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Innate immune gene expression differentiates the early avian intestinal response between Salmonella and Campylobacter

Ronan G. Shaughnessy\textsuperscript{a}, Kieran G. Meade\textsuperscript{a}, Sarah Cahalane\textsuperscript{a}, Brenda Allan\textsuperscript{b}, Carla Reiman\textsuperscript{b}, John J. Callanan\textsuperscript{c} and Cliona O’Farrelly\textsuperscript{a*}

\textsuperscript{a}Comparative Immunology Group, School of Biochemistry and Immunology, Trinity College, Dublin 2, Ireland.
\textsuperscript{b}Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, S7N 5E3, Canada.
\textsuperscript{c}School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4.

\*Corresponding author: cliona.ofarrelly@tcd.ie

Abstract

Salmonella enterica serovar Typhimurium and Campylobacter jejuni are major human pathogens, yet colonise chickens without causing pathology. The aim of this study was to compare intestinal innate immune responses to both bacterial species, in a 4-week-old broiler chicken model. Challenged and control birds were sacrificed and tissue samples taken for histopathology and RNA extraction. No significant clinical or pathological changes were observed in response to infection with either bacterial species. Expression of selected genes involved in pathogen detection and the innate immune response were profiled in caecal tissues by quantitative real-time PCR. TLR4 and TLR21 gene expression was transiently increased in response to both bacterial species ($P <0.05$). Significant increases in TLR5 and TLR15 gene expression were detected in response to S. Typhimurium but
not to *C. jejuni*. Transient increases of proinflammatory cytokine (*IL6* and *IFNG*) and chemokine (*IL8* and *K60*) genes increased as early as 6 hours in response to *S. Typhimurium*. Minimal cytokine gene expression was detected in response to *C. jejuni* after 20 hours. *IL8* gene expression however, was significantly increased by 24-fold (*P* <0.01).

The differential expression profiles of innate immune genes in both infection models shed light on the tailored responses of the host immune system to specific microbes. It is further evidence that innate regulation of these responses is an important prerequisite to preventing development of disease.

Keywords: Chicken; Commensal, Campylobacter; Salmonella; Innate immune gene expression
1. Introduction

Constant interaction between multicellular organisms and the microbial world, and concurrent evolution of local innate immune mechanisms has given rise to systems capable of differentiating between benign and harmful microorganisms (Medzhitov, 2007). Bacterial species can be classified as commensal or pathogenic depending on the outcome of these interactions with the underlying host immune system (Hooper & Gordon, 2001). Microbial detection by pattern recognition receptors (PRRs) of the innate immune system induces a signalling cascade resulting in transcriptional changes (Palsson-McDermott & O'Neill, 2007) leading to the production of chemokines, cytokines and other proinflammatory mediators that serve to amplify and regulate cytotoxic activity against potential pathogens (Wick, 2004). These mechanisms have been characterised primarily in mammalian hosts, but are found across the animal kingdom, including in birds (Boyd et al., 2007). While many components of innate immunity are conserved, fundamental differences in local innate immune responses between species may explain why some microorganisms, including *Campylobacter jejuni*, are pathogenic in one species yet a commensal in others.

Toll-like receptors (TLRs), a major family of PRRs, are expressed in chicken intestinal tissues and the local immune cells have been shown to respond to bacterial ligands (Iqbal et al., 2005a). In the caeca of newly hatched chicks, *Salmonella enterica* serovar Typhimurium induces a proinflammatory response (Fasina et al., 2008; Henderson et al., 1999). Influxes of heterophils as well as increases in cytokines and chemokines are evident (Henderson et al., 1999; Withanage et al., 2004) and are thought to contribute to the pathology observed. However, once chicks are more than a few days old, *S. Typhimurium* persistently colonises their intestines in the absence of pathology (Jones et al., 2007), suggesting that maturity of host defences contribute to the lack of clinical signs.
Campylobacter jejuni is another economically important zoonotic bacterial species, and regarded as the leading cause of food-borne human gastroenteritis in the developed world (Hendrixson & DiRita, 2004). C. jejuni is primarily an enteric bacterial species (Beery et al., 1988), colonising the caecal crypts of chickens (Newell & Fearnley, 2003). In vitro, C. jejuni induces proinflammatory cytokines from avian macrophages and kidney cells (Smith et al., 2005), and increased chemokine gene expression has been observed in chicken primary intestinal cells stimulated with the bacterium (Borrmann et al., 2007; Li et al., 2008). However, despite being highly prevalent in the chicken intestine, C. jejuni rarely induces disease, regardless of bird age (Van Deun et al., 2007), suggesting possible modulation or evasion of host immune mechanisms.

