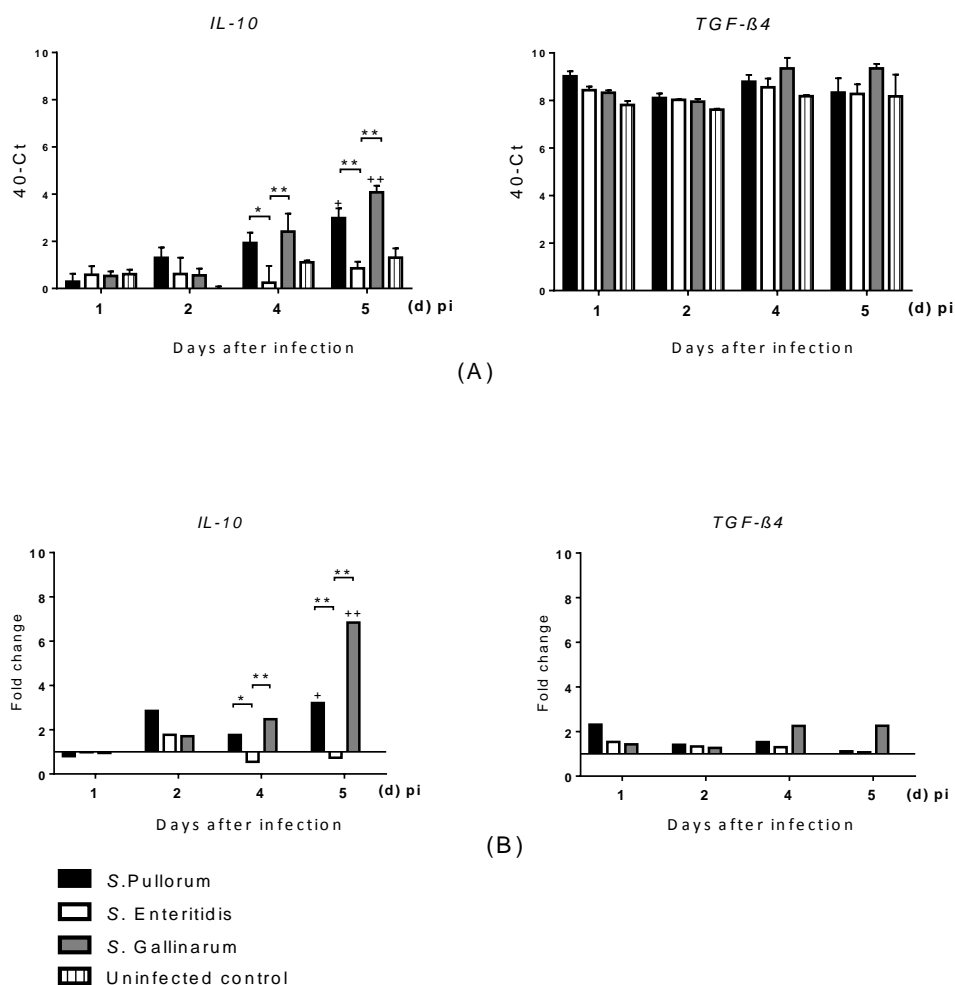
Figure 5-10. Quantification of gene expression of IL-10 and TGF-β4 in the spleen of chickens in response to *Salmonella* infection. Spleens were sampled from *Salmonella*-infected or uninfected chickens at 1, 2, 4 and 5 d pi. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3, three samples each from three chickens in one independent experiment). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from uninfected controls (shown as 1). Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between experimental groups within each time point. (+) 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, *: $P<0.05$, **: $P<0.01$.

5. 2. 3 Splenic lymphocyte proliferation in response to *Salmonella* infection *in vivo*

At 5 days after infection, spleens were taken from *S.* Enteritidis- and *S.* Pullorum-infected and uninfected chickens. CD3$^+$ (mouse anti-chicken CD3, clone CT-3 and anti-mouse IgG1: FITC) or CD4$^+$ T (mouse anti-chicken CD4: FITC, clone 2-35) cells were recognised by appropriate mAb. The CD3$^+$ and CD4$^+$ T cell population in homogenised splenocytes were determined by flow cytometric analysis. Infection with *S.* Enteritidis resulted in significant increase in the number of CD4$^+$ T cells when compared with *S.* Pullorum-infection ($P<0.05$) and uninfected controls ($P<0.01$) at 5 d pi (Figure 5-11).
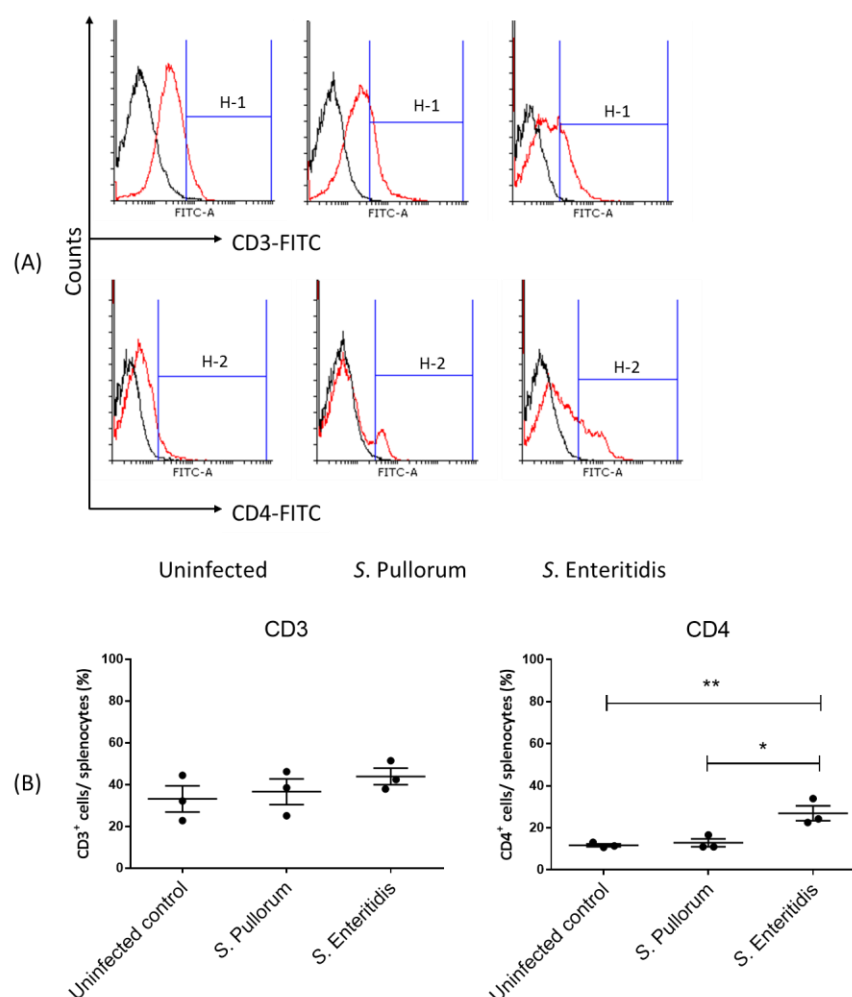
Figure 5-11. Percentage of splenic CD3+ and CD4+ T cells in chickens infected with different serovars of *Salmonella*. Spleens were taken from *S*. Enteritidis- and *S*. Pullorum-infected and uninfected chickens at 5 d pi. Mouse anti-chicken CD3 mAb (Table 2-4, #4) and anti-mouse IgG1-FITC mAb (Table 2-4, #12) recognised CD3+ T cells in the homogenised splenocytes. Mouse-anti-chicken CD4-FITC mAb (Table 2-4, #3) recognised CD4+ T cells in the homogenised splenocytes. (A): H-1 and H-2 define the proportion of CD3+ and CD4+ T cells in the spleen, respectively. Red lines: CD3+ or CD4+ population. Black lines: secondary binding (anti-mouse IgG1-FITC mAb for CD3) or isotype (mouse IgG2b: FITC, Table 2-4, #16) control. Panel (B) The data shown are the average of CD3+ and CD4+ T cells in each experimental group (n=3, three samples each from three chickens in one independent experiment). Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between percentage of CD3+ or CD4+ T cells of experimental groups within each time point. (*) indicates differences between each group, *: $P<0.05$, **: $P<0.01$.

5. 3   Chapter discussion

In this study, it was found that systemic serovars Pullorum and Gallinarum were poor colonisers of the gut. In contrast *S.* Enteritidis infection had higher counts in the intestine and rapid extra-intestinal spread to liver and spleen. These results are comparable with previous findings on the distribution of these serovars in day-old chickens (Beal *et al.*, 2004a, Withanage *et al.*, 2004, Setta *et al.*, 2012b, Matulova *et al.*, 2013). The presence of bacteria in the caeca was correlated with the profiles of pro-inflammatory cytokines produce by these organisms. *S.* Pullorum and *S.* Gallinarum did not induce the expression of inflammatory mediators in the caecal tonsils whereas *S.* Enteritidis infection was associated with a strong inflammatory response. *S.* Enteritidis displayed a strong capacity in stimulating the production of CXCLi1 and CXCLi2 from the caecal tonsils of infected chickens whereas expression of these chemokines in the caecal tonsils or spleens of *S.* Pullorum- and *S.* Gallinarum-infected chickens was equivalent to that observed in uninfected controls. In previous studies, enhanced expression of CXCLi1, CXCLi2 and IL-1β was also found in the gut of chickens infected with *S.* Typhimurium or *S.* Enteritidis (Withanage *et al.*, 2004, Cheeseman *et al.*, 2008, Setta *et al.*, 2012b, Matulova *et al.*, 2013) whereas *S.* Pullorum infection was found to down-regulate the expression of CXCLi1 and/or CXCLi2 in the ileum or caecal tonsils of day-old chicken when compared to those from *S.* Enteritidis (Chappell *et al.*, 2009, Setta *et al.*, 2012b). These together may suggest a reduced infiltration of heterophils in response to infection with *S.* Pullorum and *S.* Gallinarum. The

rapid expression of CXCLi1 and CXCLi2 in the intestine following infection with *S*. Enteritidis is indicative of subsequent inflammation and pathology as previously described for *S*. Typhimurium infection which resulted in an influx of immune cells to the intestine in chicken (heterophils) (Henderson *et al.*, 1999, Withanage *et al.*, 2004, Withanage *et al.*, 2005b) and mammalian (polymorphonuclear cells) (Zhang *et al.*, 2003) models. In this study, *S.* Enteritidis infection also up-regulated the gene expression of IL-1β, IL-6 and iNOS in the caecal tonsils at different days post-infection. This is in agreement with earlier studies which observed up-regulation of IL-1β or iNOS in the caeca of day-old chickens infected with *S*. Enteritidis (Berndt *et al.*, 2007, Matulova *et al.*, 2013). Increased levels of IL-1β mRNA were also found in the ilea and caecal tonsils of day-old chickens infected with *S*. Typhimurium (Withanage *et al.*, 2004). IL-6 production is indicative of eliciting an acute-phase responses, activation of T and B cells and development of macrophages (Kaiser and Staheli, 2013). However, IL-6 expression was found at low levels at the first two days following infection of day-old chickens with *S*. Typhimurium or *S*. Enteritidis (Withanage *et al.*, 2004, Cheeseman *et al.*, 2008). This is rather unexpected but these authors suggested that low levels might be possible in very young chickens in the early time points and that higher levels might be expected in older birds. In the current study, increased expression of IL-6 in the caecal tonsils in response to *S*. Enteritidis infection at 2 d pi may be a consequence of infection at the age of 2-days old bearing more mature immune system.

There was only a small number of *S*. Enteritidis found in the liver of individual birds, indicating a limited systemic invasion. However, significantly up-regulated gene expression of IL-1β and iNOS was detected in the spleen of *S*. Enteritidis-infected chickens at 4 d pi when compared to that of *S*. Pullorum-infected chickens or uninfected controls. In contrast to *S*. Enteritidis, the greater numbers of *S*. Pullorum and *S*. Gallinarum found in the liver at 5 d pi, indicating that systemic invasion into deeper tissue, did not elicit increased expression of pro-inflammatory mediators in the spleen, suggesting an inhibition of induction of pro-inflammatory cytokines by these serovars.

In line with the *in vitro* data the *in vivo* analysis has revealed that *S*. Enteritidis induces both Th1 and Th17-associated cytokines in the spleens and/or caecal tonsils. The increased mRNA expression of IL-12α and IL-18 at 2 d pi followed by IFN-γ mRNA detected in the caecal tonsils at 4 and 5 d pi coincided with a slight decrease of *S*. Enteritidis in the caecal contents at 5 d pi. Clearance of *S*. Enteritidis infection in the chicken is usually expected in 3-6 weeks following infection and appears to involve increased expression of IFN-γ mRNA in the gut and deeper tissues i.e. spleen and liver (Beal *et al.*, 2004b, Beal *et al.*, 2004a, Wigley *et al.*, 2005a, Withanage *et al.*, 2005b, Berndt *et al.*, 2007). IFN-γ is involved in the activation of macrophages priming cells for induction of NO and promoting intracellular killing of *Salmonella* (Mastroeni and Menager, 2003, Okamura *et al.*, 2005, Babu *et al.*, 2006).

