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Abstract

The Gastro-Intestinal (GI) tract is a unique region in the body. Our innate immune system retains a fine homeostatic balance between avoiding inappropriate inflammatory responses against the myriad commensal microbes residing in the gut while also remaining active enough to prevent invasive pathogenic attack. The intestinal epithelium represents the frontline of this interface. It has long been known to act as a physical barrier preventing the luminal bacteria of the gastro-intestinal tract from activating an inflammatory immune response in the immune cells of the underlying mucosa. However, in recent years, an appreciation has grown surrounding the role played by the intestinal epithelium in regulating innate immune responses, both in the prevention of infection and in maintaining a homeostatic environment through modulation of innate immune signalling systems. The aim of this thesis was to identify novel innate immune mechanisms regulating inflammation in the GI tract. To achieve this aim, we chose several aspects of regulatory mechanisms utilised in this region by the innate immune system. We identified several commensal strains of bacteria expressing proteins containing signalling domains used by Pattern Recognition Receptors (PRRs) of the innate immune system. Three such bacterial proteins were studied for their potentially subversive roles in host innate immune signalling as a means of regulating homeostasis in the GI tract. We also examined differential responses to PRR activation depending on their sub-cellular localisation. This was investigated based on reports that apical Toll-Like Receptor (TLR) 9 activation resulted in abrogation of inflammatory responses mediated by other TLRs in Intestinal Epithelial Cells (IECs) such as basolateral TLR4 activation. Using the well-studied invasive intra-cellular pathogen *Listeria monocytogenes* as a model for infection, we also used a PRR siRNA library screening technique to identify novel PRRs used by IECs in both inhibition and activation of inflammatory responses. Many of the PRRs identified in this screen were previously believed

not to be expressed in IECs. Furthermore, the same study has led to the identification of the previously uncharacterised TLR10 as a functional inflammatory receptor of IECs. Further analysis revealed a similar role in macrophages where it was shown to respond to intracellular and motile pathogens such as Gram-positive *L.monocytogenes* and Gram-negative *Salmonella typhimurium*. TLR10 expression in IECs was predominantly intracellular. This is likely in order to avoid inappropriate inflammatory activation through the recognition of commensal microbial antigens on the apical cell surface of IECs. Moreover, these results have revealed a more complex network of innate immune signalling mechanisms involved in both activating and inhibiting inflammatory responses in IECs than was previously believed. This contribution to our understanding of innate immune regulation in this region has several direct and indirect benefits. The identification of several novel PRRs involved in activating and inhibiting inflammation in the GI tract may be used as novel therapeutic targets in the treatment of disease; both for inducing tolerance and reducing inflammation, or indeed, as targets for adjuvant activation in the development of oral vaccines against pathogenic attack.

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List of publications

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Identification of Toll-Like Receptor 10 as a key mediator of the inflammatory response to *Listeria monocytogenes* in intestinal epithelial cells and macrophages. **Journal of Immunology** 12-03245-FLR2

Abstracts:

1. **Tim Regan**, Ken Nally, Fergus Shanahan, John MacSharry and Elizabeth Brint. (2013) “TLR10 mediates the inflammatory response to *Listeria monocytogenes* infection.” 100th American Association of Immunologists Conference, May 2013, Honolulu, Hawaii.
2. **Tim Regan**, Ken Nally, Fergus Shanahan, John MacSharry and Elizabeth Brint. (2012) “TLR10 mediates the inflammatory response to *Listeria monocytogenes* infection in intestinal epithelial cells.” Irish Society for Immunologists Conference, September 2012, Dublin, Ireland.
3. **Tim Regan**, Ken Nally, Fergus Shanahan, John MacSharry and Elizabeth Brint. (2012) “TLR10 mediates the inflammatory response to *Listeria monocytogenes* infection in intestinal epithelial cells.” European Mucosal Immunology Group Conference, October 2012, Dublin, Ireland.

Acronyms

Absent In Melanoma 2 (AIM2)

Apoptosis-associated Speck-like protein containing a CARD (ASC)

Bovine Serum Albumin (BSA)

Brain Heart Infusion (BHI)

Caspase Activation and Recruitment Domain (CARD)

c-Jun N-terminal kinase (JNK)

Class II, major histocompatibility complex, transactivator (CIITA)

Colony Forming Units (CFU)

Commensal-Associated Molecular Patterns (CAMPs)

Connexin43 (Cx43)

C-type Lectin (CLEC)

C-type Lectin Receptor (CLR)

C-X-C motif Ligand 2 (CXCL-2)

Danger Associated Molecular Pattern (DAMP)

Death Domain (DD)

Dendritic Cell (DC)

Dendritic Cell (DC)-Specific Intercellular adhesion molecule-3 Grabbing Non-integrin (DC-SIGN)

Dimethyloxaloylglycine (DMOG)

double stranded RNA (dsRNA)

Dual Oxidase 2 (DUOX2)

Dual specificity protein phosphatase 4 (DUSP4)

Early Growth Response protein-1 (EGR-1)

Epithelial cadherin (E-cadherin)

Extracellular signal-regulated kinases (ERK)

Fibroblast Stimulating Ligand-1 (FSL-1)

Forkhead box P3 (FOXP3)

Frizzled 5 (Fzd5)

Heat Shock Protein (HSP)

Heat-Killed *Listeria monocytogenes* (HKLM)

Human Beta Defensin (HBD)

Human Embryonic Kidney (HEK)

Hypoxic Inducible Factor-1 α (HIF-1 α)

Immunoglobulin A (IgA)

Inflammatory Bowel Disease (IBD)

Interferon (IFN)

Interferon Response Factors (IRFs)

Interleukin-1 receptor-associated kinase (IRAK)

Internalin A (InlA)

Internalin B (InlB)

Intestinal Epithelial Cell (IEC)

Isolated Lymphoid Follicles (ILFs)

Listeria monocytogenes adhesion protein (LAP)

Laboratory of Genetics and Physiology-2 (LGP2)

Lipopolysaccharide (LPS)

Lipoteichoic Acid (LTA)

Listeria monocytogenes (*L.monocytogenes*)

Listeriolysin O (LLO)

Loss-of-function (LOF)

Melanoma differentiation-associated antigen 5 (MDA5)

Mitochondrial antiviral signalling protein (MAVS)

Mitogen Activated Protein Kinases (MAPK)

Monocyte Chemotactic Protein-1 (MCP-1)

Mramyl Di-Peptide (MDP)

Multiplicity of infection (MOI)

MyD88 Adaptor-Like (MAL)

Myeloid Differentiation factor 88 (MyD88)

Neuronal Apoptosis Inhibitor Protein (NAIP)

NF- κ B Inducing Kinase NIK (NIK)

Nitric oxide (NO)

Nitric Oxide Synthase 2 (NOS2)

NLR family CARD-domain-containing (NLRC)

NLR family pyrin-domain-containing protein (NLRP)

NOD-Like Receptor NLR

Nuclear Factor Kappa B (NF- κ B)

Nuclear Oligomerization Domain (NOD)

Nucleotide-binding Domain Leucine-rich Repeat containing (NLR)

Oligodeoxynucleotide (ODN)

Optical Density (OD)

Pathogen Associated Molecular Pattern (PAMP)

Pattern Recognition Receptor (PRR)

Peptidoglycan (PGD)

Peripheral Blood Mononuclear Cells (PBMCs)

Phorbol myristate acetate (PMA)

Reactive Oxygen Species (ROS)

Receptor-Interacting serine/threonine Kinase (RICK)

Regulatory T cell (Treg)

Relative centrifugal force (rcf)

Retinoic acid-inducible gene I (RIG-I)

Ribonuclease L (RNase L)

RIG-I-Like Receptor RLR

Severe combined immunodeficient (SCID)

Single stranded RNA (ssRNA)

Spacious *L.monocytogenes*-containing phagosomes (SLAPs)

Spleen Tyrosine Kinase (Syk)

Sterile α and HEAT-Armadillo motifs containing protein (SARM)

Stimulator of Interferon Genes (STING)

TIR-domain-containing adapter-inducing interferon- β (TRIF)

TNF α and inducible Nitric Oxide Synthase (iNOS) producing effector Dendritic Cells (TipDCs)

TNF receptor associated factor (TRAF)

Toll/IL-1R resistance (TIR)

Toll-Like Protein A (TlpA)

Toll-Like Receptor (TLR)

Trans-Epithelial Electrical Resistance (TEER)

Trif-Related Adaptor Molecule (TRAM)

Tumour Necrosis Factor (TNF)

Vascular Endothelial Growth Factor (VEGF)

Chapter 1

General Introduction

1.1 Innate immunity

The innate immune system is our first line of defence against pathogenic attack on our body. Mammalian cells respond rapidly via the innate immune response to a diversity of danger signals. These include pathogen-derived molecules such as Lipopolysaccharide (LPS) from the outer cell membrane of Gram-negative bacteria. Such molecules are known as pathogen-associated molecular patterns (PAMPs). The body can even respond to self-derived molecular danger signals which arise from tissue damage, such as human Heat Shock Proteins (HSPs). These are known as Danger Associated Molecular Patterns (DAMPs) [1]. Recognition of these PAMPs and DAMPs is achieved by the Pattern-Recognition Receptors (PRRs) which constantly survey the extracellular space and cytoplasm for the presence of potentially harmful agents [2]. Known PRRs are divided into subfamilies including Toll-Like Receptors (TLRs) which sense PAMPs on the cell surface or in the endosomes, and Nuclear Oligodimerization Domain (NOD)-Like Receptors (Nucleotide-binding Domain Leucine-rich Repeat containing or NLRs) which recognize microbial molecules in the host cytosol, as well as playing a part in the regulation of apoptosis [2-4]. RIG-I like Receptors (RLRs) are another family of PRR of which there are 3 known members: retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated antigen 5 (MDA5) and Laboratory of Genetics and Physiology-2 (LGP2). These are RNA helicases which recognise viral RNA strands present in the host [5]. There are other forms of PRR such as C-type Lectin (CLEC) receptors (CLRs); these are proteins which contain a calcium dependent carbohydrate-binding domain and have a diverse range of functions including cell-cell adhesion and apoptosis as well as PRR function [6]. After stimulus recognition, each of these PRRs induces the activation of multiple signalling pathways, which lead to innate and adaptive immune responses [2, 4-6]. An important signal activated by TLRs, NLRs, and CLRs is the transcription factor NF- κ B, which regulates the expression of many immune and inflammatory genes. They also activate

the Mitogen Activated Protein Kinases (MAPKs), p38 and Extracellular signal-Regulated Kinase 1 (ERK1) and/or ERK2, which also play a role in enhanced gene transcription (see Figure 1.1) [7].

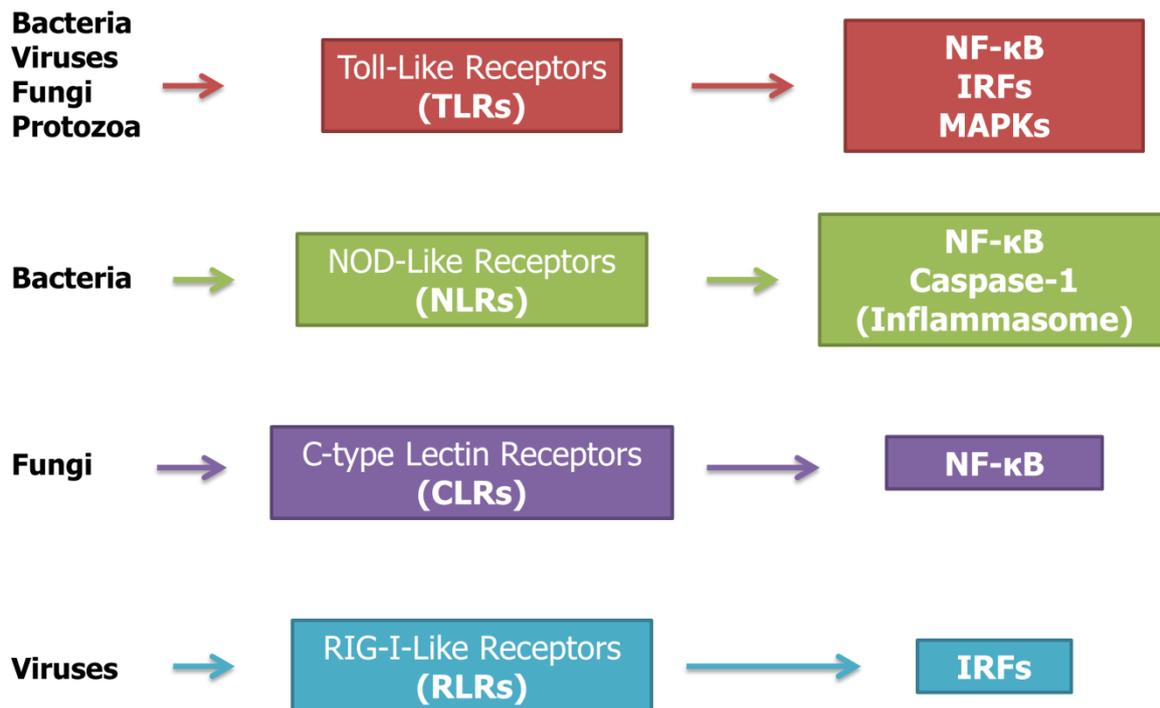


Figure 1.1 Examples of known PRRs and their function. Shown above are each of the four families of PRR. Each PRR family is known to be responsible for the detection of specific PAMPs from various pathogens as indicated. Upon recognition of each PAMP, the PRRs initiate a downstream signalling cascade leading to the eventual activation of certain transcription factors which in turn induce expression of the appropriate immune response genes. Included among these transcription factors listed above are Nuclear Factor kappa B (NF-κB), Mitogen Activated Protein Kinases (MAPKs) and Interferon Response Factors (IRFs).

Today we know of 13 mammalian TLR paralogues, (11 of which are known to be expressed in humans) [8]. These can be located at both the plasma membrane and the endosomal membrane. Because of the cellular distribution of these TLRs, pathogen

recognition can be initiated from a variety of cellular locations depending on the receptor activated. For example, the plasma membrane associated TLRs respond to components from the microbial surface, whereas the endosomal TLRs recognise various nucleic acid ligands. Crystallographic studies on TLR1–TLR2, TLR3 and TLR4 have confirmed the dimeric nature of the ligand bound receptor [9]. In the case of TLR9, it has been shown that a pre-existing dimer occurs, which presumably “tightens” upon ligand binding to create a new conformation [10]. Specific ligands for TLRs have been elucidated through use of overexpression studies, ex vivo studies and knockout mice (see Table 1.1). We know, for example, that TLR2 responds to peptidoglycan, lipopeptides, lipoteichoic acid, lipoarabinomannan, GPI anchors, phenol-soluble modulins, zymosan and glycolipids [8]. TLR2 seems unable to mediate an immune response through recognition of PAMPs independently but rather dimerises with TLR1 or TLR6 to achieve this. TLR4 recognises and binds to LPS and lipoteichoic acid from gram-positive bacteria as well as a few viral proteins and self-antigens e.g. fibronectin [8]. Flagellin, found in bacterial flagella, binds to and activates TLR5. TLR3 recognises double stranded RNA (dsRNA) from viruses while TLR7 and TLR8 both recognise single stranded RNA (ssRNA) from viruses. TLR9 recognises unmethylated CpG Oligodeoxynucleotide DNA from DNA viruses and bacteria. TLR11 exists only in a truncated form in humans and is believed to be inactive. Furthermore, we are only now uncovering the exact role TLR10 has to play. This is hindered by the lack of a murine homologue of TLR10, although we know that it is similar in sequence and structure to TLRs 1, 2 and 6. All TLRs have a conserved intracellular Toll/IL-1R resistance (TIR) domain through which they signal via the recruitment of various TIR domain-containing adapter proteins in the cytosol [11].

Receptor	Location	Ligand	Ligand origin
TLR1	Cell surface	multiple triacyl lipopeptides	Bacteria
TLR2	Cell surface	multiple glycolipids multiple lipoproteins HSP70 zymosan	Bacteria Bacteria Host Fungi
TLR3	Endosomal	double-stranded RNA (dsRNA)	Viruses
TLR4	Cell surface	lipopolysaccharide several heat shock proteins fibrinogen	Gram-negative bacteria Bacterial and host cells Host
TLR5	Cell surface	Flagellin	Bacteria
TLR6	Cell surface	multiple diacyl lipopeptides	Mycoplasma
TLR7	Endosomal	Single stranded RNA (ssRNA)	RNA Viruses
TLR8	Endosomal	Single stranded RNA (ssRNA)	RNA Viruses
TLR9	Endosomal	unmethylated CpG Oligodeoxynucleotide DNA	Bacteria, DNA Viruses
TLR10	Cell surface	?	?

Table 1.1 Intracellular location of each TLR with their known respective ligands and the origin of each ligand [12].

The 4 main adaptor proteins which bind to TLRs and facilitate signal transduction are Myeloid Differentiation factor 88 (MyD88), MyD88 Adaptor-Like (MAL), TIR-domain-containing adapter-inducing interferon- β (TRIF) and TRIF-Related Adaptor Molecule (TRAM), each displayed in Figure 1.2 interacting with their respective TLR. MyD88 is a modular protein and along with a TIR domain, it contains a Death Domain (DD) by which it recruits further downstream signalling components to the receptor complex via homotypic DD interactions [13]. These ultimately trigger the activation of immune response genes such as cytokines and chemokines (see Fig. 1.2). It is involved in mediating signal transduction for all TLRs apart from TLR3 [8]. MAL localizes to the plasma membrane where it can interact with TIR domains of activated TLR2 or TLR4 and recruit MyD88 [14]. Following activation through ligand binding, a downstream signalling cascade ensues through the cytoplasm to transcription factors in the nucleus of the cell which activate an enormous variety of specific immune response genes. The best characterised of these is probably Nuclear Factor κ B (NF- κ B) [7]. The localization of TLR4 is what determines whether the MAL-MyD88 pathway or the TRAM-TRIF pathway is activated [15, 16]. For example, TLR4, when located on the plasma membrane engages with MAL, which subsequently recruits MyD88. MyD88 then activates another signal transducing molecule, TNF Receptor Associated Factor 6 (TRAF6) which mediates NF- κ B activation. TLR4 will then traffic to endosomes, where TRAM is now engaged. TRAM subsequently recruits TRIF, which in turn recruits TRAF3. This cascade results in activation of TBK-1 which leads, in turn, to activation of viral immune response gene transcription factors, the Interferon regulatory factors (IRFs). Other TLRs using these adaptors signal in a similar signal cascading fashion to activate the appropriate response.

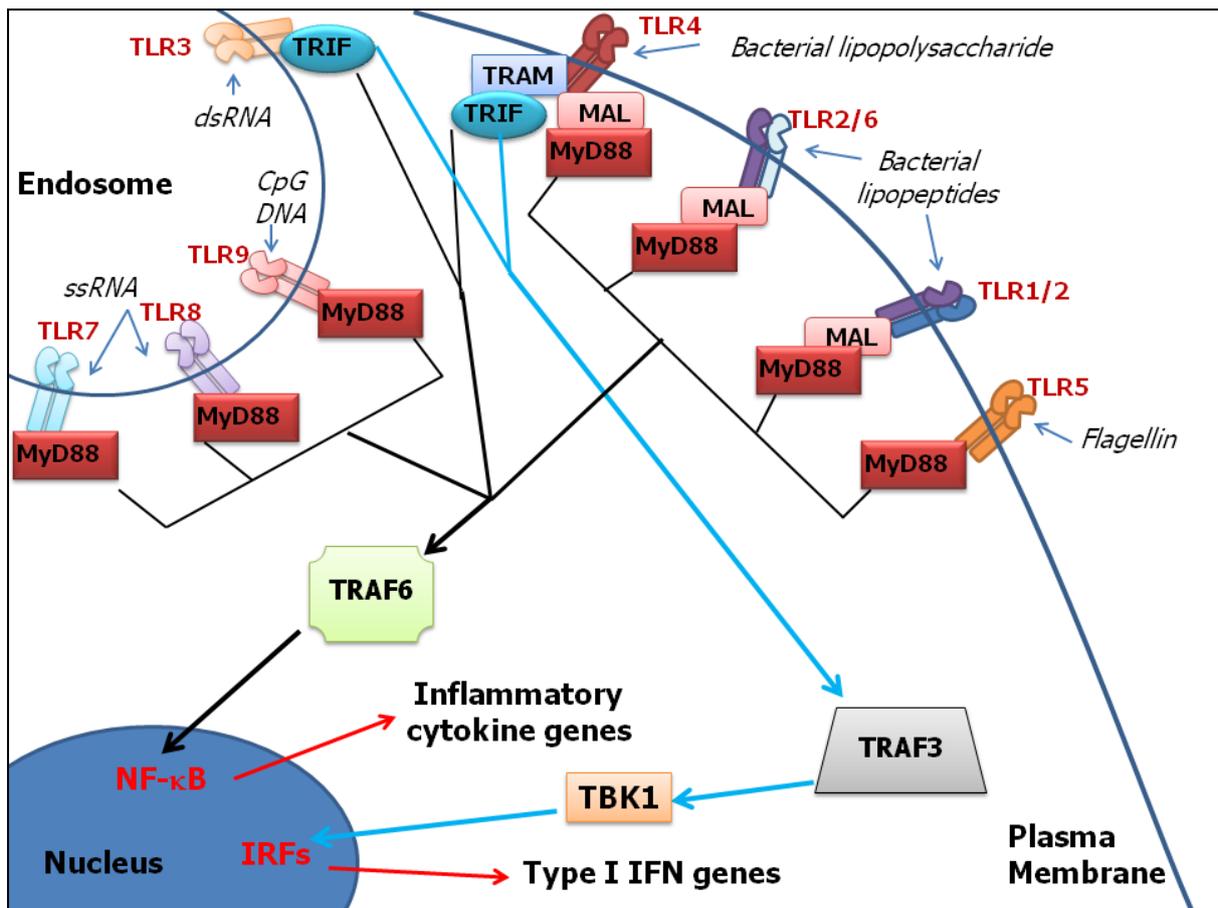


Figure 1.2 Signal transduction of TLRs following ligand recognition. Shown above are each human TLR with examples of their respective ligands. Following ligand recognition, each TLR initiates a downstream signalling cascade through their cytoplasmic TIR domains to their adjacent adaptor molecules. Every TLR is believed to signal through MyD88 with the exception of TLR3 which requires TRIF. TLR4 may also signal through TRIF with the help of another adaptor molecule, TRAM. TLR2 also requires MAL to recruit MyD88 to the complex. Recruitment of MyD88 to the TLR receptor initiates downstream activation of TNF receptor associated factor 6 (TRAF6). This in turn activates transcription factor NF-κB which results in the transcription of inflammatory cytokines. Alternatively, if TLR4 is trafficked to the endosome upon ligand recognition, it recruits TRAM, which recruits TRIF. TLR3 also recruits TRIF upon ligand binding. This initiates downstream activation of TRAF3 and subsequently TBK1 which activates transcription factors known as IRFs. These IRFs then induce production of the anti-viral interferon genes.

NLRs, located in the cytosol, comprise more than 20 members in the mammalian genome, and even more in plants and some fish species [17, 18]. They are grouped into the following sub-families: Class II, major histocompatibility complex, Transactivator, (CIITA) NOD, NLR family pyrin-domain-containing proteins (NLRPs, also called NALPs), NLR family Caspase Activation and Recruitment Domain (CARD)-domain-containing (NLRC), Neuronal Apoptosis Inhibitor Proteins (NAIP) and NLRX [19]. We are still discovering the specific ligands for each of these receptors. We know that NOD1 senses the peptidoglycan-derived peptide γ -D-glutamyl-meso-diaminopimelic acid, which is present mainly in Gram-negative bacteria [20]. NOD2 detects muramyl dipeptide, which can be found in a wide range of both Gram-positive and Gram-negative bacteria. Obviously this would indicate that the recognition specificity of NOD2 is markedly broader than that of NOD1 [20]. Evidence of cross-talk or interplay between these PRRs is apparent. For example, in a study carried out by Kim *et al* 2008 using NOD2 deficient macrophages, they were able to show that stimulation with synthetic a NOD2 ligand, Muramyl Di-Peptide (MDP) led to refractoriness to both MDP and a synthetic NOD1 agonist [21]. In addition, this NOD1 agonist induced tolerisation, not only to itself, but also to MDP. The cross-tolerisation between NOD1 and NOD2 signalling was associated with impaired NF- κ B and MAPK activation in response to pre-treatment of macrophages with MDP and the NOD1 agonists. This highlights the possibility that individuals harbouring loss-of-function (LOF) NOD2 mutations leading to reduced or loss of tolerisation to MDP may result in increased production of harmful cytokines in response to NOD1-activating bacteria [21]. In fact, several LOF NOD2 mutations have been linked to dysregulation of inflammation in the gut, leading to the onset of Inflammatory Bowel Disease (IBD) [22, 23].

Data are converging to indicate that NLRs can be categorized into functional subgroups that regulate other crucial innate immune pathways (see Figure 1.3), such as NF-

κ B, MAPK, type I interferon (IFN), cytokines, chemokines, and reactive oxygen species (ROS) as well as ribonuclease L (RNase L) activation [24]. Figure 1.3 depicts the concept that one NLR can serve multiple functions, whereas multiple NLRs can also serve similar functions. For example, NLR proteins signal through different multi-component “signalosomes”. NLR signalling modules include the NF- κ B Inducing Kinase (NIK) pathway as well as the CIITA transcriptosome, involved in major histocompatibility complex expression. The functional and physical associations of NLR proteins with the mitochondrial antiviral signalling protein (MAVS; also known as IPS-1, VISA, and Cardif) is referred to as the IFN/cytokine-inducing mito-signalosome. There is also the NF- κ B/MAPK-activating NOD1/2 complex (referred to as the nodosome), activation of which ultimately leads to the regulation of caspase-1 and NF- κ B activation. Also included are the caspase-1-activating inflammasomes.

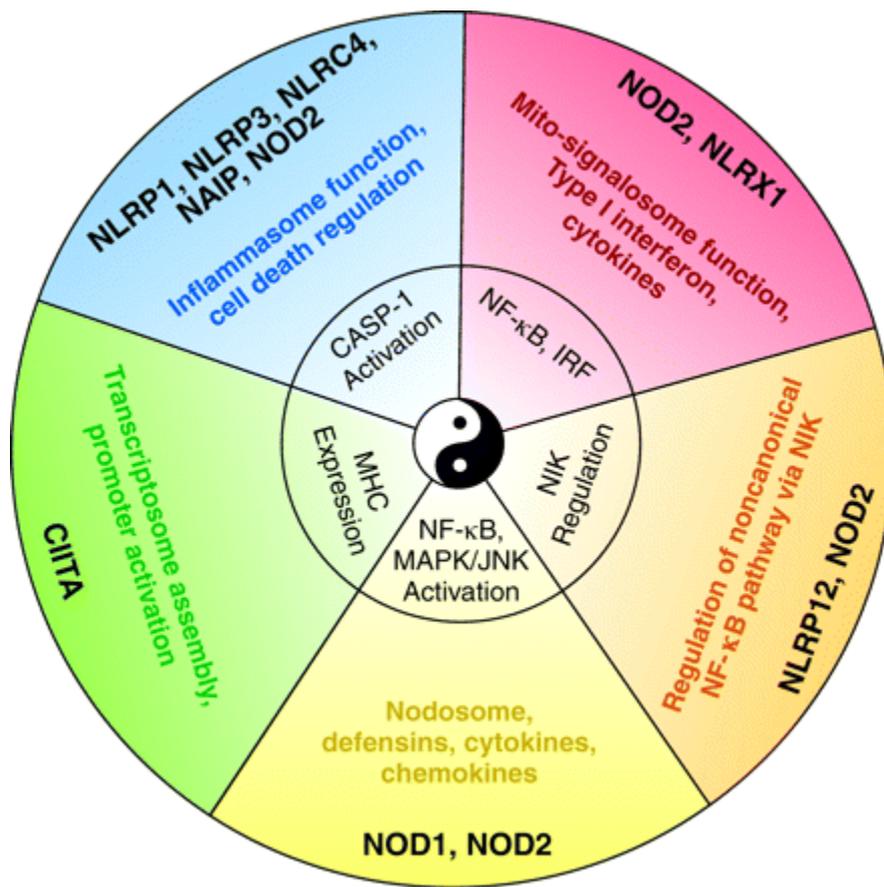


Figure 1.3 NLR Signallosomes, adapted from Ting *et al.*, 2010 [24]. This figure shows the concept that one NLR may serve multiple functions while multiple NLRs can also serve similar functions. Signalling modules here include the CIITA transcriptosome, the NF- κ B -activating NOD1/2 complex (referred to here as the ‘nodosome’), and the NIK pathway.

The term “inflammasome” is used to describe the cytoplasmic multi-protein oligomer which forms in response to a broad range of stimuli including DAMPs such as extracellular ATP, and PAMPs. It functions primarily to regulate caspase-1 activation which in turn leads to the proteolytic processing of the pro-cytokines pro-IL-1 α and pro-IL-18 [25]. In addition to this, active caspase-1 can also trigger a specific type of cell death, pyroptosis [26]. Pyroptosis is defined by its dependence on inflammasome activation and caspase-1 activity. Activation of pyroptosis ultimately leads to release of cellular contents and inflammation [27]. The exact composition of an inflammasome depends on the activator which initiates inflammasome

assembly. To date, four independent inflammasomes have been characterized: NLRP1, NLRP3 and NLRC4 (also known as IPAF), which are all members of the NLR family, as well as Absent In Melanoma 2 (AIM2) which belongs to the pyrin domain and HIN200 domain containing protein family [28] (See Figure 1.4). Each of these inflammasomes also appears to involve the adaptor protein Apoptosis-associated Speck-like protein containing a CARD (ASC) as well as pro-caspase-1 for cytokine processing. While NLRP1, NLRC4 and AIM2 recognise specific molecules; bacterial cell wall components such as muramyl dipeptide, flagellin and dsDNA respectively, the NLRP3 inflammasome responds to a much broader spectrum of stimuli. These range from pore forming toxins to ATP [29]. Along with this, it has been shown that a “priming signal” such as LPS, which upregulates NLRP3 expression, is critically required for NLRP3 activation [26], although it is yet unknown what the signal which activates NLRP3 itself might be.

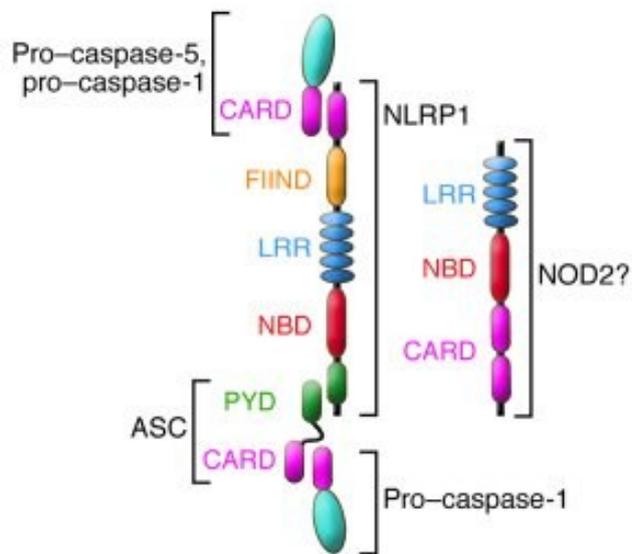
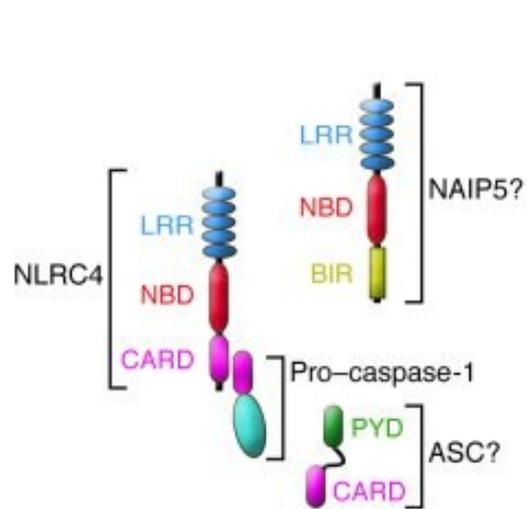
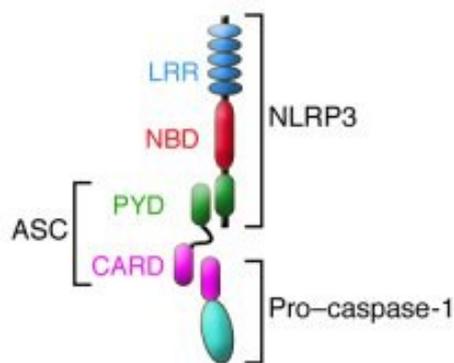
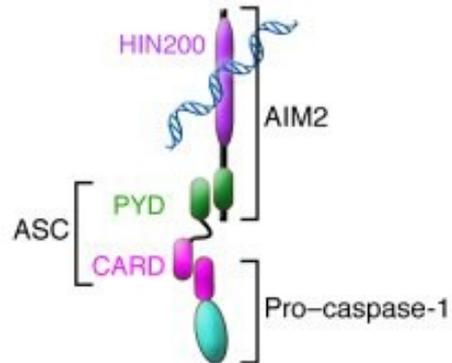
A NLRP1 inflammasome**B NLRC4 inflammasome****C NLRP3 inflammasome****D DNA inflammasome**

Figure 1.4 Graphical depiction of known inflammasomes. (A) NLRP1 contains, in addition to the NLR-typical LRR and NBD domains, a PYD, a FIIND, and a CARD. NLRP1 can recruit pro-caspase-1 and -5 and possibly forms a complex with NOD2. Recruitment of ASC enhances activation of pro-caspase-1. (B) NLRC4 contains a CARD that can directly recruit pro-caspase-1. Reports further demonstrate a role for ASC and possibly NAIP5 in NLRC4 inflammasome activation. (C) NLRP3 activates pro-caspase-1 via recruitment of ASC. (D) AIM2 is a bipartite protein consisting of a PYD and DNA-binding HIN200 domain and recognizes cytoplasmic double-stranded DNA and assembles the DNA inflammasome with ASC and pro-caspase-1. Adapted from Stutz *et al.*, 2009 [28]

C-type lectin receptors (CLRs) are a heterogeneous group of transmembrane proteins. Many are expressed in myeloid cells and signal in response to pathogen-derived or self-ligands to initiate or regulate cell activation. Sharing structural similarities with the immunoglobulin superfamily [30], the C-type lectin family of proteins encompasses upwards of 1,000 members with diverse functions including cell adhesion, regulation of natural killer function, complement activation, tissue remodelling, platelet activation, endocytosis, phagocytosis, and innate immunity [31, 32]. PAMPs recognised by CLRs are often made up of carbohydrates. Examples include glucans which are prominent constituents of the cell walls of fungi, plants, and mycobacteria; high-mannose structures expressed by some viruses, fungi, and bacteria; and fucose structures found on the surface of helminths and some bacteria [33, 34]. C-type lectins that recognize glycans, as well as others that do not bind carbohydrates have been shown to bind pathogens and to play a role in host defence. Also included in the same group are soluble defensins such as RegIII γ , which is produced in the gut and has direct microbicidal activity [35].

Evidence of synergy also exists between different PRR families in activation of inflammatory signals. For example a member of the CLR family, Dectin-1, is essential for the innate response to fungal pathogens [36]. It signals via Spleen Tyrosine Kinase (Syk) and the protein CARD9 [37]. Clear synergies between Dectin-1 and TLR2 have been reported, although the fungal ligand for TLR2 awaits definition. Syk is absolutely required for this synergy, as is MyD88 [38]. TLRs and NOD1 or NOD2 have been shown to signal to NF- κ B via the protein kinase Receptor-Interacting serine/threonine Kinase (RICK), which could be a point of synergy [39]. This is somewhat controversial, however, because there have also been reports that NOD2 ligands such as MDP inhibit signalling by multiple TLRs. This inhibitory effect was shown to involve IRF4 [40]. In addition, NOD1- and NOD2-mediated signalling has been shown to be enhanced in macrophages made tolerant to TLR activation [41]. TLRs

are also known to induce pro-IL-1 β production without causing activation of caspase-1 required for its processing to become a mature cytokine. This activation of caspase-1 in this scenario is mediated by inflammasome activation through NLR signalling.

1.2 Innate immune responses in the intestinal epithelial barrier

The intestinal epithelial barrier is a unique site in the human body, particularly in the context of innate immunity. Intestinal Epithelial Cells (IECs) line the intestinal tract and separate the underlying vascular gut mucosal tissue from the predominantly anaerobic lumen. This luminal space is packed with resident commensal microbes. In fact, there are 10 times more bacterial cells in the human gut than the total amount of human cells in the body [42]. Commensal, harmful and beneficial microbial communities co-exist with our intestine, a hospitable place for bacteria. They are provided with a stable microenvironment rich in nutrients, and these bacteria in turn provide the host with essential nutrients (such as vitamin K or biotin). Furthermore, these bacteria collaborate to aid in the digestion of food, prevent the expansion of pathogenic micro-organisms and even cooperate in intestinal development and in the modulation of the host immune responses [43]. Commensal bacteria, and specifically gut microbiota, have been given an increasing amount of attention with regard to their influence on our own bodily development and function at different levels. Areas affected include brain development and behaviour, metabolism, obesity, food digestion and overall resistance to stress and injury [44, 45]. Hence, the presence of these commensal microbes is a symbiotic relationship where both they and the host benefit from one another.

Due to the level of contact between commensal gastro-intestinal bacteria and host cells in this region, maintaining homeostasis is critically important. Homeostasis is a state of equilibrium: maintaining enough of an immune response to control microbial growth while avoiding over-activation of the immune system. The gut epithelium ensures that an effective immune response is raised to infectious micro-organisms while itself remaining predominantly unresponsive to the myriad commensal bacteria it is in contact with. This is achieved by preventing the contact of bacteria with immune cells through the formation of a physical barrier and the expression of antimicrobial peptides [46-48]. Dysregulation of

immune responses in this region can lead to breakdown of homeostasis and onset of colonic inflammation or colitis and Inflammatory Bowel Diseases such as Crohn's disease and ulcerative colitis [49]. These occur in clinically immune-competent individuals whose characteristic symptoms and signs arise from a robust, cytokine-driven, yet non-infectious inflammation of the gut [50]. Patients report gastrointestinal symptoms of abdominal pain, diarrhoea, and rectal bleeding as well as systemic symptoms of weight loss, fever, and fatigue. A better understanding of the mechanisms our body uses to maintain intestinal homeostasis would better allow us to treat patients with IBD when this balance has been dysregulated.

Homeostasis in the intestinal tract could be maintained by unique features of IECs, rather than unique features of commensal bacteria. In fact, the innate immune system, to a great extent, controls the microbiota in the intestine [43] and in addition to their role as a protective barrier at this mucosal interface, IECs have also been shown themselves to play a very important part in our body's immune response [51]. IECs are structurally and functionally polarized, with an apical surface facing the anaerobic intestinal lumen and a basolateral surface facing the adjacent cells in the lamina propria.

This polarisation gradient, leads to a differential gene expression gradient across the epithelium. This spatial organisation of the IECs allows for distinct functions at each surface utilising distinct membrane proteins [52]. The polarity and tight junctions of IECs play a particularly important part in this response, enabling the segregation of apical signals from basolateral signals in pathogen recognition. This is mediated through differentially expressed PRRs such as TLRs. TLRs are involved in regulation of the epithelial barrier through modulation of the critical mucosal antibody, Immunoglobulin A (IgA) from B cells in addition to the maintenance of intestinal integrity across tight junctions and expression of antimicrobial peptides [43]. TLR2 and TLR4, for example, have been shown to be present at

the apical surface of polarized, confluent IEC, in the cell line T84, which readily respond to acute stimulation with the corresponding bacterial ligands [53-55]. In contrast, TLR2 and TLR4 are mostly present in the cytoplasmic compartment in undifferentiated IEC and are expressed in much lower amounts than in differentiated IEC. This correlates with ligand tolerance as these cells are predominantly unresponsive to TLR2 and TLR4 ligands [53]. Furthermore, constitutively active expression of epithelial TLR4 does not induce mucosal inflammation in TLR4 transgenic mice [56, 57]. TLR2 expression in particular has been shown to play a specific role in IEC homeostasis. It controls tight-junction-associated intestinal epithelial barrier integrity in order to balance mucosal homeostasis against inflammatory-stress-induced damage. This is achieved by targeted modulation of barrier function proteins such as ZO-1 and Connexin43 (Cx43) following TLR2 activation [58, 59]. TLR2 and TLR4 have in fact been demonstrated to translocate from apical to basolateral surfaces upon stimulation by commensal bacterial associated molecular patterns [53]. TLR5 has been shown to only be expressed intracellularly or basolaterally in the colon [60], thereby avoiding an inappropriate inflammatory response to flagellated commensal bacteria present in the gut lumen. Furthermore, TLR5 knockout mice develop colitis spontaneously. This spontaneous colitis induction is protected against by cross breeding these mice with TLR4 knockout mice indicating that the mucosal inflammation is TLR4-dependent without TLR5 regulation [61]. An intracellular protein in immune cells, TLR9 has been shown to be expressed on the cell surface of IEC, both on the apical and basolateral membranes. *In vitro* studies using IEC lines in addition to several *in vivo* studies using mice have revealed distinct signalling patterns between apical versus basolateral stimulation of TLR9 [62-64]. Basolateral stimulation of TLR9 initiates an inflammatory signalling cascade while apical stimulation induces signals which inhibit inflammatory responses from basolateral activation of several TLRs. This is how, in a single IEC, TLR9 is able to induce immune tolerance from

activation by commensal bacteria in the apical surface while still activating an inflammatory response when potentially pathogenic invasive microbes are detected having crossed the barrier on the basolateral membrane. The *in vivo* model of colitis has been studied using a substance known as dextran sodium sulphate (DSS). DSS is known to disrupt the intestinal barrier and alter intestinal permeability, allowing intestinal microbiota to gain access to the intestinal mucosa [65]. Surprisingly, stimulation of TLRs 2, 3, 5 and 9 has been shown to protect against DSS induced colitis, while knock-out mice for TLR2, 4 and MyD88 are more susceptible to DSS colitis [57]. These results are the opposite of what would be expected based on the direct effects of TLR activation, and therefore suggest that TLRs limit inflammation indirectly in the intestinal epithelium.

With regard to the role of NLRs in IECs, much evidence exists supporting a protective role for these intracellular receptors in IBD. For instance, Nod1 promotes epithelial cell survival, reducing apoptosis and permeability [66]. Nod2 is known to promote inhibition of TLR signalling and inflammation in addition to enhancing regulatory T cells which curtail inflammatory signalling and, as discussed earlier, loss of function mutations have been associated with development of IBD [22, 23, 40, 67, 68]. NLRC4-deficient mice have been shown to have increased levels of CXCL1 in colitis and exhibit tumour formation. Additionally, NLRC4 is believed to be involved in differentiation between commensal and pathogenic bacteria [69, 70]. The role of NLRP3 has yet to be fully uncovered. One study has implicated DSS induced colitis in mice as being mediated by NLRP3 [71]. On the other hand, it can promote barrier integrity and help maintain intestinal homeostasis [72, 73]. It is also known to prevent colitis-associated tumourigenesis [74, 75]. NLRP6 inhibits tumour formation during colitis and excessive epithelial proliferation [76, 77]. NLRP12 controls pro-inflammatory cytokines and epithelial proliferation. It also represses canonical NF- κ B signalling and tumourigenesis [78].

RLRs play a role in innate immune recognition in IECs also. For example viral RNA sensors RIG-I and MDA-5 are known to be expressed intracellularly in IECs and are responsible for the detection of double stranded (ds) RNA viruses [79]. However, it is unlikely that they play a role in regulating homeostasis as they are specifically designed to prevent invasive viruses from hijacking our cellular machinery and do not recognise ligands from commensal microbiota. While several roles for CLR have been implicated in immune cells involved in gut inflammatory regulation, there have been few studies on CLR in IECs to date. We do know of a soluble CLR, RegIII γ , which is induced by MyD88 in IECs and is known to have bactericidal properties against invading pathogens such as *L.monocytogenes* [80]. However, little else is known on CLR expression or function, inflammatory or protective, in IECs at present.

The expression and localisation of TLRs, RIG-I receptors and NLRs in the epithelia of mice and humans is summarised in Table 1.1 [81]. This table draws attention to the fact that while some families, such as TLRs, have received a lot of attention and are quite well characterised, new information such as the functionality and expression of CLR is only just coming to light [82-84] with much investigation yet to go. CLR are, in fact, omitted from this table as they require further investigation and indeed, an update on expression/function of the more extensive NLR family's in this region would also be beneficial.

A greater need for understanding the complete function of PRRs in IECs is called for in this regard. Uncovering the mechanisms underlying dysregulated inflammatory responses would allow us to better treat such inflammatory disorders as IBD. Learning how tolerance is achieved would be beneficial for several reasons. Overcoming tolerance would further the development of convenient orally-delivered vaccines through activation of the adaptive immune system. Conversely, manipulating innate immune responses in this region may also allow us to induce tolerance against self-antigens to treat for auto-immune disorders [85]. A

simpler approach is the study of how our innate immune system responds to pathogenic infection through the intestinal epithelial interface. All work performed in this thesis involved investigating the role of PRRs in the intestine. In the final two results chapters I focussed on detection of the pathogen *L.monocytogenes* by PRRs in the intestine and as such, I aim to introduce this topic in the next section.

Receptor	Subcellular localization	Ligand	Origin of ligand
TLR2	Cell surface	Lipoteichoic acid Lipoprotein/ lipopeptides Hemoagglutinin protein Glycosyl-phosphatidylinositols	G (+) bacteria Various pathogens Viruses (Measles Virus) Parasites (<i>Toxoplasma gondii</i>)
TLR2/1 TLR2/6	Cell surface Cell surface	Triacyl lipopeptides Diacyl lipopeptides Zymosan	Bacteria and mycobacteria Mycobacteria Fungi
TLR3	Cellular compartment	dsRNA	Viruses
TLR4	Cell surface	Lipopolysaccharide Envelope proteins Glycosyl-phosphatidylinositols	G (-) bacteria Viruses (Respiratory Syncytial Virus) Parasites (<i>Toxoplasma gondii</i>)
TLR5	Cell surface	Flagellin	Bacteria
TLR7/8	Endosomal	ssRNA	Viruses
TLR9	Endosomal/cell surface	CpG-containing DNA	Bacteria and viruses
TLR11	Cell surface	Uropathogenic bacteria component Profilin	Bacteria (uropathogenic <i>Escherichia coli</i>) Parasites
NOD1	Cell cytoplasm	Meso-diaminopimelic acid	PGN of G (-) and some G (+) bacteria
NOD2	Cell cytoplasm	Muramyl dipeptide	PGN of G (+) and G (-) bacteria
RIG-I	Cell cytoplasm	5'-triphosphate-bearing RNAs	Viruses

Table 1.2 The PRRs of the Intestinal Epithelium: Subcellular localization, and recognized ligands. Adapted from Well *et al.* 2011 [81]. G (+), Gram-positive; G (-), Gram-negative; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; PGN, peptidoglycan; Meso-DAP, γ -D-glutamyl-meso-diaminopimelic acid; MDP, muramyl dipeptide NOD1, nucleotide oligomerization domain-like receptor 1; NOD2 nucleotide oligomerization domain-like receptor 2.

1.3 *Listeria monocytogenes*

Listeria monocytogenes (*L.monocytogenes*) is a foodborne Gram-positive bacterium and a facultative infectious intracellular pathogen that causes listeriosis. This may lead to life threatening diseases (such as septicaemia and meningitis) [86], particularly in immunocompromised individuals, pregnant women, and neonates [87]. Epidemics are associated with severe symptoms and high mortality rates. Pathogenic *listeriae* enter the host primarily through the intestine [88]. They cross the intestinal barrier by invading intestinal epithelial cells. This initial, subclinical step of listeriosis is actually believed to be common due to the frequent presence of pathogenic *L.monocytogenes* in food, such as meat and dairy products [88]. Between crossing the IECs and entry into the bloodstream, they reach their target organs, the liver and spleen via the lymphoid system and the blood, where they are internalized by splenic and hepatic macrophages. In the liver, *listeriae* actively multiply until the infection is controlled by an activated Dendritic Cell (DC) and adaptive immune cell-mediated immune response. During severe infections, the bacteria disseminate via the blood and cross the blood–brain barrier resulting in infections of the meninges and the brain. Furthermore it can cross the foeto-placental barrier in pregnant women which leads to infection of the foetus.

As with all pathogens, the ability to evade immune surveillance is critical to a successful infection, and *L.monocytogenes* employs many strategies by which it can evade host immune responses, thus promoting survival. *L.monocytogenes* infection of macrophages proceeds via phagocytosis followed by escape from the phagosome into the cytosol through the action of the pore-forming toxin Listeriolysin O (LLO) [89, 90]. Intracellular secretion of a pore-forming toxin appears to be unique to *L.monocytogenes* infection. Once it becomes established here, it is resistant to intracellular killing [91]. *L.monocytogenes* creates an intracellular niche dependent on its capacity to adhere to, invade and multiply within a

variety of normally non-phagocytic cells, such as enterocytes, hepatocytes, fibroblasts and endothelial cells [92]. This ability of *L.monocytogenes* to establish itself intracellularly avoiding phagocytosis aids the bacterium in evading host responses and ensures effective tissue dissemination and enhanced virulence. For a long time this mechanism of intracellular spread was thought to be the main strategy employed by *L.monocytogenes* to avoid immune detection. However, with the discovery of host recognition receptors and increased understanding of innate immune signalling pathways, it has been shown that the bacterium is also capable of avoiding detection through other mechanisms. An example of this is the modification of bacterial ligands to avoid detection by the PRRs of the innate immune system. Additionally, they are known to modulate host signalling pathways and target host immune effector cells, thereby evading host innate defences [93] (see Figure 1.5).