To date, studies of innate immune gene expression in chicken have focused on investigating the avian intestinal response to pathogenic bacterial species in order to develop strategies to control bacterial colonisation. Under non-pathogenic conditions, the role of the innate immune response to both of these bacterial species is poorly understood, especially whether such responses if present, impact on the levels of bacterial colonisation. As co-evolutionary histories of host and microbial species are likely to differ, we hypothesized that underlying innate immune gene expression would differ in response to both bacterial species and might reveal differential mechanisms for establishing the commensal state. We therefore carried out in vivo infection models with S. Typhimurium and C. jejuni to compare the local innate immune response in the caecums of challenged birds.
2. Materials and Methods

2.1 Bacterial strain preparation

Salmonella enterica serovar Typhimurium strain SL1344 was cultivated in modified N-minimal medium containing 5 mM KCL, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 100 mM Tris-HCL (pH 7.0) 38 mM glycerol, 0.1% Casamino Acids, 24 mM MgCl₂, 337 um PO₄³⁻ at 37°C with shaking at 200 rpm until an optical density at 600 nm of 0.7 was reached. The cells were harvested by centrifugation and resuspended in 0.85% NaCl to one-tenth the original volume. The number of viable bacteria present in the challenge was determined by viable cell counts on Luria-Bertani (LB) agar plates and calculated to be 1 x 10¹⁰/ml. Campylobacter jejuni strain NCTC11168 v1 was grown on Mueller Hinton (MH) agar plates. The cells were resuspended in saline and viable bacterial numbers were determined on MH agar plates (2.5 x 10⁸/ml).

2.2 Experimental challenge

Both S. Typhimurium and C. jejuni infection models were performed as previously described (Meade et al., 2008). For the analysis of the caecal immune response to both C. jejuni and S. Typhimurium challenge, 78 4-week-old birds were used from this study. 36 birds represented either mock-challenged or S. Typhimurium-challenged birds at each of three timepoints: 6 hours, 20 hours and 48 hours (n = 6 birds per timepoint). Similarly, 42 birds were used for analysis of the caecal immune response to C. jejuni challenge, representing either mock-challenged or C. jejuni-challenged birds at the same timepoints (n = 7 birds per timepoint).

2.3 Microbiology

All birds were examined for the presence of either S. Typhimurium or C. jejuni using cloacal swabs at the beginning of each experiment. To determine the presence and level of intestinal colonisation during the experiment, caecal samples
were cultured using serial dilutions. To detect S. Typhimurium, the samples were cultured on Brilliant Green Agar at 37°C for 20 hours. To detect C. jejuni, the samples were plated on Karmali agar and incubated microaerophilically at 42°C for 48 hours.

2.4 Histopathology

At post mortem, sections of caecum were sampled and preserved in liquid nitrogen and subsequently stored at -80°C. Prior to histopathological evaluations, caecal samples were brought to room temperature before being placed in formalin for routine fixation. Tissue from 2/3 animals per timepoint was trimmed, paraffin-embedded, sectioned at 5-8 μm thickness and stained with haematoxylin and eosin (H&E).