In contrast to *S*. Enteritidis, *S*. Pullorum infection in day-old chickens did not stimulate detectable expression of IFN-γ mRNA either in the caecal tonsils or

spleens. In contrast, up-regulation of type-2 cytokines (IL-4 and IL-13) were found in both organs at different time points examined in the present study. These results are in accordance with a previous study by Chappell *et al.* (2009), which proposed that, unlike *S.* Enteritidis, *S.* Pullorum can modulate host immune response away from Th1 response towards Th2-like immunity. Quantitative analysis of cytokine mRNA expression in this study did not show evidence of induction of Th1 or Th2-like response in chickens infected with *S.* Gallinarum. This may reflect that the very young bird is not an appropriate infection model for a pathogen which normally affects adult birds. Due to its higher virulence resulting in birds being humanely killed, there were insufficient *in vivo* data to characterize the early immune response to *S.* Gallinarum infection. However, infection with the live attenuated *S.* Gallinarum 9R vaccine strain resulted in mild systemic salmonellosis in three-week-old chickens, in which proliferation of splenic T cells along with an increased IFN-γ expression correlated to bacterial clearance from spleen and liver (Wigley *et al.*, 2005a). Thus, *S.* Gallinarum results in systemic infection, which is similar to its closely related avian-specific serovar Pullorum, but does not produce persistent carriage in the same way or to the same extent as *S.* Pullorum does, which may be result of the increased IFN-γ that controls and eliminates the infection, as occurs with *S.* Enteritidis.

The low levels of IFN-γ mRNA detected in the caecal tonsils, and especially the spleens, of *S.* Pullorum and *S.* Gallinarum-infected chicken may be related to an IL-10-associated anti-inflammatory response. Infection with *S.* Pullorum

and *S.* Gallinarum resulted in a higher level of IL-10 mRNA in these tissues when compared with the levels detected in *S.* Enteritidis-infected or uninfected chickens. IL-10 is an anti-inflammatory cytokine which suppresses IFN-γ production and decreases host damage (Kaiser and Staheli, 2013). It is possible that the effects are different in the different infections; with *S.* Pullorum it may conceivably contribute to establishment of the carrier state whereas in *S.* Gallinarum infection it may result in a relatively severe uncontrolled inflammatory response. Expression of IL-10 were described in infection with *S.* Enteritidis (Setta *et al.*, 2012b) and *S.* Typhimurium (Uchiya *et al.*, 2004) and was shown to inhibit hyper-production of inflammatory mediators such as IFN-γ (Kaiser *et al.*, 2006). Infection of 1 week old chickens with *S.* Typhimurium infection has been shown to increase expression of TGF-β4 in the intestine early post infection. This may have inhibited upregulation of inflammatory responses and high levels of pathology observed in very young birds (Withanage *et al.*, 2004, Withanage *et al.*, 2005b). In contrast, reduced expression of IL-10 was found in the gut of newly-hatched chickens infected with *S.* Typhimurium (Fasina *et al.*, 2008). In a recent study, infection of day-old broilers with *S.* Enteritidis induced significantly increased expression of TGF-β4 in the caeca from 4 d pi, which was suggested to contribute to the persistent colonization of the gut by *S.* Enteritidis (Kogut and Arsenault, 2015). In this study, infection of 2-day-old layer chickens with *S.* Enteritidis did not change IL-10 or TGF-β4 mRNA expression and the absence of anti-inflammatory response probably contributed to the early acute

inflammation. Possible reasons for these contradictory observations could be due to differences in genetic background and/or the age of birds.

In this study, along with the increased expression of IFN-γ mRNA in the spleen, there was a greater increased percentage of splenic CD4$^+$ T cells in response to infection with *S.* Enteritidis when compared to that of *S.* Pullorum-infected chicken at 5 d pi. This result is in accordance with previous studies that have shown that infection with *S.* Enteritidis or *S.* Typhimurium resulted in increased lymphocytes found in the liver or spleen while macrophage inflammatory proteins (MIP) family chemokines and IL-6 were suggested to play a role in recruiting these cells to the gut (Babu *et al.*, 2004, Beal *et al.*, 2004a, Withanage *et al.*, 2005b). *Salmonella* flagella have been shown to stimulate proliferation of splenocytes in young chickens (Okamura *et al.*, 2004). Thus the absence of flagella may be the reason for the smaller number of CD4$^+$ T cells detected *in vitro* (Figure 4-4 and Figure 4-5) and less percentage of splenic CD4$^+$ T cells *in vivo* (Figure 5-11) in response to the avian-specific serovar Pullorum. The observed increases of CD4$^+$ T cells could be an indication of a helper function for these cells immune defence against *Salmonella* infection. It has been found that the peak of CD4$^+$CD8$^-$ cell count in blood was followed by a remarkably elevated population of CD8$^+$ cells of cytotoxic defence mechanisms (Berndt and Methner, 2001). Therefore, proliferation of IFN-γ-producing CD4$^+$ T cells indicated an important role of Th1 adaptive immunity on clearing gut infection and limiting systemic

distribution of *Salmonella*, as shown during *S.* Typhimurium infection in mice (Mastroeni and Menager, 2003).

Time-dependent gene expression in the caeca of *S.* Enteritidis-infected chickens has also identified early expression of IL-17 as well as prolonged high level expression of IFN-γ (Crhanova *et al.*, 2011, Matulova *et al.*, 2013), suggesting that IL-17 and IFN-γ may function at different stage of inflammatory response. Although inhibited expression of IL-17 or IFN-γ by *S.* Pullorum did not show this clearly in the current study, it would be interesting to investigate the expression of IL-17 or IFN-γ during a long period of infection. The functional role of IL-17 in avian salmonellosis is undefined. In 17A$^{-/-}$ mice infected with *S.* Enteritidis, recruitment of neutrophils was significantly compromised which correlated with a reduced clearance of *S.* Enteritidis (Schulz *et al.*, 2008). Although the CXCLi1/CXCLi2 data discussed above suggested a difference between *S.* Pullorum and *S.* Enteritidis in heterophil recruitment, avian IL-17 may also function in a similar manner to promote inflammatory responses.

Chapter 6   Immunological function of the virulence plasmids of *S. enterica*

6. 1   Introduction

6. 1. 1   General introduction

Among the more than 2500 serovars of *S. enterica*, only a few, including *S. Enteritidis*, *S.* Typhimurium and *S.* Gallinarum/*S.* Pullorum, are known to harbour large plasmids involved in the virulence of the host serovars. An 8kb region designated the *spv* (*Salmonella* plasmid virulence, encoding the *spvRABCD* genes) locus, common to the virulence plasmids of different serovars, is essential in enabling systemic infection in animal models (Gulig and Doyle, 1993). The systemic phase of infection with these *Salmonella* serovars is characterized by survival and proliferation of the bacteria inside macrophages. Induction of cytopathology and actin depolymerisation during *Salmonella* infection in human monocyte-derived macrophages were found to be *spvB*-dependent (Libby *et al.*, 2000, Browne *et al.*, 2002). NADPH oxidase interacts with actin filaments and *Salmonella* appear to inhibit NADPH oxidase recruitment to the phagosome involving SPI-2 function (Vazquez-Torres *et al.*, 2000b). Thus, SpvB translocated by TTSS-2 (Browne *et al.*, 2002, Browne *et al.*, 2008, Mazurkiewicz *et al.*, 2008) may interfere with the phagolysosome killing of *Salmonella* by mediating actin depolymerisation, which is probably the reason that the virulence plasmids were suggested to increase the intra-macrophage growth rate of *Salmonella* during systemic infection of mice (Gulig and Doyle, 1993).

The virulence plasmids may also affect the interaction of *Salmonella* with the host immune system. The *spvC* gene reduced the production of pro-inflammatory cytokines TNF-α and IL-8 from *S.* Typhimurium-infected J774 macrophages and Hela cells, respectively (Mazurkiewicz *et al.*, 2008). A *S.* Typhi plasmid, designated as pR$_{ST98}$, present in over 80% of Typhi isolates and is involved in multi-drug resistance. *SpvR* and *spvB* on pR$_{ST98}$ was shown to carry genes which are 99.8% homologous to *spvR* and *spvB* on the virulence plasmid in *S.* Typhimurium (Huang *et al.*, 2005). Compared to a pR$_{ST98}$ mutant, the parent *S.* Typhi, containing pR$_{ST98}$, was later demonstrated to suppress IL-12 and IFN-γ production while up-regulating the secretion of IL-10 in infected DCs (Wei *et al.*, 2012).

There is *in vivo* and *in vitro* evidence from chapters 3, 4 and 5, which supports our hypothesis that the host immune response to *S.* Pullorum infection was skewed away from a protective Th1 immunity. *S.* Typhi and *S.* Pullorum are both host-adapted serovars inducing persistent infection in humans and chickens, respectively. We therefore considered that the *S.* Pullorum virulence plasmid might contribute to the manipulation of host immune response in a similar manner.

6. 1. 2   Chapter aims and objectives

In order to characterize the function of the *S.* Pullorum virulence plasmid in modulating the immune response compared with that in non-persistent serovars Enteritidis and Gallinarum, parent strains, plasmid-cured strains and

plasmid-restored strains of these three serovars listed in Table 2-3. Were used in infection studies using the avian macrophage-like cell line (HD11) and avian peripheral blood monocyte-derived macrophages (chMDM). This was followed by determination of intracellular survival, NO production and gene expression of cytokines and chemokines in infected macrophages.

6. 2   Results

6. 2. 1   Invasion of HD11 by *Salmonella* serovars in response to the presence of the virulence plasmid

The parent, plasmid cured and plasmid-restored strains of each *Salmonella* serotype showed similar abilities in terms of invasion and subsequent intracellular survival (Figure 6-1). For all the strains, the intracellular bacterial counts were stable and then fell after 6 h pi. Plasmid curing in *S.* Pullorum did not change its intracellular survival within HD11 cells at any time points pi ($P>0.05$). Intracellular bacterial counts of plasmid-cured strains of *S.* Enteritidis were found to be lower than those of the parent strain at 48 h pi ($P<0.05$). Reduced intracellular counts of *S.* Gallinarum plasmid-cured strains in infected HD11 cells was only observed at 2 h pi when compared to its parent and plasmid-restored strains ($P<0.05$).
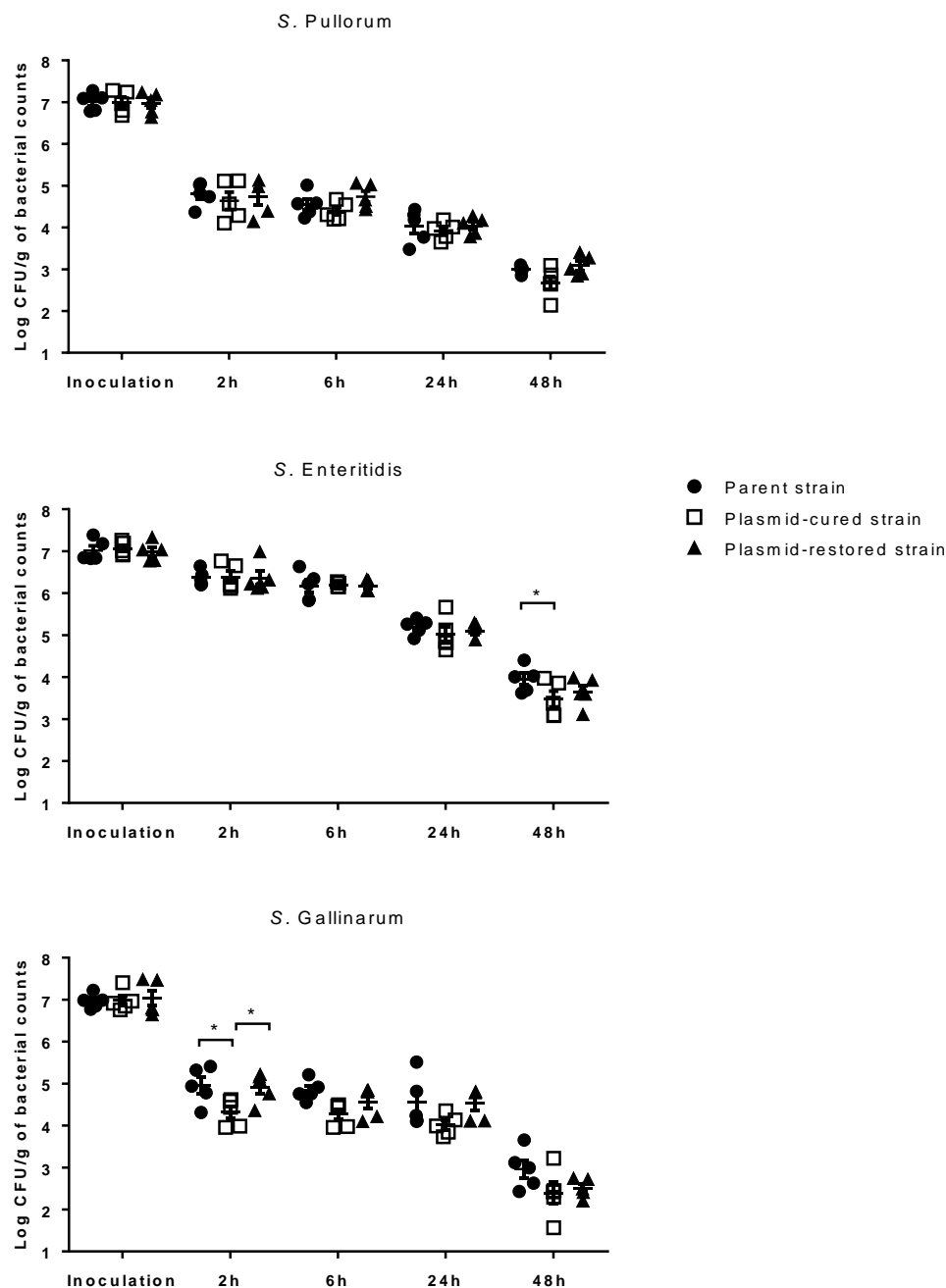
Figure 6-1. Effect of large virulence plasmids of different *Salmonella* serovars on intracellular survival within HD11 cells at different time points post infection. At 2, 6, 24 and 48 h pi, HD11 cells infected with different strains of *Salmonella* were lysed to quantify the intracellular bacterial counts (n=5). Viable colony counts were shown as Log_{10} CFU/ml. Data were analysed by two-way ANOVA followed by Tukey's multiple comparisons test to compare the bacterial counts between different strains at each time points. (*) indicates statistical differences between different serovars (*$P<0.05$, **$P<0.01$).