L.monocytogenes has more recently been shown to modulate the host immune signalling network by a downstream approach through epigenetic manipulation. Epigenetics is the study of heritable changes in gene expression or cellular phenotype, caused by mechanisms other than changes in the underlying DNA sequence. Chromatin, the highly organised structure into which our DNA is packed, is made up of nucleosomes; an octamer of a family of proteins called histones. The structure of the chromatin may be remodelled by proteins which perform acetylation, phosphorylation, methylation and ubiquitination of these histones and the DNA [94-96]. This opens up/closes off parts of the genome for transcription and thus regulation of gene expression may be controlled by these histone modifying proteins [96]. *L.monocytogenes* has been shown to induce dephosphorylation of histone H3 and deacetylation of histone H4 by secretion of LLO prior to invasion – leading to reduced transcription of some key host immunity genes [95]. Among these are IRF3, C-X-C motif Ligand 2 (CXCL2), which encodes a proinflammatory chemotactic chemokine, and Dual specificity protein phosphatase 4 (DUSP4), a gene required for MAPK signalling [95]. In this

way, *L.monocytogenes* can reduce initial host inflammatory signalling by blocking TLR2 and NOD1 induced responses such as p38 and ERK MAPK pathways [95].

L.monocytogenes manipulates the endocytic and many host-cell signalling cascades in order to replicate and avoid detection. Some of these have been highlighted in Figure 1.5. However, host cells possess surveillance systems at different cellular compartments capable of detecting *L.monocytogenes* infection and activating defence pathways which in most cases might control infection [97].

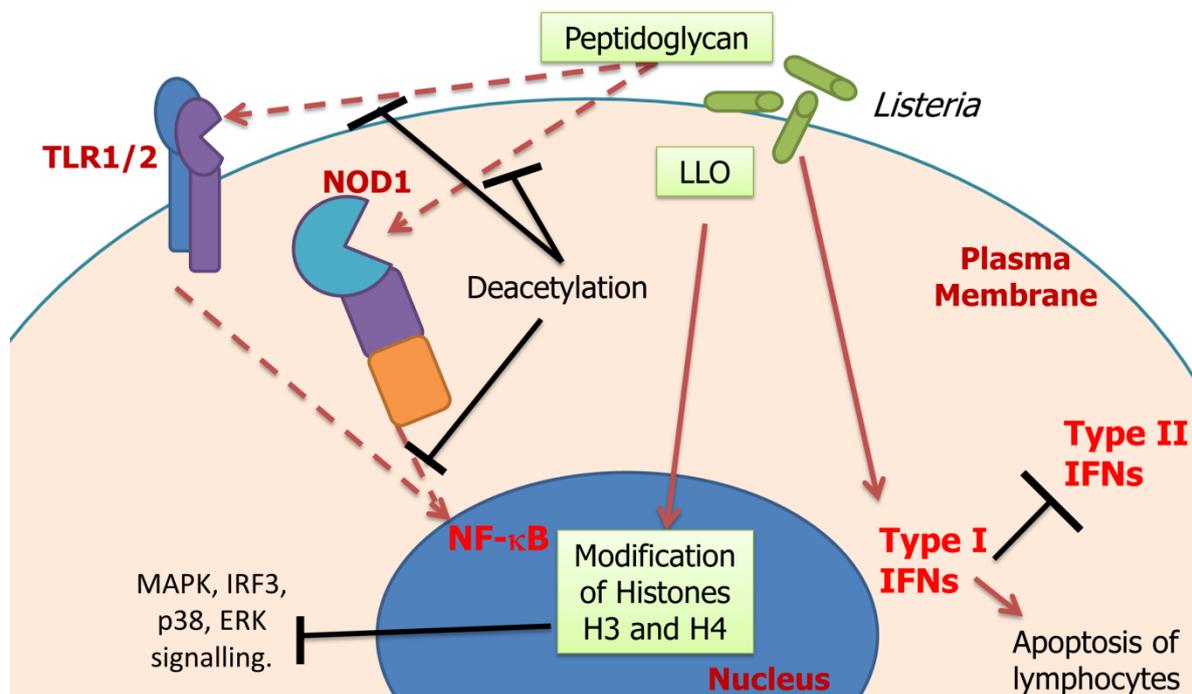


Figure 1.5 Mechanisms by which *L.monocytogenes* evades innate immune recognition.

The figure above illustrates some known mechanisms by which *Listeria* evades innate immune responses. In addition to establishing itself intracellularly avoiding phagocytosis, *L.monocytogenes* releases the toxin LLO prior to invasion. This is involved in the modification of histones H3 and H4 which in turn leads to reduced signalling by MAPK, IRF3, p38 and ERK. Additionally, *L.monocytogenes* deacetylates its peptidoglycan (PGN) to avoid recognition by TLR2 and NOD1. It is also thought to induce type I IFNs in order to reduce type II IFN signalling and increase apoptosis of lymphocytes.

1.4 Systemic innate immune responses to *L.monocytogenes* infection.

In humans, infection normally initiates at the intestinal epithelial interface. *L.monocytogenes* can invade epithelial cells through interactions of its virulence proteins with host receptors. For example, Internalin A (InlA) from *L.monocytogenes* interacts with Epithelial cadherin (E-cadherin) to mediate entry via a membrane bound vacuole [98, 99]. Internalin B (InlB) on the other hand, binds the host receptor tyrosine kinase, c-Met (natural receptor for hepatocyte growth factor HGF) thereby making use of multiple mammalian cell types (including murine liver cells) for invasion [100, 101]. Another important *L.monocytogenes* virulence factor known to be important in infection is the *L.monocytogenes* adhesion protein (LAP). Human Hsp60 has previously been identified as the epithelial receptor for LAP [37]. Following escape from the phagosome through LLO, the bacteria may now replicate to high numbers in the cytosol. Cytosolic *L.monocytogenes* express ActA which can induce host-cell actin polymerisation and facilitates direct cell to cell spreading; this again aids the bacteria to escape extracellular immune detection [102, 103].

Intestinal *L.monocytogenes* infection is not efficient in mice. This has been attributed to poor interactions between InlA and E-cadherin on murine intestinal epithelial cells [104]. Hence, due to the difficulties surrounding invasion of the bacteria when administered orally in mice, studies investigating immune responses to *L.monocytogenes* infection *in vivo* have focussed more heavily on systemic infection through intraperitoneal or intravascular injection, thus bypassing the intestinal epithelium. Furthermore, the ability of the pathogen to invade and multiply within phagocytic cells has shifted the focus of bacteria/host interactions away from IECs and more towards immune cells in order to further elucidate its evasion strategies.

Autophagy is a cytosolic self-digestion pathway which also plays a part in innate immunity against intracellular pathogens. Components in the cells cytoplasm are sequestered

into double membrane structures known as autophagosomes and degraded [105]. *L.monocytogenes* has been reported to evade autophagy in macrophages in addition to escaping autophagic vacuoles and replicating cytosolically [106]. Another study using severe combined immunodeficient (SCID) mice reported *L.monocytogenes* within liver granuloma macrophages to be replicating within vacuoles which were termed spacious *L.monocytogenes* -containing phagosomes (SLAPs) [107]. The formation of these SLAPs is thought to result from an attempt to prevent bacterial escape into the cytosol by autophagy of damaged phagosomes. However, LLO prevents maturation of these phagosomes thereby establishing a more steadfast infection by allowing bacterial replication and survival within these compartments [107].

We know that when *L.monocytogenes* enter the bloodstream from the gut lumen, they move to their target organs; the liver and the spleen where they continue to multiply. However, there is an abundance of sentry cells in the bloodstream armed with a vast array of various PRRs. Within 1-2 minutes of entering the bloodstream, *L.monocytogenes* is taken up by various myeloid cells and other tissues [108]. Resident myeloid cells in the marginal zone of the spleen, such as dendritic cells, macrophages and neutrophils, function to filter the bacteria out of the blood [109, 110]. A secreted *L.monocytogenes* protein, p60, has been found to bind DCs and stimulate them to robustly activate NK cells in the absence of other *L.monocytogenes* factors in an IL-18 dependent manner [111, 112]. Production of IFN- γ induces bacteriocidal activity in macrophages and promotes maturation of monocytes to the main effector cells, the TNF α and inducible Nitric Oxide Synthase (iNOS) producing effector Dendritic Cells (TipDCs). Some of these phagocytic cells manage to protect the host by killing engulfed bacteria, while others create an environment for rapid *L.monocytogenes* growth and dissemination. While patrolling neutrophils phagocytose relatively few bacteria initially [108, 109], their extremely effective killing has been shown to reduce

L.monocytogenes burdens in the liver from 10-100-fold within 12-24 hours post-infection [113]. However, these effects are not seen in the spleen or peritoneal cavities.

Most reports seem to agree that initial detection occurs at the cell surface or, in the case of phagocytic cells, in phagosomes by TLR2 and, following internalisation, in the cytosol by NOD-like receptors (NOD1, NOD2) as well as NALP3 and NLRC4 (IPAF) [41, 114, 115], which can lead to NF- κ B-dependent pro-inflammatory gene expression. However, there remains some controversy and conflicting reports over which PRRs play the key effective parts in *L.monocytogenes* detection. For example, caspase-1 activation is known to be required for the clearance of *L.monocytogenes* in murine infection [116] and recent reports have suggested that *L.monocytogenes* activates caspase-1 through multiple NLRs. However, although much work has been carried out in this area, there are conflicting reports regarding the contributing roles of NLRP3, AIM2, and NLRC4 inflammasomes leading to caspase-1 activation in *L.monocytogenes* infected macrophages [72, 74, 76-80].

TLR2-deficient mice have been shown to have a higher susceptibility to infection with *L monocytogenes* [114]. CD14 is involved in co-recognition by TLR2 of various TLR ligands, such as peptidoglycan from *Staphylococcus aureus* and *Streptococcus pneumonia* [117], human cytomegalovirus [118] and secreted microbial products from group B *Streptococcus* [119]; lipoteichoic acid-induced cell activation also depends on CD14 [120]. By using neutralizing antibodies to CD14 in TLR2-transfected CHO cells, the induction of TNF α production by heat-killed *L.monocytogenes* (HKLM) was shown by one research team to depend, in part, on CD14 associated with TLR2 [121]. Reduced CD14 expression results in bacterial persistence, reduced NOS2 expression in the liver, and heightened mortality [122]. In addition to this, the same group discovered that mice that are both CD14 deficient and TLR2 deficient display susceptibility to infection that is comparable to that of mice deficient in either CD14 or TLR2 alone [122].

While TLR2 may sense lipoteichoic acid found on the surface of *L.monocytogenes*, the bacteria is known to carry out N-deacetylation of peptidoglycan to avoid this detection by TLR2 and NOD-like receptors [123]. Interestingly, Edelson *et al.* (2002) [124] were able to show a redundancy for TLR2 in *L.monocytogenes* recognition such that in its absence, other molecules were sufficient for its control. In macrophage cells, killing of *L.monocytogenes* was not altered by the absence of TLR signals and was found to occur through a MyD88 and TLR2 independent mechanism [124]. However, MyD88 was shown to be essential for early *L.monocytogenes* resistance and full activation of the immune response in mice. This was in part due to its role as adaptor molecule for downstream signalling in the IL-1 and IL-18 receptors but mainly due to its role as adaptor molecule in TLR signalling, i.e. MyD88-independent responses to *L.monocytogenes* alone are insufficient for *in vivo* resistance [124] indicating that MyD88 but not TLR2 may be essential for *L.monocytogenes* recognition. A more recent finding by Jones *et al.* (2011) [125] using TLR2 deficient and wild type macrophages outlined a clear role for rapid AIM2 inflammasome activation in *L.monocytogenes* and *F.novidica* infection [125]. They also explain how in similar studies using the same methods which found no link between TLR2 and inflammasome activation [126], a much higher MOI had been used which may have masked the actions of TLR2 observed in their own model of the experiment with a smaller and perhaps more realistic MOI.

Two studies involving TLR2-mediated type I IFN production in response to *L.monocytogenes* emerged quite close together. Aubry *et al.* used a mutant strain of *L.monocytogenes* $\Delta pgdA$, which is unable to modify its peptidoglycan (*pgdA* is the gene in *L.monocytogenes* responsible for deacetylation of peptidoglycan) [127]. Using this strain, they were able to demonstrate an unconventional signalling pathway to IFN- β production requiring TLR2 and bacterial internalisation. Induction of IFN- β was independent of MAL or

MyD88 but required TRIF signalling to activate transcription factors IRF3 and IRF7 in peritoneal macrophages [127]. Dietrich *et al.* performed a similar study using knockdown of *pgdA*. This led to less IFN- β production in peritoneal macrophages [128]. TLR2-dependent IFN- β induction was shown to be triggered intracellularly following ligand binding and while TLR2 or TRIF deficiency strongly reduced IFN- β synthesis, it was not enough to silence it. Furthermore, they managed to show that blocking TLR2 internalisation or endolysosomal acidification inhibited the ability of TLR2 to induce type I IFN but not pro-inflammatory responses. The group then investigated the potential contribution of intracellular, nucleic acid-dependent pathways. Intact but not DNase-treated *L.monocytogenes* DNA was shown to significantly induce IFN- β [128]. IFN- β was significantly reduced in macrophages transfected with DNase treated over non-DNase treated lysosome-digested *L.monocytogenes* [128], suggesting the involvement of another pathway.

Double deficiency in MyD88 and TRIF was shown not to affect wild-type *L.monocytogenes* induced type I interferons, suggesting the mechanism to be independent of TLR signalling [129]. RIG-I and MAVS knock-down resulted in abolishing the IFN response in epithelial cells, but the IFN response in monocytic cells remained unaffected. By contrast, knockdown of DNA sensing Stimulator of Interferon Genes (STING) in monocytic cells reduced cytosolic *L.monocytogenes*-mediated type-I-IFN induction. These results suggest that detection of *L.monocytogenes* RNA by RIG-I represents a non-redundant cytosolic immune-recognition pathway in non-immune cells lacking a functional STING dependent signalling pathway [130].

RNA interference and inhibitor experiments using human PBMCs as well as experiments in NLRP3 and RIP2 knockout bone marrow-derived macrophages were performed by Meixenberger *et al* in 2009, in order to determine the PRRs (and inflammasomes) which play key roles during *L.monocytogenes* infection. The results

indicated that the *L.monocytogenes*-induced IL-1 β release was dependent on the adaptor protein ASC, caspase-1, and NLRP3, whereas in this case, NOD2, Rip2, NLRP1, NLRP6, NLRP12, NLRC4, and AIM2 appeared to be dispensable [131].

A study carried out in 2010 by Kim *et al.* showed NLRP3 and AIM2 inflammasomes to be critical in macrophages for control of *L.monocytogenes* infection [26]. LLO, used by the bacteria in escaping phagosomal compartments, was outlined to be critical in activating the apoptosis-associated speck-like protein containing a CARD (ASC) which was in turn critical for activation of the NLRP3 inflammasome and the AIM2 inflammasome. NLRC4 and NLRP1 were reported as being dispensable i.e. activation of Caspase-1 and subsequent IL-1 β production were not critical in *L.monocytogenes* detection. These results would suggest that under conditions of predominant lysosomal damage, yet limited cytosolic replication, NLRP3 inflammasome activation would play the major role in detection of the bacteria. This might happen when *L.monocytogenes* has infected the cell and is escaping into the cytosol. Following this, in the case of high cytosolic replication, AIM2 recognition of *L.monocytogenes* DNA may predominate in driving the inflammatory signal [26].

Similar to the above findings, another group, using RNA interference studies on PBMCs found no evidence that NLRC4 is critical for caspase-1 activation in *L.monocytogenes*-infected macrophages [26, 131]. Others observed a partial role for NLRC4 in caspase-1 activation in *L.monocytogenes* infected macrophages [132]. NLRC4 (IPAF) activation is dependent on the release of cytosolic flagellin, yet flagellin expression in *L.monocytogenes* is temperature dependent with expression in some strains being switched off at 37°C [133]. In contrast with other results, it was reported by Wu *et al.* that caspase-1 processing is almost absent in LPS-non-primed NLRC4-KO macrophages infected with WT and flagellin-deficient *L.monocytogenes*. This demonstrates a critical role for NLRC4-mediated Caspase-1 activation in *L.monocytogenes*-infected macrophages in the absence of

LPS priming [134]. This could explain the seemingly contradictory results between these findings and the previous studies in which macrophages were primed with LPS prior to infection with *L.monocytogenes*. The WT *L.monocytogenes* strain (10403s) used in the experiments by Wu *et al* expresses flagellin at 37°C [133], which excludes the possibility that defective expression of flagellin under the conditions of the experiment is responsible for the failure to activate caspase-1 in NLRC4-KO macrophages. LPS priming was shown to potentiate inflammasome activation by *L.monocytogenes* in NLRC4-KO and NLRP3-KO macrophages, indicating that prior LPS priming of macrophages can bypass the requirement for NLRC4 and/or NLRP3 [134]. Recent studies documented that this LPS priming triggers de novo protein synthesis via TLR4, which is a critical prerequisite for inflammasome activation by various danger signals [135]. It is therefore likely that LPS priming may up-regulate inflammasome or inflammasome-related gene expression to bypass upstream NLRC4 signalling. LPS priming may also obscure the roles of various inflammasomes in regulating caspase-1 activation in response to *L.monocytogenes* infection, since while knockout of NLRP3 is not sufficient to abrogate caspase-1 activation by either wild-type or flagellin-deficient *L.monocytogenes*, it does reduce caspase-1 activation significantly [134]. This suggests that NLRP3 inflammasomes contribute to caspase-1 activation in response to *L.monocytogenes* infection, which is consistent with previous reports [26, 29, 131, 132]. While activation of the NLRC4 inflammasome by *L.monocytogenes* was shown to enhance innate-response mediated protection, resulting in faster clearance of primary challenge, this gives rise to a dampened secondary response causing impaired control of secondary *L.monocytogenes* challenge [136, 137].

Nitric Oxide Synthase 2 (NOS2) has been reported to be dispensable for killing of *L.monocytogenes* by IFN- γ -activated macrophages while NOS2 deficient mice are only modestly more susceptible to systemic *L.monocytogenes* infection [124, 138, 139]. In a study

by Cole *et al*, TLR2, TLR3, TLR4 and TLR9-activated macrophages were shown to have killed cell-free *L.monocytogenes* before they had invaded a host cell. However, TLR activation was found to be enhancing the efficiency of *L.monocytogenes* spread from “donor” to “recipient” macrophages. This enhancement required nitric oxide (NO) production by NOS2 [140]. NO increased *L.monocytogenes* escape from secondary vacuoles in recipient cells and increased *Listeria* survival and growth in the same cells. NO also promoted *L.monocytogenes* spread during systemic *in vivo* infection, as shown by the fact that inhibition of NOS2 reduced spread dependent *L.monocytogenes* burdens in mouse livers [140]. By contrast, when IFN- γ was added with LPS to pre-stimulate the cells, it failed to prevent NO-dependent increases in recipient cell infection with *L.monocytogenes* via cell-cell spread. This result indicates that during cell-cell spread the effects of LPS stimulation trump those of IFN- γ .

Studies on mice have shown that in the absence of type I interferon signalling in mice, *L.monocytogenes* cannot reach as high titres as wild type mice and that mice with elevated levels of type I interferons have greater bacterial loads [93, 141]. A study by Carrero *et al*. shows that type I interferons induce T cell apoptosis early during *L.monocytogenes* infection, resulting in greater IL-10 secretion by phagocytic cells which in turn dampens the innate immune response [142]. Also, it is known that IFN $\alpha\beta$ production may down regulate IFN γ R thus reducing vital IFN γ responsiveness in the host [143]. This suggested that *L.monocytogenes* might induce type I interferons to its benefit to either directly enhance its growth, or more likely, down modulate a part of the immune response that plays an important role in controlling bacterial growth by means of “misdirection” of the immune system.

More recently, however, it was revealed that the route of infection taken by *L.monocytogenes* plays an important role in determining the effects that type I interferons have on the bacteria. Kernbauer *et al*. [144] examined the effects of type I IFN signalling on

mice following intragastric versus intraperitoneal infection with *L.monocytogenes*. Until this point, almost all studies focussed on systemic infection with the bacteria through peritoneal injection due to the poor uptake of the bacteria through the mouse intestine. This intragastric route of infection was based on work by Wollert *et al.* [145] whereby they used a strain of ‘murinised’ *L.monocytogenes* i.e. the InlA gene was improved for interaction with mouse E-cadherin leading to increased invasion of *L.monocytogenes*. Mice deficient in IFN α/β receptor (IFNAR) were shown to have higher lethality and higher bacterial titres when infection was administered intragastrically. Peritoneal or bloodstream infection, however, were more easily controlled in mice deficient in type I interferons. While the type I interferons were not found to inhibit invasion of the gut mucosa or mucosa associated lymphoid tissue, absence of a type I IFN response exacerbated inflammatory pathology in livers of mice infected via the gastrointestinal route. Type I IFNs accelerated and increased proinflammatory cytokine activity after intragastric *Listeria monocytogenes* infection [144]. Most importantly, increased IFN γ production in the absence of type I IFN responsiveness was delayed and less pronounced after intragastric compared to intraperitoneal infection. Therefore, the influence of the entry route on the kinetics of IFN γ regulation by IFN-I may function as a key determinant of innate resistance.

An earlier study had managed to demonstrate another possible beneficial effect of type I IFN signalling in defence against intracellular bacterial infection [146]. It was found that in murine infections with *L.monocytogenes*, both MyD88 and type I IFN mediated signals provided overlapping contributions to Monocyte Chemoattractant Protein-1 (MCP-1) production and recruitment of monocytes. In the absence of either signalling pathway, chemokine expression levels were only partially reduced, but residual MCP-1 production was sufficient to mediate monocyte emigration from bone marrow. It was only when both MyD88 and IFNAR mediated signals were absent that MCP-1 production was significantly reduced

to impair monocyte recruitment and effector Tip-DC accumulation in the infected spleen [146].

A schematic of systemic infection and the vital role played by the innate immune system in eventual clearance of the bacteria is displayed in Figure 1.6. The bacteria escape through the intestinal epithelium to the bloodstream by LLO mediated escape from vacuoles. In the bloodstream, the bacteria quickly disseminate to the liver and spleen. They are also capable of invading phagocytic cells such as DCs, neutrophils and macrophages where they continue to multiply in the cytosol. During this stage, NALP3, AIM2 and NLRC4 are all known to be involved in inflammasome activation; resulting in an increase of intracellular killing of the bacteria. Subsequent activation of TIP DCs and lymphocytes ultimately mediates clearance of the bacteria from the host.

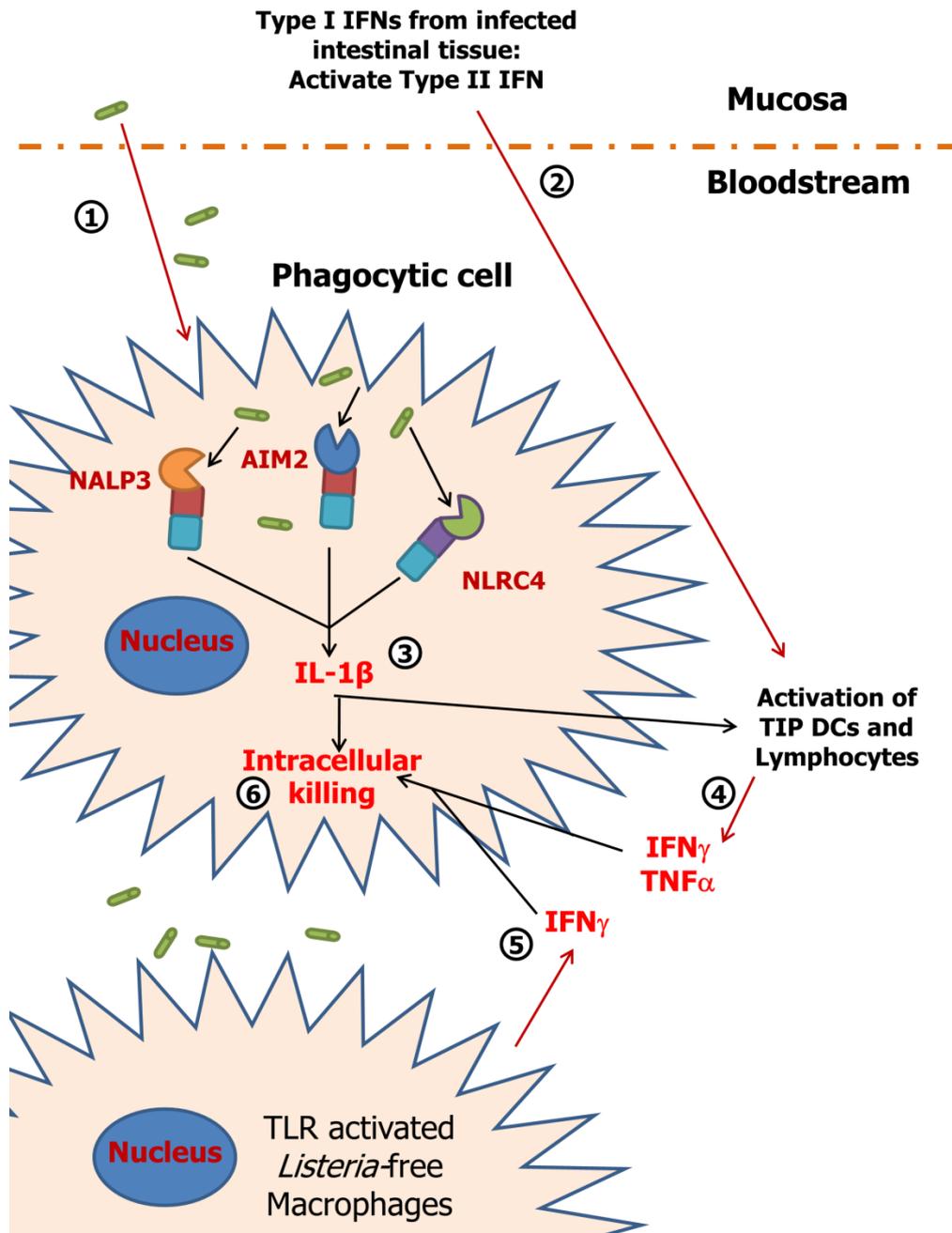


Figure 1.6 PRRs involved in the systemic innate immune response to *L. monocytogenes* infection. The figure above depicts the systemic clearance of *L. monocytogenes* through innate immune signalling. **1:** *L. monocytogenes* migrate from the intestinal epithelia through to the underlying mucosa and into the bloodstream. **2:** Type I IFNs from IEC recognition of the bacteria activates type II IFN responses in the bloodstream and activation of effector cells. **3:** *L. monocytogenes* invade and multiply within the cytosol of phagocytic cells. Here, they are recognised by NLRs and inflammasome activation leads to activation of effector cells, TIP DCs, and initiation of the adaptive immune response. **5:** Uninfected TLR-activated phagocytes produce cytokines such as type II IFNs which, together with the effector cells and inflammasome activation, induce ROS-mediated intracellular killing of *L. monocytogenes*.

1.5 Innate immune recognition of *Listeria monocytogenes* in the gut

Most natural infections with *L.monocytogenes* are acquired following ingestion and the subsequent uptake of bacteria through the mucosa of the gastrointestinal tract by intestinal epithelial cells. However, as discussed earlier, most laboratory studies characterizing immune responses to this pathogen use either intravenous or intraperitoneal inoculation to initiate infection. Consequently, most studies of infection with *L.monocytogenes* focus on immune responses to systemic infection bypassing the gut. By contrast, mucosal immune responses to infection with *L.monocytogenes* are less well characterized but are increasingly becoming the focus of investigation. This interface is where the pathogen first encounters our body's PRRs. However, as discussed earlier, there is a requirement for a review of PRR expression in Intestinal Epithelial Cells so that we may better understand their specific function in this setting.

L.monocytogenes can invade epithelial cells through interactions of its virulence proteins with host receptors. As discussed previously, InlA from *L.monocytogenes* interacts with E-cadherin to mediate entry via a membrane bound vacuole [98, 99]. Another important *L.monocytogenes* virulence factor, LAP, has been shown to promote adhesion to intestinal epithelial cells and its expression is required for full virulence in orally infected mice [36]. Human Hsp60 has previously been identified as the epithelial receptor for LAP [37]. In wild-type *L.monocytogenes*, the interaction of InlA with the epithelial receptor E-cadherin promotes invasion of intestinal epithelial Caco-2 cells, while interaction of LAP with the epithelial receptor Hsp60 mediates para-cellular transepithelial translocation [38].

As mentioned earlier, most studies investigating *L.monocytogenes* infection have focussed on systemic infection using mouse models by administering the bacteria through intravenous or intraperitoneal injection, therefore bypassing the gut epithelium. However, Kobayashi *et al.* in 2005 outlined a study whereby NOD2^{-/-} or wild type mice were infected

with *L.monocytogenes* by intravenous, intraperitoneal or intragastric administration [115]. No difference in bacterial numbers in the liver and spleen or IL-6 production was seen between the wild type mice and the NOD2^{-/-} mice challenged with intraperitoneal or intravenous injection. However, when *L.monocytogenes* was administered intragastrically, more accurately reflecting the infection scenario in humans, NOD2^{-/-} mice showed significantly greater numbers of bacteria recovered from both the liver and the spleen than did wild-type mice. Furthermore, Mice lacking NOD2 failed to generate adaptive responses to *L.monocytogenes* in the gut [115]. Hence, NOD2 is believed to have a crucial role in detecting *L.monocytogenes* in the gastro-intestinal tract.

A study by Lipinski *et al* demonstrated using Caco-2 intestinal epithelial cells that the NAD(P)H oxidase family member Dual Oxidase 2 (DUOX2) is involved in NOD2-dependent ROS production [147]. Co-immunoprecipitation and fluorescence microscopy were used to show that DUOX2 interacts and co-localises with NOD2 at the plasma membrane. Moreover, simultaneous overexpression of NOD2 and DUOX2 was found to result in cooperative protection against the bacterial cyto-invasion of *L.monocytogenes* [147]. RNAi-based studies revealed that DUOX2 is required for the direct bactericidal properties of NOD2 - highlighting ROS as effector molecules of protective cellular signalling in response to a defined danger signal carried out by a mammalian intracellular NLR system [147].

Studies following the activation of a mouse intestinal epithelial cell monolayer by *L.monocytogenes* infection demonstrated that while infection is necessary for detection of *L.monocytogenes* in IECs, chemokines may be produced by adjacent non-infected cells [148]. Immune responses to *L.monocytogenes* infection of the IEC monolayer was found to be in a NOD2-, NLRC4-, and NALP3-dependent fashion and resulted in Reactive Oxygen Species (ROS) production and subsequent MAP kinase signalling. The result involved horizontal innate communication, independent of gap junction formation, cytokine secretion, ion fluxes,

or nitric oxide synthesis, although was found to be mediated through Nox4-dependent oxygen radical formation [148]. This was found to be sufficient to induce indirect epithelial cell activation and subsequent CXCL2 production. Nox4 activation has previously been shown to be involved in TLR4-mediated NF- κ B activation in human epithelial kidney cells and monocytes [149]. ROS have also been shown to be important intracellular signal transducing molecules at subtoxic doses [150]. In accordance with these results, ROS-induced activation of MAP kinase activity was reported [151] in addition to NF- κ B activation [152], apoptosis [151], epidermal growth factor receptor signalling [153], regulation of cellular proliferation [154], and antimicrobial peptide production [155]. In addition, Nox4 can function quickly as it operates independently of cytosolic accessory proteins [148]. Initiation of Nox4-dependent ROS production upon *L.monocytogenes* infection was noted as early as 5–10 minutes after bacterial challenge. It has also been shown that inflammasome mediated release of IL-1 β is dependent on ROS activation [156-158].

We know of an effective role for NOD2 in the detection and downstream effector signalling following *L.monocytogenes* infection at the intestinal epithelial interface. However, NOD2 has remained the only well characterised PRR involved in detection of *L.monocytogenes* in IECs to date. The role of other PRRs in the setting of the epithelia requires further investigation. We also know how some of the PRRs expressed in the epithelia, listed in Table 1.1, function to detect *L.monocytogenes* elsewhere in the body. This provides us with some starting points to examine their roles in the setting of IECs. The importance of small messenger molecules such as ROS in this setting is becoming apparent and requires further clarification.

Following internalisation into IECs, escape to the cytosol is mediated by LLO and the bacteria migrate further. Infection may spread laterally across the epithelia, as well as disseminating through the bloodstream. The intestinal epithelium is in close proximity to a

large number of gut-associated lymphoid cells, such as Isolated Lymphoid Follicles (ILFs) and most notably perhaps, the Peyer's Patches [159]. Herein lie specialised PRR expressing antigen-sampling cells; the M-cells which take up antigens and specific binding proteins by endocytosis. These cells then release chemokines in response to the antigens and attract antigen presenting cells, quite often being the Dendritic Cells DCs, to the apical area of the lymphoid follicles. Lacking lysosomes, the antigens may be passed on to antigen presenting cells relatively intact and presented to lymphoid cells lying in close proximity to the epithelial basal membrane [81].

To summarise, we now have a schematic of infection and the vital role played by the innate immune system in eventual clearance of the bacteria (see Figure 1.7). We know that infection predominantly occurs via the intragastric route. Here, the bacteria mediate entry to the intestinal epithelium. At this point, TLR2, NOD2 and RIG-1 are suggested to be involved in ROS signalling as well as type I IFN induction. This is believed to send early alarm signals to the innate immune effector cells and ready a type II IFN response to the infection. The bacteria then escape through to the bloodstream by LLO mediated escape from vacuoles. In the bloodstream, the bacteria quickly migrate to the liver and spleen. They are also capable of invading phagocytic cells such as DCs, neutrophils and macrophages where they continue to multiply in the cytosol. During this stage, NALP3, AIM2 and NLRC4 are all known to be involved in inflammasome activation; resulting in an increase of intracellular killing of the bacteria. Subsequent activation of T1P DCs and lymphocytes ultimately mediates clearance of the bacteria from the host.

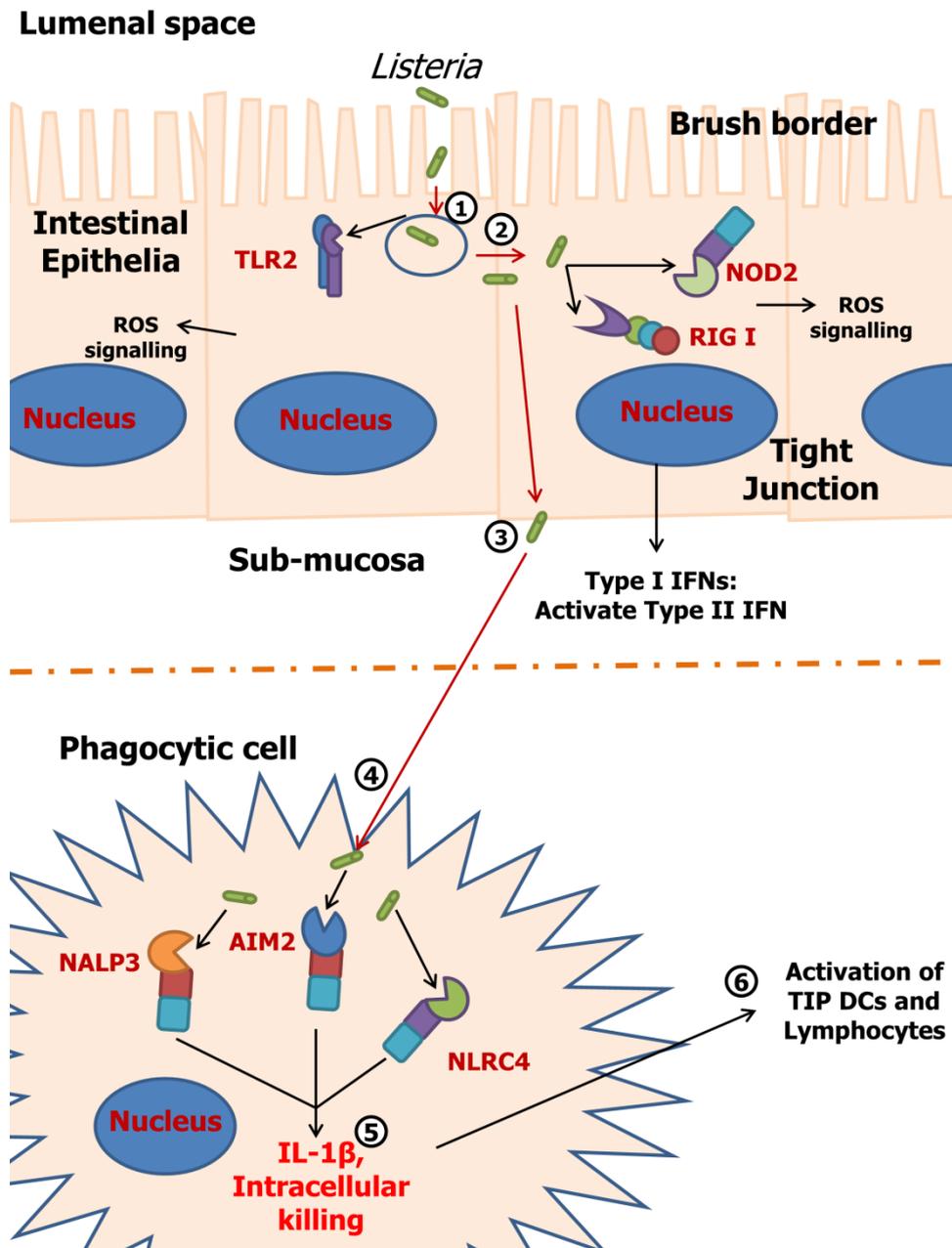


Figure 1.7 Mechanisms of innate immune mediated detection and clearance of *L.monocytogenes*. 1: Bacteria mediate entry to the intestinal epithelium. 2: Escape from vacuoles mediated by LLO. TLR2, NOD2 and RIG I are believed to be involved in ROS signalling to adjacent cells and activating a type I IFN response. 3: Bacteria escape through the sub-mucosa to the bloodstream to reach the liver and the spleen. 4: Here they invade and multiply within phagocytic cells such as DCs, neutrophils and macrophages. 5: While in the cytosol, NALP3, AIM2 and NLRC4 are all known to be involved in inflammasome activation; resulting in an increase of intracellular killing of the bacteria. 6: TIP DCs and lymphocytes are ultimately involved in clearance of the bacteria from the host.

Through discussing the route of infection and clearance of pathogenic *L.monocytogenes*, I hope to have highlighted the differential responses that take place throughout the body from various innate immune genes. How PRRs function elsewhere in the body is certainly not a definite indicator of how we expect them to operate in the intestinal epithelia for example. Yet, an understanding of both is necessary for us to get an overall picture of how the innate immune system functions so that we may better treat inflammatory disorders through manipulation of the immune system. Additionally, it will help us treat infectious diseases where we have identified the methods by which pathogens have evaded our host defence systems; this would allow us to specifically target pathogens by preventing such evasion. Overall, any further understanding of PRR function in this mucosal area is vital for the progression of oral vaccine development.

The following thesis presents several studies in which I have aimed to investigate novel innate immune mechanisms regulating inflammation in the gastro-intestinal tract. Gut homeostasis is known to be maintained through complex interactions between the commensal microbes and host cells in addition to the polarisation of IECs and highly regulated expression of PRRs across this gradient and within the cells. Furthermore, in some cases PRR activation has been directly linked to maintaining homeostasis. With this knowledge, I chose three main strategies through which aimed to achieve uncovering novel innate immune mechanisms regulating inflammation in the gastro-intestinal tract. Firstly, I investigated the expression of TIR-domain containing proteins in commensal bacteria strains with the aim to subsequently study their potential innate immune subversion tactics through biomimicry. Secondly, I examined the hypothesis that direct stimulation of apical TLR9 on IECs has the ability to abrogate inflammatory signalling from further TLR activation. Thirdly, I identified several novel PRRs involved in detection of pathogenic *L.monocytogenes* in IECs, both those involved in activating and regulating an inflammatory response. The same study has led to

the identification of the previously uncharacterised TLR10 as a functional inflammatory receptor of IEC and macrophages responding to intracellular pathogens such as *L.monocytogenes* and *S.typhimurium*.

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Chapter 2

Materials and Methods

2.1 The following buffers were utilised in the laboratory:

TBE buffer

90mM Tris
90 mM Boric Acid
2mM EDTA

RIPA lysis buffer

50mM Tris-HCL pH 8.0
150mM NaCl
0.1% SDS
0.5% Sodium deoxycholate
1% Triton X-100
1mM phenylmethylsulfonyl

PBS (pH adjusted to 7.4)

137mM NaCl
2.7mM of KCl
10mM of Na₂HPO₄·2H₂O
2mM KH₂PO₄

SDS sample buffer (50ml)

5ml glycerol
10ml 10% SDS
10mg bromophenol blue
6.25 ml 1M Tris pH 6.25
28.75 ml H₂O

Stacking gel for Western blot (6ml)

1ml 30% bisacrylamide mix

0.75 ml 1 M Tris pH 6.8

60µl 10% ammonium persulphate

6µl TEMED made up to 6ml with H₂O

Resolving polyacrylamide gel for Western blot (15ml)

30% bisacrylamide mix

3.75ml 1.5 M Tris pH 8.8

150µl 10% (w/v) ammonium persulphate

6µl TEMED made up to 15 ml with H₂O

Unless stated otherwise, all reagents were obtained from Sigma-Aldrich, Dorset, UK.

2.2 Cell culture

Caco-2 (ATCC number HTB-37), C2Bbe1 (ATCC number CRL-2102), HCA-7 (Sigma-Aldrich), HEK 293 cells (ATCC number CRL-1573) and RAW 264.7 murine macrophages (a gift from Dr. R. J. Carmody, UCC) were maintained in DMEM (Thermo Scientific, Leicestershire, UK), HT-29 (ATCC number HTB-38D) in McCoy's 5A (Thermo Scientific), T84 cells (ATCC number CCL-248) were grown in DMEM F12 (Sigma-Aldrich, Dorset, UK) and THP-1 cells (ATCC number TIB-202) in RPMI (Thermo Scientific), supplemented with 10% FCS (Thermo Scientific) and 10 µg/ml of penicillin and streptomycin (Sigma-Aldrich) and cultured in a humidified incubator at 37°C with 5% CO₂. C2Bbe1 cell media was also supplemented with 10µg/ml transferrin (Sigma-Aldrich). For continuing cell culture, cells were seeded at $\sim 1 \times 10^5$ /ml and subcultured every 3-4 days up until passage number 24. In all cases cell viability was determined using the dye Trypan blue (Invitrogen, Paisley, UK) which is excluded from healthy cells but taken up by non-viable cells. Cells were counted using the Countess cell counter (Invitrogen). THP-1 cells were differentiated into adherent, well-spread macrophages by the addition of phorbol myristate acetate (PMA) (Sigma) (5 nM) and maintained for 3 days at 37°C, 5% CO₂. Differentiated THP-1 cells were allowed to adhere over 48 hours prior to utilization in experiments.

To obtain polarized epithelial-cell monolayers, T84 or Caco-2 IECs were grown on microporous filter inserts (0.4 µm pore size, Transwell; Costar, Washington D.C, USA) for 21 days to obtain a differentiated monolayer. This was after transepithelial electrical resistance (TEER) reached at least 600 Ω cm² as measured with WPI's EVOM Epithelial Voltohmmeter.

The ligands Pam3Cys (50µg/ml); PGN (10µg/ml); LTA (10µg/ml); FSL-1 (5µg/ml); LPS (10ng/ml); Poly(I:C) (20µg/ml); TNFα (10ng/ml) (All Invitrogen) were used for cell stimulation assays as were bacteria (below).

Additionally, TLR9 stimulation involved the use of a range of concentrations of ODN 2006 from Invitrogen or obtaining constructs of ODN 2006 CpG from MWG (Eurofins, Germany). Constructs were as follows where upper-case indicates phosphodiester bases and lower case indicates **phosphorothioate bases**:

Invivogen construct:	5'-TcgTcgTTTTGTcgTTTTGTcgTT-3' – Indicated in text as “Invivogen.”
CpG as used in Lee <i>et al.</i> [1, 2]:	5'-TCGTcgTTTTGTcgTTTTGTcgTT-3' - Indicated in text as “Lee.”

2.3 Hypoxia simulation

The HIF-1 α gene was stabilised by adding Dimethyloxaloylglycine (DMOG) (a gift from the lab of Prof. Cormac Taylor, UCD, Dublin, Ireland) to a concentration of 500 μ M in the THP-1 or HT-29 cell culture medium 24 hours prior to analysis/further treatment in studies investigating effects of hypoxic conditions.

2.4 Generation of competent bacterial cells for plasmid transformation

The bacterial strain DH5 α (a gift from Dr. S. Joyce, UCC) was grown in 10mls of LB broth (Sigma) and cultured overnight shaking at 37°C. 150 μ l of this culture was then transferred to fresh 10ml of LB broth and cultured for ~2 hours until an absorbance of 0.6 at 600nm was reached. The cells were then centrifuged at 10,000 rcf for 10 minutes at 4°C and resuspended in 5 ml of ice cold 50 mM CaCl₂. The cells were then incubated on ice for ~90 minutes, aliquoted, frozen in liquid N₂ and stored at -80°C.

2.5 Bioinformatics

All BLAST searches and sequence alignments were performed using UniProt (<http://www.uniprot.org/>) unless stated otherwise.

2.6 Subcloning of bacterial TIR proteins into plasmid vectors

TIR gene sequences from bacteria were subcloned into the vectors pcDNA3.1 A Myc/His and pEF4/Myc-His A (both gifts from the lab of Dr. R. J. Carmody, UCC) the sequence of the constructs were confirmed by sequence analysis. A map of pcDNA3.1 A and its restriction site may be seen in the Appendix Figure 8.1. The cloning of BifTIR, EschTIR and POTTIR into the expression vector pcDNA3.1 myc-His A was performed using the restriction enzymes Hind III and Xho I (New England Biolabs, Hitchin, Herts, UK) and the primers used to amplify each TIR gene before insertion are as follows:

BifTIR

Forward primer: 5'-GGG AAG CTT AG GTC ATG GCA CAC AAG ACT TTC ATC TCA- 3'

Reverse primer: 5'-CCC CTC GAG AAG CAG TTT TGT GAG GTC GTA- 3'

EschTIR

Forward primer: 5' -GCG AAG AAG CTT GG GTC ATG TAT AAT ATC CTT TTC TTC ATC- 3'

Reverse primer: 5' -GC CTC GAG TCT TCT CCT GTA TGA TAT TTC- 3'

POTTIR

Forward primer: 5'-CGCG AAG CTT GG GTC ATG GAA AAT ACT GTA TTT TTT- 3'

Reverse primer: 5'-GC CTC GAG CAT ATC ATC AGA GAA ATT ACA- 3'

Each primer is shown with an underlined Hind III restriction site in the forward primer and an Xho I restriction site in the reverse primer. PCR was performed to amplify each of the TIR inserts using the Expand High Fidelity PCR system (Roche, West Sussex, UK). Inserts were then run on a 1% Agarose TBE gel and viewed using Ethidium Bromide under UV light. Using the PCR and Agarose gel Cleanup Kit (Roche), samples were then purified and ~1µg of DNA was cut with 1 unit of each restriction enzyme in the buffer at 37°C for 1 hour. The plasmid was purified following this ligation reaction using the High Pure PCR Clean Up Micro Kit (Roche). Following this, a ligation reaction was performed at room temperature

overnight with the insert and the vector using 1 unit of T4 ligase (New England Biolabs) at a ratio of 4:1 insert:vector.

2.7 Transformation of ligation reactions

Ligation reactions were incubated with 100µl of competent bacteria on ice for 10 minutes. Bacteria were then subjected to 30 seconds heat shock at 42°C before being placed back on ice for a further 5 minutes. The mixture was then plated out on pre-warmed LB-agar plates, allowed to air-dry, inverted, and incubated at 37°C overnight. The resulting colonies from the transformation were streaked onto an agar plate and grown overnight at 37°C and thereafter stored at 4°C.

2.8 Mini-preparations of plasmid DNA

DNA from bacterial colonies was isolated using Wizard[®] Plus SV Minipreps DNA purification system (Promega, Madison WI, USA). Briefly, bacterial cultures were grown in a 5ml LB medium before being centrifuged and resuspended in 250µl Cell Resuspension Solution. Cells were incubated for 5 minutes with the addition of 250µl Cell Lysis Solution before addition of 350 µl Neutralization Solution. The mixture was centrifuged at 13,000 rcf for 10 mins at room temperature. The cleared lysate was transferred to a spin column inserted into a 2ml collection tube and the supernatant was centrifuged for 1 minute at room temperature and then washed with 750µl, then 250µl of Column Wash solution. The DNA was finally eluted by addition of 100µl Nuclease Free water. Insertion of the PCR product into the vector was then confirmed by restriction digest and running on a gel.

