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Characterisation of the Innate Immune Response to
Campylobacter jejuni Colonisation in the Chicken
Intestine



A thesis submitted to the University of Dublin for the degree of Doctor of
Philosophy

2011

Ronan Shaughnessy, B.Sc
School of Biochemistry and Immunology
Trinity College Dublin

Supervisor: Professor Cliona O' Farrelly

Declaration

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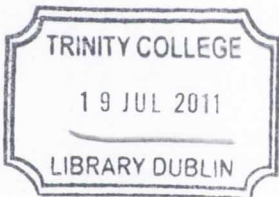


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Acknowledgements

There are many people who have guided and inspired me throughout this journey. Foremost, I need to thank my supervisor, Professor Cliona O' Farrelly, for providing advice, encouragement, criticism and patience over the last few years. Cliona, I feel very privileged to have been one of your students. I am very grateful to Dr Kieran Meade, for guiding me throughout the first two-thirds of this project, and continuing to provide advice from afar at Teagasc. I would also like to thank our bioinformatics guru, Dr. Andrew Lloyd, for mind-broadening conversations. Thanks to Dr. Brenda Allan for her hospitality during my visit to VIDO.

A special thank-you goes out to current and former graduate students of the Comparative Immunology research group. It has been an honour to serve with such a talented group of people. Fernando Narciandi, you have been a constant encyclopaedia of scientific and trivial information. Thanks to Cormac Whelehan, the eternal enthusiast, who has inflicted a constant barrage of scientific debate and laughter upon me. Thanks to Aspinas Chapwanya for comradery: from the beginning, we have been through thick and thin together. Thanks to Nollaig, Aideen, Aoife and the other Ronan, particularly for adding atmosphere and noise to the lab.

Thanks to the other current and former members of the lab, specifically Liz, Nigel, Catherine, Sarah, Emma, Tanya, Anne, Paul and the doctors. You have all provided me with help and nuggets of useful information, at one point or another.

Finally, I would like to show my family some appreciation. Thanks to my parents for sharing with me their genes and memes. You both have been important in shaping my abilities. And last, I acknowledge my three (younger) sisters for providing my life with many chaotic character building experiences!

Publications

Shaughnessy, R. G., Meade, K. G., Cahalane, S., Allan, B., Reiman, C., Callanan, J. J. & O'Farrelly, C. Innate immune gene expression differentiates the early avian intestinal response between *Salmonella* and *Campylobacter*. **Veterinary Immunology and Immunopathology**

Abbreviations

°C	Degrees Celsius
Aa	Amino acid
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
APC	Antigen presenting cell
APP	Acute phase protein
APR	Acute phase response
ARE	AU-rich element
AvBD	Avian beta-defensin
BBSRC	Biotechnology and Biological Sciences Research Council
BLAST	Basic local alignment search tool
BLAT	BLAST-like alignment tool
bp	Base pairs
BRB	Biometric Research Branch
C1QTNF7	CIq and tumor necrosis factor related protein 7
CARD	Caspase-activating and recruitment domain
CD	Cluster of differentiation
cDNA	Complementary DNA
CDT	Cytolethal distending toxin
CFU	Cell forming units
CKC	Chicken kidney cell
CPS	Capsular polysaccharide
CR	Complement receptor
CR	Common reference
CRP	C-reactive protein
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
dH ₂ O	distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotides triphosphate
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated kinases
EST	Expression sequence tag
FDR	False discovery rate
GALT	Gut associated lymphoid tissue
GBS	Guillain-Barré syndrome
GO	Gene ontology
H ₂ O ₂	Hydrogen peroxide
HCL	Hydrochloric acid
HDP	Host defence peptide
HEK	Human embryonic kidney
Hp	Haptoglobin
ICAM	Intracellular adhesion molecule

IEL	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
Iκ-β	I-kappa- β
IKK	I κ B kinase
IL	Interleukin
ILF	Isolated lymphoid follicle
IPAF	Ice protease-activating factor
IRAK	Interleukin-1 receptor associated kinase
IRF	Interferon- γ regulatory factor
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
K	Thousand
LB	Luria-Bertani
LBP	LPS-binding protein
LFA	Lymphocyte function-associated antigen
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
M	Molar
MAL	MyD88-adaptor like
MAPK	Mitogen-activated protein kinase
Mb	Megabase
MH	Meuller Hinton
MHC	Major histocompatibility complex
min	minute
ml	millilitre
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene (88)
NALP	Nacht LLR protein
NaOH	Sodium hydroxide
NET	Neutrophil extracellular trap
NFATC	Nuclear factor of activated T-cells
NF-κB	nuclear factor kappa-B
ng	nanogram
NK	Natural killer cell
NLR	Nod-like receptor
nM	Nanomolar
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NOX1	NADPH oxidase 1
ORF	Open reading frame
PAMP	Pathogen associated molecular protein
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PGN	Peptidoglycan
pH	Power of hydrogen
pi	post infection
PLA2	Phospholipase A2
PlgR	Polymeric Ig receptor

PMB	Polymyxin B
PMN	Polymorphonuclear
PMT	Photo-multiplier tube
PP	Peyer's patches
PRR	Pattern recognition receptor
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RLR	Rig-like receptor
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
SAA	Serum Amyloid A
SCC	Saline-sodium citrate
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
STAT	Signal transducers and activator of transcription
TAK	Transforming growth factor-activated protein kinase
TCR	T cell receptor
TGFB	Transforming growth factor B
TIGR	The Institute for Genomic Research
TIR	Toll/interleukin-1 receptor
TOPK	Lymphokine-activated killer T-cell-originated protein kinase
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
Tollip	Toll interacting protein
TRAF	TNF-receptor associated factor
TRAM	TRIF-related adapter molecule
TRIF	TIR-domain-containing adapter-inducing interferon- β
UMIST	University of Manchester Institute of Science and Technology
VCAM	Vascular cell adhesion molecule
VIDO	Vaccine and Infectious Disease Organization
μg	microgram
μl	microlitre
μM	micromolar

Abstract

Campylobacter jejuni is the leading cause of gastroenteritis in humans, and chickens are the primary vector for infection. The microbe colonises the caeca of birds as a commensal, and it is hypothesised that intestinal defences regulate colonisation. Identifying birds with enhanced abilities to clear *C. jejuni* would enable the selective breeding of colonisation resistant birds. However, the caecal immune response to *C. jejuni* has been poorly characterised to date and is the basis of this thesis.

Initially, to study the immune response, expression of genes associated with bacterial recognition and innate immune response was profiled in the caeca of *C. jejuni*-challenged birds across a 48 h timecourse. A *S. Typhimurium* infection model was used as a comparison. In response to *S. Typhimurium*, *TLR4*, *TLR5*, *TLR15* and *TLR21* were transiently increased while substantial cytokine and chemokine increases were evident at each interval. In contrast, only *TLR4* and *TLR21* were transiently increased (at 6 h) in response to *C. jejuni* while proinflammatory cytokine increases were not evident until 20 h post-challenge, in which high *IL8* with minor *IL1 β* and *K60* increases were evident. *IL8* was reduced at 48 h and comparable to minor *IL1 β* and *IL6* increases evident at this interval.

Whole-genome microarrays were used to characterise further the caecal immune response to *C. jejuni* at 20 h, at which time 270 genes were found to be differentially expressed. Fold-changes were small and represented a wide range of biological processes including cell growth, nutrient metabolism and immunological activity. Immunological processes were validated by qRT-PCR, in which showed only minor *NOX1* (2.3-fold) and *VCAM* (1.5-fold) increases were evident. Other means of normalisation and analysis confirmed the lack of immunological processes although *IL17* (3.6-fold), *IL21* (2.1-fold) and *TOPK* (1.9-fold) increases were evident upon validation. Further qRT-PCR analysis revealed significantly increased *IL7R* (4-fold) and *CTLA4* (2.5-fold) gene expression ($P < 0.05$). Thus, *C. jejuni* had minimal effects on global gene expression at 20 h post-challenge but potentially induced subtle T cell activity.

The lack of increased caecal pro-inflammatory gene expression in response to *C. jejuni* suggests that the microbe evades or subverts avian defences, possibly via

several of its components. To explore this hypothesis, chicken cells were challenged with *C. jejuni* recombinant cytolethal distending toxin (CDT), capsular polysaccharide (CPS) and ultrapure lipooligosaccharide (LOS). LOS was obtained from *C. jejuni* grown at both 37°C and 42°C. LOS induced cytokine (>700-fold) gene expression in chicken HD11 macrophages, which was a result of NF- κ B, p38, ERK and JNK activation. LOS also upregulated *TLR15* gene expression (15-fold) in HD11 cells, through ERK and JNK activation. Chicken embryonic fibroblast (CEF) cells responded differently to the LOS types; 37°C LOS, but not 42°C LOS, induced proinflammatory gene expression. Thus, in a 42°C environment, such as the chicken intestine, *C. jejuni* may fail to induce proinflammatory gene expression in certain cell types. Furthermore, CPS failed to induce cytokine and chemokine gene expression in either HD11 or CEF cells, which suggest that capsulated *C. jejuni* evades PRR detection. CDT was also poorly inductive of proinflammatory responses in chicken cells however, 80% of HD11 cells had undergone apoptosis at 48 h post toxin challenge. It is thus improbable that *C. jejuni* expresses this toxin in the chicken caecum, considering that commensalism is evident.

In conclusion, *C. jejuni* activates minimal immunological processes during the early stages of caecal colonisation. Gene expression increases for key mediators of innate immune response and bacterial clearance are lacking. Global gene expression analysis further reveals minimal immunological activity although subtle innate T cell responses are possibly initiated. The lack of immunopathology is likely due to structural and behavioural aspects of *C. jejuni*. Capsulated *C. jejuni* are not likely to activate intestinal PRRs as this outermost layer is immunologically inert. The LOS component activates TLR4, but may evade an additional PRR. CDT is not a PAMP although chicken cells are susceptible to its cytotoxic effects. The fact that cytotoxicity is not evident *in vivo* suggests that *C. jejuni* exhibits a commensal lifestyle during caecal colonisation. Whether birds have evolved immune systems which evade the effects of *C. jejuni* or whether *C. jejuni* has evolved in order to survive as a commensal in its avian host will require detailed interactive studies

Chapter 1

General Introduction

Chapter 1: General Introduction

1.1 Microbiology of the intestine

Immediately after birth, humans and other animals are exposed to a diverse range of microbial species. Through consumption of ordinary food and water; microbes such as nematodes, fungi, protozoa and bacteria enter the alimentary canal. Most ingested species are benign, have minimal interactions with intestinal tissue, and occupy the gut lumen only temporarily. Their inability to colonise is partially due to host factors including pH, gastric and bile secretions, gut peristalsis, mucins and nutrient availability (Manson *et al.*, 2008).

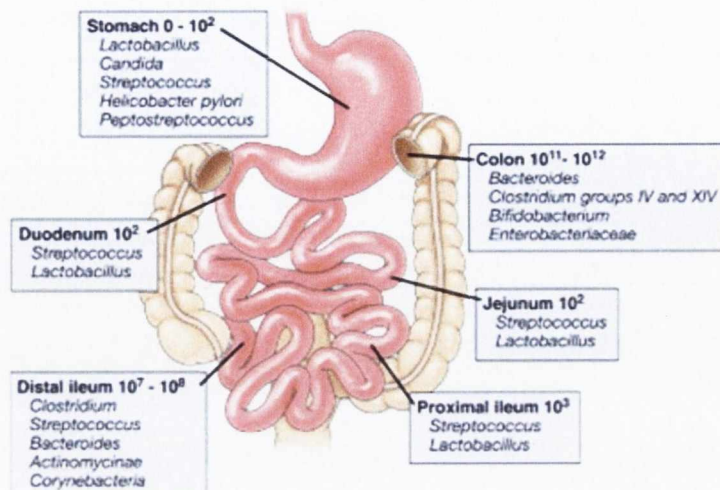
Other benign bacterial species appear to have overcome the intestinal environment, and colonise the mucosal surfaces of the gut, particularly the large intestine. Collectively they are known as the “microbiota”, which consists of approximately 10^{14} cells, more than the sum of all the host cells (Manson *et al.*, 2008). Despite such high numbers, at least 80% of the intestinal microflora cannot be easily cultivated outside the host (Hattori & Taylor, 2009), which illustrates their dependence and adaptation to the intestinal environment. Over 500 bacterial species have been identified as colonisers of the human intestine to date (Pai & Kang, 2008), and the Human Microbiome Project (HMP), funded by the National Institutes of Health (NIH), aims to identify the total number of species (Peterson *et al.*, 2009). It is, however, estimated that 99% of the microflora consists of only 35 species, mainly representing the *Bacteroides*, *Bifidobacteria*, *Eubacteria*, *Fusobacteria* and *Lactobacilli* genera (Guarner, 2006; Manson *et al.*, 2008). There is evidence that many of these microorganisms promote intestinal health. Some bacterial species can ferment carbohydrates to produce short chain fatty acids (Tuohy *et al.*, 2006), while others can synthesise vitamin K and B12 (Hill, 1997). Commensal bacteria also deter pathogenic colonisation through competition for space and nutrients. Furthermore, commensals produce bacteriostatic molecules, known as bacteriocins, which are capable of inhibiting the growth of rival commensal and pathogenic bacterial species (Guarner, 2006). Administration of broad-spectrum antibiotics reduces the enteric microflora and renders the intestine more prone to pathogenic assault (Viswanathan *et al.*, 2009). The residential microflora constantly interacts with the intestinal immune

system. Such interactions result in the intestine being in a “controlled state of inflammation” under homeostatic conditions (Fiocchi, 2003), in which optimal performance of the gut-associated lymphoid tissue (GALT) is apparent (Kelly *et al.*, 2005).

Pathogenic bacterial species do not reside solely in proximity to mucosal surfaces. In addition, they can penetrate intestinal tissues, inducing inflammation and damage in the process. Pathogenic species include *Listeria monocytogenes*, *Salmonella enterica*, Enterohemorrhagic *Escherichia coli* and *Shigella flexneri*. Unlike residential microflora, these species have clusters of virulence genes, known as “pathogenicity islands”, on their chromosomes. (Dobrindt *et al.*, 2003). These virulence genes encode adhesins that enable attachment to the host cell and thus colonisation, invasion mechanisms that enables microbes to traverse the intestinal barrier and toxins that disturb normal cellular organisation and functions (de Souza, 2003). Such bacterial strategies result in the destabilisation of the intestinal environment and contribute to the clinical signs of gastroenteritis (Viswanathan *et al.*, 2009).

However, the status of a microorganism as a commensal or a pathogen does not necessarily depend alone on the absence or presence of virulence genes. Many bacterial species are commensals in their normal animal hosts but are pathogenic to humans. The primary hosts of many of these microorganisms are domestic farm animals. Interactions with such microbes are associated with the consumption of undercooked or contaminated food and therefore are not normally encountered on a regular basis. *Campylobacter jejuni* is regarded as a commensal in chickens yet is the leading cause of gastroenteritis in humans (Hendrixson & DiRita, 2004).

(A)



(B)

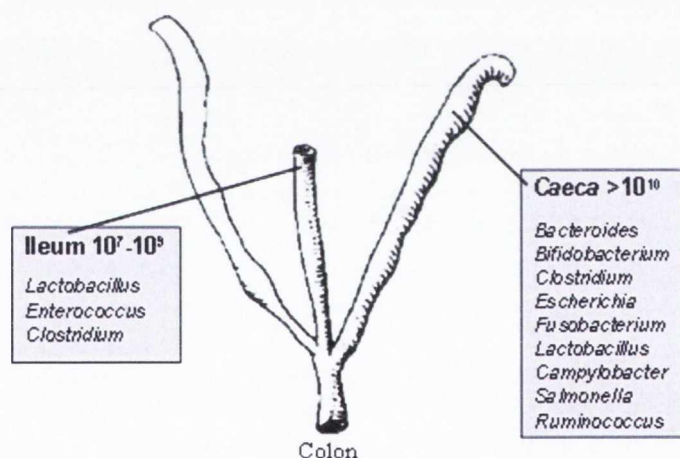


Figure 1.1 Similar commensal bacterial organisms colonise both human and chicken gastro-intestinal tract regions albeit some differences are evident. (A) is adapted from Sartor, 2008 (B) depicts bacterial levels in the chicken ileum (10^7 - 10^9 CFU) and caeca (10^{10} CFU) (Apajalahti *et al.*, 1998; Bjerrum *et al.*, 2006), as well as bacterial groups (Amit-Romach *et al.*, 2004; Bjerrum *et al.*, 2006; Gong *et al.*, 2007).

1.2 *Campylobacter jejuni*

Campylobacter (Greek word meaning “spirally curved”) is an enteric bacterial genus that first gained notoriety in the 1970s due to the ability of its members to cause gastroenteritis (Fig. 1.2 A). Members of the genus are gram-negative and have unipolar or bipolar flagella. They are obligate microaerophiles, utilising no more than 5-10% oxygen (Crushell *et al.*, 2004). To date, 17 species have been identified. *C. coli* and *C. jejuni* are pathogenic to humans with the latter species being responsible for the majority of clinical cases (Humphrey *et al.*, 2007). As few as 800 viable *C. jejuni* cells are capable of causing infection (Black *et al.*, 1988). The clinical signs of infection include fever, nausea, abdominal pain and diarrhoea. In severe cases, the stools commonly contain blood, pus or mucus (Crushell *et al.*, 2004).

C. jejuni is often carried asymptotically in the intestines of pigs, cattle, sheep, and birds (Humphrey *et al.*, 2007). Poultry are considered natural hosts as *C. jejuni* grows optimally at 42°C, the metabolic temperature of birds. Also, *C. jejuni* is highly adapted to the avian intestine, primarily in the mucus layer associated with the caecal crypts (Beery *et al.*, 1988). As little as 30 CFU of *C. jejuni* can generate 10¹⁰ CFU/g of caecal contents in the absence of clinical signs (Newell, 2001). The bacterial species is transmitted horizontally between chickens, and not vertically from mother to offspring prior to hatching (Newell & Fearnley, 2003). Entire flocks can be almost completely colonised within days (Corry & Atabay, 2001). Thus, approximately 90% of commercial flocks are colonised by *C. jejuni* (Newell & Fearnley, 2003). At slaughter, contamination of poultry products can occur, making chickens a major vector for human infection (Humphrey *et al.*, 2007). Considering that poultry products are particularly popular, *C. jejuni* is now considered the leading cause of food-borne gastroenteritis in the developed world (Hendrixson & DiRita, 2004). Campylobacteriosis is therefore a considerable burden to the poultry industry and national healthcare systems. For example, more than 2.5 million cases in the US and 80,000 cases in the Netherlands are reported annually, resulting in economic costs of US\$4.3 billion and €21 million respectively. Considering that only 10% of infections are reported, incidences and economic costs are likely to be even higher (Humphrey *et al.*, 2007).

In comparison to other enteric microbes, little is known about *C. jejuni*'s molecular biology and its behaviour during its asymptomatic or pathogenic states. Sequencing the *C. jejuni* genome in 2000 has somewhat helped to characterise the microorganism. The genome of *C. jejuni* is particularly small (1.6 Mb in size), in comparison to other microorganisms (Parkhill *et al.*, 2000). However, high genetic variability is evident, which is a result of horizontal DNA exchange among different *C. jejuni* strains (Wilson *et al.*, 2003). Analysis of the genome has also revealed a number of virulence traits, despite the organism's asymptomatic nature in its primary hosts.

The outermost layer of *C. jejuni* consists of capsular polysaccharide (CPS) (Karlyshev *et al.*, 2000), which has roles in cell adhesion and cell invasion in mammals (Bachtiar *et al.*, 2007). This capsular component is also required for optimal *C. jejuni* colonisation of the chicken intestine (Bachtiar *et al.*, 2007; Jones *et al.*, 2004). The underlying cell membrane contains an endotoxin component known as lipooligosaccharide (LOS), which consists of oligosaccharide (OS) linked to toxic lipid A (Moran & Penner, 1999) (Fig. 1.2 C). LOS promotes adhesion to and invasion of human epithelial cells but does not appear to influence colonisation levels in the chicken intestine (Fry *et al.*, 2000).

The outer membrane of *C. jejuni* also consists of a number of adhesin proteins, which are important for attachment to human epithelial cells. These include FlpA (Konkel *et al.*), JlpA (Jin *et al.*, 2001), CapA (Ashgar *et al.*, 2007) and CadF (Konkel *et al.*, 1997). The latter two adhesins are critical for chicken intestinal attachment as CadF and CapA mutants are unable to colonise caecum (Ashgar *et al.*, 2007; Ziprin *et al.*, 1999).

C. jejuni produces a number of virulence proteins associated with human epithelial cell invasion including VirK (Novik *et al.*, 2009), *Campylobacter* invasion antigen B (Konkel *et al.*, 1999) and CiaC (Christensen *et al.*, 2009). CiaB is also required for *C. jejuni* colonisation of chicken caecum (Ziprin *et al.*, 2001). *C. jejuni*'s flagella, which primarily enable motility (Hendrixson, 2008), may also be considered as a virulence component as it has also been implicated in mammalian cell invasion and chicken colonisation (Young *et al.*, 2007).

C. jejuni is known to produce cytolethal distending toxin (CDT), a heat-labile protein common to many other pathogenic gram-negative bacteria including *Shigella* species, *Helicobacter* species and *Salmonella enterica* serovar Typhi (Ge *et al.*,

2008a). The toxin is composed of three subunits, known as CdtA, CdtB and CdtC. CdtB is the active subunit, which is delivered into mammalian cells by CdtA and CdtC (Smith & Bayles, 2006). CdtB, which has DNase I-like properties, reaches the nucleus and induces chromosomal damage, such as double-strand-breaks (Fig. 1.2 C). As a consequence, cell division is halted until DNA damage is repaired. Different cell cycle phases are, however, arrested with different cell types (Ge *et al.*, 2008b; Lara-Tejero & Galan, 2002). Recombinant *C. jejuni* CDT arrests epithelial (HeLa and Caco-2) cell division at the G₂ phase between 24 hours and 48 hours post-challenge (Whitehouse *et al.*, 1998). In contrast, human fibroblasts undergo cell arrest at both G₁ and G₂ phases upon encountering *C. jejuni* CDT (Hassane *et al.*, 2003). Cell death subsequently follows, regardless of the cell division phase arrested (Ge *et al.*, 2008b). Apoptosis is evident when human monocytes are exposed to *C. jejuni* CDT, as a result of caspase-8 and caspase-9 activation (Hickey *et al.*, 2005). Other leukocytes are also likely targeted, considering that CDT from other bacterial species induce apoptosis in B and T lymphocytes (Cortes-Bratti *et al.*, 2001; Shenker *et al.*, 2001). It is unknown whether *C. jejuni* CDT has cytotoxic effects on chicken cells.

While CDT toxin from *Shigella* contributes to clinical signs in the mouse colon, little is known about the role of CDT in *C. jejuni* pathogenesis (Duong & Konkel, 2009). It is a mystery that *Campylobacter* species have retained such a toxin considering that it is benign in nature in its primary avian, swine and bovine hosts (Young *et al.*, 2007). CDT transcripts are detectable in the caeca of colonised chickens but with an absence of CDT specific antibodies in the serum of such birds (Abuoun *et al.*, 2005). Mutating each of the corresponding three *cdt* genes does not affect colonisation levels in the caecum, suggesting that CDT does not have a role in colonisation (Biswas *et al.*, 2006). The role of CDT in the development of the commensal state in chickens has not been investigated.

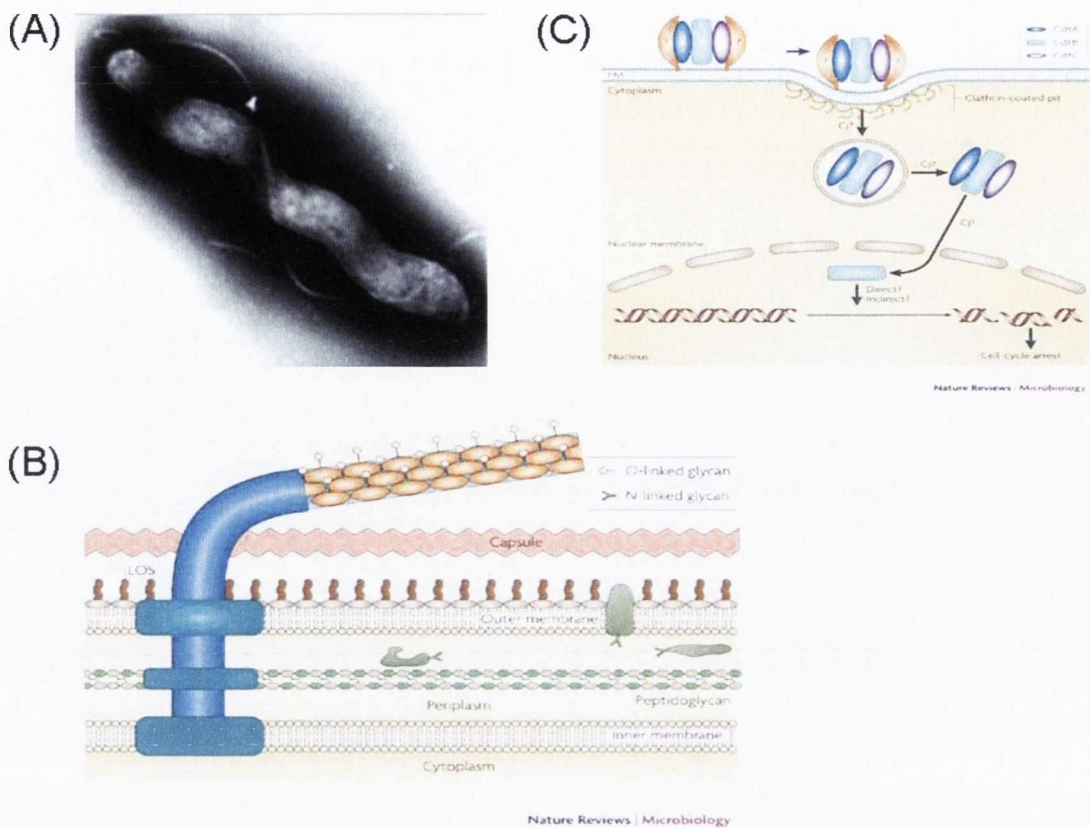


Figure 1.2 *Campylobacter jejuni*. (A) Electron microscope image of *C. jejuni* from www.sanger.ac.uk/Projects/C_jejuni/ depicts the microbe's spirally-curved morphology (B) Major surface components of *C. jejuni* include the flagellum, capsule, LOS, lipopeptides and peptidoglycan. (C) CDT docks to an unknown receptor on the host cell surface via CdtA and CdtC, and is subsequently internalised. CdtB reaches the nucleus and induces chromosomal damage, namely double-stranded breaks DNA. Cj? indicates the molecular interactions that have been discovered using CDT from bacterial species other than *C. jejuni*. (B) and (C) are adapted from Young *et al.*, 2007.

1.3 The immune response

While a microorganism's level of pathogenicity is largely dependent on its effect upon its host, the immune response encountered has a profound effect on whether that microorganism is deemed a pathogen or a non-pathogen (Casadevall & Pirofski, 2003). Initially, microbial species are detected by the host innate immune system.

1.3.1 Pattern recognition receptors

Conserved microbial components, known as pathogen-associated molecular patterns (PAMPs), are sensed by the innate immune system through families of germ-line encoded pattern recognition receptors (PRRs) (Carpenter & O'Neill, 2007). These include the Toll-like receptors (TLRs), the Nod-like receptors (NLRs) and the Rig-like receptors (RLRs). The latter family has been shown to recognise only viral components while TLRs and NLRs are both important in the detection of bacterial and viral components (Mogensen, 2009).

1.3.1.1 Toll-like receptors

The TLRs have been well characterised in mammals. TLR3, TLR7 and TLR9 are located intracellularly in endoplasmic vesicles and recognise viral nucleic acids (Kawai & Akira, 2008). TLR9 recognises bacterial CpG, and thus detects intracellular bacterial species. TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cell surface, and mainly detect bacterial components. TLR2 can dimerise with either TLR1 or TLR6 to recognise triacyl lipopeptides and diacyl lipopeptides respectively (Kumar *et al.*, 2009). TLR4 together with its accessory molecules, CD14 (which binds to LPS) and MD2 (required for responsiveness), recognise lipopolysaccharide (LPS), a major constituent of enteric gram-negative bacteria (Shimazu *et al.*, 1999; Wright *et al.*, 1990). TLR5 detects flagellin and thus, is a key sensor flagellated bacterial species (Mogensen, 2009).

Each TLR is composed of a leucine-rich repeat (LRR) domain (either located extracellularly or within endosomes), which confers ligand recognition. This domain is linked by a transmembrane domain to an intracellular Toll/IL-1 region (TIR), which propagates intracellular signalling (Carpenter & O'Neill, 2007). Ligand recognition results in structural changes of the TIR region, allowing the recruitment of TIR domain containing adapter molecules MyD88, MyD88-adapter like (MAL), TIR-domain-containing adapter-inducing interferon- β (TRIF) and TRIF-related adapter molecule (TRAM) (Akira *et al.*, 2006). All TLRs use the MyD88 pathway except TLR3, which only uses TRIF. TLR4 also has a MyD88 independent pathway, in which it uses TRAM and TRIF sequentially (Kumar *et al.*, 2009). These TRIF dependent pathways for TLR3 and TLR4 lead to the activation of Interferon regulatory factor (IRF) 3 and IRF7, which results in Type 1 Interferon (IFN) upregulation (Mogensen, 2009). Regarding the MyD88 dependent pathways, MyD88 recruits IRAK family members, which subsequently associates with TRAF6 (Kumar *et al.*, 2009). TRAF6 mediates the activation of transforming growth factor-activated protein kinase 1 (TAK1) which is subsequently involved in two different pathways (Mogensen, 2009). Firstly, TAK1 activates the IKK complex that results in phosphorylation of I κ B in the cytoplasm (Watters *et al.*, 2007). Phosphorylation of I κ B leads to its polyubiquitination and subsequent degradation (Hayden & Ghosh, 2004). Degradation of I κ B allows the translocation of NF- κ B to the nucleus where it binds to the promoter regions of proinflammatory genes (Hayden & Ghosh, 2004). Secondly, TAK1 activation also leads to activation of mitogen-activated protein kinase (MAPK) pathways, namely ERK, JNK and p38, which also contribute to proinflammatory cytokine upregulation (Fig. 1.3) (Kumar *et al.*, 2009).

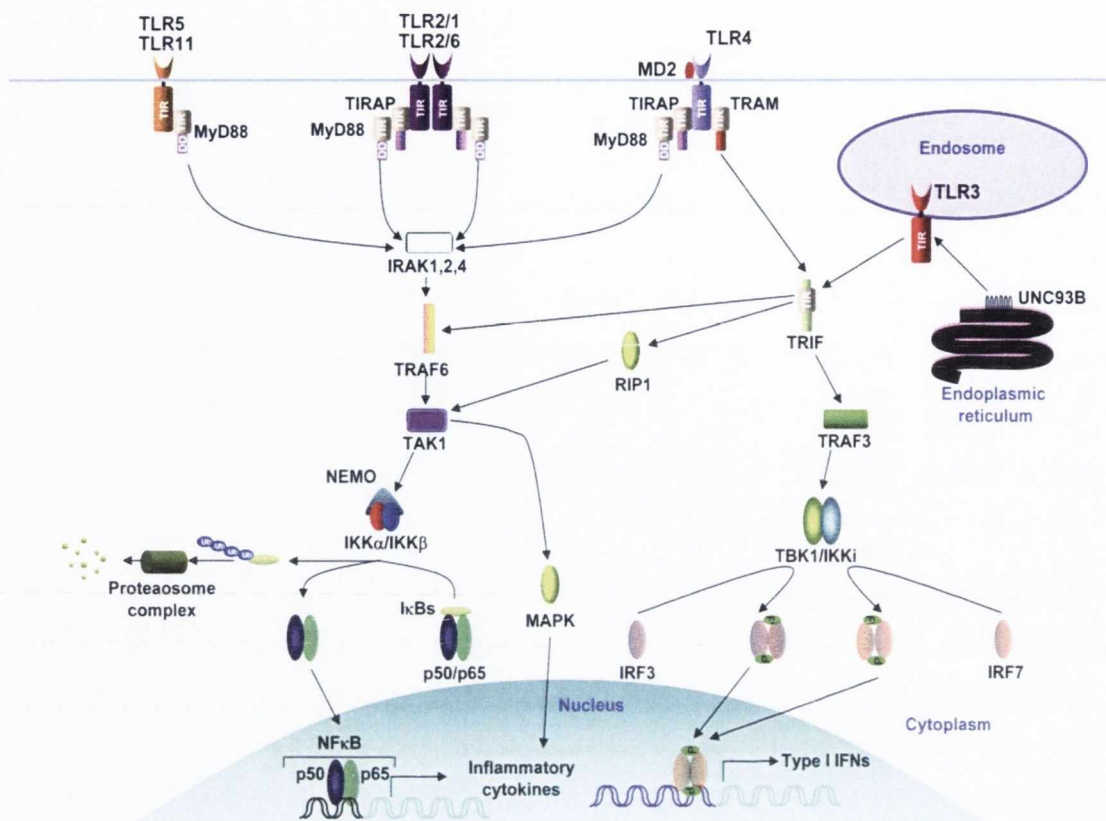


Figure 1.3 TLR signalling pathways. TLR activation may initiate MyD88 or MyD88-independent signalling pathways. The former pathway results in the activation of NF-κB and MAPK, which results in proinflammatory cytokine gene expression upregulation. The latter pathway primarily results in the activation of IRF transcription factors, resulting in type I IFN gene expression upregulation. Adapted from Kumar *et al.* 2009.

1.3.1.2 Nod-like receptors

The Nod-like receptors (NLRs) are another important PRR family. These are cytosolic proteins that are regarded as key detectors of intracellular microorganisms (Mogensen, 2009). Nucleotide-binding oligomerization domain (NOD) 1 and NOD2 are the best characterised NLR proteins. They have been implicated in the recognition of intracellular bacterial species. NOD1 recognises iE-DAP, a structural component of peptidoglycan (PGN) produced by gram-negative invasive bacteria such as *S. flexneri* and *E. coli* (Fritz *et al.*, 2006). However, non-intracellular species may also be recognised. For example, *Helicobacter pylori* activates NOD1 when it injects PGN into host cells using its Type IV secretion system (Viala *et al.*, 2004). NOD2 recognises muramyl dipeptide, a peptidoglycan component of both gram-positive and gram-negative bacteria (Kaparakis *et al.*, 2007). Each of the NODs consists of a LRR region that is required for microbial recognition, a NOD domain with a role in self-oligomerization and N-terminal regions involved in downstream signalling (Kaparakis *et al.*, 2007). Activation of NOD1 and NOD2 pathways results in NF- κ B and MAPK activation (Fig. 1.4 A) (Kaparakis *et al.*, 2007).

Nacht LRR protein (NALP) 1, NALP3 and Ice protease-activating factor (IPAF) are another important group of NLRs, and are components of multiprotein complexes known as inflammasomes. NALP1 and IPAF recognise MDP and flagellin respectively (Kumar *et al.*, 2009; Mogensen, 2009). NALP3 responds to a range of ligands including MDP, toxins, as well as bacterial and viral RNA (Kumar *et al.*, 2009). Activation of these NLRs results in the conversion of pro-caspase-1 to its active form, caspase 1 (Carneiro *et al.*, 2008). This enzyme is important for the cellular secretion of important proinflammatory cytokines, namely interleukin-1 β (IL-1 β) and IL-18. NF- κ B activation by TLRs and other NLRs leads to the production of pro-IL-1 β and pro-IL-18 proteins, which are retained within the cells. Caspase-1 cleaves these proteins to their mature forms, allowing their cellular release (Fig. 1.4 B) (Martinon *et al.*, 2002).

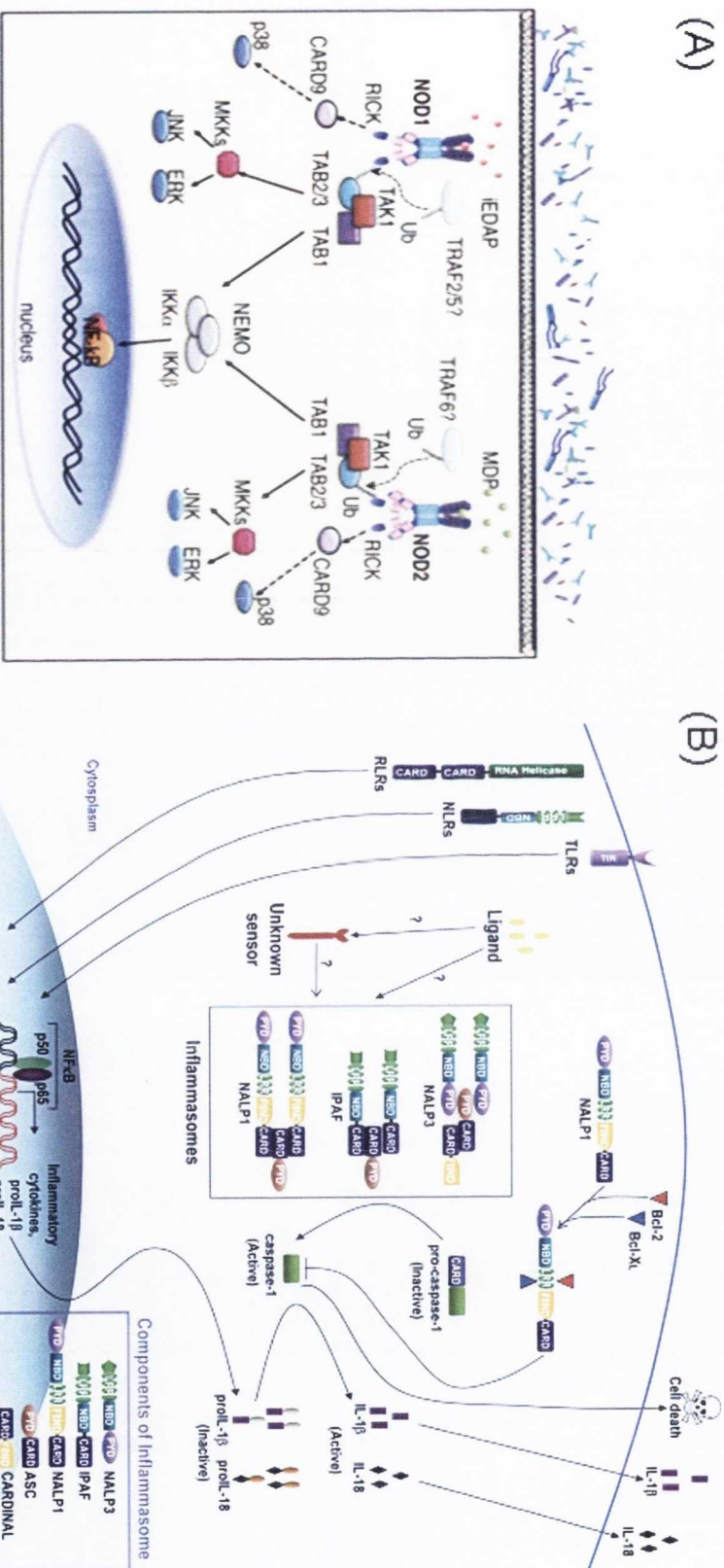


Figure 1.4 NLR signalling pathways. **(A)** NOD1 and NOD2 activation consequently results in the activation of NF-κB and MAPK, resulting in proinflammatory cytokine gene expression upregulation. Adapted from (Shaw *et al.*, 2008) **(B)** NALP1, NALP3 and IPAF activation enables pro-caspase-1 to be converted to caspase-1. Caspase 1 cleaves pro-IL-1β and pro-IL-18 into their mature forms, which are biologically active. Adapted from Kumar *et al.*, 2009.

1.3.2 Effectors of the innate immune response

Multiple innate immune effectors are transcribed and translated as a result of microbial recognition. One group, the cytokines, are key components of the innate immune response. These are a group of diverse proteins that mediate communication between cells. They have autocrine, paracrine and endocrine targets. Cytokines engage their respective receptors, which leads to intracellular signalling cascades that result in the upregulation or downregulation of specific gene expression. A diverse range of changes can occur including cellular proliferation and differentiation, chemotaxis of cells, further cytokine production, transient expression of molecules that promote inflammation and antimicrobial activity of infected tissues, as well as measures to control the level of inflammation (Janeway Charles, 2001). Cytokines associated with the inflammatory response include Tumour Necrosis Factor- α (TNF- α), IL-1 β , IL-6 and IL-8 while IL-10 and TGF- β are considered key anti-inflammatory cytokines.

1.3.2.1 Tumour Necrosis Factor- α

TNF- α is induced through MAPK pathways, such as p38 and ERK. Macrophages and monocytes release high levels of this cytokine in response to bacterial LPS. TNF- α has multiple proinflammatory effects which include neutrophil and macrophage activation, E-selectin and ICAM-1 upregulation (collectively resulting in endothelial activation), enhancement of vascular permeability and induction of other proinflammatory cytokines. It also induces anti-inflammatory IL-10, which in turn suppresses TNF- α production (Papadakis & Targan, 2000).

1.3.2.2 Interleukin-1 beta

The release of mature IL-1 β from cells requires the co-activation of NF- κ B and the inflammasome (Fig. 1.4 B) (Martinon *et al.*, 2002). Macrophages, endothelial cells, epithelial cells and fibroblasts are sources of IL-1 β that is involved in response to various stimuli including LPS, TNF- α and IL-1 β itself (Moller & Villiger, 2006).

IL-1 β induces endothelial cell activation and neutrophil activation, while also having pyrogenic effects. Secreted IL-1 β is regulated by several decoy receptors such as IL-1Ra. IL-1Ra binds to IL-1 β and neutralises its proinflammatory effects (Allantaz *et al.*, 2007).

1.3.2.3 Interleukin-6

LPS, IL-1 β and TNF- α are potent inducers of IL-6 (Park & Pillinger, 2007). Sources include fibroblasts, monocytes, macrophages and activated endothelial cells (Moller & Villiger, 2006). IL-6 induces B-cell differentiation and also has pyrogenic effects. It stimulates hepatocytes in the liver to produce acute phase proteins, namely C-reactive protein and serum amyloid A, which are major mediators of inflammation (Park & Pillinger, 2007). IL-6 also has anti-inflammatory effects. It inhibits proinflammatory IL-1 β and TNF- α while activating anti-inflammatory IL-1Ra and IL-10 (Papanicolaou *et al.*, 1998).

1.3.2.4 Interleukin-10

IL-10 is from a family of cytokines with anti-inflammatory properties. IL-10 is capable of inhibiting proinflammatory cytokine (including IL1 β and IL-6) and chemokine (including IL-8) production from mononuclear phagocytes. The cytokine can also induce the expression of cytokine antagonists (Moore *et al.*, 2001). IL-10 promotes the degradation of inflammatory gene mRNA by targeting AU-rich elements (ARE) in the 3' untranslated region of the message (Kishore *et al.*, 1999). IL-10 is also capable of stabilising IL-1Ra (Cassatella *et al.*, 1994).

1.3.2.5 Transforming growth factor- β

Transforming growth factor-beta 1 (TGF- β 1) is a pleiotropic cytokine involved with regulating growth, differentiation and apoptosis of cells. It targets diverse cells types associated with processes such as embryonic development, wound

healing (Herpin *et al.*, 2004) and maintenance of the neuromuscular system (McLennan & Koishi, 2002). TGF- β 1 is also an essential regulator of the proinflammatory immune response, as mice lacking TGF- β 1 are susceptible to severe inflammation (Shull *et al.*, 1992). TGF- β 2 and TGF- β 3 have also been identified in mammals although both have fewer anti-inflammatory roles than TGF- β 1 (Li *et al.*, 2006).

While TGF- β 1 is best characterised as a regulator of cells of the adaptive immune response, the cytokine can also target components of the innate immune response. TGF- β 1 blocks LPS-induced activation of proinflammatory cytokines by downregulating CD14 gene expression in macrophages (Imai *et al.*, 2000). TGF- β 1 also promotes the degradation of MYD88 at the protein level, thus interfering with TLR mediated NF- κ B activation (Naiki *et al.*, 2005).

1.3.2.6 Interleukin-8

Chemokines are a family of cytokines that play a role in chemotaxis. While some family members are constitutively expressed fulfilling homeostatic roles, others are transiently induced and recruit inflammatory cells to sites of infection or injury (Laing & Secombes, 2004). IL-8 is an important inducible chemokine of the innate immune response, which is induced through several pathways including NF- κ B, ERK and p38. It is induced by a range of stimuli including LPS, H₂O₂, IL-1 β and TNF- α , and is upregulated by a variety of cells including endothelial, epithelial, monocytes, macrophages and neutrophils (Hoffmann *et al.*, 2002). IL-8 primarily recruits proinflammatory cells such as monocytes, neutrophils and T cells to sites of its induction upon binding to cell surface receptors, CXCR1 and CXCR2 (Hay & Sarau, 2001).

1.3.2.7 Host defence peptides

Host defence peptides (HDPs), also known as antimicrobial peptides (AMPs), are important effectors of the innate immune response. While some are constitutively expressed, others are inducible in response to direct PRR recognition (Linde *et al.*,

2008). The MAPK and NF- κ B pathways have been implicated as mediators of HDP upregulation. In human gingival epithelial cells, HDP upregulation in response to *Fusobacterium nucleatum* (a commensal bacterial species) was shown to be dependent on the p38 and JNK pathways (Krisanaprakornkit *et al.*, 2002). In intestinal epithelial cells challenged with peptidoglycan, HDP upregulation is mediated by both the JNK and NF- κ B pathways (Vora *et al.*, 2004). In addition, HDPs can be induced in response to proinflammatory cytokines. TNF- α and IL-1 β induce HDP upregulation in nasal, tracheal and bronchial cells (Harder *et al.*, 2000). IL-17 induces HDP production through the JAK/STAT and NF- κ B pathways (Kao *et al.*, 2004). Translation of HDP genes results in the synthesis of inactive pro-peptides, which are retained within the cell. Proteolytic processing of the pro-peptides is required for cellular release of the biologically active forms (Lai & Gallo, 2009).

The mature peptides are between 12 and 50 amino acids in length and consist of a number of positively charged residues (Lai & Gallo, 2009). Cathelicidins and defensins are the two main types of HDPs in animal species with the latter divided into α -defensins, β -defensins and θ -defensins (Sang & Blecha, 2008). Cathelicidins are found in a range of vertebrates including sheep, goats, rodents, primates and birds (Tomasinsig & Zanetti, 2005). θ -defensins are exclusive to non-human primates while β -defensins are evident in all animal species, and are believed to have given rise to α -defensins (Semple *et al.*, 2006). α -defensins are found only in mammals of the super-order Euarchontoglires (Patil *et al.*, 2004) and therefore, are evident in primates and rodents but not domestic farm animals which are generally in the super-order Laurasiatheria.

Cathelicidins are produced by neutrophils, macrophages and epithelial cells (Bals & Wilson, 2003). α -defensins are produced mainly by neutrophils and Paneth cells while β -defensins are produced by epithelial cells, and leukocytes including neutrophils, natural killer cells, monocytes and macrophages (Holzl *et al.*, 2008).

HDPs have antimicrobial properties against fungal, protozoal, viral and bacterial organisms (Sang & Blecha, 2008). Using electrostatic forces, cationic HDPs can insert themselves into negatively charged bacterial membranes, resulting in the displacement of lipids, and subsequent pore formation. This membrane disruption results in cell death (Brown & Hancock, 2006). HDPs can also inhibit the nucleic acid synthesis, protein synthesis and enzymatic activity of bacterial species (Brogden, 2005). HDPs also have indirect effects on microbial infection. A human cathelicidin,

LL-37, binds to formyl peptide receptor-like 1 (FPRL1) on the cell surface of monocytes, neutrophils, mast cells and T cells. As a result, such cells are recruited to sites of LL-37 induction (Holzl *et al.*, 2008). HDPs can also modulate immune responses. Several HDPs induce proinflammatory cytokine (including IL-2 and IFN- γ) and chemokine (including IL-8) production, while others prevent the transcriptional upregulation of proinflammatory cytokines like IL-6 and TNF- α (Brown & Hancock, 2006). Many other functions continue to be ascribed to HDPs, some of which are illustrated in figure 1.5.

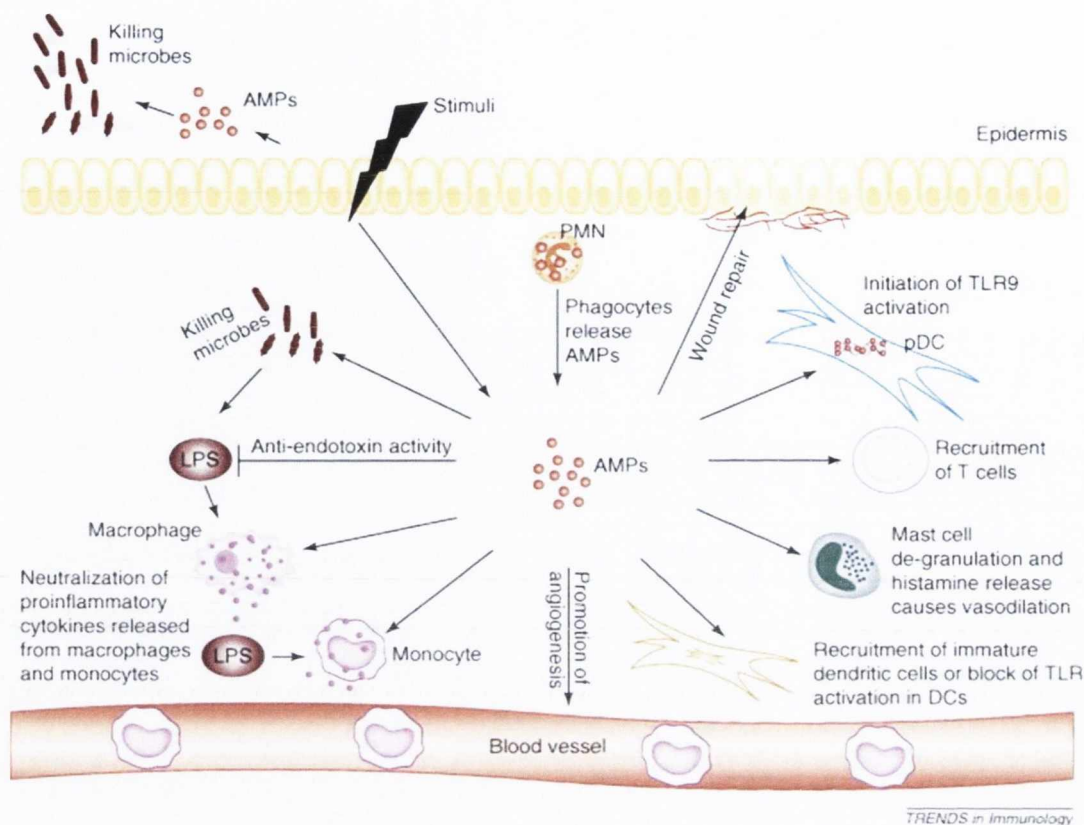


Figure 1.5 Multiple functions of HDPs during infection. HDPs (also known as AMPs) are primarily associated with microbial killing but also have roles in angiogenesis promotion, wound healing, leukocyte recruitment, leukocyte activation and proinflammatory cytokine initiation and inhibition. Adapted from Lai & Gallo, 2009.

1.3.2.8 Acute phase proteins

TNF- α , IL-1 β and IL-6 that enter the circulation during inflammation, stimulate liver hepatocytes to produce glycoproteins known as acute phase proteins (APPs). Subsequently, serum increases of these APPs by greater than 25% has systemic effects on the host, serving to contain and resolve local infection and inflammation (Gruys *et al.*, 2005).

C-reactive protein (CRP), serum amyloid A (SAA) and haptoglobin (Hp) are three APPs. CRP binds phosphocholine of a range of bacterial species but also phospholipids and other components of damaged host cells resulting in their opsonisation, thus having roles in both bacterial clearance and damage resolution respectively (Gabay & Kushner, 1999; Murata *et al.*, 2004). Monocytes stimulated with CRP secrete tissue factor and proinflammatory cytokines (Gabay & Kushner, 1999). CRP also has anti-inflammatory effects on neutrophils, preventing their recruitment and ROS production (Mortensen & Zhong, 2000). SAA binds LPS and recruits T lymphocytes. It also has inhibitory effects on neutrophils, similar to CRP. Free haemoglobin is required for bacterial growth, proinflammatory prostaglandin synthesis and NO mediated vasodilation. Hp binds haemoglobin resulting in inhibition of these processes (Sadrzadeh & Bozorgmehr, 2004).

1.3.3 Cells of the innate immune response

1.3.3.1 Macrophages

Macrophages are professional phagocytes that are resident in almost every tissue, and are constantly replenished by monocytes, their blood-residing precursors. Their primary function under homeostatic conditions is to engulf cellular debris (Mosser & Edwards, 2008). Additionally, large numbers of macrophages are recruited by chemokines to sites of infection, where they have essential roles in both innate and adaptive immune responses (Mosser & Edwards, 2008; Smith *et al.*, 2005b).

Macrophages initially engage microbial targets via their extracellular PRRs. Additionally, they can indirectly detect complement and antibody coated microbes through their CR1 and Fc cell surface receptors respectively. Following detection, macrophages internalise such microbes into vacuoles known as phagosomes, which subsequently fuse with cellular organelles known as lysosomes. Lysosomes have degradative, oxidative and acidic substances that digest the microbe present in the newly formed phagolysosome (Flannagan *et al.*, 2009). Subsequently, macrophages use Major Histocompatibility Complex (MHC) Class II receptors to present degraded microbial components to naïve T cells (Hume, 2008). Macrophages stimulated with TNF- α or IFN- γ stimulation have enhanced abilities to phagocytose and to antigen present (Mosser & Edwards, 2008).

Macrophages also produce toxic substances such as nitric oxide (NO), and reactive oxygen species (ROS) including Hydrogen peroxide (H₂O₂) and superoxide dismutase (Salzman *et al.*). These substances kill microbes but also harm the host, mainly through their cytotoxic effects. Furthermore, NO is involved in vasodilatation, necrosis, modulation of cytokine responses and the downregulation of adhesion factors (VCAM and ICAM-1) on activated endothelium (Tripathi, 2007).

1.3.3.2 Neutrophils

Neutrophils are polymorphonuclear phagocytes that primarily function to eliminate microbes (Urban *et al.*, 2006). Unlike macrophages, they have a short life span and are absent from healthy tissue (Kobayashi *et al.*, 2005). These cells circulate in the bloodstream under homeostatic conditions but migrate to sites of infection and inflammation in response to the chemoattractant properties of IL-8 (Urban *et al.*, 2006). This process also requires the simultaneous activation of the endothelium by proinflammatory cytokines. This enables the attachment of neutrophils to the walls of post-capillary venules and subsequent neutrophil transmigration into the sites of IL-8 induction (Kobayashi *et al.*, 2005).

In common with macrophages, neutrophils have mechanisms of microbial detection, phagocytosis, NO production and ROS production (Kobayashi *et al.*, 2005; Tripathi, 2007). However, neutrophils exclusively produce extracellular fibrous structures composed of granular proteins (e.g. myeloperoxidase) and nuclear

components (e.g. chromatin DNA), which are known as neutrophil extracellular traps (NETs). NETs function to contain bacterial species within sites of infection, subsequently killing them with high concentrations of antimicrobial substances (Wartha *et al.*, 2007).

1.3.3.3 Dendritic cells

Dendritic cells (DCs) are important phagocytic immune cells present in low numbers in a range of tissues. They are found in the thymus, spleen, lymph nodes, blood, skin and the intestinal mucosa (Wan & Dupasquier, 2005). In their inactive immature form, they constantly traffic between lymphoid tissues and nearby non-lymphoid tissues. They express PRRs which recognise microbes, followed by microbial internalisation, which results in DC activation. DCs can also be activated by proinflammatory cytokines such as IL-1 β and TNF- α (Kelsall & Leon, 2005). Mature DCs are also a source of cytokines such as IL-10 and IL-12 in response to bacterial infection (Scott *et al.*, 2005). DCs that have phagocytosed microbial components display such antigenic components on their surface via MHC class I or MHC class II molecules. Subsequently, DCs enter lymphoid tissue and prime naïve T cells with their MHC molecules containing microbial peptides. Thus, DCs are important to linking innate and adaptive immune responses. Furthermore, each organ has distinct populations of DCs with specialised functions (Wan & Dupasquier, 2005).