6. 2. 2 NO production by *Salmonella* serovars in response to the presence of the virulence plasmid

NO production from HD11 cell in response to infection with all the *Salmonella* strains used was significantly greater than that in the uninfected control groups at 24 and 48 h pi (*P*<0.001), with maximum production observed at 48 h pi (Figure 6-2). Infection with the plasmid-cured strain of *S.* Enteritidis did not elicit higher levels of NO production compared with its parent and the plasmid-restored strain at any of the time points examined (Figure 6-2, b). However, a significantly higher level of NO production was detected in HD11 cells infected with the plasmid-cured strains of *S.* Gallinarum at 24 h pi when compared to its parent (P<0.01) and plasmid-restored (P<0.05) strain. *S.* Pullorum infection induced a similar pattern of NO production as *S.* Gallinarum did, but there was no significant difference observed between the *S*. Pullorum strains (Figure 6-2, a and c).
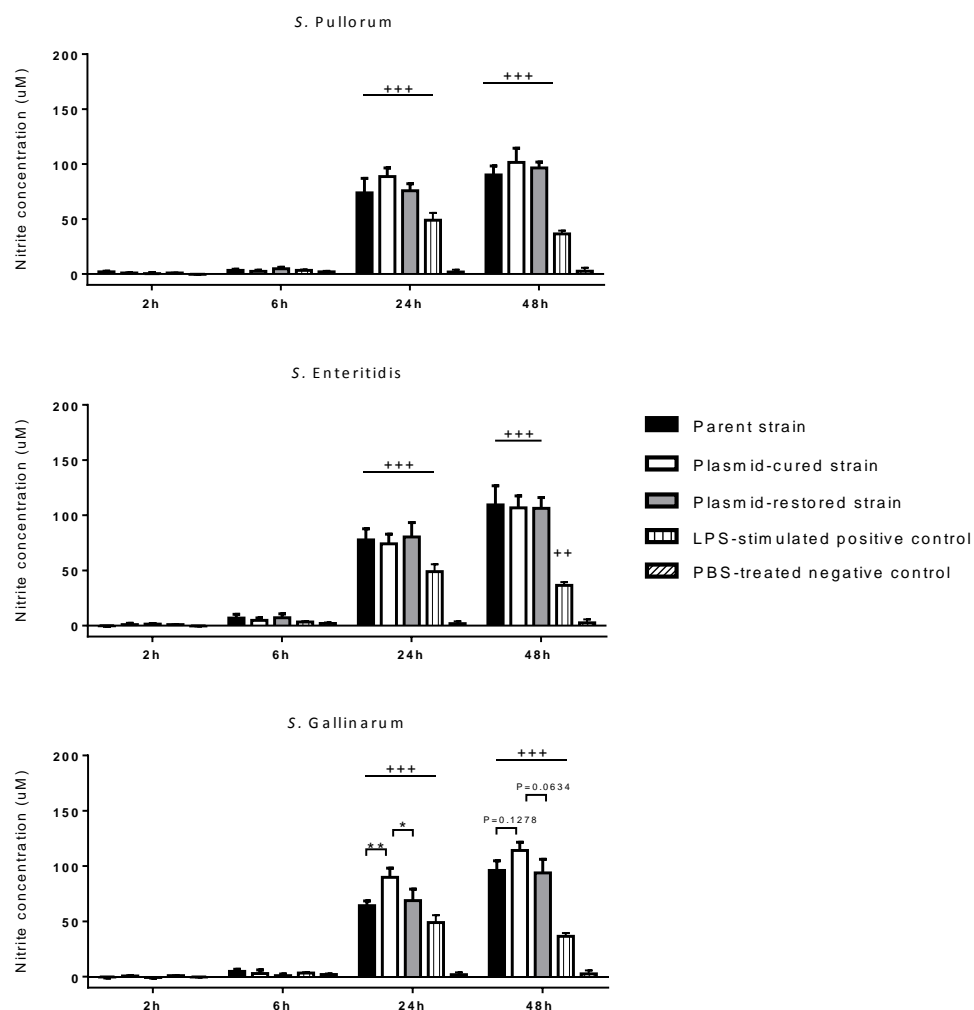
Figure 6-2. Effect of large virulence plasmids of different *Salmonella* serovars on NO production by HD11 cells. At 2, 6, 24 and 48 h pi, supernatant was collected from HD11 cells in different infection or treatment groups to determine the nitrite concentration using Griess assay. The results shown are expressed as means (nitrite concentration, μM) ± SEM of independent experiments (n=5). Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between the treated groups for each serovar. (+) shows significance from the negative controls (+++, *P*<0.001), (*) indicates significant difference of plasmid-cured strains compared with either parent or plasmid-restored strain of each serovar (*, *P*<0.05; **; *P*<0.01).

6. 2. 3   Effect of *Salmonella* virulence plasmids on HD11 macrophage viability

The percentage of viable HD11 cells was measured at 2-48 h after infection with different *Salmonella* serovars used in this study (Figure 6-3). Following infection, approximately 90% of HD11 cells remained to be alive until 6 h pi but there was a significant reduction in the percentage of viable cells when compared with that of uninfected HD11 cells at 24 h (about 75%) and 48 h (about 50%) pi ($P<0.01$). However, infection with the plasmid-cured strain of each serovar did not significantly affect the percentages of viable cells ($P>0.05$).
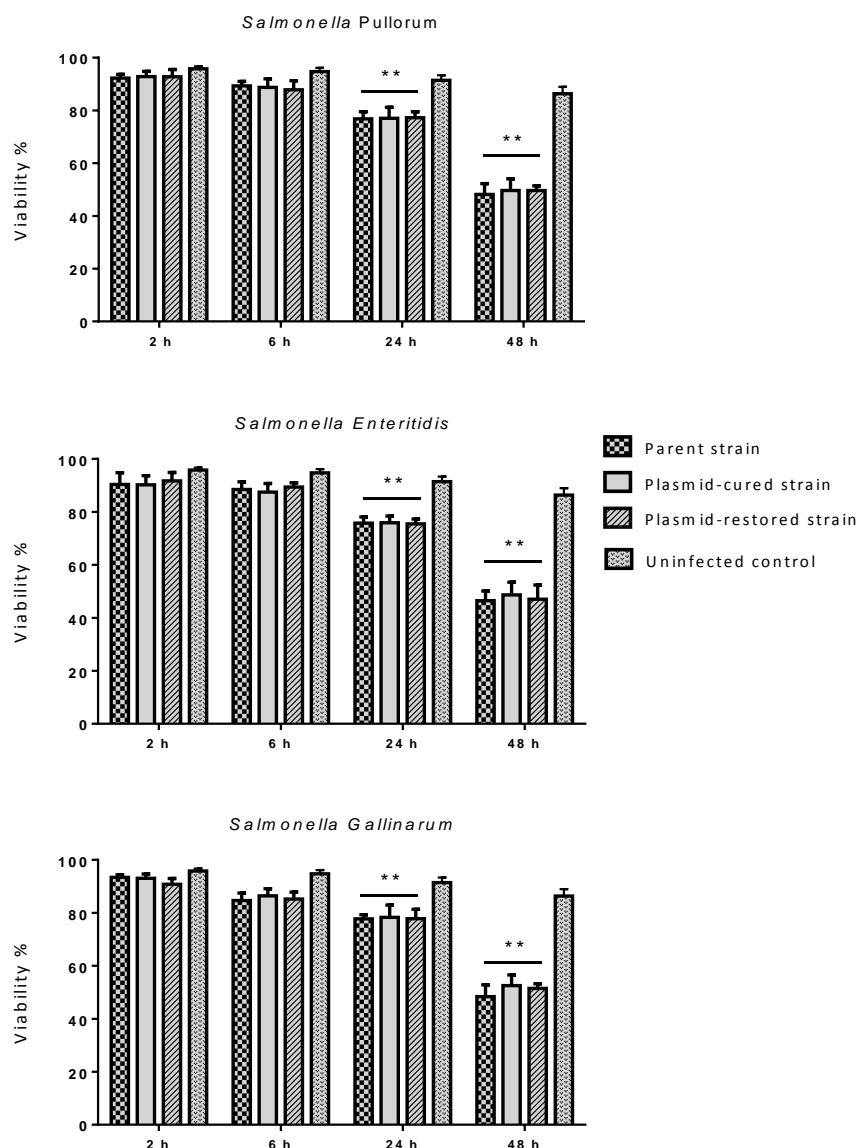
Figure 6-3. Viability of HD11 cells in response to the presence of virulence plasmids in different *Salmonella* serovars. At 2, 6, 24 and 48 h pi, the percentage of viable HD11 cells infected with *S*. Pullorum, *S*. Enteritidis and *S*. Gallinarum respectively were determined using PI. Data shown are means (viable HD11 cells %) ± SEM from three independent experiments. Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparisons test to detect difference between the treated groups for each serovar. (*) indicates significant difference from the uninfected negative controls.(*, *P*<0.05; **; *P*<0.01).

6. 2. 4 Quantification of gene expression in HD11 cells by *Salmonella* serovars in response to the presence of the virulence plasmid

The *in vitro* immune response to the presence and absence of *Salmonella* virulence plasmids was studied by quantifying the gene expression of pro-inflammatory chemokines CXCLi1 and CXCLi2 and cytokines IL-1β, IL6 and iNOS from *Salmonella*-infected HD11 cells at 6 h pi. Compared with the uninfected control, all the *Salmonella* strains induced higher mRNA expression of these pro-inflammatory mediators in HD11 cells at 6 h pi ($P<0.05$ or $P<0.01$). Moreover, in comparison with the plasmid-positive *S.* Gallinarum strain infection with the plasmid-cured mutant resulted in higher levels of IL-1β ($P<0.05$) and iNOS ($P<0.01$) mRNA (Figure 6-4).