2.9 Maxi-preparations of plasmid DNA

Plasmids were purified following the protocol for using the GenElute™ HP Plasmid Maxiprep Kit (Sigma). Transfected bacterial cultures were inoculated into LB media containing selection antibiotic e.g. 100 µg/ml of ampicillin. Bacterial cultures were grown, shaking at 275 rpm overnight at 37 °C. Each sample was prepared by harvesting 150 ml of bacterial culture by centrifugation at 5,000 rcf for 10 minutes. The pellets were thoroughly resuspended in 12 ml of Resuspension Solution and then subjected to a modified alkaline-SDS lysis by the addition of 12 ml of Lysis Solution. The lysates were neutralized with the addition of 12 ml of Neutralization Solution. The lysates were transferred to individual filters and incubated at room temperature for 5 minutes. The lysates were drawn through the filters by vacuum resulting in a clarified lysate. The clarified lysates were prepared for loading onto the binding columns by the addition of 9 ml of Binding Solution. Binding columns were prepared by attaching them to a standard laboratory vacuum manifold followed by the addition of 12 ml of Column Preparation Solution, which was drawn through by vacuum. The lysate mixtures from above were loaded onto the binding columns and drawn through by vacuum. Contaminants were removed from the columns by washing with 12 ml of Wash Solution 1, followed by 12 ml of Wash Solution 2. The columns were allowed to dry for 10 minutes while still under vacuum and then transferred to individual collection tubes. Finally, the plasmid DNA was eluted by the addition of 3 ml of endotoxin-free water and centrifugation at 3,000 rcf for 5 minutes. The plasmid concentrations of recovered eluates were then determined by taking absorbance readings at 260 nm. Total yield (ng/µl) was calculated by multiplying the concentration by the volume of recovered eluate. The purity of the plasmid DNA was determined by calculating the ratio of absorbance at 260 nm/280 nm. Samples were run on a 1% TBE agarose gel at 100 volts with a DNA ladder to reference size

and visually inspected to confirm their yield and purity. All sequencing was performed by Eurofins, MWG, Germany.

2.10 Luciferase Assays

HEK 293 or RAW cells were seeded at 2.5×10^4 /well in a 96 well plate 24h prior to transfection. Cells were transfected using Turbofect (Fermentas, Thermo Fisher Scientific, Waltham, MA, United States of America) according to the manufacturer's protocol using 50ng of ISRE or NF- κ B luciferase plasmid and 5ng of Thymidine kinase Renilla luciferase along with varying amounts of either MyD88, Mal, TLR1, TLR2, TLR4, TLR6 or TLR10 (gift from Prof. L. O'Neill, Trinity College Dublin, Ireland). 24 hours post transfection cells were infected with *L.monocytogenes* for 8 hours. Extracts were prepared by lysing the cells for 15 minutes in 50 μ L Passive Lysis Buffer (Promega) and measured for Firefly luciferase and Renilla luciferase activity. Firefly luminescence readings were corrected for Renilla activity and expressed as fold stimulation over unstimulated empty vector (EV) control.

2.11 Bacterial culture

L. monocytogenes (wild-type, strain EGD-e serotype 1/2a) was a gift from Prof. Cormac Gahan, (UCC, Cork, Ireland). Bacteria were grown to the logarithmic growth phase in brain heart infusion (BHI) broth (Merck, NJ, USA) at 37°C shaking at 200 rpm. The Heat-Killed *Listeria monocytogenes* (HKLM) used for stimulation were prepared by heating the bacteria to 70°C for 1 hour prior to washing in PBS. The *Salmonella typhimurium* strain SJW1103 (wild type) was a kind gift from Prof. P. O'Toole (UCC, Cork, Ireland). Bacteria were grown to the logarithmic growth phase in LB broth (Sigma) at 37°C shaking at 200 rpm. Bacteria were subsequently washed with PBS by two steps of centrifugation (4000 rcf for 5 min) and diluted in PBS for infection at multiplicity of infection (MOI) of 10:1.

2.12 Determining the growth curve of *L.monocytogenes*

Bacteria were streaked on a Brain Heart Infusion (BHI) agar plate and incubated at 37°C inverted overnight. The following day, a single colony was selected from the plate to inoculate 10ml of BHI broth. This was grown with shaking at 37°C overnight. Serial dilution was performed on the culture in sterile PBS and spotted in triplicate on an agar plate. The plate was then incubated overnight at 37°C and was labelled T=0. This was repeated taking an aliquot every hour until T=8. Colonies were counted and Colony Forming Units (CFU) per ml was calculated as follows: CFU per ml = no. of colonies X 100 X dilution factor. The growth curve generated with this data was graphed against Optical Density (OD) at Absorbance 595 and used as validation when determining precise CFU at a given OD.

2.13 Live/Dead staining of bacteria

L. monocytogenes was seeded overnight and cultured (2%) under the time and culture conditions specified (either 1 hour treatment with Gentamicin, no treatment or 1 hour treatment with Formalin); aerobically at 37°C in BHI broth (Merck). Following culture bacteria were washed twice in sterile PBS solution analysed for viability using Live/Dead BacLight viability and counting system (Invitrogen) in 0.85% sterile NaCl solution on an Accuri C6 Flow Cytometer (Becton Dickinson Biosciences, NJ, USA). Each strain was evaluated and specific gates were set for each strain; based on the viability profile bacteria were then provided at the required CFU in a blinded fashion to the researcher. Plate counts were also performed for each strain with the respective agar plates.

2.14 Infection of THP-1 and HT-29 cell lines

L. monocytogenes was grown overnight (1 colony from a plate was grown in 10ml BHI broth, 37°C, shaking). The following morning, 200µl was taken and diluted in 10ml BHI broth (1/50) and incubated for further growth. From the CFU/OD validation curve, an accurate CFU count could be calculated at Early (3 hour culture), Mid (4 hours culture) and Late (6 hours culture) log phases. In each case, an appropriate amount of culture was removed (e.g. OD600, 1 ml = $\sim 2 \times 10^9$ CFU) and spun at 1000 rcf for 5 minutes. Following this, the bacteria were washed 3 times in sterile PBS. Subsequently, a serial dilution of the bacteria was made up and spotted on an agar plate in order to record CFU the following day to ensure accuracy of bacterial counts. The remainder of the culture was then subject to serial dilution such that 5µl of bacteria suspended in PBS would contain the appropriate MOI when added to a 96 well plate e.g. 4×10^4 cells on a plate at MOI 10 = 4×10^5 bacteria in 5µL from a stock of 8×10^7 CFU./ml. For Heat Killed *Listeria monocytogenes* (HKLM), the bacteria were incubated at 70°C for 1 hour prior to washing in PBS. Following the addition of the bacteria, the tissue culture plate was spun at 250 rcf for 3 minutes. This was found to increase contact between the bacteria and the cells for efficient, reproducible infection. The cells were washed at 1 hour post-infection with antibiotic free cell culture medium and supplemented with cell culture medium containing Gentamicin (Invitrogen) (50µg/ml). Cells were then incubated for the times indicated in the results section prior to analysis.

2.15 Bacterial survival assays in HT-29 and THP-1 cells

For bacterial survival assays, the HT-29 or THP-1 cells were washed at 8 hours post-infection and treated with 0.2% Triton X-100 PBS. The lysates were then subjected to serial dilution and spotted on a BHI agar plate. CFU/lysate of internalised bacteria was then determined. Following infection, cells were washed and treated with 0.2% Triton X-100 PBS

in order to lyse the cells. The lysates were then subject to serial dilution and spotted on an agar plate to achieve CFU counts of internalised bacteria. In this way, bacterial internalisation efficiency was measured under different MOIs (10, 50 and 100), different bacterial growth phases (early log, mid log and late log) and for different infection times (1 hour infection + 1 hour Gentamicin treatment or 2 hours infection and 1 hour gentamicin treatment).

2.16 Confocal Microscopy on THP-1/HT-29 infected with *L. monocytogenes* to examine Internalisation

Bacteria were labelled with CellTrace CFSE (Invitrogen) as per standard protocol (i.e. 2µl CFSE added to 1×10^9 bacteria and incubated at room temperature in the dark for 15 min and bacteria were then washed three times in PBS). After final wash step, bacteria were resuspended in PBS pH 8.0. To biotinylate the bacteria, one microtube of No-Weigh™ Sulfo-NHS-Biotin (Pierce, Rockford, IL, USA) per ml of bacterial suspension was incubated at room temperature for 30 min. The bacteria were washed three times with PBS + 100mM glycine (0.75g in 100ml PBS) to quench and remove excess biotin reagent and by-products. After final wash step, bacteria were resuspended in 100µl PBS and 4µl (1:25 dilution) Streptavidin-Allophycocyanin (Pierce) was added for 30 min on ice. For co-incubation, human cells were seeded in 6-well plates containing a poly-L-Lysine coated glass coverslip with 3ml at 1×10^6 cells/ml/well (THP-1) or 3ml at 0.5×10^6 cells/ml/well (HT-29). CFSE-biotin-labelled bacteria were added to each well to give a 10:1 or 100:1 bacteria to cell ratio appropriately. Cells and bacteria were co-incubated for 2 hours at 37°C following a spin at 300 rcf for 3 minutes. Unbound cells were then aspirated with the media and the coverslips were washed once with PBS. The bound cells were fixed with formalin (~200µl/coverslip) for 20 min. Coverslips were washed three times with PBS. For each coverslip, 100µl of streptavidin-APC in 1% BSA was added (0.1g Bovine Serum Albumin (Sigma) in 10ml PBS)

and incubated for 30 min at 4°C. Cells were washed once with PBS and 0.2% Triton X-100 (20µl in 10ml PBS) was added to each coverslip for 5 min to permeabilise the cells. Coverslips were washed once with PBS. F-actin (Pierce) was stained red with fluorescent phallotoxins. Vial contents were dissolved in 1.5ml methanol to give a stock concentration of 6.6µM. For each coverslip to be stained, 5µl of 6.6µM stock solution was diluted in 200µl of PBS containing 1% BSA. Coverslips were incubated with the staining solution for 20 min at room temperature in the dark and were then washed with PBS and mounted on a slide with fluorescent mounting medium, DakoCytomation (S3023, Dako, Glostrup, Denmark). Slides were viewed using the FITC settings for the CFSE-labelled bacteria, the Rhodamine-Phalloidin settings for the phalloidin-labelled actin in the HT-29 or THP-1 cells, and the AlexaFluor 647 settings for the streptavidin-allophycocyanin.

2.17 siRNA transfection

All siRNA transfections were performed with SMARTpool siRNAs (Thermo Scientific). Sequences are contained in Appendix Table 1. For the TLR siRNA screen, cells were seeded at 1×10^4 /well in a 96 well plate the day before transfection. DharmaFECT 4 (Thermo Scientific) was then used to transfect the cells with siRNA (100nM) according to the manufacturer's instructions 48 hours prior to infection. THP-1 siRNA transfections were performed using the Amaxa (Lonza, Basel, Switzerland) system according to the manufacturer's protocol. Cells were cultured for a further 48 hours prior to infection.

2.18 Lentiviral transfection and clonal selection

HT-29 cells were grown to 50% confluency on a 12 well plate. The following day, 5µg of Polybrene (Santa Cruz biotechnology, Heidelberg, Germany) were added to the cells with 100,000 transfection units of lentiviral particles. The TLR10 shRNA and copGFP lentiviral particles were also obtained from Santa Cruz. The control lentiviral particles

contained sequences encoding for expression of the GFP protein as an added control to ensure transfection had taken place. 16 hours after transfection, fresh media was added to the cells due to the toxic nature of the polybrene. 24 hours later, the cells were split 1:3 and 4µg/ml Puromycin (Invivogen, Toulouse, France) was supplemented into the medium of transfected cells. “Parental” HT-29 cells were grown in parallel without puromycin as an untreated control. Following 2 further passages, positive selection on the lentiviral particle transfected cells using puromycin had occurred and cells were split 1:100 in order to achieve single cell colonies. Following another week of growth, colonies were selected using a pipette tip and cultured for further analysis.

2.19 Western blot analysis

Preparation of cell extracts by radioimmune precipitation (RIPA) method

Prior to Western blot analysis, cells were washed with ice-cold PBS followed by centrifugation at 1500 rcf for 5 minutes. Pellets were resuspended in 1 ml PBS and transferred to eppendorfs. The samples were centrifuged at 12,000 rcf for 5 minutes at 4°C and the pellets were resuspended in 100µl of RIPA buffer with 10% Protease Inhibitor Cocktail (Sigma). The cells were then disrupted by sonication before incubation on ice for a further 30 minutes. The samples were then centrifuged at 14,000 rcf for 20 minutes at 4°C. The supernatants (total cell lysates) were then assayed for protein concentration using the BCA Protein Assay Kit (ThermoScientific) according to the manufacturer’s instructions. Aliquots containing equal amounts of protein were mixed with 5µl SDS sample buffer. 250µl β-mercaptoethanol was added per ml of sample loading buffer immediately prior to use. Samples were then incubated at 95°C for 5 minutes prior to resolving them on Sodium Dodecylsulphate (SDS) polyacrylamide gel using a constant current of 30 mA per gel. Samples were first run through a stacking gel and then resolved according to size using a

10% polyacrylamide resolving gel. A prestained molecular weight marker (New England Biolabs) was also used to confirm the size of the protein being examined. The following antibodies were used: anti-TLR10 (H-165) (sc-30198, Santa Cruz) and β -actin antibody (AC-15) (Sigma). Proteins were detected using the Fujifilm LAS-3000 Imager (R&D Systems, Abingdon, UK).

2.20 Gene expression analysis using qRT-PCR

Following treatment, cells cultured in 96 well plates were washed in PBS. 25 μ l of Sidestep Lysis Buffer (Agilent Technologies, Geneva, Switzerland) was then added to each sample of and mixed well by pipetting. The SideStep technology achieves cell lysis and nucleic acid stabilization in the same buffer, eliminating the need for RNA purification. 5 μ l of a 1/5 dilution of this lysate was then prepared in DEPC treated H₂O prior to reverse transcriptase. For all other plate types used (12, 24 and 6-well plates), total RNA was isolated using the RNeasy Kit (Qiagen, West Sussex, UK) and 1 μ g of total RNA was used for reverse transcriptase. The reverse transcription for first strand cDNA synthesis from the mRNA present in the cell lysate was performed using the following protocol per reaction (all reagents from Roche): 4 μ l of 5x Transcriptor Buffer was added to 5 μ l DEPC treated water with 3 μ l of Random Primer, 2 μ l dNTP (ATGC) mix (10mM) and finally, 5 μ l of the lysate dilution. This reaction was run using a Veriti 96 well Thermal Cycler (Applied Biosystems) for 10 mins at 25°C, 30 minutes at 55°C, 5 mins at 85°C before cDNA being cooled to 4°C.

Individual PCR primer pairs and probes were designed using the Roche Universal Probe Library Assay Design Centre (<https://www.roche-applied-science.com/sis/rtPCR/upl/adc.jsp>). PCR reactions were performed using Sensi Mix II (Bio Line, London, UK), 900 nM of each primer and 250 nM of probe mix in the LightCycler480 System (Roche). Thermal cycling conditions were as recommended by the manufacturer

(Roche). 95°C for 5 min followed by 40 amplification cycles consisting of 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min. Linear amplification ranges were determined for all primer sets. Data analysis was carried out using the LightCycler® 480 Real-Time PCR System (Roche) software. β -actin was used as a housekeeping gene, the $2^{-\Delta\Delta C_t}$ method [3] was used to calculate relative changes in gene expression. Relative expression in each figure refers to induction levels of the gene of interest relative to the housekeeper control, beta actin, and then compared to an untreated control calibrator sample. Table 1 contains the primer sequences used (all designed for human genes).

Table 1: Sequences of primers used in qRT-PCR analysis

	Left Primer	Right Primer	UPL #
β-actin	5' attggcaatgagcggttc 3'	5' tgaaggtagtttcgtggatgc 3'	11
GAPDH	5' agccacatcgctcagacac 3'	5' gcccaatacagaccaaacc 3'	64
Fzd5a	5' accccaggggagagaaaact 3'	5' tgcaaattgggggaagtaag 3'	83
Wnt5a	5' actcggccaccacacaag 3'	5' cattgcgcacgcagtagt 3'	23
Cx43	5' gcctgaactgccttttcat 3'	5' ctccagtcacccatgttgc 3'	88
TNFα	5' cagcctcttctccttctgat 3'	5' gccagagggctgattagaga 3'	40
EGR-1	5' ggatggacattgcaggagt 3'	5' ctccacttccacgtgctc 3'	22
IL-1β	5' tacctgtctcgcgtgtgaa 3'	5' tctttgggtaattttgggatct 3'	78
IL-8	5' agacagcagagcacacaagc 3'	5' atggctcctccggtggt 3'	72
IL-23	5' tgttccccatattccagtgtg 3'	5' ggatcctttgcaagcagaac 3'	76
CCL-1	5' ttgctgctagctgggatgt 3'	5' ctggagaagggtacctgcat 3'	56
CCL-20	5' gctgctttgatgctagtgct 3'	5' gaagaatacggctctgtatccaa 3'	39
MAPK1	5' ccgtgacctcaagccttc 3'	5' gccaggccaaagtcacag 3'	62
TLR10	5' cttttgccaccaacctgaa 3'	5' ccctctctcacatctccttttg 3'	34
TLR1	5' aggggacaatccattccaa 3'	5' ttggtctatattttgacaaattctcc 3'	100
TLR2	5' tgtcattctttctcctgctaaga 3'	5' ctaggtaggacagagaatgccttt 3'	82
TLR6	5' tgaacagctctctttgagtaaatgc 3'	5' tccatttgggaaagcagagt 3'	17
MyD88	5' tgctcgagctgcttaccaa 3'	5' cctgctgctgcttcaagata 3'	33
Caspase-1	5' agggacgctgggactctc 3'	5' aagcttgacattcccttctgag 3'	17
VEGF	5' ttaacgaacgtacttgcatg 3'	5' gagagatctggttcccgaag 3'	12

Primers were generated by Eurofins MWG.

2.21 Flow Cytometry

Antibodies against TLR1, TLR2 and TLR6 (IMG-5012, IMG-6720A, IMG-304A) were from Imgenex (San Diego, CA, USA) and the TLR10 antibody was from Santa Cruz Biotechnology (TLR10 H-165). The secondary antibodies used were Alexa Fluor-488 goat anti-mouse IgG (Invitrogen) and PE-conjugated goat anti-rabbit IgG H+L (Imgenex). The respective isotype controls for each antibody were also from Imgenex. On the day of analysis,

1×10^6 cells were harvested using 0.2% EDTA PBS and washed twice in running buffer (PBS 0.5% BSA, 1% FBS 0.1% Sodium Azide). For intracellular staining, cells were fixed in BD Cytofix Cytoperm (Becton Dickinson) for 30 minutes on ice. For extracellular staining, cells were not fixed. Each primary antibody was used at $0.5 \mu\text{g/ml}$ for 30 minutes on ice. Each wash step for intracellular staining was performed using BD Perm/Wash buffer (Becton Dickinson) while the running buffer was used for extracellular staining washes. The secondary antibodies were used at a 1:1000 dilution for 20 minutes in the dark on ice and cells were analysed using the Accuri C6 flow cytometer (Becton Dickinson). The gating method employed was designed to include the live cell population only. Population gates were set using negative controls and IgG isotype controls. The results were expressed as the percentage or median fluorescence intensity (MFI) of the total live cell population after subtracting the MFI of control cells stained with the appropriate isotype control antibodies.

2.22 ELISA analysis of supernatants for cytokine production

CCL-20 levels were quantified using the anti-CCL20 kit (Meso Scale Discovery (MSD), Maryland, USA) according to the manufacturer's instructions. Concentrations were determined using the MSD Sector Imager 2400. IL-8 levels were quantified using the Ready-Set-Go!® Human IL-8 ELISA kit (eBiosciences, San Diego, USA) according to the manufacturer's instructions.

2.23 NF- κ B Activity

SEAP reporter HEK-Blue-MD2-CD-14 cells (Invivogen) were used according to the manufacturer's instructions. Briefly, cells were maintained in selective antibiotics and seeded at 2×10^4 cells/well on a 96 well plate the day before transfection. Cells were then transfected

and infected with *L.monocytogenes*. Supernatants were collected 8 hours later and NF- κ B activity was measured using QUANTI-Blue™.

2.24 Statistics

qRT-PCR results from the PRR siRNA screen in 5.12 - 5.15 were analysed using the Mann Whitney U test. This test is relatively stringent and was useful for analysing significant changes in gene induction across a large group of treatments with a relatively large number of samples/treatment. All other results were statistically evaluated using the relatively less stringent Student's t test with Welch's correction which is useful for discerning significant effects on gene induction following cell treatments. Values of $p < 0.001$ are indicated by three asterisks (***). Values of $p < 0.01$ are indicated by two asterisks (**). Values of $p < 0.05$ are indicated by one asterisk (*).

2.25 References

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Chapter 3

Identification of TIR domain-containing Proteins from Commensal Bacteria and analysis of their potential immunomodulatory properties

3.1 Introduction

The innate immune system's sentinels in the first line of defence against infection by invading microorganisms are its Pattern Recognition Receptors (PRRs). Of these, the Toll-Like Receptor (TLR) family was one of the first to be identified and as such is now well characterised. TLR signalling elicits a pro-inflammatory response which is characterized by the secretion of cytokines such as tumour necrosis factor (TNF), type I and II interferons (IFNs), and chemokines. In turn, these control the recruitment of inflammatory cells to infected tissues and also function to cross-talk with the adaptive immune system [1].

TLRs are part of a larger superfamily of receptors which include the IL-1 Receptor family members. All members of this family are characterised by a 200-residue intracellular domain termed the Toll-IL-1-Receptor (TIR) domain. This TIR domain has been shown to be critical in the activation of signalling pathways from these receptors, such as MAP kinases and the transcription factor NF- κ B, which is known to be involved in the activation of approximately a thousand immune and inflammatory genes. This conserved TIR domain is also contained in the IL-1R/TLR adaptor proteins MyD88, MAL, TRIF and TRAM. These adaptor molecules directly interact with the activated TLR in order to relay downstream signalling for appropriate immune response/transcription factor activation. Sequence conservation of this TIR domain is primarily confined to three short motifs; (Boxes 1, 2, and 3) located at amino acid residue positions 10 (Box 1), 60 (Box 2), and 170 (Box 3) and with consensus sequences: Box 1 – FDAFISY; Box 2 - GYKLC—RD—PG; and Box 3 - a conserved W surrounded by basic residues [2] (see Figure 3.1). These 3 regions map to the hydrophobic core of the domain structure as well as a long “BB” loop which had been proposed as the primary protein-protein interaction site [2]. While mutations in all three lead to a loss of surface expression, only mutations in Box 1 and Box 2 cause a direct loss of signalling activity [3]. No clear differences were found between the effects of Box 1 and Box

2 mutations in various signalling assays, although Box 2 mutations appeared to have stronger effects on signalling [3].

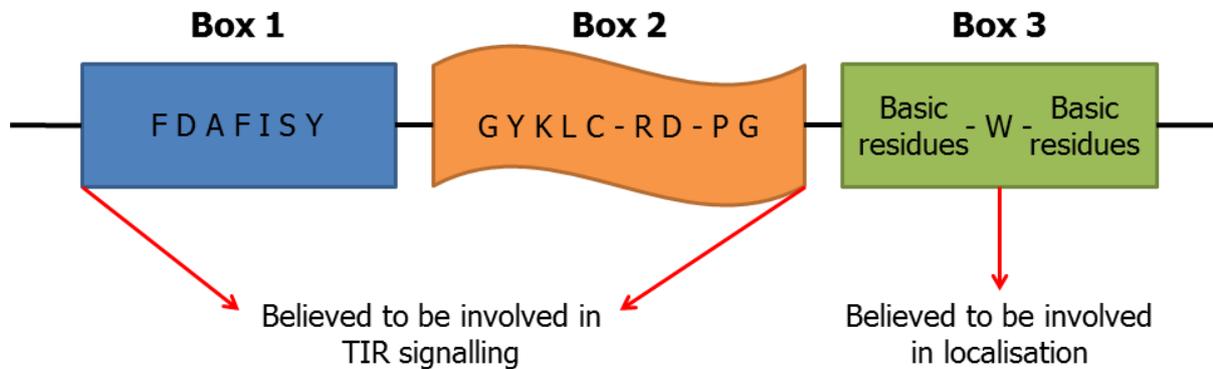


Figure 3.1 TIR domain consensus Box motifs. Shown above are the three highly conserved regions among the different members of the TIR family. These motifs are located at amino acid residue positions 10 (Box 1), 60 (Box 2), and 170 (Box 3). While Box 1 and Box 2 are believed to be more crucial for TIR:TIR signalling, the conserved W surrounded by basic residues in Box 3 is believed in directing localisation of the TIR protein [3].

Initial indications that the TIR domain was critical for TLR signalling were realised when a proline to histidine point mutation within the domain at position 712 of the polypeptide chain was shown to confer lipopolysaccharide (LPS) unresponsiveness to C3H/HeJ mice [4]. Further molecular analysis of signalling from the TIR domain has revealed that it requires the homo- or heterodimerisation of their extracellular leucine-rich repeats region, to be mediated by the microbial pathogen-associated molecular patterns (PAMPS). Following this, dimerization of the receptor cytoplasmic TIR domain occurs [5]. Only in this active conformation are the receptor TIR domains capable of a functionally productive interaction with TIR domains of adaptor molecules; MyD88, MAL, TRIF and TRAM. Competition at the level of the TIR domain has been suggested as an efficient mechanism to control TLR signalling [3]. In fact, a truncated splice variant of MyD88 has

been identified named MyD88s. MyD88s has been shown to compete with wild-type MyD88 for TLR:TIR domain interactions and inhibits an inflammatory response as MyD88s is unable to mediate any further downstream signalling [6]. More specifically, another TIR adaptor, SARM, has been identified as the only known TIR inhibitor molecule and functions as a negative regulator of TRIF signalling [7].

Several pathogens have recently been found to evade our immune system by using genes which mimic components of immune signalling pathways. Pathogens have been identified which produce TIR domain containing proteins which, in some cases, have been shown to interfere with TLR signalling, thereby limiting the immune response. The first example of this was the protein A46R, found in *vaccinia* virus which suppresses signalling from multiple TLRs and the type 1 IL-1 receptor [8]. A46R also blocked IRF3 activation by TRIF mediated signalling and thus prevented the secretion of IFN- β , which functions to suppress viral replication [8]. Using an intranasal infection model, the authors showed that A46R-deficient virus had diminished virulence, consistent with the above observations. A46R was shown to be able to form protein complexes with host TIR domain containing adaptor proteins; MyD88, MAL, TRIF, or TRAM. This suggests that A46R specifically targets IL-1R/TLR signalling through interaction with adaptor proteins [8]. An example of how this mechanism may occur is displayed in Fig. 3.2.

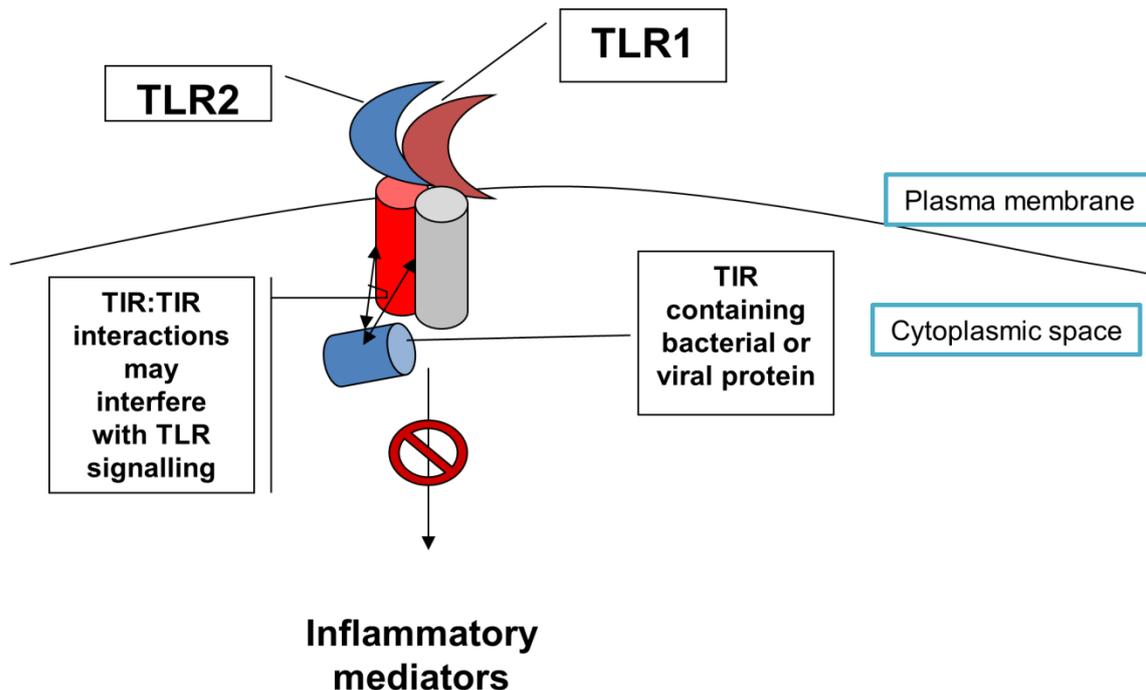


Figure 3.2 Possible Mechanism for bacterial/viral TIR domain containing protein-mediated inhibition of TLR signalling. TLR1 and TLR2 hetero-dimerise at the plasma membrane. These are prevented from initiating signal transduction through their TIR domains due to the TIR:TIR interaction between the TIR domain of the TIR containing bacterial/viral protein and the TLR's.

More recently similar targeting of the TIR domain has been identified as an immune evasion mechanism in bacteria. The *Salmonella enterica* protein, Toll-Like Protein A (TlpA) was shown to be required for suppression of NF- κ B activation and IL-1 β production during *Salmonella* infection. Mice infected with wild-type bacteria had a significantly higher mortality than those infected with a TlpA-deficient strain [9]. There are detectable sequence similarities between TlpA and TIR domains from human and other bacterial proteins, particularly at the Box 1 and Box 2 regions. However, direct interaction with any of the human TIR domain signalling proteins has not been demonstrated [9].

Two TIR-containing proteins have also been identified in *Brucella* bacteria. TcpB and Btp1 are identical proteins except for a single mutation within the predicted α -helix of their TIR domains [10, 11]. Both proteins have been shown to abrogate TNF α production by macrophages [10] and dendritic cells [11]. Infection with Btp1-deficient bacteria did not, however, alleviate symptoms of murine brucellosis [11], suggesting other mechanisms of immune evasion are used by this bacteria. Because of the delayed maturation of dendritic cells upon *Brucella* infection, it was proposed that *Brucella* might play a role in immune tolerance induction and chronic infection [11], although the exact role of Btp1 or other *Brucella* proteins in this process is not yet well understood. Recent studies suggest that TcpB targets signalling by MAL (the TLR2 and TLR4 specific adaptor protein) in at least two ways. Firstly, Radhakrishnan *et al.* demonstrated that TcpB associated with phosphoinositides (PtdIns) at the plasma membrane [12]. MAL is known to be recruited to the plasma membrane through the binding of its N-terminus to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂). This membrane localization is necessary for MAL to act as a bridging adapter between MyD88 and TLR2 or TLR4 [13]. Nevertheless, the implicit mimicry of MAL by TcpB has not been substantiated by direct binding of TcpB with MAL partner proteins such as MyD88 or TLR4, either through their TIR domains or otherwise. A new study by Sengupta *et al.* showed that TcpB promoted polyubiquitination and subsequent degradation of MAL, and this degradation process seemed more efficient for phosphorylated MAL [14]. This raises the question as to whether TcpB is an adapter for other proteases, is a protease itself, or contains E3 ligase activity. The two mechanisms outlined above for TcpB mediated MAL inhibition are not necessarily independent of each other either – for example, localization of TcpB to the plasma membrane can facilitate the binding, ubiquitination, or degradation of MAL by TcpB or other associated ubiquitination enzymes or proteases.

TcpC from uropathogenic *E. coli* strain CFT073 was identified in a bacterial genomic sequence search [10]. Despite minimal sequence similarity to the human TIR domain proteins, the typical Box 1, 2, and 3 sequences are all present within the C-terminal of TcpC. Following various pull down assays, TcpC was found to interact directly with full length MyD88, presumably through their TIR domains [10]. It was also shown to directly impair TLR2 and TLR4 signalling, reduce pro-inflammatory cytokine secretion, and increase virulence as well as bacterial burden in murine urinary tract and renal tissue infection models. The significance of TcpC as a virulence factor was highlighted also by the fact that most uropathogenic *E. coli* strains seemed to express TcpC, based on clinical samples [10]. Despite TcpC not containing a typical secretion signal, it was shown to be secreted by bacteria and taken up by macrophages - leading to abrogated cytokine production [10]. Since the mammalian TIR domain proteins are only known to be present in the cytosol, this finding suggests a novel shuttling ability of TIR domain proteins through the lipid membrane, possibly aided by undiscovered localization signalling and/or other chaperone transport proteins.

Further application of genomic sequence mining led to a TIR domain containing protein PdTLP being identified in *P. denitrificans* [15]. Similar to the previous studies highlighted above, GST pull-down and co-immunoprecipitation studies demonstrated direct binding of the PdTLP TIR domain (PdTIR) with the human and mouse MyD88 TIR domain [15, 16]. The crystal structure of PdTIR was the first among microbial TIR domains to be determined [16]. It adopts a typical TIR domain fold, with an exposed BB loop that was proposed to be the site of heterotypic interaction with partner TIR domains, such as that from MyD88 [16]. The function of PdTLP as a virulence factor has not been characterized, as it is unclear whether its ability to interact with MyD88 dampens signalling from TLRs, or offers any advantage for bacterial infection, replication, or dissemination. Finally, another bacterial

TIR-domain protein (Tdp) was identified from *Yersinia pestis* (highly pathogenic bacteria responsible for the plague) and named YpTdp [17]. Although its removal from the bacteria had no obvious effect on virulence, when over-expressed *in vitro*, YpTdp has been shown to disrupt immune signalling pathways [17].

In a 2009 review by Spear *et al*, the distribution of TIR domain proteins among bacteria, fungi, archaea and viruses was discussed and evaluated [18]. These authors proposed that due to the wide distribution of TIR domains across species, it is likely that they act simply as protein-protein interaction domains put to diverse uses and are not specific for immune evasion. However, another paper released in 2010 by Zhang *et al*. addresses this controversy in a study relating the TIR domain containing negative regulator of TLR signalling, SARM, to bacterial TIR domains. By use of close phylogenetic analysis, SARM was shown to be closely related to bacterial proteins with TIR domains suggesting that perhaps this family has a different evolutionary history from animal TIR-containing adaptors, possibly emerging via lateral gene transfer from bacteria to animals [19]. An ancient origin of the TIR protein is suggested by the generally separate animal, plant, fungi, archaea, and bacterial branches of TIR domain found in nature. Interestingly, the study also shows that 68% of the bacterial genomes analysed that do contain TIR proteins only have one such protein. Since its role is presumably to form complexes with other TIR domains, such proteins have no obvious partners to form complexes with [19]. They highlight the previously addressed paper by Spear *et al* [18] and agree that the broad distribution of TIR-domain containing proteins in bacteria challenges the view of their primary function as virulence factors, but go on to hypothesize that bacterial TIR-domain containing proteins may play important roles in commensal or mutualistic interactions between bacteria and eukaryotes [19].

The aim of the following study was to investigate the hypothesis that some commensal microbes may use TIR domain-containing proteins in order to avoid host immune detection, preventing the hosts' inflammatory response. Use of the immuno-modulatory properties of the TIR domain might, in this instance, help maintain homeostasis in the gut and contribute to the intestinal epitheliums' general tolerance to the presence of commensal bacteria. To date, such immune evasion has only been shown in pathogenic microbes, but we wished to study commensal bacteria for the presence of these proteins in order to identify possible mechanisms by which they maintain inflammatory homeostasis in the unique environment of the microbe-filled human gastro-intestinal tract. In order to test this hypothesis TIR domain containing proteins in commensal bacteria were identified and their potential role as immuno-evasive agents examined. In particular, we focussed on potential interaction between these candidates and TIR containing proteins involved in human innate immune signalling such as TLRs and their adaptor proteins such as MyD88 and MAL.

3.2 Results

3.2.1 Identification of bacterial TIR proteins

A bioinformatics search was performed by a colleague in the Alimentary Pharmabiotic Centre (APC) Core 4 lab (Dr. Marcus Claesson). This search examined the presence of TIR domains as well as other conserved domains known to be important in PRR signalling (DEATH/CARD/NACHT domains) in bacterial proteins. PFAM and Interpro databases were searched using the conserved regions of these domains. The results of this search may be seen in Table 3.1. Of the 591 bacterial proteins found to contain TIR domains, 17 were found to also have signal-peptide domains – a feature which could potentially allow the protein to be translocated from the bacteria to the cytoplasm of a host where it could possibly interact with host TIR proteins.

Domain	No. of Bacterial Strains	No. of PRR signalling domain-containing proteins
TIR	319	591
DEATH	21	21
CARD	4	12
NACHT	35	35

Table 3.1 Results of signalling domains found in proteins of bacteria. Shown above are the results of the bioinformatics search to identify proteins in bacteria containing a TIR/DEATH/CARD/NACHT signalling domain. The results were obtained using the Interpro database. The numbers of proteins containing the corresponding domain are shown in the rows above along with the number of bacterial strains found to contain that domain.

These results were followed by an extensive analysis of both bacterial strains and the proteins themselves in an effort to identify the most likely target proteins. Many of the strains from the list generated were not known to interact with humans. We focussed our search to

examine only the known commensal strains of bacteria. Furthermore, we focussed on strains which carried only one TIR domain-containing protein to minimise the likelihood of these proteins interacting with other TIR proteins from the same strain - since the bacteria contains no other TIR protein for it to interact with. From the remaining candidates, we examined sequence alignments with known human TIR proteins, such as TLRs and their adaptor molecules. Following these analyses, two proteins were selected, containing TIR domains from commensal bacterial strains *Bifidobacterium longum subsp. infantis* CCUG 52486 and *Escherichia sp. 3_2_53 FAA*. The TIR domain containing proteins from these strains were named BifTIR and EschTIR respectively (A full list of all TIR domain containing strains identified is shown in Appendix Table 2).

Bifidobacterium longum subsp. infantis is a Gram-positive anaerobic rod-shaped bacterium found in the intestines of infant humans. *Escherichia* is a genus of Gram-negative, facultatively anaerobic, rod-shaped bacteria. A number of the species of *Escherichia* are pathogenic, however, in those species which are inhabitants of the gastrointestinal tract of warm-blooded animals, such as the strain we had selected, may benefit their host in a number of ways [20]. Each strain also contained only one TIR protein, therefore decreasing the likelihood for involvement in internal signalling interactions within the bacteria. One of these proteins, (EschTIR) contains a signal peptide domain which, as mentioned earlier, could be used to allow the protein to be translocated from the bacteria into the cytoplasm of a host. Both have been identified as commensal bacteria and there was prior in-house experience of working with Bifidobacterial strains. Another reason for the selection of these proteins from the list generated was their relatively strong sequence homology (22 – 28%) to host PRRs TLR1, TLR2, TLR6 and TLR10. Figure 3.3 displays alignment of these bacterial proteins to TLR1 and TLR6 - to which both bacterial proteins showed 27-28% sequence alignment. This was better than the slightly lower homology seen between TLR2 and TLR10 with 26% and

28% for EschTIR and 22% and 28% for BifTIR respectfully. While both BifTIR and EschTIR can be seen to have quite strong homology in the Box 1 region of the TIR domain, very little or no homology exists in the Box 2 and Box 3 regions.

Fig. 3.3 A

TLR1 and BifTIR

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TLR1  LQFHAFISYSGHDSFWVKNELLPNLEKEGMQICLHERN-----FVPGKSIVENIITCIEK 689
BifTIR MAHKTFISYKYSESRFYRDQILDALGDDAVFYKGETSDSPNLTDTTTENIKNHLKQMIYD 60
      :  :::*****.  :* :  ::::*  *  ::::  .  :  ..  .*  :::  *  .

TLR1  SYKSIFVLSPNFVQSEWCHYELYFAHNNLFHEGSNSLILILLEPIQPQYSIPSSYHKLKS- 114
BifTIR TTVTIIVLSPNMLQSNWIDWEISYSLKNISRDRGRTSHTDGLLGVIPPFYGNYSWFISEIN 120
      :  :::*****::**:*  ::::  ::  :::  ::*  .*  **  **  :  *:.  :

TLR1  -----LMARRTYLEWPKEKSKRG-----LFWANLRAAIN--I 778
BifTIR HPDGHVTVSYNEELTFPIMKANRGNQKPKVYACPCQSIDKLSGSYLSYVKMEDFVNNPS 180
      :  .  *  :*  *::**  *  ::::.  :*

TLR1  KLTEQAKK----- 786
C5ECT5 RYIDNAYDKSLNADNNYDLTKLL 203
      :  ::*  .

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TLR6 and BifTIR

```

TLR6  NLQFHAFISYSEHDSAWVKSELVPYLEKEDIQICLHERNFVP-----GKSIVENIINCI 692
BifTIR -MAHKTFISYKYSESRFYRDQILDALG-DDAVFYKGETSDSPNLTDTTTENIKNHLKQMI 58
      :  :::*****.  :* :  :::::  *  :*  :  *  .  *  ..*  ::*  :::  :  *

TLR6  EKSYKSIFVLSPNFVQSEWCHYELYFAHNNLFHEGSNNLILILLEPIQPONS-----IP 745
BifTIR YDTTVTIIVLSPNMLQSNWIDWEISYSLKNISRDRGRTSHTDGLLGVIPPFYGNYSWFISE 118
      .:  :::*****::**:*  ::::  ::  :::  ::*  ..  **  **

TLR6  NKYHKLKALMTQRTYLQWPKEKSKRG-----LFWANI RAAFN- 782
BifTIR INHPDGHVTVSYNEELTFPIMKANRGNQKPKVYACPCQSIDKLSGSYLSYVKMEDFVNN 178
      :  .  .  .:  .  *  :*  *::**  *  ::::.  .*

TLR6  -----MKLTLVTENNDVKS--- 796
BifTIR PSRYIDNAYDKSLNADNNYDLTKLL 203
      .  :*  ::**  :

```

---- = **Box 1**
 ---- = **Box 2**
 ---- = **Box 3**

A third bacterial TIR protein from a strain of *Lactobacillus salivarius* was identified by an APC colleague. Their research on this bacterium had suggested that it was using some form of innate immune subversion and a bioinformatics search revealed it to have a significant TIR domain containing protein (O'Toole *et al.* unpublished). For the purposes of this report this protein is termed LacTIR and was included with BifTIR and EschTIR in subsequent experiments to investigate possible interference of host innate immune signalling by the TIR domains of these proteins.

3.2.2 Subcloning of selected bacterial TIR proteins

TIR sequences were amplified by PCR from genomic bacterial DNA and inserted into the pcDNA 3.1 myc-HisA vector as described in the methods section 2.6. The corresponding restriction enzyme nucleases used for cloning were also used to digest the plasmids following insertion. The samples were run on a 1% Agarose gel to confirm insertion of the bacterial TIR sequences (Fig. 3.4). The sequence of the construct was confirmed by sequence analysis.

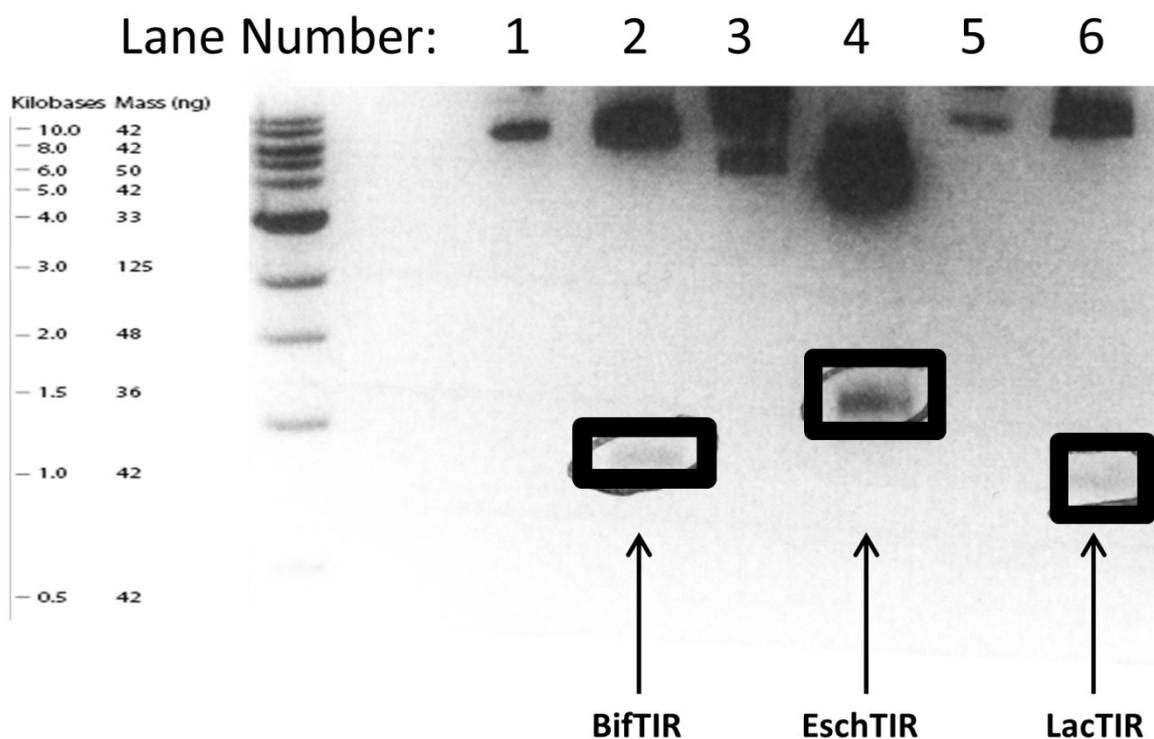


Figure 3.4 Confirmation of insertion of TIR sequence into pcDNA3.1 myc-His A vector. Shown above is a photo of an agarose gel containing the plasmids for each of the three TIR proteins listed. Lane 1 contains the undigested BifTIR plasmid. Lane 2 contains the BifTIR plasmid digested with Xho I and HindIII. Lane 3 contains the undigested EschTIR plasmid. Lane 4 contains the EschTIR plasmid digested with Xho I and HindIII. Lane 5 contains the undigested LacTIR plasmid. Lane 6 contains the LacTIR plasmid digested with Xho I and HindIII. Circled on the photo and indicated with arrows are the TIR domain-containing bacterial protein inserts having been cut out of the vectors by the restriction digest.

3.2.3 Attempts at detection of protein expression by western blot and PCR

The bacterial TIR protein sequences, now ligated into the pcDNA3.1 vector, should also contain a Myc tag attached to the C terminus, resulting in a total protein size of between 25 and 35 kDa. Expression of the TIR proteins in transfected host cells should have been detectable by use of Western Blot. However, following several transfections using increasing amounts of each bacterial TIR protein plasmid into the Human Embryonic Kidney (HEK) 293 cells; this was found not to be the case (Fig. 3.5). This was despite clear detection of a positive control plasmid used (Myc-tagged p53). Transfecting into different cell lines, U373 and HT-29 cells, also resulted in no detection of the bacterial TIR proteins (data not shown).

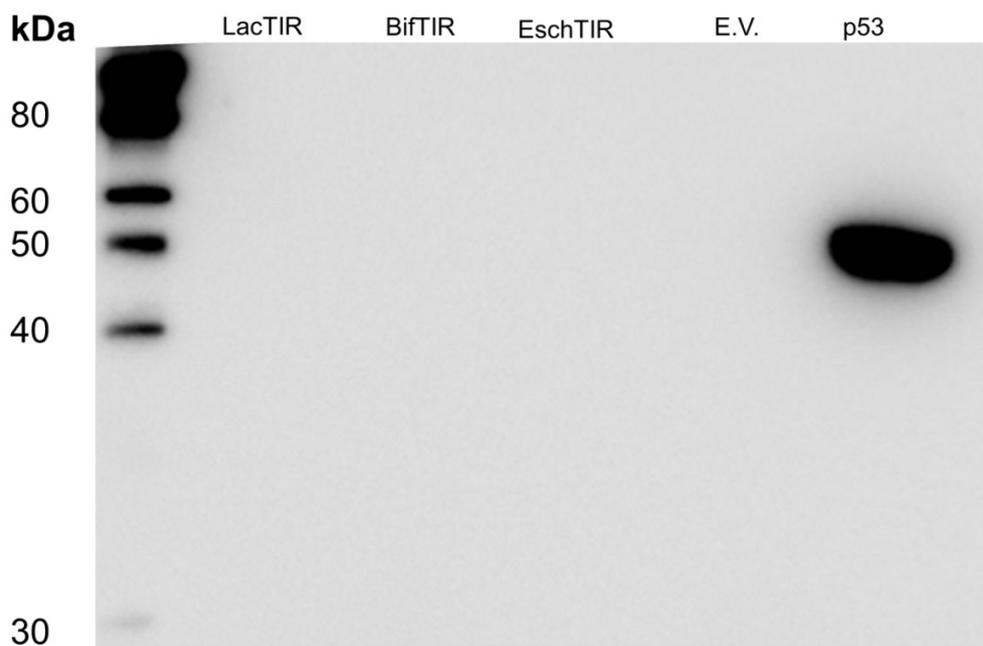


Figure 3.5 Western Blot for Myc tagged bacterial TIR proteins in HEK cells transfected with LacTIR, EschTIR, BifTIR or p53 pcDNA3.1 Myc plasmids. HEK cells were seeded on a 12 well plate and transfected with 2 μ g of each of the plasmids indicated as above (with E.V. standing for empty pcDNA3.1 Vector) as described in the methods section. Following 48 hours, cells were lysed and treated for Western blot analysis.