2.5 RNA extraction, cDNA synthesis & real time quantitative RT-PCR

Total RNA was extracted from tissue samples using a combination of (TriReagent®, the MELT™ Total Nucleic Acid Isolation System (Ambion). All samples were DNase treated. RNA yield and quality was then assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Two μg of total RNA from each sample was reverse transcribed into cDNA with oligo-dT primers using SuperScript III® first strand synthesis SuperMix kit according to the manufacturer’s instructions (Invitrogen Ltd, Paisley, UK). A total of 40ng cDNA, quantified using a NanoDrop® ND-1000 spectrophotometer, was subsequently used for each real time qRT-PCR reaction. Primers for real time qRT-PCR were designed, intron-spanning where possible, using Primer3 software (Rozen & Skaletsky, 2000) and commercially synthesised (Invitrogen Ltd, Paisley, UK). Primer sets are described in Supplementary table 1. Each reaction was carried out in duplicate, in a total volume of 25 μl with 2 μl of cDNA (20 ng/μl), 12.5 μl PCR master mix (Stratagene Corp, La Jolla, CA) and 10.5 μl primer/H2O. Optimal primer concentrations were determined by titrating 100, 300 and 900 nM final concentrations and dissociation curves were examined for the
presence of a single product. Real time qRT-PCR was performed using a MX3000P®
quantitative PCR system (Stratagene Corp, La Jolla, CA) using the following cycling
parameters: 95°C for 30 s, 60°C for 1 min and 72°C for 30 s followed by amplicon
dissociation. All gene amplifications were normalised to Ribosomal protein L7
\(RPL7\) which was selected as the most stably expressed gene across samples in both
challenge models from a panel of potential normalisers \(ACTB, GAPDH, LDHA\) and
\(RPL7\) using GeNorm version 3.4 (Vandesompele et al., 2002).

2.6 Data analysis

Data analysis was carried out using the \(2^{-\Delta\Delta CT}\) method (Livak & Schmittgen,
2001). Analyses were carried out within, and not between infection models to prevent
the introduction of stochastic error due to the variation between individual animals.
Statistical analyses were performed using non-parametric Mann-Whitney U test as
implemented in version 5.01 of StatView (SAS Institute Inc. Cary, NC, USA).
3. Results

3.1 Caecal colonisation

Levels of *S. Typhimurium* and *C. jejuni* colonisation were determined in a range of tissues and have been previously described (Meade *et al.*, 2008). Two challenged chickens (one infected with *S. Typhimurium* and one with *C. jejuni*) were culture negative across all tissues and were excluded from subsequent gene expression analysis. Specific to the caecum, persistent colonisation was detected in response to both bacterial challenges across the 48-hour timecourse. High levels of *S. Typhimurium* colonised the caecums of challenged birds at all three timepoints. A mean of $2.12 \times 10^7$ cfu/g was detected at 6 hours, a mean of $1.29 \times 10^7$ cfu/g at 20 hours, and a mean of $1.15 \times 10^7$ cfu/g at 48 hours. Levels of *C. jejuni* colonisation in the caecum were higher than with the *S. Typhimurium* challenge across the timecourse: 6 hours (a mean of $2.35 \times 10^8$ cfu/g; 10 fold higher), 20 hours (a mean of $2.25 \times 10^{11}$ cfu/g; 10,000 fold higher) and 48 hours (a mean of $8.58 \times 10^{11}$ cfu/g; 10,000 fold higher). Control animals were negative for both bacterial species. Results demonstrate consistent bacterial colonisation of the caecum in both challenge models (Meade *et al.*, 2008).

3.2 Histopathological analysis

Histopathological analysis of caecal samples obtained throughout the time course of challenge revealed only slight differences between both infection models that were not interval dependent. Regarding inflammation, there were no notable differences between both bacterial challenges and their uninfected controls. Crypt architecture was similar between controls and infected in both challenge models. Regions of gut associated lymphoid tissue (GALT) were prominent in many of the samples selected for examination and consequently, varying degrees of lymphocyte infiltration of the lamina propria were observed but not thought to be in response to
either of the bacterial species. Only subtle increases in heterophils were detected in
response to S. Typhimurium at 6, 20 and 48 hours post-challenge. Heterophil
migration was not detected in response to C. jejuni at any time point (data not shown).

3.3 Gene expression of pan-leukocyte marker CD45

CD45, also known as protein tyrosine phosphatase receptor type C, is a cell
surface marker on all immune cells of haematopoietic origin. Measuring the mRNA
expression of this marker is one way of determining cellular changes at the site of
caecal infection (Fig. 1). There was a significant but transient increase of CD45 gene
expression to both S. Typhimurium (2.8 fold; \(P=0.004\)) and C. jejuni (2.6 fold;
\(P=0.025\)) at 6 hours post challenge. In contrast, there was a significant decrease in
CD45 (-2.2 fold; \(P=0.004\)) in response to S. Typhimurium 20 hours post-challenge
but CD45 was not differentially expressed in response to C. jejuni at this time point.
Profiling CD45 gene expression 48 hours post-challenge did not reveal any significant
changes in response to either bacterial species (Fig. 1).