Removing the virulence plasmids from the host of any of the serovars did not affect the gene expression of IFN-γ, IL-18, IL-12α, IL-4, IL-10 and TGF-β4 in infected HD11 cells (Figure 6-5). Increased gene expression of IFN-γ, IL-12α and IL-18 were only observed in HD11 cells stimulated with LPS at 6 h pi ($P<0.01$). The expression of IL-4 mRNA was very low while no expression of IL-13 mRNA was detected in HD11 cells infected with any *Salmonella* strain tested in this study. In terms of IL-10 and TGF-β4, *Salmonella* infection was unable to induce gene expression of TGF-β4 in HD11 cells at 6 h pi ($P>0.05$) whereas upregulation of IL-10 mRNA was found in HD11 cells infected with different serovars with or without the plasmid ($P<0.01$).
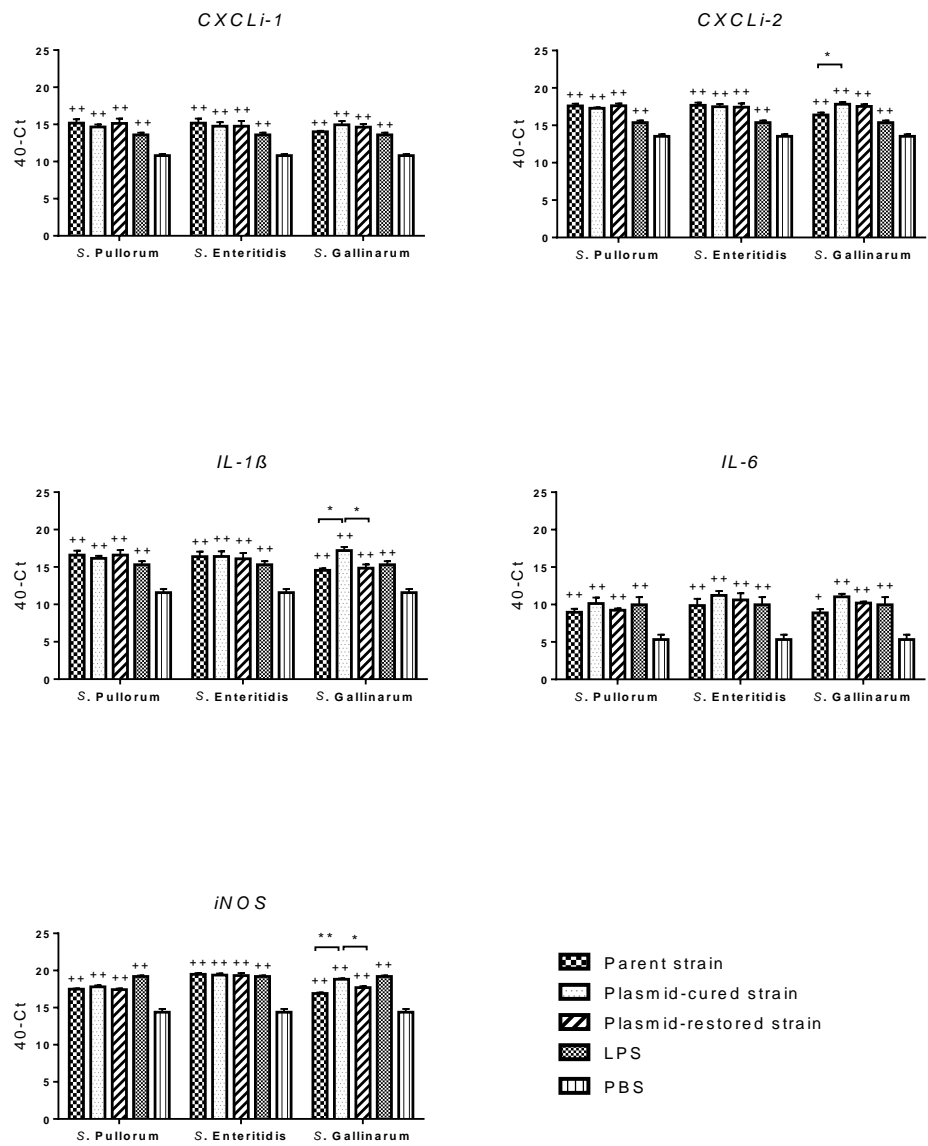
(A)
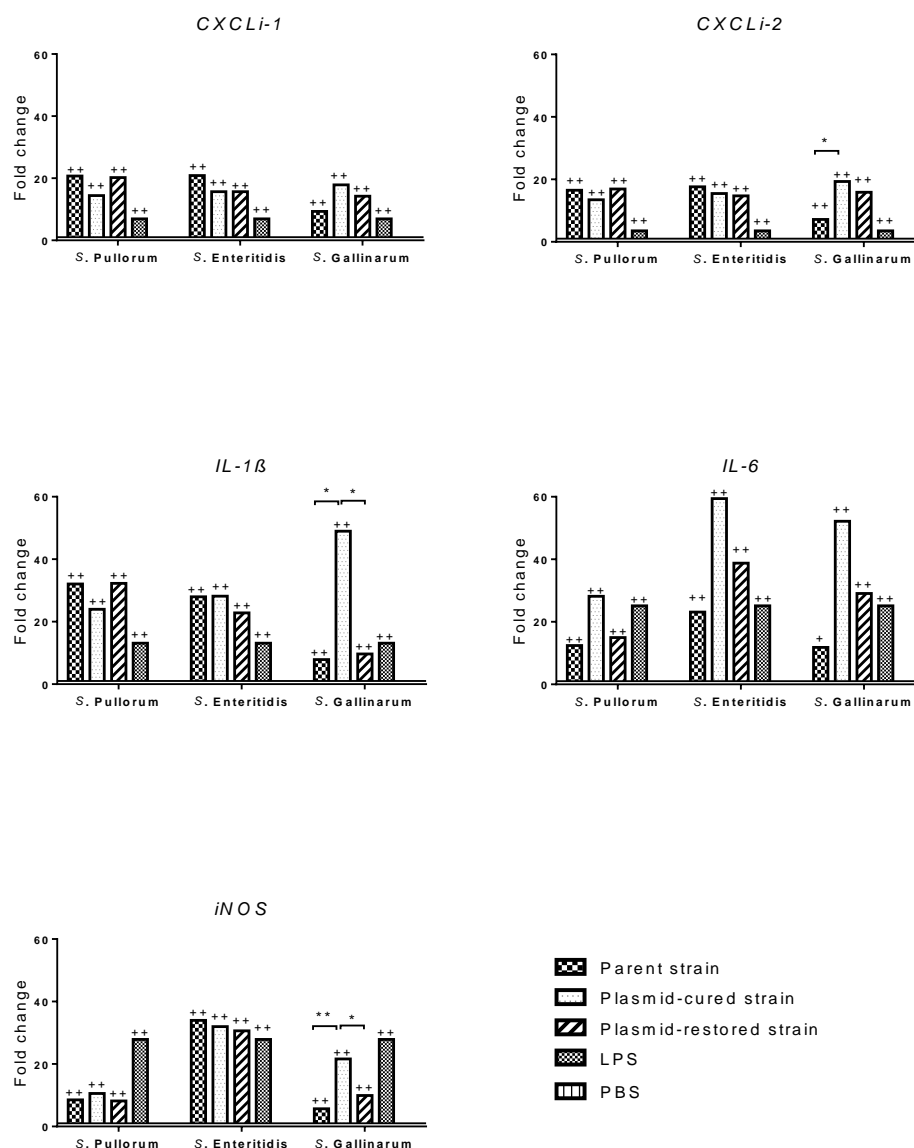
(B)

Figure 6-4. Effect of *Salmonella* infection on gene expression of pro-inflammatory mediators in HD11 cells at 6 h post-infection. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from PBS-treated uninfected controls (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 strains within each serovar group, *: P<0.05, **: P<0.01.

(A)

(B)

Figure 6-5. Effect of *Salmonella* infection on gene expression of IFN-γ, IL-18, IL-12α, IL-4, IL-10 and TGF-β4 in HD11 cells at 6 h post-infection. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3). B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from PBS-treated uninfected controls (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 strains within each serovar group, *: $P<0.05$, **: $P<0.01$.

6. 2. 5 Quantification of gene expression in chMDM by *Salmonella* serovars in response to the presence of the virulence plasmid

To confirm that the data we observed was not affected by the use of the HD11 cell line we used primary chMDM to reassess repossess for *S.* Pullorum and *S.* Enteritidis with and without plasmids. We generally observed similar profiles of pro-inflammatory mediators as those of HD11 cells in response to the presence of virulence plasmids of *S.* Pullorum and *S.* Enteritidis. Increased gene expression of the pro-inflammatory mediators, CXCLi1, CXCLi2, IL-1β, IL-6 and iNOS, were observed in chMDM infected with each strain of *Salmonella* at 6 h pi when compared to uninfected controls ($P<0.01$). A higher level of IL-6 mRNA was detected in chMDM infected with the plasmid-cured mutant of *S.* Pullorum than that in response to its parent strain ($P<0.05$) (Figure 6-6).

The presence of virulence plasmids in *S.* Pullorum and *S.* Enteritidis did not change the gene expression of IFN-γ, IL-18, IL-12α, IL-4, IL-10 and TGF-β4 in chMDM at 6 h pi (Figure 6-7) when compared with those observed in *Salmonella*-infected HD11 cells. In comparison with *S.* Enteritidis, which increased expression of IL-12α, IL-18 and IFN-γ, *S.* Pullorum infection increased the expression only of IL-18, but not IL-12α or IFN-γ mRNA in infected chMDM. Gene expression of IL-4 in all groups was very low. Moreover, compared with uninfected controls, all the strains were found to increase mRNA expression of IL-10 whereas gene expression of TGF-β4 was not up-regulated in any of the infected chMDM (Figure 6-7).

(A)