This procedure was repeated by a colleague from the O'Toole lab using the LacTIR-Myc plasmid. This group also failed to show expression of the construct with the PcDNA3.1 myc-his plasmid. To avoid concerns over the plasmid used, they cloned the LacTIR sequence into a new vector, pEF4/Myc-His A. This was sequenced several times to ensure the insert had been ligated into the correct region of the vector with the tag attached and with a good Kozak sequence, required for efficient expression. This was similarly not detectable by Western Blot following transfection into the cell line HEK293 (Fig. 3.6).

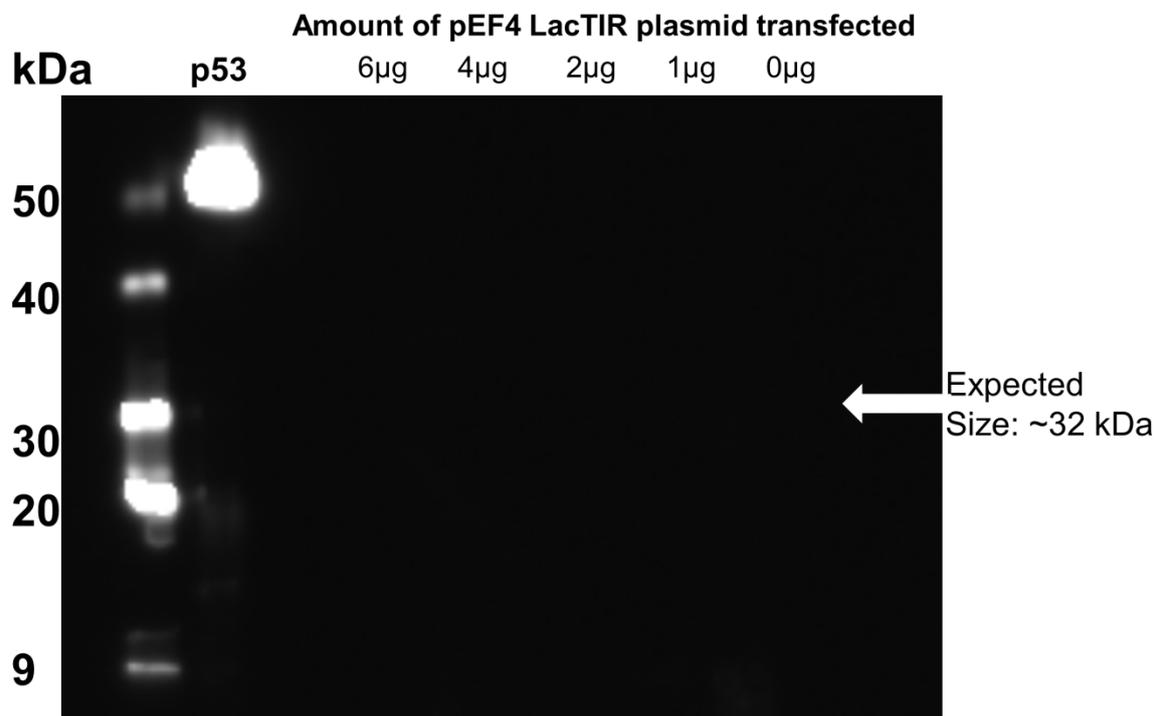
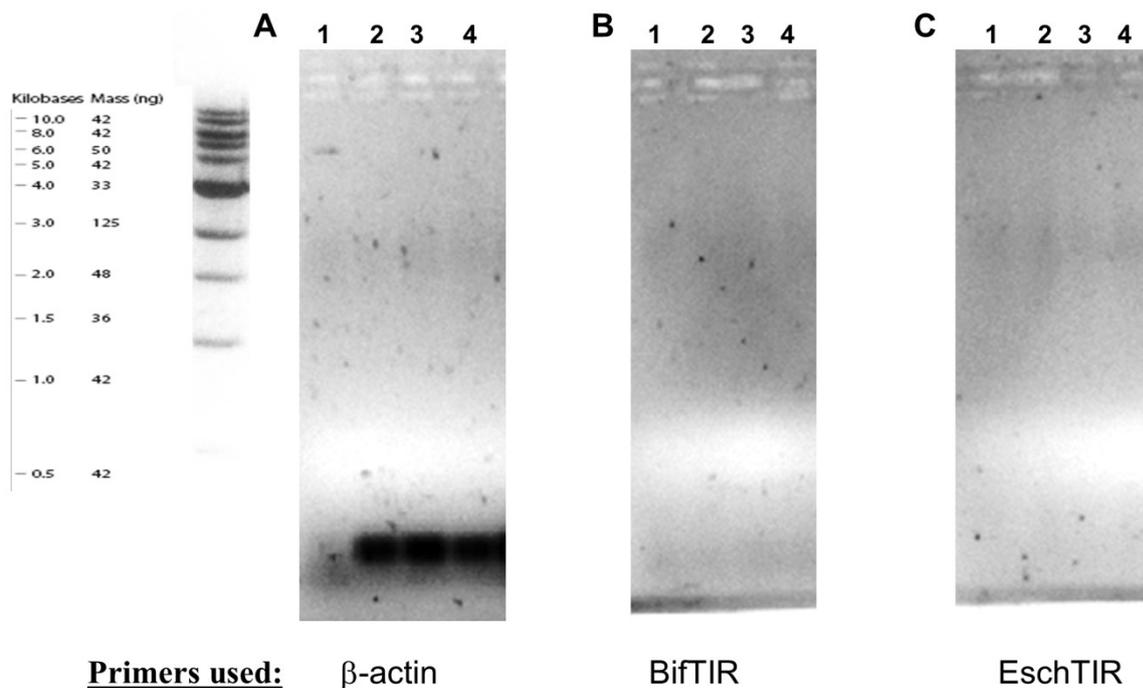


Figure 3.6 Western Blot for Myc tag in HEK 293 cells transfected with LacTIR pEF4 plasmids with p53 pEF4 plasmid as a positive control. HEK 293 cells were seeded on a 12 well plate and transfected with varying amounts of the LacTIR plasmid indicated as above. Following 48 hours, cells were lysed and treated for Western Blot analysis as described in the methods section.

Finally, HEK cells were transfected with empty vector, BifTIR, EschTIR or LacTIR plasmids and mRNA was prepared from the cell lysates the next day. cDNA was generated using reverse transcription and PCR was performed to detect the presence of mRNA for the BifTIR or EschTIR proteins. Primers were unavailable for LacTIR detection as this protein was the intellectual property of the Paul O'Toole laboratory. Figure 3.7 displays an agarose gel with PCR products from these reactions. An mRNA sample which had not been treated with reverse transcriptase was used as a negative control (lane 1). The presence of similar mRNA levels of housekeeper gene, β -actin, was confirmed for each of the lysates (Fig. 3.7A). However, the presence of the BifTIR mRNA was not detected in lane 3, where the BifTIR plasmid had been transfected, nor was it detected in any other sample. Similarly, EschTIR was not detected in lane 4, containing cDNA from a lysate transfected with EschTIR plasmid, nor was EschTIR mRNA detected in any other sample.



cDNA of HEK transfected with:

1. Negative control (-RT)
2. Empty Vector
3. BifTIR
4. EschTIR

Figure 3.7 Agarose gel analysis of PCR products of cDNA from HEK cells transfected with TIR plasmids. HEK cells were transfected with an empty vector, BifTIR plasmid, EschTIR plasmid or LacTIR plasmid as described in the methods section. The next day, mRNA was isolated from the HEK lysates and cDNA was generated using reverse transcription. PCR was then performed on the cDNA using primers for housekeeper gene β -actin (A), BifTIR (B) and EschTIR (C) to investigate the presence of these genes in the cDNA. The products of these PCR reactions were then ran on an agarose gel as indicated.

3.2.4 Effects of bacterial TIR proteins on TLR mediated NF- κ B activation by luciferase assay

Despite the failure to detect the protein by Western Blot or PCR, the extensive sequence analysis of the plasmids revealed that the insert had been ligated in the correct position of each corresponding vector. We hypothesised that the protein might be expressed at low levels and as such we proceeded with some functional analyses of the constructs, specifically investigation of the effect of the bacterial TIR domains on TLR induced NF- κ B activation. The murine macrophage RAW264 cell line was selected due to their responsiveness to ligand stimulation. These cells were transfected with each of the bacterial TIR proteins and stimulated with LPS. Effects on NF- κ B activity were monitored using an NF- κ B luciferase reporter assay with a plasmid for the NF- κ B subunit p65 as a positive control for NF- κ B activation. Figure 3.8 shows that although no statistically significant change was recorded in NF- κ B activity between the LPS stimulated RAW264 cells, there is a trend towards lower NF- κ B activity in the TIR protein transfected samples.

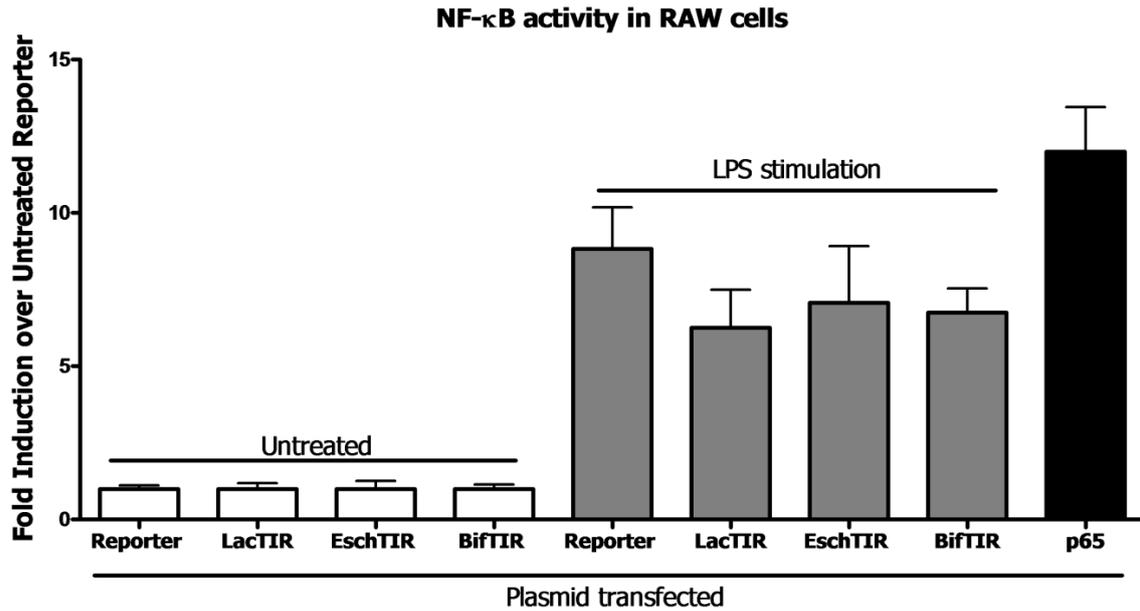


Figure 3.8 NF-κB luciferase reporter assay in RAW cells. RAW264 cells were transfected with TK Renilla and NF-κB luciferase reporter plasmids. Some samples were transfected with 100ng of the TIR plasmids or p65 plasmid as indicated on the graph. All transfections were normalised to a total of 200ng DNA with empty vector. 24 hours later, LPS was added as indicated at 100ng/ml for 8 hours. Cells were then lysed and luciferase activity was measured relative to the untreated control as described in the methods section. Values are shown as Mean ± SEM relative to the unstimulated empty vector control from three separate experiments, each carried out in triplicate. Statistics were carried out using t test with Welch's correction.

A level of variation in NF-κB was seen between experiments and as such we performed subsequent experiments in the HEK 293 cell line. Although these cells do not respond to most TLR ligands, they are highly transfectable so NF-κB may be activated by over-expressing plasmid constructs expressing TLRs or their adaptor proteins. In Figures 3.9, 3.10 and 3.11 NF-κB activation was driven by over-expression of TLR4, MyD88 or MAL respectively in HEK 293 cells. The HEK 293 cells were then transfected with increasing amounts of the bacterial TIR proteins in order to measure any abrogation of the NF-κB activity, driven by the TLR4, Mal or MyD88 constructs, from cross-talk between the TIR domains of these constructs and the bacterial TIR proteins.

In Figure 3.9A, TLR4 increased NF- κ B activity 4-fold over untransfected and this was reduced to 50% by BifTIR in a dose-dependent manner. Despite a visible decrease in TLR4-induced NF- κ B activity due to the transfection of the EschTIR protein at lower doses and an increase at higher doses, these differences were not found to be statistically significant (Fig. 3.9B). Similar to the effect seen by BifTIR in Fig. 3.9A, transfection of the LacTIR plasmid results in a highly significant decrease in TLR4-driven NF- κ B activity. However, this decrease is also less than 50% and there was no significant effect when the plasmid was transfected at the highest dose (50ng).

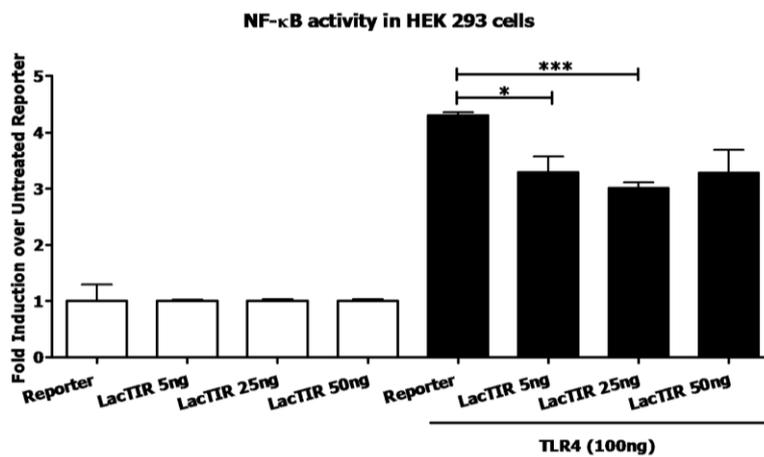
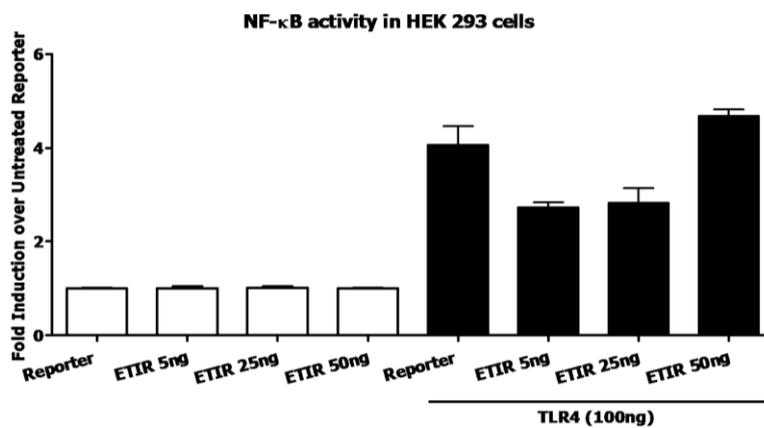
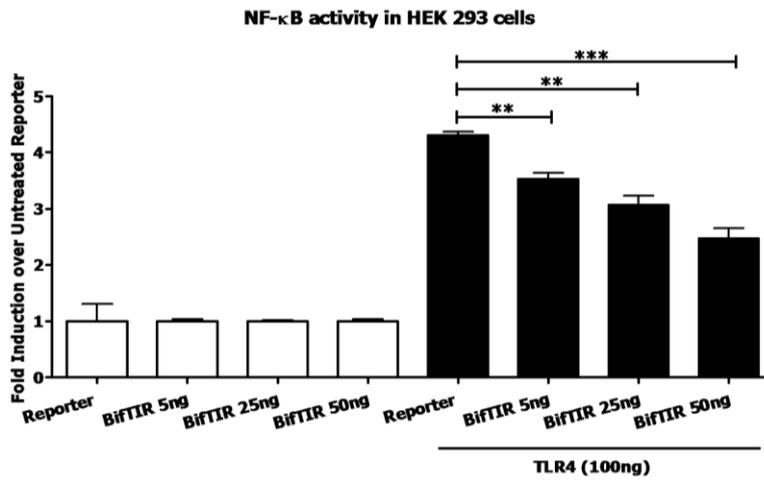


Figure 3.9 NF-κB luciferase reporter assay in HEK 293 cells following over-expression of TLR4. All cells were transfected with NF-κB reporter plasmid as described in the methods. Varying amounts of the TIR plasmids BifTIR (A), EschTIR (B) or LacTIR (C) were also transfected as indicated. NF-κB was activated by overexpressing TLR4. All transfections were normalised to 200ng DNA using the empty vector. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Statistics were carried out using t test with Welch's correction.. Values are shown as Mean \pm SEM relative to the unstimulated Empty Vector reporter control. n=3.

Figure 3.10 displays a consistent 3-fold increase in NF- κ B activity following over-expression of MAL. While this activation is significantly decreased when 5ng of the BifTIR plasmid is transfected, this effect is not observed at higher doses. No changes were observed in MAL-driven NF- κ B activation at all concentrations of EschTIR plasmid transfection and the minimal decrease visible from LacTIR transfection was not found to be significant.

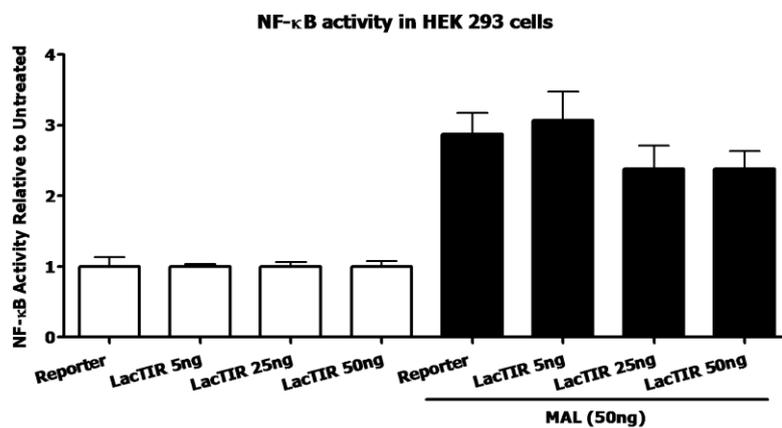
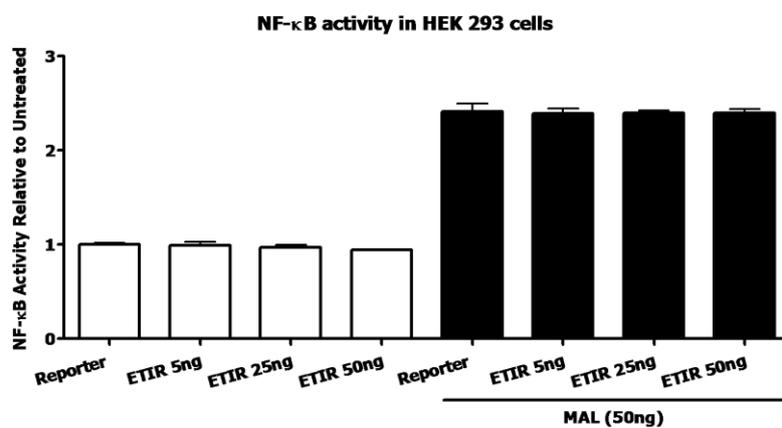
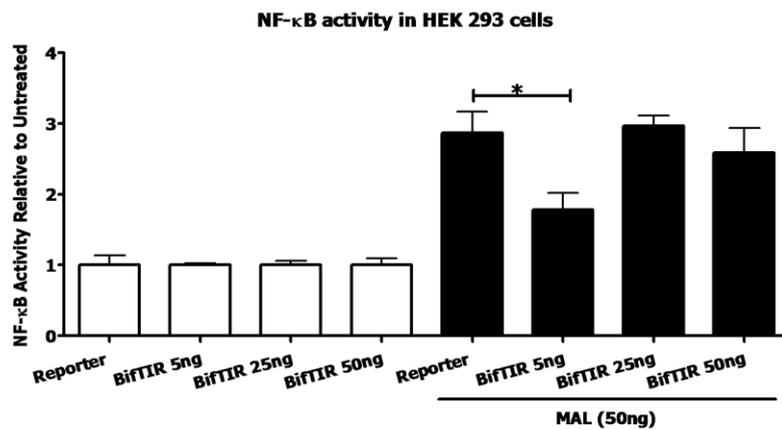


Figure 3.10 NF-κB luciferase reporter assay in HEK 293 cells following over-expression of Mal. All cells were transfected with NF-κB reporter plasmid as described in the methods. Varying amounts of the TIR plasmids BifTIR (A), EschTIR (B) or LactTIR (C) were also transfected as indicated. NF-κB was activated by overexpressing MAL. All transfections were normalised to 200ng DNA using the empty vector. * P<0.05, ** P<0.01 and *** P<0.001, Statistics were carried out using t test with Welch's correction. Values are shown as Mean ± SEM relative to the unstimulated Empty Vector reporter control. n=3.

Over-expression of MyD88 was used to drive NF- κ B activation in Fig. 3.11. Fig. 3.11 A and C display a 7-fold increase in NF- κ B activation relative to the reporter control. A 5-fold increase is seen in Fig. 3.11B. However, despite slight increases in NF- κ B activity observed when MyD88 is transfected with all 3 of the bacterial TIR protein plasmids, particularly LacTIR and BifTIR, these increases were not statistically significant.

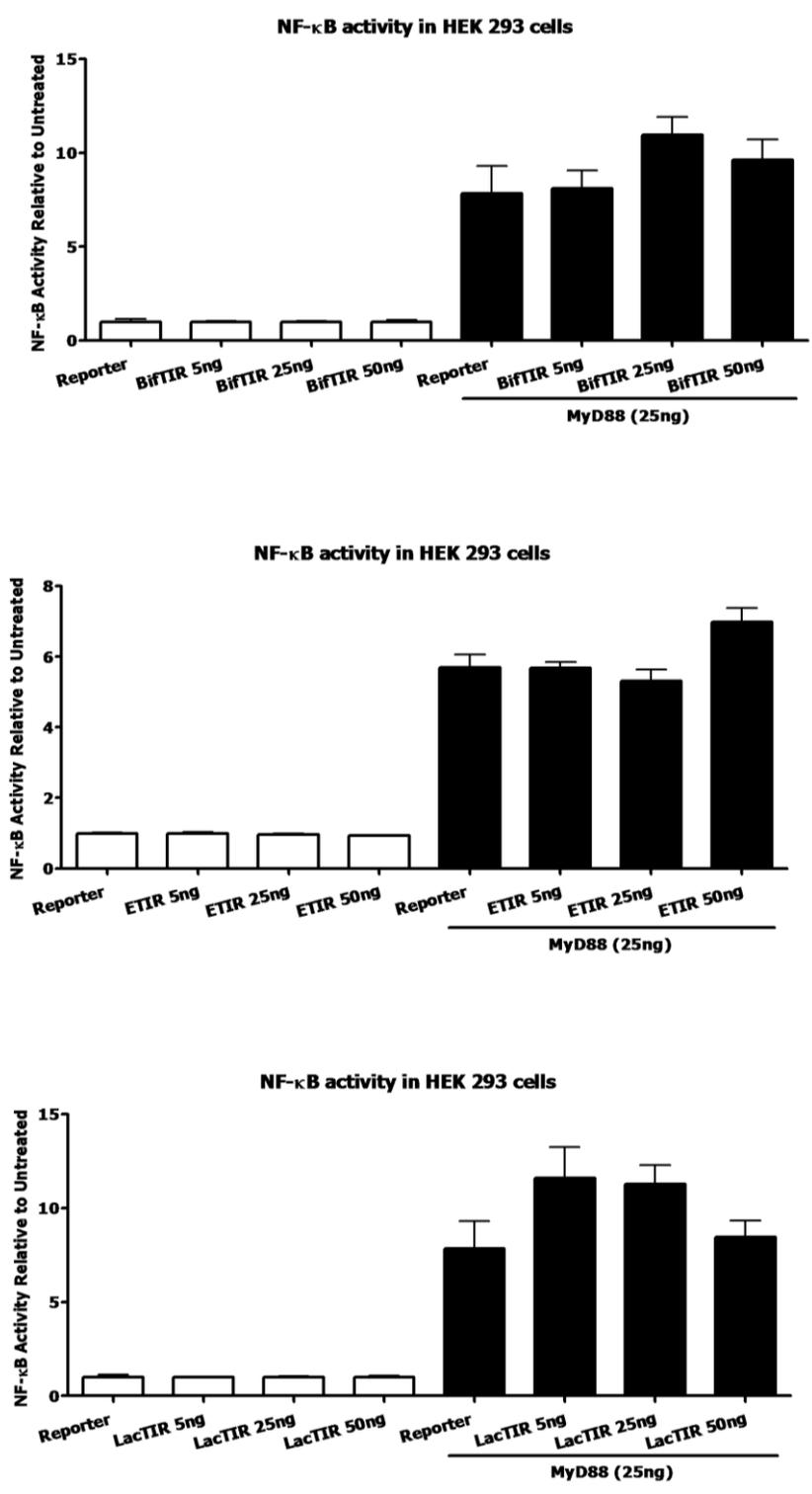


Figure 3.11 NF-κB luciferase reporter assay in HEK 293 cells following over-expression of MyD88. All cells were transfected with NF-κB reporter plasmid as described in the methods. Varying amounts of the TIR plasmids BifTIR (A), EschTIR (B) or LacTIR (C) were also transfected as indicated. NF-κB was activated by overexpressing MyD88. All transfections were normalised to 200ng DNA using the empty vector. * P<0.05, ** P<0.01 and *** P<0.001, Statistics were carried out using t test with Welch’s correction. Values are shown as Mean ± SEM relative to the unstimulated Empty Vector reporter control. n=3.

3.3 Discussion

Interest has grown in the use of mimicry by microbes to evade immune responses through modulation of host TIR:TIR signalling by use of their own TIR domain containing proteins. The advances that arise from studying such interactions not only include a better understanding of how microbes evade our immune system, but offer such additional benefits as potential cancer and auto-immune treatments [21]. Highly potent TLR signalling inhibitors have already been generated based on the A46R protein from *vaccinia* virus [22]. Many more such immune evading proteins have been discovered in pathogenic bacteria since the discovery of A46R which use host signal domain bio-mimicry [21] offering the potential for further exploitation at a clinical level. To date, however, no extensive study has been performed on the existence or use of such proteins in commensal bacteria of the human gastro-intestinal tract. The human intestine itself is known to differentially express its receptors on the surface of cells facing into the gut lumen to avoid an over-active immune response [23]. It has even been shown to respond directly to specific Commensal-Associated Molecular Patterns (CAMPs) from commensal microbes to further drive this expression differentiation [24]. We wished to investigate if some organisms might utilise bacterial:host TIR:TIR signalling with their own proteins in order to create a habitable niche for themselves in this environment through direct subversion of innate immune signalling. Furthermore, we wished to investigate the possibility that these interactions may facilitate the general state of immune unresponsiveness that the intestinal epithelial layer maintains to the presence of commensal bacteria. Inflammatory Bowel Diseases (IBD) leads to dysregulation of inflammation in the gut. Targeting any specific part of the immune system can be quite difficult without having undesired side-effects. However, if commensal bacteria used microbe:host TIR:TIR interactions in order to reduce immune signalling in the gut, it would likely be in a very site-directed and specific manner. This opens the door for potentially using

either the microbes or their TIR proteins themselves as anti-inflammatory treatment in diseases such as IBD.

Bifidobacterium infantis is a Gram-positive commensal bacteria. It inhibits the growth of Gram-negative bacteria by producing lactic acid, and helps digest complex sugars in human breast milk [25]. It has been proven to help in cases of digestive disorders [26-29] and is believed to work in enhancing healthy homeostatic properties in the gastro-intestinal environment by influencing both the microbial and host physiology [30, 31]. We wished to investigate if the selected strain of *Bifidobacterium* used its TIR domain-containing protein to reduce TLR signalling in the gut as a potential mechanism for immuno-regulation in this region. While *Escherichia* bacterial strains are perhaps better known for their pathogenic ability, however, species which are inhabitants of the gastrointestinal tract of warm-blooded animals, such as the strain we had selected, have been known to offer protective attributes to the intestinal epithelia also [32]. *Escherichia* species provide a portion of the microbially derived vitamin K for their host [20]. Furthermore, specific commensal strains of *Escherichia coli* have been shown to reduce intestinal epithelial inflammatory signalling, *in vitro*, through repression of NF- κ B signalling [33]. This is achieved through inhibiting the ubiquitination of the NF- κ B subunit I κ B. Similarly, we wished to investigate if the selected commensal strain of *Escherichia* used its TIR domain-containing protein to reduce inflammatory TLR signalling in this region also, in order to maintain a homeostatic environment in the gut. Furthermore, a signal peptide was identified on the EschTIR protein which may allow for the protein to be translocated to a specific region to act upon host signalling if the protein were secreted by the bacteria.

Similar to the previously characterised bacterial TcpB and TlpA proteins, known to modulate TLR and adaptor molecule signalling, the two proteins we had selected, EschTIR and BifTIR, were found to have strong Box 1 homology while lacking Box 2 and Box 3

regions [14]. Similarly also, they shared higher sequence similarities with the gene cluster of TLRs 1, 2, 6 and 10, in particular with TLR1 and TLR6. Additionally, the sequence of the EschTIR protein was quite similar to the subversive immune evading TcpC protein identified in uropathogenic *E.coli* [10]. However, an important point to note here is that when TIR domains with marginal sequence similarity are identified, such as between microbes and humans, some of the proteins with certain TIR domain signature sequences may turn out to differ in their tertiary structure folding, which may affect their interactions [34].

Although sequence analysis of the plasmids into which we had cloned these two bacterial TIR proteins confirmed that insertion had taken place correctly, we were unable to detect expression of the proteins through western blotting when these plasmids were transfected into human cell lines. Positive controls were used in such analyses in order to confirm the functionality of the transfection and blotting procedures. Similarly, PCR reactions were performed to detect the presence of bacterial TIR protein mRNA in HEK cells transfected with TIR plasmids although none was detected. It was hypothesised that the small proteins from these plasmids may not be detectable by Western Blot or PCR. This could be due to very low expression efficiency of the proteins in the plasmid. Furthermore, smaller proteins are more difficult to detect than larger proteins by Western blotting and the milk solution used in the blocking stage of the Western blot technique may have resulted in non-specific binding to the bacterial proteins – resulting in a lack of detection. It would be worth using other known Western blotting blocking agents such as BSA or casein to investigate if this is the case.

Despite the difficulties in protein detection, NF- κ B activation luciferase assays were performed in order to investigate if expression of these proteins resulted in abrogation of TLR induced NF- κ B activation. This was achieved through TLR4 activation using LPS stimulation as well as over-expression of TLR4 and adaptor molecules MyD88 and MAL.

The BifTIR protein, in addition to the LacTIR protein appeared to have the most potential for inhibiting TIR-mediated, NF- κ B activation. Both exhibited a trend for reduced NF- κ B activity in response to a challenge with endotoxic LPS in RAW macrophage cells. These cells were used due to their high transfectability and endogenous expression of TLRs. However, they are a murine cell line and it is likely that any potential modulation of TIR signalling by the bacterial TIR proteins studied here would be specific to TIR signalling in the human host. With the use of the human HEK cell line, both BifTIR and LacTIR proteins were also able to significantly reduce NF- κ B activity caused by overexpression of TLR4. These results were similar to effects were seen following expression of bacterial proteins TcpB and TcpC [34]. Unlike these two proteins, however, neither of the two proteins in this study was able to abrogate NF- κ B activation driven by TLR4 adaptor proteins MyD88 and MAL indicating that they may exert their potential immuno-modulatory effects by inhibiting directly at the level of the receptor.

The EschTIR protein however, did not appear to have any consistent or significant effect on the inflammation processes investigated. This protein was found to contain a signal peptide, which may be used to deliver the protein to a specific part of the host for signalling. Additionally, it is possible that its function may not be uncovered by over-expression within the cell; it may need to be isolated and added to the cell to fully reveal its function. Since we did not explore every method of TIR signalling interfaces upon which these proteins might act, it is possible that this protein evades innate immune surveillance through TIR interactions we have not explored. In addition to NF- κ B, other known downstream effectors in the TLR signalling cascade could also be affected from interactions between these bacterial TIR proteins and our innate immune signalling systems; such as Extracellular signal-Regulated Kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK) [35]. The same may, of course, be said of the LacTIR and BifTIR proteins. Furthermore, these proteins might behave

differently in the setting of IEC or gut macrophages known to have distinct signalling mechanisms. An *in vivo* study using isolated forms of each protein might prove to yield more information on this. However, using a mouse model, one should be conscious of the fact that these TIR proteins have been isolated from human commensal bacteria. Investigation of effects on TLR2 signalling would also be a worthwhile area in which to study these proteins further. Through BLAST analysis, the BifTIR and EschTIR proteins were found to share the best homology with TLR1 and TLR6 which both require TLR2 for signalling. It could be that the proteins bind to TLR2 – preventing it from forming a dimer with TLR1 or TLR6 through which it can mediate an inflammatory signal. Based on the results from this study, it is likely that TLR4-driven NF- κ B activation is limited somewhat by the BifTIR and LacTIR proteins due to their binding to the receptor itself; both proteins had little or no effect on NF- κ B activation driven by over-expressing either MAL or MyD88, two adaptor molecules required for TLR4 signalling, indicating that any interference in TIR:TIR interactions likely to be happening upstream of the adaptors at the level of the receptor itself. If this effect was observed with TLR4, it is quite possible that the same could be true for the closely related TLR2 or that EschTIR may play a role in modulating signalling here.

Many obstacles were encountered surrounding plasmid cloning and detection of expression throughout the study. Furthermore, inconsistencies were associated with studies using the RAW murine macrophage cell line and the non-quantitative luciferase assays. Due to time constraints, it was impractical to progress this work any further because of these obstacles and difficulties. A definitive result regarding the expression of the TIR proteins following transfection of the plasmids is crucial before any direct result can be taken from this work. Furthermore, there remains the possibility that any decreases seen in NF- κ B activity following transfection may be due to transfection artefact when measured using the luciferase reporter assay. A purification of the TIR proteins may be performed and added to

cells in culture to observe such effects and avoid the possibility of ambiguous data due to transfection artefact.

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Chapter 4

An investigation into colonic homeostasis induced by TLR9 signalling

4.1 Introduction

The gut epithelium has an important function in the maintenance of intestinal immune homeostasis. It ensures that an effective immune response is raised to infectious microorganisms while itself remaining predominantly unresponsive to the myriad of commensal bacteria it is in contact with. This is achieved by preventing the contact of bacteria with immune cells through the formation of a physical barrier and the expression of antimicrobial peptides [1-3]. At the frontline of this interface, the Intestinal Epithelial Cells (IECs) line the gut epithelium. It is possible that homeostasis in the intestinal tract could in fact be maintained by unique features of IECs, rather than unique bacterial features. They are structurally and functionally polarized, with an apical surface facing the intestinal lumen and a basolateral surface facing the adjacent cells in the lamina propria. This spatial organisation of the IECs allows for distinct functions at each surface utilising distinct membrane proteins [4]. In addition to their role as a protective barrier at this mucosal interface, IECs have also been shown themselves to play a very important part in our body's immune response [5]. The polarity and tight junctions of IECs play a particularly important part in this response, enabling the segregation of apical signals from basolateral signals in pathogen recognition. This is mediated through differentially expressed Pattern Recognition Receptors (PRRs) such as Toll-Like Receptors (TLRs).

IECs are known to express mRNA for all known TLRs, TLR1-10 [6], and have also been shown to express TLR1-9 protein [7-12]. The expression and function of TLR2 [13], TLR4 [14], TLR5 [15], and TLR9 [6, 16, 17] has been best characterised in IECs. Certain TLRs are expressed on the apical vs. the basolateral membranes. For example, TLR5 is expressed intracellularly or basolaterally in the colon [15], thereby avoiding an inappropriate inflammatory response to flagellated commensal bacteria present in the gut lumen, (apical side of the epithelia). TLR2 and TLR4 have been shown to be present at the apical surface of

polarized, confluent IEC, in the cell line T84, which readily respond to acute stimulation with the corresponding bacterial ligands [6, 9, 13]. In contrast, TLR2 and TLR4 are mostly present in the cytoplasmic compartment in undifferentiated IEC and are expressed in much lower amounts than in differentiated IEC. This correlates with ligand tolerance as IECs are predominantly unresponsive to TLR2 and TLR4 ligands [13]. TLR2 expression in particular has been shown to play a specific role in IEC homeostasis. It controls tight-junction-associated intestinal epithelial barrier integrity in order to balance mucosal homeostasis against inflammatory-stress-induced damage. This is achieved by targeted modulation of barrier function proteins such as ZO-1 and Connexin43 (Cx43) following TLR2 activation [18, 19]. TLR9 is also involved in maintenance of intestinal homeostasis. Several groups have shown that TLR9 knockout mice have increased severity in cases of artificially induced colitis [20, 21]. Furthermore, feeding mice with CpG Oligodeoxynucleotides (ODNs), the ligand for TLR9, resulted in inhibition of induced colonic proinflammatory cytokines and chemokines such as IL-8 in addition to colitis symptoms [22, 23].

In addition to differential expression, differential regulation of TLRs between the apical and the basolateral surfaces of these cells may allow activation of signalling pathways necessary for TLR mediated homeostasis but not TLR-mediated pro-inflammatory effects. A study performed by Lee *et al.* further highlighted a role for TLR9 in intestinal homeostasis through differential regulation of TLR signalling. HCA-7 cells, an IEC line, when grown to a polarised monolayer, produced IL-8 in response to basolateral, but not apical, stimulation of TLR3, TLR5 and TLR9 while only responding to apical and not basolateral stimulation of TLR2 [12]. Apical TLR9 stimulation was, however, found to confer intracellular tolerance to subsequent TLR challenges [12]. Moreover, apical TLR9 stimulation was also shown to inhibit IL-8 production in response to apical TLR2 and basolateral TLR3 or TLR5 stimulation [12]. Taken together, these data suggest that this “polarising-tolerising” ability of

IECs represents a unique mechanism, which helps restrain inflammatory responses in a bacteria rich environment.

The mechanism by which TLR9 signalling maintains this homeostasis and in addition, how apical TLR9 signalling may inhibit inflammatory responses from other TLRs was investigated further by Lee *et al.* [12]. Microarray analysis revealed that the most prominent apical TLR9-specific target gene was Frizzled5 (Fzd5) [12]. Fzd5, a major gut receptor for Wnt, a signalling glycoprotein, is involved in the maturation of Paneth cells in the small intestine, which produce anti-microbial peptides such as cryptidins and defensins [24]. In concordance with this *in vitro* data, Fzd5 mRNA levels, together with its target genes, cryptidins and defensins, were downregulated in the small intestines of TLR9^{-/-} mice compared with wild-type mice [21]. β -catenin, an intracellular signalling intermediate located on the Wnt signalling pathway, can physically complex with NF- κ B, resulting in a reduction of NF- κ B DNA binding, transactivation activity, and target gene expression [25]. Activated β -catenin has been found to inhibit the expression of NF- κ B target genes. Since Wnt signalling activates β -catenin, and Fzd5 is involved in this pathway, these authors proposed that, by using this pathway, apical TLR9 signalling may mediate an inhibitory effect on NF- κ B. This is in clear contrast with data showing that TLR2 and 4 induce expression of Wnt5a and Fzd5 in macrophages, T cells and B cells in an NF- κ B dependant manner and that this induction of Wnt5/Fzd5 subsequently enhanced IL-12 and IFN γ production by *M. tuberculosis*, also in an NF- κ B dependant fashion [26]. Given the conflicting evidence between these reports and the data of Lee *et al* showing that TLR9 strongly upregulates Fzd5 in a non-NF- κ B dependant manner, it is clear that further studies are needed to clarify the cross-talk between Wnt and TLR signalling. In addition, there has been no study performed identifying the direct effects of TLR signalling on the Wnt/ β -catenin pathway in IECs.

In the following study, I aimed to identify novel mechanisms behind the maintenance of tolerance in the gastro-intestinal tract through TLR9 regulation and signalling. Specifically, I aimed to characterise apical TLR9 signalling in IECs and determine the involvement of known signalling intermediates in apical TLR9 signalling to elucidate what signalling pathways were used to activate Fzd5 while inhibiting IL-8 production. In addition, I aimed to examine the role of TLR inhibitory proteins in the control of both proinflammatory and homeostatic signalling in IECs in addition to identifying novel TLR interacting proteins in IECs. To do this, I used different intestinal epithelial cell lines grown in the polarising transwell system to allow differentiation of both apical and basolateral surfaces to initially confirm the results of Lee *et al.* This involved an attempt to induce tolerance to TLR-induced inflammatory signalling through apical TLR9 stimulation with its ligand, CpG, in a monolayer of IEC using the transwell system. I then investigated pro-inflammatory gene activation and also induction of Fzd5 α . I further aimed to elucidate the mechanism through which this occurs by investigating the Fzd5/Wnt/ β -catenin signalling cascade through TLR9 stimulation.

4.2 Results

4.2.1 Validation of monolayer formation in a Caco-2 cell clone

Initially, we wished to define the optimal time-frame required for an effective polarized monolayer of intestinal epithelial cells to be generated. This was to ensure efficient polarisation of the cells with the aim of producing differential TLR expression and signalling mechanisms, on the apical and basolateral surfaces of the cells. C2Bbe1 (C2B) cells, a clone of the Caco-2 cell line, were used as they have been reported to form monolayers more efficiently than other IEC lines. Trans-Epithelial Electrical Resistance (TEER) measures the resistance between the apical and basolateral chambers through the transwell membrane and hence correlates to the formation of a monolayer of cells, as described in the methods section 2.2. The tighter and more complete the monolayer formed, the higher the TEER value. The graph in Figure 4.1 was constructed by measuring the TEER over time of several transwells (graph shown representative of 12 wells in 2 different plates).

The graph demonstrates that a resistance of $1,000\Omega$ is reached after 12 days from which time it remains relatively constant – confirming that the formation of a monolayer of cells is achieved after 12 days. This information was utilised in ensuring complete formation of a monolayer in future experiments in order to achieve an efficiently polarised epithelial monolayer.

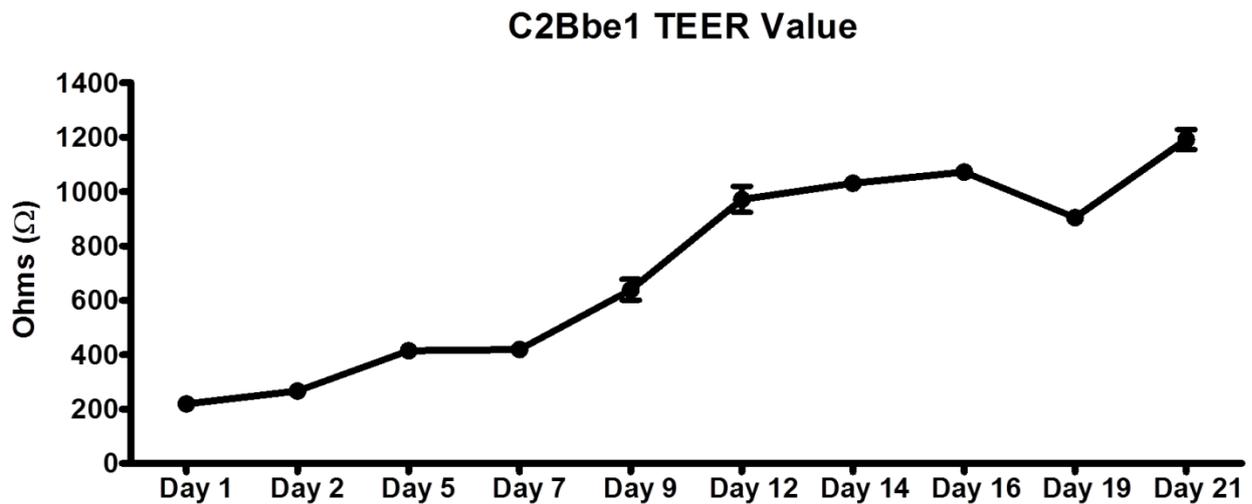


Figure 4.1 Trans-Epithelial Electrical Resistance measured over C2Bbe1 cells in a transwell system. C2Bbe1 cells were grown in a transwell system while trans-epithelial electrical resistance was measured over a duration of 3 weeks. Values are shown as Mean \pm SEM, n=3

4.2.2 Response to TLR ligands using C2Bbe1 Intestinal Epithelial Cell lines

The aim of our initial experiments was to reproduce the observations of Lee *et al.*, whereby a twofold induction of Fzd5 was observed following apical stimulation with TLR9 ligand (CpG) in IECs while IL-8 production was only induced following basolateral CpG stimulation [12]. Prior to attempting to reproduce this effect in C2Bbe1 cells, their ability to produce IL-8, Fzd5 α and TNF α in response to TLR9 stimulation was initially examined. Figure 4.2 displays qRT-PCR data from C2Bbe1 lysates which had been grown to a monolayer on transwells prior to stimulation with TLR9 ligand. This stimulation, however, failed to induce any significant changes in IL-8, Fzd5 α or TNF α expression, as seen in Figure 4.2 A, B and C respectively, with a surprising 2-fold decrease in TNF α induction from basolateral stimulation seen in Figure 4.2C.

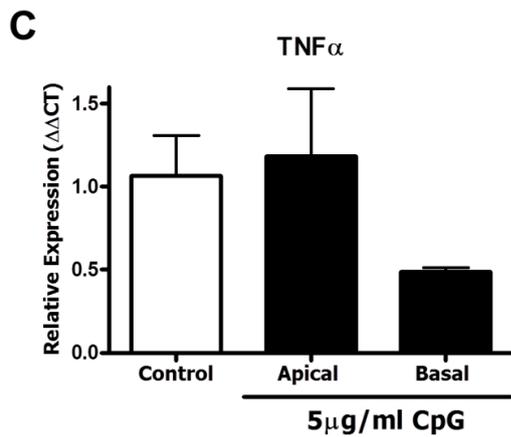
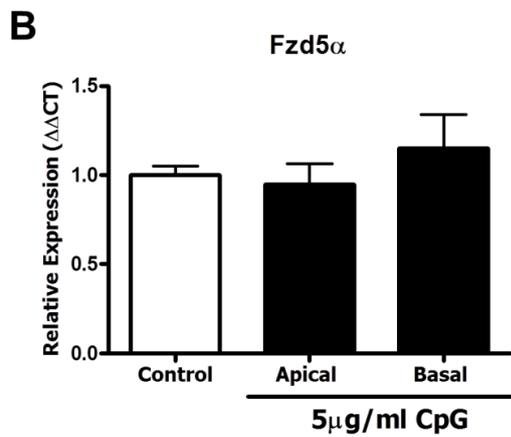
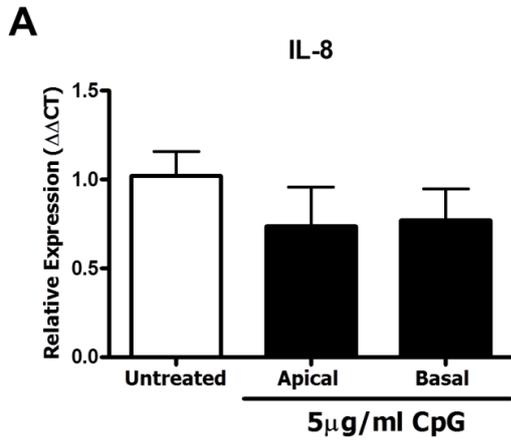


Figure 4.2 Response to apical or basolateral TLR9 stimulation in a C2Bbe1 monolayer.

Polarized C2BBe1 cells were stimulated with CpG (ISS ODN; 5 μ g/ml; TLR9); apically or basolaterally (basally) as indicated for 8 hours. mRNA levels were measured by qRT-PCR for relative expression of IL-8 (A), TNF α (B) or Fzd5 α (C). Values are shown as Mean \pm SEM, n=3. Statistics were carried out using t test with Welch's correction.

4.2.3 Response to TLR ligands *in vitro* using T84 and Caco-2 Intestinal Epithelial Cell lines

Having failed to observe a measurable response in the C2Bbe1 cells when subjected to TLR9 ligand stimulation, we sought to use other Intestinal Epithelial cell lines. Caco-2 (Figure 4.1) and T84 (Figure 4.2) Intestinal Epithelial Cells (IECs) were grown to a confluent monolayer over 21 days with the aim of achieving polarisation on a transwell system. The cells were then stimulated with a range of TLR ligands; LPS (TLR4), Flagellin (TLR5) and CpG DNA (TLR9). qRT-PCR was used to measure induction of IL-8 and TLR9 response gene, *Fzd5*, in response to these ligands. We also attempted to reproduce the findings of Lee *et al.* showing that apical stimulation of IECs with CpG was able to abrogate subsequent TLR stimulation [12]. This result was of particular interest to us as the aim of this project was to identify the mechanistic basis underlying the homeostatic role of TLR9 in IECs. For this investigation, some of the cells were stimulated apically with TLR9 ligand prior to basolateral stimulation with TLR4, TLR5 and TLR9 ligands. LPS was selected as a negative control for IL-8 induction as polarised IECs are broadly unresponsive to extracellular LPS following apical stimulation [13]. Flagellin was selected as a positive inflammatory control as IECs have been shown to produce pro-inflammatory cytokines following basolateral but not apical stimulation [12].