1.3.4 Bacterial mediated subversion of host defences

Bacterial species have developed a wide range of strategies to counter host innate immune defences. The strategies include evasion of PRR detection, interference with intracellular signalling pathways and antagonism of cytokine functions.

Flagellin is relatively conserved between bacterial species. Members of the α and ϵ Proteobacteria, such as pathogenic *Helicobacter pylori* and *Bartonella bacilliformis*, have mutations in their flagella that enables them to evade TLR5

recognition (Andersen-Nissen *et al.*, 2005). As these bacterial species require their flagella to invade mucosal tissue, these mutations were probably selected to allow TLR evasion (Galkin *et al.*, 2008). *Brucella*'s LPS and flagellin is structurally distinct, and evades TLR4 and TLR5 detection respectively, resulting in reduced TNF- α , IL-1 β and IL-6 increases in response to the microbe (Tsolis *et al.*, 2008).

Other bacterial species have developed strategies to prevent the NF- κ B mediated upregulation of innate immune genes. *S. salivarius*, an oral commensal, is capable of inhibiting NF- κ B activation by preventing the nuclear translocation of the P65 subunit (Cosseau *et al.*, 2008). Similarly, non-virulent strains of *Salmonella* interfere with the ubiquitination of I κ B- α in polarised epithelial cells, preventing its degradation. As a result, NF- κ B cannot migrate to the nucleus (Neish *et al.*, 2000). Similarly, *Lactobacillus casei* can increase the stability of I κ B- α in epithelial cells, which subsequently prevents *Shigella flexneri*-mediated induction of proinflammatory responses in such cells (Tien *et al.*, 2006). Other non-pathogenic bacterial species (e.g. *Bacteroides vulgatus*) are capable of inducing NF- κ B activation in intestinal epithelial cells (Haller *et al.*, 2002), suggesting that the lack of subsequent pathology may be controlled downstream of cell signalling.

Streptococcus pyogenes cleaves the C terminus of the IL-8 protein, inhibiting neutrophil recruitment to sites of infection (Edwards *et al.*, 2005). PTx, a virulence factor from *Bordetella pertussis*, has been shown to inhibit the chemotactic recruitment of neutrophils across endothelial cells (Kirimanjeswara *et al.*, 2005). Similarly, B-hemolysin, a *Staphylococcus aureus* protein, is capable of inhibiting neutrophil migration across endothelial cells by downregulating *IL8* expression at the mRNA level in endothelial cells (Tajima *et al.*, 2008).

1.4 The Intestinal epithelium

Innate immune responses eliminate microbes but often give rise to host damage in the process. Considering that the host is colonised by a range of non-pathogenic microorganisms, particularly in the intestine, measures must be in place to reduce harmful innate immune responses to the microbiota, while regulating the level of colonisation and still being capable of mounting an effective response to virulent

microbial species. One important physiological structure for mediating this activity is the intestinal epithelium.

The intestinal epithelium consists mainly of a single layer of polarised columnar epithelial cells with brush-border villi. Tight junctions are responsible for linking the adjacent cells and maintaining polarity. These tight junctions enable the epithelium to separate the luminal contents from the underlying intestinal layers (Fig. 1.6 and Fig 1.7). Several dietary and protective roles have been ascribed to the intestinal epithelium that work in synergy with its barrier function (Viswanathan *et al.*, 2009). The epithelium can prevent harmful substance uptake while simultaneously allowing the absorption of digested nutrients. The epithelial barrier also enables epithelial mediated fluid regulation in the lumen, which is a prerequisite for optimal nutrient digestion and absorption (Barrett, 2008).

Regarding the high volumes of immunogenic microbial species present in the intestinal lumen, the epithelium is of great importance in preventing microbial access to the underlying intestinal layers. Other than providing a physical barrier as protection, the columnar enterocytes together with other intestinal epithelial lineages, namely goblet cells and paneth cells, produce substances that regulate microbial colonisation and growth.

Stem cells at crypt bases give rise to goblet cells, which are short lived and apically secrete glycoproteins known as mucins. These mucins, together with inorganic salts, form a layer of mucous that overlies the epithelium (Lievin-Le Moal & Servin, 2006). The mucous layer provides an additional physical barrier for microbes to overcome. It also reduces microbial adhesion to the epithelium (Schenk & Mueller, 2008). However, mucins also provide a niche for commensal colonisation as they contain nutrients, such as saccharides, that are beneficial for bacterial growth (Lievin-Le Moal & Servin, 2006).

Paneth cells are located at the bases of crypts of the small intestine during homeostatic conditions. During chronic inflammation, they are also evident in the large intestine (Schenk & Mueller, 2008). They secrete a number of antimicrobial substances from their apical surface into the intestinal lumen (Fig. 1.7). One of these is an enzyme known as secretory phospholipase A2 (PLA2). PLA2 degrades the membrane phospholipids of gram-negative and gram-positive bacterial species (Muller *et al.*, 2005). Paneth cells also produce lysozyme, which hydrolyzes peptidoglycan, and is therefore bactericidal towards gram-positive bacterial species.

PLA2 and lysozyme are both constitutively expressed and their expression is not influenced by microbial stimuli (Mukherjee *et al.*, 2008). Paneth cells are the only epithelial lineage that produces α -defensins. α -defensins are both constitutively expressed and inducible in response to microbial stimuli. They have been implicated in the reduction of bacterial colonisation in the small intestine (Keshav, 2006; Mukherjee *et al.*, 2008). In host species that lack Paneth cells, it is likely that other intestinal epithelial cells have complementary antimicrobial roles. For example, enteric lysozyme is produced by villus epithelial cells in chickens (Nile *et al.*, 2004). These cells also produce β -defensins and cathelicidins (Muller *et al.*, 2005).

The epithelium that overlies the lymphoid aggregates (Peyer's patches) has cells specialized for luminal sampling known as M (microfold)-cells (Fig. 1.6 and Fig. 1.7). In comparison with other enterocytes, the overlying mucus layer is either thin or absent. M cells also have a distinct basolateral intra-epithelial pocket (Kyd & Cripps, 2008). These cells endocytose a range of antigenic substances including bacterial toxins, carbon particles and liposomes. Larger particles and bacteria are phagocytosed. The antigens are then transported in endocytic vesicles and are subsequently released into the intraepithelial pocket via exocytosis. In the pockets, the antigens encounter residential antigen presenting cells (APCs) (macrophages and dendritic cells) and lymphocytes (B and T cells). Such interactions either result in inflammatory or protective immune responses (Corr *et al.*, 2008). Also, M cells are targeted by pathogenic species. In comparison to other enterocytes, M cells are more susceptible to invasion by species such as *S. flexneri*, *Vibrio cholerae*, *L. monocytogenes* and *E. coli* (Corr *et al.*, 2008; Kyd & Cripps, 2008).

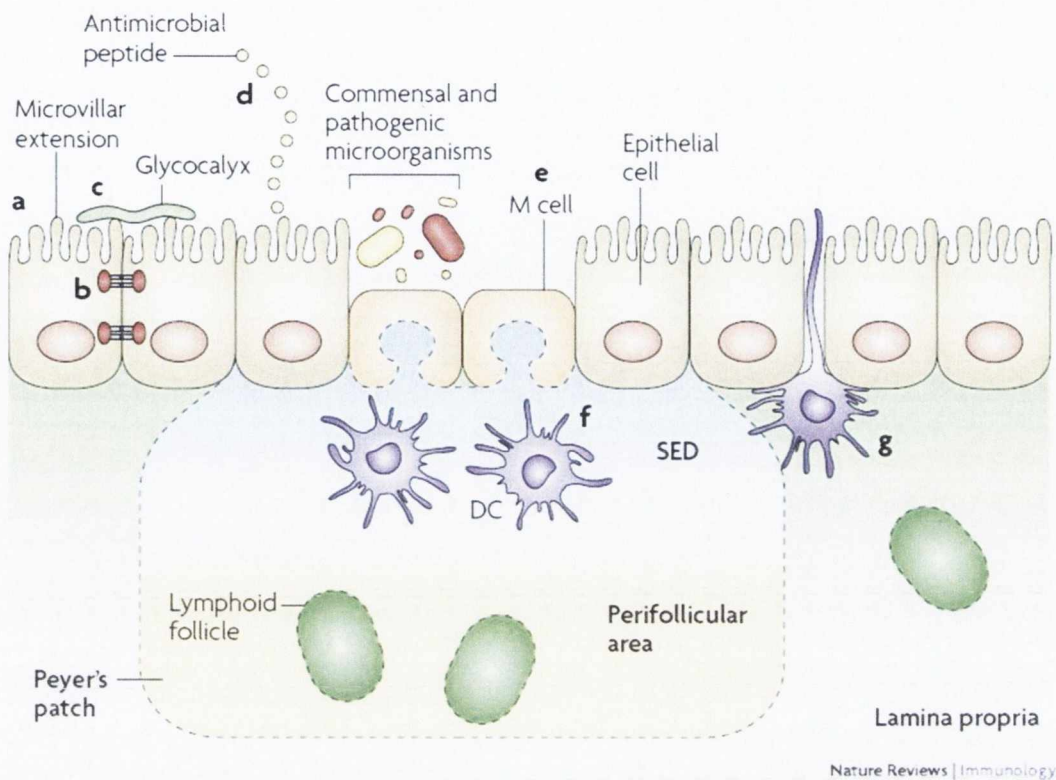


Figure 1.6 The intestinal epithelium. A single layer of enterocytes forms a barrier between the intestinal lumen and the underlying intestinal layers. Enterocytes produce antibacterial substances including glycocalyx and antimicrobial peptides (Host Defence Peptides). M cells overlie Peyer's patches and function in luminal sampling. Adapted from Artis, 2008.

1.5 PRRs of epithelium

The epithelium and underlying cells have PRRs to detect pathogenic microorganisms they encounter. Recognition of pathogens results in the induction of proinflammatory cytokines, which serve to resolve intestinal infection, but also contribute to pathology. For example, IL1 β and TNF- α production have multiple roles in innate defence, but both these cytokines also destabilise the intestinal epithelium (Papadakis & Targan, 2000; Sansonetti *et al.*, 1995). Considering that the epithelium constantly encounters approximately 10^{14} residential bacterial cells in the absence of substantial pathology, and that commensal bacterial species have similar PAMPs (e.g. LPS, flagellin and lipoteichoic acid) to pathogenic species, epithelial cells must have strategies for regulating immune responses towards different threat levels.

It is now becoming clear that the PRRs of epithelial cells are strategically distributed (Fig. 1.7) (Artis, 2008). Intestinal epithelial cells have low *TLR4* gene expression, in comparison to human dermal microvessel endothelial cells (HMECs), and lack a MD2 co-receptor. As a result, epithelial cells are unresponsive to LPS (Abreu *et al.*, 2001). Similarly, TLR2 and TLR6 have low gene expression in intestinal epithelial cells, in comparison to monocytes, and are thus unresponsive to TLR2/TLR6 specific ligands (Melmed *et al.*, 2003). A reduction of these surface PRRs contributes to reduced extracellular epithelial recognition of gram-positive and gram-negative bacterial species, resulting in reduced proinflammatory responses. In response to pathogens, surface PRRs might be upregulated by some unknown mechanisms. *TLR4* gene expression is higher in epithelial cells during inflammatory conditions (Fukata & Abreu, 2007).

Extracellular TLR expression and function also differs between the apical and basolateral surfaces. TLR5 is expressed on the basolateral surfaces of colonic epithelial cells, but not on the apical surfaces (Fig. 1.7). As a result, flagellated bacterial species need to traverse the epithelium to activate TLR5 (Gewirtz *et al.*, 2001), a characteristic more apparent in pathogenic species. Most remarkably, TLR9 protein expression is evident on apical and basolateral surfaces of intestinal epithelial cells, as opposed to its intracellular location in other cells. Basolateral stimulation results in NF- κ B activation. This transcription factor fails to be activated by apical

stimulation. Instead, apical stimulation has been shown to inhibit TLR9 mediated activation of NF- κ B and ERK (Lee *et al.*, 2008).

Also, several proinflammatory cytokines (e.g. IL-1 β) are produced in inactive forms in response to TLR activation, and require additional intracellular inflammasome activation to produce their active forms (as previously mentioned). In epithelial cells, inflammasome activation would likely require bacterial invasion or internalisation of bacterial products. Therefore a reduction in the levels of active proinflammatory cytokines would be evident in response to non-pathogenic species.

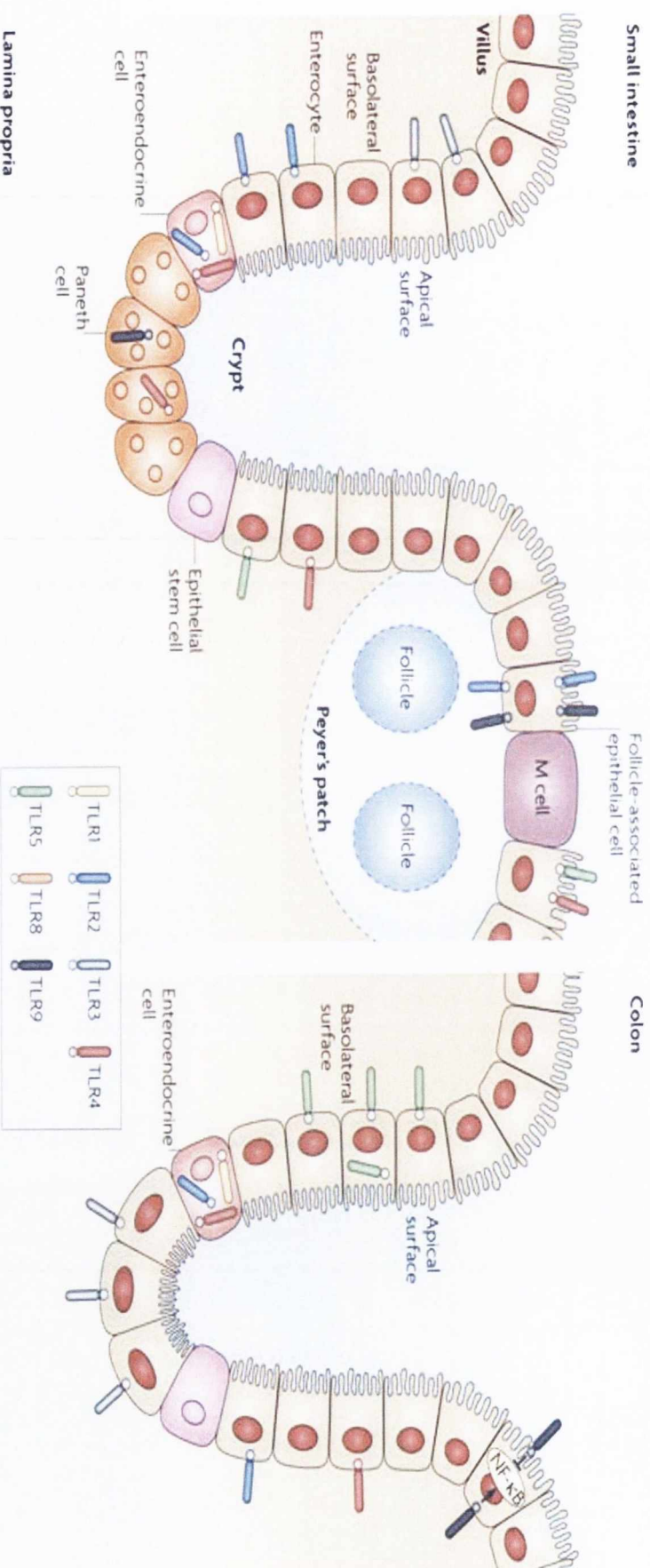


Figure 1.7 The apical surface of mammalian intestinal epithelium lacks TLR expression. TLRs are predominantly expressed on the basolateral surface of intestinal epithelial cells. Thus, the epithelium is hyporesponsive to commensals in the lumen while invasive microbial species induce proinflammatory responses upon encountering intracellular and basolateral PRRs. Adapted from Abreu, 2010.

1.6 Intra-epithelial lymphocytes

The basolateral surface of the intestinal epithelium contains populations of intraepithelial lymphocytes. In mammals, one IEL is evident between every 5-10 columnar epithelial cells (Cheroutre, 2005). These lymphocytes are predominantly CD8⁺ T cells. Both $\alpha\beta$ and $\gamma\delta$ T cell populations are evident although up to 40% of IELs are $\gamma\delta$ T cells, which is the highest concentration of such cells in the body (Kunisawa *et al.*, 2007). All IELs present at the epithelium are in an activated state, suggesting that they have initially been presented with antigen (Cheroutre, 2005). IELs have roles in regulating innate and adaptive immune responses. In response to epithelial stress, $\alpha\beta$ ⁺ IELs can trigger the maturation of underlying dendritic cells and can downregulate CD4⁺ and CD8⁺ T cell responses (Cheroutre & Madakamutil, 2004). $\gamma\delta$ ⁺ IELs have been shown to produce keratinocyte growth factor (KGF), which promotes epithelial growth and tissue repair (Kunisawa *et al.*, 2007). Therefore, IELs may also have roles in maintaining intestinal homeostasis.

1.7 Lamina propria

The lamina propria contains mesenchymal cells and myofibroblasts, which give rise to the structural integrity of the villi (Illemann *et al.*, 2004). Under homeostatic conditions, the lamina propria also contains large numbers of leukocytes namely activated T cells, B cells, plasma cells, mast cells and dendritic cells (Fig. 1.8). On this basis, the intestine is regarded as being in a state of “physiological inflammation” (Platt & Mowat, 2008). These cells serve to eliminate microbes that breach the epithelial barrier through phagocytosis and the induction of innate and adaptive immune responses, while minimising pathological immune responses with the aid of regulatory T cells (Sartor, 2008).

Macrophages residing in the lamina propria lack expression of multiple innate receptors including LFA-1 and CD14. Like conventional macrophages, this population phagocytoses microbes but lack the ability to produce proinflammatory and anti-inflammatory cytokines (Smythies *et al.*, 2005). This may be a strategy for

dealing with microflora that reach the lamina propria without inducing intestinal inflammation (Platt & Mowat, 2008).

During pathogenic encounters, CC chemokines recruit larger numbers of monocytes to the site of infection. CC chemokines are produced by a range of cells including epithelial cells and are also induced by proinflammatory cytokines such as IL-1 β and TNF- α under pathological conditions (Smith *et al.*, 2005b). The resulting activated macrophages express substantial levels of CD14 (Grimm *et al.*, 1995). Higher TLR2 and TLR4 expression is detectable in intestinal macrophages during inflammation, in comparison to the intestinal macrophages that operate under homeostatic conditions (Hausmann *et al.*, 2002).

1.8 Gut associated lymphoid tissue

The lamina propria is adjoined by regions of gut-associated lymphoid tissue (GALT) that underlie the epithelium. These are predominantly located in the small intestine and are known as Peyer's patches (Fig. 1.6 and Fig. 1.8) (Forster *et al.*, 2008; Kyd & Cripps, 2008). Other lymphoid aggregates known as isolated lymphoid follicles (ILFs) are also found. They are evident in both the small and large intestine (Izcue *et al.*, 2006). Peyer's patches and isolated lymphoid follicles are both overlain by M cells and contain antigen presenting cells (APCs) namely dendritic cells and macrophages, but also B and T lymphocytes (Forster *et al.*, 2008; Izcue *et al.*, 2006). M cell transcytosis of antigens from the intestinal lumen to the basolateral epithelial surface enables DCs of the GALT to detect and subsequently internalise such antigens (Tsuji & Kosaka, 2008). Additionally, DCs of the GALT (and the lamina propria) can extend their dendrites between two adjacent epithelial cells and into the lumen, enabling sampling of luminal antigens (Coombes & Powrie, 2008). In both circumstances of antigen detection, DCs present antigens to naïve lymphocyte populations in the PPs or the mesenteric lymph nodes (Tsuji & Kosaka, 2008). The primary functions of PPs and ILFs appear to involve generating protective immune responses while maintaining homeostasis. DCs of the intestine produce high IL-10, in comparison to splenic DCs (Coombes & Powrie, 2008). Intestinal DCs are more likely to activate several types of regulatory T-cells, which produce anti-inflammatory

cytokines, such as IL-10 and TGF- β 1. Both these cytokines target components of innate and adaptive immune responses, reducing inflammation (Tsuji & Kosaka, 2008). TH1 and TH17 cell responses resulting in proinflammatory cytokine production in response to pathogenic challenge are evident but are tightly regulated (Peron *et al.*, 2009).

Following their uptake of commensal microorganisms, DCs also activate naïve B cells in the PPs. Activated B cells subsequently give rise to plasma cells, many of which will migrate to the lamina propria. These plasma cells predominantly produce dimeric antigen-specific IgA, which is essential for bacterial deterrence and maintaining homeostasis (Macpherson *et al.*, 2005). Secreted IgA binds to polymeric Ig receptor (pIgR) located on the basolateral epithelial surface, which enables the antibodies to translocate to the apical surface and be secreted into the lumen. IgA coats specific microbes in the lumen, trapping them in the mucus and minimises their contact with the epithelium (Cerutti & Rescigno, 2008). IgA can also neutralise bacterial toxins, such as cholera toxin. Therefore, IgA responses are also important in limiting pathogenic microbes (Macpherson *et al.*, 2008). Microbes that do manage to breach the epithelium become coated with IgA. IgA coated bacterial species are targeted for destruction by phagocytes expressing FCR1. Additionally, IgA coated microorganisms in the lamina propria are transported back into the lumen via pIgR (Cerutti & Rescigno, 2008).

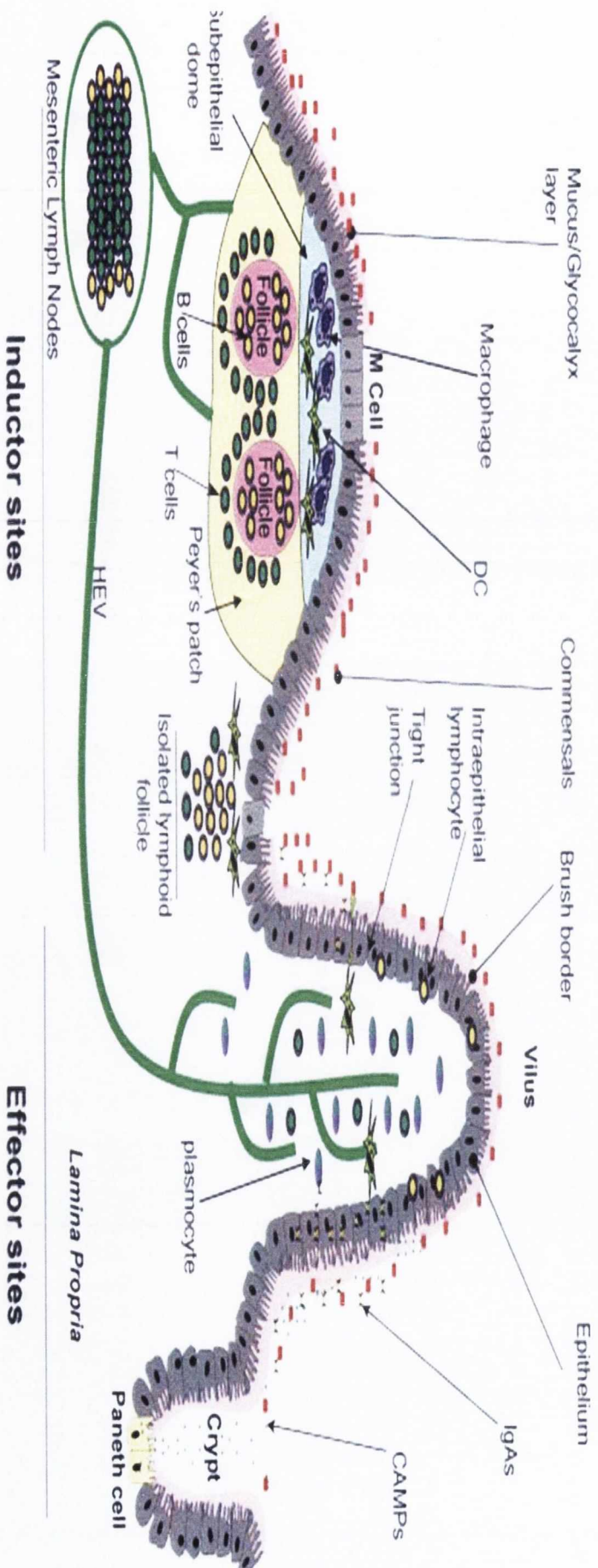


Figure 1.8 Intestinal immune system. The epithelium forms a physical barrier between the gut lumen and the lamina propria. Host defence peptides are primarily secreted into the lumen, via Paneth cells, and serve to regulate bacterial colonisation. M cells sample luminal commensals, transporting them to the underlying Peyer's patches (Kyd & Cripps), which are rich in DCs and lymphocytes cells. Thus, adaptive immune responses are initiated against commensals, resulting in the production of antimicrobial IgA. Adapted from Magalhaes *et al.*, 2007 .

1.9 *C. jejuni* mediated immune response and pathology in mammals

In the intestinal lumen of mammals, *C. jejuni* initially encounters the mucus layer, which appears to both deter and promote infection. MUC2, the predominant mucin of the mucus layer, inhibits *C. jejuni* growth. However, the microbe upregulates pathogenicity genes including *flaA*, *cadF*, *ciaB* and *cdtABC* in the presence of this mucin (Tu *et al.*, 2008). The mucus layer has also recently been shown to promote *C. jejuni* adhesion to and invasion of human goblet-like cells (Alemka *et al.*). *C. jejuni* traverses the intestinal epithelium through both transcellular and paracellular routes (Alemka *et al.*, 2010a; Bras & Ketley, 1999). Following the latter route, the epithelium is invaded through the basolateral surface (Monteville & Konkel, 2002). Subsequent to invasion, the epithelial barrier is destabilised through increased permeability and necrotic cell death of epithelial cells (Chen *et al.*, 2006; Kalischuk *et al.*, 2007; Wine *et al.*, 2008). Epithelial cell death is considered to occur in a CDT-independent manner (Kalischuk *et al.*, 2007).

Proinflammatory responses to the microbe may also contribute to barrier destabilisation. *C. jejuni* activates NF- κ B, ERK, JNK and p38 in human epithelial cells (Chen *et al.*, 2006; Watson & Galan, 2005), resulting in the production of an array of cytokines and chemokines including TNF- α , IFN- γ , IL-10, IL-6, CCL2, CCL4, CCL20, CXCL10, CXCL5 and especially IL-8 (Al-Salloom *et al.*, 2003; Bakhiet *et al.*, 2004; Friis *et al.*, 2009; Johanesen & Dwinell, 2006; Zheng *et al.*, 2008). TNF- α and IL-8 are released predominantly from the basolateral surface (Zheng *et al.*, 2008). *C. jejuni* mediated production of IL-8 from epithelial cells is associated with invasion (Beltinger *et al.*, 2008; Hickey *et al.*, 1999; Zilbauer *et al.*, 2007) although boiled *C. jejuni* extracts are also capable of inducing the chemokine (Mellits *et al.*, 2002; Mellits *et al.*, 2009). Epithelial cells also produce hBD2 and hBD3 in response to *C. jejuni*, and both peptides have bactericidal effects against the microbe (Zilbauer *et al.*, 2005).

Such proinflammatory responses to *C. jejuni* have recently been shown to be initiated by several PRRs. Boiled cell extracts and lysed *C. jejuni* activate TLR2, more so than live *C. jejuni* (Al-Sayeqh *et al.*, 2010; de Zoete *et al.*, 2010a). The precise TLR2 ligands have yet to be identified. TLR4 has also been shown to be activated by the microbe's lysate, more evidently than live *C. jejuni*, resulting in the

activation of MyD88-dependent and MyD88-independent pathways (de Zoete *et al.*, 2010a). Specifically, TLR4 has recently been shown to recognise the LOS component (Kuijf *et al.*, 2010; van Mourik *et al.*, 2010). TLR5 is unable to recognise *C. jejuni*, as a result of the distinct amino acid sequence of the microbe's flagella (Andersen-Nissen *et al.*, 2005; Johanesen & Dwinell, 2006) and it is unknown whether IPAF recognises *C. jejuni* flagellin. TLR9 is also evaded, due to the low GC content of the microbe's genome (Dalpke *et al.*, 2006; de Zoete *et al.*, 2010a). Other non-TLR PRRs have, however, been shown to sense *C. jejuni* and its components. Heat shock protein (Hsp) 90 α , a cell surface receptor, activates NF- κ B and p38 in epithelial cells upon contact with the *C. jejuni* JlpA lipoprotein (Jin *et al.*, 2003). Epithelial cell invasion by the microbe results in NOD1, but not NOD2 activation, resulting in *IL8* and *hBD2* gene expression upregulation (Zilbauer *et al.*, 2007). NOD2 can, however, activate NF- κ B in response to live *C. jejuni* in transfected human embryonic kidney (HEK) cells (Al-Sayeqh *et al.*, 2010). It is unknown precisely what *C. jejuni* components are recognised by these NLRs. Conversely, *C. jejuni* CDT induces IL-8 in epithelial cells (Hickey *et al.*, 2000) through an unknown PRR. *C. jejuni*-specific interactions with human PRRs are illustrated in figure 1.9.

Largely as a result of epithelial cytokine and chemokine production, an initial influx of neutrophils to the underlying lamina propria followed by mononuclear cells, is evident in response to *C. jejuni* infection in humans (van Spreeuwel *et al.*, 1985). Appropriate animal models of infection are lacking although ferrets and IL-10^{-/-} mice challenged with *C. jejuni* show similar neutrophil and mononuclear cell infiltration of the lamina propria (Mansfield *et al.*, 2007; Nemelka *et al.*, 2009).

Neutrophils and macrophages both produce NO, which can kill the microbe. *In vitro* experiments have shown that macrophages upregulate *NOS2*, a gene responsible for NO production, in response to *C. jejuni*. Macrophages with *NOS2*^{-/-} are less efficient at killing the microbe, in comparison to wild-type *NOS2* macrophages (Iovine *et al.*, 2008). *In vivo*, mice were shown to have increased *iNOS* (inducible nitric oxide synthase) gene expression in liver cells, splenocytes and macrophages in response to *C. jejuni* (Tarantino *et al.*, 2009). These studies suggest that reactive nitrogen species are important in response to the microbe.

Recruited neutrophils and mononuclear cells are also likely to be important producers of proinflammatory mediators in *C. jejuni* infection. Live and heat-killed *C. jejuni* induce comparable levels of IL-1 α , IL-1 β , IL-6, and IL-8 from THP1

monocytes (Jones *et al.*, 2003). Similarly, increased *IL1 β* and *IL8* gene expression is evident in challenged Mono Mac 6 (MM6) monocytes. With MM6 cells, lysed *C. jejuni* are more inductive of proinflammatory gene expression than live *C. jejuni* (de Zoete *et al.*, 2010a). Furthermore, murine macrophages induce IL-6 in response to *C. jejuni* LOS (Kuijf *et al.*, 2010). Dendritic cells also induce proinflammatory cytokines but more importantly, link the innate and adaptive immune responses during infection. Live *C. jejuni* are phagocytosed by dendritic cells, which subsequently produce TNF- α , IL-1 β , IL-6, IL-8, IL-10, IL-12p40 and IFN- γ . The LOS component alone is sufficient to induce these cytokines (Hu *et al.*, 2006; Kuijf *et al.*, 2010). Maturation markers are also upregulated, namely CD40, CD80, CD86 and HLA-DR (Hu *et al.*, 2006; Kuijf *et al.*, 2010) while LOS upregulates CD80 (Kuijf *et al.*, 2010). In murine dendritic cells, cytokine production and maturation in response to *C. jejuni* has been shown to be mediated through TLR2 and TLR4 (both MyD88 and MyD88-independent) activation (Rathinam *et al.*, 2009).

Mature dendritic cells subsequently promote the adaptive immune response. Supernatants from *C. jejuni* LOS challenged dendritic cells are capable of proliferating naïve B cells (Kuijf *et al.*, 2010) while Th1 cell priming is also evident, as *C. jejuni* activated dendritic cells induce IFN- γ production from CD4⁺ T cells (Rathinam *et al.*, 2009).

IgA and IgM producing plasma cells as well as lymphocytes have been observed in the lamina propria of campylobacteriosis patients, seven days-post onset of symptoms (van Spreeuwel *et al.*, 1985). *C. jejuni*-specific IgA is found in stool samples of *C. jejuni* infected individuals while specific IgM, IgG and IgA are also detectable in the serum (Blaser & Duncan, 1984; Cawthraw *et al.*, 2002; Tribble *et al.*, 2010). These antibodies target various *C. jejuni* components including flagellin, outer membrane proteins and LOS (Cawthraw *et al.*, 2002; Panigrahi *et al.*, 1992).

In humans, the immune response to *C. jejuni* is successful in containing infection to the intestine and results in complete clearance of the microorganism in the majority of cases within 2 weeks (Young *et al.*, 2007). However, with the majority of cases, the immune response contributes greatly to the clinical signs of campylobacteriosis. Inflammation of the small intestine, colon and rectum is a common occurrence, which contributes to the symptoms of cramping and diarrhoea. These symptoms last for 3 to 4 days. In one-third of cases, individuals experience severe intestinal inflammation, which results in bloody diarrhoea (Janssen *et al.*,

2008). In certain incidences (about 30% of cases), influenza-like symptoms namely fever, malaise, dizziness and headache are evident prior to the manifestation of gastrointestinal symptoms (Crushell *et al.*, 2004; Janssen *et al.*, 2008).

A number of complications can occur during and after *C. jejuni* infection. Campylobacteriosis has been linked to abortion and premature birth in humans (Crushell *et al.*, 2004). In immunocompromised individuals, bacteremia may occur (4 in every 1000 cases)(Janssen *et al.*, 2008).

C. jejuni infection has been linked to several autoimmune disorders namely Guillain-barré syndrome (GBS), Miller-Fisher syndrome (a subtype of GBS that affects eye movement) and reactive arthritis (van Doorn *et al.*, 2008). LOS from several *C. jejuni* strains, which bears striking resemblance to human neuronal gangliosides, initiates GBS. Antibodies generated to LOS of *C. jejuni* LOS cross-react with host gangliosides of the peripheral nervous system (Young *et al.*, 2007). Host genetic factors also determine disease susceptibility, as GBS incidences are relatively low, with only one in 1000 individuals who have had a recent *C. jejuni* infection, developing the disorder. The severity of symptoms can vary, ranging from mild numbness and weakening of the limbs to acute paralysis and respiratory failure. However, with appropriate treatment, the majority of afflicted individuals make a full recovery (van Doorn *et al.*, 2008). Reactive arthritis affects the joints shortly after the resolution of campylobacteriosis but does not usually require medical intervention (Janssen *et al.*, 2008).

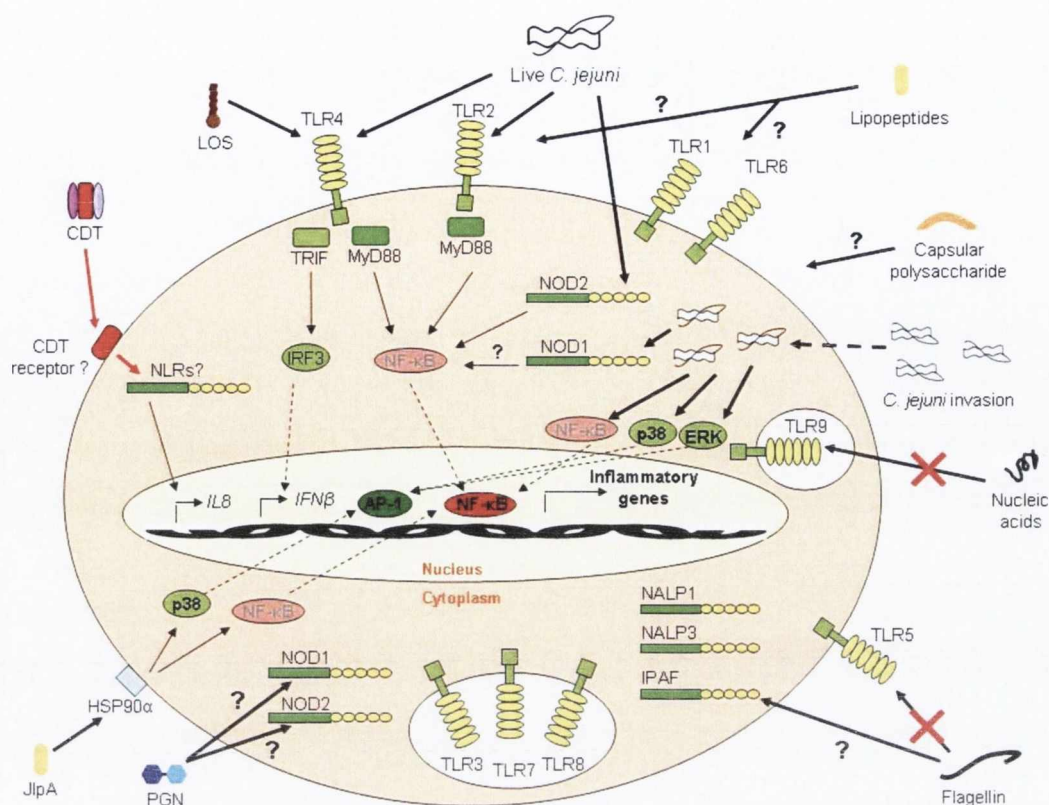


Figure 1.9 Human cellular immune responses to *C. jejuni* and its components. TLR2 and TLR4 recognise live *C. jejuni*. The precise ligands for TLR2 are unknown, but are likely to be lipopeptides. TLR4 specifically recognises *C. jejuni* LOS, resulting in the activation of both MyD88-dependent and MyD88-independent pathways. The microbe's flagellin and nucleic acids fail to activate TLR5 and TLR9 respectively. Live *C. jejuni* activates NOD1 and NOD2, but the precise ligands are unknown. CDT induces IL-8 although it is unknown what PRR initiates the response. *C. jejuni* JlpA lipoprotein activates p38 and NF-κB through the heat shock protein (HSP) 90α. Invasion is important for *C. jejuni*-mediated NF-κB, p38 and ERK activation.

1.10 The avian immune response

Several pathogens are associated with chicken mortality, including *Eimeria tenella*, Influenza A, and *Salmonella Gallinarum* (Huberman & Terzolo, 2008; McDonald & Shirley, 2009; Pantin-Jackwood & Swayne, 2009). Considering that commercial chickens are housed in close proximity to each other (often more than 1000 birds in a house), disease can spread rapidly within flocks. This is a major concern for the poultry industry. As a result, strategies are being sought to control the spread of disease, which involve bird vaccination programs and selective breeding of disease resistant birds (Pinard-van der Laan *et al.*, 2009; Schroer *et al.*, 2009). The successes of these strategies require a thorough understanding of the avian immune response. In comparison to mouse and human immunology, avian immunology is not well-characterised. Despite 300 million years of divergence between the avian and mammalian lineages, sequencing the chicken (*Gallus gallus*) genome has revealed that the innate immune system is generally conserved between the two lineages (Boyd *et al.*, 2007; Kaiser *et al.*, 2005). However, the immune gene repertoires differ somewhat between mammals and birds (Kaiser, 2007). Also, some immune cells and proteins may have slightly different functions. As a result, it is likely that there are differences regarding the avian and human immune responses to *C. jejuni*. It is therefore necessary to understand the difference between avian and mammalian immunology.

1.10.1 Chicken pattern recognition receptors

The evolutionary divergence between mammalian and avian lineages has resulted in similar and distinct components of the TLR repertoires. Humans have 10 TLRs (TLR1-10) while chickens have TLR1.1, TLR1.2, TLR2.1, TLR2.2, TLR3, TLR4, TLR5, TLR7, TLR15 and TLR21 (Fig. 1.10) (Boyd *et al.*, 2007). TLR1.2 and TLR2.1 predominantly dimerise to recognise peptidoglycan and diacylated BLP while all TLR1 with TLR2 combinations recognise triacylated BLP and Pam(3)CysSK(4) (Higuchi *et al.*, 2008). The ligands for TLR3, TLR4, TLR5 and TLR7 are similar for

mammalian and avian species (Boyd *et al.*, 2007). TLR8 is a pseudogene in chickens (Philbin *et al.*, 2005). TLR9 has not been identified in avian species. Instead, TLR21, which is orthologous to genes in fish and amphibians (Temperley *et al.*, 2008), is located in intracellular endosomes and recognises CpG motifs (Brownlie *et al.*, 2009; Keesstra *et al.*, 2010). *TLR15* was recently discovered, and its mRNA was shown to be upregulated in response to *Salmonella enterica* serovar Typhimurium in chicken caeca (Higgs *et al.*, 2006). Recently, *S. enterica* serovar Enteritidis has been shown to increase *TLR15* gene expression in chicken heterophils (the avian equivalent of neutrophils) (Nerren *et al.*, 2009; Nerren *et al.*, 2010) and the caeca of newly-hatched chicks (Mackinnon *et al.*, 2009). Increased *TLR15* gene expression is also evident in heterophils challenged with gram-positive *Enterococcus gallinarum* (Nerren *et al.*, 2010). These studies suggest that TLR15 is important in the response to gram-negative bacterial infections however, the precise ligands for this TLR have yet to be identified.

It is unclear what roles NLRs play in avian infection as these receptors have yet to be characterised in chickens (Fig. 1.10). *In silico*, *NOD1*, *NOD3*, *NOD4* and *NOD5* have been identified but *NOD2* is absent from the genome. A single NALP ortholog is evident which closely resembles human *NALP1*, *NALP3*, *NALP10* and *NALP12* (Laing *et al.*, 2008). To date functional studies on NLRs have not been reported in avian species.

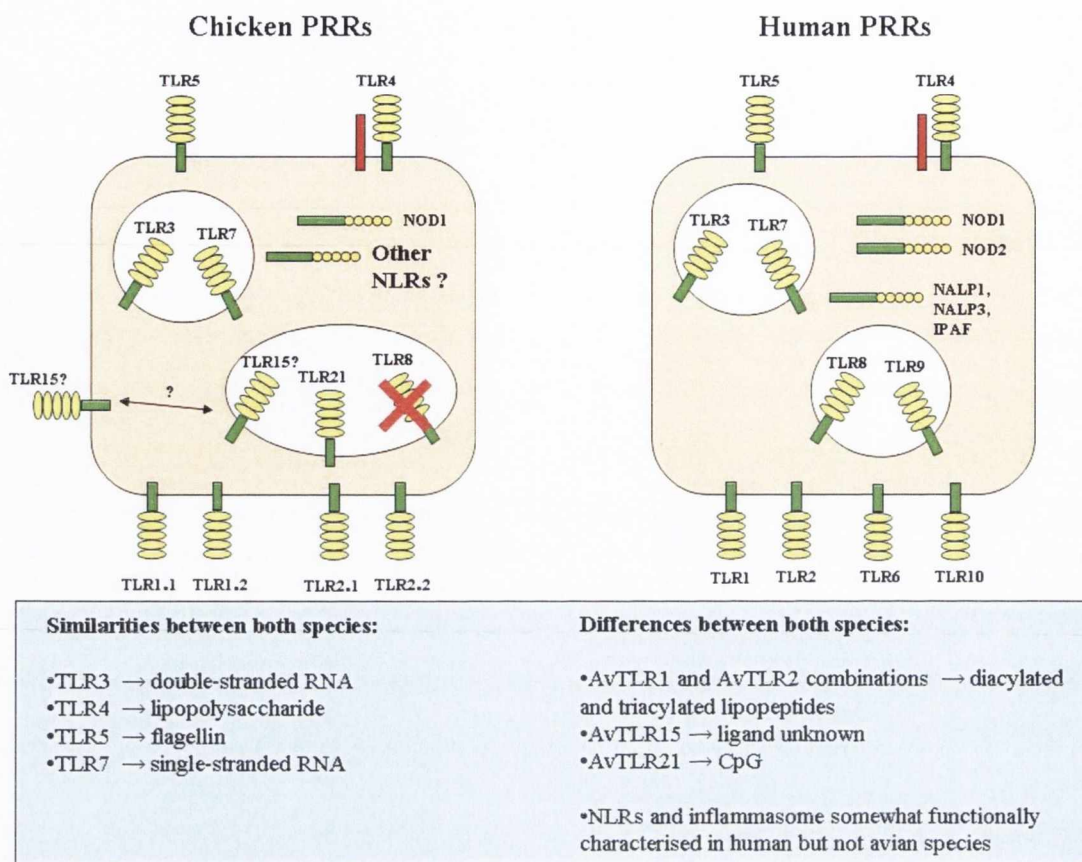


Figure 1.10 Similarities and differences in PRR repertoires identified to date between birds and humans. Avian *TLR1* and *TLR2* have been duplicated but there are no orthologs for *TLR6* and *TLR10*. *TLR3*, *TLR4*, *TLR5* and *TLR7* are conserved between the species. *TLR8* is disrupted in chickens. *AvTLR21* is functionally equivalent to human *TLR9*. *TLR15* is avian specific. It is unknown whether this receptor is located on the cell surface or within endosomes. *NOD1* is evident in birds, but not *NOD2*. Other avian NLRs are not well characterised.

1.10.2 Chicken cytokines and chemokines

The chicken genome was sequenced in 2004. It revealed that the avian repertoire of cytokines and chemokines differs somewhat to the mammalian repertoire. Only 79 cytokines are evident in the chicken genome, in comparison to the 129 cytokines in the human genome (Kaiser, 2007). Genes conserved between the two species include *IL1 β* , *IL6*, *IL10*, *IL12*, *IFNG*, *IL15* and *IL17* (Kaiser *et al.*, 2005). For most of these cytokines, there is approximately 30% amino acid identity with their mammalian counterparts. Consequently, there are very few cross-reactive antibodies available for the quantification of avian cytokines (Kaiser *et al.*, 2005). This makes studying their functions in birds a considerable challenge. However, some progress has been made in determining the function of these effectors during the chicken immune response through analysis of the chicken genome, profiling cytokine gene expression in response to various stimuli and through the synthesis of recombinant cytokine proteins. IL- β , IL-6, IL-8, IL-10 and TGF- β 4 appear to be important to the avian innate immune response and are among the better characterised cytokines.

Chicken *IL1 β* gene expression is increased in response to LPS stimuli and its protein has confirmed proinflammatory effects (Weining *et al.*, 1998). Chicken IL-6 functions are also conserved as it induces B cell proliferation and has inflammatory roles *in vivo* (Schneider *et al.*, 2001). Like IL-1 β and IL-6, TNF- α is regarded as a key component of immune responses in several species. However, while several TNF family members are evident in the chicken genome, an ortholog of TNF- α has yet to be discovered. However, a TNF- α receptor is evident in the chicken genome and TNF- α like activity is apparent in the avian immune response. Therefore, it is likely that a TNF- α homolog is present (Kaiser, 2007). Of course, the complete chicken sequence only covers 90% of the genome, so TNF- α may be lurking in the part that is hard to sequence.

Regarding chemokines, the chicken genome has two orthologs of mammalian IL-8, namely K60 (also known as CXCLi1) and IL-8 (also known as 9E/CEF4, CAF and CXCLi2). They are adjacent to each other on chromosome 4 and are presumably a result of tandem duplication. Respectively, these chemokines have 72% (Hughes & Bumstead, 2000) and 52% (Kaiser *et al.*, 1999) amino acid identity with human *IL8*. CXCR1 has been identified as the receptor for both chemokines and is expressed on

the surfaces of heterophils, which are the avian equivalence to neutrophils. Both chicken IL-8 and K60 proteins are capable of attracting heterophils although K60 is the more efficient chemoattractant (Poh *et al.*, 2008). Chicken IL-8 is also capable of recruiting monocytes, unlike human IL-8 (Barker *et al.*, 1993).

Regarding anti-inflammatory cytokines, homologs of IL-10 and TGF- β are evident in the chicken genome. Like its mammalian equivalent, chicken IL-10 is also likely to have similar anti-inflammatory properties. Chicken IL-10 has been shown to downregulate IFN- γ in stimulated splenocytes at both the mRNA and protein levels (Rothwell *et al.*, 2004). Chickens have 3 TGF- β genes known as *TGF β 2*, *TGF β 3* and *TGF β 4*. *TGF β 4* is regarded as an ortholog to human *TGF β 1*, and has similar anti-inflammatory properties (Kaiser, 2007).

1.10.3 Chicken host defence peptides

Avian species lack α -defensins but have β -defensins and cathelicidins. Fourteen avian β -defensins, AvBD1-14, have been discovered to date (Lynn *et al.*, 2007). They are expressed in a range of healthy tissues including bone marrow, testis, ileum and colon although the basal level of gene expression differs between such tissues while different tissues express different AvBDs (Lynn *et al.*, 2004). Increased AvBD gene expression is evident, *in vivo*, in response to microbial components (Akbari *et al.*, 2008; Mageed *et al.*, 2008). Whether such increases are a result of PRR activation or indirectly as a result of cytokine production remains to be determined. To date, mainly the antimicrobial properties of AvBDs have been investigated. For example, AvBD1 and AvBD2 are both active against *L. monocytogenes* and *E. coli* (Sugiarto & Yu, 2004). AvBD4, AvBD7 and AvBD9 are active against *S. Typhimurium* and *S. Enteritidis* (Milona *et al.*, 2007). AvBD6 is also bactericidal towards *S. Typhimurium*, but also toward *E. coli* and *C. jejuni* (van Dijk *et al.*, 2007). AvBD11 is active against *L. monocytogenes* and *S. Typhimurium* (Higgs *et al.*, 2005). Alternative functions for chicken AvBDs have yet to be reported. However, duck AvBD2, which has 83% amino acid identity with chicken AvBD2, has been shown to attract chicken B cells (Soman *et al.*, 2009). It is therefore likely that some chicken AvBDs have similar chemotactic properties.

To date, four cathelicidins have been identified in chickens, namely Cath-1, Cath-2, Cath-3 and Cath-B1. *CathB1* gene expression is evident in epithelial cells that overlie the bursa. However, mature Cath-B1 peptides released by epithelial cells, travel to the basolateral surface of bursal M cells. Cath 1-3 are expressed in the bone marrow and lung (Goitsuka *et al.*, 2007). Recently, Cath-2 was also found to be expressed at the protein level in the granules of heterophils but not in other peripheral blood leukocytes or intestinal epithelial cells. Mature Cath-2 is released from heterophils upon encountering *S. enterica* specific LPS. The peptide was also shown to be antibacterial towards both gram-positive and gram-negative pathogens, namely *S. enterica*, *Clostridium perfringens*, *Staphylococcus aureus* and *Bacillus cereus* (van Dijk *et al.*, 2009b). Chicken Cath-2 is also capable of inhibiting TNF- α , IL-6 and IL-10 production but not IL-8 production from LPS stimulated human PBMCs (van Dijk *et al.*, 2009a). Cath-2 may therefore have anti-inflammatory properties through reducing bacterial numbers and dampening proinflammatory responses.

1.10.4 Avian acute phase response

Acute phase proteins (APPs) such as SAA, CRP and Fibrinogen have been identified in chicken, and function similar to their mammalian counterparts. However, other APPs, namely transferrin and Hp, differ in their levels during the APR. During the APR, Transferrin is decreased in mammals but increased in chickens. Conversely, Hp is increased in mammals but decreased in chickens (Gruys *et al.*, 2005).

1.10.5 Avian phagocytic cells

Chicken macrophages appear to have similar functions to their mammalian equivalents (Qureshi *et al.*, 2000). It is unclear whether the chicken intestine has residential macrophage populations to regulate inflammation. The polymorphonuclear cell repertoire of chickens differs from mammals as they lack eosinophils and neutrophils. Heterophils are the avian equivalent of neutrophils. Like their mammalian counterparts, heterophils are recruited to sites of infection where they phagocytose and kill bacteria (Henderson *et al.*, 1999). Neutrophils and heterophils

differ in their granule content. Also, heterophils lack myeloperoxidase and have little hydrogen peroxide production (Harmon, 1998).

1.11 The chicken caecum

Chickens have a tendency to explore the floor of their housing for food, which also contains faeces from other birds in the flock. As a result, through the faecal-oral route, the intestines of birds are constantly in contact with high levels of bacterial species. The avian intestine readily becomes colonised with many of the bacterial species it encounters. In particular, the highest level of bacterial colonisation occurs in the caecum. Here, bacterial colonisation can reach 10^{11} CFU/g, which is largely due to the structure of the caecum. The caecum consists of an opening at the junction of the small and large intestine, which leads to a blind-ended tube-like structure, and is histologically similar to the small intestine (Fig. 1.11). Microbes that enter the caecum are retained by the blind-ending. Villi, which form a meshwork at the proximal end of the caecum, allow the uptake of fluids and fine food particles while simultaneously preventing entry of larger particles. Bacterial genera considered indigenous to the caecum include *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Escherichia*, *Fusobacterium*, *Lactobacillus* and *Ruminococcus* (Fig. 1.1 B.) (Amit-Romach *et al.*, 2004; Bjerrum *et al.*, 2006; Gong *et al.*, 2007). Many of these commensals, such as *Bifidobacterium*, have digestive roles and therefore, the caecum's primary function appears to be digestion (Clench & Mathias, 1995).

Importantly, the caecum is often a site of pathogenic assault from organisms such as *Eimeria tenella* and *S. Pullorum*. Additionally, the caecum is asymptotically colonised by two major human pathogens, *C. jejuni* and *S. Typhimurium*. Therefore, the caecum has implications for chicken and human health. As a result, considerable research interest has been focused on the chicken caecum. In particular, the caecal immune response has been studied. *TLR2*, *TLR4* and *TLR5* gene expression is detectable in the chicken caecum (Abasht *et al.*, 2008). However, it is unknown whether caecal epithelial cells express these TLRs or whether caecal TLR gene expression can be attributed to the professional phagocytic cells of the lamina propria. Nevertheless, invasive bacterial species stimulate proinflammatory immune

responses in the mucosa of the caecum. These are characterised by chemokine and cytokine gene expression increases along with macrophage, heterophil and $\gamma\delta$ T cell influxes to the lamina propria in the early stages of infection. Such responses contribute greatly to clinical signs (Berndt *et al.*, 2007; Chappell *et al.*, 2009). Regarding the high levels of commensals restricted to the caecal lumen, innate immune responses are likely to be evident but low key, and probably involve AvBDs to regulate levels of colonisation (Bar-Shira & Friedman, 2006).

M cells are also evident in the caecal epithelium. Therefore it is likely that microbes in the caecal lumen are transcytosed to underlying lymphoid tissues (Kitagawa *et al.*, 1998). There are two GALT tissues in the caecum, namely caecal tonsils and lymphoid nodules. The caecal tonsils are a defined region of lymphoid tissue at the proximal end of the caecum. They are the largest aggregates of GALT (Lillehoj & Trout, 1996), and are likely analogous to mammalian mesenteric lymph nodes. Like mammalian GALT, the caecal tonsils contain DCs (Del Cacho *et al.*, 2009), B cells and T cells (Lillehoj & Trout, 1996). Caecal tonsils are believed to initiate adaptive or regulatory immune responses to the caecal microflora following sampling (Lillehoj & Trout, 1996).

Lymphoid nodules are also evident in the lamina propria and are equivalent to mammalian Peyer's patches, and thus also have a role in immunosurveillance (Kitagawa *et al.*, 1998). Additionally, lymphoid nodules are also evident deeper in the lamina propria and submucosa, where they are surrounded by columnar epithelial cells. Some of these columnar enterocytes have intra-epithelial lymphocytes. There is also evidence of goblet cells and M cells overlying the lymphoid nodules (Jeurissen *et al.*, 1999).

It is clear that the chicken caecum has well developed innate and adaptive immune systems to deal with pathogens and commensals. It is unknown whether such responses control *C. jejuni* colonisation of the caecum.