(B)
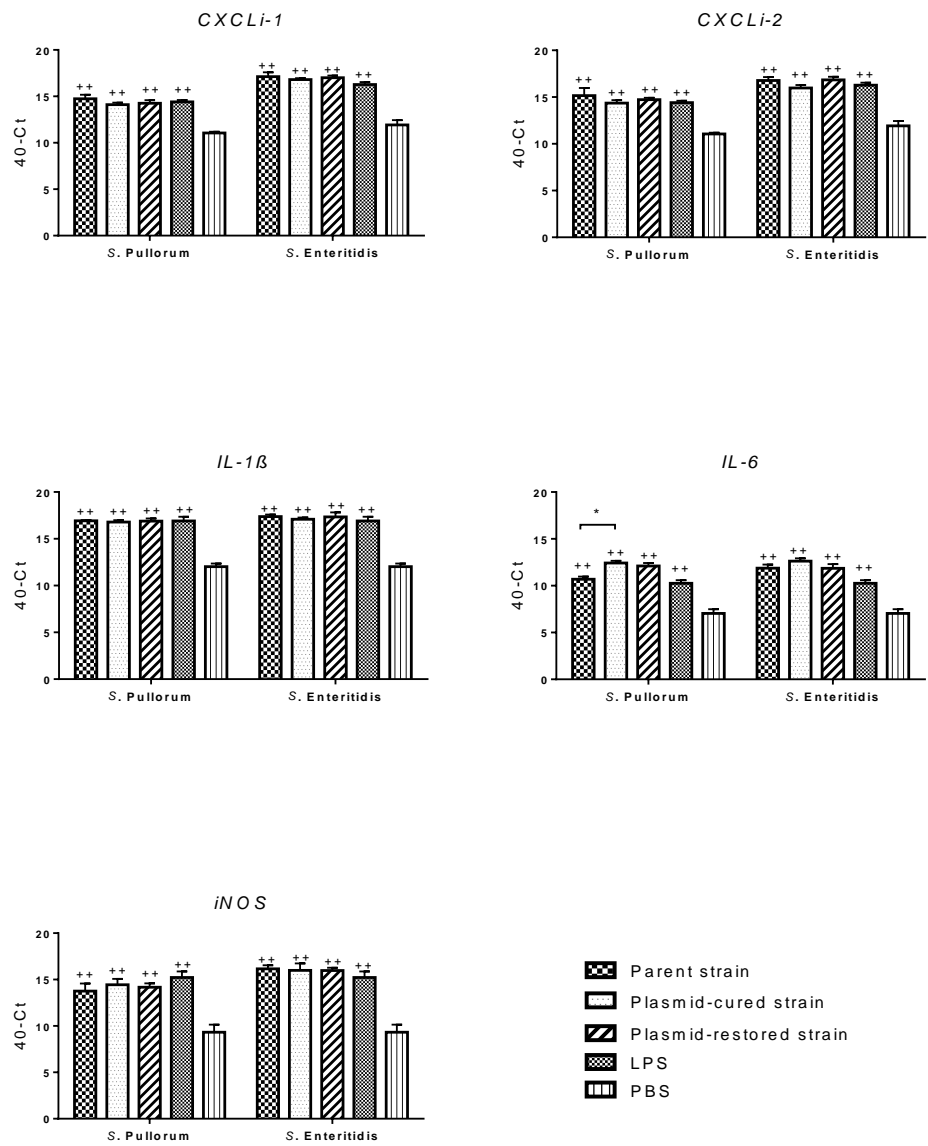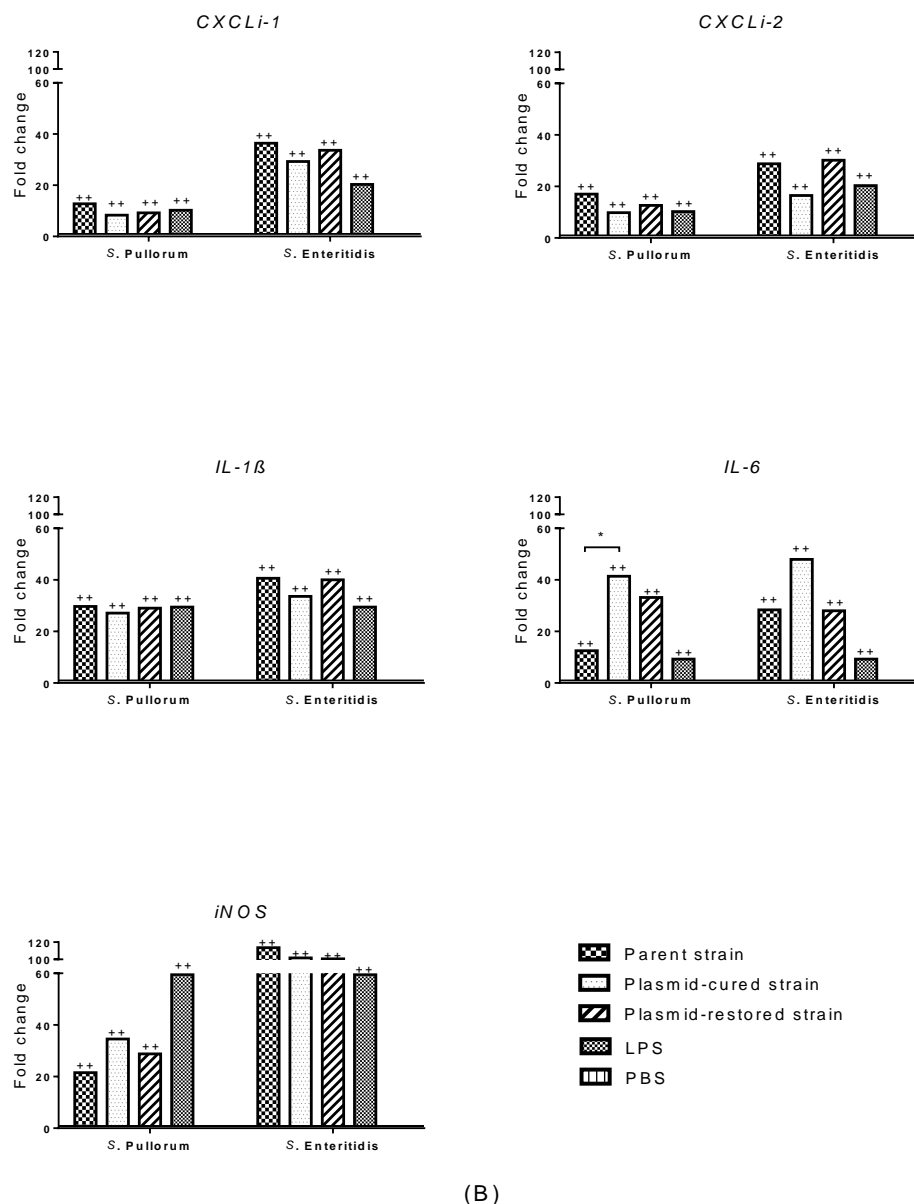
Figure 6-6. Effect of *Salmonella* infection on gene expression of pro-inflammatory mediators in chMDM at 6 h post-infection. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from PBS-treated uninfeen controls (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 strains within each serovar group, *: $P<0.05$, **: $P<0.01$.
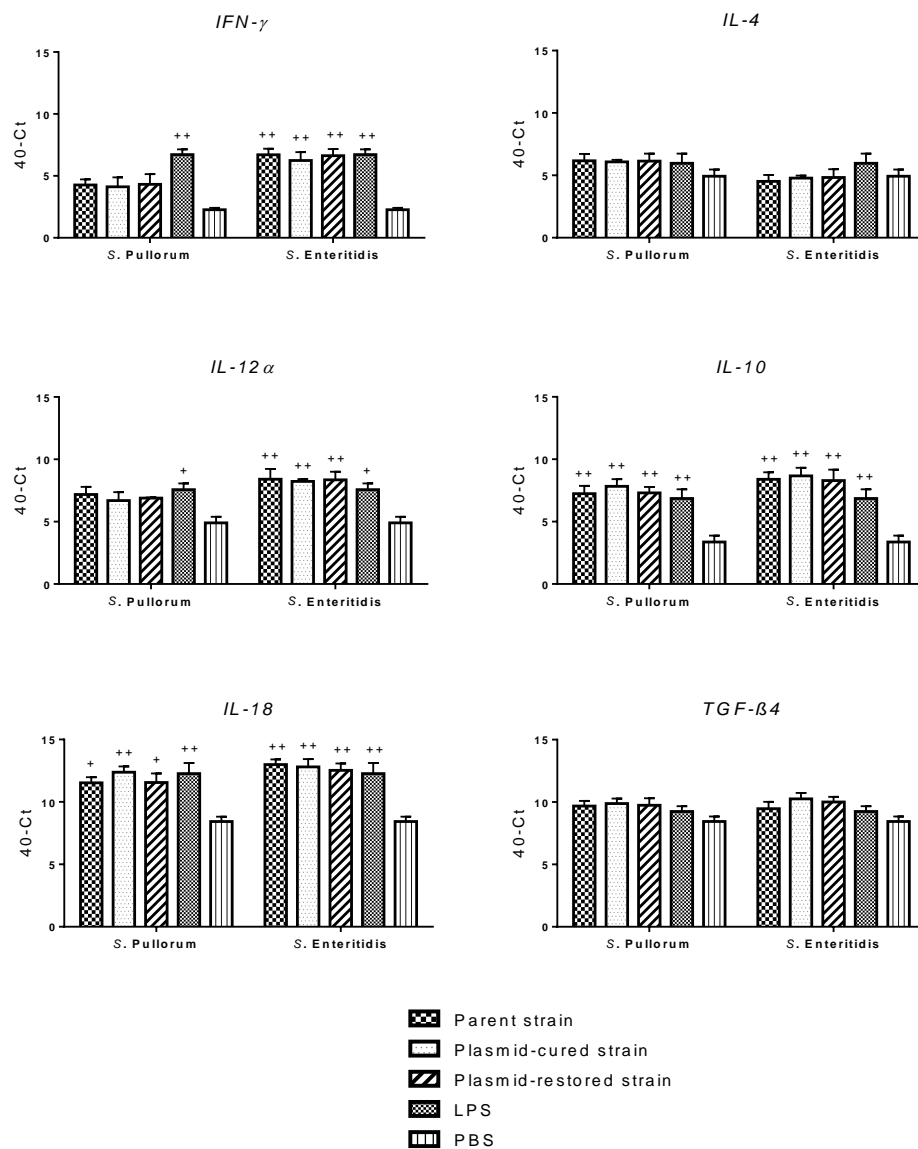
(A)

(B)

Figure 6-7. Effect of *Salmonella* infection on gene expression of IFN-γ, IL-18, IL-12α, IL-4, IL-10 and TGF-β4 in chMDM at 6 h post-infection. (A) The corrected crossing threshold (Ct) values deducted from 40 (the negative end point) after normalization with reference gene (28S) for input RNA. Data shown as averages (40-Ct) ± SEM (n=3). (B) The corrected Ct values shown as fold change in the mRNA level of cytokines in comparison to those from PBS-treated uninfected controls (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 strains within each serovar group, *: *P*<0.05, **: *P*<0.01.

6. 3 Chapter discussion

Salmonella requires the action of several virulence factors during infection. Large plasmids have been found to be essential for several serovars of *Salmonella* that routinely produce systemic diseases (Jones *et al.*, 1982, Terakado *et al.*, 1983, Barrow *et al.*, 1987b, Barrow and Lovell, 1988, Halavatkar and Barrow, 1993).

In the present study, a role for the plasmid-associated virulence in growth in phagocytes was suggested by the significantly reduced survival of the *S.* Gallinarum plasmid (pSG090)-cured mutant in HD11 cells, although small reductions were also seen with *S.* Pullorum and *S.* Enteritidis. The plasmid-encoded *spv* locus has previously been shown to be crucial for intracellular proliferation of *S.* Typhimurium and *S.* Dublin (Gulig and Doyle, 1993, Libby *et al.*, 2000). A corresponding increased NO production at 24 and 48 h pi and iNOS expression at 6 h pi from HD11 cells infected with this plasmid-cured *S.* Gallinarum strain was observed when compared to its parent strain. A smaller effect on NO production from the presence of the plasmid was also seen with *S.* Pullorum, although this was not statistically significant. The NO concentration determined in HD11 cells in response to each strain was very low until 6 h pi which is a typical NO response to *Salmonella* infection (Mastroeni *et al.*, 2000b) and perhaps correlates with the host-specific nature of *S.* Gallinarum and *S.* Pullorum infection. The lower bacterial counts of the *S.* Gallinarum plasmid-cured strain found in HD11 cells at 2 h pi might be related to the reduced efficiency of initial invasion with the increased levels of NO

production in response to this plasmid-minus strain which may contribute to the host clearance of the bacteria from infected HD11 cells. The contribution of *S*. Gallinarum virulence plasmid pSG090 towards virulence of fowl typhoid in chickens has been demonstrated previously (Barrow *et al.*, 1987b). In that study, the plasmid-cured strain of *S*. Gallinarum was eliminated from the alimentary tract of 3-week-old chickens 24 h after oral inoculation and failed to penetrate to the liver and spleen. In the current study, infection with parent, plasmid-cured and plasmid-restored strains of *S*. Pullorum produced a similar pattern of intracellular survival and NO production to those detected with *S*. Gallinarum strains, but it is not statistically confirmed to be related to its large plasmid pBL001. However, the plasmid pHH001 in *S*. Enteritidis was not shown to be involved in manipulating the NO production *in vitro*, again perhaps reflecting that it is not a pathogen which typically produces typhoid in birds but does so in mice.

The differences between these virulence plasmids in contributing towards the virulence of their respective host strains has also been demonstrated before *in vivo*. The association between the large virulence plasmid and the virulence of the host strain is more obvious in chickens infected with *S*. Gallinarum than the other serovars. This may be because *S*. Gallinarum produces typhoid in adult chickens and is more virulent than the other two serovars which only produce systemic disease in very young chickens. For the relevant typhoid serovars in the host to which they are adapted (Typhimurium in mice and Gallinarum in chickens) the virulence plasmid makes a major contribution to

the overall virulence of the strains. In contrast, where the bacterial pathogen is less virulent (Pullorum, Typhimurium or Enteritidis in chickens) the plasmid plays a less significant role in virulence (Barrow and Lovell, 1989, Barrow *et al.*, 1994). However, it seems clear that the main contribution to virulence from the virulence plasmid is in terms of long term survival in the macrophages. TTSS-2 is essential for plasmid-mediated virulence of *Salmonella* while SpvB, encoded by the virulence plasmid, is crucial for intracellular proliferation of *S.* Typhimurium and *S.* Dublin in human monocyte-derived macrophages (Libby *et al.*, 2000). During infection, SpvB-induced actin depolymerisation in host cells requires a functional TTSS-2. SpvB and SpvC are translocated from *Salmonella* in the SCV into the host cell by TTSS-2 (Browne *et al.*, 2002, Browne *et al.*, 2008, Mazurkiewicz *et al.*, 2008). TTSS-2 and the Spv proteins are both essential for caspase-dependent apoptosis of *Salmonella*-infected human macrophages (Browne *et al.*, 2002). TTSS-2 (SPI-2) also contribute to the virulence of S. Gallinarum (Jones *et al.*, 2001) and to more persistent infection in *S.* Pullorum (Wigley *et al.*, 2002b) but how these interact with the *spv* genes on the virulence plasmid in each serovar remains to be discovered.

There was no evidence derived from this study to demonstrate the association between the possession of large virulence plasmids in *S.* Gallinarum, *S.* Enteritidis or particularly, *S.* Pullorum, with the potential to manipulate host immunity away from Th1 immunity, as occurred with related genes in *S.* Typhi (Wei *et al.*, 2012). The evidence was that the gene expression of cytokines IL-12α, IL-18, IFN-γ (Th1-related), IL-4 (Th2-related) or

IL-10 in HD11 cells and chMDM were not affected by the elimination of the plasmid in these serovars. However, the induction of mRNA expression of IL-1β and iNOS in HD11 cells was different between the parent and plasmid-cured strains of *S*. Gallinarum. The non-flagellate serovars Pullorum and Gallinarum are not recognized by TLR5, which is believed to play a key role in the avoidance of stimulating an inflammatory response during their initial invasion. *S*. Gallinarum did not induce the gene expression of CXCLi1, CXCLi2 or IL-6 in the caecal tonsils of infected chickens (Setta *et al.*, 2012b). *S*. Pullorum infection led to down-regulation of CXCLi1 and CXCLi2 in the ileum (Chappell *et al.*, 2009). These suggested a reduction in heterophil infiltration in response to initial infection with *S*. Pullorum and *S*. Gallinarum. *S*. Gallinarum usually affects adult birds and produce fowl typhoid. In murine J774 macrophages, typhoid serovars including Typhimurium and Enteritidis were also found to inhibit iNOS production (Hulme *et al.*, 2012). Thus, the suppression of pro-inflammatory cytokine production associated with the possession of the virulence plasmid might be an important virulence mechanism of *S*. Gallinarum to produce systemic infection in adult chickens without initiating strong inflammatory response. It is probably relevant that Barrow *et al.* (1987b) and Rychlik *et al.* (1998) showed that the *S*. Gallinarum virulence plasmid did contribute to the brief early intestinal phase during infection.