In Figure 4.3A, IL-8 expression was increased 2.5-fold over untreated control following apical CpG stimulation, although apical TLR4 and TLR5 stimulation with LPS and flagellin respectively resulted in no increased IL-8 expression. This was in contrast to previously published results [12, 13]. 3-fold induction of IL-8 was observed following basolateral LPS stimulation, however. In addition, basal stimulation of TLR5 resulted in a 1.5-fold level of IL-8 induction, similar to previous findings [15]. A 50% reduction in IL-8 induction was observed over the untreated cells in cells subjected to basolateral LPS and CpG

stimulation following pre-treatment with apical TLR9 stimulation. However, this was not on a similar level to the total abrogation observed by Lee *et al* [12]. Figure 4.3B shows a 3-fold induction of Fzd5 α following apical TLR9 stimulation but also 5-fold from basolateral TLR4 stimulation. Furthermore, none of these changes in expression were found to be statistically significant.

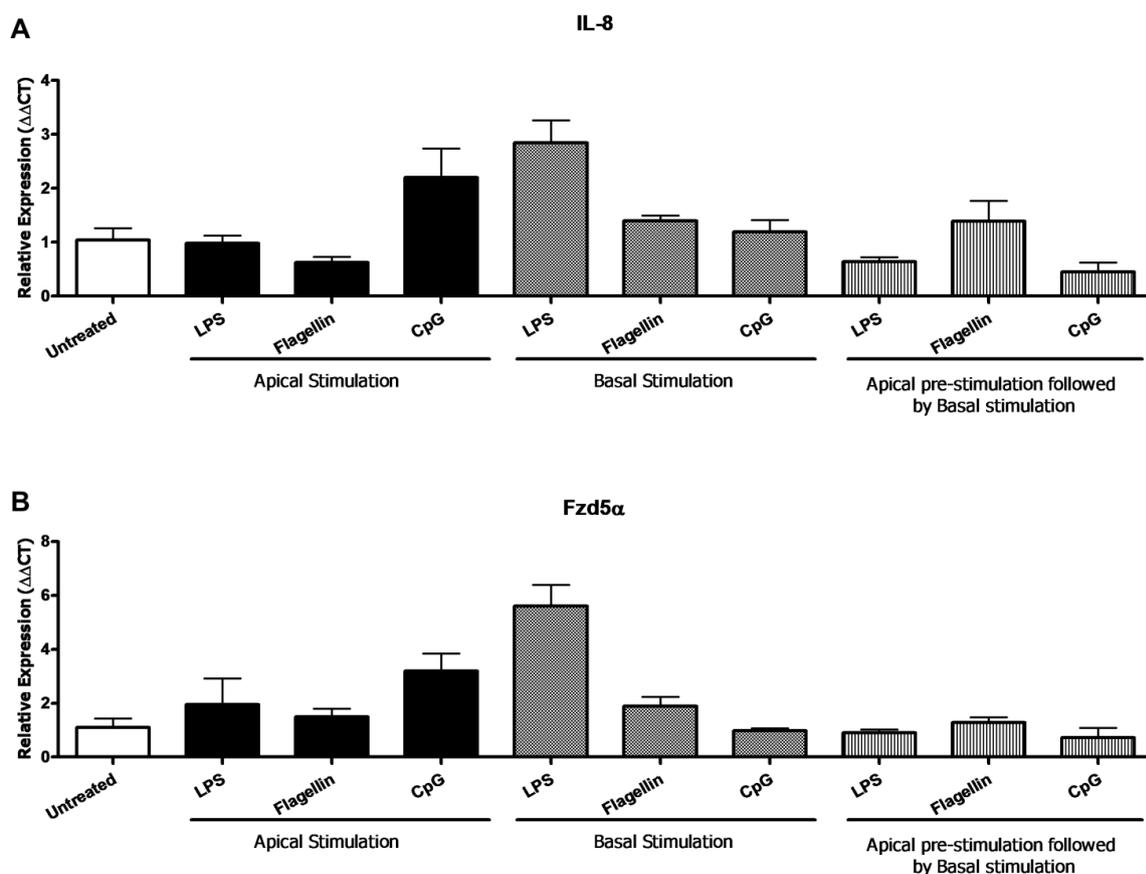


Figure 4.3 Apical/Basolateral stimulation of polarised Caco-2 cells with TLR ligands.

Polarized Caco-2 cells were stimulated with the indicated TLR ligands: LPS (100 ng/ml; TLR4); Flagellin (10 ng/ml; TLR5); CpG (ISS ODN; 5 μ g/ml; TLR9); apically or basolaterally as indicated for 16hrs. Apical pre-stimulation involved stimulating the cells apically with CpG (ISS ODN; 5 μ g/ml; TLR9) for 8 hours before stimulating basolaterally with the indicated TLR ligands for a further 16 hours. mRNA levels were measured by qRT-PCR for relative expression of IL-8 (A) or Fzd5 α (B). Statistics were carried out using t test with Welch's correction.

This experiment was subsequently repeated in the other selected IEC line, T84. Figure 4.4A displays no measurable IL-8 induction in T84 cells through stimulation with the negative control LPS, possibly due to lack of surface TLR4 expression [27]. Basal stimulation with flagellin and CpG DNA did, however, induce IL-8 expression, 3-fold over untreated cells. Although in contrast with previous reports, induction of 7-fold and 4-fold respectively was also seen over untreated controls following apical stimulation with these ligands. This result indicates that the IEC monolayer may not have been polarised correctly since it implies apical surface expression of TLR5, previously published to only be expressed basolaterally [12, 28], although these studies did not use the T84 IEC line. Apical pre-stimulation of TLR9 did seem to abrogate IL-8 induction by subsequent basolateral stimulation of TLR9, in keeping with results seen by Lee *et al.* [12]. This apical pre-stimulation, however, had no effect on basolateral LPS or flagellin-induced IL-8 induction, measured at 3-fold and 7-fold over untreated cells respectively. Furthermore, IL-8 expression levels from apical CpG stimulation alone were higher than those seen by basolateral stimulation. Similarly, 7-fold induction of Fzd5 α was observed not only through TLR9 stimulation as expected (Figure 4.4B) but also through TLR5 stimulation following both apical and basolateral stimulation. Furthermore, none of these changes in expression were found to be statistically significant.

While a more differential response had been observed between apical and basolateral stimulation of the Caco-2 cells, some of the data obtained was not in line with several previous published findings and all the data obtained showed a general lack of consistent level of activation between experiments. Experiments performed to generate the data in Figures 4.3 and 4.4 displayed a high level of discrepancy in terms of the level of response to TLR ligands and the high levels of variation between apical and basolateral stimulation. This indicates that there may have been issues associated with the ligands used; model chosen or

cell lines selected in reproducing the data of Lee *et al.* As a result, subsequent experiments were performed on the HCA-7 cell line, which was used by Lee *et al.* in the studies examining the differential effect of TLR9 signalling in IECs [12, 21].

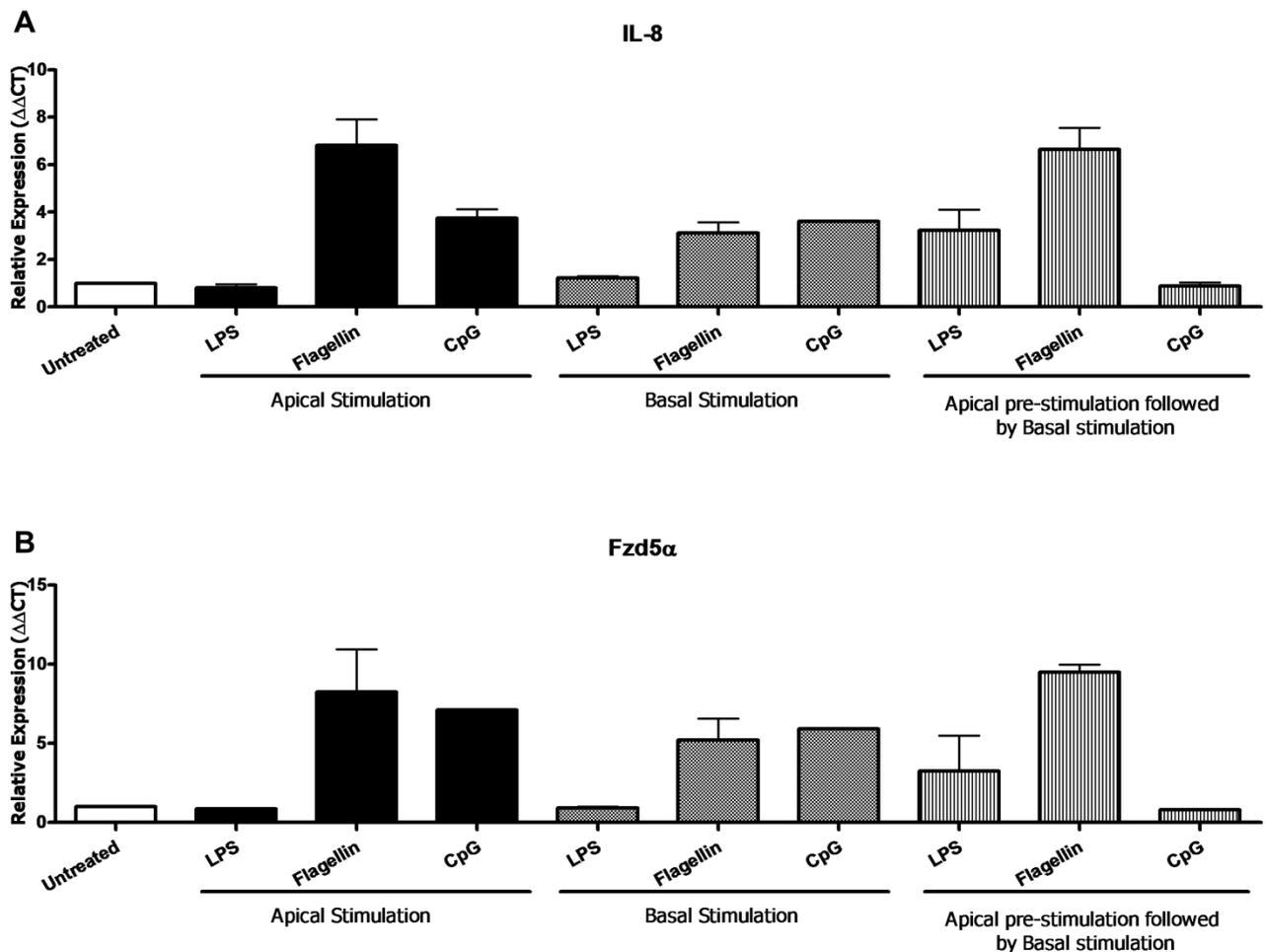


Figure 4.4 Apical/basolateral stimulation of polarised T84 cells with TLR ligands.

Polarized T84 cells were stimulated with the indicated TLR ligands: LPS (100 ng/ml; TLR4); Flagellin (10 ng/ml; TLR5); CpG (ISS ODN; 5 μ g/ml; TLR9); apically or basolaterally as indicated for 16hrs. Apical pre-stimulation involved stimulating the cells apically with CpG (ISS ODN; 5 μ g/ml; TLR9) for 8 hours before stimulating basolaterally with the indicated TLR ligands for a further 16 hours. mRNA levels were measured by qRT-PCR for relative expression of IL-8 (A) or Fzd5 α (B). Statistics were carried out using t test with Welch's correction.

4.2.4 Response to TLR ligands using HCA-7 Intestinal Epithelial Cell lines

The ability of HCA-7 cells to produce IL-8, Fzd5 α and TNF α in response to TLR9 stimulation was subsequently examined. Figure 4.5 displays IL-8 induction from HCA-7 cells which had been grown to a monolayer on transwells prior to apical or basolateral stimulation with TLR9 ligand. The positive control of a dual stimulation with TNF α and IFN γ was also used in order to verify a measurable IL-8 response could be generated and also to confirm the formation of an efficient monolayer. CpG treatment failed to induce any IL-8 expression, following either apical or basolateral stimulation. Basolateral but not apical stimulation with TNF α and IFN γ however, resulted in a 3.5-fold increase in IL-8 induction over untreated cells; similar results had been reported in other studies [29]. However, this change in expression was not found to be statistically significant.

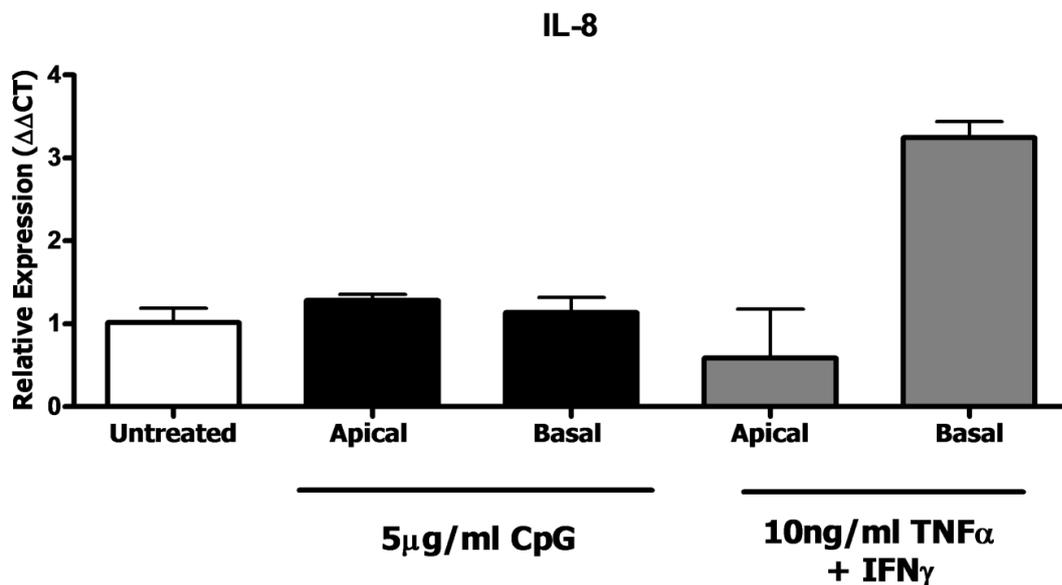


Figure 4.5 IL-8 expression in response to apical or basolateral TLR9 stimulation in a HCA-7 monolayer. Polarised HCA-7 cells were stimulated with CpG (ISS ODN; 5 μ g/ml; TLR9); apically or basolaterally as indicated for 8 hours. mRNA levels were then measured by qRT-PCR for relative expression of IL-8. TNF α and IFN γ were used apically or basolaterally as positive inflammatory controls and added at 10ng/ml each together as indicated for 8 hours. Statistics were carried out using t test with Welch's correction.

A possible explanation for the results obtained so far was that the mRNA induction was being measured 8 hours post-stimulation. This timepoint might be too late to measure effective mRNA responses to TLR ligand stimulation. To assess this, we measured protein production by MSD ELISA. No measureable IL-8 mRNA induction was observed in response to CpG stimulation in Figure 4.5. As this was in direct contrast to data shown by Lee *et al.* we subsequently quantified the pro-inflammatory cytokines IL-6, TNF α and IL-8 from the supernatants of the cells used in Figure 4.5. The mRNA for these cytokines may not have been present 8 hours post-stimulation although the cytokines themselves may have accumulated and remained in the supernatant. Figure 4.6 displays the production levels of these cytokines accumulated over the 8-hour stimulation from the supernatants of apical and basolateral chambers from each of these transwells.

No significant increase in IL-6 or IL-8 production was observed from each of the graphs in Figure 4.6 (A to D) in response to apical or basolateral stimulation with 5µg/ml CpG. However an increase was observed in the level of IL-8 production from the apical and basolateral supernatants of Figure 4.6 (A and B) following stimulation with positive controls TNFα and IFNγ. Moreover, Figure 4.6C displays a 2-fold increase in IL-6 in response to apical TNFα and IFNγ stimulation in the apical chamber. A significant increase in IL-6 production was also observed in response to basolateral TNFα and IFNγ stimulation in the basolateral chamber (Figure 4.6D).

Taken together, Figure 4.5 and Figure 4.6 indicate no activation of pro-inflammatory cytokines in response to TLR9 stimulation. However, stimulation with TNFα and IFNγ resulted in high inflammatory responses as can be seen in TNFα production levels of >5000 pg/ml (Figure 4.6E and F). This suggests that inflammatory stimulation is occurring in the cells although it is not apparent in response to TLR9 stimulation. Furthermore, these data confirm that polarisation had occurred and formation of a monolayer had taken place; this is seen through lack of inflammatory cytokines detected in the apical compartment of cells stimulated basolaterally with TNFα and IFNγ and *vice versa*. Ultimately, these data implied that the cells were forming a monolayer and were responsive to other ligands but not the TLR9 ligand used.

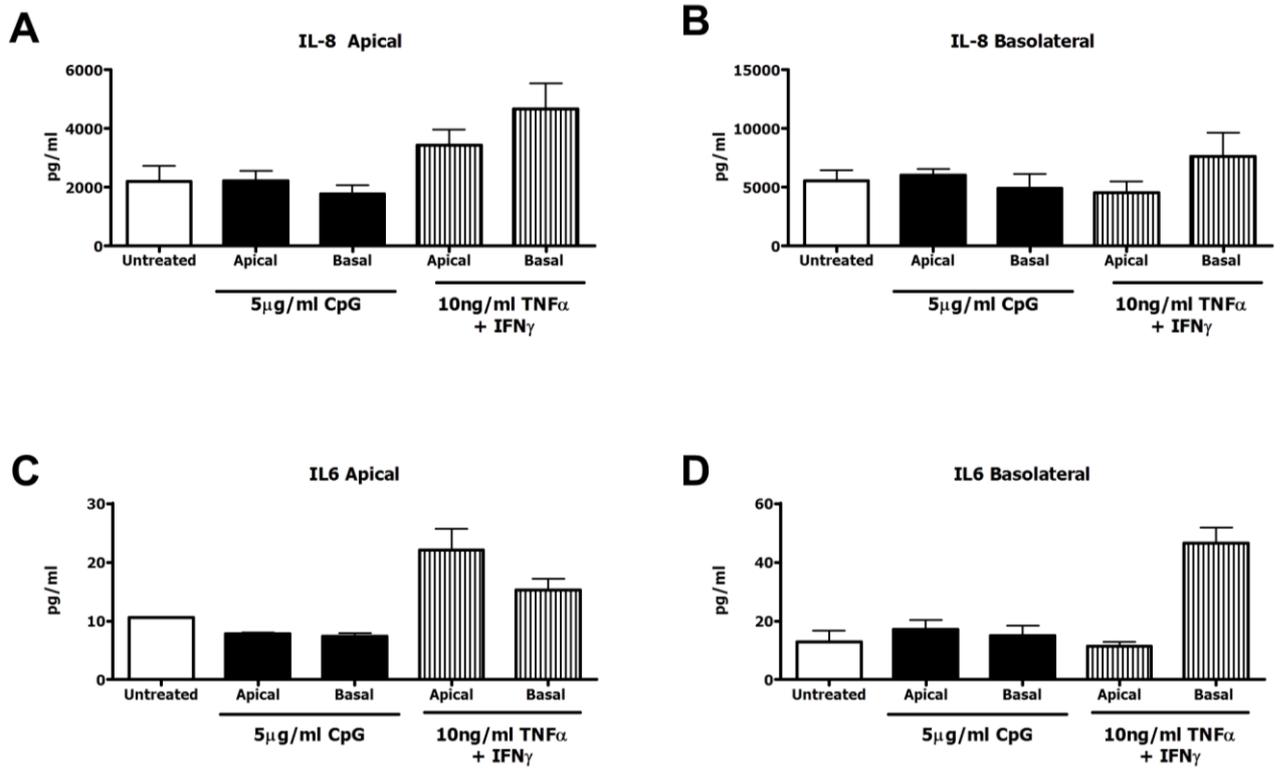


Figure 4.6 Cytokine production in response to apical or basolateral TLR9 stimulation in a HCA-7 monolayer. Supernatants from transwell samples in Figure 4.5 were measured for inflammatory cytokine production by use of an MSD plate. IL-8 and IL-6 levels were measured from both the apical and basolateral chambers as indicated. Statistics were performed using student's t-test.

4.2.5 Treatment of HCA-7 with different CpG ODN constructs

A possible explanation for the results obtained so far and the divergent results displayed by Lee *et al.* was in the nature of the oligodeoxynucleotide (ODN) TLR9 ligands used. To address this potential issue, the identical ODN construct as used by Lee *et al.* was obtained. A dose response was performed using this construct (indicated as Lee in Figure 4.7A), and the construct which had been used up until now (indicated as Invivogen in Figure 4.7A), on un-polarised HCA-7 cells and IL-8 expression levels were measured by qRT-PCR (Figure 4.7). Neither construct showed an increase IL-8 mRNA induction whilst the positive control (TNF α and IFN γ) showed a low but consistent increase in IL-8 expression (Figure 4.7A and B). These results demonstrated that the lack of responsiveness observed in HCA-7 cells when challenged with TLR9 stimulation, contradictory to the result reported by Lee *et al.* [12] was not due to the CpG ODN constructs used.

In addition to measuring IL-8 induction to monitor an inflammatory response, Fzd5 α levels were also measured since this gene should be induced following TLR9 activation in HCA-7 cells, as described by Lee *et al.* [12]. Over timepoints of 0, 1, 3 or 6 hours, stimulation with the TLR9 ligand (Lee) did not strongly induce IL-8 expression (<1.5-fold over untreated) as seen in Figure 4.7C. Induction of TLR9 target gene, Fzd5 α , mRNA levels following TLR9 stimulation was seen to rise between 1 and 6 hours with a peak of ~4-fold induction over unstimulated at 3 hours. This suggests that TLR9 stimulation may have been occurring, although but a measurable pro-inflammatory response was not observable following TLR9 stimulation. However, the pro-inflammatory response was reported to be from basolateral TLR9 stimulation and these cells had not been polarised

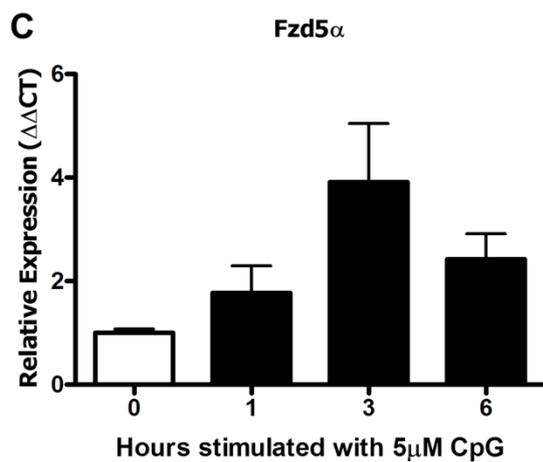
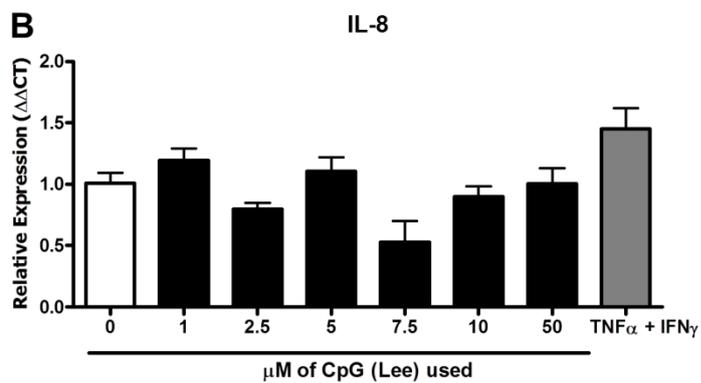
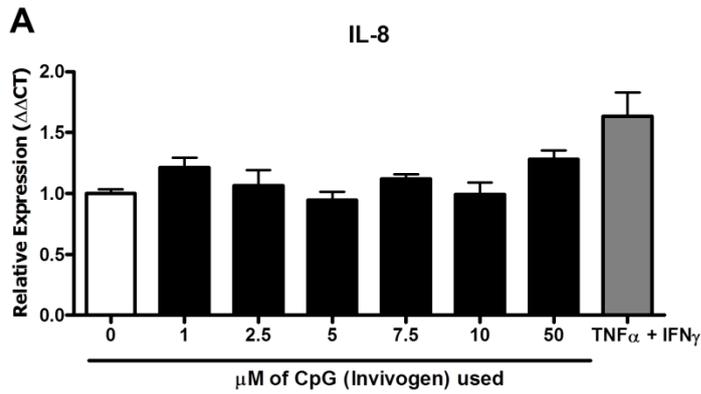


Figure 4.7 IL-8 induction in unpolarised HCA-7 cells following stimulation by increasing concentrations of TLR9 ligand. Unpolarised HCA-7 cells were treated with varying concentrations of the different ODN constructs as indicated on the graph for 8 hours (A and B) or with CpG (ISS ODN, Lee; 5 μ M) for the times indicated (C) and IL-8 mRNA levels were quantified by qRT-PCR (see methods section). These data are representative of 3 such experiments. Statistics were carried out using t test with Welch's correction.

Despite the lack of responsiveness observed in Figure 4.7, a final experiment was performed. In Figure 4.8, polarised HCA-7 cells were stimulated with TLR9 ligand in order to observe any differential activation in IL-8 expression using exactly the same cells, ligand constructs and parameters as used by Lee *et al.* Figure 4.8A and B display no significant levels of IL-8 or TNF α expression induced following 1, 3 or 6 hour stimulation with 5 μ M of the CpG construct. The 2-3 fold increase in expression of IL-8 and TNF α seen following stimulation with TNF α and IFN γ confirms that induction of these genes was measurable but not in response to TLR9 stimulation. Figure 4.8C displays no increase, and indeed a decrease, in what Lee *et al.* outlined as a TLR9 target gene Fzd5 α over the 6 hour time period. These data suggest that TLR9 stimulation was not likely to be occurring in this instance.

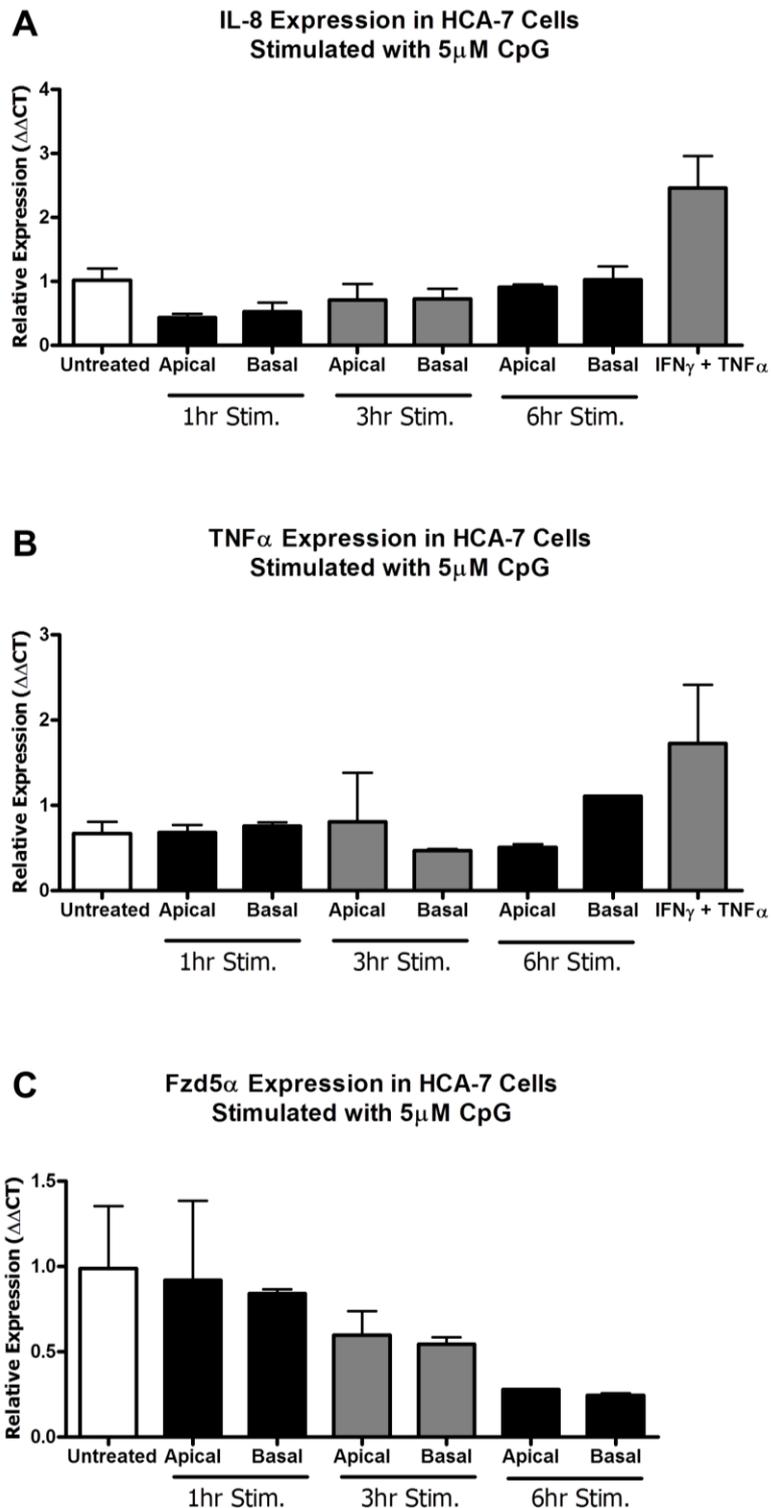


Figure 4.8 Response of polarised HCA-7 cells to TLR9 stimulation. HCA-7 cells which had been grown to a confluent monolayer on transwells as described in methods were stimulated either apically or basolaterlly with TLR9 ligand CpG (ISS ODN, Lee; 5 μ M) in a, b and c or TNF α with IFN γ at 10ng/ml each as a positive inflammatory control (a, b), for the time indicated. mRNA levels of the indicated genes were then measured by qRT-PCR. Statistics were carried out using t test with Welch's correction.

4.3 Discussion

TLR9 has been reported to be located on both apical and basolateral surfaces of the IECs [12, 30]. Recent work by Lee *et al* shows that the ability of the TLR9 ligand CpG DNA to activate NF- κ B depends on which surface of the IEC is stimulated [12]. The study by Lee *et al* describes differential effects of TLR9 ligation on polarized vs. unpolarised HCA-7 IECs. Pre-stimulation of apical TLR9 on HCA-7 cells induced tolerance to subsequent basolateral stimulation with TLR9. However, these tolerised cells did not reduce NF- κ B activation through ubiquitination and subsequent degradation of regulatory I κ B α but instead induced inhibitory molecules towards NF- κ B such as IRAK-M and Tollip. Furthermore, apical TLR9 stimulation was found to confer intracellular tolerance to subsequent TLR challenges. Apical TLR9 stimulation was also shown to inhibit IL-8 production in response to apical TLR2 and basolateral TLR3 or TLR5 stimulation. In contrast, apical stimulation with TLR3 or TLR5 ligand failed to induce tolerance to subsequent basolateral TLR signalling. IECs in TLR9-deficient mice, when compared with wild-type and TLR2-deficient mice, displayed a lower NF- κ B activation threshold and these mice were highly susceptible to experimental colitis. Microarray analysis performed by this group showed that apical stimulation of TLR9 resulted in activation of a different subset of genes when compared to basolateral stimulation, with only a 40% overlap in genes activated between the two surfaces. This indicates that there is likely to be activation of alternative signalling pathways in IECs by TLRs depending on which surface is stimulated by the TLR ligand.

In order to explore this theory, we wished to examine activation of novel TLR regulated genes which appear to have a role in TLR induced IEC homeostasis rather than a TLR induced pro-inflammatory role. Hence, we aimed to further characterise TLR induction of proteins such as Fzd5, identifying precisely which TLRs can induce these proteins, whether they can be induced through apical and/or basolateral stimulation and what TLR

signalling intermediates are necessary for induction of these proteins. We wished to characterise the regulatory and signalling mechanisms by which TLRs achieve their dual roles in IECs with the intention to further elucidate potential mechanisms that may be dysregulated in disease states such as IBD. We also hypothesised that different TLR signalling molecules and co-receptors may be utilised in intestinal epithelial cells compared with leukocytes in order to activate specific signalling pathways and achieve the divergent pro-inflammatory or homeostatic roles played by TLRs in these cells. As inappropriate activation of TLRs on IECs has been implicated in the development of several gastrointestinal disorders including colon cancer [31], colitis [32] and celiac disease [33], a greater understanding of the mechanism of action of TLRs in IECs under normal conditions and how these mechanisms are disrupted during inflammation is essential for the development of effective therapies against these diseases.

As the initial aim of this study was to identify a differential signalling pathway existed between apical and basolateral activation of TLR9, we initially wished to replicate data by Lee *et al.* This was attempted by use of the transwell system to achieve a polarised monolayer of IECs and stimulation of TLRs by synthetic ligands. We examined TLR9 stimulation in several IEC lines. Using C2Bbe1 and also Caco-2 cells, a general lack of response to the TLR9 ligand was observed by both apical and basolateral stimulation. This is similar to reports from de Kivit *et al* [34]. These authors also investigated IEC responsiveness to TLR9 stimulation. Using Caco-2 and C2Bbe1 cells, however, they reported that apical exposure to TLR ligands had no observable effect on an immune response when activated PBMC were co-cultured with Caco-2 cells. They highlight in this study that in comparison to HT-29 cells, Caco-2 cells show markedly less TLR expression, especially TLR9 [17, 35]. This can also perhaps explain the results seen in the data presented here where Caco2 and their subclone, C2Bbe1 cells, were used. While this study used unpolarised HT-29 cells and polarised T84

IECs in co-culture with activated PBMC, they reported that apical stimulation of T84 cells with TLR9 ligand resulted in enhanced IFN- γ and immune-regulatory IL-10 secretion by activated PBMC in co-culture. A similar effect was observed using unpolarised HT-29 cells, where a significant decrease in IL-13 was also observed. IL-6, IL-12 and TNF α were also measured using both HT-29 and T84 but no significant change was observed following stimulation with TLR9 ligand. Conversely, despite the absence of polarisation in the HT-29 co-cultures, apical and basolateral exposure to TLR9 ligand elicited differential responses in their study. Ultimately, their results showed that only apical exposure resulted in a Th1 response that coincided with regulatory IL-10 secretion. This was lost upon basolateral TLR9 ligand exposure, which resulted in enhanced secretion of pro-inflammatory cytokines including TNF α [34]. We did not investigate responses in HT-29 as these cells do not polarise in the transwell system.

Similarly, a study by Pederson *et al* also found that CpG ODN stimulation completely failed to induce a significant increase in IL-8 protein secretion in normal primary colonic epithelial cells [17]. They stated that fully differentiated human colonic epithelial cells, unlike HT-29, are completely unresponsive to TLR9 ligand stimulation *in vitro* despite spontaneous TLR9 gene expression. They also measured expression levels of TLR9 in Caco-2 compared to HT-29 cell lines in addition to the primary intestinal epithelial cell line, DLD-1. The results displayed a comparable level of expression in the HT-29 and DLD-1 cells but with relatively very low expression in the Caco-2 cell line. Furthermore, at least five isoforms of TLR9 protein exist and the same study reported that two of these isoforms were present in freshly isolated colonic epithelial cells, while only one isoform was seen in HT-29 cells. As the functional role of these different isoforms is unknown, further investigations are required to elucidate whether CpG ODN responsiveness is correlated to the presence of distinct isoforms of TLR9. While the expression levels of TLR9 or its different isoforms were not

investigated in my own study, it is another possible reason why I did not observe the same results as Lee *et al.*, when repeating their work using the Caco-2 or C2Bbe1 cell lines.

Finally we used HCA-7 cells as the model cell line for our study, in keeping with the exact procedure used by Lee *et al* [12]. While these cells responded to stimulation by TNF α and IFN γ , there were few significant or consistent responses to stimulation of TLR9. This lack of sensitivity to TLR9 ligand was investigated further. It was found, however, that while no inflammatory response was seen, the TLR9 target gene Fzd5a was responding to TLR9 stimulation. Further to the difficulties which arise when comparing different cell lines of the same cell type [34], phenotypes of cell lines are also known to differ between laboratories. Cell culture conditions in different labs can affect the responsiveness of cells. In addition, the aforementioned studies of Pederson *et al* highlighted the significance of different isoforms of TLR9 and possible different signalling pathways. It could well be that isoforms are expressed or not in cell lines between different laboratories over time.

In contrast to my data and also that of de Kivit *et al.*, however, oral administration of TLR9 agonists has been shown to provide protective effects in an IL-10-dependent manner [36] in animal models of colitis [22, 23]. In one study, Rachmilewitz *et al* found that in all models of experimental and spontaneous colitis examined, TLR9 ligand ISS-ODN administration ameliorated clinical, biochemical, and histologic scores of colonic inflammation in addition to inhibiting the induction of colonic pro-inflammatory cytokines and chemokines [22]. A couple of years later, the same group reported that the intragastric administration of probiotic and *E. coli* DNA ameliorated the severity of DSS-induced colitis in mice [23]. Methylated probiotic DNA, calf thymus DNA, and DNase-treated probiotics on the other hand had no effect. This reduction in severity of colitis was also observed to the same extent through intragastric administration of nonviable γ -irradiated or viable probiotics. Administering these γ -irradiated probiotics was also shown to significantly decrease the

severity of DSS-induced colitis in TLR2 and TLR4 deficient mice, whereas, in TLR9-deficient mice, γ -irradiated had no effect. However, while these protective anti-inflammatory effects may be seen through TLR9 stimulation *in vitro*, caution should be exercised when dealing with *in vitro* studies using cell lines. It has been demonstrated several times that transformed intestinal cell lines are not fully representative for the differentiated human colonic epithelium with regard to phenotypic markers of differentiation [37-39]. While similar results to those observed by Lee *et al.* [12, 21] have been reported in other cell lines such as T84 and HT-29 [12, 34], our study also highlights the importance of variations within different cell lines and their phenotypes, even among different laboratories.

To summarise, throughout the course of my experiments I was unable to reproduce results reported by Lee *et al* regarding differential TLR9 signalling in polarised IECs. As outlined above, this may be due to differential expression and signalling mechanisms of TLR9 between different cell lines in different laboratories. Comparable to what was encountered by de Kivit *et al*, I found that results from Lee *et al* could not be reproduced using IEC lines [12, 34]. My inability to reproduce the data published by Lee *et al* prevented me from further pursuing the project.

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Chapter 5

Identification of novel Pattern Recognition Receptors involved in detection of *L.monocytogenes* in intestinal epithelial cells by use of siRNA screening

5.1. Introduction

Listeria monocytogenes (*L. monocytogenes*), a Gram-positive bacterium and a facultative intracellular pathogen, has the potential to cause life-threatening diseases such as septicaemia and meningitis [1], particularly in immuno-compromised individuals, pregnant women, and neonates [2]. Pathogenic *listeriae*, being a foodborne pathogen, enter the host primarily through the intestine [3]. They cross the intestinal barrier by invading intestinal epithelial cells (IECs). From here, they enter the bloodstream, to reach their target organs, the liver and spleen, where they are internalized by hepatic and splenic macrophages. They then multiply until infection is finally controlled by the TNF α and iNOS producing effector Dendritic Cells (TipDCs). These effector cells require infected cells to signal to activate them but are not themselves infected [4].

L.monocytogenes is known to evade host immune responses by several means in order to promote its survival. It is able to invade non-phagocytic cells and is resistant to intracellular killing by macrophages after phagocytosis [5]. *L.monocytogenes* invades and multiplies within non-phagocytic cells, such as enterocytes, hepatocytes, fibroblasts and endothelial cells [6]. This ability of *L.monocytogenes* to establish itself intracellularly avoiding phagocytosis aids the bacterium to avoid host responses and ensures effective tissue dissemination and enhanced virulence. For a long time this mechanism of intracellular spread was thought to be the main strategy employed by *L.monocytogenes* to avoid immune detection. With the discovery of host pattern recognition receptors (PRRs) and increased understanding of innate immune signalling pathways, however, it has been shown that the bacterium is also capable of avoiding detection through other mechanisms. *L. monocytogenes* can de-acetylate its peptidoglycan to avoid detection by TLR2 [7]. Effective immune responses to *L.monocytogenes* infection relies on co-ordinated innate and adaptive immune

responses, with the first line of innate defence being mediated by detection of the invading bacterium by these PRRs. Several families of these receptors have been identified including the transmembrane Toll-Like Receptors (TLRs), the cytosolic NOD-Like Receptors (NLRs) and RIG-I like Receptors (RLRs) and in more recent years C-type Lectin Receptors (CLRs) [8] (see Introduction Section 1.1).

The activation of innate immunity by PRRs in response to *L.monocytogenes* infection is still not fully understood. RNA interference and inhibitor experiments using human Peripheral Blood Mononuclear Cells (PBMCs) were performed by Meixenberger *et al* in 2010 in order to determine which PRRs and inflammasomes play key roles during infection. The results indicated that the Listeria-induced IL-1 β release was dependent on the adaptor protein ASC, caspase-1, and NLRP3, whereas in this case, NOD2, RIP2, NLRP1, NLRP6, NLRP12, NLRC4, and AIM2 appeared to be dispensable [9]. However, mice lacking NOD2 fail to generate adaptive responses to *L. monocytogenes* in the gut [10]. RNAi-based studies revealed that a producer of Reactive Oxygen Species (ROS), called Dual Oxidase 2 (DUOX2), is required for the direct bactericidal properties of NOD2. This highlights ROS as effector molecules of protective cellular signalling in response to a defined danger signal by the intracellular NLRs of IECs [11]. *L.monocytogenes* has also been shown to be recognised by TLR2 [12, 13] and NOD1 [14, 15] in macrophage and endothelial cells, resulting in NF- κ B activation and pro-inflammatory gene expression. The role of TLR5 in detection of *L.monocytogenes* remains unclear; although flagellin from *L.monocytogenes* activates TLR5, bacterial mutants deficient in flagellin display an unaltered virulence [16, 17]. More recent studies have identified several inflammasome components responsible for recognition of the bacterium such as NLRC4 and AIM2 in macrophages. Recognition of *L.monocytogenes* through these receptors results in activation of caspase-1 and cleavage of IL-1 β [18]. *L.monocytogenes* infection has also been shown to induce a type I interferon response,

potentially mediated by TLR2 and the adaptor protein TRIF in peritoneal macrophages [19]. Most recently the IFN- β response has been shown to be triggered by nucleic acids released from *L.monocytogenes* acting through the intracellular sensor RIG-I in macrophage cells [20]. Much of these studies contradict the Meixenberger study in 2009, which used PBMCs and suggested many of these PRRs were dispensable or redundant during infection [9]. It is important to note here, however, that functionality of PRRs may differ from cell type to cell type.

Epithelial cells line the enteric mucosal surface providing barrier function against microbial invasion. Similar to immune cells, IECs express many of these receptors of the innate immune system and are the first site of bacterial recognition in the intestine. Characterisation of the innate immune responses to *L.monocytogenes* infection has been studied in several cells types, including endothelial cells, PBMCs, and macrophages [7]. However, no extensive screen has been performed on innate and PRR mediated mechanisms of detection of *L.monocytogenes* in the intestinal epithelia. To date, NOD2 is the only PRR directly shown to play a role in detecting *L.monocytogenes* in the intestinal epithelium [10]. Recently in fact, a study by Kernbauer *et al.* using an *in vitro* mouse model of oral *L.monocytogenes* infection revealed that the type I IFN response increased bacterial clearance when infection occurred intra-gastrically [21]. Previous studies worked with intraperitoneal infection models for *in vitro* mouse studies and type I IFN responses were believed to be detrimental to bacterial clearance; however, this detrimental effect of type I IFNs is only the case when infection occurs through a route other than intra-gastric, highlighting the need for further study of intestinal epithelial innate immunity.

In addition to the lack of understanding surrounding the immune response in the context of IECs, the importance of further investigating the role of the main effector PRRs involved in early detection of *L.monocytogenes* along with identifying PRRs which the

bacteria may use to subvert the immune system requires further clarification. The aim of this study was to identify novel PRRs involved in the early detection of *L.monocytogenes* in IECs by use of siRNA screening. This involved optimisation of infection parameters and siRNA transfection in addition to inflammatory outputs. I have identified several novel PRRs which appear to negatively regulate the inflammatory response upon detection of the bacteria in addition to discovering that TLR10 plays a role in activating an inflammatory response to the bacteria in IECs.

5.2 Results

5.2.1 Identification of *L. monocytogenes* growth phases and CFU/ml to be used in invasion assays

There exists some discrepancy in the literature regarding the methodology used in performing *in vitro* infection assays using *L. monocytogenes*; therefore optimisation of infection assays was required for THP-1 (monocyte) and HT-29 (epithelial) cells. Several variables such as; multiplicity of infection (MOI), growth rate prior to infection and incubation time of the bacteria with the host cells were examined. A reference growth curve of the bacteria over time, by counts of the colony forming units (CFU)/ml of culture, made relative to Optical Density (OD) was required (Figure 5.1). This ensured accuracy in counting for MOI when using *L. monocytogenes* for all invasion assays and also in the reproducibility of each experiment. Furthermore, it also allowed for the identification of the time points and OD values for the Early, Middle and Late log growth phases, indicated on the graph.

Listeria monocytogenes OD vs. CFU/ml

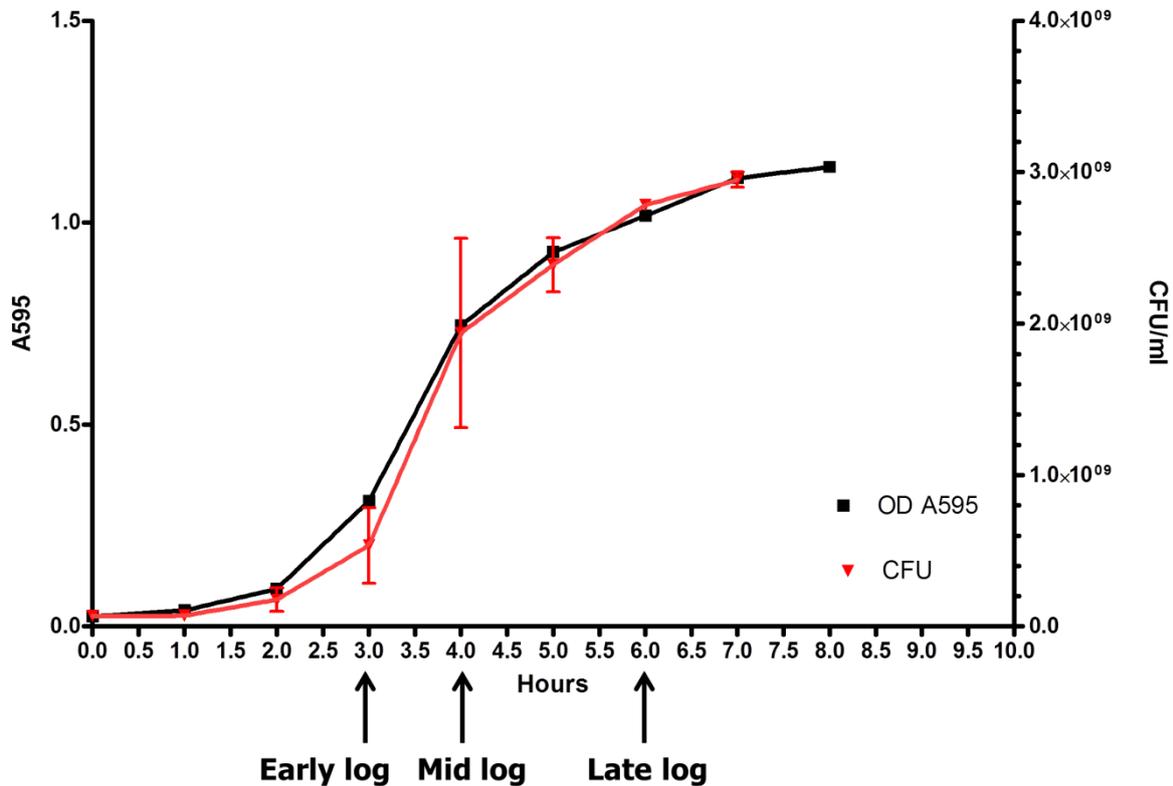


Figure 5.1 Optimisation of growth curve and log phases for *L. monocytogenes* vs. Optical Density (OD) at A595 A single colony of *L. monocytogenes* was selected and cultured overnight at 37°C. The next day, a 1/50 dilution of the culture was prepared. An aliquot was taken from this new culture every hour (including time-point zero) for 8 hours. With each aliquot, an OD measurement was made as well as serial dilutions of the culture. These dilutions were then spotted on an agar plate to determine CFU/ml for each aliquot as described in the methods section 2.12. The graph above is representative of 3 such experiments carried out on separate days.

We also wished to identify which growth phase resulted in the most efficient invasion during infection. HT-29 cells were chosen for this investigation over the monocytic THP-1 cells as the epithelial HT-29 cells should be less efficient at internalisation. This allowed the most efficient growth phase to use for infection to be more distinguishable. The results of this may be seen in Table 5.1.