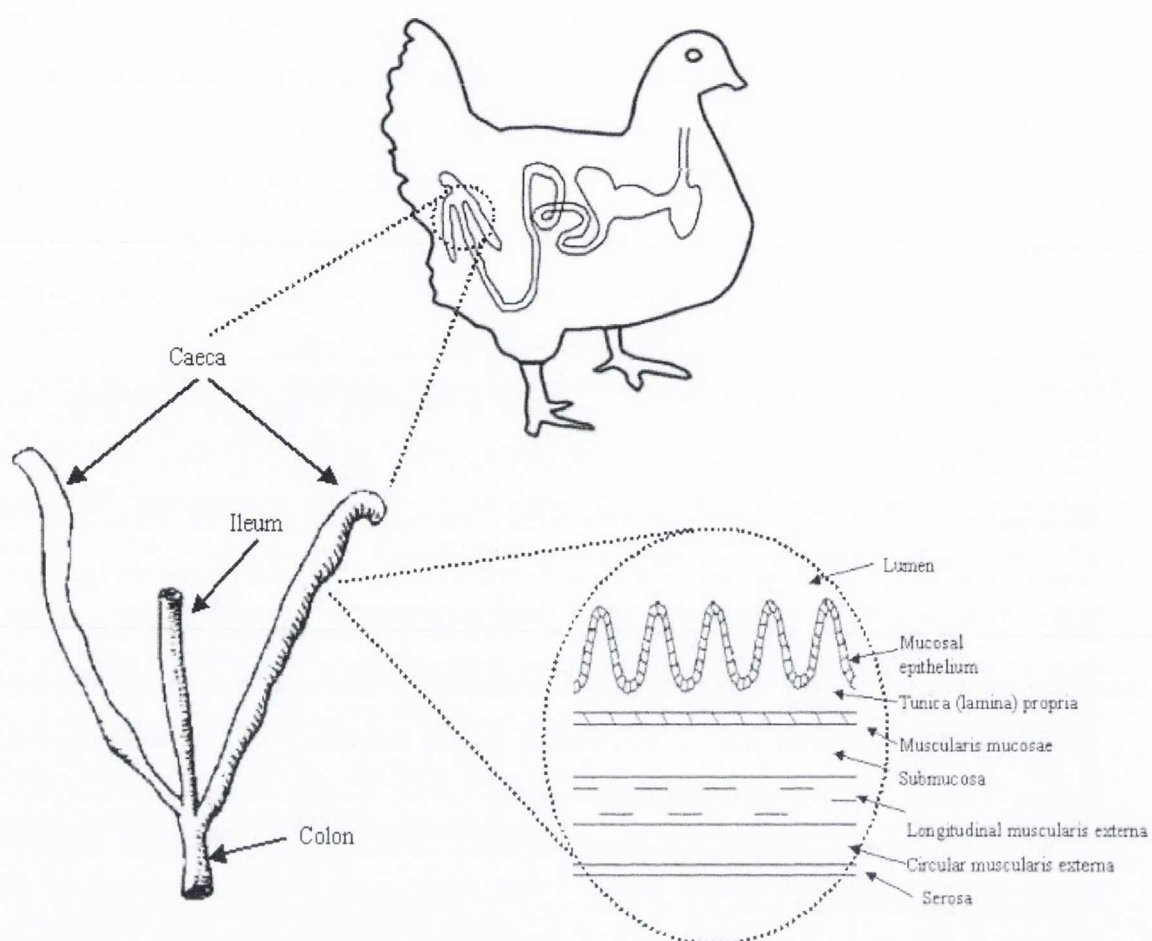


Figure 1.11 The avian caecum. The caecum is located at the junction between the ileum and colon, and consists of paired blind-ended tube-like structures, which are histologically similar to the ileum.

1.12 The avian immune response to *C. jejuni*

As with human infection, *C. jejuni* initially encounters the mucus layer overlying the intestinal epithelium. Isolated chicken mucus has no negative impact on *C. jejuni* viability (Byrne *et al.*, 2007). Instead, the microbe preferentially propagates in chicken mucus, as opposed to within caecal contents alone (Van Deun *et al.*, 2007). The mucus layer functions as a barrier, as small and large intestinal mucin, but not caecal mucin, is capable of reducing *C. jejuni* invasion of human epithelial cells (Alemka *et al.*, 2010b).

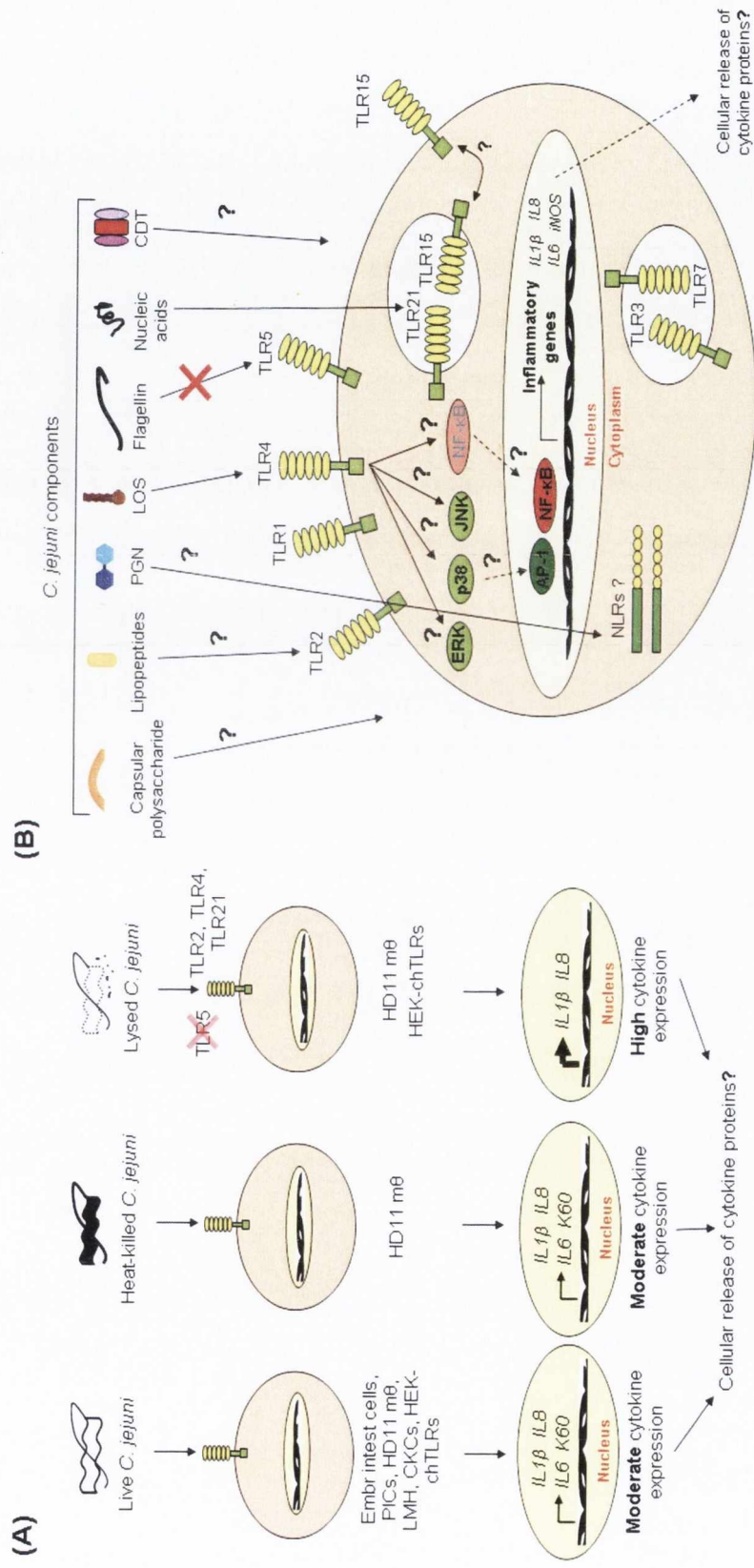
There is also evidence that *C. jejuni* invades chicken caecal epithelium. The microbe invades primary chicken intestinal cells, *in vitro*, but is not capable of surviving within such cells for long durations (Byrne *et al.*, 2007; Van Deun *et al.*, 2007). Chicken hepatocellular carcinoma (LMH) epithelial cells are also susceptible to *C. jejuni* invasion. In contrary to mammalian infection, chicken epithelial cell necrosis is not evident in response to the microbe, *in vitro* or *in vivo* (Beery *et al.*, 1988; Van Deun *et al.*, 2007). *C. jejuni* invasion does, however, induce proinflammatory cytokines in such cells, at least at the mRNA level. *IL1 β* , *IL6*, *K60* and *IL8* gene expression is induced in chicken embryonic intestinal cells (Li *et al.*, 2008c) and increased *IL8* gene expression is also evident in chicken primary intestinal cells (Borrmann *et al.*, 2007). Furthermore, increased *IL8* and *K60* gene expression, but not proinflammatory cytokines, are evident in LMH cells that have been invaded by *C. jejuni*. However, the supernatants from invaded LMH cells fail to be chemoattractant toward heterophils (Larson *et al.*, 2008). This reflects *in vivo* *C. jejuni* infection in chickens, in which heterophil and mononuclear influxes to the lamina propria are not evident. Furthermore, there is no evidence of morphological changes, necrosis, tissue damage or other signs of aberrant inflammation in the caecal mucosa of *C. jejuni*-colonised birds (Beery *et al.*, 1988; Smith *et al.*, 2008; Van Deun *et al.*, 2007) suggesting a lack of proinflammatory activity. With newly-hatched and two-week old chickens, *IL8* and *K60* gene expression increases are evident across a seven day timecourse while *IL1 β* and *IL6* increases are somewhat lacking (Smith *et al.*, 2008). Recent microarray analysis on the caeca of two different chick lines (line A and line B), seven days post-*C. jejuni* challenge, failed to show increased proinflammatory indicators. Despite line A and line B having more than 690

differentially expressed genes, only 17 and 22 of these, respectively, were immune genes. These included *IL1 β* , *IL8*, *K60*, *Ficolin 2* and *SOCS3* genes, which were decreased slightly in response to the microbe, in both lines (Li *et al.*, 2010).

A lack of a strong intestinal immune response to *C. jejuni* possibly enables the microbe to disseminate to deeper tissues, as it is often isolated from the spleen and liver (Cox *et al.*, 2005; Cox *et al.*, 2009; Knudsen *et al.*, 2006). It is unclear whether the microbe induces immunopathology in these tissues. *C. jejuni* has been isolated from healthy livers but also from livers with necrotic lesions (Cox *et al.*, 2009) while the microbe is associated with hepatic lesion formation in quail (Misawa *et al.*, 1996). Also, *in vitro* studies show that the microbe is capable of inducing proinflammatory gene expression in non-intestinal cells. Live *C. jejuni* induce *IL1 β* , *IL6*, *K60* and *IL8* gene expression in chicken HD11 macrophages although greater expression increases are evident in response to lysed *C. jejuni* (de Zoete *et al.*, 2010a; Smith *et al.*, 2005a). Primary chicken kidney cells also upregulate proinflammatory gene expression in response to viable *C. jejuni* (Smith *et al.*, 2005a).

C. jejuni interactions with avian pattern recognition receptors are only beginning to be described. As with its mammalian counterpart, chicken TLR5 is unable to recognise *C. jejuni* flagellin (de Zoete *et al.*, 2010a; de Zoete *et al.*, 2010b). Chicken TLR21, however, is capable of recognising DNA from the microbe, unlike its mammalian equivalent. Lysed, but not live, *C. jejuni* activate chicken TLR2 and TLR4 (de Zoete *et al.*, 2010a). The specific *C. jejuni*-components required for chicken TLR2 activation have yet to be described while LOS has recently been shown to activate chicken TLR4 (van Mourik *et al.*, 2010). *IFN β* gene expression is not upregulated in HD11 cells in response to LOS, which suggests that the MyD88-independent pathway is not activated (de Zoete *et al.*, 2010a). It is, however, unclear whether TLR2 and TLR4 initiate an innate immune response against *C. jejuni* in the caecum.

Nevertheless, an adaptive immune response to the microbe is evident. *C. jejuni*-specific IgM and IgG are detectible in the serum of colonised birds, 15 days after challenge (Larson *et al.*, 2008). Maternal antibodies to *C. jejuni*'s flagellin, LOS and other surface antigens are evident in newly-hatched chicks (Sahin *et al.*, 2001; Shoaf-Sweeney *et al.*, 2008).



1.13 Rationale for study

As the caecum is an important site of immune responses in the chicken, and that intestinal immune responses are critical in controlling bacterial colonisation, it is likely that such processes are the key determinants of *C. jejuni* colonisation resistance. In particular, the innate immune system is the first line of defence and therefore, is the initial strategy to deter *C. jejuni* colonisation. However, little is known about innate immune mechanisms that are characteristic to detection and immune response to *C. jejuni*. Considering that innate immune gene expression profiles differ with each specific microbial challenge, and that *C. jejuni* somehow avoids immune mediated clinical signs in chickens, it is likely that the avian innate immune response to *C. jejuni* is distinct, and may involve novel aspects. Therefore, identification of innate immune mechanisms that confer *C. jejuni* colonisation resistance forms the basis of this thesis.

The specific aims of this thesis are:

- To characterise the early innate immune response to *C. jejuni* (and at what time interval) at the primary site of colonisation, the caecum, and to compare that response to another bacterial species, *S. Typhimurium*.
- To identify the global transcriptional profile and immune gene signature in response to *C. jejuni* in the caecum.
- To examine the abilities of specific *C. jejuni* components to modulate the chicken innate immune mechanisms *in vitro*.

Chapter 2

Materials and Methods

Chapter 2: Materials and Methods

2.1 Chicken infection models

Using 4-week-old commercial broiler chickens, *S. Typhimurium* and *C. jejuni* infection models were carried out at Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan, Saskatoon, Canada by Dr. Brenda Allan (VIDO), Dr. Kieran Meade (UCD), Carla Reiman (VIDO) and Sarah Cahalane (UCD).

2.1.1 Bacterial strain preparation

S. 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 and 24 mM MgCl₂, 337 µm 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 1×10^{10} /ml. *C. jejuni* strain NCTC11168 v1 was grown microaerophilically (85% N₂, 10% CO₂, 5% O₂) on Mueller Hinton (MH) agar plates at 42°C. The cells were resuspended in saline and viable bacterial numbers were determined on MH agar plates (2.5×10^8 /ml).

2.1.2 Experimental challenges

All animal experiments were conducted according to the guidelines provided by the Canadian Council on Animal Care. On separate dates, 49 and 56 broiler chickens (Ross 308, provided by Avigen) were obtained from a commercial hatchery

in Saskatchewan, Canada, at 1 day of age. These were used for *S. Typhimurium* and *C. jejuni* infection models, respectively. They were reared in an isolation room in VIDO, until 4 weeks of age. All birds had cloacal swabs taken before commencing animal infection experiments in order to ensure they were free from *Campylobacter* and *Salmonella* species. Seven birds were removed to provide unchallenged controls for the *S. Typhimurium* infection model. Twenty-one birds were then orally challenged with 0.5 ml of *S. Typhimurium* suspended in 0.85% NaCl. At each time-point (6, 20 and 48 h post-infection), seven *S. Typhimurium*-challenged birds were euthanized by cervical dislocation. In parallel, 21 birds were challenged with 0.5 ml of 0.85% NaCl, and seven birds were euthanized at each time-point. These birds were used as positive controls for the *S. Typhimurium* infection model. Eight birds were removed to provide unchallenged controls for the *C. jejuni* infection model. Twenty-four birds were challenged with 0.5 ml of *C. jejuni* suspended in normal saline. At each time-point (6, 20 and 48 h post-infection), eight *C. jejuni*-challenged birds were euthanized by cervical dislocation and processed. Twenty-four saline-challenged birds were euthanized (eight at each time-point) to provide mock-challenged controls (Fig. 2.1). From each bird sacrificed, intestinal tissue samples were aseptically removed and immediately frozen in liquid nitrogen, prior to long-term storage at -80°C.

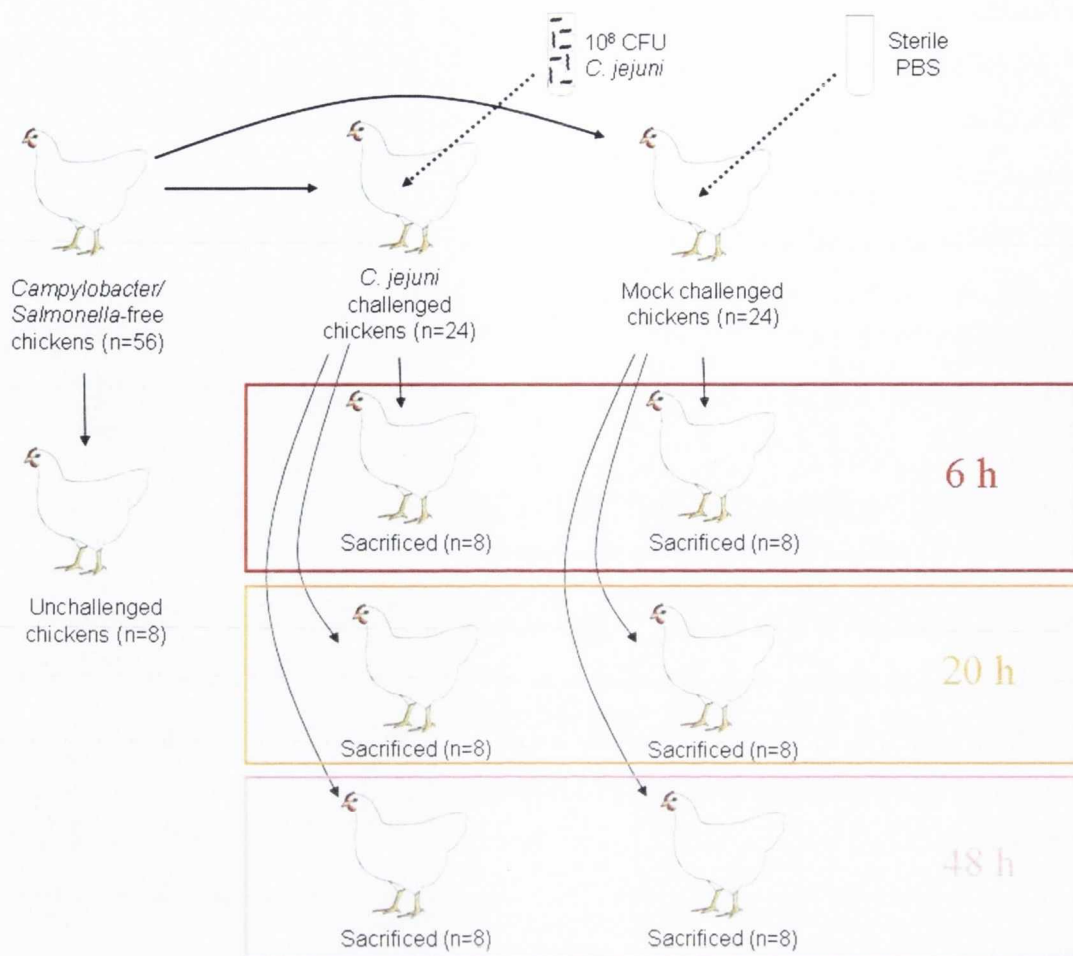


Figure 2.1 *C. jejuni* infection model carried out at VIDO. *C. jejuni*-challenged (10^8 CFU) and mock-challenged broiler chickens were sacrificed 6 h, 20 h and 48 h post-infection.

2.1.3 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, tissue samples were cultured using serial dilutions. To detect *S. Typhimurium*, the samples were cultured on Brilliant Green Agar at 37°C for 20 h. To detect *C. jejuni*, the samples were plated on Karmali agar and incubated microaerophilically at 42°C for 48 hours.

2.2 Innate immune gene expression profiling *in vivo*

2.2.1 Selection of birds for innate immune gene expression analysis

For the analysis of the caecal immune response to both *C. jejuni* and *S. Typhimurium* challenges, samples from 78 chickens from the experimental challenges (section 2.1.2) were used. Thirty-six of these birds represented *S. Typhimurium*-challenged chickens or their respective mock-challenged controls at 6 h, 20 h and 48 h post-infection (n = 6 birds per time-point). Similarly, caecal samples from 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 time-point) (Fig. 2.1).

2.2.2 Histopathology

Caecum samples were removed from -80°C storage and thawed at room temperature before being placed in formalin for routine fixation. Tissue from 2/3 animals per time-point was trimmed, paraffin-embedded, sectioned at 5-8 µm thickness and stained with haematoxylin and eosin (H&E). Histopathological analysis was carried out by Professor John Callanan (School of Agriculture, Food Science and Veterinary Medicine, University College Dublin).

2.2.3 RNA extraction from tissues

Total RNA was extracted from tissue samples using the RNeasy[®] Mini Kit (Quiagen). Briefly, liquid nitrogen-frozen tissue samples were homogenised on ice in 600 µl RLT buffer with 1% β-mercaptoethanol (β-ME) for 5 min. These homogenates were mixed with 700 µl 70% ethanol, and transferred to RNeasy spin columns, which were centrifuged at 8000 × g for 15 s. RW1 wash buffer (350 µl) was added to the columns followed by column centrifugation at 8000 × g for 15 s. Eighty µl of rehydrated DNase (Quiagen) was added to each column for 15 min. Columns were subsequently washed with 350 µl RW1 and centrifuged as before. Each column was washed twice with 500 µl RPE buffer (containing four volumes of ethanol) which involved centrifugation after each wash. Columns were placed in new collection tubes, which were centrifuged at 8000 × g for 1 min to remove residual ethanol. Subsequently, spin columns were placed in 1.5 ml collection tubes and 40 µl DNase/RNase-free distilled H₂O was added to the columns. Columns were centrifuged at 8000 × g for 1 min. The flow-through, which contained eluted RNA, was immediately stored at -80°C. RNA yield and quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies).

2.2.4 cDNA synthesis

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). cDNA yield was quantified using a NanoDrop[®] ND-1000 spectrophotometer. cDNA was subsequently diluted to 20 ng/µl and stored at -20°C.

2.2.5 Real time quantitative RT-PCR

Primers for real time qRT-PCR were designed, intron-spanning where possible, using Primer Express® v3.0 software (Applied Biosystems) and commercially synthesised (Invitrogen Ltd, Paisley, UK). Primer sets are described in table 2.1. 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 for 40 cycles, followed by amplicon dissociation.

Table 2.1 Real time qRT-PCR primer sequences, Genebank accession number and optimal primer concentrations for all genes analysed (continued on the next page).

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Accession number	Primer conc.
<i>CD45</i>	TATTCTGGTGTCTTGATGTTGTG	CTGTACAAGGCTGATGACTTCA	NM_204417.1	300
<i>TLR4</i>	AGTCTGAATGTGCTGAGCTCAAT	GCGACGTTAAGCCATGGAAG	NM_001030693.1	300
<i>TLR5</i>	CCTTGTGCTTTGAGGAACGAGA	CACCCATCTTTTGAGAAACTGCC	NM_001024586	300
<i>TLR15</i>	GTTCTCTCTCCAGTTTGTAAATAGC	GTGGTTCATTGGTTGTTTTAGGAC	NM_001037835	300
<i>TLR21</i>	TGCCCCCTCCACTGTGTCACCT	AAAGGTGCCTTGACATCCT	NM_001030558.1	300
<i>IL1β</i>	TCGACATCAACCAGAAAGTGC	GAGCTTGTAGCCCTTGATGC	DQ393267	900
<i>IL6</i>	AGAAATCCCTCCTCGCCAAT	AAATAGCGAACGGCCCTCA	NM_204628.1	900
<i>IFNG</i>	AGCTCCCGATGAACGACTTG	ATCTCCTTGAGACTGGCTCCTT	NM_205149	300
<i>IL8</i>	GTTTCAGCTGCTGTGCGCAA	GGCGTCAGCTTCACATCTTGA	DQ393272	900
<i>K60</i>	GCCAGTGCATAGAGACCATTTC	TGCCATCTTTCAGAGTAGCTATGACT	NM_205018	300
<i>RPL7</i>	ACATGTACAGACAGAGATCCGCA	AAGCTGTAAACACCTTCCGGACCTT	NM_001006345	300
<i>ACTB</i>	CCACAGCTGCCCTAGCTCTTC	AGGACTCCATACCCAAAGAAAGATG	NM_205518	900
<i>LDHA</i>	CCTGAAGGCTCTTCATCCAG	TTCAGCTAGATCTGCCACAGAA	NM_205284	900
<i>GAPDH</i>	AGCTTACTGGAATGGCTTTCCG	ATCAGCAGCAGCCTTCACTACC	NM_204305	900
<i>TGFB4</i>	GGATGGACCCCGATGA	CGTTGAACACGAAAGAAGATGCT	M31160	900
<i>IKKB</i>	AGTTGTACCCGGAGACTGAGAGAGAA	TTGAGCATAAATGACTCGGACCTT	NM_001031397	300
<i>TOPK</i>	GGGAGATGATGACCCTGTCTGT	GGGTTCCCAAAAGCTGCATAA	NM_420011	300
<i>NOX1</i>	CGTCATCCACGGTGTGCT	CACGTGTTGCCCTGCACCTCTT	NM_001101830	300
<i>CIQTNF7</i>	TTCTGTCTAAGCACAGTACCTGCAT	TTCCTTCAGTGTGCTGCCCTACAG	XM_420778	300
<i>NFATC1</i>	CCAAATGCCCAAGCGATCAC	TTCAGAGTTGCAGCTTCTTGAA	FG356199	300
<i>MAPK9</i>	AGAAGCTCTCTCCACCTCAAAATTT	CCACACCATTCCTTGCTTCTTCTT	NM_205095	300
<i>PLA2G2E</i>	ACCTGCGTTCCCCACCTT	ACACTCTGCTGCCCTTCTTCA	XM_424364	300
<i>VCAM1</i>	GAGATTGTTTGTGTGCCAGATT	TGGAGATGCATTAAATGAAGTATTTT	XM_422310	300
<i>TCEB1</i>	GTGTGAGGGGCCAGATGCT	CCTGGTCCACTCAACATAGCTTT	NM_001007888	300
<i>IL17F</i>	AAAAATTCCTCCAAACTGTGAGA	GGTCTCATCGATCCTGTAATCC	NM_204460	300
<i>IL21</i>	CAACTTCACCAAAAGCAATGAAAT	ATCCATCCCCCAGGGTTTCT	NM_001024835	300
<i>CTLA4</i>	CTGTCCCGGTGCAGGAAT	CCTTGCTGTGGCCAGTAC	NM_001040091	300
<i>IL7R</i>	CAAAAAGGAGGCAAAATGAATATCTCA	TTATGCTCTTCCAAGTGCTTTCTTC	NM_001080106	300
<i>KK34</i>	GACCAAGTTGGAATCTCCGTATATG	TGCTCTGATCTCTGTGCAGGAA	AY321512	300
<i>AvBD1</i>	GATCCTCCAGGCTCTAGGAAG	GCCCCATATTCTTTTGC	NM_204993	900
<i>AvBD2</i>	GGTGTCTTCGCCCCGGCGGGA	TTATGCAITCCAAAGGCCAATTTG	NM_204992	900

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Accession number	Primer conc.
<i>AvBD3</i>	GAAC TGCCACTCAGTGCAGAAT	ATGGGGTTGTTTCCAGGAGC	NM_204650	900
<i>AvBD4</i>	TCATCGTGTCTCCTCTTTGTG	AATACTTGGGACGGCATAGC	NM_001001610	300
<i>AvBD5</i>	GCTGTCCCTTGCTCGAGGATT	GGAAATACCATCGGCTCCGGC	NM_001001608	900
<i>AvBD6</i>	GTCAGCCCTACTTTTCCAGC	GCCCACTGTTCCTCACAC	NM_001001193	900
<i>AvBD7</i>	ACCTGTGTCTGTCTCTCCTC	TGCACAGCAAGAGCCTATTCT	NM_001001194	900
<i>AvBD8</i>	TTCTCCTCACTGTGCTCCAA	AAGGCTCTGGTATGGAGGTG	NM_001001781	900
<i>AvBD9</i>	GCTTACAGCCCAAGAACGCT	GGAGCTAGGTGCCCATTTGCA	NM_001001611	900
<i>AvBD10</i>	GGCTCAGCAGACCCACTTTTCC	CTGCGCCGGAATCTTGGCAC	NM_001001609	900
<i>AvBD11</i>	GGTCTCGGCTTGCCCAAGAC	ATGGAAGTCTGATGATGTCTC	AY621313	300
<i>AvBD12</i>	GGAACCTTTGTTTCGTGTTCA	GAGAAATGACGGGTTCAAAAGC	AY534898	900
<i>AvBD13</i>	GATCTCCAGCTGCTCTTTG	AGTGCCCATGTTGTTCCT	AY701473	900
<i>AvBD14</i>	CATATTCTCTCTGTTCTTGTCTC	GCCAGTCCATTGTAGCAGGT	AM402954	900
<i>API</i>	CCTGTCCCTATTGACATGGA	TCCAACCTGGCAATCCTTTC	NM_001031289.1	300
<i>IRF7</i>	CGCTGAAGAAGTGCAAGGT	GCAAAACACCCCTGAAGTCGAA	NM_205372.1	300
<i>p65</i>	CGCTGCGTGCACAGTTTC	CTCCTCCATTGGCACATTGA	NM_205129.1	300
<i>STAT1</i>	CGGATGGGCTTCTATCTTATGG	AACTGCCAGCTCAGAACTTCAGA	NM_001012914.1	300
<i>IFNA</i>	ACGCCAAAGCCTCCTCAAC	TTTTTGATGGTGAGGTGAAGGTT	NM_205427.1	300
<i>IFNB</i>	TTCTCCTGCAACCATCTTCGT	TGGAAAAGTCACGTCCTTGTGTG	NM_001024836.1	300
<i>CD14</i>	TGCAGCACCTGGATCTCAGC	TGCGAGCACATGGTCCCAAG	AM933591	900
<i>MD2</i>	CATCTGGCACGCTGCTGTTA	CCGCTGCAAGAACTTGTTC	XM_418301.2	300
<i>MyD88</i>	AAGAAAGTGTGGAGGATGGT	CGGAATCAGCCGCTTGAG	NM_001030962.1	300
<i>p50</i>	TGTCACAAAGGCCCTCTGAGAGA	GAATGCCAAGACCCAGCTTTTC	NM_205134.1	900
<i>IRF1</i>	GCA TGAGACCCCTGGTTGGA	CCATGTCCCAGCCATGCT	NM_205415.1	300
<i>IRF4</i>	CCCGATTGAGCCCAAGCATAA	CAGCCTTCAGGACTAGAAGTTGTCA	NM_204299.1	300
<i>Hs IL1β</i>	CAAGGAGAAAGAAATGACAAAATACC	TGGGATCTACACTCTCCAGCTGTA	NM_000576.2	300
<i>Hs IL6</i>	AAAAAGGCAAGAAATCTAGATGCAA	TCCTTAAAGCTGGCAGAGAATG	NM_000600.3	300
<i>Hs IL8</i>	CACTGCGCCCAACACAGAAAT	TGAATTCTCAGCCCTCTTCAAAA	NM_000584.2	300
<i>Hs GAPDH</i>	CCACCCATGGCAAAATTCC	CAGCATCGCCCCACTTG	NM_002046.3	300

2.2.6 Selection of a stably expressed normalisation gene

Gene expression quantification requires multi-step processes: RNA extraction, cDNA synthesis via reverse transcription and finally qRT-PCR. During these processes, experimental errors are common place. RNA quality and quantity, reverse transcriptase enzyme efficiencies and qRT-PCR reaction efficiencies can vary considerably between samples, and thus can result in inaccurate gene expression quantification between two experimental groups. To minimise this, each gene of interest is amplified in each sample in parallel with an internal reference gene. A reference gene is assumed to be stably expressed under any experimental condition, and thus acts as a normaliser. Traditionally, either *GAPDH* or *ACTB* would have been used. However, it has since been shown that certain experimental conditions can affect the expression of these genes (Dheda *et al.*, 2005). Therefore, in each experimental condition, it is critical to select a reference gene that is stably expressed. geNorm is a Visual Basic Application (VBA) for Microsoft Excel that is used to determine the most stable reference gene from a panel of potential reference genes, in a given experiment. The algorithm assumes that the expression ratios of the two best reference genes are identical across all samples. Using this principal, a gene stability measure “*M*” is calculated for each reference gene. The lower the *M* value, the more stable the reference gene (Vandesompele *et al.*, 2002). Using geNorm, the stability of four putative reference genes namely *RPL7*, *GAPDH*, *ACTB* and *LDHA* were investigated across samples for both bacterial-challenged and mock-control birds, in the *C. jejuni* and *S. Typhimurium* infection models.

Genes that have *M* values < 1.5 are considered stable. For both infection models, all four reference genes had *M* values < 0.9 indicating that neither *C. jejuni* nor *S. Typhimurium* challenge affect the stability of these genes. Regarding the *C. jejuni* infection model, *RPL7* was found to be the most stable reference gene in the caecum, caecal tonsil and liver. *RPL7* was also stably expressed in caecum samples of the *S. Typhimurium* infection model. On this basis, *RPL7* was chosen as the most appropriate reference gene for transcriptional profiling in the *C. jejuni* and *S. Typhimurium* infection models.

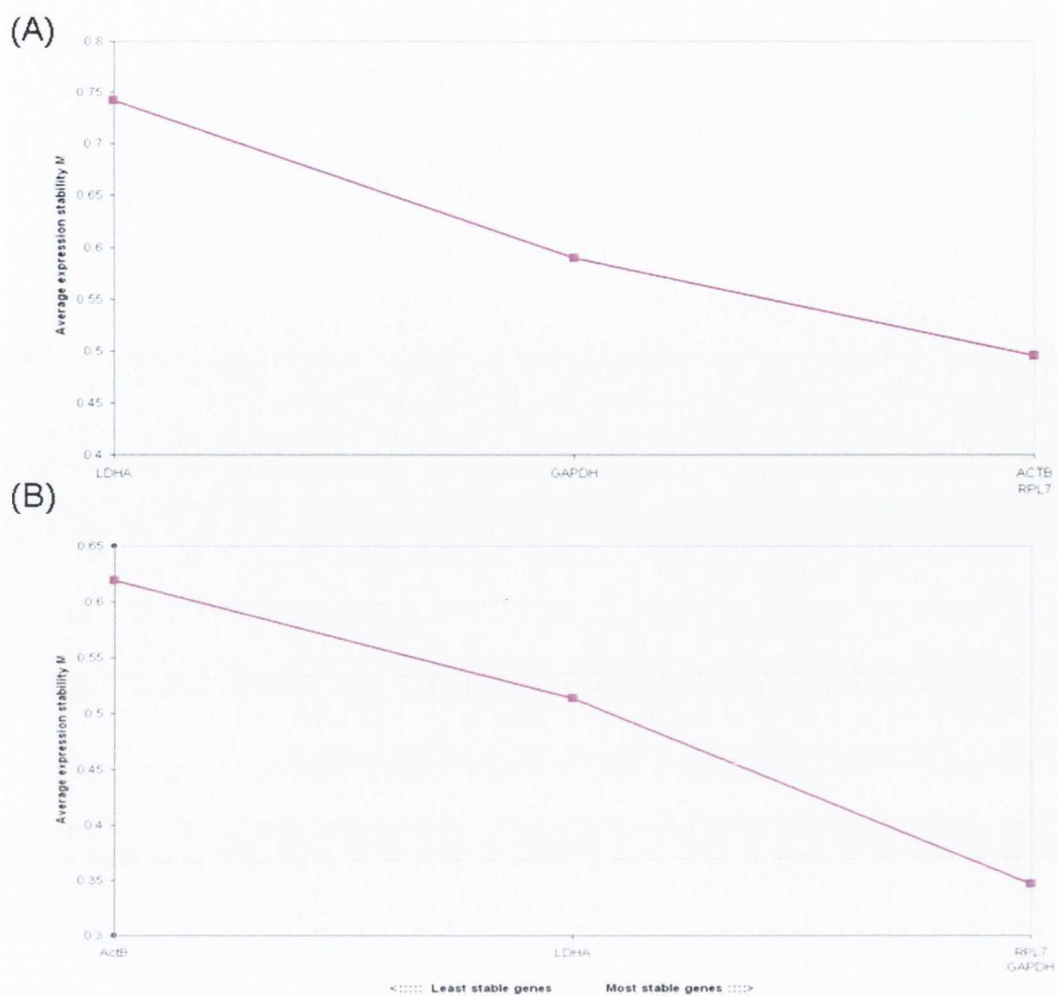


Figure 2.2 Stability of reference genes across caecal samples of *C. jejuni*-challenged birds and their mock-challenged controls. **(A)** Stability of reference genes across caecal tonsil samples at 6 h post-challenge **(B)** Stability of reference genes across caecum samples at 6 h post-challenge.

2.2.7 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).

2.3 Global gene expression analysis *in vivo* in response to *C. jejuni*

2.3.1 Selection of birds for global gene expression analysis

Caecum samples from 22 birds from the *C. jejuni* infection model (section 2.1.2) were used, representing seven *C. jejuni*-challenged (20 h post-challenge), seven mock-challenged (20 h post-challenge) and eight unchallenged chickens (Fig. 2.1 and Fig. 2.4).

2.3.2 Microarray experimental design and cDNA labelling

Gene expression between *C. jejuni*-challenged and mock-challenged birds was compared using a common-reference design (CRD), which involves hybridising each different biological sample to a common sample (Churchill, 2002). The common reference sample consisted of pooled RNA that had been extracted from the caeca of unchallenged birds. This design involves hybridizing the common reference sample with both the *C. jejuni*-challenged and mock-challenged samples enabling flexible data comparisons, and avoiding artificial direct pairings between animals from each treatment group (Fig. 2.3). To achieve this, 8 µg of common reference RNA and 10 µg sample RNA were reverse transcribed into cDNA using the Superscript™ Plus Direct cDNA Labelling System (Invitrogen) according to manufacturers instructions.

The common reference sample was labelled with Alexa Fluor[®] 647 while the challenge/mock-challenged samples were labelled with Alexa Fluor[®] 555. cDNA concentrations and dye incorporation were determined using a Nanodrop[™] 1000 Spectrophotometer (Thermo Scientific).

Chicken specific oligonucleotide microarrays containing 24,182 (20K) genes/transcripts, supplied by VIDO, University of Saskatchewan, were used in this study. The gene/transcript library was similar to the ARK-Genomics *G. gallus* 20K v1.0 library (www.ark-genomics.org/microarrays/bySpecies/chicken) except the arrays were spotted by a gene array facility located in the Prostate Centre at Vancouver General Hospital (www.microarray.prostatecentre.com).

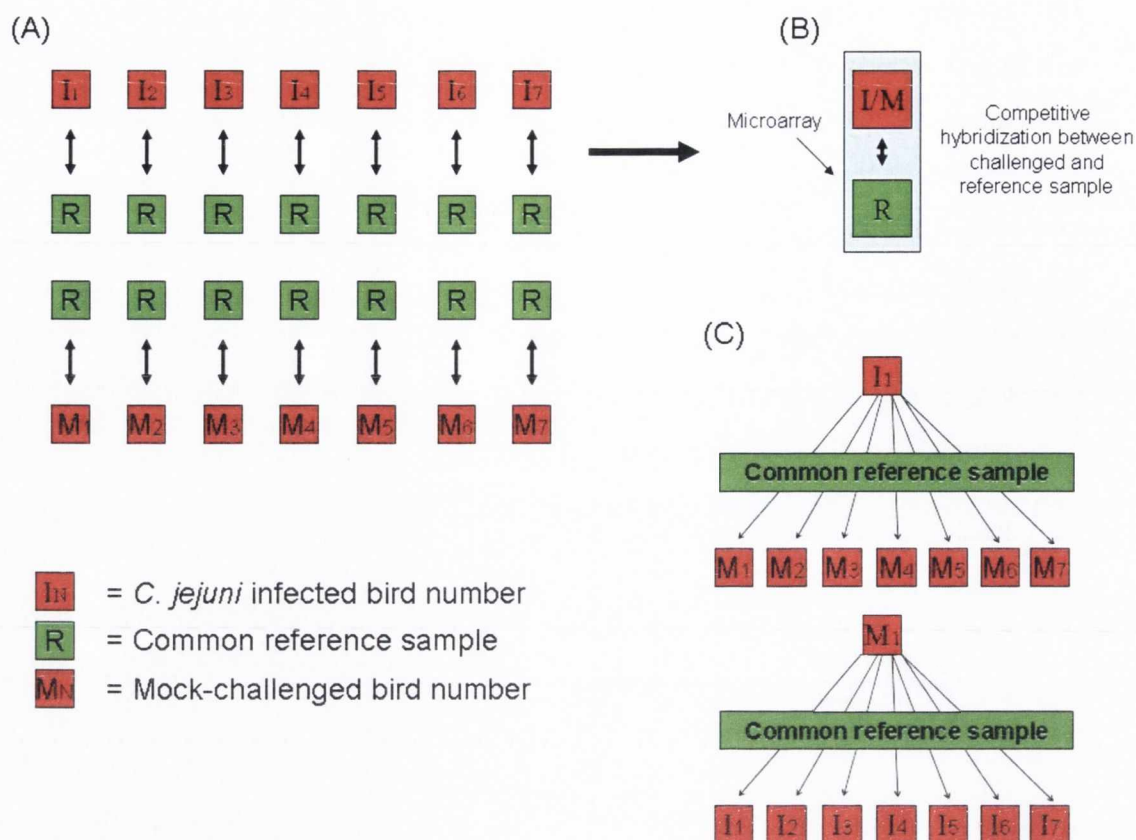


Figure 2.3 Common reference design. **(A)** Fluorescent labelled cDNA samples from *C. jejuni*-infected birds and their mock-challenged controls are each compared to a common reference sample containing a different fluorescent label. **(B)** Competitive hybridization to a microarray takes place between challenged samples and the common reference sample. **(C)** The common reference design enables flexible comparisons between the *C. jejuni*-infected birds and their mock-challenged controls.

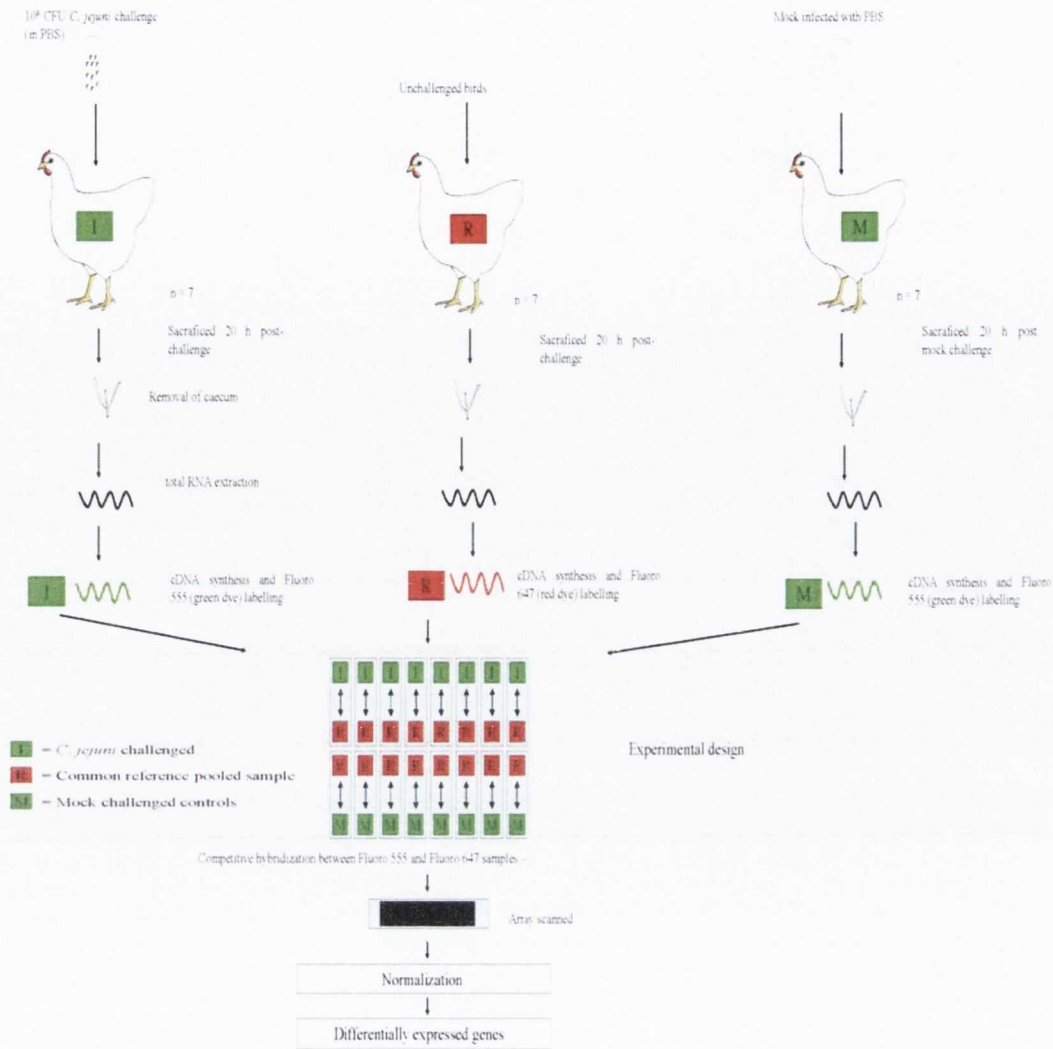


Figure 2.4 Microarray experiment flow-through

2.3.3 Microarray hybridizations and array scanning

Each labelled reference sample was combined with a labelled sample in addition to 90 µl SlideHyb Glass Hybridization Buffer #3 (Ambion Inc.) to form the probe. 100µl of probe was hybridised to 20K oligonucleotide chicken microarrays using the following program on an automated HS 400™ Pro Hybridization Station (Tecan UK Ltd.) – WASH (4X SCC/ 0.2% SDS): 75°C, Runs 1, Wash 10 s, Soak 20 s; PROBE INJECTION: 85°C, Denaturation: 95°C, 2 min; HYBRIDISATION: 42°C, Agitation Frequency Medium 4 hours; HYBRIDISATION: 35°C, Agitation Frequency Medium 4 hours; HYBRIDISATION: 30°C, Agitation Frequency Medium 4 hours; WASH (2X SCC/ 0.1% SDS): 37°C, Runs 2, Wash 10 s, Soak 20 s; WASH (0.2X SCC/ 0.1% SDS): 25°C, Runs 2, Wash 15 s, Soak 30 s; WASH (0.2X SCC): 25°C, Runs 2, Wash 20 s, Soak 40 s and SLIDE DRYING: 25°C, 2 min N₂ at 2.7 bar. Each array was subsequently scanned using a Genepix 4000B scanner (Molecular Devices Ltd.) at photo-multiplier tube (PMT) voltages to avoid the saturation of spots. Spots that were deemed unreliable were flagged as bad, which would be taken into consideration during subsequent data analysis.

2.3.4 Data pre-processing, normalization and analysis

The median background intensities were subtracted from the median foreground intensities to establish the working signal intensities for subsequent analysis using BRB-ArrayTools version 3.8.0 (Simon *et al.*, 2007). Prior to analysis of raw gene expression from a microarray experiment, the feature values must be adjusted to account for technical variations such as dye biases, dye incorporation inefficiencies and spatial intensity variations that may interfere with the true caecal gene expression ratios between *C. jejuni*-challenged and mock-challenged cohorts (Smyth & Speed, 2003). A number of normalization strategies have been devised to tackle different components of technical variation. These assume that the majority of genes on the array are not differentially expressed or there is a balance between genes that are upregulated and downregulated. Based on this assumption, a normalization

factor is calculated and applied to the intensity values, in order to balance the fluorescent intensities between the two hybridized samples (Quackenbush, 2001). Normalizations and subsequent data analyses were also carried out using BRB-ArrayTools. The raw data was adjusted using Median (Zien *et al.*, 2001), locally weighted scatterplot smoothing (Lowess) and Lowess print-tip normalizations (Yang *et al.*, 2002). Prior to and following normalizations, the data was visualised using MA plots, which plot the difference in average log intensities against the average of log intensities ($M = \log_2 R - \log_2 G$; $A = \frac{1}{2}(\log_2 R + \log_2 G)$)(Yang *et al.*, 2002). Class comparisons (univariate parametric and non-parametric tests) between the *C. jejuni*-challenged and mock-challenged data sets were subsequently carried out to compare the three normalization strategies. To study the false discovery rate (FDR), class comparisons were repeated with misclassified data sets.

2.3.5 Characterisation of differentially expressed genes

Following class comparisons, genes with alpha levels of $P < 0.01$ were deemed significantly differentially expressed. The 70 nucleotide probe of each differentially expressed gene was compared against the chicken genome using BLAST (basic local alignment search tool), to initially annotate the genes. GO Slim using the Protein Information Resource (PIR) subset of GO terms was used to assign known biological functions to each significantly differentially expressed gene. cDNA synthesis, qRT-PCR and data analysis were carried out for validation as previously described in sections 2.2.4, 2.2.5 and 2.2.7 respectively.

2.4 Avian cell interactions with *C. jejuni* components *in vitro*

2.4.1 Cell culture

All cell lines were routinely subcultured and subsequently incubated under 5% CO₂ at 37°C. Chicken embryonic fibroblasts (CEF) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated foetal

calf serum (FCS), 50 units/ml penicillin (Invitrogen) and 50 µg/ml streptomycin (Invitrogen). The HD11 chicken macrophage cell line was grown in Roswell Park Memorial Institute (RPMI)-1640 medium with the addition of 10% heat-inactivated FCS and 50 µg/ml gentamicin (Invitrogen). HD11 cells were trypsinised prior to subculturing and plating. THP1 (Human acute monocytic leukemia cell line) cells were cultured in RPMI supplemented with 10% heat-inactivated FCS, 50 units/ml penicillin and 50 µg/ml streptomycin. THP1 cells were also differentiated into macrophages in which 15×10^6 cells were transferred to T-175 tissue culture flasks with 40 ml THP1 media supplemented with 10 ng/ml Phorbol 12-myristate 13-acetate (PMA) (Invivogen). THP1 cells were differentiated for 72 h under 5% CO₂ at 37°C. Subsequently, unattached (undifferentiated and dead) cells were washed away with sterile Dulbecco's PBS (1x), and standard THP1 media was added to the flasks, which were subsequently incubated for 48 h under 5% CO₂ at 37°C. THP1 macrophage cells were trypsinised prior to subculturing and plating.

2.4.2 Bacterial cell components and reagents

Ultrapure LPS from *E. coli* (K12 or 0111:B4) was purchased from Invivogen (www.invivogen.com). Purified capsular polysaccharide (CPS) and ultrapure LOS from *C. jejuni* strain 81116 were obtained from Professor Anthony Moran (School of Natural Sciences, National University of Ireland, Galway). CPS had been isolated from *C. jejuni* grown at 37°C and subsequently subjected to RNase A, DNase II and proteinase K treatments to remove bacterial components other than polysaccharides (Corcoran *et al.*, 2006). Ultrapure LOS had been extracted from *C. jejuni* grown at both 37°C and 42°C using the hot phenol-water extraction procedure, and subsequently purified via enzymatic digestion (RNase A, DNase II and proteinase K) and ultracentrifugation (Moran *et al.*, 1991a). Recombinant *C. jejuni* CDT was provided by Professor Hidde Ploegh (Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA). CDT subunits had been expressed separately in an *E. coli* expression system followed sequentially by affinity purification of subunits, subunit co-refolding, further affinity purification and size exclusion. Recombinant chicken IL-1β and IL-6 cytokines were purchased from AbD Serotec (www.abdserotec.com). Inhibitors for JNK (SP600125), p38 (SB203580),

ERK (PD98059) and I κ B- α (Bay11-7082) were purchased from Invivogen (www.invivogen.com). Polymyxin B (PMB), a cyclic cationic polypeptide antibiotic which neutralises LPS, CLI-095 (TAK-242), a cyclohexane that suppresses TLR4-mediated signalling, and OxPAPC (1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine), an oxidized phospholipid that inhibits both TLR2 and TLR4 activation were also purchased from Invivogen (www.invivogen.com).

2.4.3 *In vitro* effects of microbial components

Challenges involving bacterial cell components were conducted in antibiotic-free media. HD11, THP1 monocyte and THP1 macrophage cells were added at 1×10^6 cells/ml to wells of 12-well tissue culture plates, 1 h prior to challenges. CEF cells (1×10^6 cells/ml) were seeded into 12-well plates 24 h before challenges, and were washed with Dulbecco's PBS (1x) at 37°C, followed by the addition of 1 ml of fresh media to each well, 1 h prior to challenges. *E. coli* LPS and *C. jejuni* LOS were added to final concentrations of 1 μ g/ml and 5 μ g/ml. To neutralise endotoxin contaminants, 10 μ g/ml PMB was added to cells 1 h prior to challenges with *C. jejuni* CPS and *C. jejuni* recombinant CDT. CPS was added to cells at 1 μ g/ml and 10 μ g/ml while CDT was added at 1 μ g/ml and 5 μ g/ml. All challenges were conducted for 4 h, unless otherwise stated.

2.4.4 *In vitro* effects of proinflammatory stimuli

For chicken recombinant cytokine challenges, 10 μ g/ml PMB was added to HD11 cells (1×10^6 cells/ml), 1 h prior to challenges. IL-1 β and IL-6 were added to final concentrations of 10 ng/ml, 1 μ g/ml or 5 μ g/ml. Each challenge was performed in duplicates or triplicates and controls consisted of unchallenged cells. Challenges were conducted for 4 h. For supernatant based challenges, 1×10^6 HD11 cells in 1 ml of antibiotic-free medium per well were challenged with *E. coli* LPS at final concentrations of 1 μ g/ml. At 4 h post challenge, supernatants (900 μ l) from unchallenged and *E. coli* LPS-challenged cells were removed. Subsequently, PMB was added to the supernatants at final concentrations of 100 μ g/ml for 1 h at 37°C.

Subsequently, 1×10^6 HD11 cells (in 1 ml media per well) were stimulated with 900 μ l of challenged or unchallenged supernatants for 4 h.

2.4.5 Signalling pathway dissection

Chicken HD11 macrophages cells were added at 1×10^6 cells/ml to wells of 12-well tissue culture plates for 1 h prior to challenge. Signalling pathway inhibitors were dissolved in dimethyl sulfoxide (DMSO). SP600125, SB203580, PD98059 and Bay11-7082 were added to final concentrations of 100 μ M, 20 μ M, 5 μ M and 10 μ M respectfully, 1 h prior to *C. jejuni* LOS (1 μ g/ml) challenges. Controls consisted of cells challenged with 0.1% DMSO and cells challenged with LOS (with 0.1% DMSO). In wells of challenged and unchallenged, DMSO concentrations were no greater than 0.1%. Each challenge was performed in triplicate. Statistical analysis was carried out between LOS challenged and LOS challenged in the presence of each inhibitor using unpaired t-tests (GraphPad Prism 5).

HD11 cells (1×10^6 cells/ml) and THP1 monocytes (1×10^6 cells/ml) were incubated with 300 nM, 1 μ M or 3 μ M CLI-095 for 1 h prior to challenge. Controls consisted of cells challenged with 0.1% DMSO. Subsequently, CLI-095 treated cells were challenged with *C. jejuni* LOS or *E. coli* LPS at final concentrations of 1 μ g/ml for 4 h. For OxPAPC challenges, the phospholipid was added to HD11 cells (1×10^6 cells/ml) at final concentrations of 15 μ g/ml or 30 μ g/ml 1 h prior to challenge. Subsequently, OxPAPC-treated cells were challenged with 1 μ g/ml *E. coli* LPS for 4 h. Controls consisted of unchallenged cells.

2.4.6 RNA extraction from cells

Total RNA was extracted from cells using TRIZOL[®] Reagent (Invitrogen) according to the manufacturer's recommendations. Briefly, cell samples were lysed in 1 ml TRIZOL reagent at room temperature over a 5 min period. Next, 200 μ l chloroform (Sigma-Aldrich) was added to the lysates in 2 ml tubes. Tubes were shook vigorously for 15 s followed by incubation at room temperature for 3 min. Samples were subsequently centrifuged at $12,000 \times g$ for 15 min at 4°C. The colourless upper

aqueous layer of each sample was carefully removed and mixed with 500 μ l isopropanol (Fisher Scientific). Samples were incubated at room temperature for 10 min followed by centrifugation at $12,000 \times g$ for 10 min at 4°C, which resulted in the formation of RNA pellets. Supernatants were removed and 1 ml of 75% ethanol was added to each of the pellets followed by vortexing of samples and centrifugation at $7,500 \times g$ for 5 min at 4°C. Supernatants were removed and cell pellets were dried for 5 min. Subsequently, cell pellets were dissolved in 20-35 μ l RNase-free distilled H₂O for 5 min at 60°C. Samples were immediately stored at -80°C. RNA yield was quantified using a NanoDrop® ND-1000 spectrophotometer.

2.4.7 cDNA synthesis, qRT-PCR and data analysis

RNA samples from challenged and unchallenged cells were reverse transcribed into cDNA as indicated in section 2.2.4 while qRT-PCR was carried out as indicated in section 2.2.5. *RPL7* and *GAPDH* were chosen as the normalisation genes for chicken and human cell challenges, respectively. Subsequently, data analysis was carried out using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Primer sets are described in table 2.1.