In contrast, this strategy does not seem to be essential for *S*. Pullorum because it interacts with the immature immune system in young chickens

although infection with the *S*. Pullorum plasmid-cured strain increased the expression of IL-6 in avian macrophages highlighting the difference between very young and mature chickens and the epithelial versus macrophage phase during infection. Compared to *S*. Enteritidis and *S*. Typhimurium, reduced expression of pro-inflammatory cytokines by *S*. Pullorum or *S*. Gallinarum was observed in both epithelial cells and HD11 macrophages (Kaiser *et al.*, 2000, Setta *et al.*, 2012a, Freitas Neto *et al.*, 2013). However, it seemed that the absence of flagella from these avian-adaptive serovars was only related to the differences observed in epithelial cells (CKC) (Freitas Neto *et al.*, 2013), which illustrated the evasion of flagella-induced inflammatory responses in assisting initial systemic invasion of *S*. Pullorum and *S*. Gallinarum from the gut epithelium. Reduced expression of pro-inflammatory cytokines in macrophages may involve the virulence plasmids which probably carry anti-inflammatory properties.

Chapter 7 General discussion and future work

7. 1 Introduction

Salmonellosis remains a major disease in animal and humans worldwide. The majority of *Salmonella* serovars generally cause gastrointestinal disease of varying severity in a range of hosts. In contrast, a small number of host-adapted typhoid serovars, including *S.* Typhi, *S.* Dublin, *S.* Pullorum, *S.* Gallinarum and *S.* Abortusovis, cause systemic typhoid-like infections in a restricted range of hosts. The disease can produce a high mortality and extensive use of antibiotics with these infections has led to an increasing problem with antibiotic resistance. One of the features of the infection produced by these typhoid serovars is disease-free persistent infections, primarily within macrophages in lymphoid tissues, in a proportion of convalescents. This results in localization in the gall bladder and spleen leading to faecal shedding by carriers for many years (*S.* Typhi in man and *S.* Dublin in cattle) (Sojka *et al.*, 1974, Wray and Sojka, 1977, House *et al.*, 2001) or localization in the reproductive tract leading to abortion (*S.* Dublin) or vertical transmission through hatching eggs to progeny (*S.* Pullorum). These persistently infected individuals serve as a significant reservoir for disease transmission and pose significant economic and public health problems. The immunological basis of the carrier state in *S.* Pullorum as representative of this typhoid group is the subject of this thesis.

In this model, *S*. Pullorum was observed to persist within splenic macrophages *in vivo* for several months in the presence of high titres of circulating anti-*Salmonella* IgG (Wigley *et al.*, 2001, Wigley *et al.*, 2005b) and it has been found to induce much lower levels of splenic IFN-γ than occurs during infection by the related serovar *S*. Enteritidis (Chappell *et al.*, 2009). However, the full nature of the serovar-specific interaction with macrophages, the associated immune modulation and its impact on outcome of infection is unclear.

We evaluated the *in vitro* immune response to *Salmonella* in either macrophages alone and also in T cells cultured with infected macrophages. We also studied the immune response to *Salmonella in vivo* in newly-hatched chickens infected with *S.* Pullorum and related non-persistent serovars (*S. Enteritidis* and *S.* Gallinarum). The comparison between *S*. Pullorum and the related non-persistent serotypes (*S.* Enteritidis and *S.* Gallinarum) in terms of the various biological and immunological parameters investigated in this study are summarized in Appendix. 5.

7. 2   Discussion

7. 2. 1   Immune modulation in the present study

The results derived from *in vitro* experiments, using primary avian macrophages and CD4$^+$ T cells in co-culture, are largely consistent with the observations in infection of 2-day-old chickens *in vivo*. In contrast to the strong Th1/Th17 responses associated with *S*. Enteritidis infection *ex vivo* and

in vivo, *S*. Pullorum infection did not enhance Th1 and/or Th17 related cytokine expression *in vitro* in avian macrophages splenocytes, co-cultured CD4$^+$ T cells. This was also the case *in vivo* in the caeca and spleen of infected chickens. Although modulation of adaptive immunity by *S*. Pullorum towards a non-protective Th2-like response was only evident *in vivo*, these results support our hypothesis that the mechanisms that underline persistent infection with *S*. Pullorum involve a manipulation of adaptive immune responses away from a protective IFN-γ-producing Th17 response. This may enable *S*. Pullorum to evade immune clearance resulting in persistent carriage. In addition, regulatory effector cells and suppressed lymphocyte proliferation, rather than induction of clonal anergy or involvement of the virulence plasmid, may have contributed to its persistence to some extent, indicating that the interaction with the host is complex. The fact that *S*. Enteritidis also caused considerable cellular toxicity *in vitro* is an additional factor in the difference of the effect of the pathogen on the host which may have had a direct effect on the nature of the immune response induced.

The good correlation between the *in vivo* data with the results derived from co-cultured avian macrophages and CD4$^+$ T cells *in vitro* on the gene expression of Th1/Th17 cytokines indicates that this is an approach which will contribute considerably to reduced animal use. The *in vivo* data showing cytokine expression and splenic lymphocyte populations were derived from total mRNA or splenocytes derived from the caecal tonsils and spleens. However, the caecal tonsils and spleens of young chickens of less than one-

week old were too small to isolate sufficient macrophages and CD4^{+} T cells. Therefore, we were unable to work with specific individual cell populations from these samples. Some of the discrepancies between the *in vitro* and in vivo results may thus have resulted from the tissue samples containing cells other than pure macrophage and CD4^{+} T cell populations. In addition, Enzyme-linked immunospot assay (ELISPOT) is a potential method to define cellular source of cytokines and chemokines by measuring secreted cytokine molecules from single cells (Stenken and Poschenrieder, 2015). In studies reported in this thesis we have used avian macrophages as a model because previous studies reported that *S*. Pullorum and *S*. Typhimurium persist within macrophages to produce chronic infection in chickens and mice respectively (Wigley *et al.*, 2001, Monack *et al.*, 2004, Wigley *et al.*, 2005b). However, DCs have long been known as important professional APCs, effectively activating T cells once mature (Liu and MacPherson, 1993, Kaiser, 2010, Kaspers and Kaiser, 2013). In fact, DCs are able to present antigen orders of magnitude higher than macrophages. Therefore, there could be functional differences between DCs and macrophages which determine the nature of the initial response to *S*. Pullorum. A difference has been observed in bovine DCs and macrophages in response to *S*. Typhimurium infection. Bovine peripheral blood-derived DCs infected with *S*. Typhimurium up-regulated expression of MHCI, MHCII and co-stimulatory molecules whereas infected macrophages showed only a slight increase in CD40 (Norimatsu *et al.*, 2003). In this study enhanced TNF-α, IL-1β, IL-6 and iNOS mRNA expression was observed in both

DCs and macrophages following infection with *S*. Typhimurium but DCs also up-regulated expression of GM-CSF and IL-12β mRNA while expression of IL-10 mRNA was only increased in macrophages (Norimatsu *et al.*, 2003). Thus, although we recognize that for very early stage infection DCs might have been useful macrophage are the cells in which *Salmonella* are generally found in systemic disease (Dunlap *et al.*, 1992, Wigley *et al.*, 2001) and are thus a relevant model system.

7. 2. 2 Altered activities of immune cells in persistent infection

Salmonella are able to maintain infection within macrophages, and in the case of serovars such as *S*. Pullorum and *S*. Dublin this gives rise to persistent infection. Macrophages are a sentinel for innate defence against infection and are also mediators that direct the adaptive immune response (Hoebe *et al.*, 2004). Two functionally different types of macrophages, designated M1 and M2, have been isolated in mammals. M1 macrophages are inflammatory and have microbicidal activity while M2 macrophages are immunomodulatory (Mills *et al.*, 2000). In a murine model of persistent infection, *S*. Typhimurium infection preferentially associated with M2 macrophages activated by Th2 cytokines (Eisele *et al.*, 2013). Quantitative analysis of *in vivo* cytokine expression in the present study revealed a type 1 to type 2 cytokine switch by *S*. Pullorum, which was in line with reduced expression of inflammatory cytokines in *S*. Pullorum-infected avian macrophages. Although it is not yet clear whether M1/M2 macrophage polarization promoting Th1/Th2

responses occurs in avian, involvement of macrophages resembling the M2 polarization seems to occur during persistent carriage of *S*. Pullorum infection.

Although we did not observe an induction of the type 2 anti-inflammatory cytokines, IL-4 or IL-13, in *S*. Pullorum-infected macrophages, related observations have been made with the intracellular bacterial pathogens *Yersinia enterocolitica* and *Yersinia pestis*. It has been well established that the two alternative pathways of arginine metabolism involving the enzymes iNOS (M1 macrophages) or arginase (M2 macrophages) utilized by macrophages can alter the outcome of infection in opposite ways (Munder *et al.*, 1999, Mills *et al.*, 2000). Susceptible BALB/c mice infected with *Yersinia enterocolitica* result in arginase activation within macrophages, leading to the modulation of M2 macrophages, and increased production of TGF-β1 and IL-4 whereas lower levels of NO and reduced IFN-γ production in the peritoneal macrophages or splenic lymphocytes occurred in the early phase of infection (Tumitan *et al.*, 2007). This M2 polarization was reversed in murine macrophages infected with bacteria defective for the type III secretion system (Mills *et al.*, 2000, Hoffmann *et al.*, 2004). We showed *S*. Pullorum is a less robust stimulus for iNOS mRNA expression in chMDM in comparison with *S*. Enteritidis, where arginine may be partially metabolised by the arginase pathway. LcrV is a protein found in *Yersinia pestis*, which stimulates M2 polarization probably by up-regulating IL-10 (Brubaker, 2003). The involvement of IL-10-related regulation will be discussed below.

The importance of Th1 responses, particularly the involvement of IFN-γ, in clearance of *Salmonella* infection in chicken has been widely reported (Beal *et al.*, 2004a, Beal *et al.*, 2004b, Wigley *et al.*, 2005a, Withanage *et al.*, 2005b, Berndt *et al.*, 2007). *S.* Pullorum may inhibit directly the protective role of macrophages by interfering with the IFN-γ/IL-12 pathway to produce persistent infection. IL-12 that stimulates Th1 differentiation has been found to be critical in controlling the early exponential growth of *S.* Typhimurium in the spleen and liver of the mice (Mastroeni *et al.*, 1998) while the later control of persistent infection required IFN-γ (Monack *et al.*, 2004). Similarly in this study, in comparison with *S.* Enteritidis, failure of *S.* Pullorum to increase IL-12α expression in the spleen at 1d 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. *Salmonella*-induced reduction of IL-12 secretion was also observed in murine macrophages infected with *S.* Dublin. Despite being a potent stimulus for secretion of the IL-12 subunit p40 from infected murine macrophages, *S.* Dublin showed a limited ability to induce secretion of the bioactive p70 heterodimer (Bost and Clements, 1997). The outer membrane protein Omp25 of another intracellular bacterial pathogen, *Brucella suis*, was shown to inhibit the production of TNF-α in human macrophages, leading to reduced production of IL-12 (Dornand *et al.*, 2002). Avian TNF-α has not yet been identified but LITAF (LPS-induced TNF-α factor) was not significantly changed in HD11 cells infected with *Salmonella*, including serovars Pullorum, Gallinarum and

Enteritidis (Setta *et al.*, 2012a) suggesting the suppression of IL-12 expression by *S.* Pullorum may be independent of LITAF expression in infected macrophages.

Salmonella can actively invade cells and *Salmonella* virulence factors which contribute to pathogenesis have been studied extensively (McGhie *et al.*, 2009). However, it is unclear which of these or other factors could interfere with M1-(like) polarization, resulting in persistent infection. The SPI-2 type III secretion system maintains replication and survival of *Salmonella* within macrophages and is essential for inducing systemic infection in chickens (Jones *et al.*, 2001, Jones *et al.*, 2007). SPI-2 was shown to inhibit trafficking of NADPH oxidase to the phagosome, thus interfering with the oxidative microbicidal activity of macrophages (Vazquez-Torres *et al.*, 2000b). It has been reported that intact PhoP is required to reduce iNOS expression in murine J774 macrophages and is associated with reduced nuclear translocation of *NF*-Kb and AP-1 (Hulme *et al.*, 2012). Similarly, *Mycobacterium bovis* also demonstrated its ability to prevent iNOS recruitment to the phagosome, inhibiting NO release (Miller *et al.*, 2004). A study by Bispham *et al.* (2001) reported that enteric infection of calves by *S.* Dublin required the SPI-2 effector (SseD) while another study reported that the wild type *S*. Gallinarum SPI-2 effector protein (SsaU) was required to induce typhoid in chicken (Jones *et al.*, 2001) and to enable persistent infection of chickens by *S*. Pullorum (Wigley *et al.*, 2002b). These studies highlight the difficulty in separating the factors required to produce systemic

infection compared to persistent infection. Studies on persistent *S.* Typhimurium infection has found that a SPI-2-dependent effector SseI blocked migration of macrophages and DC in part by associating with the host factor IQ motif containing GTPase activating protein 1 (IQGAP1), an important regulator of the cytoskeleton and cell migration (McLaughlin *et al.*, 2009). This interfered with the ability of the adaptive immune system to interact with these cells and correlated with lower numbers of DCs and CD4^{+} T cells in the spleen, when comparison with an *sseI* mutant (McLaughlin *et al.*, 2009).