Log Phase	Early	Mid	Late
Invasion Efficiency (CFU recovered from lysate post-infection)	$0.25 \pm 0.06\%$	$0.08 \pm 0.05\%$	$0.1 \pm 0.06\%$

Table 5.1 Optimisation of growth phase of *L. monocytogenes* to be used in invasion assays with HT-29 cells. *L. monocytogenes* was grown to the growth phases indicated, based on the graph from Figure 5.1. A known number of bacteria could then be used for infection of HT-29 cells at an MOI of 100. Infection was performed as described in 2.15 of the methods section and lysate of host HT-29 cells were used to spot on agar plates to count CFU post-infection. This total CFU count recovered/lysate was calculated as a percentage of the total number of bacteria used to infect the cells. Using this method, an internalisation or invasion efficiency of the bacteria was calculated. The table above represents the mean values with the standard error of the mean for 4 such assays.

Following the addition of the bacteria to the host cells, gentamicin, which is not believed to enter host cells, was added to prevent further bacterial replication without killing intracellular bacteria. Verification that the gentamicin was not killing the intracellular bacteria but was preventing their growth was required since controversy on this issue, regarding the uptake of gentamicin by host cells, is also found throughout the literature [22, 23]. Figure 5.2 displays a live/dead staining of bacteria treated with Gentamicin or formalin for 1 hour using flow cytometry. This demonstrates that the gentamicin did not kill all of the bacteria, as the formalin treatment did. The gentamicin treatment did, however, prevent further bacterial replication or growth; since no colonies were recovered from the agar plates spotted with the gentamicin-treated culture despite more than half of the bacteria remaining viable following the gentamicin treatment.

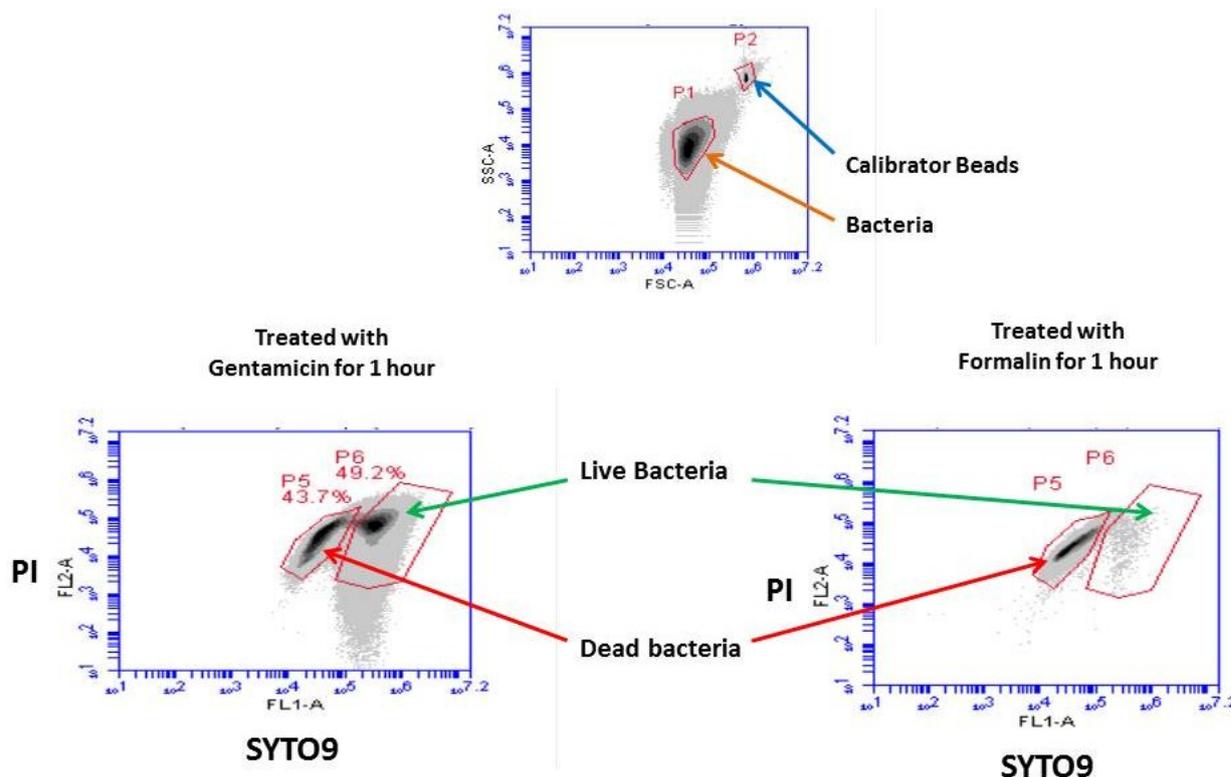


Figure 5.2 Verification of Gentamicin induced inhibition of bacterial replication without resulting in bacterial cell death. A culture of *L. monocytogenes* was grown overnight. The following day, half of the cells were washed and treated with 50 μ g/ml of Gentamicin in BHI broth for 1 hour, while the rest were treated with 4% formalin for 1 hour. A live/dead screen was then performed as described in the methods section 2.13 in order to determine what percentage of the bacteria had survived following the gentamicin or formalin treatment. Binding of SYTO9 to DNA is dependent on active transport, so SYTO9 diffuses through the intact cell membrane and binds cellular DNA of viable cells only, while PI binds DNA of damaged cells only. Thus, the X axis represents SYTO9 bound DNA, or live bacterial staining, and the Y axis represents PI-bound DNA or non-viable cells. In addition to the live/dead screen, bacteria were spotted on an agar plate to determine how much growth of *L. monocytogenes* took place following gentamicin treatment.

The results in Figure 5.2 above show that only half of the bacteria treated with gentamicin were dead/unviable. An overnight culture of bacteria such as this contains a number of dead bacteria already, as the cells enter death phase following stationary phase of growth naturally. The killing effect of the gentamicin was relatively mild compared to the formalin treated cells where all of the bacteria are dead. However, no colonies from either of

these cultures were observed confirming that the gentamicin treatment had prevented further bacterial growth.

The optimal incubation time of the bacteria with the host cells, and the MOI used was next determined. Figure 5.3 displays internalisation efficiency of *L. monocytogenes* at various MOI using either an incubation period of 1 or 2 hours for the bacteria with the host cells before the addition of gentamicin. It was concluded from these results that 2 hours incubation prior to addition of antibiotic was the optimal incubation time.

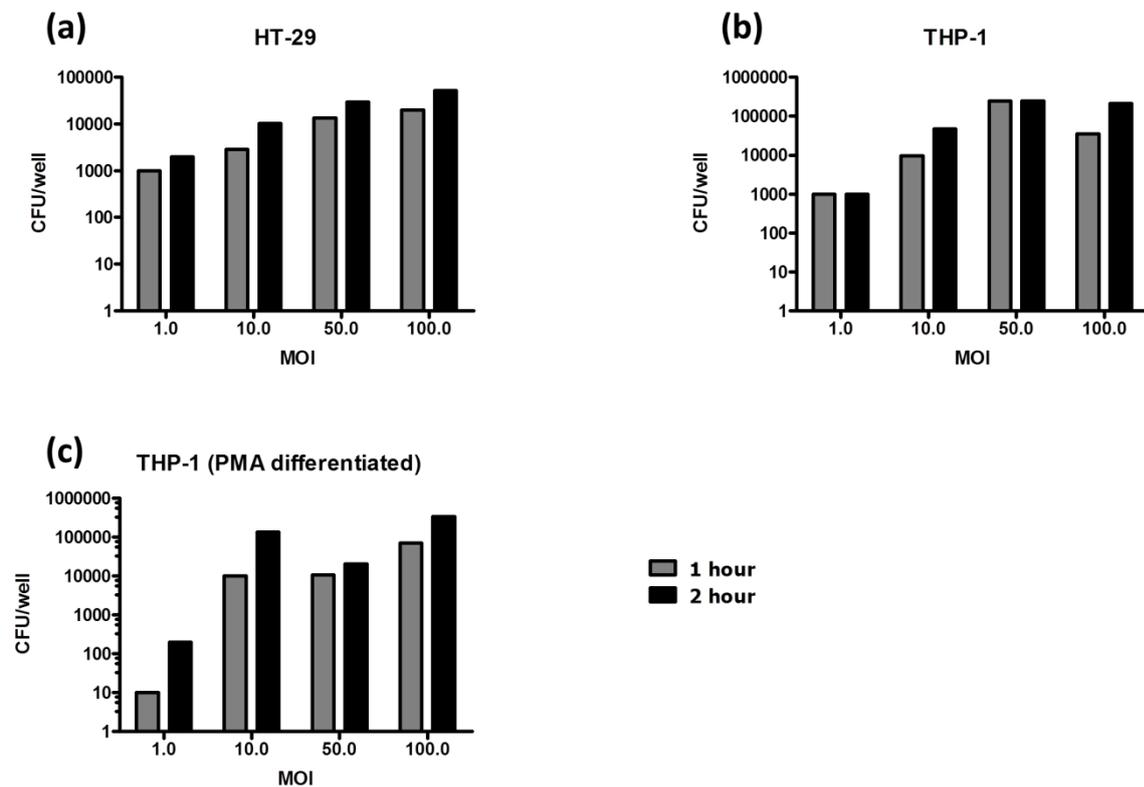


Figure 5.3 Optimisation of MOI and incubation time of *L. monocytogenes* with the host cells. *L. monocytogenes* was grown to Early Log Phase growth. HT-29 (a), THP-1 (b), or THP-1 cells pre-treated with PMA (c) were then subject to infection at various MOI. Following either 1 or 2 hours incubation with the bacteria, media was supplemented with 50µg/ml gentamicin for a further hours. Subsequently, cells were lysed and the number of internalised bacteria was calculated by counting the CFU from the lysates as described in the methods section 2.15.

Confocal microscopy was used to verify internalisation of *L. monocytogenes* and to exclude the possibility of external adherence of the bacteria to the cell. Internalisation of the bacteria is vital in order to measure the potential response of any cytoplasmic PRRs, such as NLRs. As is evident in Figure 5.4, after 2 hours incubation with the host cells, *Listeria* are internalised by the cells and also appear to adhere externally.

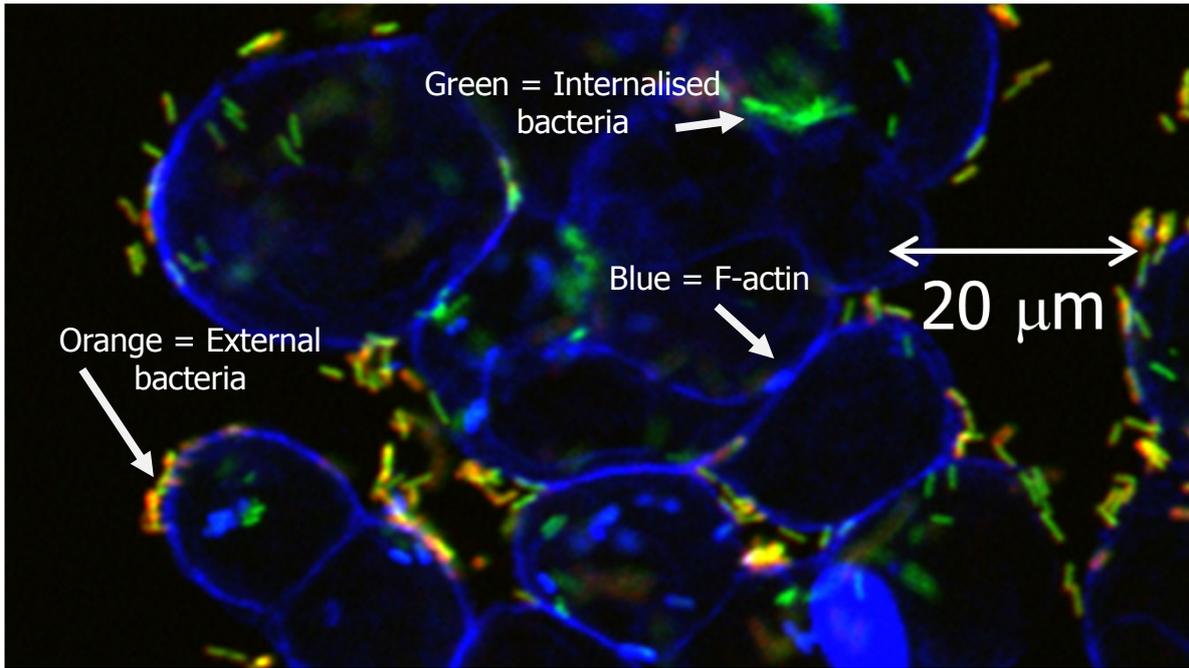


Figure 5.4 (a) HT-29 cells infected for 2 hours at MOI 100

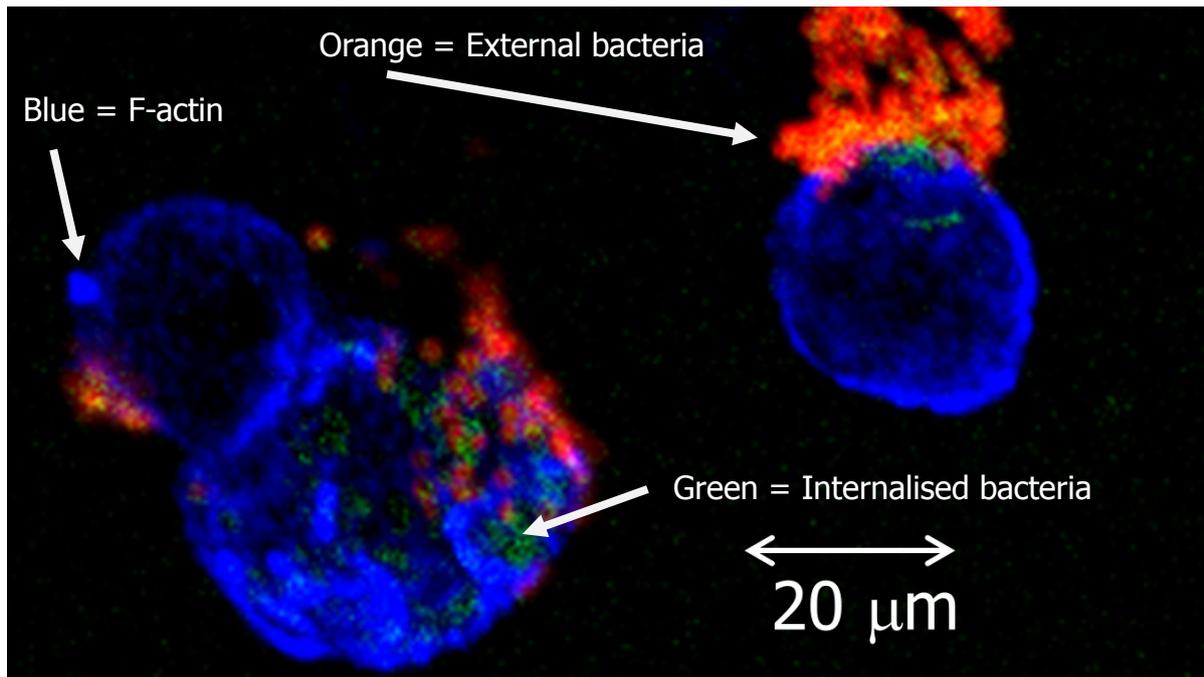


Figure 5.4 (b) THP-1 cells infected for 2 hours at MOI 100

Figure 5.4 Confirmation of cellular internalisation of *L. monocytogenes*. Host cells were prepared for confocal microscopy as described in the methods section 2.16. Bacteria were CFSE (green) labelled and biotinylated. Infection was then carried out as described previously at the MOI of 100 for 2 hours. Cells were fixed and host F-actin was stained with fluorescent phallotoxins (blue). Streptavidin-allophycocyanin was used to bind to the biotinylated bacteria such that all bacteria were CFSE labelled but only extracellular bacteria had also taken up the streptavidin-allophycocyanin dye (red). Figure 5.4 (a) shows HT-29 cells while (b) contains THP-1 cells, each stained blue with internalised bacteria shown as green and external bacteria as orange (green + red).

Based on this optimisation data, the parameters chosen were for *L. monocytogenes* to be in early log phase at the time of infection, MOI of 50, and incubated with host cells for 2 hours, prior to gentamicin treatment for 1 hour. The next step was to characterise the host cell immune response to infection.

5.2.2 Measuring the host inflammatory response to internalised *L. monocytogenes* in THP-1 and HT-29 cells

As the overall aim of the assay was to measure the innate immune response mediated by PRRs in response to *L. monocytogenes*, an early time-point of infection was selected at 3 hours based on the data above. qRT-PCR was used to measure mRNA of select cytokines expressed following infection at this timepoint. A range of cytokines and chemokines from the literature believed to be involved in epithelial inflammatory signalling were measured for induction in HT-29 cells following the 3 hour infection. The cytokines measured were CCL-1, CCL-2, CCL-5, CCL-17, CCL-19, CCL-20, IL-1 β , IL-6, IL-8, IL-12, IL-17, IL-18, IL-23, Caspase-1 and EGR-1. Of these, CCL-2, CCL-5, CCL-17, CCL-19, IL-6, IL-12 and IL-17 were undetectable by qRT-PCR. However, IL-8, CCL-1, CCL-20 and EGR-1 were induced 10, 17, 75 and 30-fold respectively over uninfected controls (Figure 5.5). Based on this, we used the early transcription factor, EGR-1 and the chemotactic agents CCL-20 and IL-8 in the measurement of an inflammatory response to infection with *L. monocytogenes* in HT-29 cells.

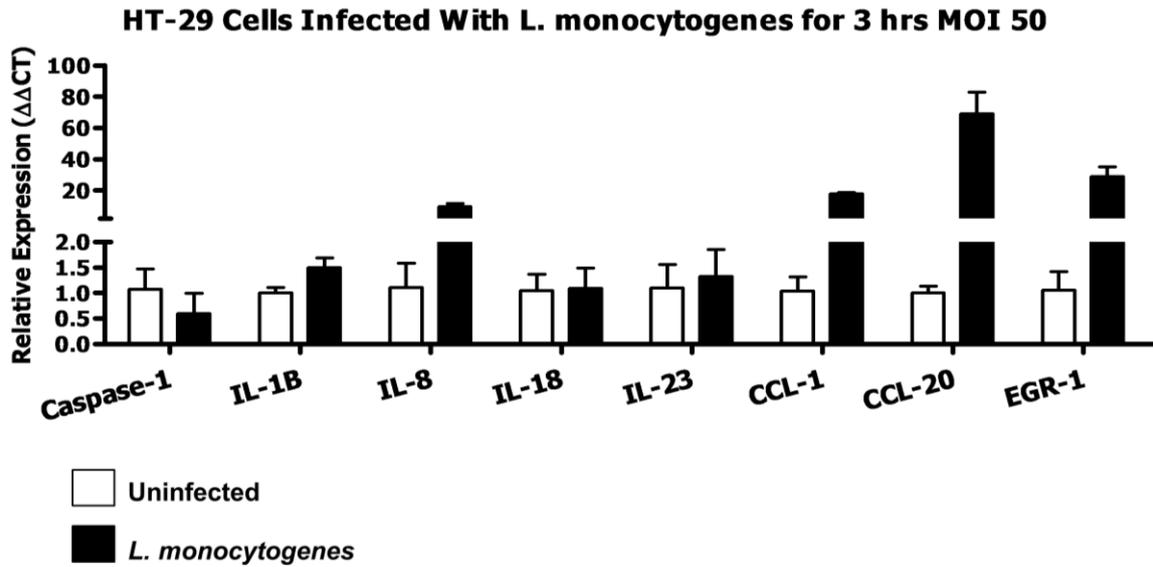


Figure 5.5 Cytokine and chemokine induction in HT-29 cells following *L. monocytogenes* infection. Levels of CCL-1, CCL-2, CCL-5, CCL-17, CCL-19, CCL-20, IL-1 β , IL-6, IL-8, IL-12, IL-17, IL-18, IL-23, Caspase-1 and EGR-1 induction were measured in HT-29 cells following a 3 hour infection with *L. monocytogenes*. Results from all detectable genes measured are displayed above with each induction made relative to the uninfected control. . * P<0.05, ** P<0.01 and *** P<0.001, Values are shown as Mean \pm SEM, n=3. Statistics were carried out using t test with Welch's correction.

These genes were examined further to ensure that they provided a consistently robust output in response to infection (Fig. 5.6). Each gene was significantly induced in response to infection (p<0.05). Induction of Early Growth Response protein-1 (EGR-1), Chemokine (C-C motif) Ligand 20 (CCL-20) and IL-8 over untreated controls was measured at 40, 20, 60-fold induction respectively. THP-1 cells were also examined for induction of each of these genes with the addition of IL-1 β (Figure 5.7). EGR-1 and IL-1 β were significantly induced with 10 and 6-fold induction measured respectively over the untreated cells. There was no significant increase in CCL-20 or IL-8 induction in THP-1s despite induction levels seen at 8 and 6-fold over untreated cells respectively. PMA treatment for differentiation of the THP-1 cells was

not performed at this stage due to inflammatory activation by PMA which made an *L. monocytogenes*-mediated response more difficult to differentiate.

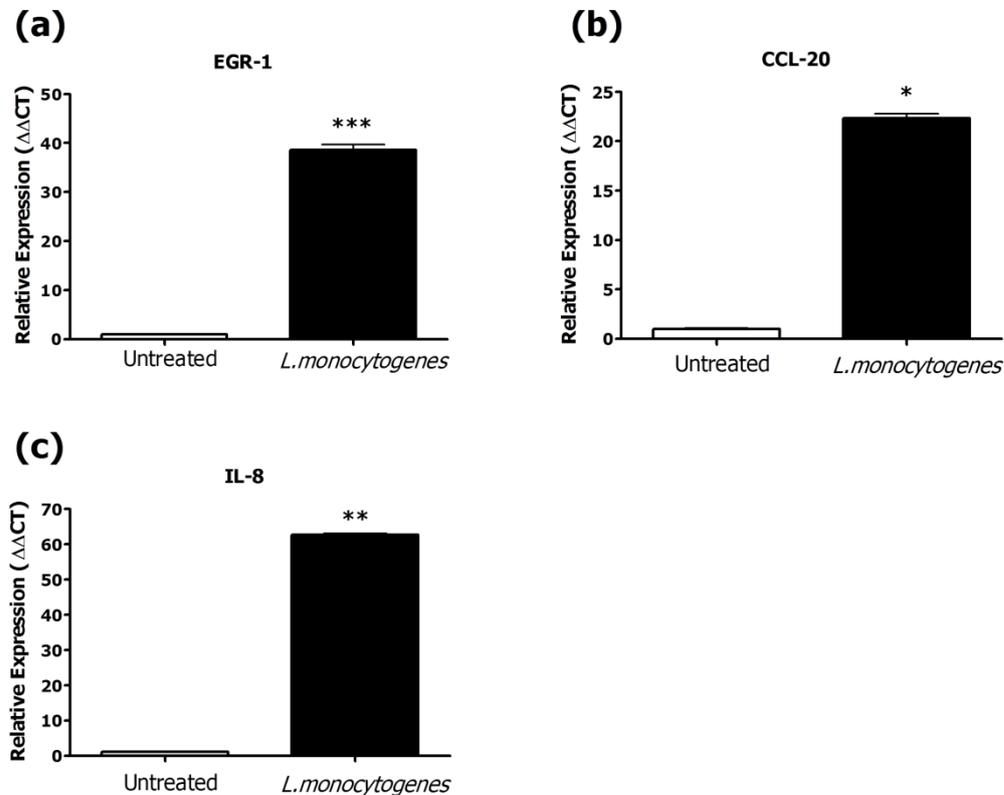


Figure 5.6 Measurement of HT-29 Response to *L. monocytogenes* infection by qRT-PCR. Shown above is the qRT-PCR data for EGR-1 (a), CCL-20 (b), and IL-8 (c) in HT-29 cells following infection with *L. monocytogenes* at MOI of 50 for 3 hours. Each infection was carried out as described in the methods section 2.14. Cells were then lysed and RNA was extracted for qRT-PCR analysis. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Values are shown as Mean \pm SEM, $n=3$. Statistics were carried out using t test with Welch's correction.

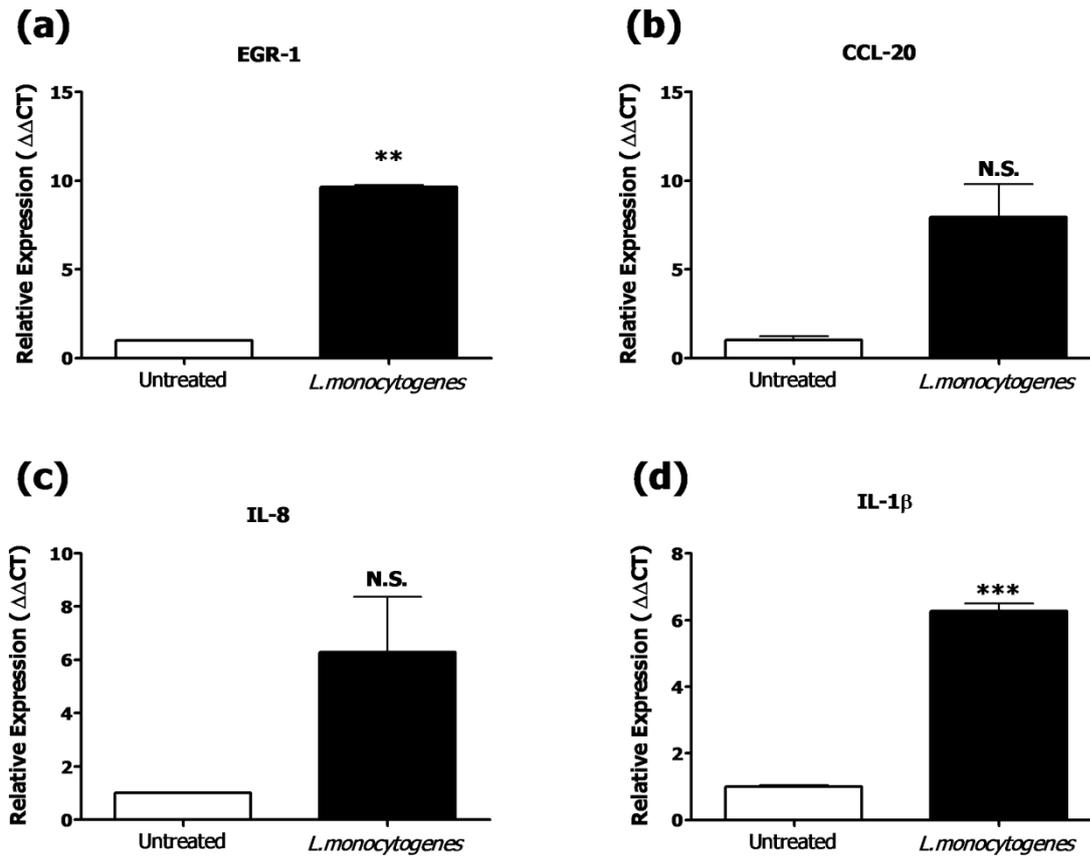


Figure 5.7 Measurement of THP-1 Response to *L. monocytogenes* infection by qRT-PCR. Shown above is the qRT-PCR data for EGR-1 (a), CCL-20 (b), IL-8 (c) and IL-1 β (d) in THP-1 monocyte cells following infection with *L. monocytogenes* at MOI of 50 for 3 hours. Each infection was carried out as described in the methods section 2.14. Cells were then lysed and RNA was extracted for qRT-PCR analysis. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Values are shown as Mean \pm SEM, $n=3$. Statistics were carried out using t test with Welch's correction.

5.2.3 Optimisation of siRNA treatment for THP-1 and HT-29 cells

In addition to having defined the genes measured for induction by qRT-PCR following infection with *L. monocytogenes* as an output for the siRNA library screen, siRNA treatment also required optimisation for each cell type. In addition to confirmation of a successful knockdown, it was also necessary to show that infection with *L. monocytogenes* did not affect siRNA treatment. MAPK1 siRNA, which was readily available in the lab, was used as a negative control. This gene is not believed to be involved in an immune response to the bacteria, so knocking down MAPK1 should not lower the response of any of the cytokines selected as an output following *L. monocytogenes* infection. CCL-20 was used as a measurement of inflammatory response for the epithelial cells since they strongly induce this chemokine in response to *L. monocytogenes*; similarly IL-1 β induction was measured for the monocytic THP-1 cells (see Fig. 5.6, 5.7). IL-8 was measured for each as it is induced in both cell types. Figure 5.8(a) confirms the silencing of MAPK1 in HT-29 cells with at least 50% reduction in MAPK1 expression following siRNA treatment. No significant change in the expression of IL-8 (b) or CCL-20 (c) was recorded between MAPK-1 and control siRNA treated samples following infection.

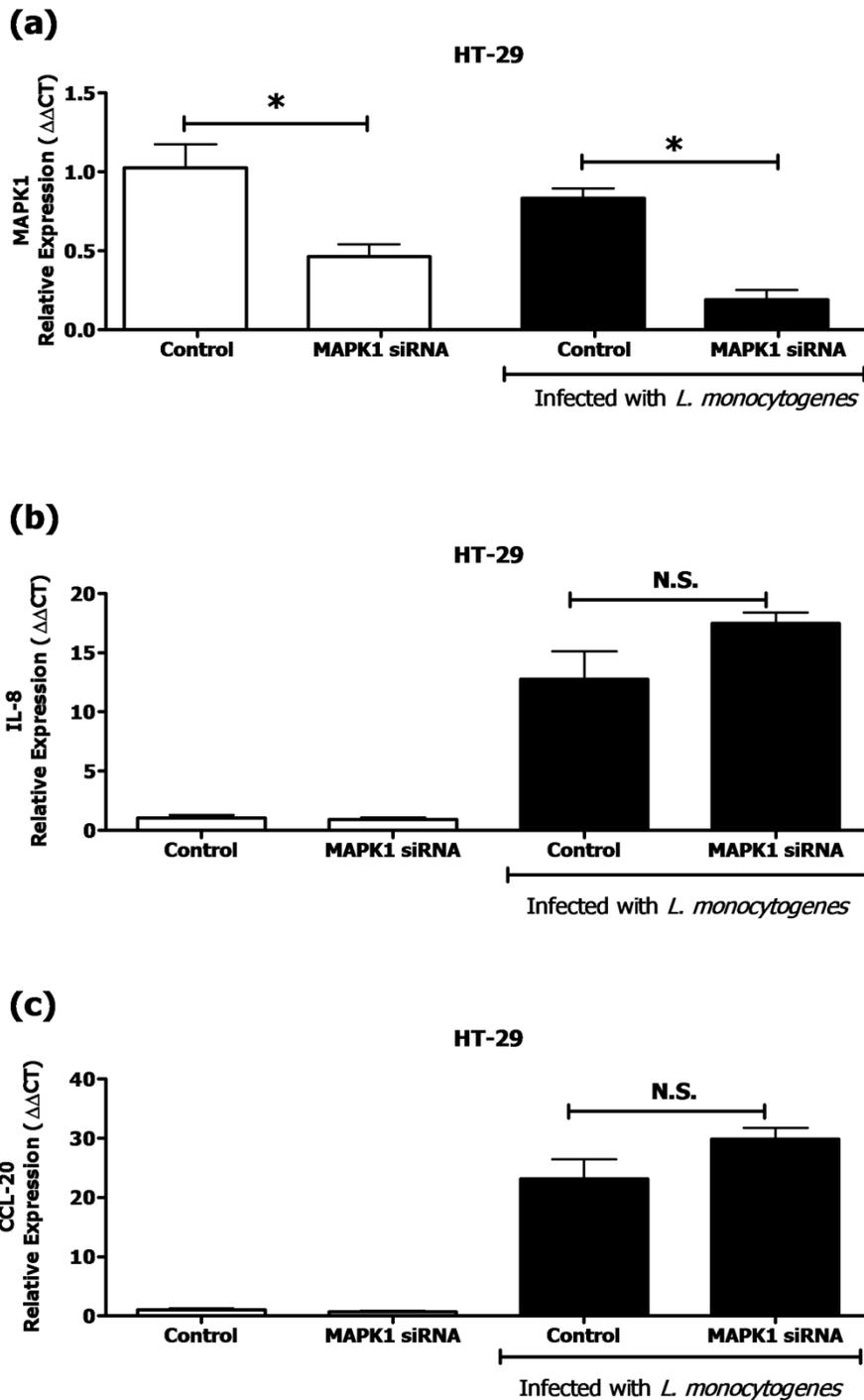


Figure 5.8 Verification of siRNA technique in HT-29 cells using MAPK-1 siRNA.

Cells were seeded in a 96 well plate. siRNA treatment was performed as described in the methods 2.17 using either a non-targeting siRNA control or MAPK-1 siRNA. Cells were then subject to infection with *L. monocytogenes* as described in the methods section 2.14. Lysates of the cells were then prepared for analysis by qRT-PCR of MAPK1 expression (a), IL-8 expression (b) or CCL-20 expression (c) made relative to the untreated control. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Statistics were carried out using t test with Welch's correction.

Similarly in THP-1s, a silencing of 50% was seen in MAPK1 induction following MAPK1 siRNA treatment (Fig. 5.9 (a)). Again, there was no change in the induction of IL-8 (Figure 5.9 (b)) or in IL-1 β induction (c) between the control or MAPK1 siRNA treated samples following *L. monocytogenes* infection. Importantly, neither cell type underwent any changes in knockdown efficiency following infection, nor were there any significant differences in the inflammatory outputs used for infection following siRNA treatment.

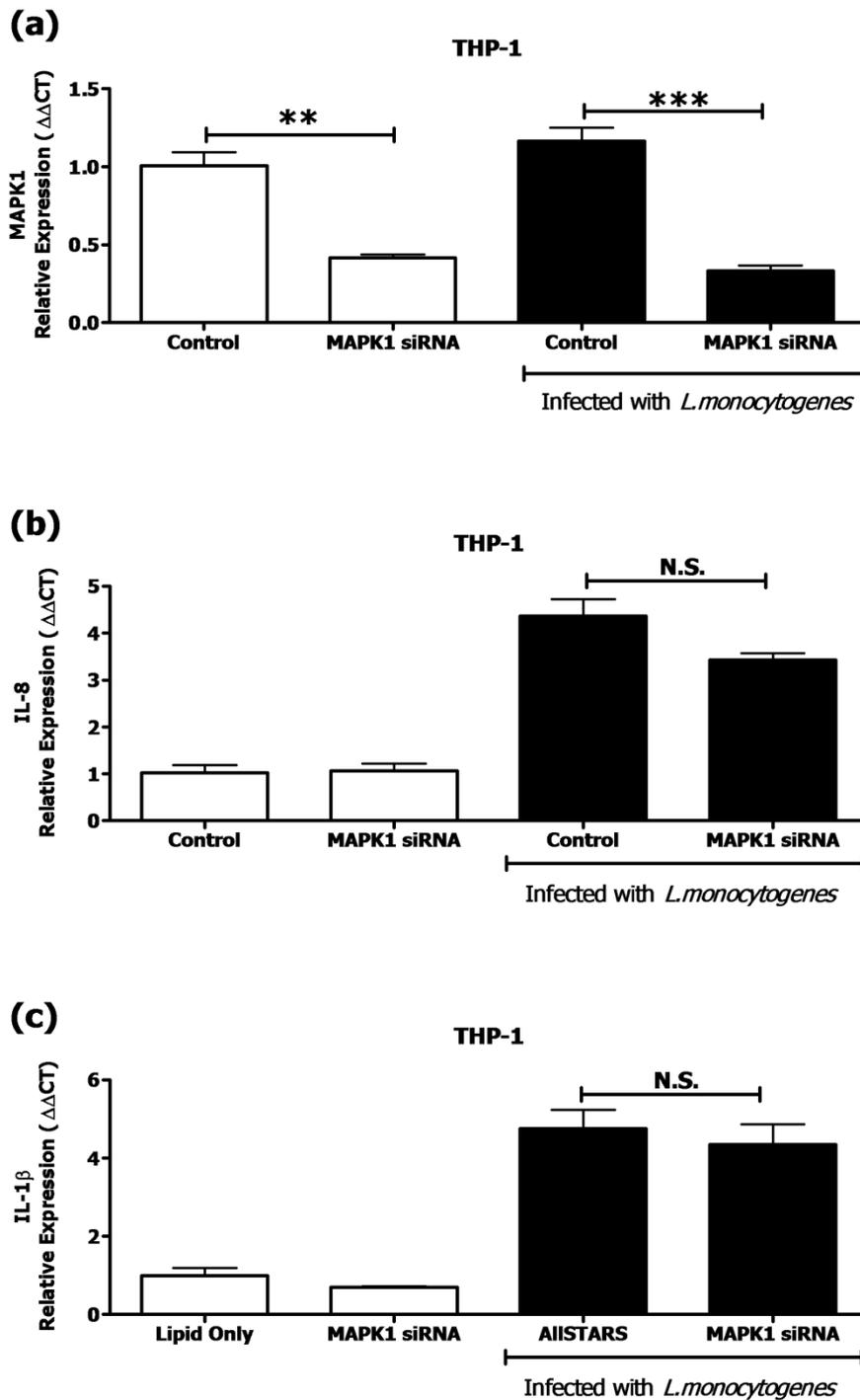


Figure 5.9 Verification of siRNA technique in THP-1 cells using MAPK-1 siRNA.

Cells were seeded in a 96 well plate. siRNA treatment was performed as described in the methods section 2.17 using either a non-targeting siRNA control or MAPK-1 siRNA. Cells were then subject to infection with *L. monocytogenes* as described in the methods section 2.14. Lysates of the cells were then prepared for analysis by qRT-PCR of MAPK1 expression (a), IL-8 expression (b) or IL-1 β expression (c) made relative to the untreated control. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Statistics were carried out using t test with Welch's correction.

Subsequently, 2 positive controls were used for siRNA treatment; RelA, a subunit of the inflammatory gene regulator, NF- κ B, and the TLR adaptor protein, MyD88. Silencing of either of these genes should lead to reduced induction of the inflammatory genes selected as an output following infection since both RelA and MyD88 are reported to be vital for detection and clearance of *L. monocytogenes* in the human system [24]. Figure 5.10 displays the successful silencing of each of these genes in HT-29 cells with 60 to 80% silencing of RelA induction (5.10a) and at least 75% reduction in MyD88 induction (5.10b) as measured by qRT-PCR. The corresponding inflammatory response following infection also revealed a decrease from 5-fold induction of CCL-20 over uninfected cells in the control siRNA treated samples, to 2-fold in the RelA siRNA treated samples and 4-fold in the MyD88 siRNA treated samples (5.10c). A similar result was obtained when IL-8 expression was examined with the same levels of induction and decreased induction across the control, RelA and MyD88 siRNA treated samples following *L. monocytogenes* infection (5.10d). It is interesting to note that RelA appears to be involved in epithelial responses but not MyD88. This implies that NF- κ B activation is necessary for induction of IL-8 and CCL-20 in response to *L. monocytogenes* but suggests that either MyD88 is dispensable or perhaps the level of knockdown achieved was not sufficient to affect the epithelial response to the bacteria as measured by CCL-20 and IL-8 induction.

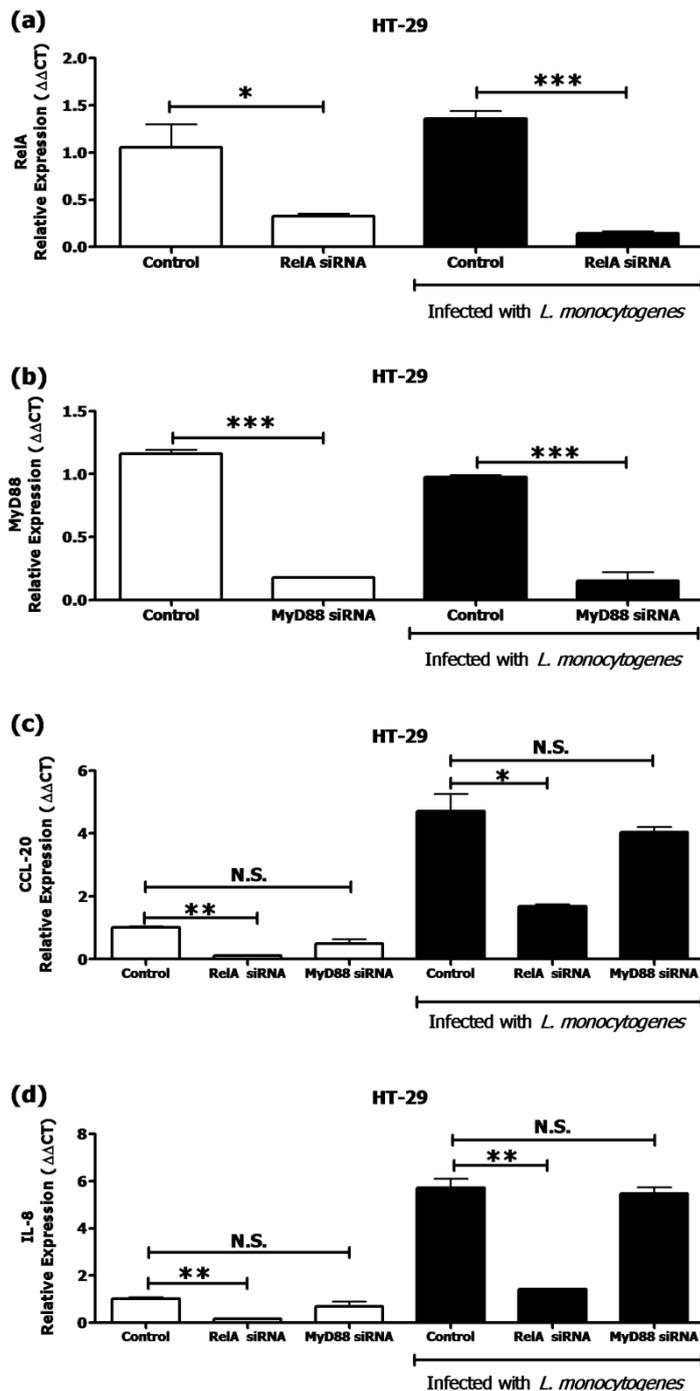


Figure 5.10 Verification of siRNA technique in HT-29 cells using MyD88 and RelA siRNA. Cells were seeded in a 96 well plate. siRNA treatment was performed as described in the methods section 2.17 using either a non-targeting siRNA control , RelA or MyD88 siRNA. Cells were then subject to infection with *L. monocytogenes* as described in the methods section 2.14. Lysates of the cells were then prepared for analysis by qRT-PCR of RelA expression (a), MyD88 expression (b), CCL-20 expression (c) or IL-8 (d) made relative to the untreated control. * P<0.05, ** P<0.01 and *** P<0.001. Statistics were carried out using t test with Welch’s correction.

In THP-1 cells, there was a 60% reduction in both RelA expression (Fig. 5.11 a) and MyD88 expression (Fig. 5.11 b) following siRNA treatment compared to the control siRNA treated samples. Neither RelA nor MyD88 siRNA had an effect on IL-8 induction (Fig. 5.11 c) over uninfected samples following infection with *L. monocytogenes*. There was, however, a significant reduction of IL-1 β expression in the uninfected cells which had been treated with RelA or MyD88 siRNA (Fig. 5.11 d). This was reflected in the significant decrease of IL-1 β levels induced in response to infection; control siRNA samples responded with 3-fold induction over uninfected while RelA and MyD88 siRNA treated samples displayed similar levels of induction to the uninfected scrambled siRNA treated control. This would seem to imply that unlike the epithelial cells, THP-1 did not require RelA or MyD88 for IL-8 induction following infection, or again, that the knockdown was not sufficient to affect the response to the bacteria. IL-1 β induction, however, was greatly reduced upon silencing RelA and MyD88, implying a more critical function for each of these genes in IL-1 β induction. This was seen both in uninfected and infected samples.

Taken together these experiments show that we were able to perform efficient knockdown in both HT-29 and THP-1 cells and also that we could affect innate immune response outputs following knockdown of known immune genes.

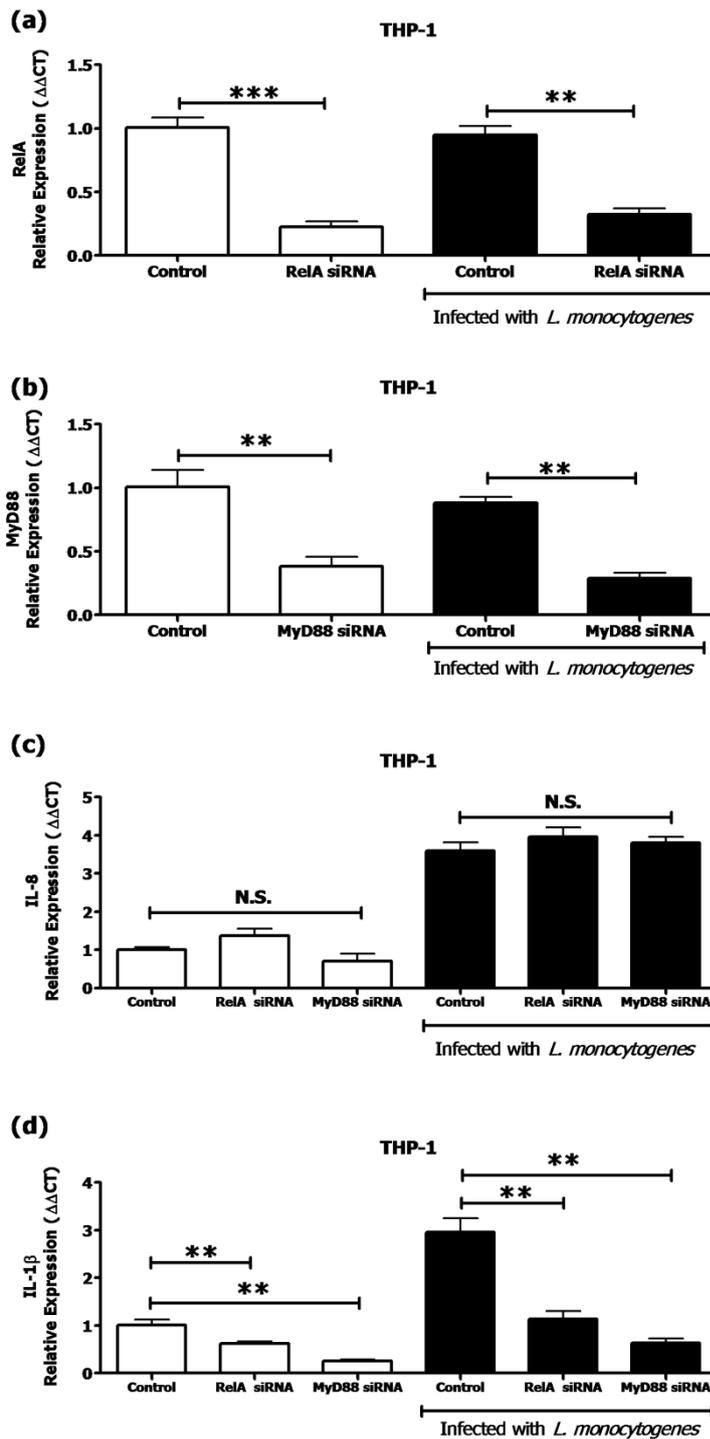


Figure 5.11 Verification of siRNA technique in THP-1 cells using MyD88 and RelA siRNA Cells were seeded in a 96 well plate. siRNA treatment was performed as described in the methods section 2.17 using either a non-targeting siRNA control, RelA or MyD88 siRNA. Cells were then subject to infection with *L. monocytogenes* as described in the methods section 2.14. Lysates of the cells were then prepared for analysis by qRT-PCR of RelA expression (a), MyD88 expression (b), CCL-20 expression (c) or IL-1 β (d) made relative to the untreated control. * P<0.05, ** P<0.01 and *** P<0.001. Statistics were carried out using t test with Welch’s correction.

5.2.4 PRR siRNA library screen used to measure changes in inflammatory response following infection with *L. monocytogenes*

Following the optimisation of the infection parameters for *L. monocytogenes* and the inflammatory response output and siRNA treatment in both THP-1 monocytes and HT-29 epithelial cells, the next phase of the study involved using the PRR siRNA library. A list of the genes involved in this screen is shown in Table 5.2 below.

Toll-Like Receptors	NOD-like receptors		C-type Lectin Receptors	
TLR1	CIITA	NLRP5	CD209	CLEC12B
TLR2	NAIP	NLRP6	CD207	CLEC6A
TLR3	NOD1	NLRP7	CLEC10A	CLEC4C
TLR4	NOD2	NLRP8	CLEC5A	CLEC4E
TLR5	NLRC3	NLRP9	CLEC7A	CLEC4A
TLR6	NLRC4	NLRP10	CLEC12A	MRC1
TLR7	NLRC5	NLRP11	CLEC1B	LY75
TLR8	NLRP1	NLRP12	CLEC9A	
TLR9	NLRP2	NLRP13		
TLR10	NLRP3	NLRP14		
	NLRP4	NLRX1		
Adaptors:	MYD88	CASP1	RIPK2	

Table 5.2 The PRRs and adaptor proteins included in the siRNA library screen

We decided to focus on using the HT-29 cell line with the silencing library as most previous studies have focussed on *L. monocytogenes* interaction with macrophage cells rather than epithelia. It is thought that *L. monocytogenes* initially invades epithelia; therefore, this may be the first site where PRR activation occurs. Figures 5.12, 5.13 and 5.14 below display the qRT-PCR results from EGR-1, IL-8 and CCL-20 expression levels respectively in response to *L. monocytogenes* infection following siRNA treatment for the genes indicated in the graphs.