2.4.8 Flow cytometry analysis of *C. jejuni* CDT activity on cells

THP1 monocytes and HD11 macrophage were added at 1×10^6 cells/ml to wells of 12-well tissue culture plates 1 h prior to stimulation. Recombinant *C. jejuni* CDT were added to final concentrations of 1 μ g/ml, and cells were subsequently incubated at 37°C for 48 h. Cell samples were then washed in 500 μ l sterile PBS and were subsequently resuspended in 100 μ l Annexin V Incubation Reagent (Trevigen®), which consisted of 10 μ l 10X Binding Buffer, 10 μ l Propidium Iodine, 1 μ l Annexin V-FITC and 79 μ l distilled H₂O. Cell samples were incubated in the dark for 15 min at room temperature, followed by the addition of 400 μ l 1X Binding Buffer. Samples were immediately processed using a CyAn™ ADP Analyzer (Dako). Subsequent analysis was performed by Barry Moran, School of Biochemistry and Immunology, Trinity College Dublin.

Chapter 3

Comparative Innate Immune Gene Expression Profiling in Response to *S. Typhimurium* and *C. jejuni* in the Chicken Caecum

Chapter 3: Comparative Innate Immune Gene Expression Profiling in Response to *S. Typhimurium* and *C. jejuni* in the Chicken Caecum

3.1 Introduction

The caeca of commercial chickens often encounter high-profile bacterial organisms that, therein, exhibit either pathogenic or commensal lifestyles. Two *Salmonella enterica* serovars, Gallinarum and Pullorum, invade the caecal mucosa and establish systemic infections, which often result in bird mortalities. *Salmonella enterica* serovar Typhimurium induces caecal pathology in to newly hatched chicks that is not evident with older birds (Chappell *et al.*, 2009). On the other hand, *C. jejuni* is a commensal in chickens, regardless of bird age (Van Deun *et al.*, 2007). Considering that clinical signs are largely associated with underlying proinflammatory responses, fundamental aspects of the caecal innate immune response must differ between *Salmonella* and *Campylobacter* species.

The innate immune response to *S. enterica* has been mainly characterised in young chicks. This bacterial species is capable of invading the epithelium, and is subsequently detectable within the lamina propria and residential macrophages (Berndt *et al.*, 2007; Henderson *et al.*, 1999). During this process, a number of PRRs are likely activated, as components of the microbe are known to activate several avian TLRs including TLR5 (Iqbal *et al.*, 2005; Keestra *et al.*, 2008) and TLR4 (Kogut *et al.*, 2005), while both receptors are associated with resistance to systemic *S. enterica* infections (Iqbal *et al.*, 2005; Leveque *et al.*, 2003). Moreover, in the caeca of challenged birds, increased *TLR15* gene expression is evident (Higgs *et al.*, 2006; Mackinnon *et al.*, 2009), although precise ligands for this receptor have yet to be identified. Consequent to PRR activation, *IL8* and *K60* chemokines are induced (Withanage *et al.*, 2004), which attract substantial numbers of heterophils and mononuclear cells to the lamina propria (Berndt *et al.*, 2007; Henderson *et al.*, 1999). Collectively, residential and recruited cells likely to produce an array of proinflammatory cytokines that contribute to inflammation such as IL-1 β , IL-6, IFN-

γ , macrophage inflammatory protein (MIP)-1 β , lipopolysaccharide-induced tumor necrosis factor- α factor (LITAF) and IL-12 as mRNA for these genes are increased in *S. enterica* infected intestinal samples (Berndt *et al.*, 2007; Fasina *et al.*, 2008; Iqbal *et al.*, 2005; Withanage *et al.*, 2004). The roles of such proinflammatory mediators has, however, not been investigated in older birds with asymptomatic *S. Enteritidis* and *S. Typhimurium* infections where maturation of innate defences possibly account for the lack of immunopathology.

Maturation of host defences alone clearly does not explain *C. jejuni*'s commensal status in the avian intestine, as even newly hatched chicks colonised with the microbe are free of clinical signs (Van Deun *et al.*, 2007). The avian innate immune response to *C. jejuni* has not been studied in any great detail and thus, one is free to suggest a number of hypotheses for the lack of pathology. While live *C. jejuni* induces proinflammatory gene expression in macrophages, primary kidney cells and epithelial cells of avian origin (Borrmann *et al.*, 2007; Li *et al.*, 2008c; Smith *et al.*, 2005a), the microbe may still have the ability to evade key avian PRRs that are required for the initiation of significant intestinal immunopathology. Alternatively, *C. jejuni* may produce components that dampen proinflammatory responses while it is also possible that the microbe adapts a more avirulent phenotype in the avian mucosa, somewhat minimising the innate immune response. Finally, as birds are regarded as the primary host, avian defences may have adapted to regulate *C. jejuni* colonisation without the necessity of proinflammatory effectors. For example, murine macrophages repeatedly exposed to LPS tend to reduce proinflammatory cytokine production in favour of antimicrobial protein increases (Foster *et al.*, 2007). Chicken antimicrobial effectors include 14 beta-defensins (Lynn *et al.*, 2007) and four cathelicidins (Goitsuka *et al.*, 2007). AvBD6 has proven antimicrobial activity towards *C. jejuni* (van Dijk *et al.*, 2007) while Cath-2 has the ability to reduce proinflammatory cytokine production (van Dijk *et al.*, 2009a).

A *C. jejuni* infection model using 4-week-old commercial broiler chickens was carried out to characterise the immune response to the microbe. To investigate the intestinal innate immune response, the first step was to determine whether genes for key TLRs, cytokines, chemokines and HDPs were differentially expressed in the caeca of *C. jejuni*-challenged birds relative to mock-challenged controls, and thus is the focus of this chapter. Innate immune gene expression was also profiled in the

caeca of *S. Typhimurium* challenged chickens, to serve as a comparison, as we hypothesised that the innate immune response to each microbe would differ.

3.2 Results

3.2.1 Caecal colonisation

S. Typhimurium and *C. jejuni* caecal colonisation quantified was performed by Dr. Brenda Allan, VIDO, University of Saskatchewan, Saskatoon, Canada. 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. Persistent colonisation was detected in response to both bacterial challenges across the 48 h time-course. High levels of *S. Typhimurium* colonised the caeca of challenged birds at all three time-points. A mean of 2.1×10^7 cfu/g was detected at 6 h, a mean of 1.3×10^7 cfu/g at 20 h, and a mean of 1.2×10^7 cfu/g at 48 h. Levels of *C. jejuni* colonisation in the caecum were higher than with the *S. Typhimurium* challenge across the time-course: 6 h (a mean of 2.4×10^8 cfu/g; >10 fold), 20 h (a mean of 2.25×10^{11} cfu/g; $>1.7 \times 10^4$ fold higher) and 48 h (a mean of 8.58×10^{11} cfu/g; >74,000 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).

3.2.2 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 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* 6 h, 20 h and 48 h post-challenge. Heterophil migration was not detected in response to *C. jejuni* at any time point (Table 3.1).

3.2.3 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. 3.1). There was a significant but transient increase of *CD45* gene expression to both *S. Typhimurium* (2.77 fold; $P=0.004$) and *C. jejuni* (2.55 fold; $P=0.025$) at 6 h post challenge. In contrast, there was a significant decrease of *CD45* gene expression (-2.23 fold; $P=0.004$) in response to *S. Typhimurium* 20 h post-challenge but *CD45* was not differentially expressed in response to *C. jejuni* at this time point. Profiling *CD45* gene expression 48 h post-challenge did not reveal any significant changes in response to either bacterial species (Fig. 3.1.).

3.2.4 Bacterial induced TLR gene expression profiling

We compared TLR gene expression changes in response to both bacterial challenges (Fig. 3.2). Of the 4 TLRs examined by qRT-PCR, all 4 genes were significantly differentially expressed in one or other infection model ($P < 0.05$).

TLR4, which detects LPS, was transiently increased by 2.29 fold ($P=0.037$) at 6 h post-challenge with *S. Typhimurium* followed by a significant reduction after 20 h (3.35 fold; $P=0.004$), and not differentially expressed after 48 h (Fig. 3.2 A). Increased *TLR4* (3.98 fold; $P=0.025$) gene expression was detected in response to *C. jejuni* after 6 h and was not differentially expressed for the remainder of the time-course (Fig. 3.2 B).

TLR5, which detects flagellin, was also transiently increased in response to *S. Typhimurium* by 1.92 fold ($P=0.053$) after 6 h, decreased significantly at 20 h (-1.29 fold; $P=0.053$), and was increased again after 48 h (2.17 fold; $P=0.055$) post-challenge (Fig. 3.2 A). *TLR5* was not differentially expressed in response to *C. jejuni* throughout the time-course.

TLR15 gene expression was decreased in response to *S. Typhimurium* at 20 h (-1.19 fold; $P=0.055$) and increased at 48 h (6.23 fold; $P=0.004$) post-challenge (Fig. 3.2 A). In contrast, *TLR15* was not differentially expressed in response to *C. jejuni* throughout the time-course.

TLR21 gene expression was increased in response to *S. Typhimurium* at 6 h (5.2-fold; $P = 0.004$), followed by a decrease at 20 h (-1.1-fold; $P = 0.050$), and was not differentially expressed at 48 h post-challenge. Increased *TLR21* (3.5-fold; $P = 0.025$) gene expression was detected in response to *C. jejuni* at 6 h and was not differentially expressed for the remainder of the time-course (Fig. 3.2 B).

3.2.5 Comparative chemokine and cytokine gene expression profiling

Expression profiling of cytokines and chemokines was performed to detect differential expression in both infection models, and characterise the predominant type of immune response across the timecourse. *IFNG* gene expression was transiently increased at 6 h (4.07 fold; $P=0.055$), not differentially expressed at 20 h, and significantly increased at 48 h (6.01 fold; $P=0.007$) post-challenge in response to *S. Typhimurium* (Fig. 3.3 A). In contrast, *IFNG* was not differentially expressed in response to *C. jejuni* throughout the timecourse (Fig. 3.3 B).

In response to *S. Typhimurium*, differential gene expression of *IL1 β* was first detected at 20 h with an increase of 2.30 fold ($P=0.050$), followed by a further increase of 7.41 fold ($P=0.004$) at 48 h post-challenge (Fig. 3.3 A). Similarly, increased *IL1B* gene expression was detected after both 20 h (5.24 fold; $P=0.005$) and 48 h (3.87 fold; $P=0.025$) in response to *C. jejuni* (Fig. 3.3 B).

IL6 gene expression was significantly increased in response to *S. Typhimurium* at 6 h (11.73 fold; $P=0.004$), 20 h (1.87 fold; $P=0.054$) and 48 h (19.39 fold; $P=0.004$) post-challenge (Fig 3.3 A). Differential gene expression of *IL6* in response to *C. jejuni* was evident only after 48 h (4.35 fold; $P=0.025$) post-challenge (Fig 3.3 B).

Increased *TGFB4* gene expression in response to *S. Typhimurium* at 6 h (1.55 fold; $P=0.004$), 20 h (2.37 fold; $P=0.055$) and 48 h (1.91 fold; $P=0.003$) post challenge. In contrast *TGFB4* gene expression was not differentially expressed in response to *C. jejuni* throughout the timecourse (Fig. 3.3).

Gene expression of the chemokine *IL8* was significantly increased in response to *S. Typhimurium* at 6 h (5.07 fold; $P=0.004$), not differentially expressed at 20 h, and again increased at 48 h (11.48 fold; $P=0.055$) post-challenge. There was a significant decrease of *IL8* (-2.91 fold; $P=0.030$) gene expression and in response to

C. jejuni after 6 h, followed by a substantial increase at 20 h (24.28 fold; $P=0.002$) and a reduction at 48 h (3.33 fold; $P=0.002$) post-challenge (Fig. 3.4 A).

Increased expression of another chemokine, *K60* was also evident in response to *S. Typhimurium* after 6 h (2.10 fold; $P=0.004$), 20 h (4.34 fold; $P=0.004$) and 48 h (4.06 fold; $P=0.043$) post-challenge. In response to *C. jejuni*, there was a transient decrease of *K60* gene expression at 6 h (-4.69 fold; $P=0.024$) followed by a transient increase at 20 h (6.64 fold; $P=0.002$) post-challenge (Fig. 3.4 B).

3.2.6 Bacterial induced AvBD gene expression profiling in the caecum

AvBD gene expression was profiled at 6 h post-challenge, in both infection models. AvBD gene expression was highly variable in both bacterial challenged and mock-challenged representatives. In some birds, expression of specific AvBD genes was not detectable, which made it difficult to accurately quantify gene expression changes. For certain AvBDs, non-specific amplification was evident, sometimes in only one of the infection models. In such circumstances, further analysis was not carried out. As AvBD profiling proved difficult, it was not possible to compare gene expression between the two infection models for every AvBD.

Significantly increased gene expression for *AvBD5* (16.8-fold; $P=0.004$), *AvBD9* (13.4-fold; $P=0.004$), *AvBD10* (12.3-fold; $P=0.004$), *AvBD12* (6.5.8-fold; $P=0.004$) and *AvBD13* (11.5-fold; $P=0.004$) were detected in response to *S. Typhimurium* (Fig. 3.5 A).

In response to *C. jejuni*, significantly increased gene expression was detected for *AvBD5* (3.8-fold; $P=0.025$), *AvBD6* (10.5-fold; $P=0.025$), *AvBD9* (23.4-fold; $P=0.025$), *AvBD12* (21.2-fold; $P=0.025$), *AvBD13* (3.9-fold; $P=0.035$) and *AvBD14* (3.5-fold; $P=0.025$) gene expression. Decreased *AvBD7* (-1.8-fold) gene expression was detected in response to *C. jejuni* ($P=0.025$) (Fig. 3.5 B).

AvBD5, *AvBD9* and *AvBD10* were also profiled at 20 h post-challenge in response to *C. jejuni*. None of the three genes were significantly differentially expressed at this time-point (Fig. 3.6).

3.2.7. *C. jejuni* induced chemokine and AvBD gene expression in the caecal tonsil

To determine if the source of the elevated *IL8* chemokine gene expression in response to *C. jejuni* was from lymphoid tissue, *IL8* and *K60* were profiled in the caecal tonsil. Increased *IL8* gene expression was detected 6 h (2.29 fold; $P=0.025$), 20 h (4.51 fold; $P=0.002$) and 48 h (7.36 fold; $P=0.025$) post-challenge. *K60* mRNA was significantly increased only at 48 h post-challenge (2.25 fold; $P=0.025$) (Fig. 3.7).

In the caecal tonsil, increased *AvBD3* (4.1-fold; $P=0.025$), *AvBD10* (2.3-fold; $P=0.025$) and *AvBD13* (2.2-fold; $P=0.025$) gene expression was detected in response to *C. jejuni* at 6 h post-challenge (Fig. 3.8).

3.3 Discussion

To date, studies of innate immune gene expression in the chicken have mainly 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 adult 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 poorly understood, especially whether such responses, if present, influence the level of bacterial colonisation. *S. Typhimurium* is capable of inducing immune mediated pathology only in newly-hatched chicks (Henderson *et al.*, 1999) but not in older birds, suggesting that maturity of host defences may be important. In contrast, *C. jejuni* is a commensal in both infant and mature chickens (Van Deun *et al.*, 2007; Young *et al.*, 2007), suggesting the involvement of novel strategies to minimise immunopathology. The hypothesis was that comparing caecal innate immune gene expression between *S. Typhimurim* and *C. jejuni* challenged birds would highlight distinct features of innate immune response to the latter bacterial species that may explain its unique commensal state.

As expected, both bacterial species persistently colonised the caecum of challenged birds across the 48 h time-course. In comparison to *S. Typhimurium* colonisation, *C. jejuni* colonisation levels were higher, most notably at 20 h and 48 h post-challenge (>17,000-fold and >74,000-fold higher respectively), 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. A previous study showed marked heterophil infiltration to the lamina propria from chicks after *S. Typhimurium* challenge (Hendrixson & DiRita, 2004). Only subtle heterophil infiltration to the caecum was detected in this study of adult birds in response to *S. Typhimurium*. Heterophil influx was clearly absent from the *C. jejuni* challenge, in agreement with recent literature (Henderson *et al.*, 1999). To further characterise cellular changes in response to infection, but at the molecular level, 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 h in response to both bacterial challenges. *CD45* is, however, upregulated on activated inflammatory cells

(Hermiston *et al.*, 2003) and therefore, such increases may represent transient activation of residential phagocytes within the lamina propria in response to the high bacterial challenges. The lack of increased *CD45* gene expression for the remainder of the time-course to either infection suggests an absence of immune cell infiltration or activation, and is in agreement with the histological observations.

The expression of genes involved with microbial detection and activation of subsequent innate immune responses were next quantified. As early as 6 h post-challenge, expression of *TLR4* and *TLR21* was transiently increased in caecal tissue in response to both bacterial challenges. It is unclear whether TLR upregulation directly correlates to TLR activation although chicken *TLR4* and *TLR21* have been shown to recognise endotoxins and chromosomal DNA respectively from both *C. jejuni* and *Salmonella in vitro* (de Zoete *et al.*, 2010a; Keesstra *et al.*, 2010; Kogut *et al.*, 2005; van Mourik *et al.*, 2010). Increased *TLR5* gene expression was detected only in response to *S. Typhimurium*, evident at both 6 h and 48 h post-challenge. This increase could possibly represent TLR activation as chicken *TLR5* is activated by *S. Typhimurium* flagellin while *C. jejuni* flagellin has recently been observed not to trigger the receptor (de Zoete *et al.*, 2010a; de Zoete *et al.*, 2010b; Iqbal *et al.*, 2005). Interestingly, *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). It is, however, unknown whether *TLR15* recognises any components from the microbe. Collectively, increased *TLR5* and *TLR15* gene expression in response to *S. Typhimurium* but not to *C. jejuni* highlights differences in the caecal immune response to each bacterial species that may be a reflection of differential TLR activation.

In newly hatched chicks, *S. Typhimurium* induces gene expression for multiple proinflammatory cytokines and chemokines (Withanage *et al.*, 2004) that are considered major mediators of the subsequent intestinal inflammation (Berndt *et al.*, 2007; Henderson *et al.*, 1999). In this study, despite a lack of aberrant inflammation and other clinical signs, proinflammatory cytokine and chemokine gene expression increases were evident in the caeca of *S. Typhimurium* challenged 4-week-old birds throughout the time course. *IFNG*, *IL6* and *IL8* gene expression changes followed a similar transient pattern, which involved significant increases at 6 h, no increase at 20 h, and significant increased expression again at 48 h. Intermittent invasion of the caecal epithelium and subsequent encounters with underlying phagocytes may

account for such observations. *K60* and *TGF β 4* gene expression increases were, however, evident at each interval while *IL1 β* gene expression gradually increased throughout the timecourse. Thus, there is a typical innate immune response to *S. Typhimurium* in maturing birds however, this response appears to be finely-tuned, potentially regulating bacterial levels without inducing excessive inflammation.

In contrast, cytokine and chemokine gene expression increases in response to *C. jejuni* were not detected until 20 h post-challenge. Initial induction of proinflammatory gene expression is likely to somewhat correlate with initial *C. jejuni* invasion or translocation of the epithelial barrier as apical intestinal epithelium tend to lack functional PRRs. It thus appears that *C. jejuni* invade the caecal epithelium at a slower rate than *S. Typhimurium*. At the 20 h interval, minor *IL1 β* and *K60* gene expression as well as high *IL8* gene expression was evident in response to *C. jejuni*. Interestingly, 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). However, at 48 h post-challenge in the present model, the *IL8* gene expression increase was much reduced and comparable to the minor *IL1 β* and *IL6* gene expression increases at this interval. Therefore it appears that high chemokine expression in response to the microbe was only transient. Epithelial cells produce chemokines in response to *C. jejuni* infection (Borrmann *et al.*, 2007; Johanesen & Dwinell, 2006; Li *et al.*, 2008c) and thus, high *IL8* gene expression followed by a reduction could signify *C. jejuni* temporarily invading the epithelium and then returning to the mucus layer. Indeed, it has been established that the microbe propagates in chicken intestinal mucus and not within chicken intestinal epithelial cells (Van Deun *et al.*, 2007). However, residential leukocytes underlying the caecal epithelium also potentially contribute to caecal *IL8* gene expression increases, should such cells encounter *C. jejuni*. To investigate this possibility, chemokine gene expression was profiled in the caecal tonsils of *C. jejuni*-challenged birds, as these defined lymphoid aggregates consist of phagocytic and lymphoid populations of cells inductive of proinflammatory responses (Del Cacho *et al.*, 2009; Haghighi *et al.*, 2008; Lillehoj & Trout, 1996). Significantly increased *IL8* gene expression was observed at 6 h, 20 h and 48 h post-challenge. Significantly increased *K60* gene expression was evident at 48 h post *C. jejuni*-challenge. These results suggest that the caecal tonsils are an important site for intestinal IL-8 production in response to *C.*

jejuni. Chicken IL-8 protein has previously been shown to attract both heterophils and monocytes (Barker *et al.*, 1993; Poh *et al.*, 2008). Yet, our histological data indicates no inflammatory cell influx in response to *C. jejuni*. Interestingly, *C. jejuni* induces IL8 gene expression in chicken LMH cells but supernatants from challenged cells are poor chemoattractants for heterophils, *in vitro* (Larson *et al.*, 2008). Thus, *C. jejuni* may have developed mechanisms to interrupt the chemotactic properties of IL8. It must, however, also be noted that other cytokines and chemokines are likely required for efficient leukocyte recruitment, and overall *C. jejuni* appears to induce minimal cytokine gene expression.

Host-defence peptides are important regulators of bacterial colonisation in the intestine (Salzman *et al.*, 2010). Thus, AvBD gene expression was also profiled in the caeca of *S. Typhimurium* and *C. jejuni* challenged birds initially at the 6 h interval. Basal AvBD gene expression was highly variable amongst the control birds of each infection model, with gene expression for certain AvBDs being undetectable in some individuals. This was also evident with challenged birds and thus, it was difficult to accurately determine AvBDs that were genuinely differentially expressed in response to infection. Nevertheless, it would appear that AvBD5, AvBD9, AvBD12 and AvBD13 are important as their gene expression was significantly increased in both infection models at 6 h. However, these increases were more significant with the *S. Typhimurium* infection model, and thus the reliability of the AvBD gene expression data in relation to the *C. jejuni* infection model came under question. To address this concern, AvBDs that had been deemed significantly increased in response to *C. jejuni* at 6 h, were also profiled at 20 h. These AvBDs (*AvBD5*, *AvBD9* and *AvBD12*) were not differentially expressed in response to *C. jejuni* at this time-point, suggesting that either AvBDs are only transiently increased in response to the bacterial species or the increases observed at 6 h are false positives. To clarify this, it would be worth to use a tissue culture model (as these are not affected by genetic variation), to determine AvBDs that are differentially expressed in response to *C. jejuni*.

The results in this chapter provide some intriguing insights into the nature of the intestinal immune gene expression patterns to both bacterial species. Furthermore, it identifies some key molecules that characterise and differentiate the immune response to *C. jejuni*. 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.

Table 3.1 Summary of histopathological findings in the caeca of unchallenged, *S. Typhimurium*-challenged (6 h, 20 h and 48 h) and *C. jejuni*-challenged (6 h, 20 h and 48 h) birds (n=2/3). Analysis was performed by Professor J. J. Callanan.

	<i>S. Typhimurium</i> challenge	<i>C. jejuni</i> challenge
Unchallenged controls	• Moderate lymphocyte infiltration. • Crypts intact	
6 h	• Moderate lymphocyte infiltration in lamina propria • Occasional heterophils	• Minimal lymphocyte infiltration in lamina propria • Crypts intact
20 h	• Moderate lymphocyte infiltration in lamina propria • Occasional heterophils	• Mild-moderate lymphocyte infiltration in lamina propria • Crypts intact
48 h	• Mild-moderate lymphocyte infiltration in lamina propria • Occasional heterophils	• Minimal lymphocyte infiltration in lamina propria • Crypts intact

Table 3.2 Real time qRT-PCR median fold change in the caeca of *S. Typhimurium*-challenged birds (n = 6) relative to mock-challenged controls at 6 h, 20 h and 48 h post-challenge. *P* values were determined using Mann-Whitney U test.

Gene Symbol	6 h post-challenge		20 h post-challenge		48 h post-challenge	
	Fold change	<i>P</i> value	Fold change	<i>P</i> value	Fold change	<i>P</i> value
CD45	2.77	0.004	-2.23	0.004	-0.03	>0.999
TLR4	2.29	0.037	-3.35	0.004	-0.01	>0.999
TLR5	1.92	0.053	-1.29	0.053	2.17	0.055
TLR15	2.54	0.200	-1.19	0.055	6.23	0.004
TLR21	5.20	0.004	-1.05	0.054	1.56	0.337
IL1B	2.29	0.335	2.30	0.050	7.41	0.004
IL6	11.73	0.004	1.87	0.054	19.39	0.004
IFNG	4.07	0.055	-2.13	0.337	6.01	0.007
TGFB4	1.55	0.004	2.37	0.055	1.91	0.003
IL8	5.07	0.004	1.95	0.333	11.48	0.055
K60	2.10	0.004	4.34	0.004	4.06	0.043

Table 3.3. Real time qRT-PCR median fold change in the caeca of *C. jejuni*-challenged birds (n = 7) relative to mock-challenged controls at 6 h, 20 h and 48 h post-challenge. *P* values were determined using Mann-Whitney U test.

Innate immune genes	6 h post-challenge		20 h post-challenge		48 h post-challenge	
	Fold change	<i>P</i> value	Fold change	<i>P</i> value	Fold change	<i>P</i> value
<i>CD45</i>	2.55	0.025	-1.75	0.180	-1.25	0.180
<i>TLR4</i>	3.98	0.025	1.61	0.180	1.27	0.406
<i>TLR5</i>	3.29	0.180	1.17	0.655	1.15	0.655
<i>TLR15</i>	-1.00	0.749	1.50	0.655	-1.04	0.655
<i>TLR21</i>	3.54	0.025	-1.01	0.655	1.95	0.180
<i>IL1B</i>	-1.82	0.097	5.24	0.005	3.87	0.025
<i>IL6</i>	1.30	0.371	1.32	0.180	4.35	0.025
<i>IFNG</i>	1.49	0.180	1.02	0.798	2.44	0.794
<i>TGFB4</i>	1.73	0.085	-1.26	0.655	1.10	0.201
<i>IL8</i>	-2.91	0.030	24.28	0.002	3.33	0.002
<i>K60</i>	-4.69	0.024	6.64	0.002	1.03	0.749

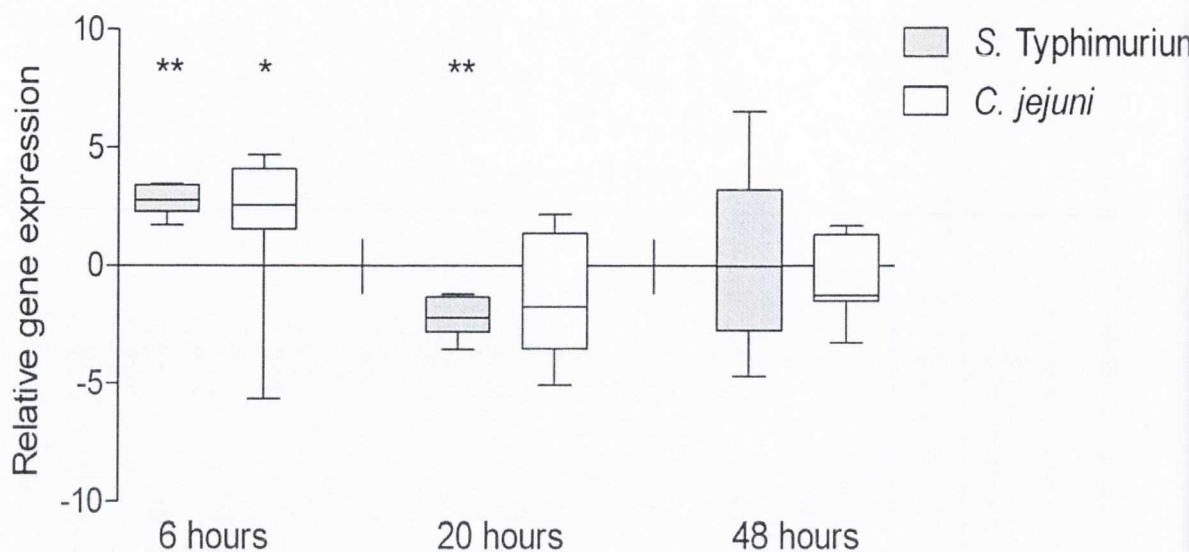


Figure 3.1 Comparative CD45 gene expression profiles in response to both *S. Typhimurium* (n=6) and *C. jejuni* (n=7) in the caecum at 6 h, 20 h and 48 h post-challenge. The horizontal line within each box plot represents the median fold changes in gene expression in infected chickens relative to the 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 * $P < 0.05$, ** $P < 0.01$).

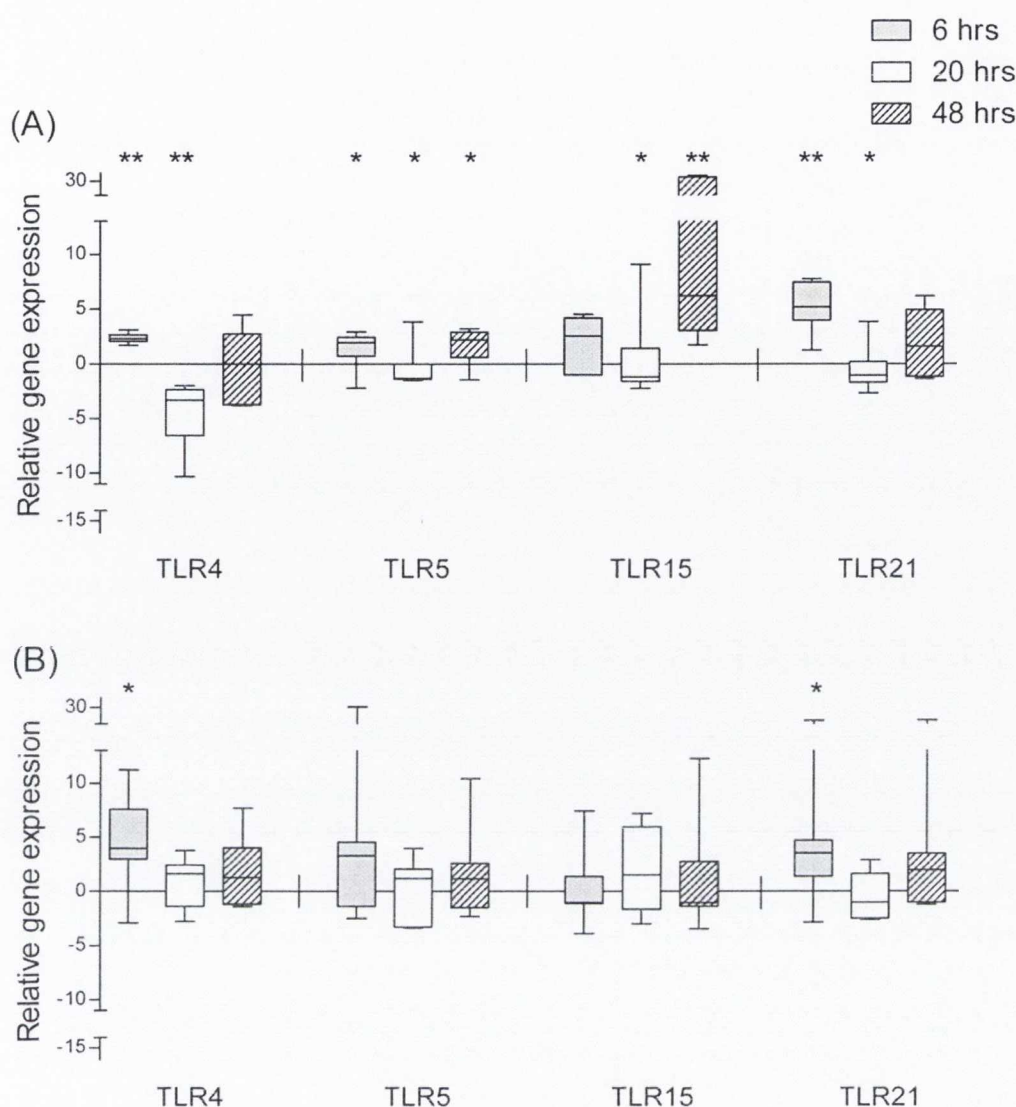


Figure 3.2 Comparative TLR gene expression profiles in response to both (A) *S. Typhimurium* (n=6) and (B) *C. jejuni* (n=7) in the caecum at 6 h, 20 h and 48 h post-challenge. The horizontal line within each box plot represents the median fold changes in gene expression in infected chickens relative 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 * $P < 0.05$, ** $P < 0.01$).

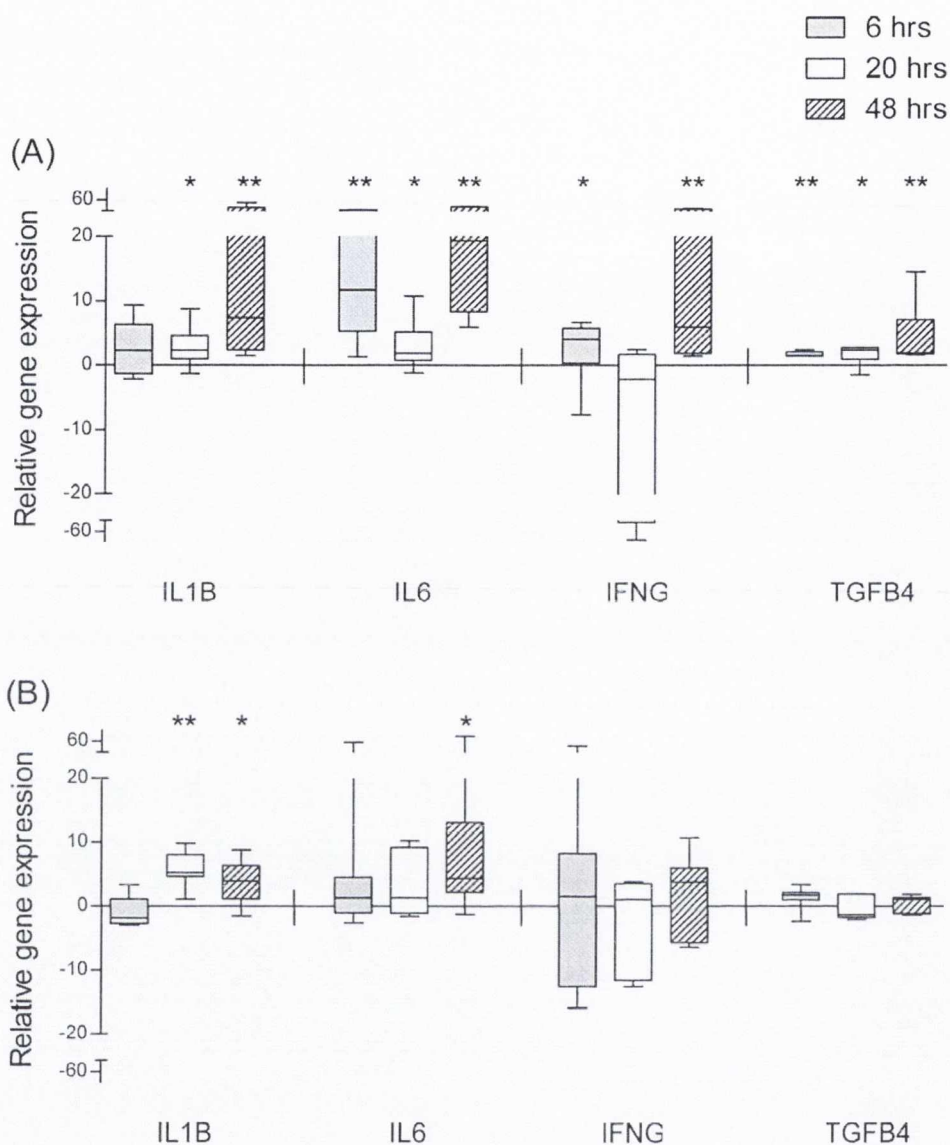


Figure 3.3 Comparative cytokine gene expression profiles in response to both (A) *S. Typhimurium* (n=6) and (B) *C. jejuni* (n=7) in the caecum at 6 h, 20 h and 48 h post-challenge. The horizontal line within each box plot represents the median fold changes in gene expression in infected chickens relative 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 * $P < 0.05$, ** $P < 0.01$).

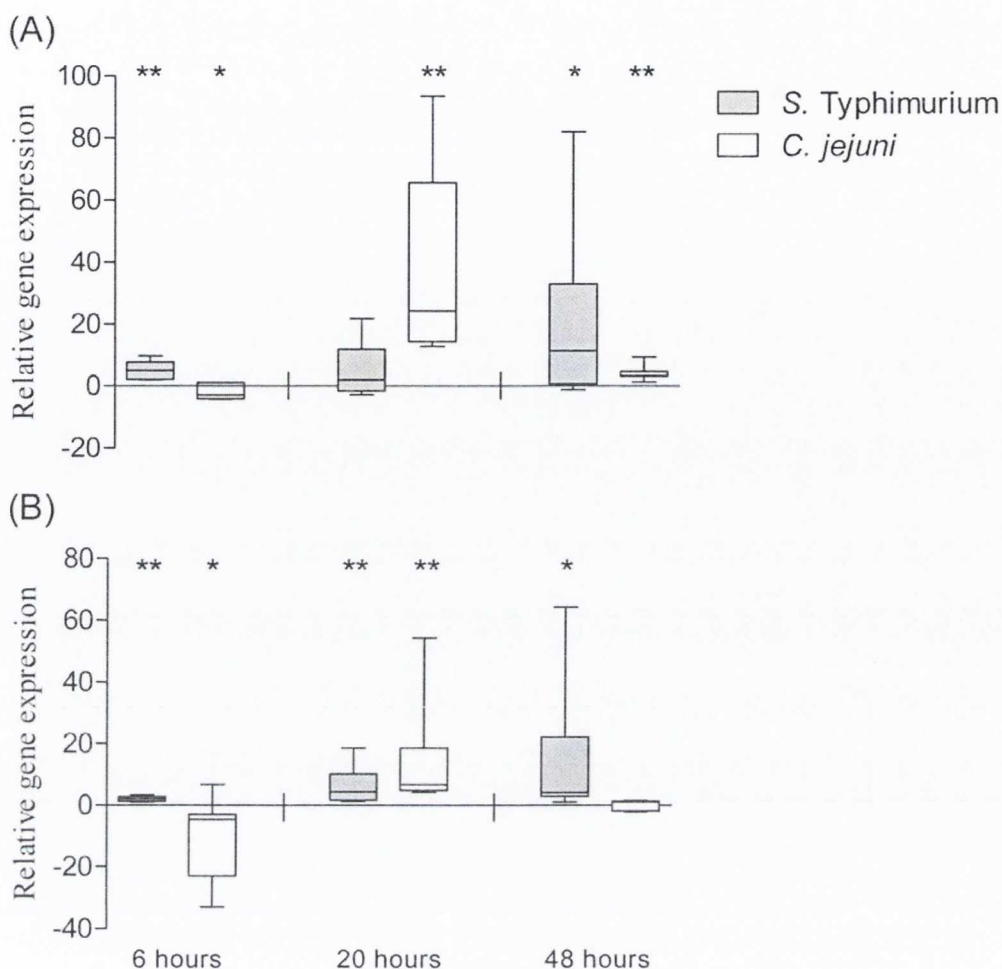


Figure 3.4 Comparative caecal chemokine gene expression profiles for **(A)** *IL8* and **(B)** *K60*, in response to both *S. Typhimurium* (n=6) and *C. jejuni* (n=7) 6 h, 20 h and 48 h post-challenge. The horizontal line within each box plot represents the median fold changes in gene expression in infected 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 * $P < 0.05$, ** $P < 0.01$).

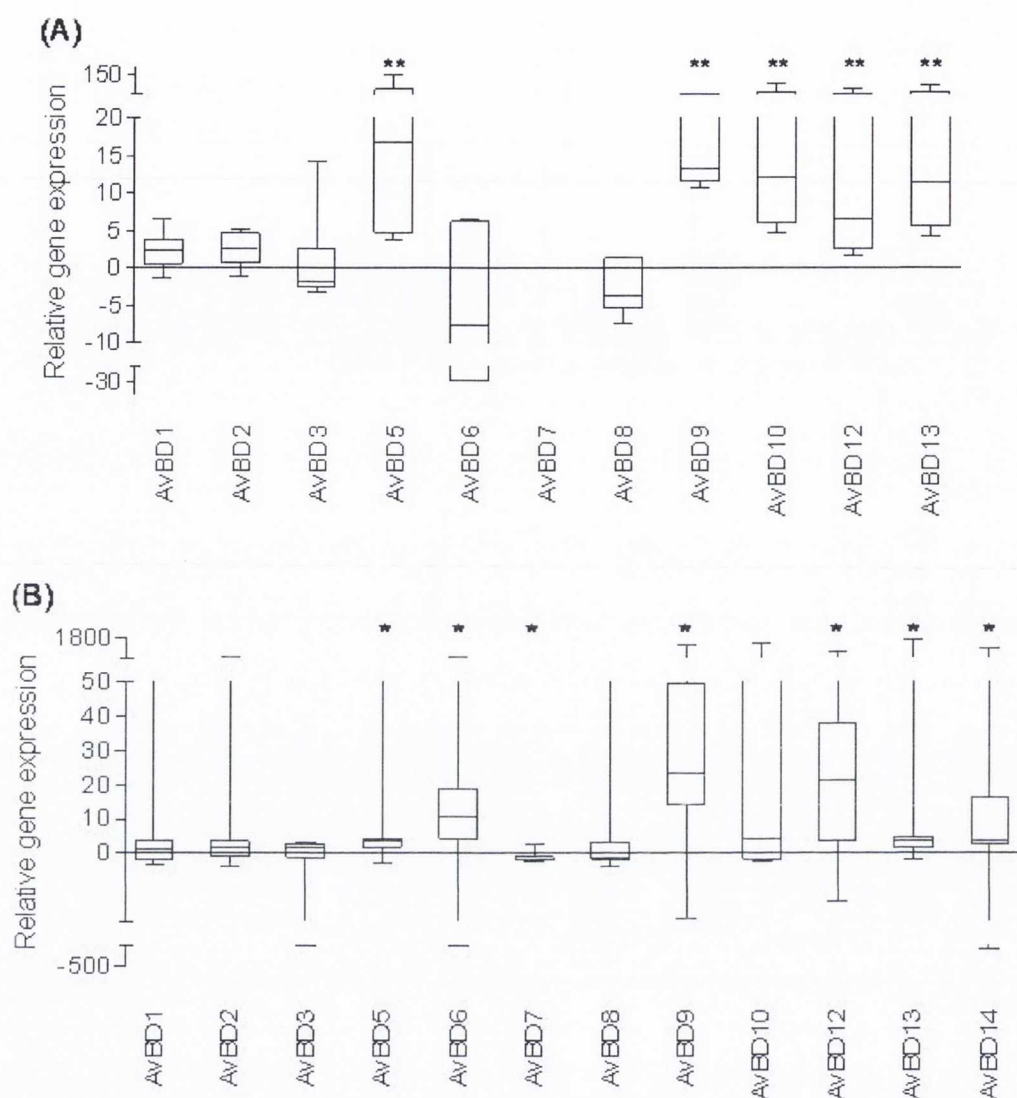


Figure 3.5 AvBD gene expression profiles in response to both **(A)** *S. Typhimurium* (n = 6) and **(B)** *C. jejuni* (n = 7) in the caecum at 6 h post-challenge. The horizontal line within each box plot represents the median fold changes in gene expression in infected chickens relative to the mock-infected controls. Statistical analysis was carried out separately for each infection model to compare challenged and control birds (significantly differentially expressed genes are highlighted * $P < 0.05$, ** $P < 0.01$).

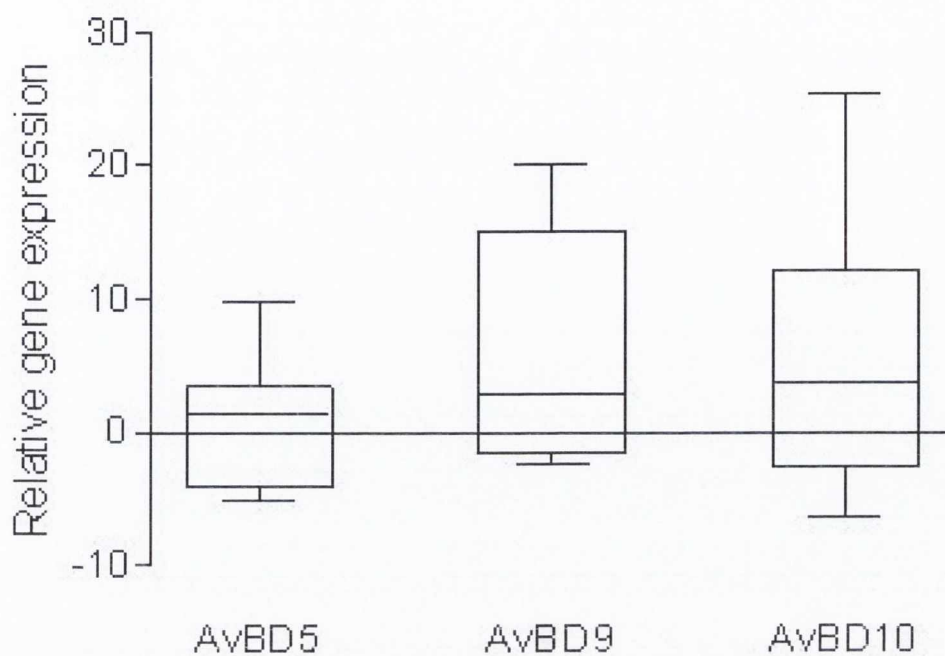


Figure 3.6 AvBD gene expression profiles in response to *C. jejuni* (n = 7) in the caecum at 20 h post-challenge. The horizontal line within each box plot represents the median fold changes in gene expression in infected chickens relative to the mock-infected controls (significantly differentially expressed genes are highlighted * $P < 0.05$, ** $P < 0.01$).

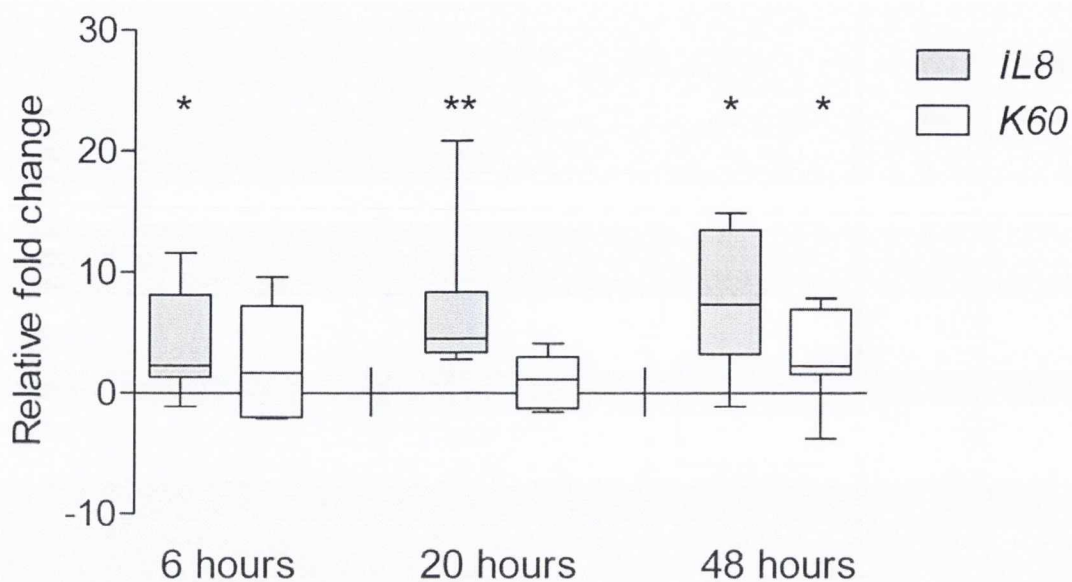


Figure 3.7 *IL8* and *K60* chemokine gene expression profiles in the caecal tonsil in response to *C. jejuni* (n=7) at 6 h, 20 h and 48 h post-challenge. The horizontal line within each box plot represents the median fold changes in gene expression in infected chickens relative to the mock-infected controls (significantly differentially expressed genes are highlighted *P < 0.05, **P < 0.01).

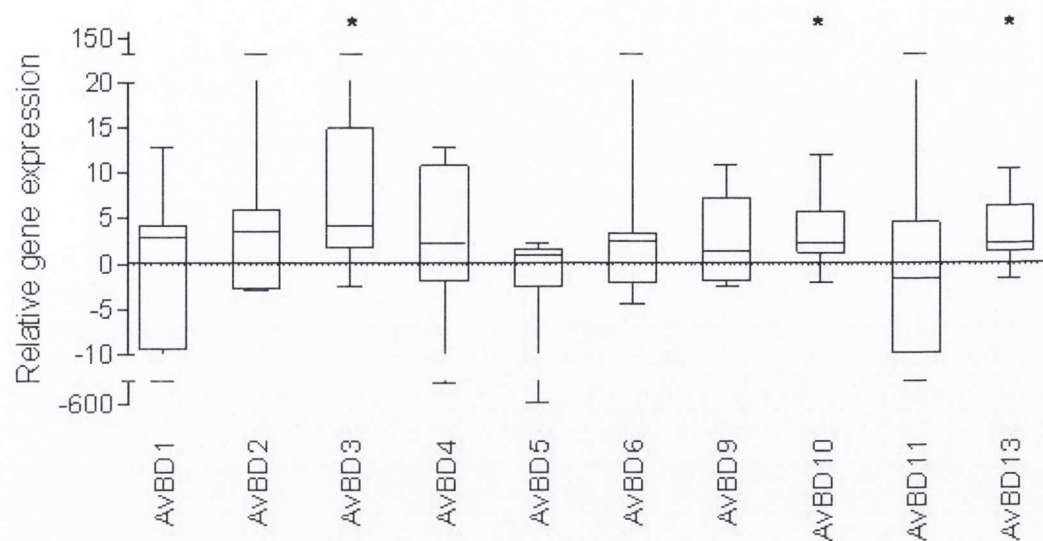


Figure 3.8 AvBD gene expression profiles in the caecal tonsil in response to *C. jejuni* (n=7) at 6 h post-challenge. The horizontal line within each box plot represents the median fold changes in gene expression in infected chickens relative to the mock-infected controls (significantly differentially expressed genes are highlighted * $P < 0.05$, ** $P < 0.01$).

Chapter 4

Global Gene Expression Analysis of Chicken Caecal Response to *Campylobacter jejuni* Challenge

Chapter 4: Gene Expression Analysis of Chicken Caecal Response to *C. jejuni* Challenge

4.1 Introduction

Profiling the expression of genes associated with a typical innate immune response in the caeca of 4-week-old *C. jejuni*-challenged birds has revealed minimal proinflammatory gene expression changes despite high levels of colonisation (chapter 3). The only prominent indication of inflammatory activity consisted of a 24-fold *IL8* gene expression increase in response to the microbe at 20 h post-challenge. In parallel, Smith *et al* observed that chemokine gene expression increases were more apparent than proinflammatory cytokine gene expression increases in the caeca of newly-hatched and two-week old chickens in response to *C. jejuni*, across a seven day timecourse (Smith *et al.*, 2008). Thus, it appears that the microbe fails to induce a traditional innate immune response in the avian intestine.

However, focusing on well-characterised proinflammatory genes to elucidate the innate immune response has its limitations. Basically, it is possible that the avian innate immune response to *C. jejuni* involves immune components that do not fit the “usual suspects” criteria. It is difficult to specifically identify such immune components considering that the intestinal immune response is complex involving multiple cell types, multiple means of microbial recognition (e.g. TLRs, NLRs and C-type lectins) and multiple proinflammatory and anti-inflammatory effectors as well as molecules that regulate cellular processes, signalling pathways and effector molecule activity. To add further complexity, the immune response somewhat differs between mammalian and avian species. Immune gene repertoires differ, and certain immune components function differently between the two species (Kaiser, 2007). Furthermore, avian-specific inflammatory effectors and regulators may operate, considering that *TLR15* is exclusive to birds (Higgs *et al.*, 2006). Therefore, global gene expression analysis may be necessary to characterise the caecal immune response to *C. jejuni*.

Whole-genome microarrays might be a useful tool for characterising *C. jejuni*-induced activity. Microarrays typically consist of thousands of microscopic single stranded cDNA or oligonucleotides bound to glass slides. The sequence of each spot represents a specific transcript or gene (Friend & Stoughton, 2002). As such,

microarrays are a high-throughput method for determining differential gene expression between two different sample classes (e.g. untreated and treated). Using mRNA as a template, cDNA is synthesised with the incorporation of different fluorescent dyes (red and green) into each sample. By using the biological property of complementary base pairing, red and green labelled cDNA samples compete for hybridisation with the bound probes. Following hybridisation, arrays are scanned to determine the ratio of red to green dye intensity, which after appropriate normalization, are a representative of the gene expression ratio between the two samples (Friend & Stoughton, 2002). Such assays allow the measurement of gene expression for thousands of genes simultaneously in response to a given stimulus (Fathallah-Shaykh, 2005). Assuming that differential gene expression signifies involvement of the biological processes associated with such genes, the global transcriptional profile could potentially identify specific signalling pathways, effector molecules, regulatory and anti-inflammatory components, targets for microbial subversion and novel immunological processes, if evident in response to a particular microbe.

A number of chicken-specific microarrays have been developed, and have had some success in characterising immune responses to various microbial species. A chicken 5K immune cDNA microarray was developed by ARK Genomics, in which innate immune genes from the chicken EST collection were combined with cDNA libraries from lymphoid rich tissues of birds challenged with bacterial, protozoan and viral pathogens (Smith *et al.*, 2006). Using this array, genes associated with multiple immunological processes including TNF activity, IFN pathways and antigen presentation were upregulated in the lungs of avian influenza challenged birds (Degen *et al.*, 2006). An avian macrophage specific cDNA microarray was constructed from 4906 chicken EST clones, and was used to investigate the interactions of blood-derived macrophages with *E. coli* and its LPS component, in which proinflammatory cytokine gene expression increases were observed (Bliss *et al.*, 2005). Whole-genome microarrays have also been deployed to study the avian immune response. Agilent have developed a 44K microarray that incorporates the chicken genome, and has been supplemented with viral transcripts (Li *et al.*, 2008a). This array has been used in several studies. In chicken heterophils, several TLR signalling components and proinflammatory effector molecules were differentially expressed in response to *Salmonella enterica* serovar Enteritidis (Chiang *et al.*, 2008) while multiple innate

and adaptive immune components were increased in response to *Clostridium perfringens* in the spleen (Sarson *et al.*, 2009). Finally, a 20K array is available from ARK Genomics, which represents ESTs from the ENSEMBL, TIGR and BBSRC UMIST Chick databases. These arrays were used to investigate the avian tracheal response to avian influenza, in which multiple antiviral genes were upregulated (Reemers *et al.*, 2010).

Considering such benefits of high-throughput immunological analyses, the overall aim of this part of the project was to use whole-genome microarrays to examine global gene expression changes in the caeca of *C. jejuni*-colonised birds at 20 h post-challenge. It was hypothesised that analysis of the global transcriptional profile would determine the extent of caecal immunological activity towards the microbe. Furthermore, non-immunological processes activated in response to *C. jejuni* colonisation could also be elucidated. Differentially expressed immune and non-immune genes identified would be viewed as potential colonisation resistance candidates.

4.2 Results

4.2.1 Comparison of normalization strategies

Different normalization strategies account for different aspects of technical variation that occur during microarray data generation. Choosing the most appropriate normalization approach was necessary for gaining an accurate representation of the gene expression ratios between *C. jejuni*-challenged and mock-challenged birds. Initially, the data from each of the hybridizations was visualised using MA-plots. With the raw data, asymmetrical plots were evident, which suggested intensity based dye biases (Fig. 4.1; panel 1). Median, Lowess and Print-Tip Lowess normalizations (Smyth & Speed, 2003) were applied to the data to determine their effects on the raw data. Asymmetrical MA-plots were retained following Median normalization indicating inappropriate normalization (Fig. 4.1; panel 2). In contrast, the application of Lowess and Print-Tip Lowess normalizations resulted in symmetrical MA-plots (Fig. 4.1; panel 3 and 4 respectively), which indicated successful removal of intensity related fluorescent dye bias.