The current understanding of SPI-1 is that its main role is in TTSS-1-mediated cell invasion. However, a microarray-based negative selection screen revealed that SPI-1-encoded invasion and translocation effectors SipB, SipC, and SipD were necessary to sustain long-term systemic infection of *S.* Typhimurium in mice (Lawley *et al.*, 2006). The SPI-1-secreted effector protein SipB induces caspase-1–dependent apoptosis during the initial interaction with macrophages (Hersh *et al.*, 1999), resulting in extracellular bacteria that potentially infect a second cell, which may aid in the establishment of systemic disease. *S.* Typhimurium could also induce epithelial extrusion accompanied with inflammatory cell death characterized by caspase-1 activation and thus escapes from its intracellular niche (Knodler *et al.*, 2010). It is likely that the ability of *S.* Typhimurium to continuously re-invade epithelial tissues is necessary to sustain a persistent intestinal colonisation. However, *sipB* (SPI-1) and *ssaV* (SPI-2) mutants of *S.* Typhimurium still persist systemically in mice although at lower levels, suggesting a contribution but

not absolute requirement of SPI-1 and SPI-2 for persistent infection (Lawley *et al.*, 2008).

Further work to identify the bacterial determinants of persistent infection in *S.* Pullorum will likely require an investigation of all the genes associated with intracellular survival and growth including SPI-2 genes and a number of metabolic function (O'Callaghan *et al.*, 1988). This might be done by mutation studies in either *S.* Pullorum if this is a positive attribute or in *S.* Enteritidis, to try to produce a *S.* Enteritidis strain which shows persistent infection, if this is a negative attribute.

Regulatory T cells play a role in maintaining the balance between immune activation and suppression (Vignali *et al.*, 2008), but their role in modulating immune activation during persistent infection is undefined. A recent study examined the development of Th1 cells and Tregs after *Salmonella* infection of resistant mice. It found that alterations in the potency of Tregs during infection reduced the effectiveness of Th1 responses, increased bacterial growth and controlled the tempo of persistent *S.* Typhimurium infection in mice (Johanns *et al.*, 2010). It is unclear whether similar alterations in Treg activities can affect the Th1 responses in susceptible mouse or in chickens. Results reported in this thesis show that expression of CTLA-4 was not increased in CD4$^+$ T cells cultured with *S.* Pullorum-infected chMDM. The suppressive properties of avian Treg cells (CD4$^+$CD25$^+$) was suggested to be IL-10-dependent (Selvaraj, 2013). In this study, *S.* Pullorum infection lead to invasion of liver and increased IL-10 expression in the spleen at 4-5 d pi. It

suggested a possible regulatory effect of IL-10 on inhibiting cytokine production and macrophage activity during systemic dissemination and possibly persist infection. In our study, taken together with the reduced type1 and increased type 2 cytokine expression, the enhanced expression of IL-10 mRNA, especially in the spleen, towards the end of the first week following infection with *S*. Pullorum, may be involved in driving host immunity further towards a Th2-like response. The early expression of IL-10 may function mainly as an immune regulator (discussed above) while up-regulation of IL-10 expression in response to *S*. Enteritidis infection may occur later following the acute phase of inflammatory response. This latter effect may be to reduce the inflammatory response thus preventing acute chronic inflammation (Uchiya *et al.*, 2004, Li *et al.*, 2009, Setta *et al.*, 2012b). We detected *in vitro* induction of IL-10 mRNA in chMDM infected with *S*. Enteritidis. The absence of strong expression of IL-10 mRNA *in vivo* may have resulted from the post-mortem examination being done early in infection during the acute phase of infection, when IL-10 would be expected to be up-regulated to suppress the overwhelmed expression of IFN-γ and would be observed at higher levels when the inflammatory response to *S*. Enteritidis infection has passed.

The differential proliferation of peripheral blood CD4^{+} T cells in response to infection with *S*. Pullorum or *S*. Enteritidis *in vitro* is in accordance with the different percentage of splenic CD4^{+} T cells detected *in vivo*. One study has shown that increased numbers of splenic T cells coincided with clearance of *S*. Enteritidis and *S*. Typhimurium from infected chickens (Beal *et al.*, 2004a).

However, definitive increased number of splenic T cells has not been shown in this study. It is unclear whether the relatively lower efficiency of *S*. Pullorum up-regulating percentage of splenic CD4$^+$ T cells is causally linked to persistent infection of *S*. Pullorum. Furthermore it is not known if the increased splenic CD4$^+$ T cells shown in this study following *S*. Pullorum infection has a Th1, Th17 or Th2 phenotype.

Both *S*. Pullorum and *S*. Gallinarum are avian-specific serovars, but *S*. Gallinarum generally more closely resembles *S*. Typhimurium infection in mice, in which infection results in a typhoid-like disease normally without persistent infection (Mastroeni and Menager, 2003). The innate immune system controls the bacterial numbers in the early stage of infection (Wigley *et al.*, 2002a, Mastroeni and Menager, 2003) and promotes adaptive immunity. In mice, T lymphocytes produce IFN-γ in response to IL-12 and IL-18 secreted from *Salmonella*-infected macrophages, which in turn leads to increased activation of macrophages, leading to clearance of *S*. Typhimurium from the tissues. The host immune response to systemic infection with the vaccine strain *S*. Gallinarum 9R in chickens mirrors this, with an increase in IFN-γ expression and increased T cell proliferation correlating to bacterial clearance from the liver and spleen of infection chickens (Wigley *et al.*, 2005a). However, the host genetic background is important and persistent infection has been observed in *S*. Gallinarum infection in a resistant inbred chicken line, with the organism persisting for more than 14 weeks with infection mainly limited to the liver and spleen (Berchieri *et al.*, 2001a). Persistent infection

involving fully virulent *S*. Typhimurium has also been shown in *Nramp1$^{+/+}$* mice, where bacteria are able to reside within macrophages in the mesenteric lymph nodes (MLN) for up to one year (Monack *et al.*, 2004).

One feature of the 'typhoid' serovars that are associated with systemic infection and persistent infection is auxotrophy (Uzzau *et al.*, 2000). Connected with that it is interesting to note that certain attenuated auxotrophic mutants of *S*. Typhimurium are able to show prolonged infection even in susceptible BALB/c mice (O'Callaghan *et al.*, 1988). This was not true of all auxotrophic attenuated mutant since although some persisted for more than 70 (*purA*) more than 70 (*aroApurA*) and more than 119 (*purE*) days respectively an *aroA* mutant was eliminated by 35 days. This suggests that it might be possible to produce systemically persistent *S*. Enteritidis infection in chicken using similar auxotrophic mutants. Certainly it would be interesting to examine the cytokine response of the infected macrophages using this *in vitro* model with these mutants to try to determine the relationship between auxotrophy and persistence.

CD4$^+$ Th cells play a central role in controlling *Salmonella* infection in mice. A strong Th1 response is effective in controlling *Salmonella* growth during the early acute phase in the mouse (Mastroeni, 2002). Exposure to inorganic lead (Pb) rendered mice susceptible to infection with *S*. Typhimurium and increased bacteria burdens, largely resulting from a shift in immune response towards a Th2-type reaction (Fernandez-Cabezudo *et al.*, 2007). In resistant mice persistently infected with virulent *S*. Typhimurium, high levels of

circulating antibody correlated with a Th2-dominant immune response. Neutralisation of IFN-γ reactivated acute infection, probably by interfering with macrophage activation (Monack *et al.*, 2004). This suggests that functional IFN-γ is still required to suppress bacterial growth during persistent infection. It also implies an increase in both Th1 and Th2 cytokines in response to infection. However, it is rational to consider that the ratio of these cytokines levels will govern the overall direction of the immune response to be mainly Th1 or Th2. In addition to Th1 and Th2 responses, Th17 cells are key contributors to inflammation. Griffin and McSorley (2011) reviewed recent research that highlighted the role of Th17 cells in the control of *Salmonella* infection. But there has been little focus on their role in avian salmonellosis. Increased expression of IL-17 was also found in the caeca of chickens infected with *S.* Enteritidis (Crhanova *et al.*, 2011) although a functional role for avian IL-17 in the mucosal inflammatory response to *Salmonella* has not yet been described. Further studies are also required to elucidate its potential role in chronic *Salmonella* infection. Recent studies have reported that chicken IL-17 might play a role in protection against *Eimeria maxima* and *Eimeria tenella* infection but appears to mediate *Eimeria tenella*-induced immunopathology during infection (Kim *et al.*, 2012, Zhang *et al.*, 2013).

The current study on *S.* Pullorum carriage and the studies on persistent *S.* Typhimurium infection in mice in favouring Th2-dominant response also mirrors the Th1/Th2 modulation for chronic infection induced by other

pathogens, such as leprosy caused by *Mycobacterium leprae*. Leprosy presents a spectrum of clinical manifestations with distinct immunologic forms, varying from slow bacterial growth with little tissue damage and a strong Th1-mediated cellular immunity at one pole (tuberculoid leprosy) to the other extreme with extensive bacillary load, considerable tissue damage with a predominantly Th2 response (lepromatous leprosy) (Sieling and Modlin, 1994, Garcia *et al.*, 2001). The spectrum of severity observed in this disease also shows the importance of host genetics in determining the outcome of infection and whether or not persistent infection/carriage takes place.

7. 2. 3 Cytokine therapy for persistent infection

Cytokine production, which may be manipulated by *Salmonella* and several other pathogens, including helminths, not only has a crucial effect on the outcome of infection but might itself theoretically be manipulated to control the course of the infection.

Immune-regulation between Th1 and Th2 type responses has been reported in a number of helminth infections the further manipulation of which may indicate a route for the control of the persistent carrier state in *S*. Pullorum infection in chickens.

In *Trichuris muris* infection in mice a Th1 or Th2 response results in susceptibility and resistance respectively. *In vivo* neutralization of IFN-γ results in expulsion of the parasite while depletion of IL-4 in resistant BALB/K mice prevents the generation of a protective Th2 response resulting in

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chronic infection with *Trichuris muris* (Else *et al.*, 1994). In murine infection with the nematode, *Nippostrongylus brasiliensis,* exacerbation or protection are related to Th1 and Th2-associated response, respectively. Administration of IL-12 increased expression of IFN-γ and IL-10, inhibited the IgE response and mRNA transcription for Th2-associated cytokines and enhanced adult worm survival and egg production in mice during early primary infections (Finkelman *et al.*, 1994). Both IFN-α and IFN-γ can increase fecundity of *Nippostrongylus brasiliensis* and delay expulsion of adult parasite, resulting in inhibited host protection against nematode infection probably by the antagonistic effects of IFN on an IL-4-mediated Th2 response (Urban *et al.*, 1993). In another study, IL-4 administration inhibited murine gastrointestinal infection with the nematode parasite *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* (Urban *et al.*, 1995).

Similar effects can be observed in bacterial infections. *In vivo* administration of supernatants from *S*. Enteritidis-immune T cells increased host resistance of day-old and 18-day-old chickens to invasion of organs by *S*. Enteritidis (Tellez *et al.*, 1993). This was thought to be mediated by increased infiltration and enhanced bactericidal activities of heterophils (Ziprin *et al.*, 1996). This indicates that the suppression of a Th2 type response by Th1-associated cytokines can have a profound effect on susceptibility to infection and inflammation. The regulatory function of chicken Th1 and Th2 cytokines on macrophage activities has been investigated *in vitro*, where chicken IFN-γ was found to prime HD11 macrophages to produce significantly higher levels of

ROS and NO while IL-4 inhibited NO production by macrophages when exposed to bacteria or microbial agonists *in vitro* (He *et al.*, 2011). In accordance with these studies, Foster *et al.* (2003a) reported that IFN-γ-was required to induce lethal ROS in *S.* Typhimurium-infected murine J774 macrophages. Other work by this group showed that vasoactive intestinal peptide (VIP) (mostly produced by Th2 cells) inhibited IFN-γ-induced ROS and killing of (a normally avirulent) *phoP* mutant of *S.* Typhimurium in J774 cells (Foster *et al.*, 2005). This indicates that the enhanced antimicrobial activity of IFN-γ-stimulated macrophages *in vivo* may reduce/eliminate *S.* Pullorum within/from splenic macrophages of infected chickens, although *S.* Typhimurium and *S.* Enteritidis were shown to be able to survive in IFN-γ activated murine or chicken macrophages (Brodsky *et al.*, 2005, He *et al.*, 2011). One further line of our enquiry would therefore be to explore the use of parenteral administration of Th1-related cytokines (IFN-γ and IL-12) to reduce persistent infection of the chicken spleen by *S.* Pullorum.

Such experiments might be extended further. There would seem to be less practical value in parenteral administration of recombinant chicken Th1 cytokines to large numbers of *S.* Pullorum infected chickens in the field but the use of such an approach might be useful for related *Salmonella* serovars which enter the carrier state such as a *S.* Typhi in man and *S.* Dublin in cattle.