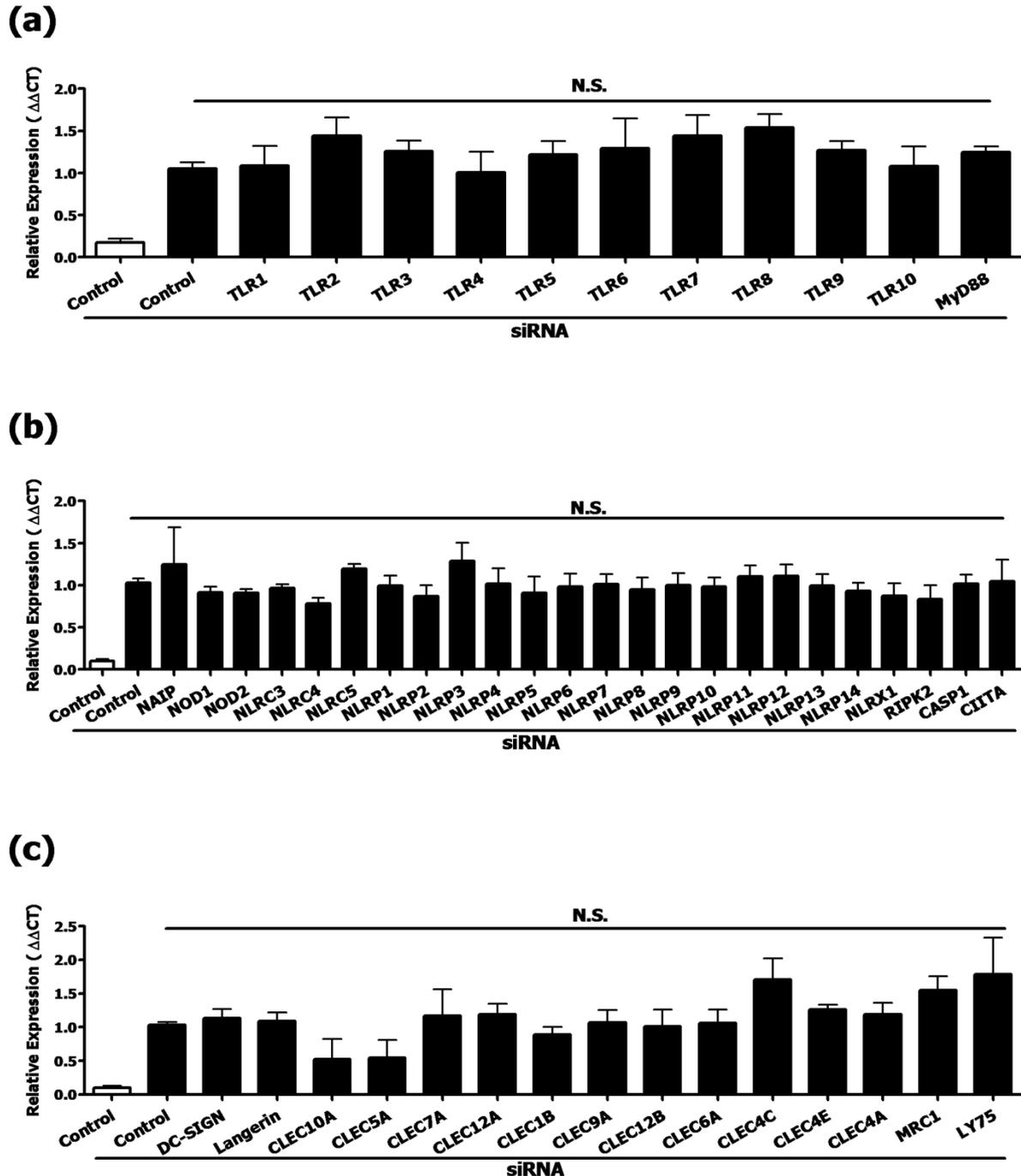


Figure 5.12 Relative mRNA expression of EGR-1 in HT-29 cells treated with PRR siRNA compared with non-targeting siRNA treated control following *L. monocytogenes* infection. Cells were treated with the PRR siRNA library as described in the methods section 2.17. Following infection with *L.monocytogenes*, lysates were analysed by qRT-PCR for EGR-1 induction. Each bar represents the expression of EGR-1 relative to the non-targeting siRNA treated infected control. For clarity, the results have been divided into samples treated with siRNA targeting TLR genes (a), NLR genes (b) and CLR genes (c). * P<0.05, ** P<0.01 and *** P<0.001. Values are shown as Mean \pm SEM, n=3. Statistics were carried out using the Mann Whitney U test.

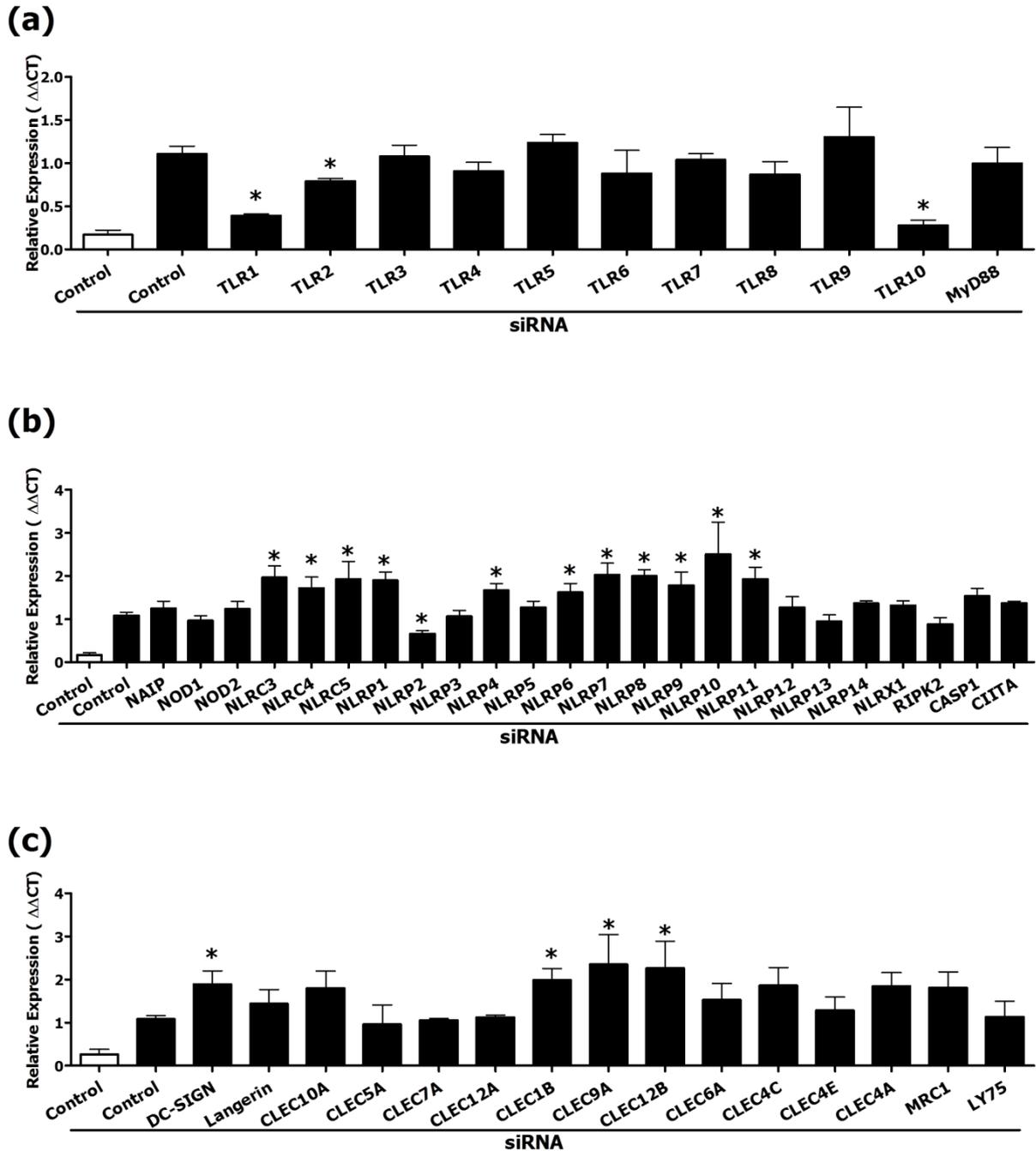


Figure 5.13 Relative mRNA expression of IL-8 in HT-29 cells treated with PRR siRNA compared with non-targeting siRNA treated control following *L. monocytogenes* infection. Cells were treated with the PRR siRNA library as described in the methods section 2.17. Following infection with *L.monocytogenes*, lysates were analysed by qRT-PCR for EGR-1 induction. Each bar represents the expression of IL-8 relative to the non-targeting siRNA treated infected control. For clarity, the results have been divided into samples treated with siRNA targeting TLR genes (a), NLR genes (b) and CLR genes (c). * P<0.05, ** P<0.01 and *** P<0.001. Values are shown as Mean \pm SEM, n=3. Statistics were carried out using the Mann Whitney U test.

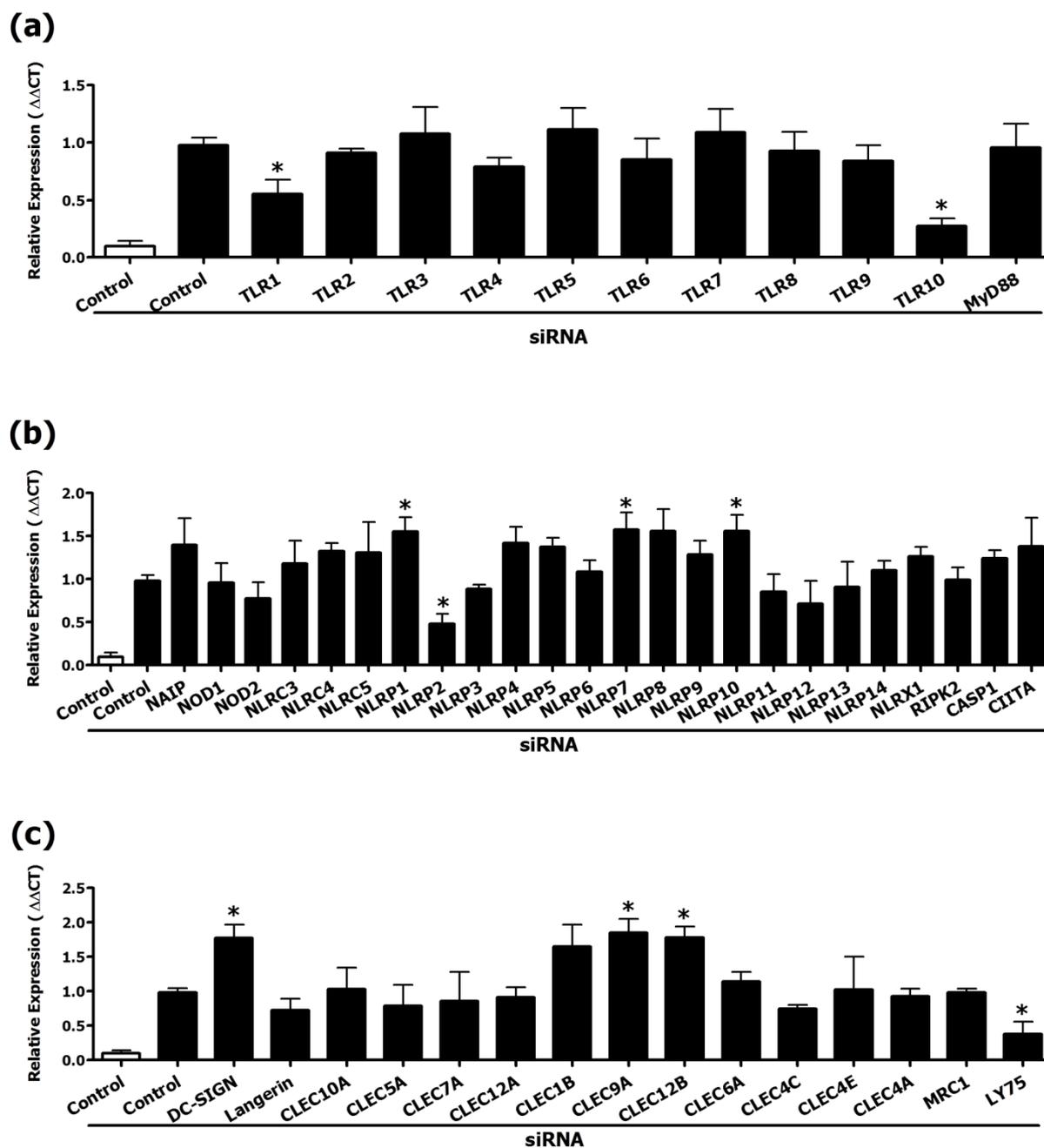


Figure 5.14 Relative mRNA expression of CCL-20 in HT-29 cells treated with PRR siRNA compared with non-targeting siRNA treated control following *L. monocytogenes* infection. Cells were treated with the PRR siRNA library as described in the methods section. Following infection with *L.monocytogenes*, lysates were analysed by qRT-PCR for EGR-1 induction. Each bar represents the expression of CCL-20 relative to the non-targeting siRNA treated infected control. For clarity, the results have been divided into samples treated with siRNA targeting TLR genes (a), NLR genes (b) and CLR genes (c). * P<0.05, ** P<0.01 and *** P<0.001. Values are shown as Mean \pm SEM, n=3. Statistics were carried out using the Mann Whitney U test.

EGR-1 expression, displayed in Figure 5.12, was increased 7-fold upon infection with *L. monocytogenes* in the control siRNA treated cells. However, it was not significantly affected by siRNA treatment with any of the 50 PRR targets in the library following infection. Figure 5.13 displays IL-8 expression, which increased 6-fold following infection with *L. monocytogenes* in the control siRNA treated cells. CCL-20 was induced 10-fold in the control siRNA treated samples following infection (Fig. 5.14). Several significant changes in IL-8 and CCL-20 induction were measured following PRR siRNA treatment. Therefore, due to the large volume of data generated from Figures 5.13 and 5.14, each of the statistically significant changes in expression are displayed on a separate figure (Figure 5.15).

Figure 5.15 displays expression levels of CCL-20 (a) and IL-8 (b) made relative to the control siRNA treated sample following infection. The samples with lowered expression of each of these genes indicate PRRs involved in early *L. monocytogenes* detection in intestinal epithelial cells have been silenced by siRNA. The samples with higher expression could reveal some regulatory PRRs involved in controlling an otherwise detrimental inflammatory response. Alternatively, they could reveal PRRs utilised by *L. monocytogenes* to evade detection by our innate immune surveillance system.

CCL-20 expression increased almost 2-fold following siRNA treatment for regulatory genes NLRP7 and NLRP10 over samples treated with non-targeting siRNA. We have observed a decrease of 50% in CCL-20 expression following treatment with NLRP2 siRNA in infected cells. The same trend was conserved in IL-8 expression levels with a 50% decrease observed following siRNA treatment for NLRP2 and a 2-fold increase after NLRP1, NLRP7 and NLRP10 were silenced. In addition, siRNA treatment for several other NLRs resulted in increased IL-8 expression in response to the bacteria. siRNA targeting NLRC3, NLRC4, NLRC5, NLRP4, NLRP6, NLRP8, NLRP9 and NLRP11 resulted in a 3-fold increase in IL-8 expression following infection. NOD1 and NOD2 are known to be involved

in detection of *L. monocytogenes* and eliciting an inflammatory response [15, 25, 26]. Although no significant change in any inflammatory genes we measured were observed in response to infection following treatment with siRNA for NOD1, NOD2 or their adaptor molecule RIPK2, there was a trend in decreased IL-8 and CCL-20 expression (Figure 5.13 b and 5.14 b).

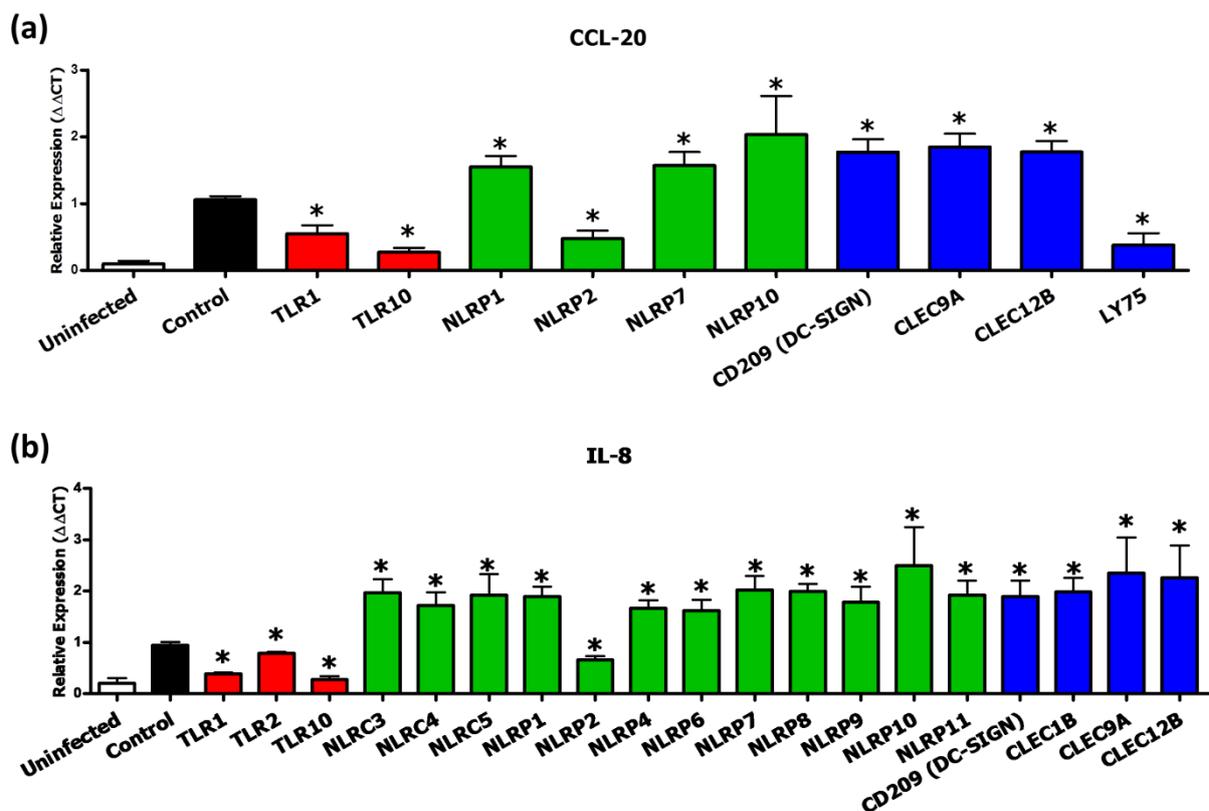


Figure 5.15 Relative expression of CCL-20 and IL-8 in HT-29 cells in response to *L.monocytogenes* infection following treatment with PRR siRNA. Due to the volume of data generated from the screen, Figure 5.15 displays only the data from Figures 5.12, 5.13 and 5.14 in which a statistically significant change in expression of the gene measured was observed following treatment with the PRR siRNA indicated. Changes in CCL-20 induction (a) in addition to IL-8 induction (b) are displayed while no significant changes were observed in EGR-1 expression following siRNA treatment. * P<0.05, ** P<0.01 and *** P<0.001. Values are shown as Mean \pm SEM, n=3. Statistics were carried out using the Mann Whitney U test.

Among the lesser studied CLR family, silencing of DC-SIGN, CLEC-9A and CLEC-12B resulted in a 2-fold increase in CCL-20 induction following infection. The same can be said of the increased IL-8 induction following silencing of each of these CLRs with the addition of CLEC1B. Silencing of LY75 resulted in a notably significant decrease in CCL-20 induction following infection with *L. monocytogenes*, although no effect was observed on IL-8 induction.

Few significant changes in IL-8 or CCL-20 induction were recorded in response to infection following treatment with TLR siRNA. Similar to the result in Figure 5.10, silencing of MyD88 had no effect on the cells ability to respond to *L. monocytogenes* infection through IL-8 and CCL-20 production, despite all TLRs with the exception of double-stranded RNA sensing TLR3 signalling via MyD88. This is reflected in the screen results (Figures 5.9, 5.10 and 5.11). It is somewhat surprising in this regard to observe significant decreases in CCL-20 and IL-8 expression upon silencing of TLR1 and TLR10, both believed to require MyD88 for signalling [27]. Since TLR10 has not yet been fully characterized, this is particularly novel. Additionally, the TLR1 siRNA treated samples showing reduced early inflammatory activation has not been reported previously. There is relatively little reduction in CCL20 and IL-8 induction due to TLR2 siRNA treatment compared to that observed when siRNA for TLR1 and TLR10 were used.

5.3. Discussion

In this study, I aimed to identify novel PRRs involved in early detection of *L.monocytogenes* through means of siRNA screening. This involved the optimisation of such parameters as bacterial growth phase, multiplicity of infection, incubation time with bacteria, use of antibiotics during infection, output of inflammatory response, and successful transfection of siRNA and achieving knockdown of the target genes. In doing so, novel information regarding the roles played by various PRRs of intestinal epithelial cells involved in activation or suppression of the immune response to *Listeria* was generated.

EGR-1 has been implicated in the regulation of a number of genes involved in inflammation, differentiation, growth, tissue repair and regeneration and development [28, 29]. Its expression has been shown to be induced by a range of pro-inflammatory stimuli, including many known to induce NF- κ B expression [30, 31]. Additionally, both transcription factors are known to synergistically be involved in TNF α -mediated gene transcription [32]. While strongly induced in response to the bacteria, EGR-1 was not affected by any of the siRNA from the PRR screen. Although the reason for this is unclear, it suggests that perhaps it is being driven by mechanisms other than PRR-mediated inflammatory responses; for example modulation of the host cell membrane from invading bacteria. It might also suggest that NF- κ B activation was not silenced sufficiently by any PRR siRNA treatment to block induction of the EGR-1 transcription factor. Since this is a very early transcription factor, it is possible that the 3 hour time-point may have been too long to measure any observable changes induced by the PRR siRNAs in response to the bacteria.

The PRRs identified in mediating the response to infection are TLR1, TLR2, TLR10, NALP2 and LY75. The PRRs identified in abrogating IL-8 and CCL-20 expression in response to infection, however, is more expansive: NLRC3, NLRC4, NLRC5, NLRP1,

NLRP4, NLRP6, NLRP7, NLRP8, NLRP9, NLRP10, NLRP11, DC-SIGN, CLEC1B, CLEC9A and CLEC12B (See Figure 5.16).

Similar to data generated here, it has been previously reported that NLRP7 and NLRP10 repress NF- κ B activation and IL-1 β production induced by other NLRs of the same family [33]. The same group also reported that NLRP2 plays a similar role in repressing NF- κ B induction from these NLRs in monocytic cells. This is similar to other reports studying this effect of NLRP2, again performed on THP-1 monocytes [34]. In contrast, however, we have observed a decrease of 50% in CCL-20 expression following treatment with NLRP2 siRNA in infected cells indicating that, NLRP2 may be essential, not inhibitory for immune activation. Our work, however, was performed in epithelial cells and this result most closely reflects other work by Ji *et al.* [35]. Their studies were performed using an invasive bacteria on gingival epithelial cells and an important inflammatory role was highlighted for NLRP2 and TLR2 in mediating an inflammatory response to the bacteria through human beta defensin production [35]. NLRP1 is known for its role in inflammasome activation [36] however there has not been any other findings to date which report an NLRP1 mediated reduction in inflammatory response to intracellular pathogenic attack, such as in our findings presented here. It is of note that most studies involving inflammasome activation have been performed on cells such as macrophages and dendritic cells and epithelial cells are not regarded to be involved in inflammasome activation so it is likely that these NLRs may have different roles here. The same trend was conserved in IL-8 expression levels decreasing following siRNA treatment for NLRP2 and increasing after NLRP1, NLRP7 and NLRP10 were silenced.

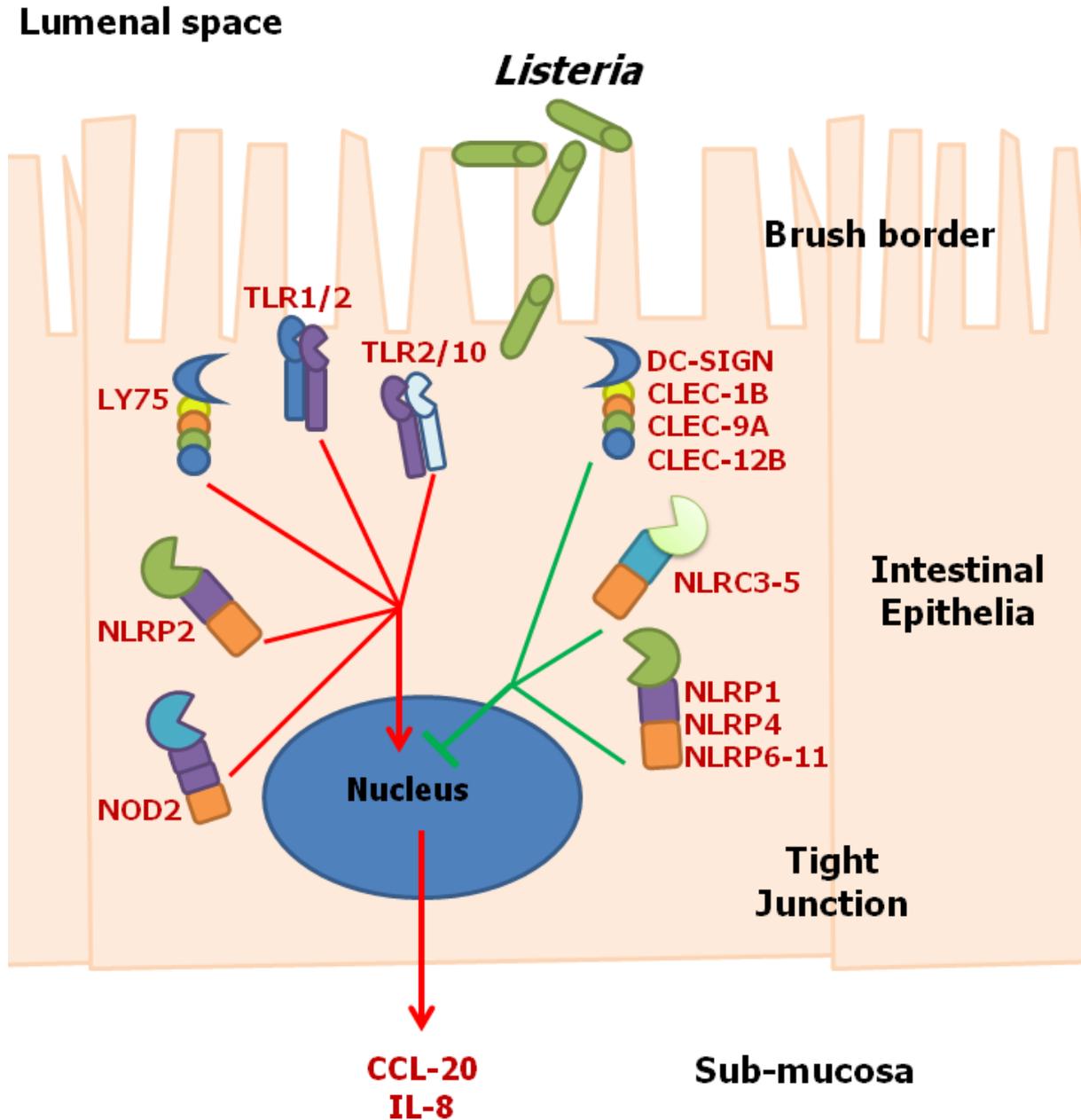


Figure 5.16 PRRs involved in regulating an inflammatory response to *L.monocytogenes* in intestinal epithelial cells identified by siRNA screening. Illustrated above is an intestinal epithelial cell (IEC) in the monolayer of cells which make up the intestinal epithelial barrier. Depicted are the tight junctions between cells in this monolayer and the apical brush border of the cell facing the microbe rich lumenal space. *Listeria* gains entry to the cells from this lumenal space where upon it is recognised by various intracellular and plasma membrane located PRRs before it makes its way into the sub-mucosa and eventually, the bloodstream. On the left-hand side are PRRs identified in our PRR siRNA screen believed to be directly involved in the production of pro-inflammatory CCL-20 and IL-8 production. On the right-hand side are PRRs believed to be involved in curtailing or regulating these responses.

Additionally, siRNA treatment for several other NLRs resulted in increased IL-8 expression in response to the bacteria. siRNA targeting NLRC3, NLRC4, NLRC5, NLRP4, NLRP6, NLRP8, NLRP9 and NLRP11 resulted in an increase in IL-8 expression following infection. While NLRP3 is known for its ability to inhibit TLR-mediated inflammatory responses in macrophages [37], it is the first time that a similar result has been reported in intestinal epithelial cell lines. NLRC4 is known for its role in inflammasome activation in response to bacterial flagellin [38, 39], however to date, its inhibitory role for *Listeria* mediated IL-8 induction has not been reported. NLRC5 has also been shown to be involved in attenuating NF- κ B activation and repressing type I IFN responses [40, 41]. However, the mechanism has not been clearly defined, and indeed in some cell population it was found to positively regulate type I IFN signalling [42]. The fact that NLRC5 siRNA attenuated IL-8 induction in response to *L. monocytogenes* may indicate an inhibitory role for NLRC5 in epithelial signalling. NLRP4 is also known for its ability to inhibit type I IFN signalling. It achieves this through targeting kinase TBK1, which is involved in NF- κ B activation, for degradation [43]. NLRP6 has already been shown to inhibit immune responses to *L. monocytogenes in vivo* [44]. Much less is known about NLRP8, NLRP9 and NLRP11 with no studies yet published on their immune function. The results from this screen however would indicate that, similar to their familial counterparts, NLRP4, NLRP6, NLRP7 and NLRP10, they too play a part in regulating immune activation in the intestinal epithelia.

Dendritic Cell (DC)-Specific Intercellular adhesion molecule-3 Grabbing Non-integrin (DC-SIGN) is known for its role in recognising a broad range of pathogen-derived ligands and self-glycoproteins, particularly, as the name suggests in dendritic cells, and presenting them to T-cells [45]. The role for DC-SIGN in epithelial cells or in negatively regulating the inflammatory response has not yet been investigated thoroughly. However, intracellular *Mycobacteria* and the Human Immuno-deficiency Virus (HIV) have previously

been shown to target DC-SIGN, both to infect DCs and to down-regulate DC-mediated immune responses [46, 47]. Furthermore, DC-SIGN activation is believed to modulate TLR-mediated inflammatory responses [48]. Silencing DC-SIGN also resulted in a significant increase in CCL-20 induction in response to the infection in IECs, suggesting a broader expression pattern and functionality than had originally been suggested – potentiating new targets for gut-associated microbial attack. Little is known of CLEC9A, in the setting of IECs or elsewhere. CLEC12B, however, is known to have the ability to counteract Natural Killer cell signalling by unconventional means and is believed to be involved in limiting the activity of monocyte-derived immune cells [49]. Due to both of these receptors being of the same family, there might be a similar novel role for them in regulating inflammatory responses in epithelial cells. The same can be said of the increased IL-8 induction following silencing of each of these CLR receptors with the addition of CLEC1B, of which there is very little known in the context of IECs. It is known, however, that CLEC1B is believed to function as an activation receptor in neutrophils [50], suggesting an alternate role in the context of IECs due to the increased IL-8 production measured upon silencing CLEC1B. LY75, also known as DEC-205, has been primarily investigated in dendritic cells where it is involved in binding to and increasing the uptake of the synthetic bacterial and viral DNA homologue, CpG ODNs. It is then believed to deliver the antigen to TLR9 [51], which sends inflammatory signals in response to CpG DNA. It is also thought to play roles not only in endocytosis/phagocytosis but also in cell adhesion and migration [52]. Silencing of LY75 resulted in a notably significant decrease in CCL-20 induction following infection with *L. monocytogenes*, although no effect was observed on IL-8 induction. This suggests a specific response for LY75, perhaps in recruiting phagocytic cells to the site of infection in IECs. NOD1 and NOD2 are known to be involved in detection of *L. monocytogenes* and eliciting an inflammatory response [15, 25, 26]. Although no significant change in any inflammatory

genes measured were observed in response to infection when cells were treated with siRNA for NOD1, NOD2 or their adaptor molecule RIPK2, there was, however, a trend in decreased IL-8 and CCL-20 expression.

The most significant reductions in inflammatory cytokine induction were seen following siRNA treatment against TLR1, TLR2, TLR10 and NALP2. TLR2 has previously been shown to be involved in *L. monocytogenes* detection and clearance [13, 53-55] although not in the specific context of IECs. NALP2 has also been demonstrated to be involved in detection of *L.monocytogenes* in gingival epithelial cells [35] although this is the first time it has been implicated in IECs also. Ligands for TLR1 such as peptidoglycan are known to be expressed by *L.monocytogenes* so it was not surprising to see a significant decrease in inflammatory gene induction in response to the bacteria following siRNA treatment for this gene. TLR10, however, having never been assigned a specific ligand or function, has never before been documented to play a role in detection of any pathogens or indeed to mediate an inflammatory response against any ligands tested. It is also of note that silencing of TLR10 resulted in the most statistically significant reduction in inflammatory cytokine induction in response to the bacteria.

Despite an observable reduction in IL-8 and CCL-20 induction following siRNA treatment for these TLR genes, no significant effect was measured following MyD88 siRNA transfection. This is surprising since each of TLR1, TLR2 and TLR10 are believed to require MyD88 for signal transduction [56]. Furthermore, the siRNA optimisation experiments revealed that RelA appeared to be involved in epithelial responses but not MyD88. This demonstrated that NF- κ B activation is necessary for induction of IL-8 and CCL-20 in response to *L. monocytogenes* but suggested that MyD88 is dispensable. It is important also, however, to consider the possibility that although 80% knockdown of MyD88 was observed following siRNA transfection, perhaps the level of knockdown achieved was not sufficient

enough to affect the epithelial response to the bacteria as measured by CCL-20 and IL-8 induction. It may be that these cells require relatively minimal amounts of MyD88 for efficient signal transduction. Moreover, it could also be possible that in addition to the well documented roles MyD88 plays in mediating inflammatory responses against pathogenic attack in IECs [57, 58], there might exist a role for MyD88 in mediating more homeostatic and less pro-inflammatory responses in IECs. This could be through the immuno-suppressive effects associated with TLR activation in IECs, for example [59].

Shortcomings in this data set include the lack of consistency and effectiveness of siRNA mediated gene silencing. As this was a large screen, it was not practical to measure each sample for quantifying the level of knockdown for each gene. Therefore, it is impossible to know how successful each siRNA transfection was. Nevertheless, much optimisation was performed in this regard prior to using the screen and the use of Smart Pool siRNA increased the chances of successful silencing. It should also be pointed out that TLR expression levels have recently been linked to circadian rhythm [60], a factor which we did not appreciate and would require future studies. Ultimately however, almost all of the data generated and almost every “hit” from the siRNA screen has been a novel find, particularly in the context of intestinal epithelial cells which have not classically been associated with PRR expression or innate immune functioning. A large amount of novel data in particular was produced involving several of the lesser known PRRs particularly with regard to regulating/abrogating the immune response to *L.monocytogenes*: in particular, members of the CLR and NLR families. While little else is known about many of these, it is important too to remember that this was only performed in one cell type and further studies involving any *in vitro* analysis should offer more interesting clues to their functionality. Fewer PRRs from the total number involved in this siRNA screen were implicated in mounting an inflammatory response to the bacteria in this study compared to the number of PRRs implicated in curtailing any

inflammatory responses. This again illustrates the importance of homeostatic regulation by PRRs in IECs. The two most novel PRRs involved in mounting an inflammatory response were LY75 and TLR10. Since silencing of TLR10, the only TLR to which a ligand has not previously been assigned resulted in the most significantly different expression levels of CCL-20 and IL-8 across all PRRs tested, I chose to focus on this candidate for the final chapter of my thesis.

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Chapter 6

Validation and further characterisation of the role of TLR10 in the immune response to *L.monocytogenes*

6.1 Introduction

Toll-like receptors (TLRs), a major family of the innate immune system's cohort of Pattern Recognition Receptors (PRRs), play a crucially important role in the early stages of infection. In addition to the induction of innate immunity, they are also pivotal in subsequent recruitment of adaptive immune response cells. In humans, 10 functional TLRs have been discovered and their ligands for each have been identified; except for in the case of TLR10 [1]. In mammals, TLR1, 6, and 10 genes are arranged closely together on the genome and seem to have arisen from a duplication event [2, 3]. TLR10 shares the highest homology with TLR1 and TLR6, both of which mediate immune responses to a variety of microbial and fungal components in cooperation with TLR2 [4]. In addition to the ability of TLR10 to homodimerise [5], it can also form a heterodimer with TLR1 or TLR2 and like other TLRs, it is believed to transmit an intracellular signal via the adaptor protein MyD88 [6]. However, multiple gaps and retroviral insertions have rendered the TLR10 receptor a pseudogene in mice; hence the useful tool of a TLR10 deficient mouse strain has not been available to help us elucidate its function and discover its ligand [6].

The structure of TLR10 has been well characterised in an attempt to fully understand its signalling potential. Similar to the rest of its family, TLR10 has multiple Leucine-Rich Repeats (LRRs) for recognising and binding to Pattern Associated Molecular Patterns (PAMPs) and a Toll/interleukin-1 receptor (TIR) domain for downstream signalling. The crystal structure of the cytoplasmic TLR10 TIR domain has been used as a structural model for TIR domain signalling [5]. In fact, it was this study which revealed the dimeric nature of the ligand-bound TLR TIR domains which are required for further downstream signalling. Recent crystallographic studies of the TLR dimers TLR1/2, TLR2/6 in addition to TLR4, and TLR3 homodimers have provided an explanation for *in vivo*, *in vitro*, and clinical

observations [7]. While crystallographic analysis of TLR10 has not offered as much information, it has been used to predict TLR10 signalling and ligand binding. A study by Govindaraj *et al.* used computer modelling of these crystal structures in order to identify the potential sites of interaction between the homo (TLR10/10) and heterodimers of TLR10 (TLR10/2 and TLR10/1) in addition to attempting to determine a specific ligand for each of these complexes [8]. Figure 6.1 displays an alignment of the LRRs contained in TLR1, TLR2 and TLR10 which was generated in this study for helping determine TLR10 ligand recognition. Their computer modelling data revealed that the binding orientations of the TLR10 heterodimer were found to be similar to other TLR2 family members. The binding orientation of the TLR10 homodimer, however, was shown to be different from the heterodimer due to the presence of negatively charged surfaces at the LRR11-14 (Fig. 6.1), providing a specific cavity for ligand binding [8]. They went on to show that the TLR1/2 ligand, Pam3Cysk4, might be the ligand for the TLR10/2 complex. They also suggested that PamCysPamSK4 a di-acylated peptide, might activate the TLR10/1 hetero and TLR10 homodimers [8].

N-term domain

LRRNT	TLR10	RE LMTNCSNMSLRKVPADLTPA	49
	TLR2	SLSCDRNGICKGSSGSLNSIPSGLTEA	53
	TLR1	SEFLVDRSKNGLIHVPKDLSQK	46

Consensus **LxxLxLxxNxLxxLxxxxLxxLxx**

LRR1	TLR10	TTTLDLSYNNLFQSQSDFHSVSK	73
	TLR2	VKSLDLSNNRITYISNSDLQRQVY	77
	TLR1	TTILNISQNYISELWTSDFLSL	70

LRR2	TLR10	LRVLI LCHNR IQQLDLKTFEFNKE	97
	TLR2	LQALVLTSMGINTIEEDSFSSLG	101
	TLR1	LRI LIISHNR IQYLDISVFKNQ	94

LRR3	TLR10	LRYL DLSNNR LKSVTWYLAG	118
	TLR2	LEHL DLSYNNLSNLSSSWFKPLSS	125
	TLR1	LEYLDLSHNLKLVKISCHPTVN	115

LRR4	TLR10	LRYL DLSFNDFDTMPICEEAGNMSH	143
	TLR2	LTF LNLGPNPYKT LGETS LFSHLTK	150
	TLR1	LKHL DLSFNADFALPICKEFGNMSQ	140

Central domain

Consensus **LXXLX**

LRR5	TLR10	LEI LGLSGAKIQKDFQKIAHLH	166
	TLR2	LQI LRVGNMDFTKIQRKDFAGLTF	175
	TLR1	LKFLGLSTTHLEKSSVLPPIAHLN	163

LRR6	TLR10	LNTVFLGFRTLPHYEEGSLPILN	189
	TLR2	LEELEIDASDLQSYEPKSLKSIQN	199
	TLR1	ISKVLLVGLGETYGEKEDPEG LQDFN	188

LRR7	TLR10	TTKLHI VLPMDTNFVLLRDIKT	213
	TLR2	VSHLILHMKQHIL LLEIFVDTSS	223
	TLR1	TESLHIVFPTNKEFHFI L DVS VKT	212

LRR8	TLR10	SKI LEMTNI DGKSQFVSYEMQRNLSLENAK	243
	TLR2	VECLELRD TDLDTFHFSE LSTGETNSL	250
	TLR1	VANLELSNICKVLEDSKCSYF LSI LAKLQTNPK	245

LRR9	TLR10	TSVLLLNKVDLLWDDLFLILQFVWHTS	270
	TLR2	IKKFTFRNVKITDES L FQVMKLLNQISG	278
	TLR1	LSNLT LNNIETT WNSFIRILQLVWHTT	272

LRR10	TLR10	VEHFQIRNVT FSGKAYLDHNSFDYSNTV	298
	TLR2	LLELEFDDCT LNGVGNFRASDNDRVIDPGK	308
	TLR1	VWSSISNVK LQGLDFRDFDYSGTS	298

C-term domain

Consensus **LxxLxLxxNxLxxLxxxxLxxLxx**

LRR11	TLR10	MRTIKLEHVFHRYFYTCODKDYI LLTKMD	327
	TLR2	VETLTI RRLHIPRFYLFYDLSTLYSLTER	337
	TLR1	LKALS L HQVSDVFGFPDYSYIYIFSNMN	327

LRR12	TLR10	IENLTI SNAQKHMLFPNYPTK	349
	TLR2	VKRITVENSQVFLVPCLLSQHLKS	361
	TLR1	IKNFTVSGTQKHMLCPSKISP	349

LRR13	TLR10	FQYLNFAANNLIDELFRTIQLPH	373
	TLR2	LEYLDLSENL MVEEY LKNSACE DAWPS	388
	TLR1	F LHLDFSNL LIDVFEINOGHLTE	373

LRR14	TLR10	LKT LILNGN LLETL S LVS CFANMTP	398
	TLR2	LQT LILRQNH LASELEKTGETLLTKN	414
	TLR1	LET LILQMN LLELSKIAEMTTQMK	399

LRR15	TLR10	LEHLDLSQNL LQHKNDENCSWPET	422
	TLR2	LTNIDISKN SFHSMPE TCQWPEK	437
	TLR1	LQQLDISQNSVSYDEKKGDCSWTKS	424

LRR16	TLR10	VVMNLSYNNK LSDSVFRCLPKS	444
	TLR2	MKY LNLSSTRIHSVTGCI PKT	458
	TLR1	L LSNMSSNI LDTIFRCLPPR	446

LRR17	TLR10	IQI LD LNNNQIQTVPKETIHLMA	467
	TLR2	LEI LDVSNMNLN LFS LNL PQ	478
	TLR1	IKVLDLHSHN KIKSIPKQVMKLEA	469

LRR18	TLR10	LRE LNIAFNF LTDLPGCSHF SR	489
	TLR2	LKE LYI SRNK LMTLPDASLLPM	500
	TLR1	LQE LN VASNLK SVDPGIFDR L TS	493

LRR19	TLR10	LSV LNIE MNF I LSPS LDFVQSQQE	513
	TLR2	LLV LKISRNLK SVDPGIFDR L TS	524
	TLR1	LQKIWLHTNP WDCSCPRI DYLSRWLNKN	521

LRRCT	TLR10	VKTLNAGRNPFRCTCE LKNFIQLETYSEVMMV	577
	TLR2	GWSDSYTCEYPLNLRGR LKDVHLHE LSCNTA	
	TLR1	LQKIWLHTNP WDCSCPRI DYLSRWLNKNSQKE	

		QGS AKCSGSGKPVRSIICP	575
		SQKEQGS AKCSGSGKPVRSIICP	544



Figure 6.1 Structure based sequence alignments of TLR1, TLR2 and TLR10. The TLR10, 2 and 1 sequences are aligned based on their structures. Conserved leucines and asparagine ladder are written in red and green, respectively. The positions of β -strands are shown above the consensus patterns .

An *in vitro* study attempting to identify the specific ligand sensed by TLR10, and the consequent immune response it may activate, was performed using chimeric receptors of TLR1 and TLR10. TLR1 was chosen based on the strong homology shared by TLRs 1 and TLR10, and the fact that TLR1 was already well characterised. These chimeras were produced by fusing the extracellular recognition domain of TLR10 and the intracellular signalling domain of TLR1 (TLR10x1) and were compared with a receptor which had the extracellular recognition domain of TLR1 and the intracellular signalling domain of TLR10 (TLR1x10) [9]. Thus, by stimulating the TLR10x1 receptor with a variety of ligands, one could measure typical TLR1 responses to test for ligand responsiveness. Similarly, by stimulating TLR1x10 with known TLR1 ligands, typical TLR activation responses such as NF- κ B activity could be measured, giving an indication as to what downstream signalling TLR10 activation leads to. Investigations using TLR10x1 revealed that TLR10 is likely to sense triacylated lipopeptides and a wide variety of other microbial-derived agonists shared by TLR1, but not TLR6 [9]. TLR10 was revealed in this study to colocalise with TLR2 in the phagosome and physically interact in an agonist-dependent fashion. Although prior to our studies, TLR10, alone or in cooperation with TLR2, had not been shown to activate typical TLR-induced signalling, including NF- κ B-, IL-8-, or IFN- β -driven reporters [9].

Polymorphisms in TLR10 have been linked to several disease states. It has been reported that a haplotype of TLR10 is associated with an increase in the risk of nasopharyngeal carcinoma in a Cantonese population [10]. Mutations in the TLR10 gene have also been linked to asthma [11] and Crohn's disease [12, 13], independent of the well-recognised NOD2 mutation associated with this disease [14]. Studies on single nucleotide polymorphisms in the TLR1-6-10 gene cluster have suggested that mutations within this cluster may also be associated with a risk of asthma [15], prostate cancer [16-18], sarcoidosis [19] or non-Hodgkin lymphoma [20]. These reports are for the most part based on sequence

mutation analysis without investigating the altered function of TLR10 in each case. Further understanding of the natural function of TLR10 would allow us to better predict phenotypes associated with each mutation for these illnesses and determine its altered function. This, of course, could potentially provide invaluable information towards creating better treatment strategies for each of these diseases.

In this chapter, we initially verified the effect of reduced inflammatory response to *L.monocytogenes* in HT-29 IECs upon TLR10 silencing using PRR siRNA screening, outlined in Chapter 4. We explored this effect in other IEC lines and in THP-1 macrophage cells and further attempted to elucidate the function and signalling mechanism of TLR10 in these cell types. We also explored the effects of simulated hypoxia on TLR10 expression and microbial sensing in HT-29 and THP-1 cells through stabilisation of HIF-1 α , achieved through stimulation with a substance known as Dimethyloxaloylglycine (DMOG).

6.2 Results

6.2.1 Validation of the specificity of TLR1, TLR2 and TLR10 siRNAs

Following our observations of a reduced inflammatory response in HT-29 cells upon treatment with siRNA targeting TLR1, TLR2 and TLR10, we wished to further investigate the effects of TLR1, TLR2 and TLR10 siRNA on HT-29 responsiveness to *L.monocytogenes*. However, prior to any further analysis using siRNA mediated knockdown, we wished to verify the specificity and efficiency of the siRNAs used. These genes, along with TLR6, are very closely related, each located together in a gene cluster on chromosome 4, as well as being structurally similar. Since problems of “off-target effects” have been reported using siRNAs in the past, the specificity of the TLR siRNA used were investigated. qRT-PCR measurements were made to quantify relative expression levels of each of these genes following siRNA treatment against TLR1, TLR2 and TLR10 (Fig. 6.2). THP-1 macrophage cells were used to determine this due to the higher expression levels of TLRs found in these cells compared to cell lines of the intestinal epithelium. TLR1 induction was significantly affected (~70% reduction) by TLR1 siRNA alone (Fig. 6.2A). Similarly, TLR2 siRNA treatment alone had an effect on TLR2 expression with a reduction of 80% measured in TLR2 expression levels (Fig. 6.2B). The same was true of TLR10 expression; only reduced by TLR10 siRNA (Fig. 6.2D). Importantly, TLR6 induction was unaffected by any of the siRNA for TLR1, 2 or 10. Additionally no “off-target” effects were measured on any of these closely related genes following siRNA treatment. This confirmed the specificity and efficacy of the TLR siRNA used.