Different normalization methods can result in varying numbers of significantly differentially expressed genes, and can influence the numbers of false-positive and false-negative genes discovered. Significantly differentially expressed gene lists resulting from each of the three normalization strategies used were compared for overlapping genes. At the $P < 0.01$ level, 250, 270 and 188 genes were significantly differentially expressed using Median, Lowess and Print-Tip Lowess normalizations respectively (Fig. 4.2 A and B). Only 64 genes were common to all three methods, although there was strong overlap in resulting genesets (193 genes) between Median and Lowess normalizations (Fig. 4.2 A).

To gain insight into the false discovery rate (FDR) for each of the normalization strategies, *C. jejuni*-challenged and mock-challenged microarrays (n=14) were randomly classified so that classes consisted of both *C. jejuni*-challenged and mock-challenged arrays. Class comparison was subsequently performed and in this regard, significantly differentially expressed genes identified would be expected to represent false-positive genes. For Median, Lowess and Print-Tip Lowess normalizations, 88, 84 and 103 falsely discovered genes were evident respectively.

When compared to the analyses based on true classifications, FDRs of 35%, 31% and 54% respectively were apparent (Fig. 4.2 B). Considering, the lower FDR and the appropriate MA-plot adjustments, Lowess was chosen as the most appropriate normalization strategy for subsequent reliable determination of true significant differential gene expression. Lowess normalization applies a local regression curve to the raw data that addresses intensity-dependent dye biases (Yang *et al.*, 2002).

4.2.2 Characterisation of global differential gene expression in response to *C. jejuni*

Global gene expression was compared between *C. jejuni*-challenged and mock-challenged birds at 20 hours post-inoculation. This timepoint previously revealed evidence of high *IL8* gene expression in response to *C. jejuni* (chapter 3) and thus was a suitable interval for microarray analysis. At the $P < 0.01$ level, 270 genes were significantly differentially expressed between the two groups with only minor fold changes evident. Initial characterisation involved 70 nucleotide probe sequences corresponding to differentially expressed genes being aligned against the chicken genome using BLAST. Fifty-three hits corresponded to hypothetical proteins, 115 hits mapped to poorly annotated regions of the genome while 102 hits corresponded to known or predicted genes (Table 4.1). Of the annotated genes, 17 were increased greater than 1.5-fold and 25 were decreased by more than 1.5-fold.

GO Slim using the Protein Information Resource (PIR) subset of GO terms was used to provide gene ontology for the significantly differentially expressed genes. A wide range of biological processes were evident (Table 4.2). Metabolic activities were highly represented such as macromolecule metabolic processes (159 gene products) and nitrogen compound metabolic processes (102 gene products). Immune response terms were also identified, namely immune system processes (21 gene products) and cytokine production (6 gene products). In total, 104 biological processes correlated to the differentially expressed data (Table 4.2).

4.2.3 Validation of significantly differentially expressed genes

Differentially expressed genes associated with immunological processes were chosen for validation by qRT-PCR. Of eight genes investigated, four were confirmed to be significantly differentially expressed (Fig. 4.3). Expression of genes encoding Vascular cell adhesion molecule 1 (1.5-fold; $P=0.002$) and NADPH oxidase 1 (2.3-fold; $P=0.002$) were increased while mitogen-activated protein kinase 9 (-1.3-fold; $P=0.002$) and C1q tumor necrosis factor related protein 7 (-3.19-fold; $P=0.025$) were decreased in *C. jejuni* challenged birds.

As normalization occasionally removes truly differentially expressed genes, immune genes identified through the other normalization approaches were also potentially significant. Specifically, there was evidence of T cell activity in the immune response to *C. jejuni* colonisation and such genes were also validated. Significantly increased *TOPK* (1.9-fold; $P=0.025$), *IL17* (3.6-fold; $P=0.002$) and *IL21* (2.1-fold; $P=0.025$) gene expression was evident in response to *C. jejuni* in the chicken caeca (Fig. 4.4). Further investigation of additional molecules involved in T cell activity via qRT-PCR revealed significantly increased *KK34* (1.64-fold; $P=0.004$), *IL7R* (3.97-fold; $P=0.003$) and *CTLA4* (2.54-fold; $P=0.003$) gene expression in *C. jejuni* challenged birds.

4.3 Discussion

Whole-genome microarrays are potentially useful tools for characterising the complete caecal transcriptional response to *C. jejuni*. In turn, global analysis of such transcriptional changes would, in theory, reveal detailed information on biological processes activated in response to microbial colonisation.

However, this high-throughput data acquisition approach comes with the price of being susceptible to numerous types of technical variations that occur during microarray data generation processes. Such technical variations include RNA yield inconsistencies, dye labelling inefficiencies, dye biases and scanning errors. All of these impact on the reliability of microarray data (Yang *et al.*, 2002). Thus, in order to obtain an accurate representation of differential caecal gene expression between the *C. jejuni*-challenged and mock-challenged cohorts, the data first needed to be “normalized”. The raw data from each of the microarray hybridizations was initially visualised using MA plots to determine the most appropriate normalization strategy. These are scatterplots in which each dot represents a spot (gene) on the microarray. For each gene, the fluorescent intensity ratio of the red channel to the green channel (M ; on the Y axis) is plotted against the average intensity (A ; on the X axis) (Dudoit *et al.*, 2000). M values for the majority of genes were close to 0, which indicates that the hybridizations were successful and that dye biases were minimal. The scatterplots were, however, unsymmetrical and thus, an indication that there were intensity dependent variations between the red and green channels (Yang *et al.*, 2002). As a result, normalization of the data was clearly warranted. For the identification of the most appropriate adjustment strategy, Median, Lowess and Lowess Print-tip normalizations were initially applied to the data. Each of these methods comes with their own advantages and disadvantages for correcting different aspects of technical variation, which thus impacts on the numbers of false-positive and false-negative differentially expressed genes. For each microarray hybridization, median normalization generates a single normalization factor that is subsequently applied to the data to balance the red and green channels, and is thus useful for addressing dye biases (Zien *et al.*, 2001). Applying this normalization strategy to the data failed to improve the symmetry of the MA plots, indicating that intensity-dependent dye variations had not been removed. However, applying Lowess normalization removed

such variations, as was evident by the resulting symmetrical MA plots. This normalisation method is noted for its ability to remove intensity based dye biases by applying a “local regression curve” to the raw data (Yang *et al.*, 2002). Equally, Lowess Print-tip normalisation produced symmetrical MA plots. This strategy is basically Lowess normalization, in which normalization factors are calculated and applied separately to each block of a microarray. This method is commonly used to correct spatial intensity biases (Yang *et al.*, 2002).

Gene lists arising from each of the three normalization strategies were compared, in which genes with a $P < 0.01$ were considered significantly differentially expressed. Surprisingly, only 64 genes were common to all three normalization methods despite there being greater than 180 significantly differentially genes evident to each individual gene list. However, on closer inspection, it was clear that there was some agreement between Median and Lowess normalizations, as 193 genes were commonly differentially expressed. Thus, there were major differences between choosing global (Lowess and Median) and local (Lowess Print-tip) means of normalization, but it was unclear how many false discoveries were evident to each strategy. False positive are notorious with microarray experiments, as a result of unadjustable technical variations. For example, a P -value cut-off of 0.05 usually implies that 5% of differentially expressed genes are truly false positives (Breitling, 2006). Choosing a more stringent P -value threshold serves to control the number of FDRs (Tsai *et al.*, 2003), but does correct for the false discoveries arising from inappropriate normalization. A crude but useful method for calculating the false discovery rate (FDR) is to determine the number of differentially expressed genes when the experimental classes are randomly misclassified. As deliberate misclassification has occurred, these genes are all false-positives. Subsequent comparison of the numbers of such falsely significantly differentially expressed genes (misclassification) with the numbers of true significantly differentially expressed genes (between *C. jejuni*-challenged and mock-challenged groups) determines the FDR percentage. Using this method, it was found that the FDR exceeded 30% with all three methods of normalization, which indicates that at least 30% of the significantly differentially expressed genes to each method are false positives. Interestingly, Lowess Print-tip normalization was found to have the highest FDR (54%). While spatial intensity effects were evident in the arrays, it appears that using local (Print-tip) normalization introduces error and that the global normalization methods are

more reliable for this data set. Global normalization strategies assume that the majority of genes on an array are not differentially expressed or there is a balance between genes upregulated or downregulated (Quackenbush, 2001). Assuming this principal, normalization factors are calculated based on the entire spots of the array and subsequently applied to the data. In this experiment >20,000 spots are used to calculate global normalization factors for each array. Local normalization strategies use the same principle but instead calculate the normalization factor from each independent block. In these avian arrays there are about 400 genes per block. Therefore, it appears that the normalization factor is more reliable when calculated from >20,000 genes, as opposed to 400 genes. Considering all of the above, Lowess was chosen as the most appropriate normalization strategy; although it was also clear that there was no perfect means of normalization.

At 20 h post-challenge, 270 genes were differentially expressed in the caeca in response to *C. jejuni*. The majority of fold-changes were, however, minor (<1.5-fold), suggesting that *C. jejuni*-colonisation does not have a profound effect on caecal processes. Nevertheless, gene ontology (GO) analysis was conducted to assign biological functions to the differentially expressed genes. However, the majority of these genes had no chicken-specific GO terms so in addition, human and mouse GO databases were searched. As a result, in some circumstances, avian genes were assigned the biological functions of their mammalian counterparts. A wide variety of biological processes including cell growth, nutrient metabolism, neurological response and immune response were represented. With the hypothesis that intestinal defences were regulating *C. jejuni* colonisation, a primary focus was, however, placed on the immunological processes evident. Yet, only eight of the significantly differentially expressed genes had links to inflammatory processes, and only four of these could be validated, in which only minor qRT-PCR fold-changes were evident. These were NADPH oxidase 1 (*NOX1*), which is associated with the production of reactive oxygen species from phagocytic cells (Sumimoto, 2008); vascular cell adhesion molecule 1 (*VCAM1*), a marker for endothelial activation (Wittchen, 2009); inhibitor of kappa light polypeptide gene enhancer in B-cells (*IKBKB*), a component of NF- κ B signalling (Rothwarf *et al.*, 1998); and complement-C1q tumor necrosis factor-related protein isoform 2 (*C1QTNF2*). While it is possible that oxidative burst and endothelial activation were components of the intestinal response to *C. jejuni*, there was no other evidence of such processes among the differential gene expression.

Recent microarray analysis on the caeca of two different chick lines (line A and line B), seven days post-*C. jejuni* challenge has further revealed absences of key proinflammatory indicators. Despite line A and line B having greater than 690 differentially expressed genes, only 17 and 22 of these, respectively, were immune genes. These included *IL1 β* , *IL8*, *K60*, *Ficolin 2* and *SOCS3* genes, which were decreased slightly in response to the microbe, in both lines (Li *et al.*, 2010). Thus, it appears that *C. jejuni* fails to induce a detectable caecal immune response.

However, before we can accept this conclusion, the reliability of microarrays for thoroughly studying the caecal immune response needs to be scrutinised. In the previous chapter, innate immune gene expression profiled using qRT-PCR revealed high *IL8* with minor *IL1 β* and *K60* gene expression increases in the caeca of *C. jejuni*-challenged and mock-challenged birds at 20 h post-inoculation. Surprisingly, microarray analysis failed to identify differential expression of these genes. cDNA failing to hybridize to its respective probes (Lee *et al.*, 2000) and the fact that microarrays have limited detection of lowly abundant mRNA (Venkatasubbarao, 2004) including cytokines may collectively explain such false-negatives. In one study, increased *IL1 β* and *IL8* gene expression was evident in the trachea of influenza A-challenged birds, but whole-genome microarray analysis failed to detect such increases (Reemers *et al.*, 2010). Furthermore, normalization sometimes removes truly differentially expressed genes (Eddy & Storey, 2008). To explore this possibility, datasets normalised using Median and Print-tip Lowess approaches were also investigated for evidence of *IL8* gene expression. *IL1 β* , *IL8* and *K60* were, however, not differentially expressed in these two datasets. In this regard, this microarray generated gene expression data may possibly be too noisy to accurately reveal truly differentially expressed genes.

In a final attempt to identify immunological processes activated in response to *C. jejuni*, differentially expressed immune genes from datasets arising from the other normalization approaches were validated via qRT-PCR. Interestingly, significant lymphokine-activated killer T cell-originated protein kinase (*TOPK*), *IL17* and *IL21* gene expression increases were detected. *TOPK* is expressed by activated T lymphocytes (Abe *et al.*, 2000), while IL-17 and IL-21 are cytokines produced by such cells (Bhave & Carson, 2009; Miossec *et al.*, 2009). Thus, collectively, there was evidence of T cell responses. To further investigate such responses, markers of T cell activity were also profiled by comparing *C. jejuni*-challenged and mock-challenged

birds by qRT-PCR. IL7R α (CD127) and CTLA4, receptors expressed on the surface of various T cell types (Racape *et al.*, 2009; Rudd *et al.*, 2009), were significantly increased at the mRNA level. Interestingly, IL7R α is associated with proinflammatory responses (Racape *et al.*, 2009) while CTLA4 regulates such responses (Rudd *et al.*, 2009). In this regard, proinflammatory T cell responses would regulate *C. jejuni* colonisation while anti-inflammatory T cell responses might reduce immunopathology. We can hypothesise that innate T cell responses are more probable than adaptive T cell responses, considering the timepoint is 20 h post-challenge in birds which have not previously encountered *C. jejuni*. $\gamma\delta$ T cells are abundant in the chicken caecum and mediate responses towards intestinal bacteria (Berndt *et al.*, 2007). Mammalian $\gamma\delta$ T cells have been shown to produce IL-17 (O'Brien *et al.*, 2009; Sutton *et al.*, 2009) while other $\gamma\delta$ T cell subsets have been shown to limit pathology in the gut (Ziegler, 2004). Hence, $\gamma\delta$ T cell populations may have an important role in *C. jejuni* colonisation in the chicken caeca.

In conclusion, this chapter proposes that *C. jejuni* activates minimal proinflammatory processes at its primary site of colonisation, the caecum. Microarrays have, however, not been particularly useful for identifying global caecal transcriptional changes to the microbe, largely due to the high number of false-positives and false-negatives. Thus, they may have failed to detect several key immunological processes. Nevertheless, we have identified evidence of innate T cell activity that we would not have otherwise hypothesised.

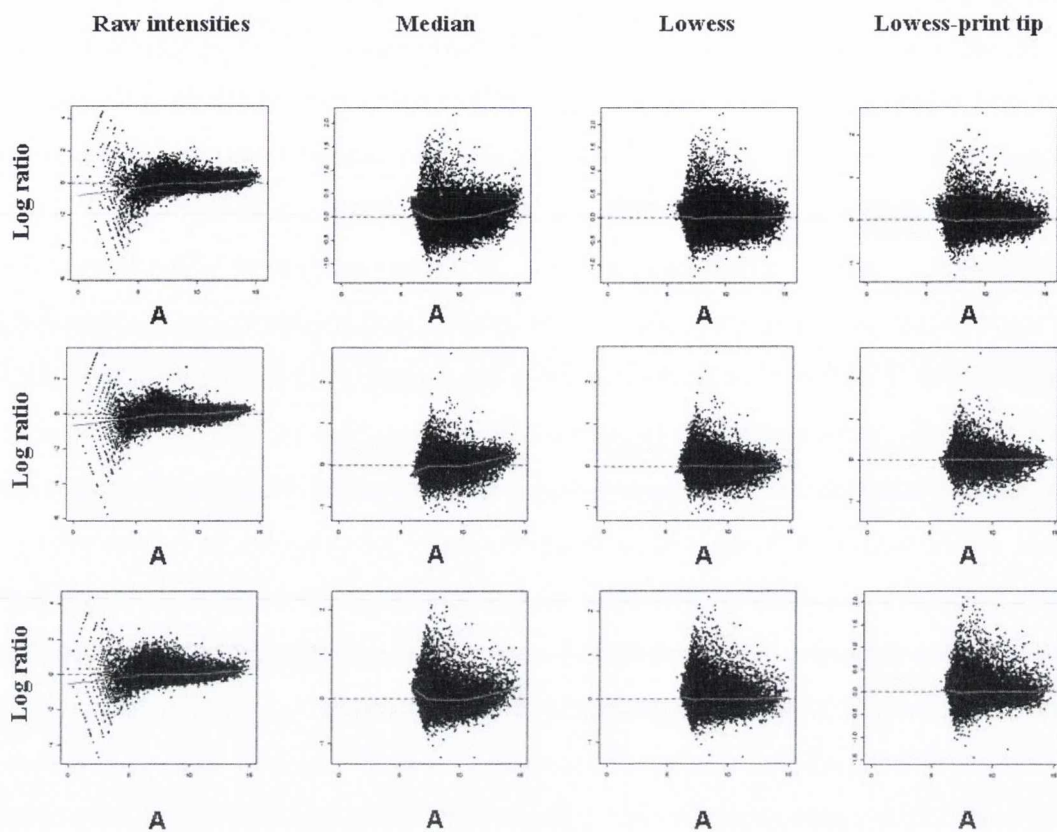


Figure 4.1 MA plots pre and post Median, Lowess and Print-Tip Lowess normalizations. Three of the 14 hybridizations are represented. “A” designates average intensity.

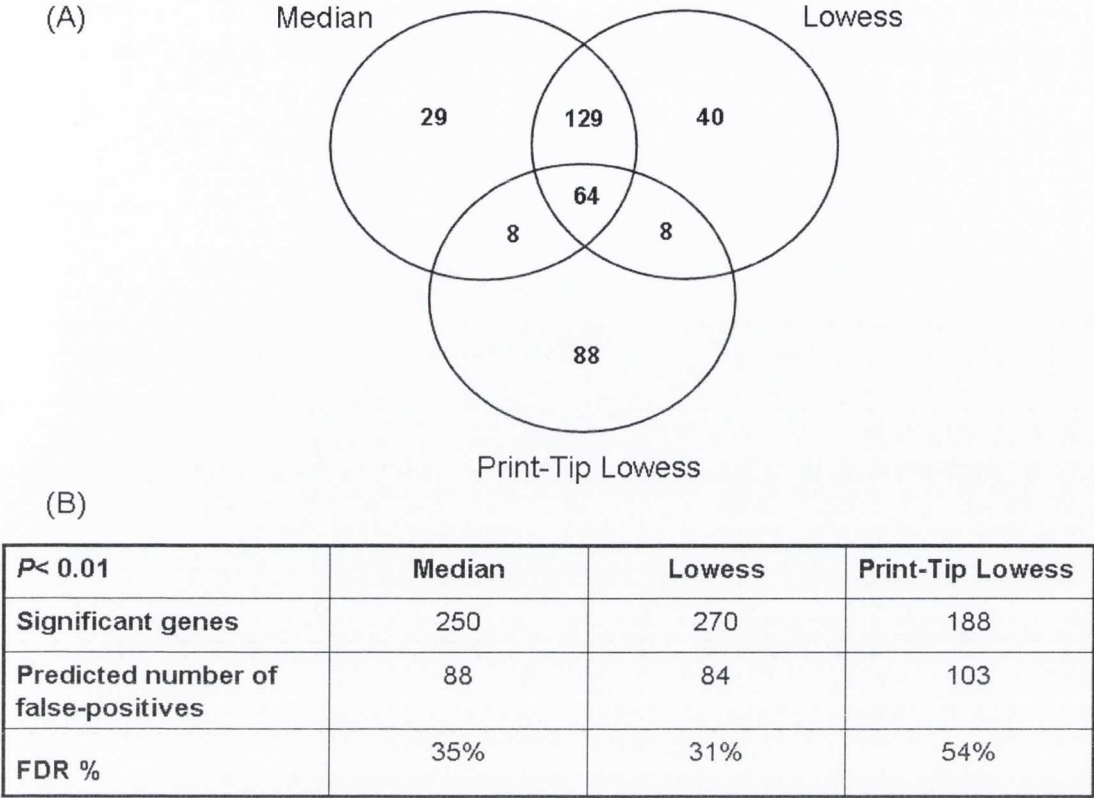


Figure 4.2 Comparison of Median, Lowess and Print-Tip Lowess normalizations. **(A)** The Venn diagram illustrates overlaps and differences between the three normalization strategies for differentially expressed genes ($P < 0.01$) between *C. jejuni*-challenged and mock-challenged chickens at 20 h. **(B)** False discovery rates were generated from jumbling the *C. jejuni*-challenged and mock-challenged groups and subsequently performing analysis.

Table 4.1 List of significantly differentially expressed genes ($P < 0.01$) in the caeca between *C. jejuni*-challenged ($n = 7$) and mock-challenged chickens at 20 hours ($n = 7$). List was generated using Lowess normalization followed by class comparison (BRB Array tools) (list is continued on the next two pages).

P-value	Fold-change	Gene name	Gene description
0.0008	-1.4	HOXA3	Homeobox A3
0.0011	1.4	RHEB	Similar to RAS-homolog enriched in brain
0.0013	1.4	BHLHE40	Similar to putative phytoene synthase
0.0014	-1.5	IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
0.0014	1.3	PAQR3B	Similar to phosphopantothienoylcysteine synthetase
0.0014	1.3	TCEB1	Transcription elongation factor B (SIII), polypeptide 1 (15kDa, elongin C)
0.0016	-1.5	RIC8A	Resistance to inhibitors of cholinesterase 8 homolog A
0.0018	1.2	DNAJA5	Similar to DnaJ homology subfamily A member 5
0.0019	-1.9	H3F3A	H3 histone, family 3B
0.0021	-1.3	NOX1	Similar to NADPH oxidase homolog 1 long form (NOX 1)
0.0021	-1.7	N4BP2	Similar to Nedd4 binding protein 2
0.0022	-1.6	LRCH3	Similar to Leucine-rich repeats and calponin homology (CH) domain containing
0.0022	1.5	LIMK2	LIM domain kinase 2
0.0023	-1.4	GRK6	Similar to G protein-coupled receptor kinase 6
0.0024	-1.3	GLDN	Similar to gliomedin
0.0025	-1.3	EXPH5	Similar to exophilin 5
0.0025	-1.4	SDR16C5	Short chain dehydrogenase/reductase family 16C, member 5
0.0026	-1.3	FIGN	Similar to fidgetin
0.0027	2.2	TEF	Vitellogenin binding protein (VBP), beta/beta isoform
0.0027	-1.6	CXORF20	Similar to chromosome X open reading frame 20
0.0029	-1.5	NEU3	Similar to ganglioside sialidase
0.0030	-1.6	GPBP1	Similar to vasculin
0.0030	1.5	CUL2	Cullin 2
0.0031	1.2	PAX2	Paired box gene 2
0.0033	-1.4	ADORA2B	Adenosine receptor 2b
0.0037	-1.3	B4GALT1	Similar to beta-1,4-galactosyltransferase (beta-1,4-GalT) V
0.0038	-1.4	TMC7	Transmembrane channel-like 7
0.0038	-1.9	REXO1L1	Similar to REX1, RNA exonuclease 1 homolog (<i>S. cerevisiae</i>)
0.0039	1.5	PRP8	Similar to splicing factor Prp8
0.0040	-1.6	LRP6	Similar to low density lipoprotein receptor-related protein 6
0.0040	-1.3	C1QTNF2	Similar to complement-c1q tumor necrosis factor-related protein isoform 2
0.0042	1.5	LOC776626	Similar to MLTK-alpha
0.0042	-1.4	PKB	Similar to MAPKK like protein kinase
0.0042	-1.4	IGSF9	Immunoglobulin superfamily, member 9B
0.0043	2.0	KCNE1	Similar to potassium voltage-gated channel subfamily E member 1
0.0043	1.2	EIF4G3	Similar to eukaryotic translation initiation factor

0.0044	1.5	SLC39A10	4 gamma, 3 Similar to solute carrier family 39 (zinc transporter)
0.0044	-1.3	SPEG	Similar to striated muscle-specific serine/threonine protein kinase
0.0046	1.5	ATG4A	Similar to putative autophagy-related cysteine endopeptidase
0.0046	-1.3	TESK1	Similar to TESK1
0.0047	-1.3	METAP1D	Similar to mitochondrial methionine aminopeptidase 3
0.0050	1.4	ALK	Similar to anaplastic lymphoma kinase receptor
0.0052	-1.5	TXK	TXK tyrosine kinase
0.0052	1.3	NRIF	Similar to nuclear receptor-interacting factor
0.0052	-1.7	PLA2G3	Similar to group IIE secreted phospholipase A2
0.0055	-1.5	LOC396098	LOC396098
0.0055	1.3	NPY1R	Neuropeptide Y receptor Y1
0.0056	-1.7	ZNF593	Zinc finger protein 593
0.0058	-1.2	BAG4	Similar to silencer of death domains
0.0060	1.3	NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
0.0060	1.2	SOX30	Similar to SRY (sex determining region Y)-box 30
0.0060	1.3	CALD1	Caldesmon 1
0.0061	1.9	Slbp	Similar to histone stem-loop binding protein
0.0061	1.2	MCCC2	Similar to Methylcrotonoyl-Coenzyme A carboxylase 2 (beta)
0.0061	1.4	RHBDL3	Similar to ventrhold transmembrane protein
0.0062	-1.7	TUBA3E	Similar to Tubulin alpha-3/alpha-7 chain
0.0064	1.3	MTERF	Similar to transcription termination factor-like protein
0.0065	-1.3	LOC428141	Similar to Trappin-6
0.0066	1.2	COX10	Similar to COX10 homolog
0.0066	1.4	VCAM1	Similar to vascular cell adhesion molecule (VCAM1)
0.0066	-1.3	TBC1D20	Similar to TBC1 domain family, member 20
0.0066	1.4	CR2	Complement component (3d/Epstein Barr virus) receptor 2
0.0067	-1.3	MYF6	Myogenic factor 6 (herculin)
0.0068	-1.5	NCOR1	Similar to nuclear receptor co-repressor 1
0.0068	-2.1	VPS13B	Similar to vacuolar protein sorting 13B
0.0069	-1.9	NVP2	Similar to neural visinin-like Ca2+-binding protein type 2
0.0070	-1.6	Slc28a2	Similar to Na+-dependent purine-selective nucleoside transporter
0.0072	-1.8	C9ORF5	Similar to chromosome 9 open reading frame 5
0.0072	1.4	FGF4	Fibroblast growth factor 4
0.0075	1.2	PDE8A	Similar to cAMP-specific cyclic nucleotide phosphodiesterase PDE8A1
0.0079	-1.3	RHOBTB2	Rho-related BTB domain containing 2
0.0079	1.3	CRHR2	Corticotropin releasing hormone receptor 2
0.0081	-1.9	HAS3	Similar to hyaluronic acid synthase 3
0.0081	1.3	GINS3	Similar to GINS complex subunit 3 (Psf3 homolog)
0.0081	1.4	STRA13	Similar to Stra13
0.0083	1.3	Aldh1a1	Aldehyde dehydrogenase 1A1
0.0084	-1.5	SLC30A8	Similar to solute carrier family 30 (zinc transporter), member 8
0.0084	-1.5	PTC2	Similar to patched-2
0.0085	-1.3	EHMT1	Similar to histone methyltransferase
0.0086	1.3	ERI1	Similar to lysophospholipase I

0.0086	-1.9	CMAS	Similar to cytidine monophospho-N-acetylneuraminic acid synthetase
0.0086	2.6	TGFBRAP1	Similar to Transforming growth factor, beta receptor associated protein 1
0.0087	2.3	DNAH3	Similar to axonemal heavy chain dynein type 3
0.0087	-1.4	COL6A1	Collagen, type VI, alpha 1
0.0087	1.4	CNOT10	CCR4-NOT transcription complex, subunit 10
0.0088	-1.3	SMARCA1	Similar to SWI/SNF
0.0088	2.7	ZNF367	Zinc finger protein 367
0.0089	2.3	EHMT1	Similar to G-protein coupled receptor (putative)
0.0089	1.6	PAQR3	Similar to progesterin and adipoQ receptor family member III
0.0091	1.5	BPIL2	Similar to bactericidal/permeability-increasing protein-like 2
0.0091	1.3	RIF1	Similar to receptor-interacting factor 1
0.0091	1.5	USP3	Similar to ubiquitin specific peptidase 3
0.0092	1.8	CHN1	Chimerin (chimaerin) 1
0.0096	-1.4	SLC18A2	Similar to synaptic vesicle monoamine transporter
0.0096	1.4	OTOG	Similar to otogelin
0.0096	-1.4	ITFG2	Similar to Integrin alpha FG-GAP repeat containing 2
0.0097	1.3	LRRFIP2	Similar to LRR FLI-I interacting protein 2 isoform 1
0.0097	1.6	ARFGAP2	Zinc finger protein 289, ID1 regulated
0.0097	1.2	CACNA1C	Similar to calcium channel, voltage-dependent, L type, alpha 1C subunit
0.0099	1.4	B3GALT1	Similar to beta 1,3-galactosyltransferase polypeptide 1
0.0100	-1.6	GALNTL6	Similar to polypeptide N-acetylgalactosaminyltransferase 17
0.0100	1.4	CSRNP3	Similar to TGF-beta induced apoptosis protein 2

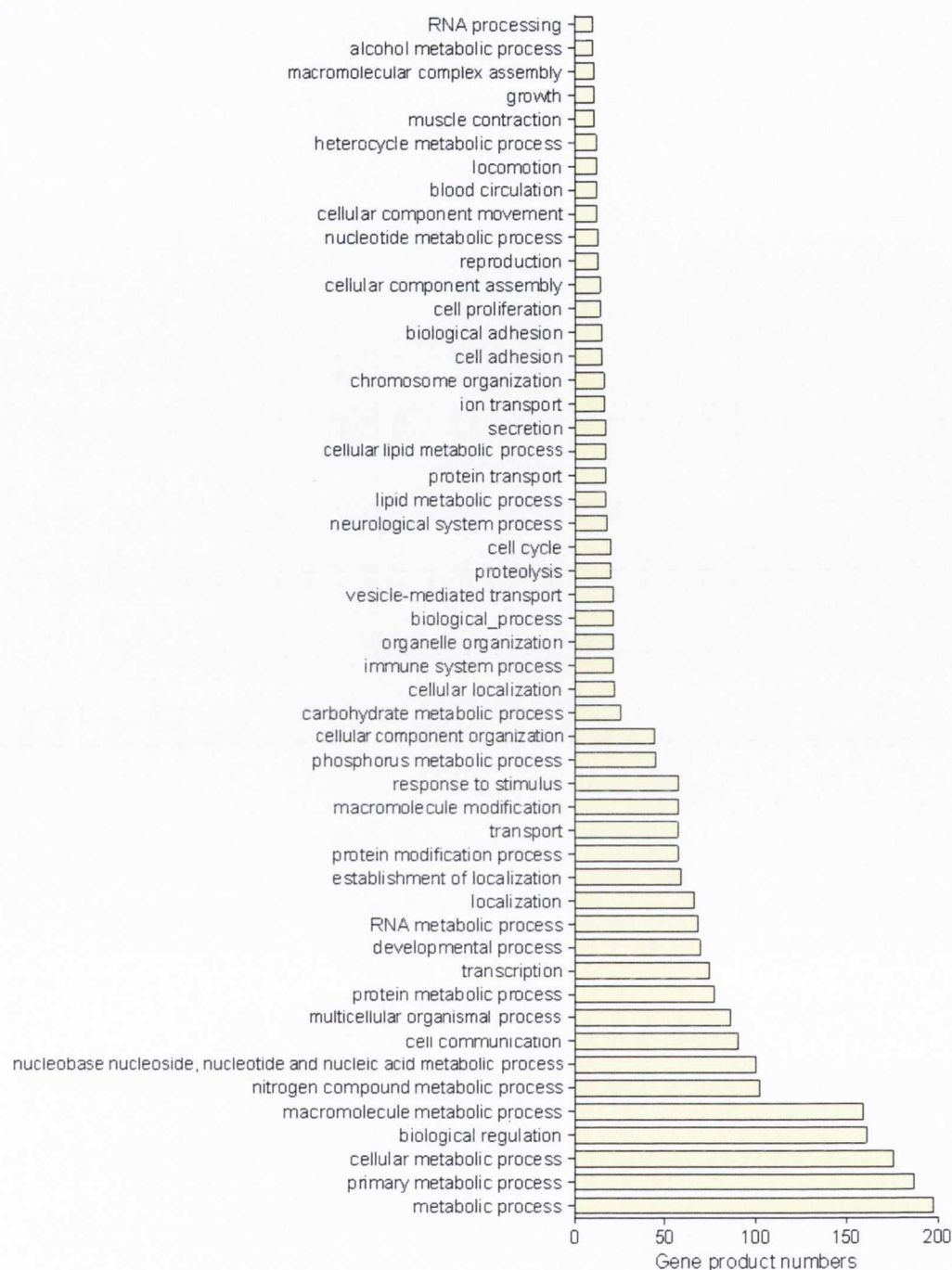


Figure 4.3 GO Slim using the Protein Information Resource (PIR) subset of GO terms reveals that global gene expression in response to *C. jejuni* in the caeca correlates to a wide range of biological processes. Gene product numbers refer to the number of differentially expressed genes that correlate to each GO term. The most highly represented biological processes are shown.

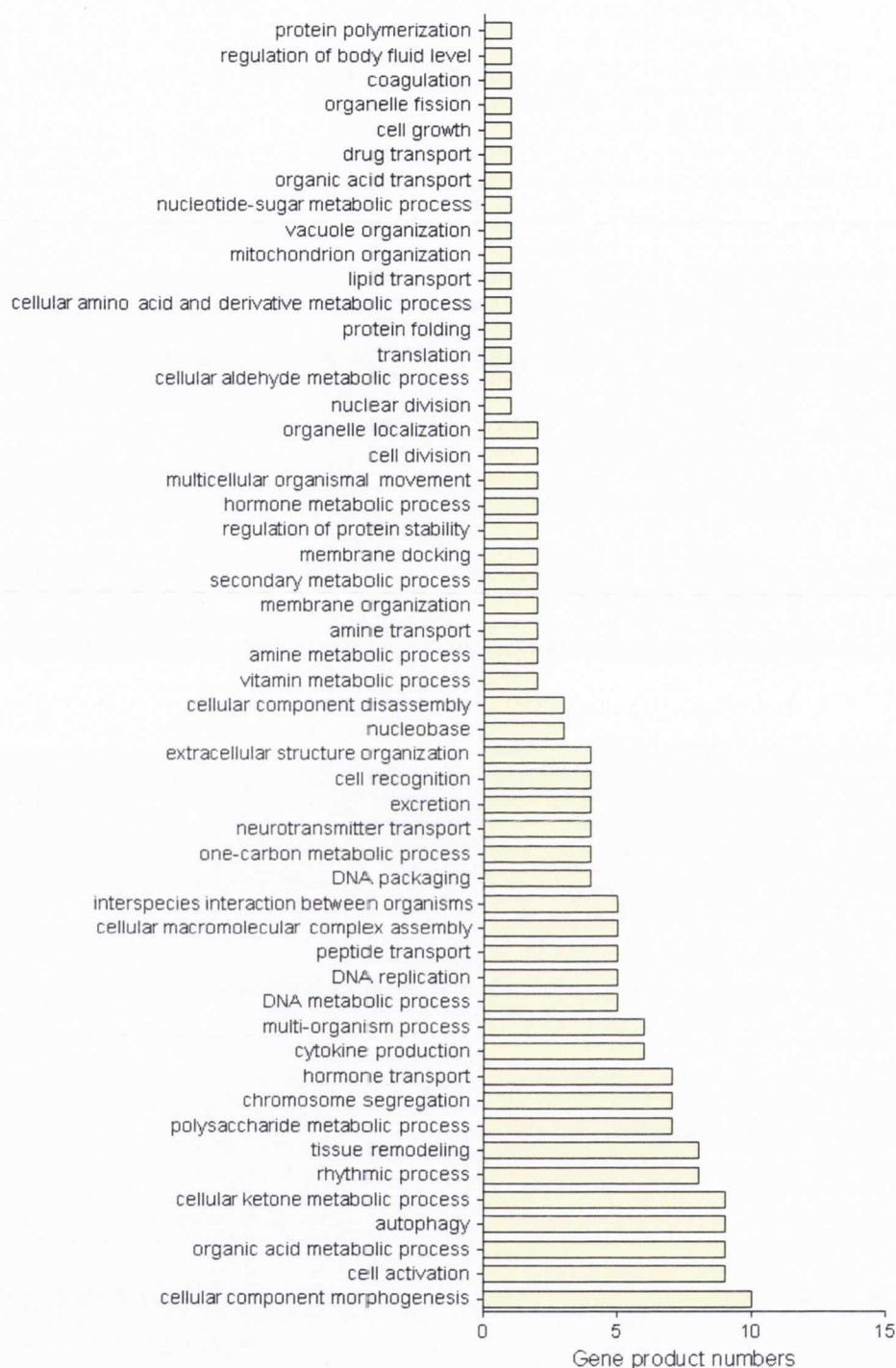


Figure 4.4 GO Slim using the Protein Information Resource (PIR) subset of GO terms reveals that global gene expression in response to *C. jejuni* in the caeca correlates to a wide range of biological processes. Gene product numbers refer to the number of differentially expressed genes that correlate to each GO term. The lowest represented biological processes are shown.

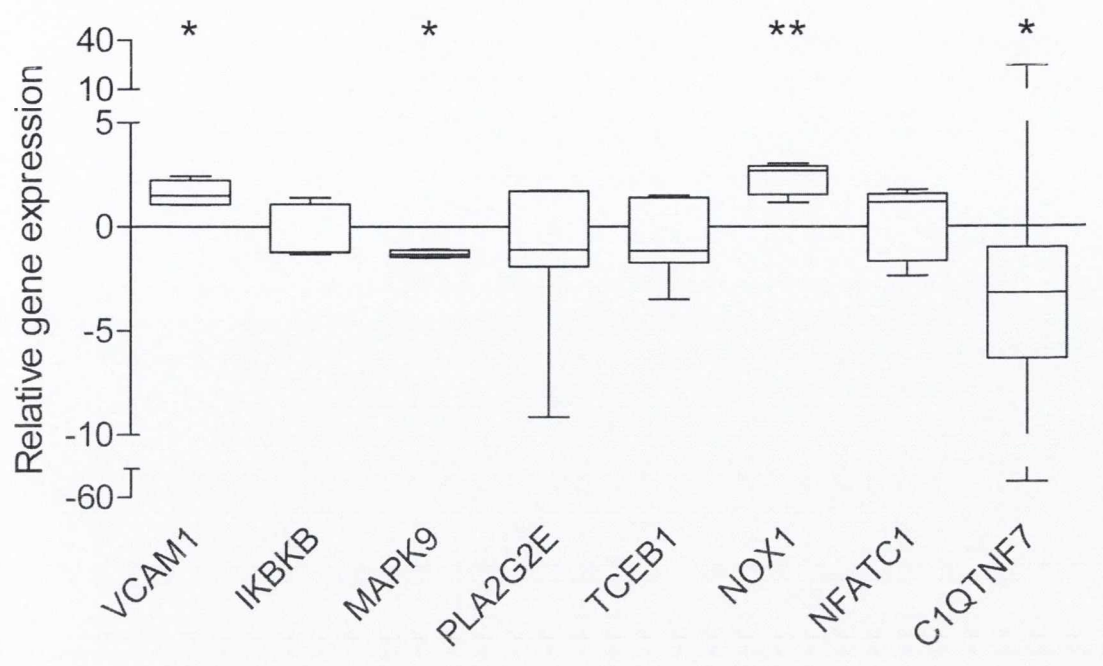


Figure 4.5 Validation of immune gene expression via qRT-PCR. The horizontal line within each box plot represents the median fold changes in caecal gene expression in *C. jejuni*-challenged chickens relative to mock-challenged controls (* $P < 0.05$, ** $P < 0.01$).

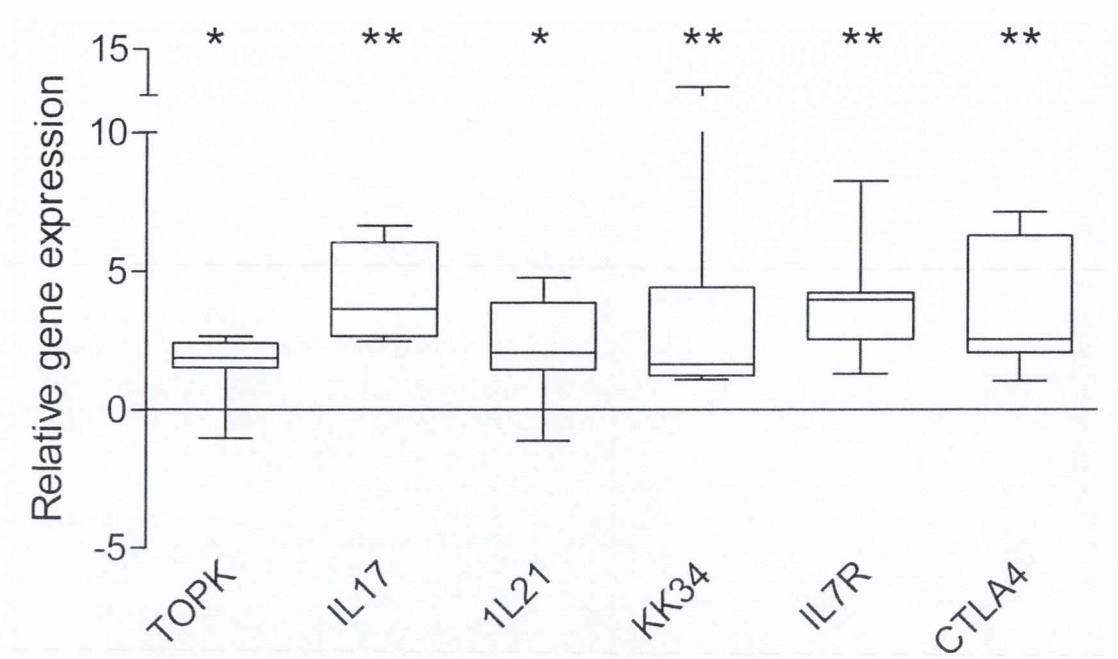


Figure 4.6 T cell related gene expression in response to *C. jejuni* in the chicken caeca at 20 h. The horizontal line within each box plot represents the median fold changes in caecal gene expression in *C. jejuni*-challenged chickens relative to mock-challenged controls (* $P < 0.05$, ** $P < 0.01$).

Chapter 5

Avian Cell Responses to *C. jejuni* Capsular Polysaccharide, Lipooligosaccharide and Cytolethal Distending Toxin

Chapter 5: Avian Cell Responses to *C. jejuni* Capsular Polysaccharide, Lipooligosaccharide and Cytolethal Distending Toxin

5.1 Introduction

On the basis of innate immune gene expression profiling (chapter 3) and global gene expression analysis (chapter 4) of caeca from *C. jejuni*-challenged chickens, it appears that the microbe fails to induce a substantial proinflammatory innate immune response within the avian intestine. This explains the observed lack of aberrant inflammation, leukocyte influx and other signs of intestinal immunopathology (chapter 3). This is, however, a stark contrast to *C. jejuni* infection in humans, in which intestinal biopsies of campylobacteriosis patients reveal epithelial ulceration, neutrophil infiltration and oedema within the lamina propria (van Spreeuwel *et al.*, 1985). Onset of such signs are believed to be associated with colonic cytokine (TNF- α and IL-12) increases, as is evident with a murine-*C. jejuni* infection model (Lippert *et al.*, 2009). Considering the immunopathological differences between human and chicken infections, *C. jejuni* must be able to evade or subvert the avian immune response.

C. jejuni is composed of multiple potentially immunogenic components including peptidoglycan (PGN), lipopeptides, lipooligosaccharide (LOS), capsular polysaccharide (CPS), nucleic acids, flagellin and cytolethal distending toxin (CDT) (Young *et al.*, 2007). Many of these components would be considered as PAMPs, and in theory, capable of activating PRRs common to both humans and birds. PRR evasion is, however, also evident as both human and avian TLR5 fail to detect *C. jejuni* flagellin (Andersen-Nissen *et al.*, 2005; de Zoete *et al.*, 2010a). As birds are considered the primary host of *C. jejuni*, with commensalism being evident, the microbe may have evolved to evade a range of avian PRRs. It is also possible that certain *C. jejuni* components subvert the establishment of proinflammatory responses.

The outer layer of *C. jejuni* consists of capsular polysaccharide, which separates underlying PAMPs including lipooligosacchade and lipopeptides from the extracellular environment (Young *et al.*, 2007). It is unclear whether *C. jejuni* CPS induces proinflammatory responses in human or avian cells although in general,

bacterial CPSs are not perceived to be PRR agonists. Thus, capsulated *C. jejuni* are likely to be poor inducers of proinflammatory responses. Bacterial CPS has also been reported to subvert the innate immune response as *Neisseria meningitidis* CPS binds to human CD14, which prevents TLR4-mediated recognition of the microbe's LOS (Kocabas *et al.*, 2007). Considering this, *C. jejuni* CPS may specifically subvert the innate immune response.

Of all the *C. jejuni* components, LOS is likely to be the most potent proinflammatory cytokine inducer. *C. jejuni* grown at 37°C and 42°C, the body temperatures of humans and birds respectively, produce structurally different types of LOS (Personal Communication from Professor Anthony Moran, NUI, Galway; unpublished data). LOS isolated from the microbe grown at 37°C has recently been shown to activate both human and chicken TLR4 (Kuijf *et al.*, 2010; van Mourik *et al.*, 2010), resulting in the induction of proinflammatory gene expression (de Zoete *et al.*, 2010a). Proinflammatory responses to the 42°C LOS variation have not been investigated. Temperature-driven structural changes are likely to affect the immunopotency of *C. jejuni* LOS, as this is evident with LPS from *Yersenia pestis* (Kawahara *et al.*, 2002).

CDT is considered to be a key *C. jejuni* virulence component (Smith & Bayles, 2006). The toxin is associated with increased *C. jejuni*-colonisation and gastric lesion formation in NF- κ B deficient mice (Fox *et al.*, 2004) although its role in human infection has not been determined (Smith & Bayles, 2006). *In vitro*, CDT induces cell cycle arrest in a range of human cells including intestinal epithelial cells (Whitehouse *et al.*, 1998) and fibroblasts (Hassane *et al.*, 2003), followed by cell death (Ge *et al.*, 2008), while leukocytes undergo apoptosis (Hickey *et al.*, 2005). The toxin also induces IL-8 from intestinal epithelial cells (Hickey *et al.*, 2000). Thus, CDT is likely to contribute to intestinal pathology through induction of both cell death and proinflammatory responses. The effects of CDT, however, have not been investigated *in vitro* or *in vivo* in birds. Hypothetically, the failure of *C. jejuni* CDT to induce apoptosis and proinflammatory responses in chicken cells may alone explain the microbe's commensal status in birds.

The aim of this chapter was to characterise chicken macrophage and fibroblast responses to the CPS, LOS and CDT components of *C. jejuni*. Components identified as inducers of avian proinflammatory response would help subsequently identify receptors, pathways and effectors involved (genes for colonisation resistance studies).

Furthermore, identification of avian responses to the components that differ to their mammalian equivalents would be a step towards explaining *C. jejuni* commensalism in birds.

5.2 Results

5.2.1 Macrophage responses to *C. jejuni* CPS

Macrophages are appropriate for initially investigating the immunostimulatory properties of bacterial components, as these cells express a wide range of PRRs and respond by producing high levels of proinflammatory mediators. There is evidence to suggest that bacterial capsular polysaccharides bind to innate receptors (Zamze *et al.*, 2002) and thus, it is possible that proinflammatory responses ensue. Initially, it appeared that *C. jejuni* CPS (1 µg/ml) induced low *IL1β* (42-fold) and *IL6* (46-fold) gene expression increases in HD11 cells at 4 h post-challenge. There was, however, a possibility that this response was caused by low LOS contamination in the CPS preparation and thus, challenges were repeated with the addition of Polymyxin B (PMB), an antibiotic that neutralises lipid A toxicity (Cardoso *et al.*, 2007). In the presence of PMB (10 µg/ml), cytokine gene expression increases in response to CPS were eliminated, which indicates that *C. jejuni* CPS is not immunostimulatory towards avian macrophages (Fig. 5.1 A and B).

5.2.2 Macrophage responses to *C. jejuni* LOS

At 4 h post-challenge, ultrapure *E. coli* LPS (1 µg/ml) induced high *IL1β* (~300-fold) and *IL6* (~1500-fold) gene expression in chicken HD11 macrophages. Ultrapure LOS (1 µg/ml) from *C. jejuni* grown at 37°C, however, induced higher *IL1β* (~400-fold) and *IL6* (~5200-fold) gene expression (Fig. 5.1 A and B). Conversely, *E. coli* LPS induced higher proinflammatory gene expression than *C. jejuni* LOS in human macrophages. *E. coli* LPS (1 µg/ml) induced ~400-fold *IL1β* and ~300-fold *IL8* gene expression increases in THP1 macrophages while *C. jejuni* LOS (1 µg/ml) induced *IL1β* and *IL8* gene expression increases by >200-fold (Fig. 5.1 C and D).

Varying the *C. jejuni* LOS and *E. coli* LPS doses in each challenge revealed differences in the response to each endotoxin. Gradual cytokine gene expression increases in both chicken HD11 macrophages and human THP1 macrophages were evident in response to sequential 100 ng/ml, 1 µg/ml and 5 µg/ml LOS challenges

(Fig. 5.1 C). In contrast, increasing cytokine gene expression in response to LPS reached a plateau at 1 $\mu\text{g/ml}$, while cytokine gene expression in response to 5 $\mu\text{g/ml}$ LPS was much reduced, and comparable to gene expression increases induced by 100 ng/ml LPS (Fig. 5.1 D).

To determine whether TLR4 was involved in *C. jejuni* LOS recognition, challenges were performed in the presence of TLR4-specific inhibitors. CLI-095, an inhibitor of TLR4 signalling (Kawamoto *et al.*, 2008), abolished *IL1 β* and *IL8* gene expression increases in *E. coli* LPS-challenged THP1 monocytes. CLI-095 also reduced *C. jejuni* LOS-mediated *IL1 β* and *IL8* gene expression increases, but only by about 50% (Fig. 5.2 A and B). In contrast, the inhibitor failed to reduce *IL6* and *IL8* gene expression increases in *E. coli* LPS-challenged chicken HD11 macrophages, indicating that CLI-095 has no inhibitory effects on chicken TLR4-signalling (Fig. 5.2 C). On this basis, HD11 cells were challenged with *E. coli* LPS in the presence of another TLR4 inhibitor, OxPAPC (1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine) (Erridge *et al.*, 2008). Similarly, though, OxPAPC failed to suppress *IL6* and *IL8* gene expression increases in such cells (Fig. 5.3 A). To confirm that chicken cells were responding to the lipid A component of *C. jejuni* LOS, challenges were conducted in the presence of PMB. This antibiotic, at 10 $\mu\text{g/ml}$ concentration, completely prevented the induction of *IL1 β* gene expression in HD11 cells in response to *E. coli* LPS. In contrast, PMB reduced LOS-mediated induction only by about 60% (Fig. 5.3 B).

To further investigate avian responses to *C. jejuni* LOS and *E. coli* LPS, TLR gene expression was profiled in challenged cells. Responses were similar to both endotoxins, in which *TLR15* upregulation (15-fold) was evident while *TLR2*, *TLR4*, *TLR5* and *TLR21* were not differentially expressed (Fig. 5.4 A). Profiling *TLR15* gene expression in HD11 cells in response to LOS across a 4 h timecourse revealed that the receptor was upregulated as early as 30 min post-challenge (Fig. 5.4 B).

5.2.3 Signalling pathways activated in HD11 cells in response to *C. jejuni* LOS

To investigate the signalling pathway to *C. jejuni* LOS, gene expression analysis of TLR4 co-receptors and pathway components was carried out. Upregulation of *p50* (5-fold), *AP1* (3-fold) and *IRF1* (5-fold) gene expression was evident in response to *C. jejuni* LOS while *CD14* gene expression was downregulated (-6-fold). A similar gene expression pattern was evident in response to *E. coli* LPS, suggesting similar pathway activation between the two endotoxin challenges (Fig. 5.5 A).

To further characterise signalling pathways, LOS challenges against HD11 cells were carried out in the presence of I κ B- α , p38, ERK and JNK inhibitors. I κ B- α , p38, ERK and JNK inhibitors significantly reduced *IL6* gene expression in LOS challenged cells ($P < 0.05$), indicating activation of NF- κ B and MAPK pathways (Fig. 5.5 C). The JNK inhibitor profoundly reduced *TLR15* gene expression while I κ B- α and ERK were also important for optimal *TLR15* gene expression increases in response to LOS. In contrast, significantly increased *TLR15* was evident to LOS in the presence of the p38 inhibitor (Fig. 5.5 B).

5.2.4 HD11 challenges with recombinant cytokines and supernatants

Mammalian *TLR2* gene expression has previously been shown to be upregulated directly by recombinant IL-1 β , and negatively regulated by the p38 pathway (Sakai *et al.*, 2004). On this basis, it was hypothesised that *TLR15* gene expression increases in response to *C. jejuni* LOS and *E. coli* LPS were indirectly a result of proinflammatory cytokine stimuli. To test this hypothesis, HD11 cells were challenged with either recombinant chicken IL-1 β or IL-6. *TLR15* was not differentially expressed to either recombinant cytokine at 4 h post-challenge (Fig. 5.6). Varying the dosage of each recombinant cytokine had no impact on *TLR15* gene expression.

It was, however, possible that other proinflammatory effectors in the supernatants of endotoxin-challenged cells mediated *TLR15* upregulation. To address

this hypothesis, supernatants collected from *E. coli* LPS-stimulated HD11 cells were treated with PMB, and subsequently used to challenge fresh HD11 cells. Supernatants induced minor *IL6* (23-fold) gene expression and high *IL8* (>1000-fold) gene expression but *TLR15* was not differentially expressed, at 4 h post challenge (Fig. 5.6).

5.2.5 CEF responses to *C. jejuni* LOS and CPS

Fibroblast-like cells are major constituents of the intestinal lamina propria that may or may not upregulate proinflammatory responses to *C. jejuni* components. On this basis, chicken embryonic fibroblasts were challenged with *C. jejuni* CPS or LOS for 4 h. CPS induced *IL8* gene expression although with addition of PMB, the polysaccharide failed to induce proinflammatory gene expression. Similarly, *E. coli* LPS failed to induce proinflammatory responses in CEF cells. In contrast, *IL8* gene expression was upregulated by 12-fold and 27-fold in response to 1 µg/ml and 5 µg/ml LOS concentrations respectively (Fig. 5.8 A). Further investigation revealed that, at the mRNA level, CEF cells express *TLR2*, *TLR5*, *TLR15* and *TLR21* but lack *TLR4* (Fig. 5.8 B). Thus, *C. jejuni* LOS is, in part, possibly recognised by an avian PRR, other than TLR4. Subsequent TLR gene expression profiling revealed *TLR15* gene expression increases, but not *TLR2*, *TLR5* and *TLR21* gene expression increases, in response to LOS (Fig. 5.9 A).

To investigate the signalling pathway to *C. jejuni* LOS in CEF cells, gene expression analysis of TLR4 co-receptors and pathway components was carried out. There was no clear indication of upregulation of *p50* and *AP1* gene expression upregulation although *IRF7* gene expression was upregulated by 8-fold (Fig. 5.9 B). Considering that IRF7 activation results in the induction of type I interferons (Genin *et al.*, 2009), *IFNα* and *IFNβ* gene expression was investigated. However, neither IFN gene was differentially expressed in response to *C. jejuni* LOS at 4 h post-challenge (Fig. 5.9 C).

5.2.6 HD11 and CEF responses to LOS from *C. jejuni* grown at 42°C

While LOS from *C. jejuni* grown at 37°C clearly induced proinflammatory responses in avian macrophages and fibroblasts, it was unknown whether LOS from the microbe grown at 42°C induces comparable responses. On the basis of recent unpublished data (Molecular Biochemistry Lab, NUI, Galway), it has been shown that *C. jejuni* grown at 37°C and 42°C produce structurally different types of LOS. Thus, challenges were repeated with the latter LOS type. In chicken HD11 macrophages, 42°C LOS induced *IL1 β* (800-fold), *IL6* (3500-fold), *IL8* (600-fold) gene expression, which was comparable to the 37°C LOS challenge at 4 h post-challenge (Fig. 5.7). However, unlike 37°C LOS, 42°C LOS failed to induce proinflammatory gene expression increases in CEF cells (Fig. 5.8 A), which suggests that this LOS type evades an as yet unidentified PRR.