Chronic infection with *S.* Typhi is known to be associated with shedding via the gall bladder although the spleen, and, by extension, probably also the liver, are known to be infected (Vogelsang and Boe, 1948, Young *et al.*, 2002,

Nath *et al.*, 2010). In *S*. Dublin infection in cattle, persistent shedding can occur from the gut, possibly involving the gall bladder, and also from the udder but the spleen is also affected (Sojka *et al.*, 1974, Hinton and Williams, 1977, Wallis *et al.*, 1995) while *S*. Pullorum is known to persist mainly within splenic macrophages *in vivo* (Wigley *et al.*, 2001, Wigley *et al.*, 2005b). It may be that systemic colonisation of *Salmonella* of the mammalian spleen and MLN is accompanied by infection of the liver which itself may be responsible for localisation in the gall bladder resulting in shedding into the intestine from the bile. Thus persistent infection within the splenic macrophages may also be the key infection site of other typhoid serovars producing chronic infections. If a cytokine therapy approach to controlling persistent *S*. Pullorum infection could be effective there would be huge value in extending such studies to *S*. Typhi and *S.* Dublin in humans and cattle respectively.

7. 3   Future work

Future work will first focus on the utilization of avian IFN-γ and IL-12 as immune therapeutics to modulate a non-protective Th2 type response back towards a protective Th1 response *in vivo* and to reduce or eliminate *S*. Pullorum within the splenic macrophages of infected chickens. There are also advantages of comparing behaviour in the chicken with persistent infection in the mouse model.

We will also use macrophages and CD4$^+$ T cells in co-culture *in vitro* as a model to extend our understanding of persistent infection of other typhoid serovars such as *S*. Dublin and *S*. Abortusovis.

It is clear now that *S*. Pullorum modulates the cytokine production from infected macrophages and encountered T cells, but further studies are also required to find out the bacterial determinants of this characteristics.

Appendix

Appendix. 1

Slide agglutinating test for selected *Salmonella* strains

Serovars	'O' antigens†	Phase 1 'H' antigen†	Phase 2 'H' antigen†
S. Enteritidis	1, 9, 12	g,m	-
S. Pullorum	(1), 9, 12	-	-
S. Gallinarum	1, 9, 12	-	-
S. Typhimurium	1, 4, 5, 12	i	1, 2
S. Dublin	1, 9, 12	g, p	-

†Remel™ Agglutinating Sera (Thermo Fisher Scientific, UK)

Appendix. 2

Phosphate-Buffered Saline (PBS)

 1 × PBS tablet added to 500 ml of Dh_2O

MACS buffer

0.5 % BSA and 2 Mm EDTA in PBS, PH 7.2

FACS buffer

1 % BSA in PBS

HD11 cell culture medium

RPMI 1640 (Gibco, Paisley, UK), containing:
5% foetal bovine serum (FBS; Gibco; v/v)
5% chicken serum (v/v)
10% tryptose phosphate broth (TPB; v/v)
1% of L-glutamine (2Mm; Gibco, UK; v/v)

PBMC-derived macrophage (chMDM) culture medium

RPMI 1640, containing:
10% FCS (v/v)
20Mm Hepes (Sigma-Aldrich, UK)
50µg/ml gentamicin sulphate (Sigma-Aldrich, UK)
10 units/ml streptomycin/penicillin (Gibco, Paisley, UK)
1.25 µg/ml fungizone (Gibco)
2Mm L-glutamine (Gibco)

*Antibiotics-free culture medium

RPMI 1640, containing:
10% FCS (v/v)
20Mm Hepes (Sigma-Aldrich, UK)
2Mm L-glutamine (Gibco)

Appendix. 3
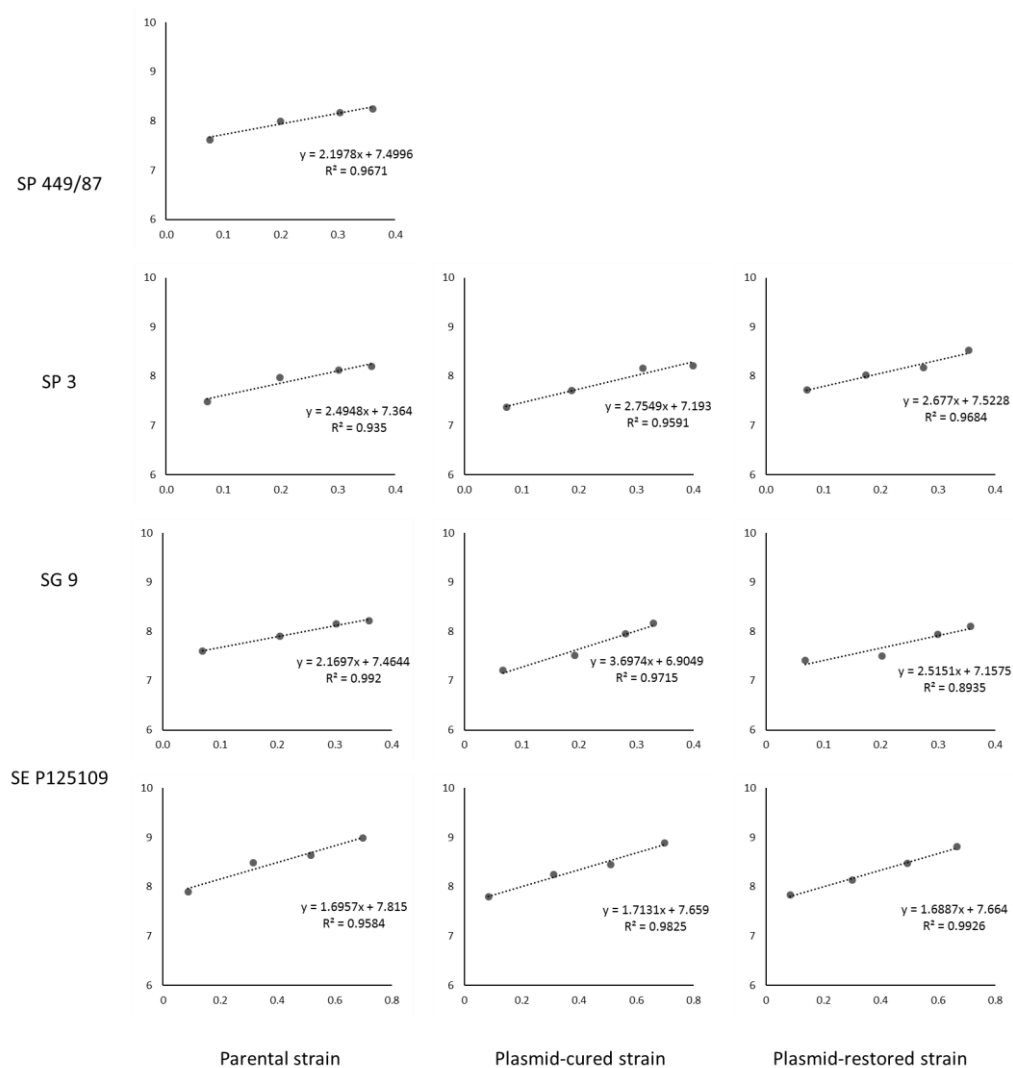
Calibration graphs between the bacterial counts and the optical density of different *Salmonella* **serovars.** SP, *S*. Pullorum, SE. *S* Enteritidis, SG, *S*. Gallinarum

Appendix. 4
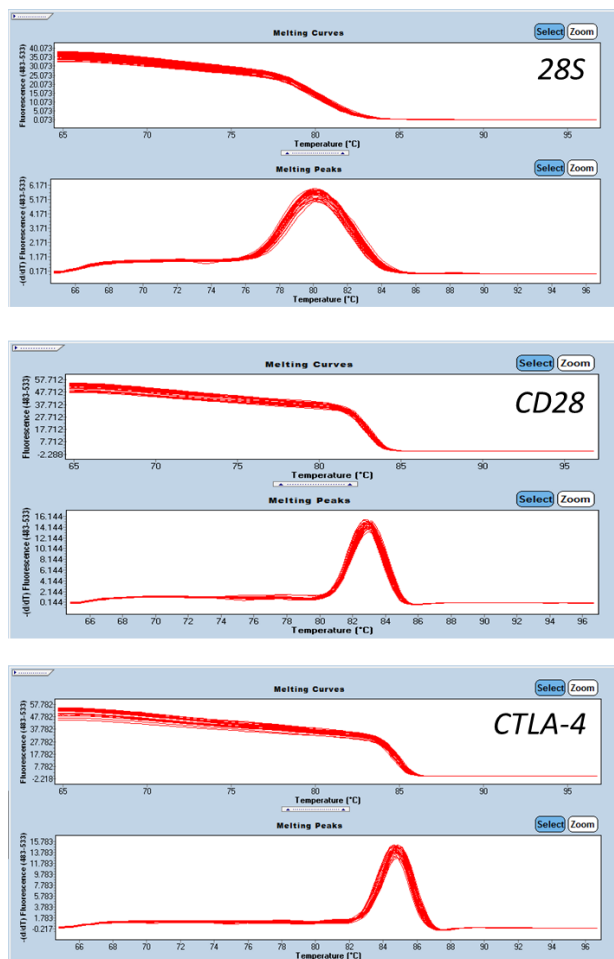
The Melting curve of primers of CD28 and CTLA-4 used for qRT-PCR.

Appendix. 5

In vitro immune responses of avian cells to infection with different serovars of *S. enterica*

Bacterial stimulus	Immune responses		
	chMDM	CD4$^+$ T cells	Splenocytes
S. Pullorum	CXCLi1 and IL-1β ↑, CXCLi2, iNOS and IL-6 ↑ ↓ IFN-γ and IL-12α → ↓, IL-18 ↑↓ IL-4 and IL-13 ↑↑ IL-10 ↑, TGF-β4 → CD40, CD80 and CD86 ↑	IFN-γ →↓ IL-17A →; IL-17F ↓ ↓ IL-4 → IL-10 and TGF-β4 → ◊; CD28↑ CTLA4→	IFN-γ and IL-12α → , IL-18 → ↓ IL-4 → IL-17F →
S. Gallinarum	CXCLi1 and IL-1β ↑, CXCLi2, iNOS and IL-6 ↑ ↓ IFN-γ → ↓, IL-12α → and IL-18 ↑ IL-4 and IL-13 → IL-10 and TGF-β4 → CD40, CD80 and CD86 ↑	IFN-γ → IL-17A →; IL-17F → ↓ IL-4 → IL-10 and TGF-β4 → ◊; CD28↑ CTLA4→	IFN-γ and IL-12α ↑, IL-18 → IL-4 → IL-17F ↑
S. Enteritidis	CXCLi1, CXCLi2, IL-1β, iNOS and IL-6 ↑ IFN-γ, IL-12α and IL-18 ↑ IL-4 and IL-13 → IL-10 ↑, TGF-β4 → CD40, CD80 and CD86 ↑	IFN-γ ↑ IL-17A →; IL-17F ↑ IL-4 → IL-10 and TGF-β4 → ◊◊; CD28↑ CTLA4→	IFN-γ, IL-12α and IL-18 ↑ IL-4 → IL-17F ↑

◊◊, high proliferating; ◊ proliferating

Compared to uninfected control: ↑, increase; ↓, decrease; →, no change

Compared to S. Enteritidis-infection: ↑, increase; ↓, decrease

Early immune dynamics of chicken in response to infection with persistence and non-persistence serovars of *S. enterica*

Bacterial stimulus	Immune mediators	
	Caecal tonsil	Spleen
S. Pullorum	CXCLi1, CXCLi2, IL-1β, iNOS and IL-6 → ↓ IFN-γ ↓ ↓, IL-12α and IL-18 → ↓ IL-17F → ↓ IL-4 and IL-13 ↑ ↑ IL-10 ↑ ↑, TGF-β4 →	CXCLi1, CXCLi2 and IL-6 →, IL-1β and iNOS → ↓ IFN-γ and IL-12α → ↓, IL-18 → IL-17F → ↓ IL-4 and IL-13 ↑ ↑ IL-10 ↑ ↑, TGF-β4 →
S. Gallinarum	CXCLi1, CXCLi2, IL-1β, iNOS and IL-6 → ↓ IFN-γ, IL-12α and IL-18 → IL-17F → IL-4 and IL-13 → IL-10 and TGF-β4 →	CXCLi1, CXCLi2, IL-1β, iNOS and IL-6 → IFN-γ, IL-12α and IL-18 → IL-17F → IL-4 and IL-13 → IL-10 and TGF-β4 →
S. Enteritidis	CXCLi1, CXCLi2, IL-1β, iNOS and IL-6 ↑ IFN-γ, IL-12α and IL-18 ↑ IL-17F ↑ IL-4 and IL-13 → IL-10 and TGF-β4 →	CXCLi1, CXCLi2, and IL-6 →; IL-1β and iNOS ↑ IFN-γ, IL-12α and IL-18 ↑ IL-17F → IL-4 and IL-13 → IL-10 and TGF-β4 →

Compared to uninfected control: ↑, increase; ↓, decrease; →, no change

Compared to S. Enteritidis-infection: ↑, increase; ↓, decrease

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