3.4 Bacterial induced TLR gene expression profiling

We compared TLR gene expression changes in response to both bacterial
challenges (Fig. 2). Of the 4 TLRs examined by qRT-PCR, all 4 genes were
significantly differentially expressed in one or other infection model (\(P <0.050\)).

\(\text{TLR4}\), which detects LPS, was transiently increased by 2.3 fold (\(P=0.037\)) at 6
hours post-challenge with S. Typhimurium followed by a significant reduction after
20 hours (-3.4 fold; \(P=0.004\)), and not differentially expressed after 48 hours (Fig.
2A). Increased \(\text{TLR4}\) (4.0 fold; \(P=0.025\)) gene expression was detected in response to
\(\text{C. jejuni}\) after 6 hours and was not differentially expressed for the remainder of the
timecourse (Fig. 2B).
TLR5, which detects flagellin, was also transiently increased in response to S. Typhimurium by 1.9 fold ($P=0.053$) after 6 hours, decreased significantly at 20 hours (-1.3 fold; $P=0.053$), and was increased again by 48 hours (2.2 fold; $P=0.055$) post-challenge (Fig. 2A). TLR5 was not differentially expressed in response to C. jejuni across the timecourse.

TLR15 gene expression was decreased in response to S. Typhimurium at 20 hours (-1.2 fold; $P=0.055$) and increased at 48 hours (6.2 fold; $P=0.004$) post-challenge (Fig. 2A). In contrast, TLR15 was not differentially expressed in response to C. jejuni across the timecourse.

TLR21 gene expression was increased in response to both S. Typhimurium (5.2 fold; $P=0.004$) and C. jejuni (3.5 fold; $P=0.025$) at 6 hours post-challenge. Differential TLR21 gene expression was not evident for the remainder of timecourse to either infection (Fig. 2A&B).

### 3.5 Comparative chemokine and cytokine gene expression profiling

Profiling of cytokines and chemokine gene expression was performed to detect differences in both infection models, and characterise the predominant type of immune response across the timecourse. IFNG gene expression was transiently increased at 6 hours (4.1 fold; $P=0.055$), not differentially expressed at 20 hours, and significantly increased at 48 hours (6.0 fold; $P=0.007$) post-challenge with S. Typhimurium (Fig. 3A). In contrast, IFNG was not differentially expressed in response to C. jejuni at any timepoint across the timecourse.

In response to S. Typhimurium, differential gene expression of IL1β was first detected at 20 hours with an increase of 2.3 fold ($P=0.050$), followed by a further increase of 7.4 fold ($P=0.004$) at 48 hours post-challenge (Fig. 3A). Similarly, increased IL1β gene expression was detected after both 20 hours (5.2 fold; $P=0.005$) and 48 hours (3.9 fold; $P=0.025$) in response to C. jejuni (Fig. 3B).
IL6 gene expression was significantly increased in response to S. Typhimurium at 6 hours (11.7 fold; $P=0.004$), 20 hours (1.9 fold; $P=0.054$) and 48 hours (19.4 fold; $P=0.004$) post-challenge (Fig. 3A). Differential gene expression of IL6 in response to C. jejuni was evident only after 48 hours (4.4 fold; $P=0.025$) post-challenge (Fig. 3B).

Increased TGFB4 gene expression in response to S. Typhimurium at 6 hours (1.6 fold; $P=0.004$), 20 hours (2.4 fold; $P=0.055$) and 48 hours (1.9 fold; $P=0.003$) post challenge (Fig. 3A). In contrast TGFB4 gene expression was not differentially expressed in response to C. jejuni throughout the timecourse.

Gene expression of the chemokine IL8 was significantly increased in response to S. Typhimurium 6 hours (5.1 fold; $P=0.004$), not differentially expressed at 20 hours, and again increased at 48 hours (11.5 fold; $P=0.055$) post-challenge (Fig. 4A). There was a significant decrease of IL8 (-2.9 fold; $P=0.030$) gene expression and in response to C. jejuni after 6 hours, followed by a substantial increase at 20 hours (24.3 fold; $P=0.002$) and a reduction at 48 hours (3.3 fold; $P=0.002$) post-challenge (Fig. 4B).