5.2.7 Macrophage and monocyte responses to recombinant *C. jejuni* CDT

Mammalian cells have been shown to produce proinflammatory mediators in response to CDT (Akifusa *et al.*, 2001; Hickey *et al.*, 2000). It is unknown whether this toxin is immunostimulatory towards avian cells. To investigate this possibility, human and chicken cells were challenged with recombinant *C. jejuni* CDT. Human THP1 monocytes showed no evidence of *IL1 β* and *IL8* gene expression increases in response to 1 μ g/ml CDT at 4 h and 24 h post-challenge. A higher CDT dose (5 μ g/ml) induced minor *IL1 β* (21-fold) increases at 4 h while minor *IL1 β* (21-fold) and *IL8* (63-fold) increases were evident at 24 h post-challenge (Fig. 5.10 A). Similarly, chicken HD11 macrophages challenged with CDT for 4 h revealed only minor *IL1 β* and *IL8* gene expression increases. Proinflammatory gene expression increases were absent in CEF cells at 4 h post-CDT challenge (Fig. 5.10 A).

Induction of distension and cytotoxicity within mammalian host cells is, however, considered to be the primary effect of exposure to *C. jejuni* CDT (Ge *et al.*, 2008). In particular, leukocytes undergo apoptosis in response to the toxin (Hickey *et al.*, 2005) and thus, the apoptotic potential of CDT was assessed in human THP1 monocytes and chicken HD11 macrophages. At 48 h post CDT-challenge, 14% and

8% of THP1 monocytes had undergone early and late stage apoptosis respectively (Fig. 5.11 A; right panel). Chicken HD11 macrophages were more susceptible to apoptosis as at 48 h post CDT-challenge, 77% of the cells were in the late stages of apoptosis (Fig. 5.11 B; right panel).

5.3 Discussion

A more reductionist approach to studying *C. jejuni* interactions with the avian immune response involves determining how specific chicken cells respond to specific *C. jejuni* components. While the microorganism consists of many distinct structures, CPS, LOS and CDT are among the best studied (Young *et al.*, 2007), and the latter two components have previously been identified as PAMPs in human infection (Hickey *et al.*, 2000; Hu *et al.*, 2006). Thus, chicken cells were initially challenged with these components to determine whether proinflammatory responses were induced.

CPS is the outermost layer of *C. jejuni* and thus, would be the first component to come in contact with avian intestinal PRRs. Human epithelial cells have been shown to produce IL-6 in response to crude *C. jejuni* CPS (Friis *et al.*, 2009). Using chicken HD11 macrophages and CEF cells, proinflammatory gene expression increases were detected in response to the purified *C. jejuni* CPS preparation. It was, however, possible that low level *C. jejuni* LOS contamination was driving this response, considering that cytokine gene expression increases were only minor, and there have been very few reports of proinflammatory responses to bacterial CPS. To investigate endotoxin contamination, CPS challenges were conducted in the presence of polymyxin B. Polymyxin B is an antibiotic produced by *Paenibacillus polymixa* that binds lipid A molecules and thus, neutralises the immunostimulatory properties of endotoxins such as *E. coli* LPS (Cardoso *et al.*, 2007). This antibiotic completely abolished CPS-mediated cytokine and chemokine gene expression responses in both HD11 and CEF cells, indicating endotoxin contamination in the CPS preparation. More importantly, these data confirm that *C. jejuni* CPS is not a PAMP to chickens. With intact *C. jejuni*, this immunologically inert CPS might shield underlying LOS and lipopeptides from interacting with the PRRs in the avian intestine. Indeed, in comparison to lysed *C. jejuni*, live *C. jejuni* poorly activate chicken TLR2 and TLR4 (de Zoete *et al.*, 2010a). Furthermore, other capsulated bacterial species are less inductive of TLR4 activation, *in vitro* and *in vivo* (Raffatellu *et al.*, 2005; Wilson *et al.*, 2008; Wu *et al.*, 2009a).

In contrast, ultrapure LOS from *C. jejuni* grown at 37°C induced substantial cytokine gene expression increases in chicken HD11 macrophages, which were

slightly higher than the cytokine increases observed in response to *E. coli* LPS challenge. As a comparison, human THP1 monocytes were differentiated into macrophages, and subsequently challenged with the *C. jejuni* and *E. coli* endotoxins. THP1 macrophages responded in a similar manner to their avian equivalents, indicating minimal differences in lipid A recognition and subsequent proinflammatory response between chicken and human macrophages. Similarly, de Zoete *et al* recently observed increased *IL1 β* gene expression in both chicken HD11 macrophages and human MM6 monocytes in response to *C. jejuni* LOS (de Zoete *et al.*, 2010a).

Varying the challenge doses, however, suggested that *E. coli* LPS and *C. jejuni* LOS are differentially recognised. With both HD11 and THP1 macrophages, a low LPS dose induced the highest cytokine gene expression while expression was reduced in response to high doses. This suggests macrophage-mediated tolerance to LPS, considering that *CD14* gene expression was downregulated by 6-fold in challenged HD11 cells. In contrast, cytokine gene expression gradually increased in response to increasing LOS doses. Possibly, *C. jejuni* LOS was being recognised by another PRR although, only in part, as it has recently been confirmed that *C. jejuni* LOS is recognised by both chicken and human TLR4 (Kuijf *et al.*, 2010; van Mourik *et al.*, 2010). Thus, to confirm the role of TLR4, LOS and LPS challenges were performed in the presence of CLI-095, an anti-sepsis substance that inhibits TLR4-mediated signalling by targeting the receptor's intracellular domain (Kawamoto *et al.*, 2008). To serve as a positive control, inhibition was initially assessed in THP1 cells, as CLI-095 has been shown to be effective on human monocytes (Kawamoto *et al.*, 2008). Indeed, in the presence of the inhibitor, cytokine and chemokine gene expression increases in response to *E. coli* LPS were undetectable, indicating complete inhibition of human TLR4 signalling. CLI-095 also reduced proinflammatory mediator gene expression increases in response to *C. jejuni* LOS albeit complete inhibition was not achieved. On this basis, the microbe's LOS appears to be partially recognised in a TLR4-independent manner. CLI-095, however, failed to reduce TLR4-mediated signalling in chicken HD11 macrophages, which indicates that this inhibitor is not specific for chicken TLR4. This is largely due to the fact there is only a 60% amino acid identity between human and chicken TLR4-TIR domains (Boyd *et al.*, 2007). Similarly, another inhibitor, OxPAPC, also failed to inhibit chicken TLR4-mediated signalling. OxPAPC inhibits TLR2 and TLR4 activation by specifically binding to MD2, LBP and CD14 (Erridge *et al.*, 2008). Considering that

there is only about 30% amino acid identity between human and chicken CD14 (Wu *et al.*, 2009b), it is plausible that OxPAPC fails to target chicken specific TLR co-receptors on the basis of species-specific differences. Therefore, using human TLR inhibitors, it was not possible to determine whether proinflammatory cytokine gene expression increases in chicken HD11 cell in response to *C. jejuni* LOS were, partially or completely, a result of TLR4 activation. However, using PMB, it was possible to show that HD11-mediated proinflammatory responses to *E. coli* and *C. jejuni* endotoxins were a result of lipid A recognition. In the presence of PMB, increases in cytokine gene expression in response to *E. coli* LPS were eliminated, and reduced in response to *C. jejuni* LOS. Such incomplete neutralisation of LOS is likely an indication that the endotoxin is somewhat resistant to PMB binding, on the basis of previous studies. PMB specifically binds to phosphate groups of lipid A but with *C. jejuni* lipid A, these groups can be substituted by diphosphorylethanolamine thus, reducing binding efficiency (Moran *et al.*, 1991b). Further evidence of resistance comes from the fact that live *C. jejuni* are resistant to PMB killing (Cullen & Trent, 2010; van Mourik *et al.*, 2010).

To further characterise the responses to *C. jejuni* LOS and *E. coli* LPS, TLRs implicated in responses to bacterial infection were profiled at the mRNA level of challenged HD11 cells relative to unchallenged cells. TLR4, which recognises both bacterial LPS (Kogut *et al.*, 2005) and *C. jejuni* LOS (van Mourik *et al.*, 2010), was not differentially expressed at the mRNA level to either endotoxin challenge. Thus, activation of a specific TLR is not necessarily indicated by the upregulation of its specific mRNA. Interestingly, *TLR15* was the only differentially expressed receptor, in which upregulation was evident to both endotoxin challenges. Consistent with this observation, *TLR15* has been shown to be upregulated by gram-negative bacterial challenges (Higgs *et al.*, 2006; Mackinnon *et al.*, 2009) yet, there is also evidence to suggest that gram positive bacterial species upregulate the receptor (Lu *et al.*, 2009; Nerren *et al.*, 2009). Thus, TLR15 appears to be upregulated as a result of both TLR4 and TLR2 activation although the pathways responsible have not been identified.

To initially identify the signalling pathways responsible for *TLR15* upregulation and cytokine increases in response to *C. jejuni* LOS, expression of genes encoding TLR4 co-receptors and intracellular signalling pathway components were investigated in challenged HD11 cells. While some key pathway components such as *MyD88*, *IKBKB* and *p65* were not differentially expressed, the *p50* and *API*

transcription factors were clearly upregulated, suggesting NF- κ B and MAPK activation respectively. To further study the activated pathways, LOS challenges against HD11 cells were conducted in the presence of I κ B- α , p38, ERK and JNK inhibitors. These inhibitors have previously been successfully used to study chicken leukocyte signalling in response to bacterial and viral challenges (Han *et al.*, 2009; Kogut *et al.*, 2008). In response to LOS, maximum upregulation of *IL6* gene expression required NF- κ B, p38 and JNK activation. To a lesser extent, ERK activation was also required. Similarly, live *C. jejuni* have been shown to activate all four pathways in human cells (Chen *et al.*, 2006; Watson & Galan, 2005), while NF- κ B and MAPK pathways are activated in response to *S. enterica* LPS in chicken cells (Kogut *et al.*, 2008). Thus, the typical pathways required for cytokine production are activated in chicken macrophages in response to *C. jejuni* LOS. Interestingly, though, *TLR15* upregulation in response to *C. jejuni* LOS was moderately reduced by I κ B- α and ERK inhibitors while a substantial reduction was evident as a result of JNK inhibition. In contrast, *TLR15* upregulation was enhanced by the p38 inhibitor. Thus, JNK and p38 pathways are key positive and negative regulators of *TLR15* gene expression respectively. Similarly, the p38 pathway has been shown to negatively regulate *TLR2* gene expression in human epithelial cells (Sakai *et al.*, 2004).

It was, however, unknown whether *TLR15* upregulation was directly induced as a result of TLR activation or whether upregulation was indirectly induced by cytokines increased in response to endotoxin challenge. Regarding the latter, it has been reported that IFN- γ and GM-CSF upregulate *TLR2* and *TLR4* gene expression in human leukocytes (O'Mahony *et al.*, 2008) while the former receptor has been shown to be upregulated in human epithelial cells via IL-1 β stimulation (Sakai *et al.*, 2004). Thus, it was hypothesised that *TLR15* upregulation, where evident, was directly induced by avian proinflammatory cytokines. Such cytokines clearly did not include chicken IL-1 β and IL-6, as their recombinant equivalents failed to upregulate the PRR in HD11 cells. It was, however, possible that other cytokines present in the supernatants of endotoxin challenged HD11 cells mediated *TLR15* upregulation considering that macrophages tend to produce a wide range of effectors in response to endotoxin challenge. To address this issue, supernatants from *E. coli* LPS-challenged HD11 cells were treated with polymyxin B, and subsequently used to stimulate unchallenged HD11 cells. Polymyxin B completely neutralizes the immunostimulatory properties of *E. coli* LPS and thus, gene expression changes as a

result of supernatant challenge would indicate a response to HD11 secreted factors and not to the initial endotoxin challenge. Supernatants from LPS-challenged cells induced moderate and high *IL6* and *IL8* gene expression respectively, indicating autocrine cytokine induction. However, supernatants failed to upregulate *TLR15*, which suggests that the receptor is directly upregulated as a result of *TLR4* activation and not indirectly in response to proinflammatory cytokine stimuli.

It has recently been discovered that *C. jejuni* grown at 37°C produce LOS that is structurally distinct to the LOS type evident with *C. jejuni* grown at 42°C (Personal Communication from Professor Anthony Moran, NUI, Galway; unpublished data). Considering that 42°C also represents the body temperature of birds, the latter LOS type is more likely to be encountered by avian defences. Thus, avian cells were challenged with 42°C *C. jejuni* LOS. This LOS type induced cytokine gene expression increases in HD11 cells that were similar in magnitude to the increases induced by 37°C LOS, suggesting that both LOS types equally stimulated proinflammatory responses in avian cells. Considering that the intestinal lamina propria also consists of fibroblast-like cells (Illemann *et al.*, 2004), CEF cells were also challenged with both *C. jejuni* LOS types. In response to LOS from *C. jejuni* grown at 37°C, increased *TLR15* and *IL8* gene expression was evident. Subsequent signalling pathway gene analysis gave no clear indication of NF-κB or MAPK activation although *IRF7* gene expression was increased by 8-fold. IRF7 activation results in type 1 IFN production (Genin *et al.*, 2009) yet, *IFNα* and *IFNβ* were not differentially expressed. In this case, increased *IRF7* gene expression could signify the priming of antiviral pathways, considering that fibroblasts are commonly targeted by viruses (Escoffier & Gerlier, 1999; Revello & Gerna, 2010). In contrast, both LOS isolated from *C. jejuni* grown at 42°C and *E. coli* LPS failed to induce proinflammatory mediator gene expression in CEF cells. Further analysis revealed that CEF cells express *TLR2*, *TLR4*, *TLR5*, *TLR15* and *TLR21* but lacks *TLR4* gene expression. Thus, 42°C LOS appears to be recognised by chicken TLR4 yet 37°C LOS can be recognised in a TLR4-independent manner. It is unclear what other PRR is recognising 37°C *C. jejuni* LOS, but is possibly TLR2, considering that it is highly expressed in CEF cells, and the LPS of a related microbe known as *Helicobacter pylori*, once considered a member of the *Campylobacter* genus, has been reported to activate both human TLR2 (Yokota *et al.*, 2007) and TLR4 (Kawahara *et al.*, 2001). TLR15 is, however, also a potential candidate as it is upregulated in 37°C LOS-

challenged CEF cells, and is most closely related to chicken TLR2 with 30% amino acid sequence identity (Higgs *et al.*, 2005). Therefore, future studies are clearly warranted to identify the alternative receptor for 37°C LOS. Regardless, though, this receptor is clearly evaded by 42°C *C. jejuni* LOS and thus, would not be activated by the microbe in the avian intestine. In such circumstances, TLR4 would recognise the LOS component although in unchallenged birds, caecal *TLR4* is lowly expressed at the mRNA level, in comparison to other PRRs including *TLR2* and *TLR15* mRNA levels (Higgs *et al.*, 2006). Thus, in response to the LOS type produced by *C. jejuni* at 42°C, TLR4 activation would be minimal while the alternative LOS PRRs would be evaded. This possibility requires further investigation but is nevertheless plausible, considering that several bacterial species have been reported to alter their endotoxin structures in order to reduce host proinflammatory responses. *Yersenia pestis* produces two distinct LPS types, one at 27°C and the other at 37°C, with the latter endotoxin type is less proinflammatory towards human macrophages (Kawahara *et al.*, 2002). Furthermore, *Porphyromonas gingivalis* produces two LPS types; one type activates TLR4 and the other type inhibits TLR4 activation (Coats *et al.*, 2007; Reife *et al.*, 2006).

There is growing evidence that bacterial exotoxins are recognised by intracellular PRRs (particularly NALP3) and are thus, inductive of proinflammatory responses (Harder *et al.*, 2009; Toma *et al.*, 2010). *C. jejuni* CDT may be recognised by similar means considering that the toxin induces IL-8 in human epithelial cells. It was unknown whether *C. jejuni* CDT induced such responses in avian cells. Initially, human THP1 monocytes were stimulated with recombinant *C. jejuni* CDT, in order to serve as a comparison. Only minor *IL1 β* and *IL8* gene expression increases were evident a 4 h and 24 h post-challenge. It has been shown that wild-type *C. jejuni* and *C. jejuni* lacking functional CDT induce similar cytokine and chemokine levels in THP1 cells (Jones *et al.*, 2003). Thus, *C. jejuni* CDT is not particularly immunostimulatory towards THP1 cells. Similarly, CDT induced only minor proinflammatory gene expression increases in HD11 cells although this was somewhat expected considering that with this cell-line, wild-type *C. jejuni* and CDT-deficient *C. jejuni* induce similar cytokine and chemokine gene expression levels (Smith *et al.*, 2005a). Furthermore, CDT failed to induce proinflammatory gene expression in CEF cells. Collectively, CDT is not likely a major PAMP in chickens. *C. jejuni* CDT, however, primarily functions to induce cell cycle arrest in host cells.

Mammalian leukocytes such as monocytes and macrophages are susceptible to such cytotoxic effects, and subsequently undergo apoptosis (Smith & Bayles, 2006). Chicken lymphocytes undergo programmed cell death in response to *C. jejuni* outer-membrane proteins (OMP) *in vitro* (Zhu *et al.*, 1999) but it is unknown whether the CDT component specifically induces apoptosis in chicken leukocytes. Thus, apoptosis was investigated in chicken HD11 macrophages challenged with the toxin. As a control, THP1 cells were initially assessed as *C. jejuni* CDT has been shown to induce apoptosis in human 28SC monocytes (Hickey *et al.*, 2005). Over 20% of such cells had undergone programmed cell death at 48 h post-CDT challenge, indicating that cytotoxic activity was retained with the recombinant protein. In contrast, the toxin induced programmed cell death in about 80% of chicken HD11 macrophages at this interval, which suggests that *C. jejuni* CDT is highly apoptotic towards chicken leukocytes. *In vivo* implications for such findings regarding pathology are somewhat debatable. Firstly, it is unclear whether *C. jejuni* produce the toxin during colonisation of the avian intestine. CDT is detectible at the mRNA level in colonised caeca yet while serum samples from such birds contain antibodies specific to various *C. jejuni* components, CDT-specific antibodies are lacking (Abuoun *et al.*, 2005). Moreover, wild-type and CDT-deficient *C. jejuni* do not differ in ability to colonise the chicken caeca and in such circumstances, intestinal clinical signs are absent to both strains (Abuoun *et al.*, 2005; Biswas *et al.*, 2006). Thus, there is a lack of evidence for CDT activity *in vivo*.

In conclusion, the results of this chapter show that *C. jejuni* components differ in their abilities to induce proinflammatory responses in chicken cells. The CPS component is immunologically inert and thus, capsulated *C. jejuni* are likely to be shielded from intestinal PRRs. Similarly, CDT fails to induce proinflammatory responses in avian cells but mediates cytotoxic effects. LOS from *C. jejuni* grown at 37°C appears to be a major proinflammatory mediator, activating TLR4 and possibly an alternative PRRs, resulting in NF- κ B and MAPK activation, giving rise to *TLR15* and proinflammatory mediator gene expression upregulation. LOS from *C. jejuni* grown at 42°C is also proinflammatory but fails to activate the “alternative PRR”, potentially reducing subsequent inflammatory responses.

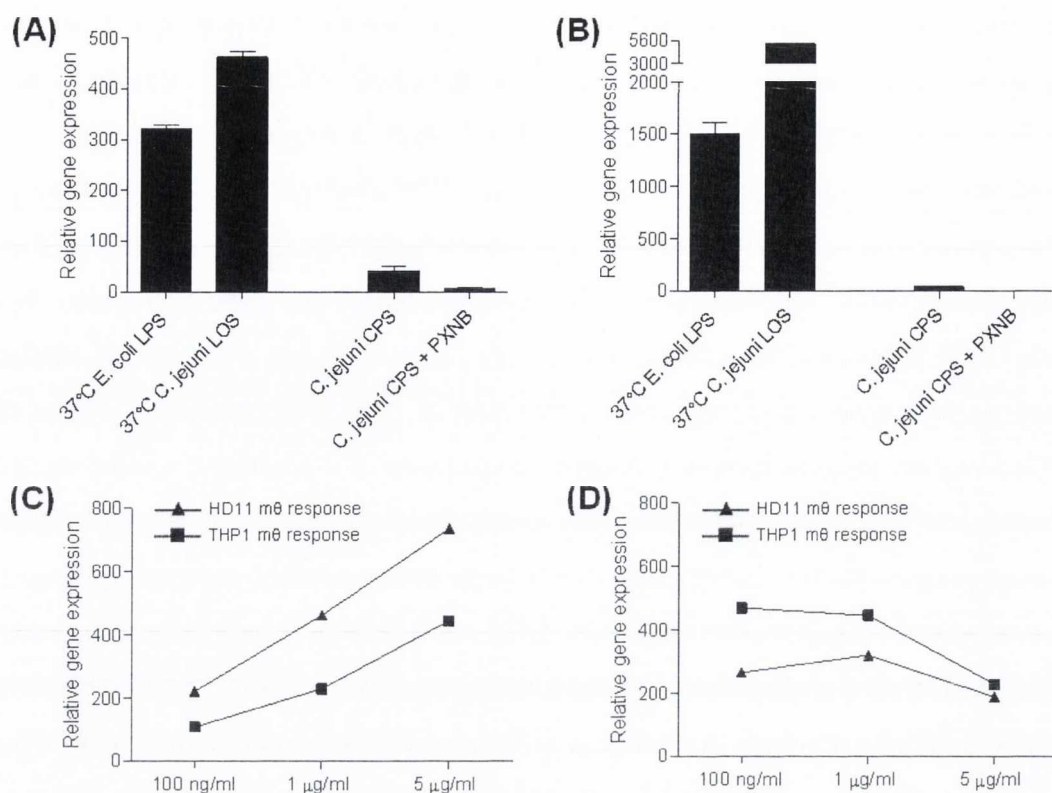


Figure 5.1 Chicken and human macrophage responses to *C. jejuni* surface components. **(A)** *IL1β* and **(B)** *IL6* gene expression is increased in chicken HD11 macrophages in response to ultrapure *E. coli* LPS (1µg/ml) and ultrapure *C. jejuni* LOS (1µg/ml) at 4 h post-challenge. Minimal increases are evident in response to 1 µg/ml purified *C. jejuni* CPS while CPS in the presence of polymyxin B (PXNB) fails to induce cytokine expression. **(C)** Gradual *IL1β* gene expression increases are evident in both HD11 and THP1 macrophages in response to increasing *C. jejuni* LOS doses. **(D)** *IL1β* gene expression increases are gradually reduced in both HD11 and THP1 macrophages in response to increasing *E. coli* LPS doses.

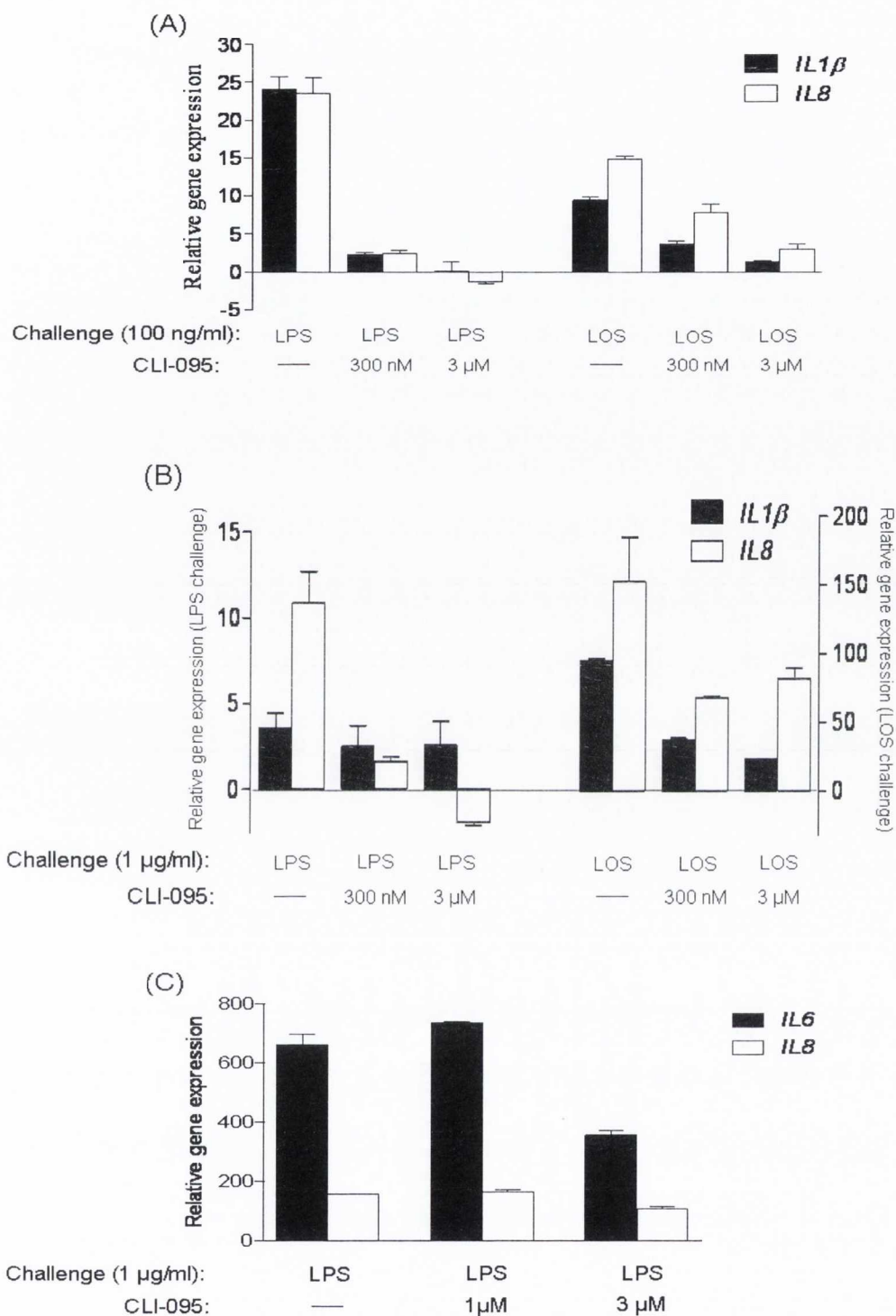


Figure 5.2 CLI-095 inhibits TLR4 signalling in human cells, but not in chicken cells. In human THP1 monocytes, CLI-095 inhibits *IL1β* and *IL8* gene expression increases in response to (A) 100 ng/ml and (B) 1 μg/ml doses of *E.coli* LPS and *C. jejuni* LOS (C) In chicken HD11 macrophages, CLI-095 fails to inhibit *IL6* and *IL8* gene expression increases in response to 1 μg/ml *E. coli* LPS. All data represents 4 h post-challenge.

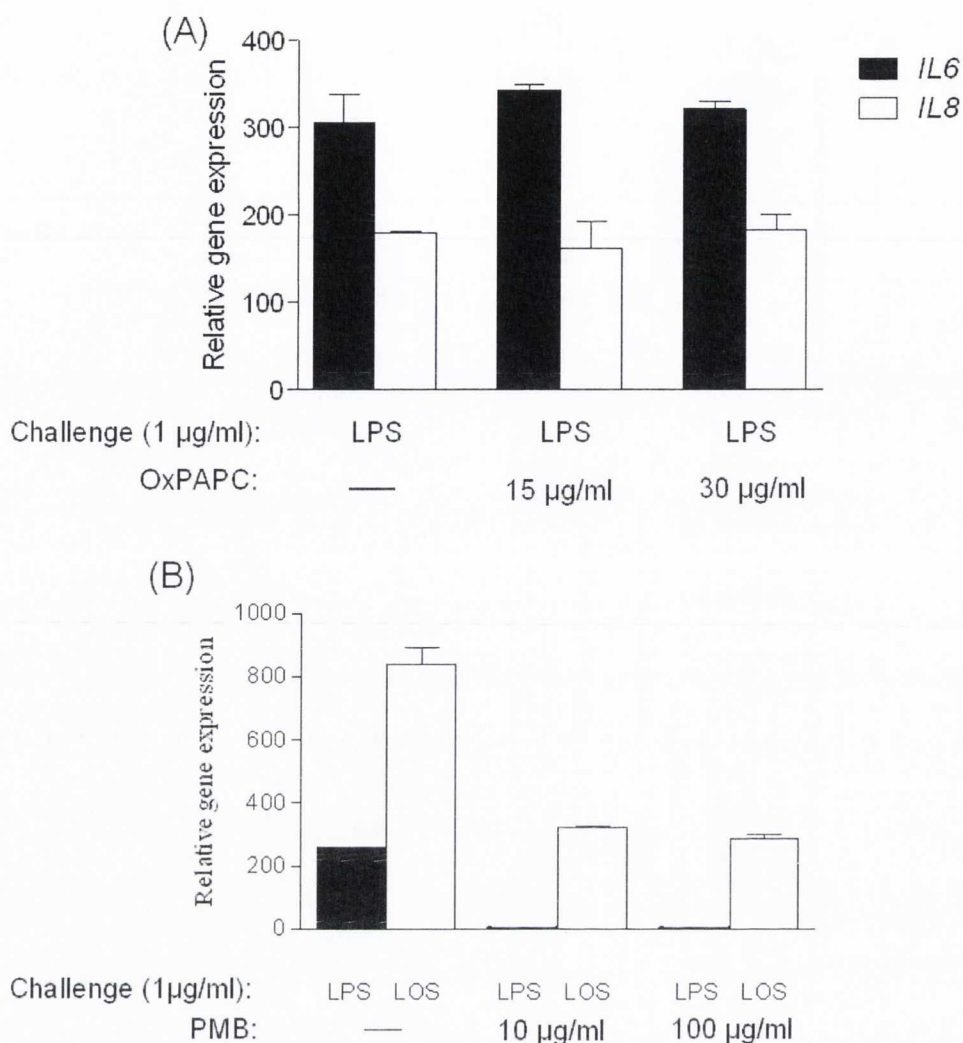


Figure 5.3 Inhibition of TLR4 signalling. **(A)** OxPAPC fails to prevent TLR4-mediated induction of *IL6* and *IL8* gene expression in chicken HD11 macrophages in response to *E. coli* LPS. **(B)** PMB completely inhibits *E. coli* LPS-mediated induction of *IL1β* gene expression in HD11 cells while *C. jejuni* LOS-mediated induction is only partially reduced. All data represents 4 h post-challenge.

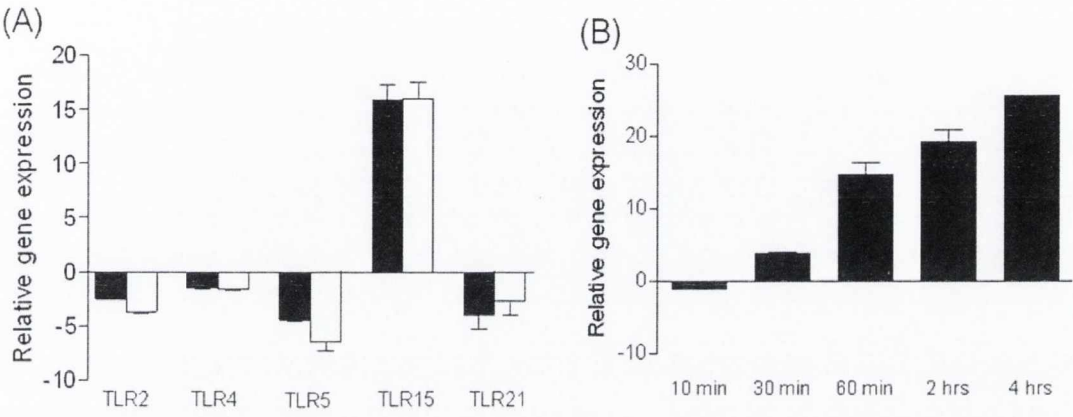


Figure 5.4 Differential pattern recognition receptor (PRR) and signalling pathway gene expression in response to 1 µg/ml *E. coli* LPS and *C. jejuni* LOS in HD11 macrophages. **(A)** LPS (■) and LOS (□) both upregulate TLR15 gene expression 4 h post-challenge. **(B)** *TLR15* mRNA upregulation is evident as early as 30 minutes post-*C. jejuni* LOS challenge.

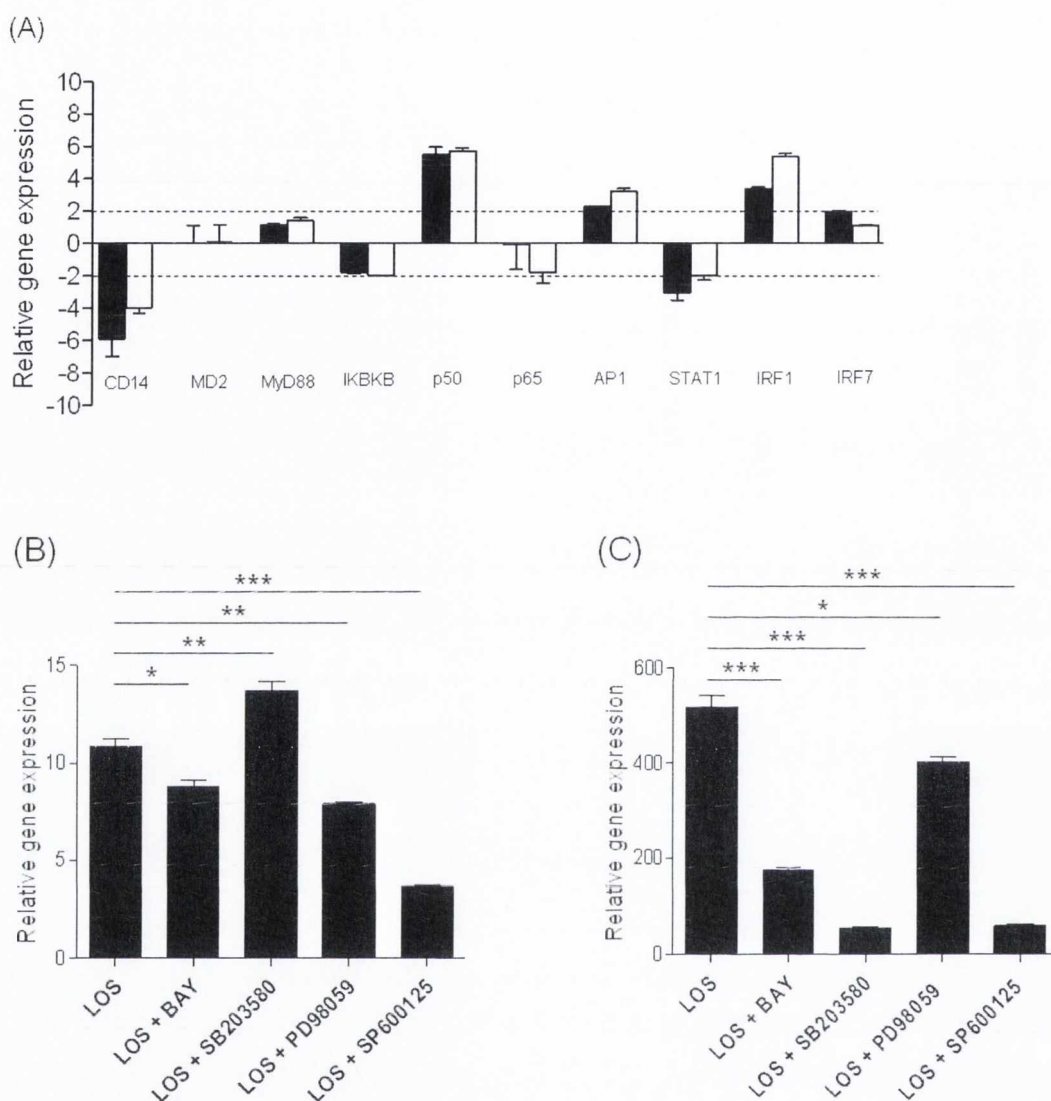


Figure 5.5 *C. jejuni* LOS activates NF- κ B and MAPK pathways in chicken HD11 macrophages. **(A)** *E. coli* LPS (■) and *C. jejuni* LOS (□) upregulate NF- κ B, MAPK and IRF pathway components at 4 h post-challenge. Subsequently, HD11 cells were incubated with either Bay11-7082 (I κ B- α inhibitor), SB203580 (p38 inhibitor), PD98059 (ERK inhibitor) or SP600125 (JNK inhibitor) for 1 h prior to LOS (1 μ g/ml) challenge. **(B)** Increased *TLR15* gene expression in response to LOS (4 h post-challenge) is further enhanced in the presence of a p38 inhibitor but significantly downregulated in the presence of I κ B- α , ERK and JNK inhibitors. **(C)** *IL6* gene expression in response to LOS (4 h post-challenge) is significantly reduced by I κ B- α , p38, ERK and JNK inhibitors. Statistical analysis was performed using unpaired t-tests (* P < 0.05, ** P < 0.01, *** P < 0.001).

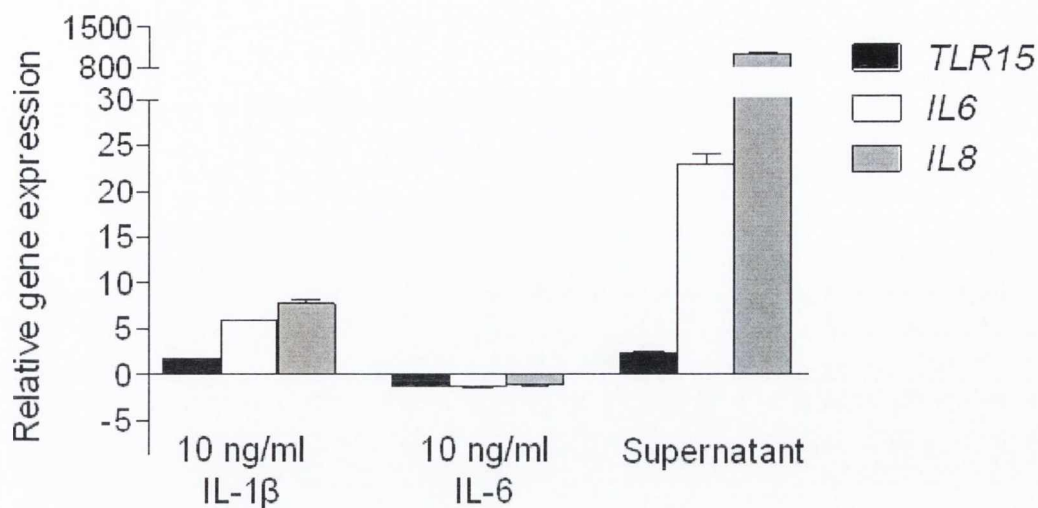


Figure 5.6 *TLR15* is not upregulated by proinflammatory cytokines in HD11 chicken macrophages. Recombinant chicken IL-1 β and IL-6 do not upregulate *TLR15*, *IL6* or *IL8*. HD11 cells stimulated with polymyxin B treated supernatant (900 μ l) of *E. coli* LPS-challenged HD11 cells also fail to upregulate *TLR15* but not *IL8* gene expression.

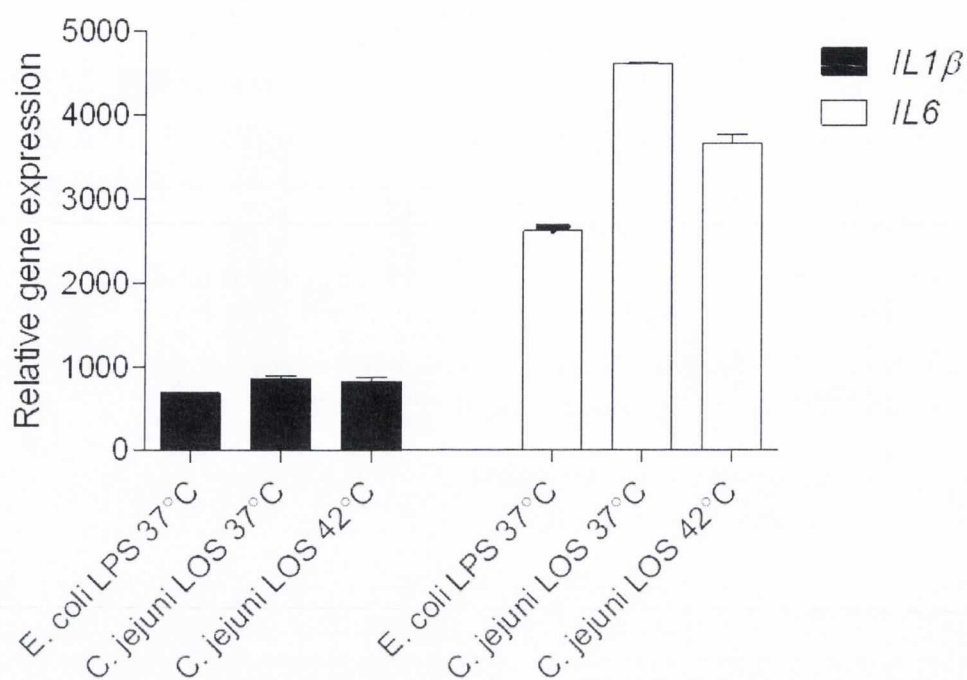


Figure 5.7 LOS (1 $\mu\text{g/ml}$) from *C. jejuni* grown at both 37°C and 42°C induce comparable *IL1 β* and *IL6* gene expression increases at 4 h post-challenge in chicken HD11 macrophages.

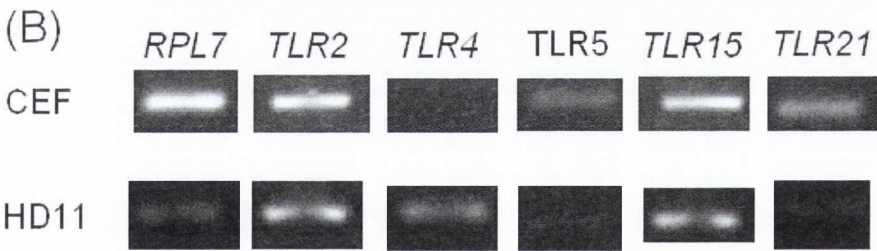
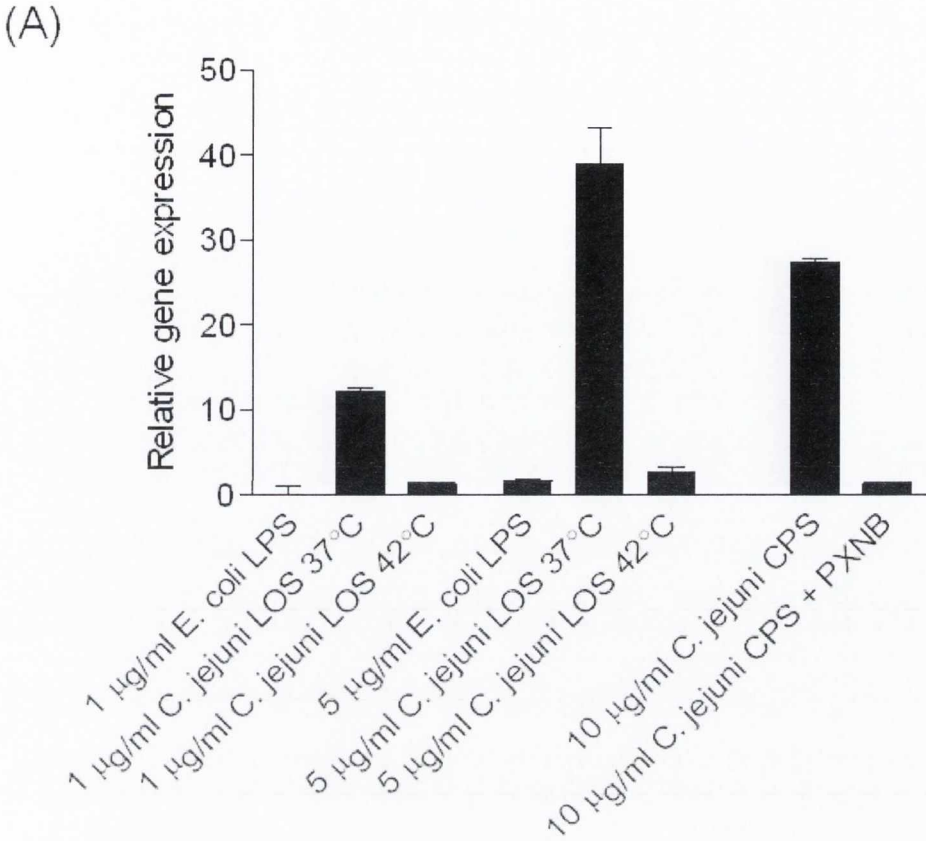


Figure 5.8 Differential PRR recognition of LOS between *C. jejuni* grown at 37°C and 42°C. **(A)** Chicken embryonic fibroblasts (CEF) induce *IL8* gene expression in response to LOS purified from *C. jejuni* grown at 37°C but not to LOS purified from *C. jejuni* grown at 42°C. *C. jejuni* CPS and *E. coli* LPS are also not inductive of *IL8* gene expression in CEFs. **(B)** In comparison to chicken HD11 macrophages, CEFs do not express basal TLR4 mRNA. *RPL7* served as a housekeeper gene.

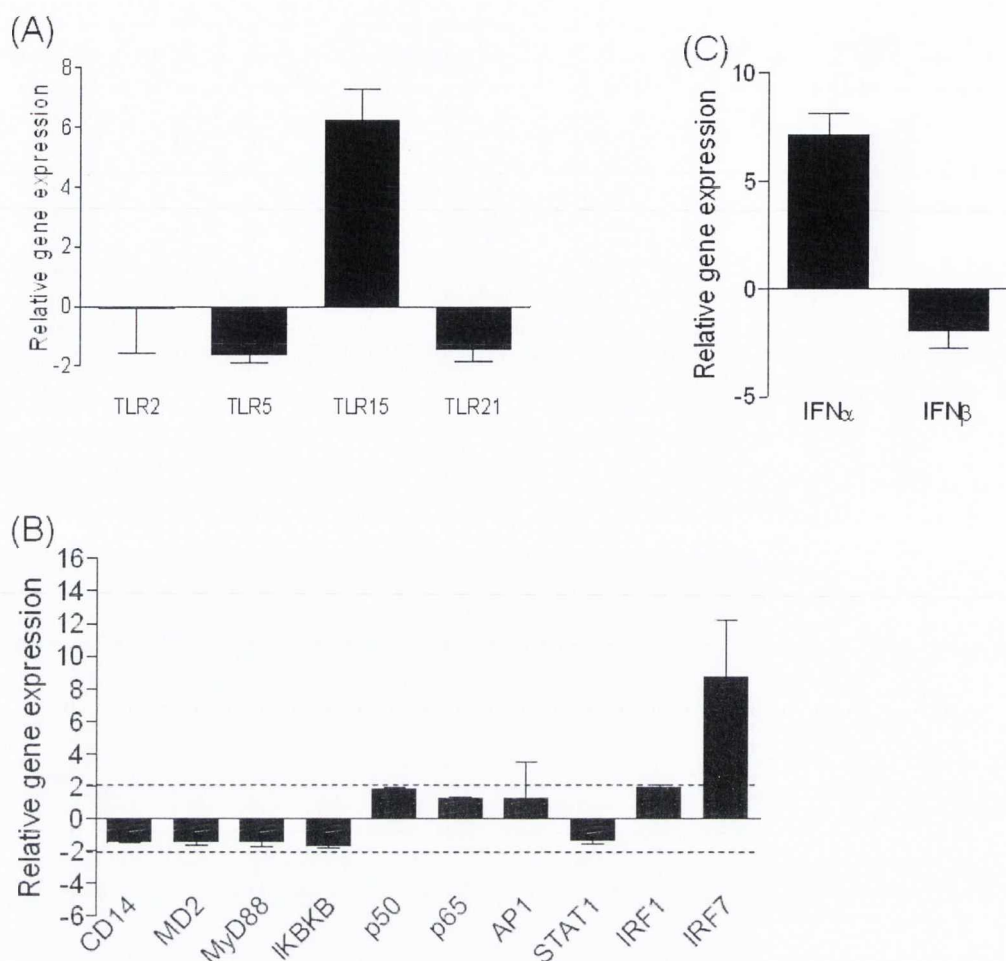


Figure 5.9 Pattern recognition receptor (PRR) and signalling pathway gene expression in response to 1 µg/ml *C. jejuni* LOS (grown at 37°C) in CEFs at 4 h post-challenge. **(A)** LOS induces *TLR15* gene expression in CEFs. **(B)** *IRF7*, a type I interferon pathway gene, is upregulated in response to LOS. **(C)** LOS induces only minor type I interferon gene expression.

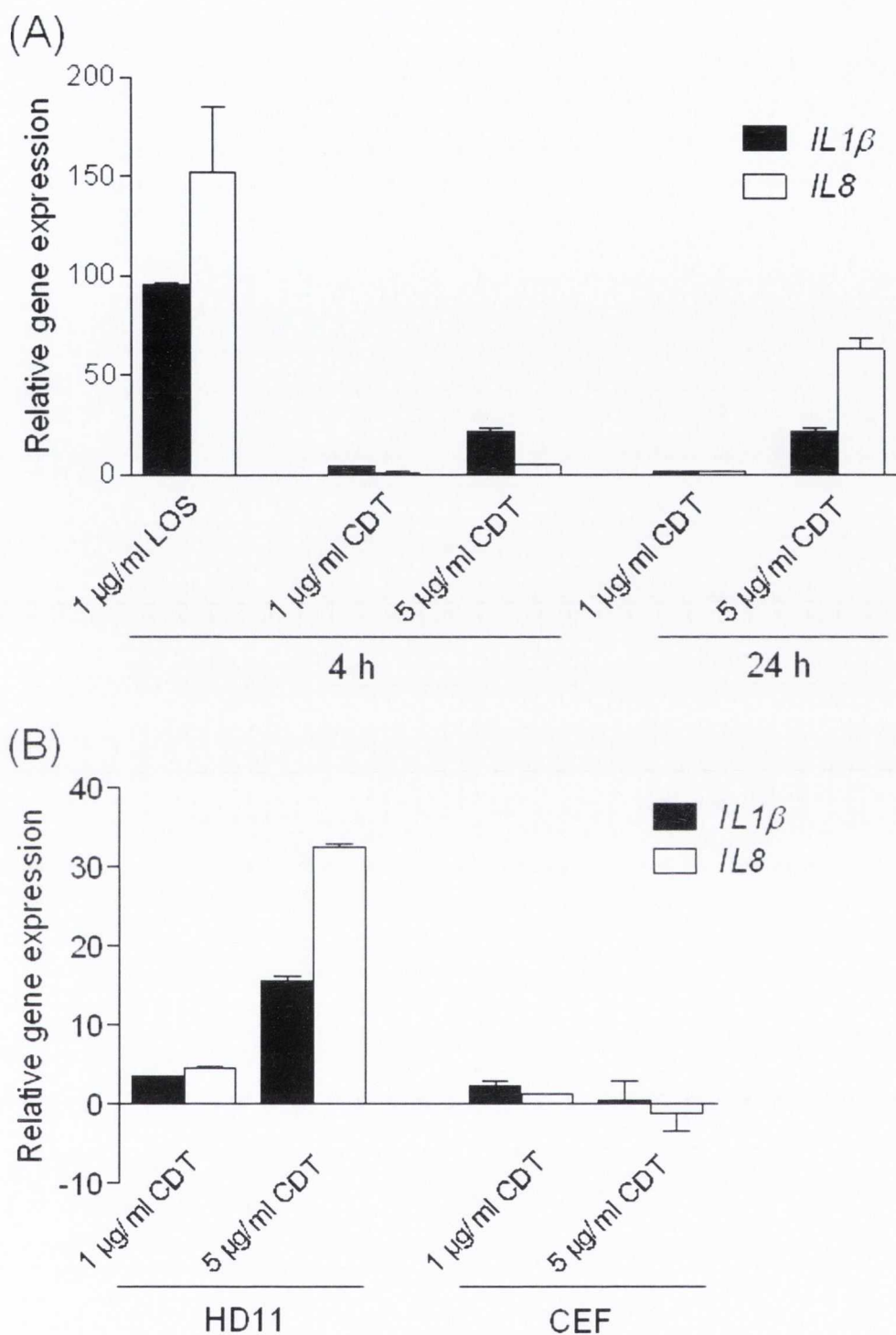


Figure 5.10 Recombinant *C. jejuni* CDT induces minimal cytokine and chemokine gene expression in human and chicken cells. **(A)** Low *IL1β* and *IL8* upregulation is evident in human THP1 monocytes (in comparison to *C. jejuni* LOS) in response to CDT at 4 h and 24 h post-challenge. **(B)** Low *IL1β* and *IL8* upregulation is evident in chicken HD11 macrophages and chicken embryonic fibroblasts (CEF) in response to CDT at 4 h post-challenge.

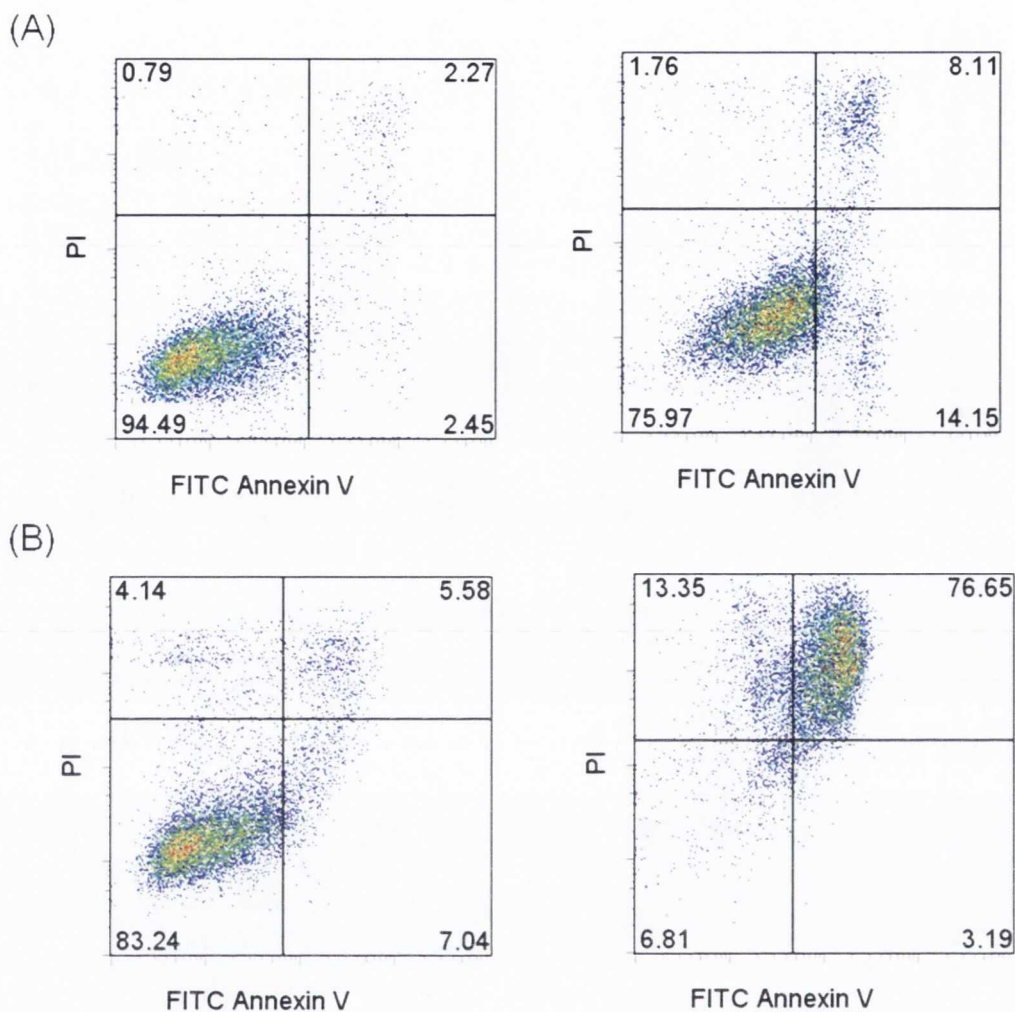


Figure 5.11 Recombinant *C. jejuni* CDT induces apoptosis in both human monocytes and chicken macrophages. **(A)** Early and late stages of apoptosis are evident with THP1 monocytes at 48 h post-CDT (1 $\mu\text{g/ml}$) challenge (right panel). The left panel represents unchallenged THP1 cells. **(B)** Late stage apoptosis is evident with HD11 macrophages at 48 h post-CDT (1 $\mu\text{g/ml}$) challenge (right panel). The left panel represents unchallenged HD11 cells.