Increased expression of another chemokine, K60 was also evident in response to S. Typhimurium after 6 hours (2.1 fold; $P=0.004$), 20 hours (4.3 fold; $P=0.004$) and 48 hours (4.1 fold; $P=0.043$) post-challenge (Fig. 4A). In response to C. jejuni, there was a transient decrease of K60 gene expression at 6 hours (-4.7 fold; $P=0.024$) followed by a transient increase at 20 hours (6.6 fold; $P=0.002$) post challenge (Fig. 4B).

3.6 C. jejuni induced chemokine gene expression in the caecal tonsil

To determine if the source of the elevated IL8 chemokine gene expression was from the lymphoid tissue in the caecal tonsil, IL8 and K60 were profiled, in response to C. jejuni (Fig. 5). Increased IL8 gene expression was detected 6 hours (2.3 fold;
$P=0.025$), 20 hours (4.5 fold; $P=0.002$) and 48 hours (7.4 fold; $P=0.025$) post-
challenge. *K60* mRNA was significantly increased only at 48 hours post-challenge
(2.3 fold; $P=0.025$).
4. Discussion

To date, studies of innate immune gene expression in the chicken have focused on the avian intestinal response to pathogenic bacterial species. However, two zoonotically important microorganisms, *S. Typhimurium* and *C. jejuni*, persist in the intestines of birds in the absence of clinical signs of disease. Under non-pathogenic conditions, the role of the immune response to both of these bacterial species is only partially known, especially whether such responses influence the level of bacterial colonisation. *S. Typhimurium* is capable of inducing immune mediated pathology in only newly hatched chicks (Henderson *et al.*, 1999) and not in 1-week-old chicks (Jones *et al.*, 2007), indicating that maturity of host defences may be important. In contrast, *C. jejuni* resides as a commensal in chickens, regardless of age (Young *et al.*, 2007), suggesting possible modulation or evasion of immune mechanisms. We therefore hypothesised that underlying differences in innate immune gene expression in response to both bacterial species may reveal mechanisms for establishing the commensal state.

As expected, both bacterial species persistently colonised the caecum of challenged birds across the 48-hour timecourse. In comparison to *S. Typhimurium* colonisation, *C. jejuni* colonisation levels were higher most notably at 20 hours and 48 hours post-challenge (10,000 fold higher), indicating *C. jejuni*’s ability to thrive in the caecal environment. Despite persistent bacterial colonisation, clinical signs of disease were not observed in either infection model. Previous studies have shown marked heterophil infiltration to the lamina propria in chicks following *S. Typhimurium* challenge (Berndt *et al.*, 2007; Henderson *et al.*, 1999). Only subtle heterophil infiltration to the caecum was detected in this study of 4 week-old birds in response to *S. Typhimurium*. Heterophil influx was clearly absent from the *C. jejuni* challenge, in agreement with recent literature (Hendrixson & DiRita, 2004). To further characterise cellular changes in response to infection, but at the molecular level, gene expression
of CD45 was also examined. CD45 is a transmembrane receptor expressed on cells of
haematopoietic origin including heterophils. We observed only minor increases of
CD45 gene expression at 6 hours in response to both bacterial challenges. CD45 is
upregulated on activated inflammatory cells (Hermiston et al., 2003) and therefore
such increases may represent transient activation of residential phagocytes of the
lamina propria in response to the high bacterial challenges. The lack of increased
CD45 gene expression for the remainder of the timecourse to either infection suggests
an absence of immune cell infiltration or activation and is in agreement with the
histological observations.