Chapter 6

Final Discussion

Chapter 6: Final Discussion

The field of avian immunology has been driven by the requirement to combat avian bacterial pathogens, such as *S. Gallinarum* and *S. Pullorum*, which have major impacts on bird mortality. Through immunological studies, it was hypothesised that bird vaccines could be devised, and it would be possible to selectively breed disease resistant birds (Calenge *et al.*, 2010). This, in turn, would ease the financial burden on the poultry industry. Another major issue for this industry is food safety, considering that commercial chickens are considered the primary vector for *C. jejuni* infection in humans. This microbe, however, was found to asymptotically colonise its avian host. Consequently, it was generally assumed that commensals did not induce immune responses. In recent times, though, discoveries in the human and murine immunology fields have shown that immune responses are required to mediate commensalism and regulate colonisation levels of commensal bacterial species, particularly at mucosal surfaces (Duerkop *et al.*, 2009). On this basis, it was hypothesised that avian defences also regulated *C. jejuni* intestinal colonisation. As there was evidence that intestinal colonisation levels in birds varied as a result of underlying genetic traits (Boyd *et al.*, 2005; Li *et al.*, 2008b), it was further hypothesised that such traits corresponded to immunological processes and thus, it may ultimately be possible to breed *C. jejuni*-resistant birds. However, such a notion would first require thorough analysis of the avian immune response to the microbe and thus, provides the rationale for this thesis.

To initially characterise the avian response, mucosal expression of key immune genes associated with microbial recognition and innate immune response were profiled in the caeca of *C. jejuni*-challenged 4-week-old chickens, across a 48 h timecourse. A similar *S. Typhimurium* infection model was used as a positive control, considering that LPS (Kogut *et al.*, 2005), flagellin (Iqbal *et al.*, 2005) and nucleic acids (Keestra *et al.*, 2010) from this microbial species have been shown to activate chicken PRRs while the live microbe is capable of inducing intestinal immune responses, at least in infant birds (Henderson *et al.*, 1999). Indeed, increased TLR, cytokine, chemokine and HDP gene expression was evident in the caeca of *S. Typhimurium*-challenged birds as early as 6 h post-challenge, and proinflammatory gene expression increases remained throughout the timecourse (Fig. 6.1 B). During

these responses, there was no evidence of histopathological changes suggesting that local caecal defences alone were sufficient to counter *S. Typhimurium*. Thus, defences appear to be finely tuned to minimise aberrant inflammation and associated pathology. *C. jejuni*, too, evaded the induction of caecal pathology although the innate immune gene expression profile was distinct from the profile induced by *S. Typhimurium*. In response to *C. jejuni*, there was no evidence of increased proinflammatory cytokine and chemokine gene expression increases in the caeca at 6 h post-challenge (Fig. 6.2 A). Furthermore, it was unclear whether Host defence peptides (HDPs) were truly increased at this interval as their expression was highly variable between individual birds. Intestinal defensins are produced primarily by the epithelium, and this layer would represent a relatively small portion of each caecal sample. As a result it was difficult to accurately compare localised HDP gene expression between challenged and unchallenged birds. At 20 h post-*C. jejuni* challenge, *IL1 β* and *K60* genes were marginally expressed in the presence of high *IL8* gene expression. At 48 h post-challenge, minor proinflammatory mediator increases were also evident while increased *IL8* gene expression was also minor. Overall, it was clear that *C. jejuni* failed to induce as active a caecal innate immune response as *S. Typhimurium*. Such differences are possibly a result of each microbe behaving differently in the caecal environment. *S. Typhimurium* may preferentially invade intestinal tissues as the microbe is often isolated from the lamina propria (Berndt *et al.*, 2007; Henderson *et al.*, 1999). On the basis of findings from mammalian *S. Typhimurium* infections, replication is likely to take place within the lamina propria (Bedoui *et al.*, 2010). As a result, *S. Typhimurium* would readily activate PRRs of the basolateral epithelial surface and of residential lamina propria leukocytes therefore, explaining increased proinflammatory cytokine and chemokine gene expression. In contrast, evidence suggests that *C. jejuni* primarily resides within caecal crypts, where it has clearly adapted to replicate in the mucus, considering that it struggles for survival in the underlying epithelial cells (Van Deun *et al.*, 2007). Therefore, *C. jejuni* may not normally invade the caecal epithelium. If so, inflammatory responses would be minimal, as the apical surface of intestinal epithelia tend to lack TLR expression (Abreu, 2010). High *IL8* gene expression at 20 h post-challenge, however, indicated invasion of the caecal epithelium (Fig. 6.2 B), considering that chicken epithelial cells upregulate this chemokine in response to *C. jejuni* invasion *in vitro* (Borrmann *et al.*, 2007; Li *et al.*, 2008c). *C. jejuni* has a range of virulence factors associated with

invasion during mammalian infections (Young *et al.*, 2007). It is possible that such virulence machinery is still active during the early stages of colonisation and may represent a *C. jejuni* survival strategy. As the microbial population increases in the mucus layer, essential nutrients required for replication would become less available. As a result *C. jejuni* may instinctively invade to search for further nutrients. This is likely to only be transient as *IL8* gene expression was much reduced at 48 h post-challenge (Fig. 6.2 C). At this interval, *C. jejuni* may have downregulated its virulence components to assume a more commensal lifestyle. On the other hand, increased *IL8* gene expression may also be somewhat a reflection of M-cell-mediated sampling of luminal *C. jejuni* as increased gene expression for this chemokine was upregulated in caecal tonsil samples all throughout the timecourse. Such increases are probably mediated by DCs, considering that they appear to be the predominant innate cell in caecal GALT (Casteleyn *et al.*, 2010; del Cacho *et al.*, 1993).

While there was clearly a lack of a typical innate immune response to *C. jejuni*, there was also the possibility that other immunological processes were being activated, particularly those that would not traditionally be considered key to innate defences. Furthermore, it was also possible that cytokine responses were being suppressed by unknown regulatory processes. To explore such possibilities, global caecal gene expression changes in response to *C. jejuni* at the 20 h interval was characterised using whole-genome microarrays. Few immunological processes were identified to be activated using this non-hypothesis driven approach. Validation only revealed weak evidence of oxidative burst and endothelial activation. Similarly, another whole-genome microarray based study revealed a lack of differentially expressed immune genes in the caeca of birds 7 days post *C. jejuni*-challenge (Li *et al.*, 2010). Microarray-based analysis, therefore, appeared to confirm a lack of immunological activity towards the microbe. However, the reliability of the data came under question. Based on validation, at least 50% of the differentially expressed genes were false-positives while the absence of differentially expressed *IL8*, *K60* and *IL1 β* genes amongst the array data, which had been demonstrated to be increased using qRT-PCR, signified the occurrence of false-negatives. Thus, the array data may not be an accurate representation of global caecal transcriptional changes in response to *C. jejuni*. This was largely a result of technical noise, as significant findings differed between normalization methods. Nevertheless, between the different normalization approaches, it was possible to validate increased *IL17*, *IL21* and *TOPK* gene

expression increases, collectively an indication of T cell activity. Thus, microarray technology failed to accurately identify complete global transcriptional changes in response to *C. jejuni* colonisation but proved somewhat useful for gene fishing. Further qRT-PCR analysis of T cell genes revealed increased *KK34*, *CTLA4* and *IL7R α* gene expression. It is also plausible that T cell responses might be specifically activated in response to *C. jejuni*, considering that the caecal lamina propria contains innate $\gamma\delta$ T cell populations that are active against bacterial species (Berndt *et al.*, 2006). While it is also possible that minor T cell gene expression increases are attributed to biological noise, considering that the caecal lamina propria contains numerous lymphoid aggregates and activated T cells (Kitagawa *et al.*, 1998). Thus, future studies are clearly warranted to confirm and characterise caecal T cell responses are initiated against *C. jejuni* in the early stages of colonisation.

While innate immune gene expression profiling and microarray analysis collectively demonstrated minimal proinflammatory activity towards *C. jejuni* in the caecum, these *in vivo* analyses failed to explain the observed commensal state. On this basis, an *in vitro* approach was taken to explore reasons for the lack of innate immune response. From an immunological perspective, the most fundamental interactions between microbe and host involve specific microbial components initiating proinflammatory responses in specific cell types. In this regard, the ability of *C. jejuni*-specific capsular polysaccharide (CPS), lipooligosaccharide (LOS) and cytolethal distending toxin (CDT) to induce inflammatory gene expression in chicken cells was assessed. As expected, CPS was confirmed to be immunologically inert (Fig. 6.3). Considering that this component forms the outermost microbial layer, with intact *C. jejuni*, the underlying LOS and lipoproteins would be shielded from PRRs of the avian intestine, resulting in reduced immunopathology. Indeed, it has been documented that capsulated bacteria are poor inducers of proinflammatory responses *in vivo* (Wilson *et al.*, 2008; Wu *et al.*, 2009a). There is, however, the possibility of capsular and acapsular phases with *C. jejuni*. The microbe may transiently shed its capsule during human infection, as the underlying LOS is required for efficient invasion of human epithelial cells (Fry *et al.*, 2000). In contrast, efficient *C. jejuni* colonisation of the avian intestine requires CPS (Jones *et al.*, 2004), but not LOS (Fry *et al.*, 2000), which suggests that the microbe is predominantly capsulated in its chicken host. Such differences in *C. jejuni* capsular expression between human and

chicken hosts may explain the stark differences in immunopathology between the two hosts (Fig. 6.4) although future studies are clearly required to clarify this notion.

Studying the responses of chicken cells to the LOS component yielded the most important findings. Ultrapure LOS from *C. jejuni* grown at 37°C induced significant proinflammatory gene expression increases in chicken macrophages. de Zoete et al made similar observations, and it has recently been determined that *C. jejuni* LOS is recognised by chicken TLR4 (de Zoete *et al.*, 2010a; Kuijf *et al.*, 2010; van Mourik *et al.*, 2010). Thus, there has been a recent interest in understanding how the avian host responds to this component. With regard to this, it has recently been discovered by our collaborators that shifting the *C. jejuni* growth temperature from 37°C to 42°C results in structural changes to the LOS component. The latter temperature is representative of the internal environment of birds and thus, human and chicken intestinal PRRs may each encounter a different LOS variant. Furthermore, chicken defences would not encounter the 37°C-LOS variant. Nevertheless, we can confirm that the LOS component of *C. jejuni* is potentially a PAMP during chicken colonisation as the 42°C variant also induced substantial proinflammatory gene expression increases in chicken macrophages. However, challenging TLR4-lacking CEF cells with each LOS variant yielded intriguing results. Responses were not detected to 42°C-LOS, which confirms that TLR4 is required to initiate intestinal proinflammatory responses to this component. In contrast, 37°C-LOS induced proinflammatory gene expression increases in CEF cells, indicating TLR4-independent recognition. Thus, there is the possibility that this LOS variant is being recognised by TLR4 and an additional PRR (Fig. 6.3). This is not unusual, considering that other microbial ligands such flagellin and nucleic acids are each recognised by more than one PRR (Vance *et al.*, 2009). The LPS component of *Helicobacter pylori* is possibly activated by both TLR2 and TLR4 in humans (Kawahara *et al.*, 2001; Yokota *et al.*, 2007). More important, is the fact that 42°C-*C. jejuni* LOS only activates TLR4. *C. jejuni* thus appears to have evolved within its avian host to avoid recognition by the alternative receptor. Possibly, this receptor is expressed throughout the avian intestine, and particularly on the apical epithelium. Such a scenario would explain the lack of intestinal proinflammatory responses to *C. jejuni*, especially when one considers that the microbe also evades TLR5 detection (de Zoete *et al.*, 2010a; de Zoete *et al.*, 2010b). While TLR4 would potentially recognise the LOS component, it is unclear whether such activation would occur in the intestine.

On the basis of mammalian studies, it is probable that TLR4 is absent from the apical epithelium (Abreu, 2010). Thus, TLR4 would be activated by *C. jejuni* invasion, but not in response to caecal crypt colonisation alone. TLR15-related data served as further indirect evidence that TLR4 was not activated during colonisation. *TLR15* gene expression was upregulated in chicken macrophages directly in response to TLR4 activation, and not indirectly in response to proinflammatory mediators. Therefore, *TLR15* upregulation, at least, gives an indication of TLR4 activation. From an intestinal perspective, this also appears to be the case, as caecal *TLR15* upregulation is evident in response to gram-negative *Salmonella* challenges (Higgs *et al.*, 2006; Mackinnon *et al.*, 2009). In this thesis, caecal *TLR15* was upregulated in response to *S. Typhimurium* but not to *C. jejuni*. Thus, it can indirectly be inferred that minimal TLR4 activation occurs as a result of colonisation by the latter bacterial species.

Cytolethal distending toxin is associated with pathogenic bacterial species and is considered a key *C. jejuni* virulence component (Ge *et al.*, 2008). This toxin has previously been shown to induce IL-8 in human epithelial cells (Hickey *et al.*, 2000), and was therefore considered a potential immunostimulatory *C. jejuni* component. CDT, however, failed to induce proinflammatory gene expression increases in chicken macrophages and fibroblasts, which indicated that the toxin was not recognised by avian PRRs. On this basis, CDT would not directly induce proinflammatory responses in the avian intestine. This also appears to be the case with human infections, as we observed minimal proinflammatory gene expression increases in human THP1 monocytes challenged with the toxin. Such cells are equipped to deal with a range of bacterial PAMPS and thus, would likely respond to CDT, should the toxin be immunostimulatory. Furthermore, in the past ten years, there have been no reports of cytokine induction in human cells in response to *C. jejuni* CDT. Regarding other toxin producing bacterial species, there is very little *in vitro* evidence of CDT-mediated cytokine induction (Smith & Bayles, 2006) except for *Actinobacillus actinomycetemcomitans* CDT, which has been shown to induce IL-1 β , IL-6 and IL-8 in PBMCs (Akifusa *et al.*, 2001). Thus, CDT is probably not recognised by avian or mammalian PRRs. It is, however, possible that the toxin indirectly contributes to intestinal proinflammatory responses, on the basis of its cytotoxic effects. CDT causes cell cycle arrest through the induction of DNA damage (Ge *et al.*, 2008). As a result, the toxin causes death in cells such as enterocytes

(Whitehouse *et al.*, 1998) and fibroblasts (Hassane *et al.*, 2003). Destruction of such cell types in an intestinal setting would facilitate the movement of *C. jejuni* across the epithelium and through the lamina propria, increasing microbial contact with intestinal PRRs (Fig. 6.4). Indeed, there is evidence to suggest that this is the case in mammalian infections. CDT-deficient *C. jejuni* colonise the intestines of severe combined immunodeficient (SCID) mice, but are less efficient at disseminating to further tissues (Purdy *et al.*, 2000), while the microbe's toxin is associated with increased *C. jejuni*-colonisation and gastric lesion formation in NF- κ B deficient mice (Fox *et al.*, 2004). Furthermore, CDT from a related bacterial species, *Helicobacter hepaticus*, is associated with the enhancement of colitis in IL-10^{-/-} mice (Pratt *et al.*, 2006). Considering that such evidence of pathology was lacking in the caeca of *C. jejuni* colonised birds we were very surprised to discover that chicken macrophages were also susceptible to CDT-mediated cytotoxic effects. At 48 h post-CDT challenge, about 80% of cells had undergone programmed cell death. This result demonstrates that *C. jejuni* CDT is capable of entering chicken cells and subsequently inducing chromosomal damage within. However, on the basis of several studies, it is unlikely that *C. jejuni* produces CDT during chicken colonisation (Fig 6.4 B). Colonisation abilities do not differ between wild-type and CDT-mutant *C. jejuni* while clinical signs are absent to both strains (Abuoun *et al.*, 2005; Biswas *et al.*, 2006). Furthermore, birds generate specific antibodies to multiple *C. jejuni* components, but not to CDT (Abuoun *et al.*, 2005; Sahin *et al.*, 2001; Shoaf-Sweeney *et al.*, 2008). In contrast, CDT-specific antibodies are readily detected in sera samples of human campylobacteriosis patients (Abuoun *et al.*, 2005). Thus, it seems that *C. jejuni* produces CDT during human infection, but not during chicken colonisation (Fig. 6.4 A and B).

In conclusion, *C. jejuni* activates minimal immunological processes at its primary site of intestinal colonisation. Key proinflammatory cytokines, chemokines and HDPs associated with innate immune response and bacterial clearance are lacking and this is reflected by histological assessment, in which there are no signs or aberrant inflammation or leukocyte influxes. Global gene expression analysis further reveals minimal immunological activity although indirectly, there is evidence of innate T cell activity. Thus, there is the possibility that innate T cell responses may regulate *C. jejuni* colonisation although further studies are required to confirm such processes. The lack of intestinal immunopathology is likely due to the structure and behaviour of

the microbe. Capsulated *C. jejuni* are not likely to activate intestinal PRRs as this outermost layer is immunologically inert. The LOS component activates TLR4, but may evade and additional PRR. CDT is not a PAMP, but chicken cells are susceptible to its cytotoxic effects. The fact that cytotoxicity is not evident *in vivo* suggests that *C. jejuni* exhibits a commensal lifestyle during caecal colonisation. Whether birds have evolved immune systems which evade the effects of *C. jejuni* or whether *C. jejuni* has evolved in order to survive as a commensal in its avian host will require detailed interactive studies.

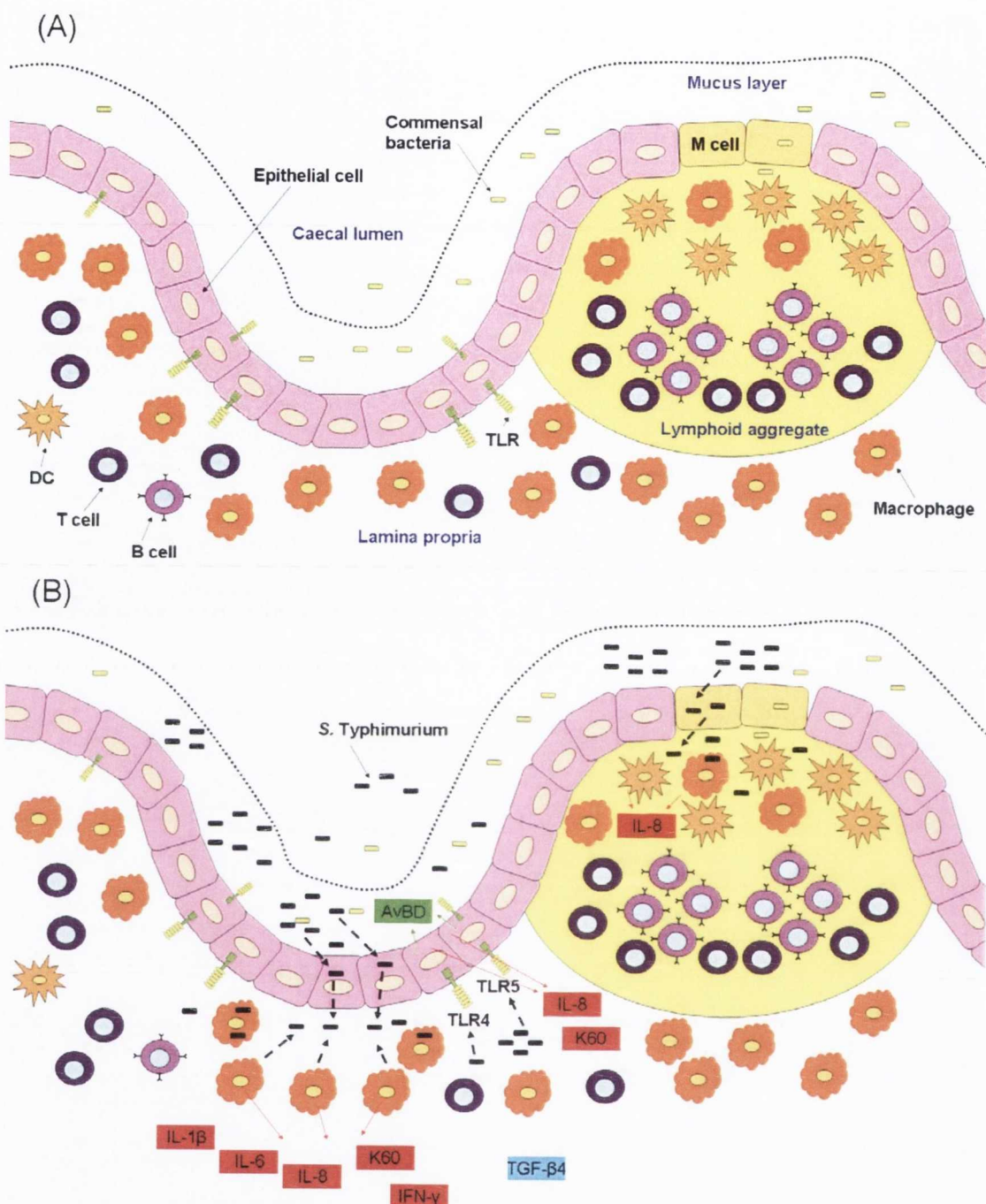


Figure 6.1 The caecal immune response to *S. Typhimurium* (A) the caecum of healthy unchallenged birds. TLRs are likely to predominantly be expressed on the basolateral surface. The lamina propria is rich in phagocytes and activated lymphocytes while adjoining lymphoid aggregates are rich in dendritic cells and lymphocytes. (B) *S. Typhimurium* induces gene expression for a range of AvBDs, cytokines and chemokines possibly as a result of invasion or mucosal sampling. Leukocyte recruitment is minimal. Thus, residential macrophages in the lamina are likely to defend against the microbe.

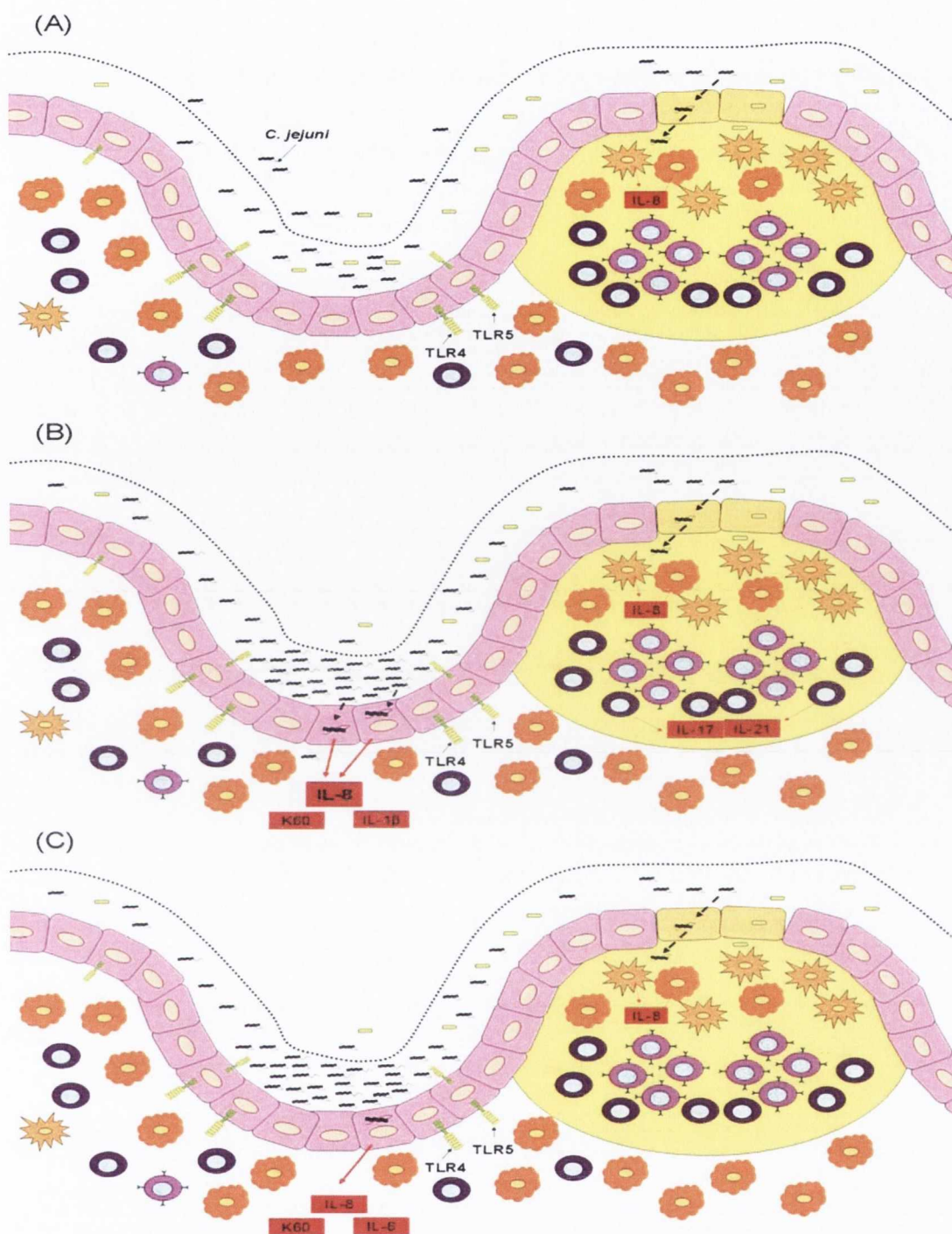


Figure 6.2 The caecal response to *C. jejuni* colonisation. **(A)** At 6 h, *C. jejuni* begins to multiply in the caecal mucus and has minimal interactions with the caecal epithelium. Thus, proinflammatory responses are avoided although mucosal sampling of *C. jejuni* takes place, resulting in IL-8 production by underlying leukocytes. **(B)** At 20 h, limited nutrients in the mucus layer may force *C. jejuni* to invade the epithelium as caecal proinflammatory gene expression increases are evident. Most notably, *IL8* gene expression increases are relatively high while microarray analysis suggests

innate T cell activity. The latter is possibly initiated as a result of mucosal sampling. (C) At 48 h post-challenge, *IL8* gene expression is much reduced while *K60* and *IL6* gene expression increases are evident, but only minor. Possibly, *C. jejuni* has adapted to the mucus layer at this interval, resulting in minimal invasion and thus, avoiding cytokine induction.

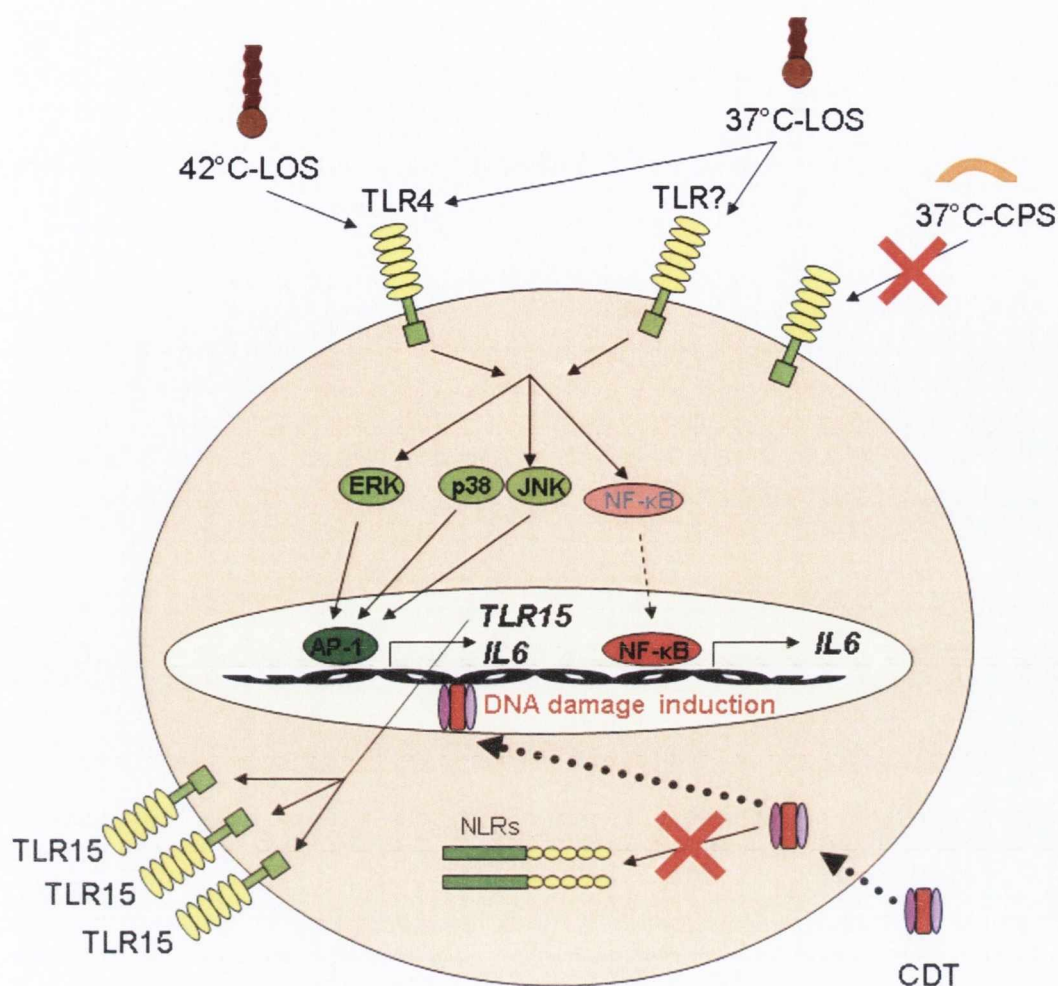


Figure 6.3 Chicken cell responses to individual *C. jejuni* components. CPS from *C. jejuni* grown at 37°C is not recognised by avian PRRs. TLR4 is required for the induction of proinflammatory gene expression in response to the 42°C. The 37°C LOS variant signals through TLR4 and possibly through an additional unidentified TLR. NF-κB and MAPK are activated to this LOS variant, resulting in cytokine gene expression upregulation. The latter pathway gives rise to TLR15 upregulation, at least at the mRNA level. CDT does not appear to be recognised by avian PRRs although chicken cells are susceptible to CDT-mediated cytotoxicity.

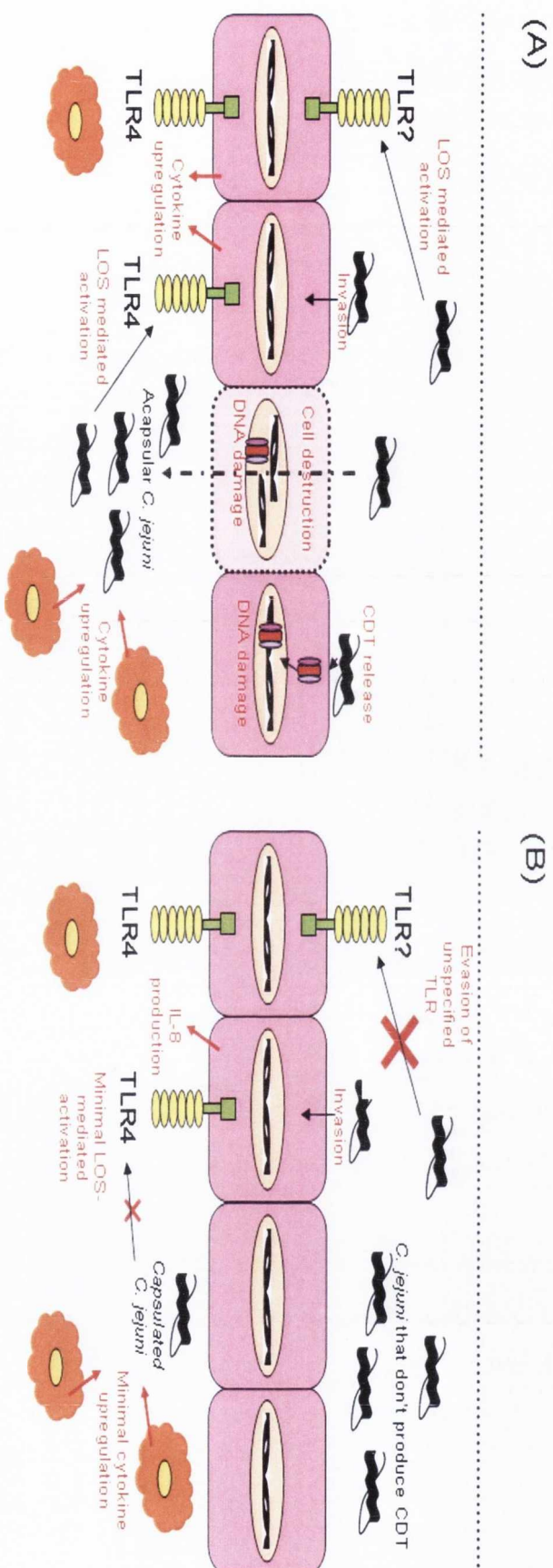


Figure 6.4 Hypothetical differences between *C. jejuni* infection in humans and *C. jejuni* colonisation in birds. (A) During human infection, *C. jejuni* invades and destabilises the intestinal epithelium. The latter occurrence may be partially mediated by CDT. Epithelial cell death would enhance access of *C. jejuni* to the lamina propria, placing the microbe in contact with residential leukocytes and PRRs of the basolateral epithelium. Should *C. jejuni* be acapsular during this phase, more PRR activation would occur. The LOS component would activate TLR4 and possibly another unspecified PRR. As a result a range of proinflammatory mediators are produced resulting in leukocyte influxes. (B) During chicken colonisation, *C. jejuni* exhibits a commensal lifestyle, residing primarily in intestinal mucus while CDT is not produced. Invasion of the epithelium may transiently occur resulting in the production of IL-8. *C. jejuni* that enter the lamina propria may potentially activate TLR4 via the 42°C-LOS variant although should the microbe be capsulated, PRR activation would be minimal.

Chapter 7

References

Bibliography

- Abasht, B., Kaiser, M. G. & Lamont, S. J. (2008).** Toll-like receptor gene expression in cecum and spleen of advanced intercross line chicks infected with *Salmonella enterica* serovar Enteritidis. *Vet Immunol Immunopathol* **123**, 314-323.
- Abe, Y., Matsumoto, S., Kito, K. & Ueda, N. (2000).** Cloning and expression of a novel MAPKK-like protein kinase, lymphokine-activated killer T-cell-originated protein kinase, specifically expressed in the testis and activated lymphoid cells. *J Biol Chem* **275**, 21525-21531.
- Abreu, M. T., Vora, P., Faure, E., Thomas, L. S., Arnold, E. T. & Arditi, M. (2001).** Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. *J Immunol* **167**, 1609-1616.
- Abreu, M. T. (2010).** Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nature reviews Immunology* **10**, 131-144.
- Abuoun, M., Manning, G., Cawthraw, S. A., Ridley, A., Ahmed, I. H., Wassenaar, T. M. & Newell, D. G. (2005).** Cytolethal distending toxin (CDT)-negative *Campylobacter jejuni* strains and anti-CDT neutralizing antibodies are induced during human infection but not during colonization in chickens. *Infect Immun* **73**, 3053-3062.
- Akbari, M. R., Haghighi, H. R., Chambers, J. R., Brisbin, J., Read, L. R. & Sharif, S. (2008).** Expression of antimicrobial peptides in cecal tonsils of chickens treated with probiotics and infected with *Salmonella enterica* serovar typhimurium. *Clin Vaccine Immunol* **15**, 1689-1693.
- Akifusa, S., Poole, S., Lewthwaite, J., Henderson, B. & Nair, S. P. (2001).** Recombinant *Actinobacillus actinomycetemcomitans* cytolethal distending toxin proteins are required to interact to inhibit human cell cycle progression and to stimulate human leukocyte cytokine synthesis. *Infect Immun* **69**, 5925-5930.
- Akira, S., Uematsu, S. & Takeuchi, O. (2006).** Pathogen recognition and innate immunity. *Cell* **124**, 783-801.
- Al-Salloom, F. S., Al Mahmeed, A., Ismaeel, A., Botta, G. A. & Bakhiet, M. (2003).** *Campylobacter*-stimulated INT407 cells produce dissociated cytokine profiles. *J Infect* **47**, 217-224.
- Al-Sayeqh, A. F., Loughlin, M. F., Dillon, E., Mellits, K. H. & Connerton, I. F. (2010).** *Campylobacter jejuni* activates NF-kappaB independently of TLR2, TLR4, Nod1 and Nod2 receptors. *Microb Pathog*.
- Alemka, A., Clyne, M., Shanahan, F., Tompkins, T., Corcionivoschi, N. & Bourke, B. (2010a).** Probiotic colonization of the adherent mucus layer of

HT29MTXE12 cells attenuates *Campylobacter jejuni* virulence properties. *Infect Immun* **78**, 2812-2822.

Alemka, A., Whelan, S., Gough, R., Clyne, M., Gallagher, M. E., Carrington, S. D. & Bourke, B. (2010b). Purified chicken intestinal mucin attenuates *Campylobacter jejuni* pathogenicity in vitro. *J Med Microbiol* **59**, 898-903.

Allantaz, F., Chaussabel, D., Banchereau, J. & Pascual, V. (2007). Microarray-based identification of novel biomarkers in IL-1-mediated diseases. *Curr Opin Immunol* **19**, 623-632.

Amit-Romach, E., Sklan, D. & Uni, Z. (2004). Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. *Poult Sci* **83**, 1093-1098.

Andersen-Nissen, E., Smith, K. D., Strobe, K. L., Barrett, S. L., Cookson, B. T., Logan, S. M. & Aderem, A. (2005). Evasion of Toll-like receptor 5 by flagellated bacteria. *Proc Natl Acad Sci U S A* **102**, 9247-9252.

Apajalahti, J. H., Sarkilahti, L. K., Maki, B. R., Heikkinen, J. P., Nurminen, P. H. & Holben, W. E. (1998). Effective recovery of bacterial DNA and percent-guanine-plus-cytosine-based analysis of community structure in the gastrointestinal tract of broiler chickens. *Appl Environ Microbiol* **64**, 4084-4088.

Artis, D. (2008). Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nature reviews Immunology* **8**, 411-420.

Ashgar, S. S., Oldfield, N. J., Wooldridge, K. G., Jones, M. A., Irving, G. J., Turner, D. P. & Ala'Aldeen, D. A. (2007). CapA, an autotransporter protein of *Campylobacter jejuni*, mediates association with human epithelial cells and colonization of the chicken gut. *J Bacteriol* **189**, 1856-1865.

Bachtiar, B. M., Coloe, P. J. & Fry, B. N. (2007). Knockout mutagenesis of the *kpsE* gene of *Campylobacter jejuni* 81116 and its involvement in bacterium-host interactions. *FEMS Immunol Med Microbiol* **49**, 149-154.

Bakhiet, M., Al-Salloom, F. S., Qareiballa, A., Bindayna, K., Farid, I. & Botta, G. A. (2004). Induction of alpha and beta chemokines by intestinal epithelial cells stimulated with *Campylobacter jejuni*. *J Infect* **48**, 236-244.

Bals, R. & Wilson, J. M. (2003). Cathelicidins--a family of multifunctional antimicrobial peptides. *Cell Mol Life Sci* **60**, 711-720.

Bar-Shira, E. & Friedman, A. (2006). Development and adaptations of innate immunity in the gastrointestinal tract of the newly hatched chick. *Dev Comp Immunol* **30**, 930-941.

Barker, K. A., Hampe, A., Stoeckle, M. Y. & Hanafusa, H. (1993). Transformation-associated cytokine 9E3/CEF4 is chemotactic for chicken peripheral blood mononuclear cells. *J Virol* **67**, 3528-3533.

- Barrett, K. E. (2008).** New ways of thinking about (and teaching about) intestinal epithelial function. *Adv Physiol Educ* **32**, 25-34.
- Bedoui, S., Kupz, A., Wijburg, O. L., Walduck, A. K., Rescigno, M. & Strugnell, R. A. (2010).** Different bacterial pathogens, different strategies, yet the aim is the same: evasion of intestinal dendritic cell recognition. *J Immunol* **184**, 2237-2242.
- Beery, J. T., Hugdahl, M. B. & Doyle, M. P. (1988).** Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Appl Environ Microbiol* **54**, 2365-2370.
- Beltinger, J., del Buono, J., Skelly, M. M., Thornley, J., Spiller, R. C., Stack, W. A. & Hawkey, C. J. (2008).** Disruption of colonic barrier function and induction of mediator release by strains of *Campylobacter jejuni* that invade epithelial cells. *World J Gastroenterol* **14**, 7345-7352.
- Berndt, A., Pieper, J. & Methner, U. (2006).** Circulating gamma delta T cells in response to *Salmonella enterica* serovar enteritidis exposure in chickens. *Infect Immun* **74**, 3967-3978.
- Berndt, A., Wilhelm, A., Jugert, C., Pieper, J., Sachse, K. & Methner, U. (2007).** Chicken cecum immune response to *Salmonella enterica* serovars of different levels of invasiveness. *Infect Immun* **75**, 5993-6007.
- Bhave, N. S. & Carson, W. E., 3rd (2009).** Immune modulation with interleukin-21. *Ann N Y Acad Sci* **1182**, 39-46.
- Biswas, D., Fernando, U., Reiman, C., Willson, P., Potter, A. & Allan, B. (2006).** Effect of cytolethal distending toxin of *Campylobacter jejuni* on adhesion and internalization in cultured cells and in colonization of the chicken gut. *Avian Dis* **50**, 586-593.
- Bjerrum, L., Engberg, R. M., Leser, T. D., Jensen, B. B., Finster, K. & Pedersen, K. (2006).** Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. *Poult Sci* **85**, 1151-1164.
- Black, R. E., Levine, M. M., Clements, M. L., Hughes, T. P. & Blaser, M. J. (1988).** Experimental *Campylobacter jejuni* infection in humans. *J Infect Dis* **157**, 472-479.
- Blaser, M. J. & Duncan, D. J. (1984).** Human serum antibody response to *Campylobacter jejuni* infection as measured in an enzyme-linked immunosorbent assay. *Infect Immun* **44**, 292-298.
- Bliss, T. W., Dohms, J. E., Emara, M. G. & Keeler, C. L., Jr. (2005).** Gene expression profiling of avian macrophage activation. *Vet Immunol Immunopathol* **105**, 289-299.

- Borrmann, E., Berndt, A., Hanel, I. & Kohler, H. (2007).** Campylobacter-induced interleukin-8 responses in human intestinal epithelial cells and primary intestinal chick cells. *Veterinary microbiology* **124**, 115-124.
- Boyd, A., Philbin, V. J. & Smith, A. L. (2007).** Conserved and distinct aspects of the avian Toll-like receptor (TLR) system: implications for transmission and control of bird-borne zoonoses. *Biochem Soc Trans* **35**, 1504-1507.
- Boyd, Y., Herbert, E. G., Marston, K. L., Jones, M. A. & Barrow, P. A. (2005).** Host genes affect intestinal colonisation of newly hatched chickens by *Campylobacter jejuni*. *Immunogenetics* **57**, 248-253.
- Bras, A. M. & Ketley, J. M. (1999).** Transcellular translocation of *Campylobacter jejuni* across human polarised epithelial monolayers. *FEMS Microbiol Lett* **179**, 209-215.
- Breitling, R. (2006).** Biological microarray interpretation: the rules of engagement. *Biochim Biophys Acta* **1759**, 319-327.
- Brogden, K. A. (2005).** Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol* **3**, 238-250.
- Brown, K. L. & Hancock, R. E. (2006).** Cationic host defense (antimicrobial) peptides. *Curr Opin Immunol* **18**, 24-30.
- Brownlie, R., Zhu, J., Allan, B., Mutwiri, G. K., Babiuk, L. A., Potter, A. & Griebel, P. (2009).** Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. *Mol Immunol*.
- Byrne, C. M., Clyne, M. & Bourke, B. (2007).** *Campylobacter jejuni* adhere to and invade chicken intestinal epithelial cells in vitro. *Microbiology* **153**, 561-569.
- Calenge, F., Kaiser, P., Vignal, A. & Beaumont, C. (2010).** Genetic control of resistance to salmonellosis and to *Salmonella* carrier-state in fowl: a review. *Genet Sel Evol* **42**, 11.
- Cardoso, L. S., Araujo, M. I., Goes, A. M., Pacifico, L. G., Oliveira, R. R. & Oliveira, S. C. (2007).** Polymyxin B as inhibitor of LPS contamination of *Schistosoma mansoni* recombinant proteins in human cytokine analysis. *Microb Cell Fact* **6**, 1.
- Carneiro, L. A., Magalhaes, J. G., Tattoli, I., Philpott, D. J. & Travassos, L. H. (2008).** Nod-like proteins in inflammation and disease. *J Pathol* **214**, 136-148.
- Carpenter, S. & O'Neill, L. A. (2007).** How important are Toll-like receptors for antimicrobial responses? *Cell Microbiol* **9**, 1891-1901.
- Casadevall, A. & Pirofski, L. A. (2003).** The damage-response framework of microbial pathogenesis. *Nat Rev Microbiol* **1**, 17-24.

- Cassatella, M. A., Meda, L., Gasperini, S., Calzetti, F. & Bonora, S. (1994).** Interleukin 10 (IL-10) upregulates IL-1 receptor antagonist production from lipopolysaccharide-stimulated human polymorphonuclear leukocytes by delaying mRNA degradation. *J Exp Med* **179**, 1695-1699.
- Casteleyn, C., Doom, M., Lambrechts, E., Van den Broeck, W., Simoens, P. & Cornillie, P. (2010).** Locations of gut-associated lymphoid tissue in the 3-month-old chicken: a review. *Avian Pathol* **39**, 143-150.
- Cawthraw, S. A., Feldman, R. A., Sayers, A. R. & Newell, D. G. (2002).** Long-term antibody responses following human infection with *Campylobacter jejuni*. *Clin Exp Immunol* **130**, 101-106.
- Cerutti, A. & Rescigno, M. (2008).** The biology of intestinal immunoglobulin A responses. *Immunity* **28**, 740-750.
- Chappell, L., Kaiser, P., Barrow, P., Jones, M. A., Johnston, C. & Wigley, P. (2009).** The immunobiology of avian systemic salmonellosis. *Vet Immunol Immunopathol* **128**, 53-59.
- Chen, M. L., Ge, Z., Fox, J. G. & Schauer, D. B. (2006).** Disruption of tight junctions and induction of proinflammatory cytokine responses in colonic epithelial cells by *Campylobacter jejuni*. *Infect Immun* **74**, 6581-6589.
- Cheroutre, H. & Madakamutil, L. (2004).** Acquired and natural memory T cells join forces at the mucosal front line. *Nature reviews* **4**, 290-300.
- Cheroutre, H. (2005).** IELs: enforcing law and order in the court of the intestinal epithelium. *Immunol Rev* **206**, 114-131.
- Chiang, H. I., Swaggerty, C. L., Kogut, M. H., Dowd, S. E., Li, X., Pevzner, I. Y. & Zhou, H. (2008).** Gene expression profiling in chicken heterophils with *Salmonella enteritidis* stimulation using a chicken 44 K Agilent microarray. *BMC genomics* **9**, 526.
- Christensen, J. E., Pacheco, S. A. & Konkel, M. E. (2009).** Identification of a *Campylobacter jejuni*-secreted protein required for maximal invasion of host cells. *Mol Microbiol* **73**, 650-662.
- Churchill, G. A. (2002).** Fundamentals of experimental design for cDNA microarrays. *Nat Genet* **32 Suppl**, 490-495.
- Coats, S. R., Do, C. T., Karimi-Naser, L. M., Braham, P. H. & Darveau, R. P. (2007).** Antagonistic lipopolysaccharides block *E. coli* lipopolysaccharide function at human TLR4 via interaction with the human MD-2 lipopolysaccharide binding site. *Cell Microbiol* **9**, 1191-1202.
- Coombes, J. L. & Powrie, F. (2008).** Dendritic cells in intestinal immune regulation. *Nature reviews* **8**, 435-446.

- Corcoran, A. T., Annuk, H. & Moran, A. P. (2006).** The structure of the lipid anchor of *Campylobacter jejuni* polysaccharide. *FEMS Microbiol Lett* **257**, 228-235.
- Corr, S. C., Gahan, C. C. & Hill, C. (2008).** M-cells: origin, morphology and role in mucosal immunity and microbial pathogenesis. *FEMS Immunol Med Microbiol* **52**, 2-12.
- Corry, J. E. & Atabay, H. I. (2001).** Poultry as a source of *Campylobacter* and related organisms. *Symp Ser Soc Appl Microbiol*, 96S-114S.
- Cortes-Bratti, X., Karlsson, C., Lagergard, T., Thelestam, M. & Frisan, T. (2001).** The *Haemophilus ducreyi* cytolethal distending toxin induces cell cycle arrest and apoptosis via the DNA damage checkpoint pathways. *J Biol Chem* **276**, 5296-5302.
- Cosseau, C., Devine, D. A., Dullaghan, E. & other authors (2008).** The commensal *Streptococcus salivarius* K12 downregulates the innate immune responses of human epithelial cells and promotes host-microbe homeostasis. *Infect Immun* **76**, 4163-4175.
- Cox, N. A., Hofacre, C. L., Bailey, J. S. & other authors (2005).** Presence of *Campylobacter jejuni* in various organs one hour, one day, and one week following oral or intracloacal inoculations of broiler chicks. *Avian Dis* **49**, 155-158.
- Cox, N. A., Richardson, L. J., Buhr, R. J. & Fedorka-Cray, P. J. (2009).** *Campylobacter* species occurrence within internal organs and tissues of commercial caged Leghorn laying hens. *Poult Sci* **88**, 2449-2456.
- Crushell, E., Harty, S., Sharif, F. & Bourke, B. (2004).** Enteric campylobacter: purging its secrets? *Pediatr Res* **55**, 3-12.
- Cullen, T. W. & Trent, M. S. (2010).** A link between the assembly of flagella and lipooligosaccharide of the Gram-negative bacterium *Campylobacter jejuni*. *Proc Natl Acad Sci U S A* **107**, 5160-5165.
- Dalpke, A., Frank, J., Peter, M. & Heeg, K. (2006).** Activation of toll-like receptor 9 by DNA from different bacterial species. *Infect Immun* **74**, 940-946.
- de Souza, C. P. (2003).** Pathogenicity mechanisms of prokaryotic cells: an evolutionary view. *Braz J Infect Dis* **7**, 23-31.
- de Zoete, M. R., Kestra, A. M., Roszczenko, P. & van Putten, J. P. (2010a).** Activation of human and chicken toll-like receptors by *Campylobacter* spp. *Infect Immun* **78**, 1229-1238.
- de Zoete, M. R., Kestra, A. M., Wagenaar, J. A. & van Putten, J. P. (2010b).** Reconstitution of a functional Toll-like receptor 5 binding site in *Campylobacter jejuni* flagellin. *J Biol Chem* **285**, 12149-12158.
- del Cacho, E., Gallego, M., Sanz, A. & Zapata, A. (1993).** Characterization of distal lymphoid nodules in the chicken caecum. *The Anatomical record* **237**, 512-517.

- Del Cacho, E., Gallego, M., Lillehoj, H. S., Lopez-Bernard, F. & Sanchez-Acedo, C. (2009).** Avian follicular and interdigitating dendritic cells: Isolation and morphologic, phenotypic, and functional analyses. *Vet Immunol Immunopathol* **129**, 66-75.
- Dheda, K., Huggett, J. F., Chang, J. S., Kim, L. U., Bustin, S. A., Johnson, M. A., Rook, G. A. & Zumla, A. (2005).** The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. *Anal Biochem* **344**, 141-143.
- Dobrindt, U., Agerer, F., Michaelis, K. & other authors (2003).** Analysis of genome plasticity in pathogenic and commensal *Escherichia coli* isolates by use of DNA arrays. *J Bacteriol* **185**, 1831-1840.
- Dudoit, S., Yang, Y. H., Callow, M. J. & Speed, T. (2000).** Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments.
- Duerkop, B. A., Vaishnava, S. & Hooper, L. V. (2009).** Immune responses to the microbiota at the intestinal mucosal surface. *Immunity* **31**, 368-376.
- Duong, T. & Konkel, M. E. (2009).** Comparative studies of *Campylobacter jejuni* genomic diversity reveal the importance of core and dispensable genes in the biology of this enigmatic food-borne pathogen. *Curr Opin Biotechnol*.
- Eddy, S. F. & Storey, K. B. (2008).** Comparative molecular physiological genomics. Heterologous probing of cDNA arrays. *Methods in molecular biology (Clifton, NJ)* **410**, 81-110.
- Edwards, R. J., Taylor, G. W., Ferguson, M. & other authors (2005).** Specific C-terminal cleavage and inactivation of interleukin-8 by invasive disease isolates of *Streptococcus pyogenes*. *J Infect Dis* **192**, 783-790.
- Erridge, C., Kennedy, S., Spickett, C. M. & Webb, D. J. (2008).** Oxidized phospholipid inhibition of toll-like receptor (TLR) signaling is restricted to TLR2 and TLR4: roles for CD14, LPS-binding protein, and MD2 as targets for specificity of inhibition. *J Biol Chem* **283**, 24748-24759.
- Escoffier, C. & Gerlier, D. (1999).** Infection of chicken embryonic fibroblasts by measles virus: adaptation at the virus entry level. *Journal of virology* **73**, 5220-5224.
- Fasina, Y. O., Holt, P. S., Moran, E. T., Moore, R. W., Conner, D. E. & McKee, S. R. (2008).** Intestinal cytokine response of commercial source broiler chicks to *Salmonella typhimurium* infection. *Poult Sci* **87**, 1335-1346.
- Fathallah-Shaykh, H. M. (2005).** Microarrays: applications and pitfalls. *Arch Neurol* **62**, 1669-1672.

Fiocchi, C. (2003). More answers and more questions in inflammatory bowel disease. *Curr Opin Gastroenterol* **19**, 325-326.

Flannagan, R. S., Cosio, G. & Grinstein, S. (2009). Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat Rev Microbiol* **7**, 355-366.

Forster, R., Pabst, O. & Bernhardt, G. (2008). Homeostatic chemokines in development, plasticity, and functional organization of the intestinal immune system. *Semin Immunol* **20**, 171-180.

Foster, S. L., Hargreaves, D. C. & Medzhitov, R. (2007). Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* **447**, 972-978.

Fox, J. G., Rogers, A. B., Whary, M. T., Ge, Z., Taylor, N. S., Xu, S., Horwitz, B. H. & Erdman, S. E. (2004). Gastroenteritis in NF-kappaB-deficient mice is produced with wild-type *Campylobacter jejuni* but not with *C. jejuni* lacking cytolethal distending toxin despite persistent colonization with both strains. *Infect Immun* **72**, 1116-1125.

Friend, S. H. & Stoughton, R. B. (2002). The magic of microarrays. *Sci Am* **286**, 44-49, 53.

Friis, L. M., Keelan, M. & Taylor, D. E. (2009). *Campylobacter jejuni* drives MyD88-independent interleukin-6 secretion via Toll-like receptor 2. *Infect Immun* **77**, 1553-1560.

Fritz, J. H., Ferrero, R. L., Philpott, D. J. & Girardin, S. E. (2006). Nod-like proteins in immunity, inflammation and disease. *Nat Immunol* **7**, 1250-1257.

Fry, B. N., Feng, S., Chen, Y. Y., Newell, D. G., Coloe, P. J. & Korolik, V. (2000). The *galE* gene of *Campylobacter jejuni* is involved in lipopolysaccharide synthesis and virulence. *Infect Immun* **68**, 2594-2601.

Fukata, M. & Abreu, M. T. (2007). TLR4 signalling in the intestine in health and disease. *Biochem Soc Trans* **35**, 1473-1478.

Gabay, C. & Kushner, I. (1999). Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med* **340**, 448-454.

Galkin, V. E., Yu, X., Bielnicki, J., Heuser, J., Ewing, C. P., Guerry, P. & Egelman, E. H. (2008). Divergence of quaternary structures among bacterial flagellar filaments. *Science* **320**, 382-385.

Ge, Z., Schauer, D. B. & Fox, J. G. (2008). In vivo virulence properties of bacterial cytolethal-distending toxin. *Cell Microbiol* **10**, 1599-1607.

Genin, P., Vaccaro, A. & Civas, A. (2009). The role of differential expression of human interferon- α genes in antiviral immunity. *Cytokine Growth Factor Rev* **20**, 283-295.

- Gewirtz, A. T., Navas, T. A., Lyons, S., Godowski, P. J. & Madara, J. L. (2001).** Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol* **167**, 1882-1885.
- Goitsuka, R., Chen, C. L., Benyon, L., Asano, Y., Kitamura, D. & Cooper, M. D. (2007).** Chicken cathelicidin-B1, an antimicrobial guardian at the mucosal M cell gateway. *Proc Natl Acad Sci U S A* **104**, 15063-15068.
- Gong, J., Si, W., Forster, R. J., Huang, R., Yu, H., Yin, Y., Yang, C. & Han, Y. (2007).** 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca. *FEMS Microbiol Ecol* **59**, 147-157.
- Grimm, M. C., Pullman, W. E., Bennett, G. M., Sullivan, P. J., Pavli, P. & Doe, W. F. (1995).** Direct evidence of monocyte recruitment to inflammatory bowel disease mucosa. *J Gastroenterol Hepatol* **10**, 387-395.
- Gruys, E., Toussaint, M. J., Niewold, T. A. & Koopmans, S. J. (2005).** Acute phase reaction and acute phase proteins. *Journal of Zhejiang University Science* **6**, 1045-1056.
- Guarner, F. (2006).** Enteric flora in health and disease. *Digestion* **73 Suppl 1**, 5-12.
- Haghighi, H. R., Abdul-Careem, M. F., Dara, R. A., Chambers, J. R. & Sharif, S. (2008).** Cytokine gene expression in chicken cecal tonsils following treatment with probiotics and Salmonella infection. *Veterinary microbiology* **126**, 225-233.
- Haller, D., Russo, M. P., Sartor, R. B. & Jobin, C. (2002).** IKK beta and phosphatidylinositol 3-kinase/Akt participate in non-pathogenic Gram-negative enteric bacteria-induced RelA phosphorylation and NF-kappa B activation in both primary and intestinal epithelial cell lines. *J Biol Chem* **277**, 38168-38178.
- Han, Y., Niu, M., An, L. & Li, W. (2009).** Upregulation of proinflammatory cytokines and NO production in BV-activated avian macrophage-like cell line (HD11) requires MAPK and NF-kappaB pathways. *Int Immunopharmacol* **9**, 817-823.
- Harder, J., Meyer-Hoffert, U., Teran, L. M., Schwichtenberg, L., Bartels, J., Maune, S. & Schroder, J. M. (2000).** Mucoid *Pseudomonas aeruginosa*, TNF-alpha, and IL-1beta, but not IL-6, induce human beta-defensin-2 in respiratory epithelia. *Am J Respir Cell Mol Biol* **22**, 714-721.
- Harder, J., Franchi, L., Munoz-Planillo, R., Park, J. H., Reimer, T. & Nunez, G. (2009).** Activation of the Nlrp3 inflammasome by *Streptococcus pyogenes* requires streptolysin O and NF-kappa B activation but proceeds independently of TLR signaling and P2X7 receptor. *J Immunol* **183**, 5823-5829.
- Harmon, B. G. (1998).** Avian heterophils in inflammation and disease resistance. *Poult Sci* **77**, 972-977.

Hassane, D. C., Lee, R. B. & Pickett, C. L. (2003). Campylobacter jejuni cytolethal distending toxin promotes DNA repair responses in normal human cells. *Infect Immun* **71**, 541-545.

Hattori, M. & Taylor, T. D. (2009). The human intestinal microbiome: a new frontier of human biology. *DNA Res* **16**, 1-12.

Hausmann, M., Kiessling, S., Mestermann, S. & other authors (2002). Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. *Gastroenterology* **122**, 1987-2000.

Hay, D. W. & Sarau, H. M. (2001). Interleukin-8 receptor antagonists in pulmonary diseases. *Curr Opin Pharmacol* **1**, 242-247.

Hayden, M. S. & Ghosh, S. (2004). Signaling to NF-kappaB. *Genes Dev* **18**, 2195-2224.

Henderson, S. C., Bounous, D. I. & Lee, M. D. (1999). Early events in the pathogenesis of avian salmonellosis. *Infect Immun* **67**, 3580-3586.

Hendrixson, D. R. & DiRita, V. J. (2004). Identification of Campylobacter jejuni genes involved in commensal colonization of the chick gastrointestinal tract. *Mol Microbiol* **52**, 471-484.

Hendrixson, D. R. (2008). Restoration of flagellar biosynthesis by varied mutational events in Campylobacter jejuni. *Mol Microbiol* **70**, 519-536.

Hermiston, M. L., Xu, Z. & Weiss, A. (2003). CD45: a critical regulator of signaling thresholds in immune cells. *Annu Rev Immunol* **21**, 107-137.

Herpin, A., Lelong, C. & Favrel, P. (2004). Transforming growth factor-beta-related proteins: an ancestral and widespread superfamily of cytokines in metazoans. *Dev Comp Immunol* **28**, 461-485.

Hickey, T. E., Baqar, S., Bourgeois, A. L., Ewing, C. P. & Guerry, P. (1999). Campylobacter jejuni-stimulated secretion of interleukin-8 by INT407 cells. *Infect Immun* **67**, 88-93.

Hickey, T. E., McVeigh, A. L., Scott, D. A., Michielutti, R. E., Bixby, A., Carroll, S. A., Bourgeois, A. L. & Guerry, P. (2000). Campylobacter jejuni cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. *Infect Immun* **68**, 6535-6541.

Hickey, T. E., Majam, G. & Guerry, P. (2005). Intracellular survival of Campylobacter jejuni in human monocytic cells and induction of apoptotic death by cytolethal distending toxin. *Infect Immun* **73**, 5194-5197.

Higgs, R., Lynn, D. J., Gaines, S., McMahon, J., Tierney, J., James, T., Lloyd, A. T., Mulcahy, G. & O'Farrelly, C. (2005). The synthetic form of a novel chicken

beta-defensin identified in silico is predominantly active against intestinal pathogens. *Immunogenetics* **57**, 90-98.

Higgs, R., Cormican, P., Cahalane, S. & other authors (2006). Induction of a novel chicken Toll-like receptor following *Salmonella enterica* serovar Typhimurium infection. *Infect Immun* **74**, 1692-1698.

Higuchi, M., Matsuo, A., Shingai, M. & other authors (2008). Combinational recognition of bacterial lipoproteins and peptidoglycan by chicken Toll-like receptor 2 subfamily. *Dev Comp Immunol* **32**, 147-155.

Hill, M. J. (1997). Intestinal flora and endogenous vitamin synthesis. *Eur J Cancer Prev* **6 Suppl 1**, S43-45.

Hoffmann, E., Dittrich-Breiholz, O., Holtmann, H. & Kracht, M. (2002). Multiple control of interleukin-8 gene expression. *Journal of leukocyte biology* **72**, 847-855.

Holzl, M. A., Hofer, J., Steinberger, P., Pfistershammer, K. & Zlabinger, G. J. (2008). Host antimicrobial proteins as endogenous immunomodulators. *Immunol Lett* **119**, 4-11.

Hu, L., Bray, M. D., Osorio, M. & Kopecko, D. J. (2006). *Campylobacter jejuni* induces maturation and cytokine production in human dendritic cells. *Infect Immun* **74**, 2697-2705.

Huberman, Y. D. & Terzolo, H. R. (2008). Fowl typhoid: assessment of a disinfectant oral dose to reduce horizontal spread and mortality. *Avian Dis* **52**, 320-323.

Hughes, S. & Bumstead, N. (2000). The gene encoding the chicken chemokine K60 maps to chromosome 4. *Anim Genet* **31**, 418-419.

Hume, D. A. (2008). Macrophages as APC and the dendritic cell myth. *J Immunol* **181**, 5829-5835.

Humphrey, T., O'Brien, S. & Madsen, M. (2007). *Campylobacters* as zoonotic pathogens: a food production perspective. *International journal of food microbiology* **117**, 237-257.

Illemann, M., Hansen, U., Nielsen, H. J., Andreasen, P. A., Hoyer-Hansen, G., Lund, L. R., Dano, K. & Nielsen, B. S. (2004). Leading-edge myofibroblasts in human colon cancer express plasminogen activator inhibitor-1. *Am J Clin Pathol* **122**, 256-265.

Imai, K., Takeshita, A. & Hanazawa, S. (2000). Transforming growth factor-beta inhibits lipopolysaccharide-stimulated expression of inflammatory cytokines in mouse macrophages through downregulation of activation protein 1 and CD14 receptor expression. *Infect Immun* **68**, 2418-2423.

- Iovine, N. M., Pursnani, S., Voldman, A., Wasserman, G., Blaser, M. J. & Weinrauch, Y. (2008).** Reactive nitrogen species contribute to innate host defense against *Campylobacter jejuni*. *Infect Immun* **76**, 986-993.
- Iqbal, M., Philbin, V. J., Withanage, G. S. & other authors (2005).** Identification and functional characterization of chicken toll-like receptor 5 reveals a fundamental role in the biology of infection with *Salmonella enterica* serovar typhimurium. *Infect Immun* **73**, 2344-2350.
- Izcue, A., Coombes, J. L. & Powrie, F. (2006).** Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol Rev* **212**, 256-271.
- Janeway Charles, A. (2001).** *Immunobiology 5 : the immune system in health and disease*. New York ; Edinburgh: Garland : Churchill Livingstone.
- Janssen, R., Krogfelt, K. A., Cawthraw, S. A., van Pelt, W., Wagenaar, J. A. & Owen, R. J. (2008).** Host-pathogen interactions in *Campylobacter* infections: the host perspective. *Clin Microbiol Rev* **21**, 505-518.
- Jeurissen, S. H., Wagenaar, F. & Janse, E. M. (1999).** Further characterization of M cells in gut-associated lymphoid tissues of the chicken. *Poult Sci* **78**, 965-972.
- Jin, S., Joe, A., Lynett, J., Hani, E. K., Sherman, P. & Chan, V. L. (2001).** JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells. *Mol Microbiol* **39**, 1225-1236.
- Jin, S., Song, Y. C., Emili, A., Sherman, P. M. & Chan, V. L. (2003).** JlpA of *Campylobacter jejuni* interacts with surface-exposed heat shock protein 90alpha and triggers signalling pathways leading to the activation of NF-kappaB and p38 MAP kinase in epithelial cells. *Cell Microbiol* **5**, 165-174.
- Johanesen, P. A. & Dwinell, M. B. (2006).** Flagellin-independent regulation of chemokine host defense in *Campylobacter jejuni*-infected intestinal epithelium. *Infect Immun* **74**, 3437-3447.
- Jones, M. A., Totemeyer, S., Maskell, D. J., Bryant, C. E. & Barrow, P. A. (2003).** Induction of proinflammatory responses in the human monocytic cell line THP-1 by *Campylobacter jejuni*. *Infect Immun* **71**, 2626-2633.
- Jones, M. A., Marston, K. L., Woodall, C. A., Maskell, D. J., Linton, D., Karlyshev, A. V., Dorrell, N., Wren, B. W. & Barrow, P. A. (2004).** Adaptation of *Campylobacter jejuni* NCTC11168 to high-level colonization of the avian gastrointestinal tract. *Infect Immun* **72**, 3769-3776.
- Kaiser, P., Hughes, S. & Bumstead, N. (1999).** The chicken 9E3/CEF4 CXC chemokine is the avian orthologue of IL8 and maps to chicken chromosome 4 syntenic with genes flanking the mammalian chemokine cluster. *Immunogenetics* **49**, 673-684.

- Kaiser, P., Poh, T. Y., Rothwell, L. & other authors (2005).** A genomic analysis of chicken cytokines and chemokines. *J Interferon Cytokine Res* **25**, 467-484.
- Kaiser, P. (2007).** The avian immune genome--a glass half-full or half-empty? *Cytogenet Genome Res* **117**, 221-230.
- Kalischuk, L. D., Inglis, G. D. & Buret, A. G. (2007).** Strain-dependent induction of epithelial cell oncosis by *Campylobacter jejuni* is correlated with invasion ability and is independent of cytolethal distending toxin. *Microbiology* **153**, 2952-2963.
- Kao, C. Y., Chen, Y., Thai, P., Wachi, S., Huang, F., Kim, C., Harper, R. W. & Wu, R. (2004).** IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-kappaB signaling pathways. *J Immunol* **173**, 3482-3491.
- Kaparakis, M., Philpott, D. J. & Ferrero, R. L. (2007).** Mammalian NLR proteins; discriminating foe from friend. *Immunol Cell Biol* **85**, 495-502.
- Karlyshev, A. V., Linton, D., Gregson, N. A., Lastovica, A. J. & Wren, B. W. (2000).** Genetic and biochemical evidence of a *Campylobacter jejuni* capsular polysaccharide that accounts for Penner serotype specificity. *Mol Microbiol* **35**, 529-541.
- Kawahara, K., Tsukano, H., Watanabe, H., Lindner, B. & Matsuura, M. (2002).** Modification of the structure and activity of lipid A in *Yersinia pestis* lipopolysaccharide by growth temperature. *Infect Immun* **70**, 4092-4098.
- Kawahara, T., Teshima, S., Oka, A., Sugiyama, T., Kishi, K. & Rokutan, K. (2001).** Type I *Helicobacter pylori* lipopolysaccharide stimulates toll-like receptor 4 and activates mitogen oxidase 1 in gastric pit cells. *Infect Immun* **69**, 4382-4389.
- Kawai, T. & Akira, S. (2008).** Toll-like receptor and RIG-I-like receptor signaling. *Ann N Y Acad Sci* **1143**, 1-20.
- Kawamoto, T., Ii, M., Kitazaki, T., Iizawa, Y. & Kimura, H. (2008).** TAK-242 selectively suppresses Toll-like receptor 4-signaling mediated by the intracellular domain. *Eur J Pharmacol* **584**, 40-48.
- Keestra, A. M., de Zoete, M. R., van Aubel, R. A. & van Putten, J. P. (2008).** Functional characterization of chicken TLR5 reveals species-specific recognition of flagellin. *Mol Immunol* **45**, 1298-1307.
- Keestra, A. M., de Zoete, M. R., Bouwman, L. I. & van Putten, J. P. (2010).** Chicken TLR21 is an innate CpG DNA receptor distinct from mammalian TLR9. *J Immunol* **185**, 460-467.
- Kelly, D., Conway, S. & Aminov, R. (2005).** Commensal gut bacteria: mechanisms of immune modulation. *Trends in immunology* **26**, 326-333.

- Kelsall, B. L. & Leon, F. (2005).** Involvement of intestinal dendritic cells in oral tolerance, immunity to pathogens, and inflammatory bowel disease. *Immunol Rev* **206**, 132-148.
- Keshav, S. (2006).** Paneth cells: leukocyte-like mediators of innate immunity in the intestine. *Journal of leukocyte biology* **80**, 500-508.
- Kirimanjeswara, G. S., Agosto, L. M., Kennett, M. J., Bjornstad, O. N. & Harvill, E. T. (2005).** Pertussis toxin inhibits neutrophil recruitment to delay antibody-mediated clearance of *Bordetella pertussis*. *J Clin Invest* **115**, 3594-3601.
- Kishore, R., Tebo, J. M., Kolosov, M. & Hamilton, T. A. (1999).** Cutting edge: clustered AU-rich elements are the target of IL-10-mediated mRNA destabilization in mouse macrophages. *J Immunol* **162**, 2457-2461.
- Kitagawa, H., Hiratsuka, Y., Imagawa, T. & Uehara, M. (1998).** Distribution of lymphoid tissue in the caecal mucosa of chickens. *J Anat* **192** (Pt 2), 293-298.
- Knudsen, K. N., Bang, D. D., Andresen, L. O. & Madsen, M. (2006).** *Campylobacter jejuni* strains of human and chicken origin are invasive in chickens after oral challenge. *Avian Dis* **50**, 10-14.
- Kobayashi, S. D., Voyich, J. M., Burlak, C. & DeLeo, F. R. (2005).** Neutrophils in the innate immune response. *Arch Immunol Ther Exp (Warsz)* **53**, 505-517.
- Kocabas, C., Katsenelson, N., Kanswal, S., Kennedy, M. N., Cui, X., Blake, M. S., Segal, D. M. & Akkoyunlu, M. (2007).** *Neisseria meningitidis* type C capsular polysaccharide inhibits lipooligosaccharide-induced cell activation by binding to CD14. *Cell Microbiol* **9**, 1297-1310.
- Kogut, M. H., He, H. & Kaiser, P. (2005).** Lipopolysaccharide binding protein/CD14/TLR4-dependent recognition of salmonella LPS induces the functional activation of chicken heterophils and up-regulation of pro-inflammatory cytokine and chemokine gene expression in these cells. *Animal biotechnology* **16**, 165-181.
- Kogut, M. H., Genovese, K. J., He, H. & Kaiser, P. (2008).** Flagellin and lipopolysaccharide up-regulation of IL-6 and CXCLi2 gene expression in chicken heterophils is mediated by ERK1/2-dependent activation of AP-1 and NF-kappaB signaling pathways. *Innate Immun* **14**, 213-222.
- Konkel, M. E., Garvis, S. G., Tipton, S. L., Anderson, D. E., Jr. & Cieplak, W., Jr. (1997).** Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. *Mol Microbiol* **24**, 953-963.
- Konkel, M. E., Kim, B. J., Rivera-Amill, V. & Garvis, S. G. (1999).** Bacterial secreted proteins are required for the internalization of *Campylobacter jejuni* into cultured mammalian cells. *Mol Microbiol* **32**, 691-701.

- Konkel, M. E., Larson, C. L. & Flanagan, R. C. (2010).** Campylobacter jejuni FlpA binds fibronectin and is required for maximal host cell adherence. *J Bacteriol* **192**, 68-76.
- Krisanaprakornkit, S., Kimball, J. R. & Dale, B. A. (2002).** Regulation of human beta-defensin-2 in gingival epithelial cells: the involvement of mitogen-activated protein kinase pathways, but not the NF-kappaB transcription factor family. *J Immunol* **168**, 316-324.
- Kuijf, M. L., Samsom, J. N., van Rijs, W. & other authors (2010).** TLR4-mediated sensing of Campylobacter jejuni by dendritic cells is determined by sialylation. *J Immunol* **185**, 748-755.
- Kumar, H., Kawai, T. & Akira, S. (2009).** Pathogen recognition in the innate immune response. *Biochem J* **420**, 1-16.
- Kunisawa, J., Takahashi, I. & Kiyono, H. (2007).** Intraepithelial lymphocytes: their shared and divergent immunological behaviors in the small and large intestine. *Immunol Rev* **215**, 136-153.
- Kyd, J. M. & Cripps, A. W. (2008).** Functional differences between M cells and enterocytes in sampling luminal antigens. *Vaccine* **26**, 6221-6224.
- Lai, Y. & Gallo, R. L. (2009).** AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends in immunology* **30**, 131-141.
- Laing, K. J. & Secombes, C. J. (2004).** Chemokines. *Dev Comp Immunol* **28**, 443-460.
- Laing, K. J., Purcell, M. K., Winton, J. R. & Hansen, J. D. (2008).** A genomic view of the NOD-like receptor family in teleost fish: identification of a novel NLR subfamily in zebrafish. *BMC Evol Biol* **8**, 42.
- Lara-Tejero, M. & Galan, J. E. (2002).** Cytolethal distending toxin: limited damage as a strategy to modulate cellular functions. *Trends Microbiol* **10**, 147-152.
- Larson, C. L., Shah, D. H., Dhillon, A. S., Call, D. R., Ahn, S., Haldorson, G. J., Davitt, C. & Konkel, M. E. (2008).** Campylobacter jejuni invade chicken LMH cells inefficiently and stimulate differential expression of the chicken CXCLi1 and CXCLi2 cytokines. *Microbiology* **154**, 3835-3847.
- Lee, J., Gonzales-Navajas, J. M. & Raz, E. (2008).** The "polarizing-tolerizing" mechanism of intestinal epithelium: its relevance to colonic homeostasis. *Semin Immunopathol* **30**, 3-9.
- Lee, M. L., Kuo, F. C., Whitmore, G. A. & Sklar, J. (2000).** Importance of replication in microarray gene expression studies: statistical methods and evidence from repetitive cDNA hybridizations. *Proc Natl Acad Sci U S A* **97**, 9834-9839.

- Leveque, G., Forgetta, V., Morroll, S., Smith, A. L., Bumstead, N., Barrow, P., Loredó-Osti, J. C., Morgan, K. & Malo, D. (2003).** Allelic variation in TLR4 is linked to susceptibility to *Salmonella enterica* serovar Typhimurium infection in chickens. *Infect Immun* **71**, 1116-1124.
- Li, M. O., Wan, Y. Y., Sanjabi, S., Robertson, A. K. & Flavell, R. A. (2006).** Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol* **24**, 99-146.
- Li, X., Chiang, H. I., Zhu, J., Dowd, S. E. & Zhou, H. (2008a).** Characterization of a newly developed chicken 44K Agilent microarray. *BMC Genomics* **9**, 60.
- Li, X., Swaggerty, C. L., Kogut, M. H. & other authors (2008b).** The paternal effect of *Campylobacter jejuni* colonization in ceca in broilers. *Poult Sci* **87**, 1742-1747.
- Li, X., Swaggerty, C. L., Kogut, M. H., Chiang, H. I., Wang, Y., Genovese, K. J., He, H. & Zhou, H. (2010).** Gene expression profiling of the local cecal response of genetic chicken lines that differ in their susceptibility to *Campylobacter jejuni* colonization. *PLoS One* **5**, e11827.
- Li, Y. P., Ingmer, H., Madsen, M. & Bang, D. D. (2008c).** Cytokine responses in primary chicken embryo intestinal cells infected with *Campylobacter jejuni* strains of human and chicken origin and the expression of bacterial virulence-associated genes. *BMC microbiology* **8**, 107.
- Lievin-Le Moal, V. & Servin, A. L. (2006).** The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota. *Clin Microbiol Rev* **19**, 315-337.
- Lillehoj, H. S. & Trout, J. M. (1996).** Avian gut-associated lymphoid tissues and intestinal immune responses to *Eimeria* parasites. *Clin Microbiol Rev* **9**, 349-360.
- Linde, A., Ross, C. R., Davis, E. G., Dib, L., Blecha, F. & Melgarejo, T. (2008).** Innate immunity and host defense peptides in veterinary medicine. *J Vet Intern Med* **22**, 247-265.
- Lippert, E., Karrasch, T., Sun, X., Allard, B., Herfarth, H. H., Threadgill, D. & Jobin, C. (2009).** Gnotobiotic IL-10; NF-kappaB mice develop rapid and severe colitis following *Campylobacter jejuni* infection. *PLoS One* **4**, e7413.
- Livak, K. J. & Schmittgen, T. D. (2001).** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408.
- Lu, Y., Sarson, A. J., Gong, J., Zhou, H., Zhu, W., Kang, Z., Yu, H., Sharif, S. & Han, Y. (2009).** Expression profiles of genes in Toll-like receptor-mediated signaling of broilers infected with *Clostridium perfringens*. *Clin Vaccine Immunol* **16**, 1639-1647.

- Lynn, D. J., Higgs, R., Gaines, S., Tierney, J., James, T., Lloyd, A. T., Fares, M. A., Mulcahy, G. & O'Farrelly, C. (2004).** Bioinformatic discovery and initial characterisation of nine novel antimicrobial peptide genes in the chicken. *Immunogenetics* **56**, 170-177.
- Lynn, D. J., Higgs, R., Lloyd, A. T. & other authors (2007).** Avian beta-defensin nomenclature: a community proposed update. *Immunol Lett* **110**, 86-89.
- Mackinnon, K. M., He, H., Nerren, J. R., Swaggerty, C. L., Genovese, K. J. & Kogut, M. H. (2009).** Expression profile of toll-like receptors within the gastrointestinal tract of 2-day-old Salmonella enteritidis-infected broiler chickens. *Veterinary microbiology* **137**, 313-319.
- Macpherson, A. J., Geuking, M. B. & McCoy, K. D. (2005).** Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria. *Immunology* **115**, 153-162.
- Macpherson, A. J., McCoy, K. D., Johansen, F. E. & Brandtzaeg, P. (2008).** The immune geography of IgA induction and function. *Mucosal Immunol* **1**, 11-22.
- Magalhaes, J. G., Tattoli, I. & Girardin, S. E. (2007).** The intestinal epithelial barrier: how to distinguish between the microbial flora and pathogens. *Semin Immunol* **19**, 106-115.
- Mageed, A. M., Isobe, N. & Yoshimura, Y. (2008).** Expression of avian beta-defensins in the oviduct and effects of lipopolysaccharide on their expression in the vagina of hens. *Poult Sci* **87**, 979-984.
- Mansfield, L. S., Bell, J. A., Wilson, D. L., Murphy, A. J., Elsheikha, H. M., Rathinam, V. A., Fierro, B. R., Linz, J. E. & Young, V. B. (2007).** C57BL/6 and congenic interleukin-10-deficient mice can serve as models of Campylobacter jejuni colonization and enteritis. *Infect Immun* **75**, 1099-1115.
- Manson, J. M., Rauch, M. & Gilmore, M. S. (2008).** The commensal microbiology of the gastrointestinal tract. *Adv Exp Med Biol* **635**, 15-28.
- Martinon, F., Burns, K. & Tschopp, J. (2002).** The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* **10**, 417-426.
- McDonald, V. & Shirley, M. W. (2009).** Past and future: vaccination against Eimeria. *Parasitology*, 1-13.
- McLennan, I. S. & Koishi, K. (2002).** The transforming growth factor-betas: multifaceted regulators of the development and maintenance of skeletal muscles, motoneurons and Schwann cells. *Int J Dev Biol* **46**, 559-567.
- Meade, K. G., Narciandi, F., Cahalane, S., Reiman, C., Allan, B. & O'Farrelly, C. (2008).** Comparative in vivo infection models yield insights on early host immune response to Campylobacter in chickens. *Immunogenetics*.

Mellits, K. H., Mullen, J., Wand, M., Armbruster, G., Patel, A., Connerton, P. L., Skelly, M. & Connerton, I. F. (2002). Activation of the transcription factor NF-kappaB by *Campylobacter jejuni*. *Microbiology* **148**, 2753-2763.

Mellits, K. H., Connerton, I. F., Loughlin, M. F., Clarke, P., Smith, J., Dillon, E., Connerton, P. L., Mulholland, F. & Hawkey, C. J. (2009). Induction of a chemoattractant transcriptional response by a *Campylobacter jejuni* boiled cell extract in colonocytes. *BMC microbiology* **9**, 28.

Melmed, G., Thomas, L. S., Lee, N. & other authors (2003). Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. *J Immunol* **170**, 1406-1415.

Milona, P., Townes, C. L., Bevan, R. M. & Hall, J. (2007). The chicken host peptides, gallinacins 4, 7, and 9 have antimicrobial activity against *Salmonella* serovars. *Biochem Biophys Res Commun* **356**, 169-174.

Miossec, P., Korn, T. & Kuchroo, V. K. (2009). Interleukin-17 and type 17 helper T cells. *N Engl J Med* **361**, 888-898.

Misawa, N., Ohnishi, T., Uchida, K., Nakai, M., Nasu, T., Itoh, K. & Takahashi, E. (1996). Experimental hepatitis induced by *Campylobacter jejuni* infection in Japanese quail (*Coturnix coturnix japonica*). *J Vet Med Sci* **58**, 205-210.

Mogensen, T. H. (2009). Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev* **22**, 240-273, Table of Contents.

Moller, B. & Villiger, P. M. (2006). Inhibition of IL-1, IL-6, and TNF-alpha in immune-mediated inflammatory diseases. *Springer Semin Immunopathol* **27**, 391-408.

Monteville, M. R. & Konkel, M. E. (2002). Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. *Infect Immun* **70**, 6665-6671.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L. & O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* **19**, 683-765.

Moran, A. P., Rietschel, E. T., Kosunen, T. U. & Zahringer, U. (1991a). Chemical characterization of *Campylobacter jejuni* lipopolysaccharides containing N-acetylneuraminic acid and 2,3-diamino-2,3-dideoxy-D-glucose. *J Bacteriol* **173**, 618-626.

Moran, A. P., Zahringer, U., Seydel, U., Scholz, D., Stutz, P. & Rietschel, E. T. (1991b). Structural analysis of the lipid A component of *Campylobacter jejuni* CCUG 10936 (serotype O:2) lipopolysaccharide. Description of a lipid A containing a hybrid backbone of 2-amino-2-deoxy-D-glucose and 2,3-diamino-2,3-dideoxy-D-glucose. *Eur J Biochem* **198**, 459-469.

- Moran, A. P. & Penner, J. L. (1999).** Serotyping of *Campylobacter jejuni* based on heat-stable antigens: relevance, molecular basis and implications in pathogenesis. *J Appl Microbiol* **86**, 361-377.
- Mortensen, R. F. & Zhong, W. (2000).** Regulation of phagocytic leukocyte activities by C-reactive protein. *Journal of leukocyte biology* **67**, 495-500.
- Mosser, D. M. & Edwards, J. P. (2008).** Exploring the full spectrum of macrophage activation. *Nature reviews* **8**, 958-969.
- Mukherjee, S., Vaishnava, S. & Hooper, L. V. (2008).** Multi-layered regulation of intestinal antimicrobial defense. *Cell Mol Life Sci* **65**, 3019-3027.
- Muller, C. A., Autenrieth, I. B. & Peschel, A. (2005).** Innate defenses of the intestinal epithelial barrier. *Cell Mol Life Sci* **62**, 1297-1307.
- Murata, H., Shimada, N. & Yoshioka, M. (2004).** Current research on acute phase proteins in veterinary diagnosis: an overview. *Vet J* **168**, 28-40.
- Naiki, Y., Michelsen, K. S., Zhang, W., Chen, S., Doherty, T. M. & Arditi, M. (2005).** Transforming growth factor-beta differentially inhibits MyD88-dependent, but not TRAM- and TRIF-dependent, lipopolysaccharide-induced TLR4 signaling. *J Biol Chem* **280**, 5491-5495.
- Neish, A. S., Gewirtz, A. T., Zeng, H., Young, A. N., Hobert, M. E., Karmali, V., Rao, A. S. & Madara, J. L. (2000).** Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. *Science* **289**, 1560-1563.
- Nemelka, K. W., Brown, A. W., Wallace, S. M. & other authors (2009).** Immune response to and histopathology of *Campylobacter jejuni* infection in ferrets (*Mustela putorius furo*). *Comp Med* **59**, 363-371.
- Nerren, J. R., Swaggerty, C. L., MacKinnon, K. M., Genovese, K. J., He, H., Pevzner, I. & Kogut, M. H. (2009).** Differential mRNA expression of the avian-specific toll-like receptor 15 between heterophils from *Salmonella*-susceptible and -resistant chickens. *Immunogenetics* **61**, 71-77.
- Nerren, J. R., He, H., Genovese, K. & Kogut, M. H. (2010).** Expression of the avian-specific toll-like receptor 15 in chicken heterophils is mediated by gram-negative and gram-positive bacteria, but not TLR agonists. *Vet Immunol Immunopathol* **136**, 151-156.
- Newell, D. G. (2001).** Animal models of *Campylobacter jejuni* colonization and disease and the lessons to be learned from similar *Helicobacter pylori* models. *Symp Ser Soc Appl Microbiol*, 57S-67S.
- Newell, D. G. & Fearnley, C. (2003).** Sources of *Campylobacter* colonization in broiler chickens. *Appl Environ Microbiol* **69**, 4343-4351.

- Nile, C. J., Townes, C. L., Michailidis, G., Hirst, B. H. & Hall, J. (2004).** Identification of chicken lysozyme g2 and its expression in the intestine. *Cell Mol Life Sci* **61**, 2760-2766.
- Novik, V., Hofreuter, D. & Galan, J. E. (2009).** Characterization of a *Campylobacter jejuni* VirK protein homolog as a novel virulence determinant. *Infect Immun* **77**, 5428-5436.
- O'Brien, R. L., Roark, C. L. & Born, W. K. (2009).** IL-17-producing gammadelta T cells. *Eur J Immunol* **39**, 662-666.
- O'Mahony, D. S., Pham, U., Iyer, R., Hawn, T. R. & Liles, W. C. (2008).** Differential constitutive and cytokine-modulated expression of human Toll-like receptors in primary neutrophils, monocytes, and macrophages. *Int J Med Sci* **5**, 1-8.
- Pai, R. & Kang, G. (2008).** Microbes in the gut: a digestable account of host-symbiont interactions. *Indian J Med Res* **128**, 587-594.
- Panigrahi, P., Losonsky, G., DeTolla, L. J. & Morris, J. G., Jr. (1992).** Human immune response to *Campylobacter jejuni* proteins expressed in vivo. *Infect Immun* **60**, 4938-4944.
- Pantin-Jackwood, M. J. & Swayne, D. E. (2009).** Pathogenesis and pathobiology of avian influenza virus infection in birds. *Rev Sci Tech* **28**, 113-136.
- Papadakis, K. A. & Targan, S. R. (2000).** Tumor necrosis factor: biology and therapeutic inhibitors. *Gastroenterology* **119**, 1148-1157.
- Papanicolaou, D. A., Wilder, R. L., Manolagas, S. C. & Chrousos, G. P. (1998).** The pathophysiologic roles of interleukin-6 in human disease. *Ann Intern Med* **128**, 127-137.
- Park, J. Y. & Pillinger, M. H. (2007).** Interleukin-6 in the pathogenesis of rheumatoid arthritis. *Bull NYU Hosp Jt Dis* **65 Suppl 1**, S4-10.
- Parkhill, J., Wren, B. W., Mungall, K. & other authors (2000).** The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* **403**, 665-668.
- Patil, A., Hughes, A. L. & Zhang, G. (2004).** Rapid evolution and diversification of mammalian alpha-defensins as revealed by comparative analysis of rodent and primate genes. *Physiol Genomics* **20**, 1-11.
- Peron, J. P., Ligeiro de Oliveira, A. P. & Rizzo, L. V. (2009).** It takes guts for tolerance: The phenomenon of oral tolerance and the regulation of autoimmune response. *Autoimmunity reviews*.
- Peterson, J., Garges, S., Giovanni, M. & other authors (2009).** The NIH Human Microbiome Project. *Genome Res* **19**, 2317-2323.

- Philbin, V. J., Iqbal, M., Boyd, Y., Goodchild, M. J., Beal, R. K., Bumstead, N., Young, J. & Smith, A. L. (2005).** Identification and characterization of a functional, alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens. *Immunology* **114**, 507-521.
- Pinard-van der Laan, M. H., Bed'hom, B., Coville, J. L. & other authors (2009).** Microsatellite mapping of QTLs affecting resistance to coccidiosis (*Eimeria tenella*) in a Fayoumi x White Leghorn cross. *BMC genomics* **10**, 31.
- Platt, A. M. & Mowat, A. M. (2008).** Mucosal macrophages and the regulation of immune responses in the intestine. *Immunol Lett* **119**, 22-31.
- Poh, T. Y., Pease, J., Young, J. R., Bumstead, N. & Kaiser, P. (2008).** Re-evaluation of Chicken CXCR1 Determines the True Gene Structure: CXCLi1 (K60) AND CXCLi2 (CAF/INTERLEUKIN-8) ARE LIGANDS FOR THIS RECEPTOR. *J Biol Chem* **283**, 16408-16415.
- Pratt, J. S., Sachen, K. L., Wood, H. D., Eaton, K. A. & Young, V. B. (2006).** Modulation of host immune responses by the cytolethal distending toxin of *Helicobacter hepaticus*. *Infect Immun* **74**, 4496-4504.
- Purdy, D., Buswell, C. M., Hodgson, A. E., McAlpine, K., Henderson, I. & Leach, S. A. (2000).** Characterisation of cytolethal distending toxin (CDT) mutants of *Campylobacter jejuni*. *J Med Microbiol* **49**, 473-479.
- Quackenbush, J. (2001).** Computational analysis of microarray data. *Nat Rev Genet* **2**, 418-427.
- Qureshi, M. A., Heggen, C. L. & Hussain, I. (2000).** Avian macrophage: effector functions in health and disease. *Dev Comp Immunol* **24**, 103-119.
- Racape, M., Vanhove, B., Soulillou, J. P. & Brouard, S. (2009).** Interleukin 7 receptor alpha as a potential therapeutic target in transplantation. *Arch Immunol Ther Exp (Warsz)* **57**, 253-261.
- Raffatellu, M., Chessa, D., Wilson, R. P., Dusold, R., Rubino, S. & Baumler, A. J. (2005).** The Vi capsular antigen of *Salmonella enterica* serotype Typhi reduces Toll-like receptor-dependent interleukin-8 expression in the intestinal mucosa. *Infect Immun* **73**, 3367-3374.
- Rathinam, V. A., Appledorn, D. M., Hoag, K. A., Amalfitano, A. & Mansfield, L. S. (2009).** *Campylobacter jejuni*-induced activation of dendritic cells involves cooperative signaling through Toll-like receptor 4 (TLR4)-MyD88 and TLR4-TRIF axes. *Infect Immun* **77**, 2499-2507.
- Reemers, S. S., van Leenen, D., Koerkamp, M. J., van Haarlem, D., van de Haar, P., van Eden, W. & Vervelde, L. (2010).** Early host responses to avian influenza A virus are prolonged and enhanced at transcriptional level depending on maturation of the immune system. *Mol Immunol* **47**, 1675-1685.

- Reife, R. A., Coats, S. R., Al-Qutub, M., Dixon, D. M., Braham, P. A., Billharz, R. J., Howald, W. N. & Darveau, R. P. (2006).** Porphyromonas gingivalis lipopolysaccharide lipid A heterogeneity: differential activities of tetra- and penta-acylated lipid A structures on E-selectin expression and TLR4 recognition. *Cell Microbiol* **8**, 857-868.
- Revello, M. G. & Gerna, G. (2010).** Human cytomegalovirus tropism for endothelial/epithelial cells: scientific background and clinical implications. *Rev Med Virol* **20**, 136-155.
- Rothwarf, D. M., Zandi, E., Natoli, G. & Karin, M. (1998).** IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. *Nature* **395**, 297-300.
- Rothwell, L., Young, J. R., Zoorob, R., Whittaker, C. A., Hesketh, P., Archer, A., Smith, A. L. & Kaiser, P. (2004).** Cloning and characterization of chicken IL-10 and its role in the immune response to Eimeria maxima. *J Immunol* **173**, 2675-2682.
- Rudd, C. E., Taylor, A. & Schneider, H. (2009).** CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunol Rev* **229**, 12-26.
- Sadrzadeh, S. M. & Bozorgmehr, J. (2004).** Haptoglobin phenotypes in health and disorders. *Am J Clin Pathol* **121 Suppl**, S97-104.
- Sahin, O., Zhang, Q., Meitzler, J. C., Harr, B. S., Morishita, T. Y. & Mohan, R. (2001).** Prevalence, antigenic specificity, and bactericidal activity of poultry anti-Campylobacter maternal antibodies. *Appl Environ Microbiol* **67**, 3951-3957.
- Sakai, A., Han, J., Cato, A. C., Akira, S. & Li, J. D. (2004).** Glucocorticoids synergize with IL-1beta to induce TLR2 expression via MAP Kinase Phosphatase-1-dependent dual Inhibition of MAPK JNK and p38 in epithelial cells. *BMC Mol Biol* **5**, 2.
- Salzman, N. H., Hung, K., Haribhai, D. & other authors (2010).** Enteric defensins are essential regulators of intestinal microbial ecology. *Nat Immunol* **11**, 76-82.
- Sang, Y. & Blecha, F. (2008).** Antimicrobial peptides and bacteriocins: alternatives to traditional antibiotics. *Anim Health Res Rev* **9**, 227-235.
- Sansonetti, P. J., Arondel, J., Cavaillon, J. M. & Huerre, M. (1995).** Role of interleukin-1 in the pathogenesis of experimental shigellosis. *J Clin Invest* **96**, 884-892.
- Sarson, A. J., Wang, Y., Kang, Z., Dowd, S. E., Lu, Y., Yu, H., Han, Y., Zhou, H. & Gong, J. (2009).** Gene expression profiling within the spleen of Clostridium perfringens-challenged broilers fed antibiotic-medicated and non-medicated diets. *BMC genomics* **10**, 260.
- Sartor, R. B. (2008).** Microbial influences in inflammatory bowel diseases. *Gastroenterology* **134**, 577-594.

- Schenk, M. & Mueller, C. (2008).** The mucosal immune system at the gastrointestinal barrier. *Best Pract Res Clin Gastroenterol* **22**, 391-409.
- Schneider, K., Klaas, R., Kaspers, B. & Staeheli, P. (2001).** Chicken interleukin-6. cDNA structure and biological properties. *Eur J Biochem* **268**, 4200-4206.
- Schroer, D., Veits, J., Grund, C., Dauber, M., Keil, G., Granzow, H., Mettenleiter, T. C. & Romer-Oberdorfer, A. (2009).** Vaccination with Newcastle disease virus vectored vaccine protects chickens against highly pathogenic H7 avian influenza virus. *Avian Dis* **53**, 190-197.
- Scott, K., Manunta, M., Germain, C. & other authors (2005).** Qualitatively distinct patterns of cytokines are released by human dendritic cells in response to different pathogens. *Immunology* **116**, 245-254.
- Semple, C. A., Gautier, P., Taylor, K. & Dorin, J. R. (2006).** The changing of the guard: Molecular diversity and rapid evolution of beta-defensins. *Mol Divers* **10**, 575-584.
- Shaw, M. H., Reimer, T., Kim, Y. G. & Nunez, G. (2008).** NOD-like receptors (NLRs): bona fide intracellular microbial sensors. *Curr Opin Immunol* **20**, 377-382.
- Shenker, B. J., Hoffmaster, R. H., Zekavat, A., Yamaguchi, N., Lally, E. T. & Demuth, D. R. (2001).** Induction of apoptosis in human T cells by *Actinobacillus actinomycetemcomitans* cytolethal distending toxin is a consequence of G2 arrest of the cell cycle. *J Immunol* **167**, 435-441.
- Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K. & Kimoto, M. (1999).** MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* **189**, 1777-1782.
- Shoaf-Sweeney, K. D., Larson, C. L., Tang, X. & Konkel, M. E. (2008).** Identification of *Campylobacter jejuni* proteins recognized by maternal antibodies of chickens. *Appl Environ Microbiol* **74**, 6867-6875.
- Shull, M. M., Ormsby, I., Kier, A. B. & other authors (1992).** Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* **359**, 693-699.
- Simon, R., Lam, A., Li, M. C., Ngan, M., Menenzes, S. & Zhao, Y. (2007).** Analysis of Gene Expression Data Using BRB-Array Tools. *Cancer Inform* **3**, 11-17.
- Smith, C. K., Kaiser, P., Rothwell, L., Humphrey, T., Barrow, P. A. & Jones, M. A. (2005a).** *Campylobacter jejuni*-induced cytokine responses in avian cells. *Infect Immun* **73**, 2094-2100.
- Smith, C. K., Abuoun, M., Cawthraw, S. A., Humphrey, T. J., Rothwell, L., Kaiser, P., Barrow, P. A. & Jones, M. A. (2008).** *Campylobacter* colonization of the chicken induces a proinflammatory response in mucosal tissues. *FEMS Immunol Med Microbiol* **54**, 114-121.

- Smith, J., Speed, D., Hocking, P. M., Talbot, R. T., Degen, W. G., Schijns, V. E., Glass, E. J. & Burt, D. W. (2006).** Development of a chicken 5 K microarray targeted towards immune function. *BMC Genomics* **7**, 49.
- Smith, J. L. & Bayles, D. O. (2006).** The contribution of cytolethal distending toxin to bacterial pathogenesis. *Crit Rev Microbiol* **32**, 227-248.
- Smith, P. D., Ochsenbauer-Jambor, C. & Smythies, L. E. (2005b).** Intestinal macrophages: unique effector cells of the innate immune system. *Immunol Rev* **206**, 149-159.
- Smyth, G. K. & Speed, T. (2003).** Normalization of cDNA microarray data. *Methods (San Diego, Calif)* **31**, 265-273.
- Smythies, L. E., Sellers, M., Clements, R. H., Mosteller-Barnum, M., Meng, G., Benjamin, W. H., Orenstein, J. M. & Smith, P. D. (2005).** Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest* **115**, 66-75.
- Soman, S. S., Arathy, D. S. & Sreekumar, E. (2009).** Discovery of *Anas platyrhynchos* avian beta-defensin 2 (Apl_AvBD2) with antibacterial and chemotactic functions. *Mol Immunol* **46**, 2029-2038.
- Sugiarto, H. & Yu, P. L. (2004).** Avian antimicrobial peptides: the defense role of beta-defensins. *Biochem Biophys Res Commun* **323**, 721-727.
- Sumimoto, H. (2008).** Structure, regulation and evolution of Nox-family NADPH oxidases that produce reactive oxygen species. *FEBS J* **275**, 3249-3277.
- Sutton, C. E., Lalor, S. J., Sweeney, C. M., Brereton, C. F., Lavelle, E. C. & Mills, K. H. (2009).** Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* **31**, 331-341.
- Tajima, A., Iwase, T., Shinji, H., Seki, K. & Mizunoe, Y. (2008).** Inhibition of Endothelial IL-8 Production and Neutrophil Transmigration by *Staphylococcus aureus* {beta}-hemolysin. *Infect Immun* **77**, 327-334.
- Tarantino, M., Dionisi, A. M., Pistoia, C., Petrucci, P., Luzzi, I. & Pasquali, P. (2009).** Involvement of nitric oxide in the control of a mouse model of *Campylobacter jejuni* infection. *FEMS Immunol Med Microbiol* **56**, 98-101.
- Temperley, N. D., Berlin, S., Paton, I. R., Griffin, D. K. & Burt, D. W. (2008).** Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss. *BMC genomics* **9**, 62.
- Tien, M. T., Girardin, S. E., Regnault, B., Le Bourhis, L., Dillies, M. A., Coppee, J. Y., Bourdet-Sicard, R., Sansonetti, P. J. & Pedron, T. (2006).** Anti-

inflammatory effect of *Lactobacillus casei* on *Shigella*-infected human intestinal epithelial cells. *J Immunol* **176**, 1228-1237.

Toma, C., Higa, N., Koizumi, Y. & other authors (2010). Pathogenic *Vibrio* activate NLRP3 inflammasome via cytotoxins and TLR/nucleotide-binding oligomerization domain-mediated NF-kappa B signaling. *J Immunol* **184**, 5287-5297.

Tomasinsig, L. & Zanetti, M. (2005). The cathelicidins--structure, function and evolution. *Curr Protein Pept Sci* **6**, 23-34.

Tribble, D. R., Baqar, S., Scott, D. A. & other authors (2010). Assessment of the duration of protection in *Campylobacter jejuni* experimental infection in humans. *Infect Immun* **78**, 1750-1759.

Tripathi, P. (2007). Nitric oxide and immune response. *Indian J Biochem Biophys* **44**, 310-319.

Tsai, C. A., Hsueh, H. M. & Chen, J. J. (2003). Estimation of false discovery rates in multiple testing: application to gene microarray data. *Biometrics* **59**, 1071-1081.

Tsolis, R. M., Young, G. M., Solnick, J. V. & Baumler, A. J. (2008). From bench to bedside: stealth of enteroinvasive pathogens. *Nat Rev Microbiol* **6**, 883-892.

Tsuji, N. M. & Kosaka, A. (2008). Oral tolerance: intestinal homeostasis and antigen-specific regulatory T cells. *Trends in immunology* **29**, 532-540.

Tu, Q. V., McGuckin, M. A. & Mendz, G. L. (2008). *Campylobacter jejuni* response to human mucin MUC2: modulation of colonization and pathogenicity determinants. *J Med Microbiol* **57**, 795-802.

Tuohy, K. M., Hinton, D. J., Davies, S. J., Crabbe, M. J., Gibson, G. R. & Ames, J. M. (2006). Metabolism of Maillard reaction products by the human gut microbiota--implications for health. *Mol Nutr Food Res* **50**, 847-857.

Urban, C. F., Lourido, S. & Zychlinsky, A. (2006). How do microbes evade neutrophil killing? *Cell Microbiol* **8**, 1687-1696.

Van Deun, K., Pasmans, F., Ducatelle, R., Flahou, B., Vissenberg, K., Martel, A., Van den Broeck, W., Van Immerseel, F. & Haesebrouck, F. (2007). Colonization strategy of *Campylobacter jejuni* results in persistent infection of the chicken gut. *Vet Microbiol* **130**, 285-297.

van Dijk, A., Veldhuizen, E. J., Kalkhove, S. I., Tjeerdsma-van Bokhoven, J. L., Romijn, R. A. & Haagsman, H. P. (2007). The beta-defensin gallinacin-6 is expressed in the chicken digestive tract and has antimicrobial activity against food-borne pathogens. *Antimicrobial agents and chemotherapy* **51**, 912-922.

van Dijk, A., Molhoek, E. M., Veldhuizen, E. J., Bokhoven, J. L., Wagendorp, E., Bikker, F. & Haagsman, H. P. (2009a). Identification of chicken cathelicidin-2 core elements involved in antibacterial and immunomodulatory activities. *Mol Immunol*.

- van Dijk, A., Tersteeg-Zijderveld, M. H., Tjeerdsma-van Bokhoven, J. L., Jansman, A. J., Veldhuizen, E. J. & Haagsman, H. P. (2009b).** Chicken heterophils are recruited to the site of Salmonella infection and release antibacterial mature Cathelicidin-2 upon stimulation with LPS. *Mol Immunol* **46**, 1517-1526.
- van Doorn, P. A., Ruts, L. & Jacobs, B. C. (2008).** Clinical features, pathogenesis, and treatment of Guillain-Barre syndrome. *Lancet Neurol* **7**, 939-950.
- van Mourik, A., Steeghs, L., van Laar, J., Meiring, H. D., Hamstra, H. J., van Putten, J. P. & Wosten, M. M. (2010).** Altered linkage of hydroxyacyl chains in lipid A of Campylobacter jejuni reduces TLR4 activation and antimicrobial resistance. *J Biol Chem* **285**, 15828-15836.
- van Spreeuwel, J. P., Duursma, G. C., Meijer, C. J., Bax, R., Rosekrans, P. C. & Lindeman, J. (1985).** Campylobacter colitis: histological immunohistochemical and ultrastructural findings. *Gut* **26**, 945-951.
- Vance, R. E., Isberg, R. R. & Portnoy, D. A. (2009).** Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. *Cell Host Microbe* **6**, 10-21.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. & Speleman, F. (2002).** Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* **3**, RESEARCH0034.
- Venkatasubbarao, S. (2004).** Microarrays--status and prospects. *Trends Biotechnol* **22**, 630-637.
- Viala, J., Chaput, C., Boneca, I. G. & other authors (2004).** Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. *Nat Immunol* **5**, 1166-1174.
- Viswanathan, V. K., Hodges, K. & Hecht, G. (2009).** Enteric infection meets intestinal function: how bacterial pathogens cause diarrhoea. *Nat Rev Microbiol* **7**, 110-119.
- Vora, P., Youdim, A., Thomas, L. S. & other authors (2004).** Beta-defensin-2 expression is regulated by TLR signaling in intestinal epithelial cells. *J Immunol* **173**, 5398-5405.
- Wan, H. & Dupasquier, M. (2005).** Dendritic cells in vivo and in vitro. *Cell Mol Immunol* **2**, 28-35.
- Wartha, F., Beiter, K., Normark, S. & Henriques-Normark, B. (2007).** Neutrophil extracellular traps: casting the NET over pathogenesis. *Current opinion in microbiology* **10**, 52-56.

- Watson, R. O. & Galan, J. E. (2005).** Signal transduction in *Campylobacter jejuni*-induced cytokine production. *Cell Microbiol* **7**, 655-665.
- Watters, T. M., Kenny, E. F. & O'Neill, L. A. (2007).** Structure, function and regulation of the Toll/IL-1 receptor adaptor proteins. *Immunol Cell Biol* **85**, 411-419.
- Weining, K. C., Sick, C., Kaspers, B. & Staeheli, P. (1998).** A chicken homolog of mammalian interleukin-1 beta: cDNA cloning and purification of active recombinant protein. *Eur J Biochem* **258**, 994-1000.
- Whitehouse, C. A., Balbo, P. B., Pesci, E. C., Cottle, D. L., Mirabito, P. M. & Pickett, C. L. (1998).** *Campylobacter jejuni* cytolethal distending toxin causes a G2-phase cell cycle block. *Infect Immun* **66**, 1934-1940.
- Wilson, D. L., Bell, J. A., Young, V. B., Wilder, S. R., Mansfield, L. S. & Linz, J. E. (2003).** Variation of the natural transformation frequency of *Campylobacter jejuni* in liquid shake culture. *Microbiology* **149**, 3603-3615.
- Wilson, R. P., Raffatellu, M., Chessa, D., Winter, S. E., Tukel, C. & Baumler, A. J. (2008).** The Vi-capsule prevents Toll-like receptor 4 recognition of *Salmonella*. *Cell Microbiol* **10**, 876-890.
- Wine, E., Chan, V. L. & Sherman, P. M. (2008).** *Campylobacter jejuni* mediated disruption of polarized epithelial monolayers is cell-type specific, time dependent, and correlates with bacterial invasion. *Pediatr Res* **64**, 599-604.
- Withanage, G. S., Kaiser, P., Wigley, P. & other authors (2004).** Rapid expression of chemokines and proinflammatory cytokines in newly hatched chickens infected with *Salmonella enterica* serovar typhimurium. *Infect Immun* **72**, 2152-2159.
- Wittchen, E. S. (2009).** Endothelial signaling in paracellular and transcellular leukocyte transmigration. *Front Biosci* **14**, 2522-2545.
- Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J. & Mathison, J. C. (1990).** CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **249**, 1431-1433.
- Wu, M. F., Yang, C. Y., Lin, T. L. & other authors (2009a).** Humoral immunity against capsule polysaccharide protects the host from magA+ *Klebsiella pneumoniae*-induced lethal disease by evading Toll-like receptor 4 signaling. *Infect Immun* **77**, 615-621.
- Wu, Z., Rothwell, L., Hu, T. & Kaiser, P. (2009b).** Chicken CD14, unlike mammalian CD14, is trans-membrane rather than GPI-anchored. *Dev Comp Immunol* **33**, 97-104.
- Yang, Y. H., Dudoit, S., Luu, P., Lin, D. M., Peng, V., Ngai, J. & Speed, T. P. (2002).** Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res* **30**, e15.

- Yokota, S., Ohnishi, T., Muroi, M., Tanamoto, K., Fujii, N. & Amano, K. (2007).** Highly-purified *Helicobacter pylori* LPS preparations induce weak inflammatory reactions and utilize Toll-like receptor 2 complex but not Toll-like receptor 4 complex. *FEMS Immunol Med Microbiol* **51**, 140-148.
- Young, K. T., Davis, L. M. & Dirita, V. J. (2007).** *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat Rev Microbiol* **5**, 665-679.
- Zamze, S., Martinez-Pomares, L., Jones, H., Taylor, P. R., Stillion, R. J., Gordon, S. & Wong, S. Y. (2002).** Recognition of bacterial capsular polysaccharides and lipopolysaccharides by the macrophage mannose receptor. *J Biol Chem* **277**, 41613-41623.
- Zheng, J., Meng, J., Zhao, S., Singh, R. & Song, W. (2008).** *Campylobacter*-induced interleukin-8 secretion in polarized human intestinal epithelial cells requires *Campylobacter*-secreted cytolethal distending toxin- and Toll-like receptor-mediated activation of NF-kappaB. *Infect Immun* **76**, 4498-4508.
- Zhu, J., Meinersmann, R. J., Hiett, K. L. & Evans, D. L. (1999).** Apoptotic effect of outer-membrane proteins from *Campylobacter jejuni* on chicken lymphocytes. *Curr Microbiol* **38**, 244-249.
- Ziegler, H. K. (2004).** The role of gamma/delta T cells in immunity to infection and regulation of inflammation. *Immunol Res* **29**, 293-302.
- Zien, A., Aigner, T., Zimmer, R. & Lengauer, T. (2001).** Centralization: a new method for the normalization of gene expression data. *Bioinformatics* **17 Suppl 1**, S323-331.
- Zilbauer, M., Dorrell, N., Boughan, P. K., Harris, A., Wren, B. W., Klein, N. J. & Bajaj-Elliott, M. (2005).** Intestinal innate immunity to *Campylobacter jejuni* results in induction of bactericidal human beta-defensins 2 and 3. *Infect Immun* **73**, 7281-7289.
- Zilbauer, M., Dorrell, N., Elmi, A. & other authors (2007).** A major role for intestinal epithelial nucleotide oligomerization domain 1 (NOD1) in eliciting host bactericidal immune responses to *Campylobacter jejuni*. *Cell Microbiol* **9**, 2404-2416.
- Ziprin, R. L., Young, C. R., Stanker, L. H., Hume, M. E. & Konkel, M. E. (1999).** The absence of cecal colonization of chicks by a mutant of *Campylobacter jejuni* not expressing bacterial fibronectin-binding protein. *Avian Dis* **43**, 586-589.
- Ziprin, R. L., Young, C. R., Byrd, J. A., Stanker, L. H., Hume, M. E., Gray, S. A., Kim, B. J. & Konkel, M. E. (2001).** Role of *Campylobacter jejuni* potential virulence genes in cecal colonization. *Avian Dis* **45**, 549-557.