We next quantified expression of genes involved with microbial detection and
activation of subsequent innate immune responses. As early as 6 hours post-challenge,
expression of TLR4 and TLR21 was transiently increased in caecal tissue in response
to both bacterial challenges. Chicken TLR4 recognises LPS and has been associated
with Salmonella infection (Kogut et al., 2005; Leveque et al., 2003) while a ligand for
TLR21 has yet to be identified. Interestingly, increased TLR5 gene expression was
detected only in response to S. Typhimurium during the timecourse (6 hours and 48
hours). TLR5 activation by flagellin from S. Typhimurium has been demonstrated to
induce increased IL1β mRNA from chicken cells (Iqbal et al., 2005) but C. jejuni’s
flagellin evades detection by human TLR5 (Andersen-Nissen et al., 2005; Johanesen
& Dwinell, 2006). The lack of increased TLR5 gene expression in response to C.
jejuni suggests a similar mechanism of evasion in the avian host. Similarly, TLR15
was increased in response to S. Typhimurium but not in response to C. jejuni. Higgs et
al. have previously shown an increase in caecal TLR15 mRNA in response to heat-
killed S. Typhimurium (Higgs et al., 2006). Unchanged expression of TLR5 and
TLR15 genes in response to C. jejuni throughout the timecourse highlights key
differences in the recognition of both bacterial challenges.
Previous studies showed that *S. Typhimurium* induces expression of a range of proinflammatory cytokines and chemokines in newly hatched chicks accompanied by clinical signs of disease (Withanage *et al.*, 2004; Withanage *et al.*, 2005). In our study, in the absence of pathology, we still found increased proinflammatory cytokine and chemokine gene expression in response to *S. Typhimurium* post-challenge in the caecum of challenged chickens. *IFNG, IL6* and *IL8* gene expression changes followed a similar pattern: transient increases at 6 hours, no increase at 20 hours, and significant increased expression evident again at 48 hours. Intermittent invasion of the caecal epithelium and subsequent encounters with underlying phagocytes may account for these observations. Significant increases of *IL1B, K60* and *TGFB4* were also evident in response to *S. Typhimurium*.

In contrast, in response to *C. jejuni*, increased expression of proinflammatory genes was not detected until 20 hours post-challenge. Therefore, the delayed response may be due to *C. jejuni* invading the caecal epithelium at a slower rate than *S. Typhimurium*. Based on mammalian studies, it is likely that the basolateral surfaces of the caecal epithelium have greater TLR expression and are thus more inductive of proinflammatory responses. Invasion is required to encounter the basolateral surface and the immunopotent phagocytes of the lamina propria. Therefore, the delayed proinflammatory response may be due to *C. jejuni* invading the caecal epithelium at a slower rate than *S. Typhimurium*. While only minor increases in *IL1B* and *K60* were evident, they were accompanied by high *IL8* gene expression. A recent study has also shown increased expression of *IL8* and *K60* genes accompanied by minimal changes in cytokine gene expression in intestinal tissues of both newly hatched and 2 week old chickens challenged with *C. jejuni* (Smith *et al.*, 2008). At 48 hours post-challenge, *IL8* gene expression remained elevated although to a much lesser extent. High *IL8* gene expression followed by reduced expression might be a result of *C. jejuni* temporarily invading the epithelium and then returning to the mucus layer as the
microorganism multiplies in mucus and not epithelial cells (Van Deun et al., 2007). As *C. jejuni* colonisation in the mucosae would become more established, epithelial invasion would be less likely.

The caecal tonsils are a defined aggregate of lymphoid tissue, rich in lymphocyte and phagocytic cells, inductive of proinflammatory gene expression in response to bacterial stimulus (Haghighi et al., 2008). We observed significantly increased *IL8* gene expression at 6 hours, 20 hours and 48 hours post-challenge. Increased *K60* gene expression was also evident at 48 hours post *C. jejuni*-challenge in the caecal tonsil. These results suggest that the caecal tonsils are an important site for intestinal IL-8 production in response to *C. jejuni*. Chicken IL-8 has previously been shown to attract both heterophils and monocytes (Barker et al., 1993; Poh et al., 2008). Yet, our histological data in combination with unchanged *CD45* gene expression in the presence of high *IL8* gene expression indicates no inflammatory cell influx in response to *C. jejuni*. These results suggest IL-8 is post-transcriptionally modified or perhaps that *C. jejuni* has developed mechanisms to interrupt the chemotactic properties of this chemokine.

The results from this study provide some intriguing insights into the nature of the intestinal immune gene expression patterns that are integral to the host response to both bacterial species. Furthermore, it identifies some key molecules that characterise and differentiate the immune response to *C. jejuni*. Future work will concentrate on the elucidation of pathways regulating the *IL8* gene expression detected in the caecum and investigate the mechanisms that may suppress the chemotactic and pathological effects of this chemokine. Uncovering the specific regulatory mechanisms activated in response to high-level *C. jejuni* colonisation may shed light on the role of the host innate immune response in the development of commensal tolerance in the chicken.
Acknowledgements

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**Figure captions**

Fig. 1. Comparative CD45 (a marker for proinflammatory cell migration) gene expression profiles in response to both S. Typhimurium (n=6) and C. jejuni (n=7) in the caecum at 6 hours, 20 hours and 48 hours post-challenge. The horizontal lines within each box plot represents the median fold changes in gene expression in infected chickens relative to the mock-infected controls. Statistical analysis was carried out separately for each infection model to compare challenged and control birds (significantly differentially expressed genes are highlighted *P <0.05, **P <0.01).

Fig. 2. Comparative TLR gene expression profiles in response to both (A) S. Typhimurium (n=6) and (B) C. jejuni (n=7) in the caecum at 6 hours, 20 hours and 48 hours post-challenge. The horizontal lines within each box plot represents the median fold changes in gene expression in infected chickens relative to the mock-infected controls. Statistical analysis was carried out separately for each infection model to compare challenged and control birds (significantly differentially expressed genes are highlighted *P <0.05, **P <0.01).

Fig. 3. Comparative cytokine gene expression profiles in response to both (A) S. Typhimurium (n=6) and (B) C. jejuni (n=7) in the caecum at 6 hours, 20 hours and 48 hours post-challenge. The horizontal lines within each box plot represents the median fold changes in gene expression in infected chickens relative to the mock-infected controls. Statistical analysis was carried out separately for each infection model to compare challenged and control birds (significantly differentially expressed genes are highlighted *P <0.05, **P <0.01).

Fig. 4. Comparative caecal chemokine gene expression profiles for (A) K60 and (B) IL8, in response to both S. Typhimurium (n=6) and C. jejuni (n=7) 6 hours, 20 hours...
and 48 hours post-challenge. The horizontal lines within each box plot represents the
median fold changes in gene expression in infected chickens relative to the mock-
infected controls. Statistical analysis was carried out separately for each infection
model to compare challenged and control birds (significantly differentially expressed
genes are highlighted * $P <0.05$, ** $P <0.01$).

Fig. 5. IL8 and K60 chemokine gene expression profiles in the caecal tonsil in
response to C. jejuni (n=7) at 6 hours, 20 hours and 48 hours post-challenge. The
horizontal lines within each box plot represents the median fold changes in gene
expression in infected chickens relative to the mock-infected controls (significantly
differentially expressed genes are highlighted * $P <0.05$, ** $P <0.01$).
References


Figure 2

A

B

Relative gene expression

6 hrs
20 hrs
48 hrs

TLR4  TLR5  TLR15  TLR21

TLR4  TLR5  TLR15  TLR21
Supplementary table 1. Real time qRT-PCR primer sequences, Genbank accession number, amplicon sizes and optimum primer concentrations for all genes analysed.

<table>
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<th>Gene Symbol</th>
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<th>Reverse primer (5’-3’)</th>
<th>Accession number</th>
<th>Amplicon Size (bp)</th>
<th>Primer Conc. (nm)</th>
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Supplementary table 2. Real time qRT-PCR median fold change in the caecums of *S. Typhimurium* infected birds (n = 6) relative to mock-infected controls at 6 hours, 20 hours and 48 hours post-challenge. *P* values were determined using Mann-Whitney U test.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>6 hours post-infection</th>
<th>20 hours post-infection</th>
<th>48 hours post-infection</th>
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Supplementary table 3. Real time qRT-PCR median fold change in the caecums of *C. jejuni* infected birds (n = 7) relative to mock-infected controls at 6 hours, 20 hours and 48 hours post-challenge. *P* values were determined using Mann-Whitney U test.

<table>
<thead>
<tr>
<th>Innate immune genes</th>
<th>6 hours post-infection</th>
<th>20 hours post-infection</th>
<th>48 hours post-infection</th>
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<td>Fold change</td>
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Supplementary table 4. Real time qRT-PCR median fold change in the caecal tonsils of *C. jejuni* infected birds (n = 7) relative to mock-infected controls at 6 hours, 20 hours and 48 hours post-challenge. *P* values were determined using Mann-Whitney U test.

<table>
<thead>
<tr>
<th>Innate immune genes</th>
<th>6 hours post-infection</th>
<th>20 hours post-infection</th>
<th>48 hours post-infection</th>
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