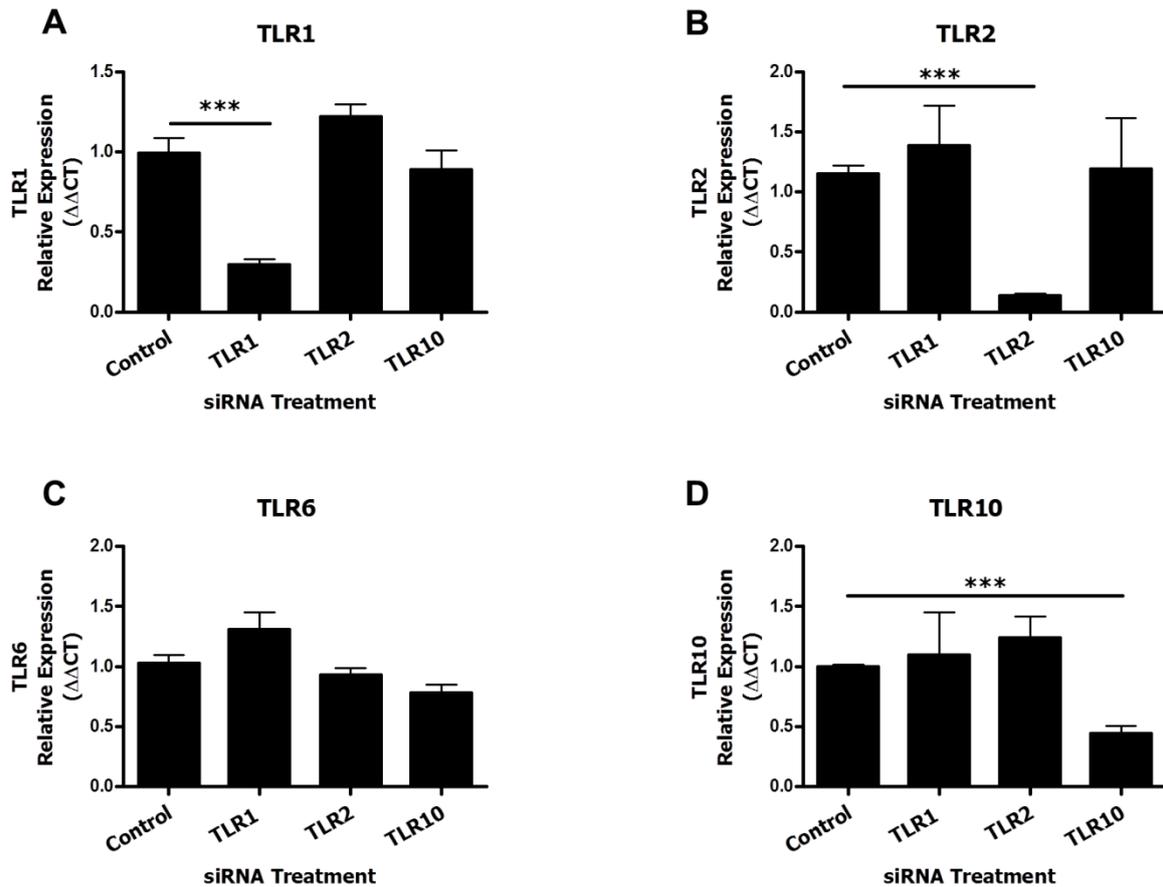


Figure 6.2 Specificity of TLR1, TLR2 and TLR10 siRNA analysis by qRT-PCR. THP-1 cells were treated with the either scrambled siRNA or siRNA against TLR1, TLR2 or TLR10 with 5ng/ml PMA for 48 hours. qRT-PCR was used to monitor expression levels of TLR1 (A), TLR2 (B), TLR6 (C) and TLR10 (D). Statistics were carried out using t test with Welch’s correction.

Given the importance of ensuring efficient knockdown of TLR10 was being observed, Western blotting was also employed using HT-29 cells in order to demonstrate endogenous expression of TLR10 and a reduction in TLR10 expression following siRNA treatment. TLR10 expression is displayed in Fig. 6.3(a). The ~75 kDa isoform is visible in the empty vector transfected sample (lane B) and the specificity of the antibody was confirmed through transfection with TLR10 in lanes C-F. Silencing of TLR10 protein levels was also confirmed through TLR10 siRNA treatment in lanes I and K. TLR1 expression was seen also at ~75

kDa (Fig. 6.3(b)) in lane A. Silencing was confirmed following TLR1 siRNA treatment (lane B) with no change seen following TLR10 siRNA treatment (lane C).

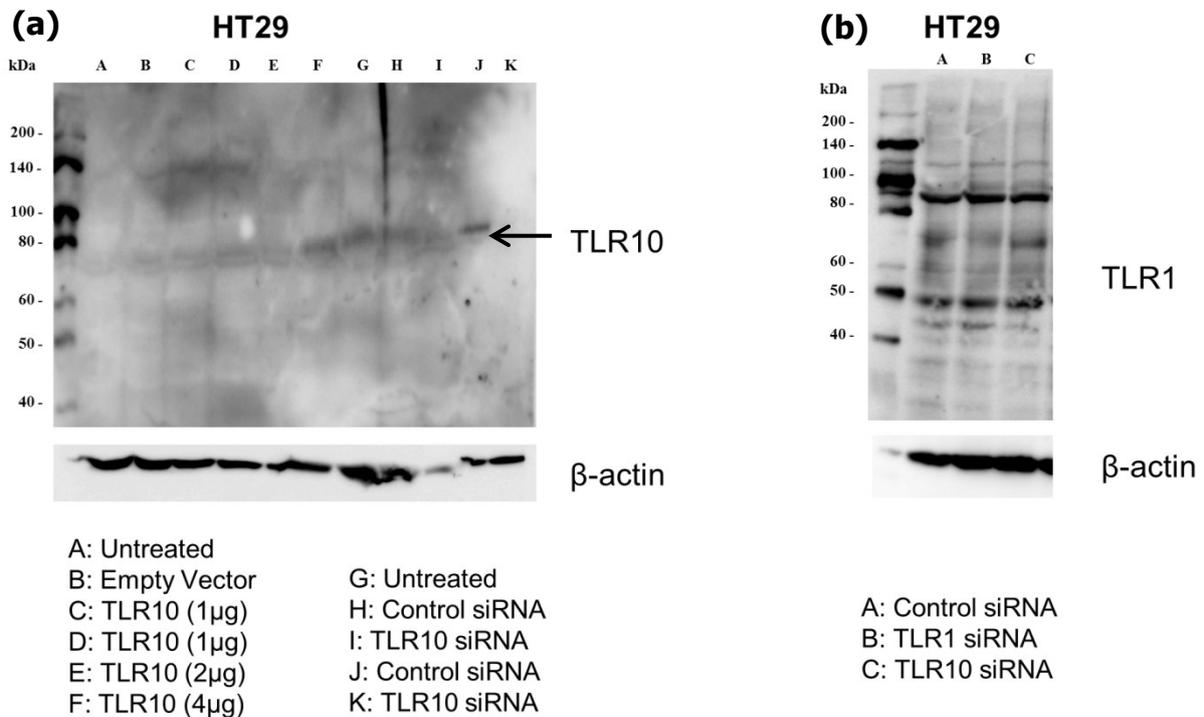


Figure 6.3 Validation of TLR1 and TLR10 siRNA efficiency by Western blot in HT-29 cells. Lysates of HT-29 cells were prepared for western blotting analysis 48 hours after transfection as described in the methods section 2.19. Each lane on the left blot against TLR10 represents the following transfection: Untreated (A), Empty Vector (B), TLR10 (1 μ g) (C), TLR10 (1 μ g) (D), TLR10 (2 μ g) (E), TLR10 (4 μ g) (F), Untreated (G) Control siRNA (H) TLR10 siRNA (I), Control siRNA (J) TLR10 siRNA (K). In addition to TLR10 expression, β -actin expression was also examined to ensure equal levels of protein were loaded on the gel. Similarly, TLR1 expression was measured on the right in HT-29 cells. Each lane represents the following siRNA treatment: Scrambled control (A), TLR1 (B) and TLR10 (C). β -actin expression was also examined to ensure equal levels of protein were loaded.

In addition to measurement by qRT-PCR and western blot analysis, flow cytometry was also used to verify the specificity of each siRNA at a protein level (Fig. 6.4). While an efficient antibody for TLR6 was unavailable, expression levels of TLR1, TLR2 and TLR10 protein were quantified following siRNA treatment for each. Gating strategies were employed (see methods section 2.21) in order to quantify the expression levels of each TLR detected over the isotype control in the control siRNA or TLR siRNA treated samples. THP-1 macrophage-like cells were used once again due to the higher levels of expression of each of the TLRs measured. Fig. 6.4A compares expression levels of TLR1, TLR2 and TLR10 following siRNA treatment with TLR1. A reduction from 3.4% to 0.5% was observed in TLR1 expression following siRNA treatment with no sizable changes in TLR2 or TLR10 expression. Fig. 6.4B displays a slight decrease in TLR1 expression following TLR2 siRNA treatment at 14.7% compared to 17.6% in control samples. TLR2 expression however, was reduced from 57.5% to 38.7% and a negligible change was observed in TLR10 expression. TLR10 siRNA treatment in Fig. 6.4C resulted in no changes for TLR1 or TLR2 expression but a 50% decrease in TLR10 expression from 2.7% to 1.3% compared to control siRNA treated samples. This confirmed that the effects seen in inflammatory gene responses to *L.monocytogenes* following TLR10 siRNA treatment were not likely to be due to “off-target” effects.

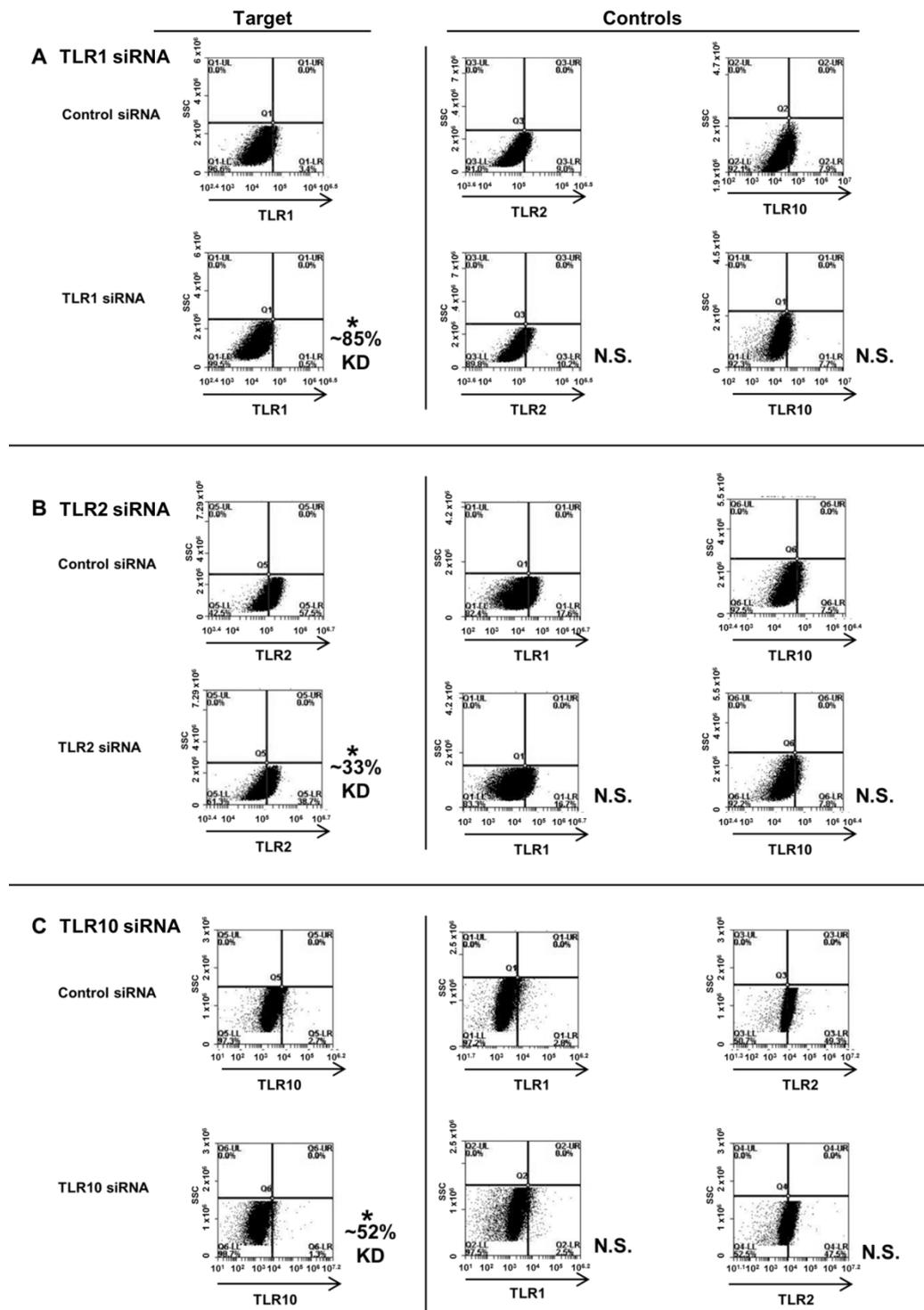


Figure 6.4 Specificity of silencing of siRNA used against TLR1, TLR2 and TLR10. THP-1 cells were treated with control (scrambled) siRNA and TLR1 siRNA (A), TLR2 siRNA (B) or TLR10 siRNA (C) with 5ng/ml PMA for 48 hours. Flow cytometry was used to monitor expression levels of TLR1, TLR2 and TLR10. Gating was performed against the isotype control and all control siRNA samples were measured against the corresponding TLR siRNA at the time of analysis to avoid intra-assay variation in staining efficiency. Representative scatter plots of flow data are shown with relative knockdown (KD) of target TLR.

6.2.2 Confirmation of reduced chemokine production following TLR10 silencing

Having confirmed the specificity and efficacy of these siRNA, we could now examine with confidence the effects of TLR1, TLR2 and TLR10 siRNA on HT-29 responsiveness to *L.monocytogenes*. We subsequently wished to examine the relative involvement of TLR10 as compared to TLR1 and TLR2 in mediating the immune response to *L.monocytogenes* (Fig. 6.5). Similar to the results seen in the screen data, TLR10 siRNA had the most significant effect on IL-8 and CCL-20 induction in response to the bacteria compared with TLR1 and 2 siRNA. CCL-20 was induced 12-fold in the control siRNA treated samples (Fig. 6.5A). This was reduced to 10-fold when TLR1 was silenced and 9-fold when TLR2 siRNA was employed. The TLR10 siRNA treatment however, reduced induction to 6-fold over the uninfected control. Similarly, in Fig. 6.5B, IL-8 was induced 8-fold over uninfected controls in control siRNA treated samples, 6-fold following TLR1 siRNA treatment, 7-fold with TLR2 siRNA and reduced to 4-fold with TLR10 siRNA.

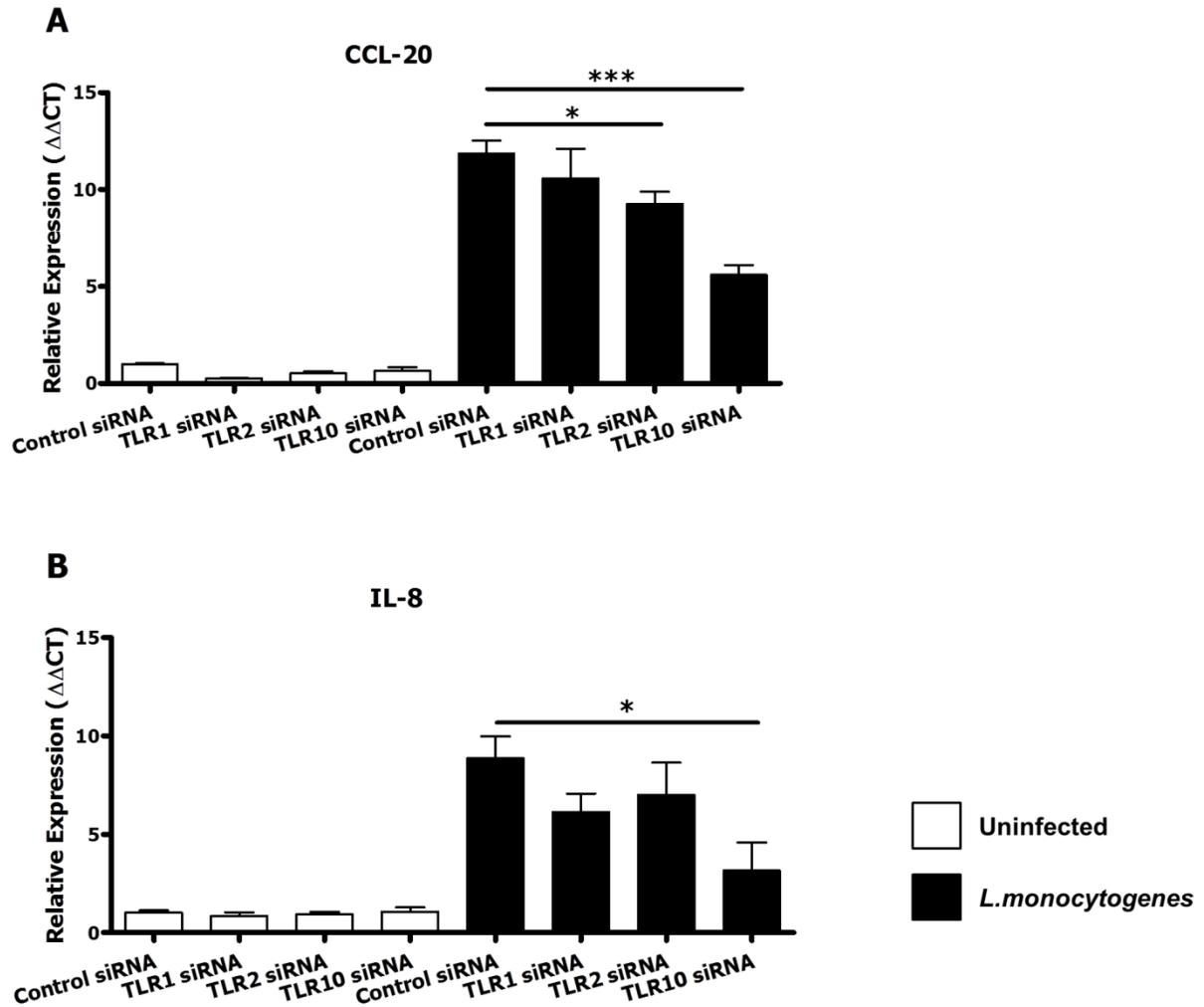


Figure 6.5 Contrast between the effects of TLR1, TLR2 or TLR10 siRNA on gene induction in HT-29 cells following infection with *L.monocytogenes*. HT-29 cells were treated with siRNA against TLR1, TLR2 or TLR10 and infected with *L.monocytogenes* for 3 hours. qRT-PCR was then used to analyse expression levels of CCL-20 (A) or IL-8 (B). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Values are shown as Mean \pm SEM, $n = 3$. Statistics were carried out using t test with Welch's correction.

Since the level of silencing for each of three TLRs 1, 2 and 10 was not quantified for Fig. 6.5, one cannot definitively claim any of the TLRs to be more involved than the other despite the result due to the inconsistency of efficacy of siRNA on their targets. Nevertheless, our studies shown in Fig. 6.2 and Fig. 6.4 validating the siRNAs used had shown roughly comparable levels of KD of all TLRs, indicating that TLR10 may be a key mediator of the

immune response of *L.monocytogenes*. Furthermore, as no direct mediation of an immune response against a specific pathogen had been identified before with TLR10, the result warranted further investigation. In addition to verifying the reduction caused by TLR10 siRNA treatment in IL-8 and CCL-20 induction following *L.monocytogenes* infection, CCL-1 induction was also measured and silencing of TLR10 mRNA expression by qRT-PCR was performed (Fig. 6.6). CCL-20 was induced 12-fold over uninfected cells in the control siRNA treated samples, compared to 6-fold in the TLR10 siRNA treated cells. IL-8 induction was also decreased significantly, from 8-fold induction in the control siRNA treated samples to 4-fold induction in the TLR10 siRNA treatment after infection with *L.monocytogenes*. This 50% reduction is comparable with the siRNA screen data also. Although a highly significant 50% reduction was observed in CCL-20 and IL-8 induction following infection in TLR10 siRNA treated samples, CCL-1 induction was less affected, only being reduced by 40% (Fig. 6.6C). As much as 60% silencing of TLR10 induction is observed in Fig. 6.6D. This effect was unchanged following infection with *L.monocytogenes*.

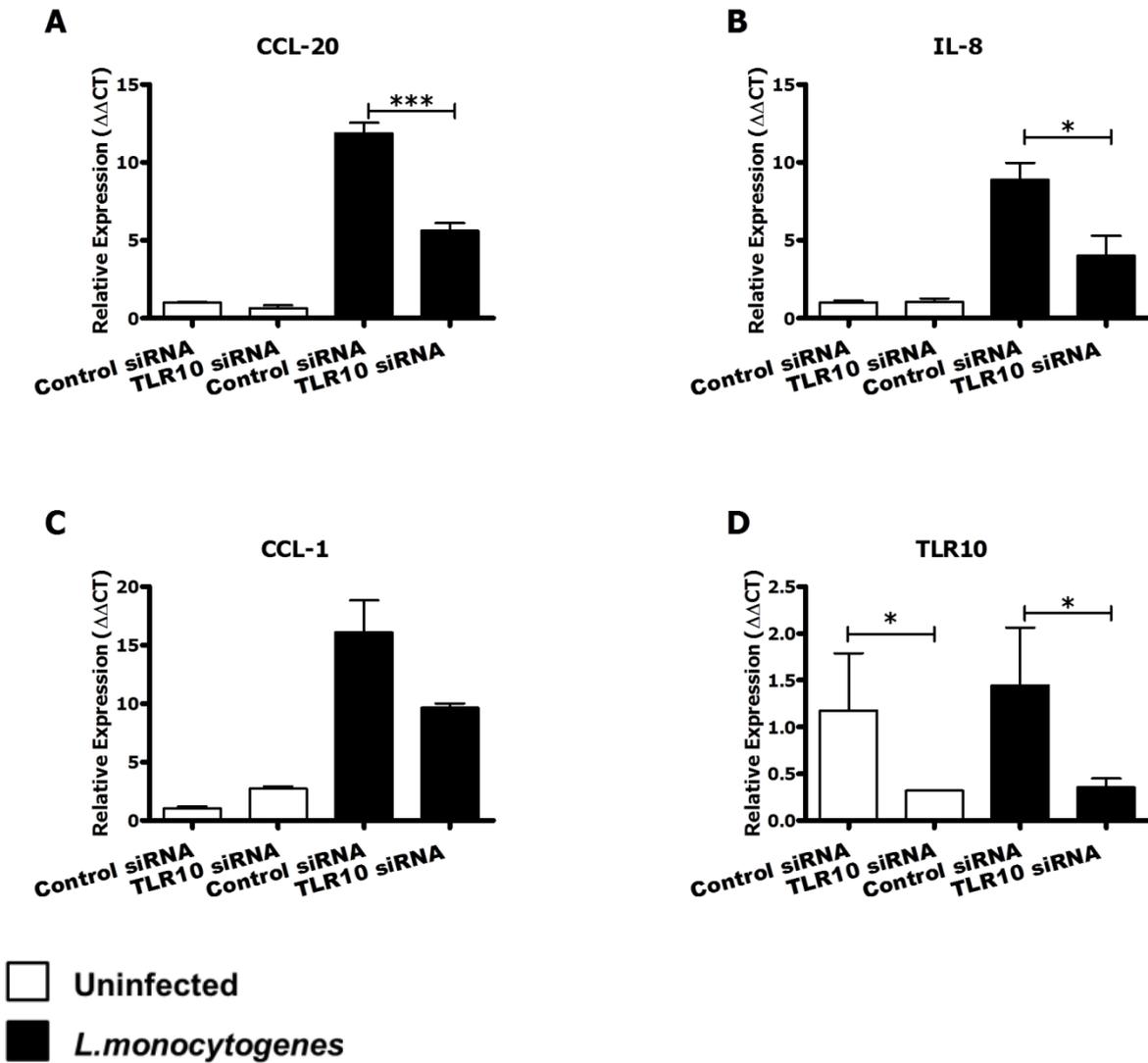


Figure 6.6 Effects of TLR10 siRNA on gene induction in HT-29 cells following infection with *L.monocytogenes* HT-29 cells were treated with TLR10 siRNA and infected with *L.monocytogenes* for 3 hours. qRT-PCR was then used to analyse expression levels of TLR10 (A), CCL-20 (B), CCL-1 (C) and IL-8 (D). * P<0.05, ** P<0.01 and *** P<0.001, Values are shown as Mean ± SEM, n=3. Statistics were carried out using t test with Welch's correction.

6.2.3 Effect of TLR10 silencing seen in both other IEC lines and Macrophages

It had initially been reported that TLR10 was exclusively expressed in B cells and lymphoid tissue [6]. However, the results from our experiments investigating the effect of TLR10 siRNA had led to detection of TLR10 mRNA in HT-29 IECs. In order to confirm that the same effect was reproducible using other IEC lines, HCA-7 cells were treated with TLR10 siRNA similar to HT-29 cell treatments and were subject to infection with *L.monocytogenes* (Fig. 6.7). Endogenous expression and silencing of TLR10 was confirmed in the HCA-7 cells by qRT-PCR (Fig. 6.7A). Levels of CCL-20, CCL-1 and IL-8 induction were similar to those results seen in HT-29 cells. TLR10 siRNA treatment reduced CCL-20 induction to a 60-fold induction over uninfected samples. CCL-1 was unaffected by TLR10 siRNA treatment, however (Fig. 6.7C), and relatively little IL-8 induction (3-fold) was recorded in response to the bacteria (Fig. 6.7D). This was, however, reduced almost 50% by TLR10 siRNA treatment. Higher levels of TLR10 mRNA were measured in HCA-7 cells compared to HT-29 cells. This may account for the higher levels of CCL-20 induction following infection; 120-fold over uninfected (Fig. 6.7B).

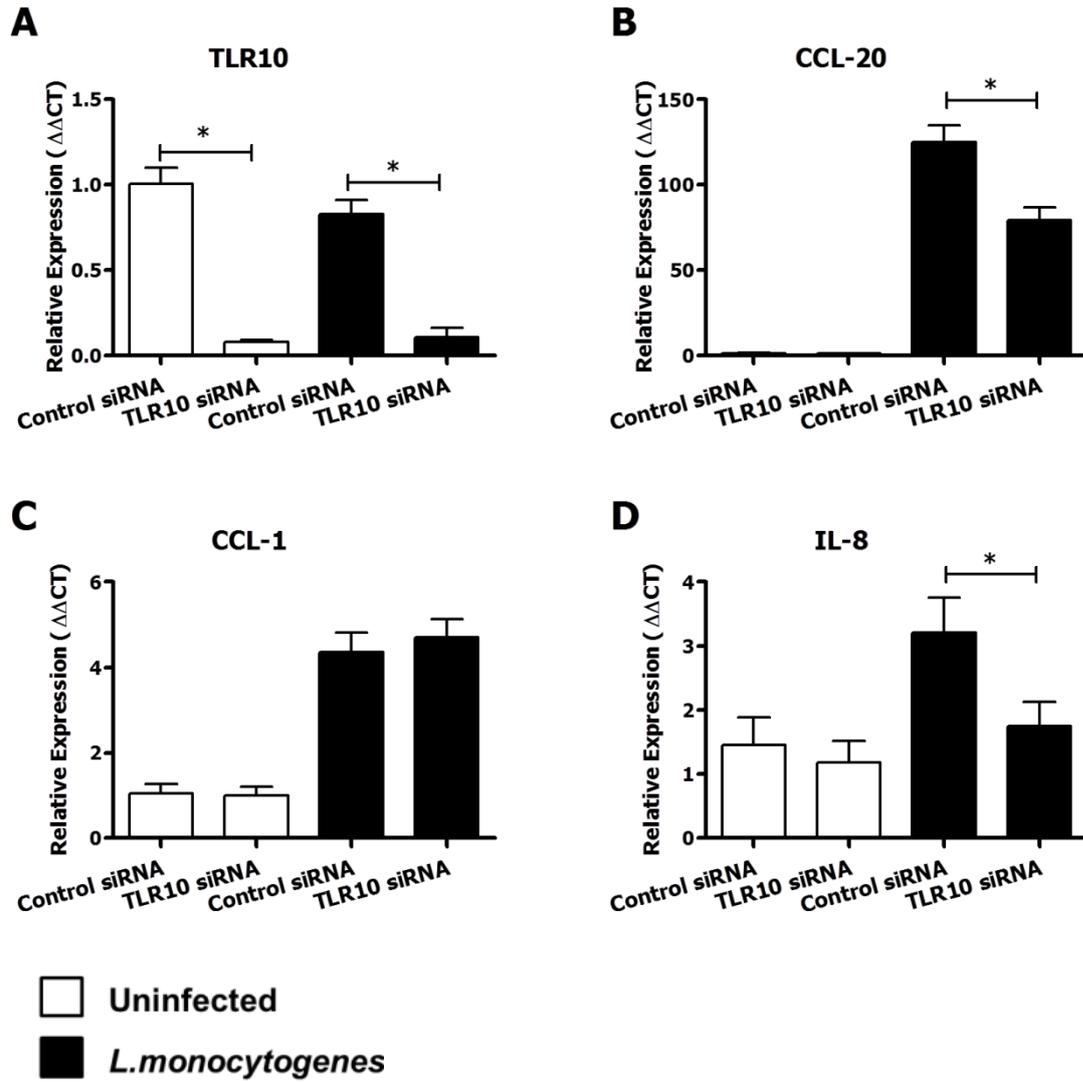


Figure 6.7 Effect of silencing TLR10 on inflammatory gene induction in HCA-7 cells. HCA-7 cells were treated with TLR10 siRNA and infected with *L.monocytogenes* for 3 hours. qRT-PCR was then used to analyse expression levels of TLR10 (A), CCL-20 (B), CCL-1 (C) and IL-8 (D). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Values are shown as Mean \pm SEM, $n=3$. Statistics were carried out using t test with Welch's correction.

All studies on the effects of TLR10 siRNA mediated silencing had until now been generated measuring mRNA levels by qRT-PCR. In order to observe this effect at a protein level, HT-29 cells were subject to TLR10 siRNA transfection and CCL-20 protein production was measured by ELISA following *L.monocytogenes* infection over a 48 hour period (Fig. 6.8A). The same was performed in the IEC line, HCT-116 cells (Fig. 6.8B). A comparable result was observed between the two cell lines, each showing a significant decrease in CCL-20 production in the TLR10 siRNA treated samples following infection.

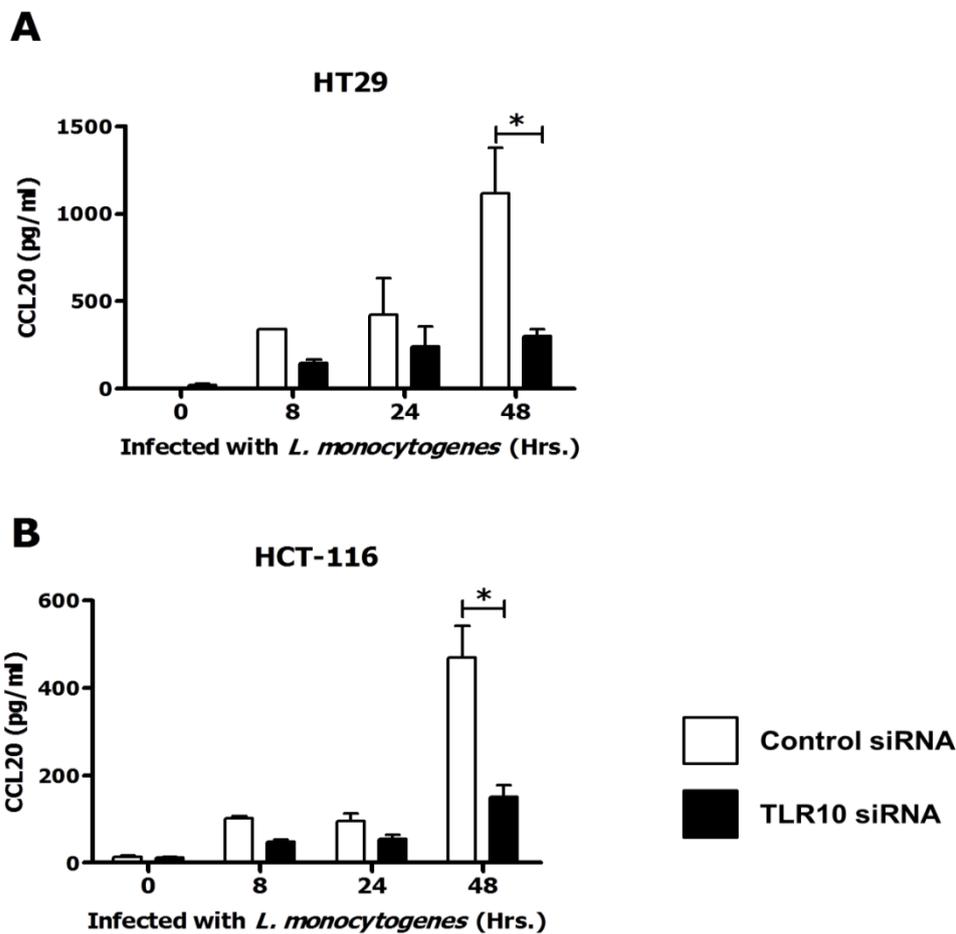


Figure 6.8 Effect of silencing TLR10 on CCL-20 production in HT-29 and HCT-116 cells. HT-29 (A) or HCT-116 (B) were treated with TLR10 siRNA and infected with *L.monocytogenes* for up to 48 hours. Supernatants were analysed for CCL-20 production by ELISA at the times indicated post-infection. * P<0.05, ** P<0.01 and *** P<0.001, Values are shown as Mean \pm SEM, n=3. Statistics were carried out using t test with Welch's correction.

Finally, in order to investigate if this effect was seen in cells other than IECs, THP-1 macrophage-like cells were treated with TLR10 siRNA and infected with *L.monocytogenes*. A dramatic decrease in CCL-20 production was observed in these cells over a 48 hour period also (Fig. 6.9).

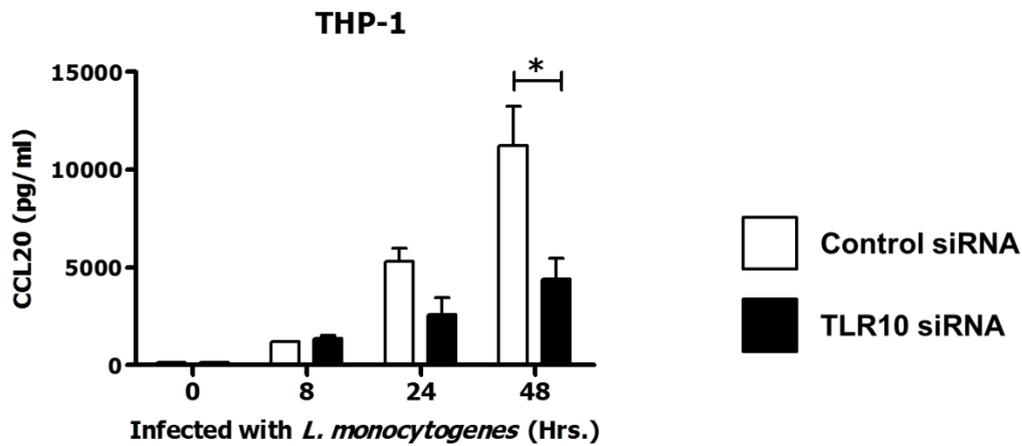


Figure 6.9 Effect of silencing TLR10 on CCL-20 production in THP-1 macrophage-like cells. THP-1 cells were treated with PMA TLR10 siRNA and infected with *L.monocytogenes* for up to 48 hours. Supernatants were analysed for CCL-20 production by ELISA at the times indicated post-infection. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Values are shown as Mean \pm SEM, $n=3$. Statistics were carried out using t test with Welch's correction.

6.2.4 TLR10 effects *L.monocytogenes* survival in IECs and macrophages

Altering expression of the PRRs NALP3 and Aim2 has been previously shown to reduce the ability of *L.monocytogenes* to survive and replicate within cells [21]. We wished to identify whether altering expression of TLR10 would have a similar effect. Survival of *L.monocytogenes* was analysed by calculating the intracellular bacterial burden in both HT-29 and THP-1 following TLR10 silencing over an 8 hour period. Increased survival of the bacteria is seen in both cell lines indicating a critical role for the receptor in antagonizing bacterial intracellular viability (Fig. 6.10A and B respectively).

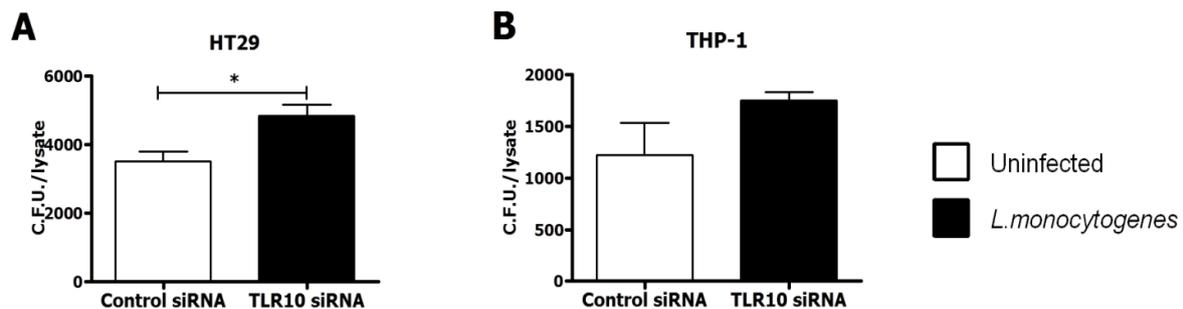


Figure 6.10 TLR10 silencing increases intracellular *L.monocytogenes* survival in HT-29 epithelial cells. HT-29 and THP1 macrophage like cells were transfected with control or TLR10 siRNA and then infected with *L.monocytogenes*,(MOI 50:1), for 8 hours before intracellular bacterial survival was then determined by plate counting (A and B). * $P < 0.05$, Values are shown as Mean \pm SEM, $n=3$. Statistics were carried out using t test with Welch's correction.

6.2.5 TLR10 requires TLR2 to mediate optimal NF- κ B activation in response to *L.monocytogenes*.

Recognition of *L.monocytogenes* by different PRRs has been shown to lead to NF- κ B-dependent pro-inflammatory gene expression, inflammasome activation and caspase-1 cleavage and IFN- β responses [22]. As both NF- κ B and IFN- β are major downstream signalling outputs of TLRs we investigated activation of these in response to overexpression of TLR constructs with or without stimulation by *L.monocytogenes*. A TLR10 construct was used for overexpression studies in either an NF- κ B reporter cell line or in conjunction with an ISRE (Interferon Stimulated Response Element) -luciferase reporter construct. As TLR10 has been shown to dimerise with TLR2 [9] a variety of combinations of TLRs1/2/10 were overexpressed in conjunction with the reporter constructs. In Fig. 6.11A it can be seen that overexpression of either TLR 1, 2 or 10 alone did not activate NF- κ B significantly above control level. Stimulation of the overexpressed constructs with *L.monocytogenes*, however, results in a twofold level of activation above control with TLR2 and significantly higher NF- κ B activation following co-transfection of TLR2 and TLR10 (5 fold above control), which was comparable to co-transfection of TLR1 and 2. This data indicates that TLR10 alone is not able to respond to *L.monocytogenes* but that together with TLR2, it facilitates optimal NF- κ B activation. ISRE activation was also measured by luciferase assay in response to *L.monocytogenes* following overexpression of combinations of TLR constructs (Fig. 6.11B). Unlike NF- κ B, no increase in the ability of any of the TLR combinations to drive an ISRE-luciferase construct was seen following *L.monocytogenes* stimulation.

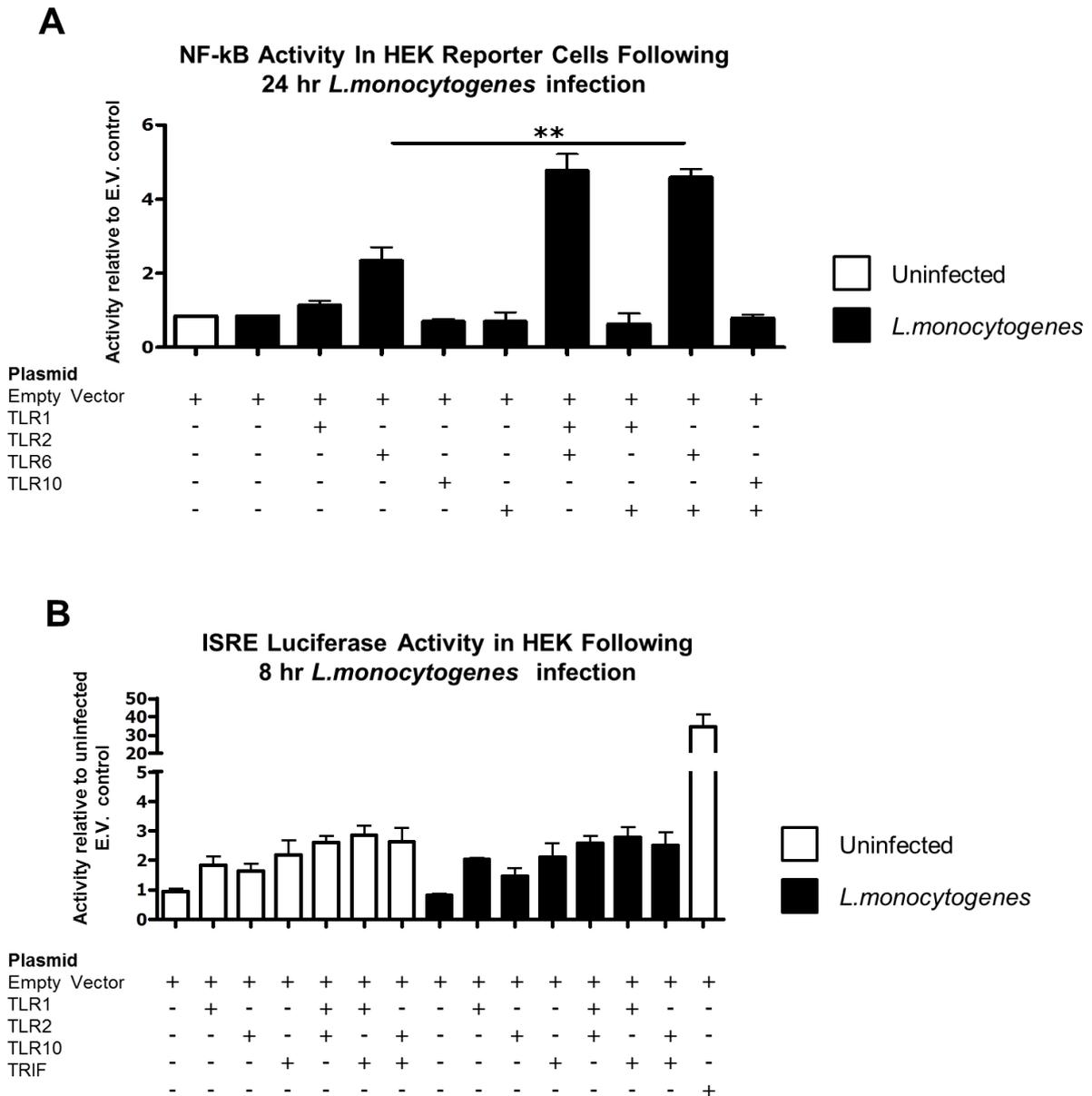


Figure 6.11 TLR10 requires TLR2 for NF-κB activation in response to *L.monocytogenes*. (A), The NF-κB SEAP reporter HEK293 cell line was transfected with the plasmid combinations indicated, and were then infected with or without *L.monocytogenes* for 8 hours. The cell free supernatants were then collected and used to quantify NF-κB activation by colorimetric QUANTI-blue™ determination (B). HEK293 cells were transfected with an ISRE-luciferase reporter, plasmid combinations indicated, and were then infected with or without *L.monocytogenes* for 8 hours. Cell extracts were then lysed and luciferase activity and corresponding luminescence was determined for ISRE activity ** P<0.01, Values are shown as Mean ± SEM, n=3. Statistics were carried out using t test with Welch's correction.

Despite the NF- κ B reporter cell line used having stably transfected CD14 and MD2, this alone was not enough to fully explain the NF- κ B activation observed in response to the bacteria when TLR2 was expressed on its own. While endogenous levels of TLR10 have been reported in data sheets pertaining to the cell line, this had never been demonstrated in a publication. Using the TLR10 plasmid construct for verification, Fig. 6.12 displays endogenous TLR10 expression in the HEK cells used (lanes A-C) with its presence confirmed by overexpression of TLR10 (lanes D-F).

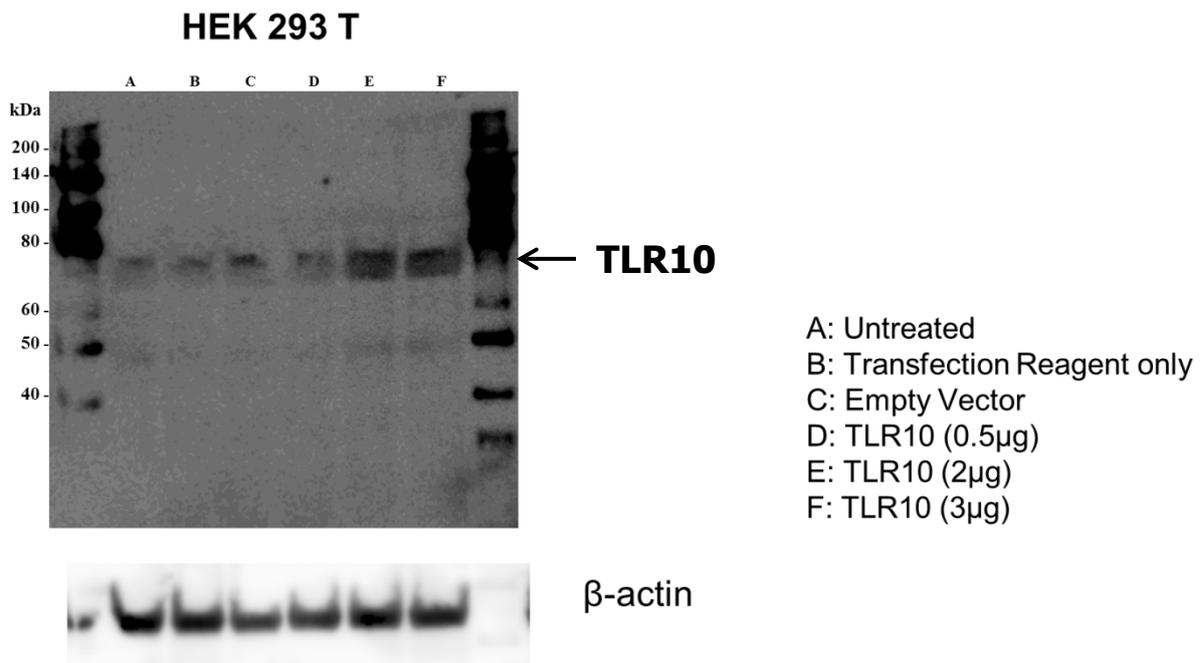


Figure 6.12 Presence of endogenous TLR10 expression in HEK 293 T cells. Lysates of HEK 293 cells were prepared for analysis by western blotting 48 hours following transfection as described in the methods section 2.19. Each lane represents the following treatment: Untreated (A), Transfection reagent only (B), Empty Vector (C), TLR10 plasmid (0.5 μ g) (D), TLR10 plasmid (2 μ g) (E), TLR10 plasmid (3 μ g) (F). In addition to TLR10 expression, β -actin expression was also examined to ensure equal levels of protein were loaded on the gel.

6.2.6 Intracellular expression of TLR10 protein in IECs

Literature concerning the subcellular location of TLR10 reported it to be predominantly based on the plasma membrane. However, it had also been reported that TLR10 expression was not present in IECs and due to the fact that we had discovered TLR10 to be present in IECs, we wished to also investigate its location in HT-29 cells. By use of intracellular and extracellular flow cytometry staining, it would appear that intracellular TLR10 expression is much higher than its expression on the plasma membrane. This is demonstrated in Fig. 6.13 with a difference of 0.3% staining by extracellular staining (Fig. 6.13A) compared to 14.7% staining observed by intracellular staining (Fig. 6.13B). Using heat-killed *L.monocytogenes* (HKLM), and live *Listeria*, HT-29 cells were subject to TLR10 siRNA treatment and stimulated with the bacteria. qRT-PCR analysis revealed that CCL-20 induction was reduced by 75% by TLR10 siRNA treatment from 100- to 25-fold over uninfected cells (Fig. 6.13C) in response to *L.monocytogenes* infection. HKLM, however, which is unable to invade the HT-29 cells, failed to induce any major increase in CCL-20 induction over uninfected cells. A similar result was observed in IL-8 induction (Fig. 6.13D). A reduction of over 50% in IL-8 induction was observed in response to live bacteria following TLR10 siRNA treatment but the HKLM once again failed to induce IL-8. Taken together these data imply that intracellular TLR10 might be responsible for detecting the invasive *L.monocytogenes*.

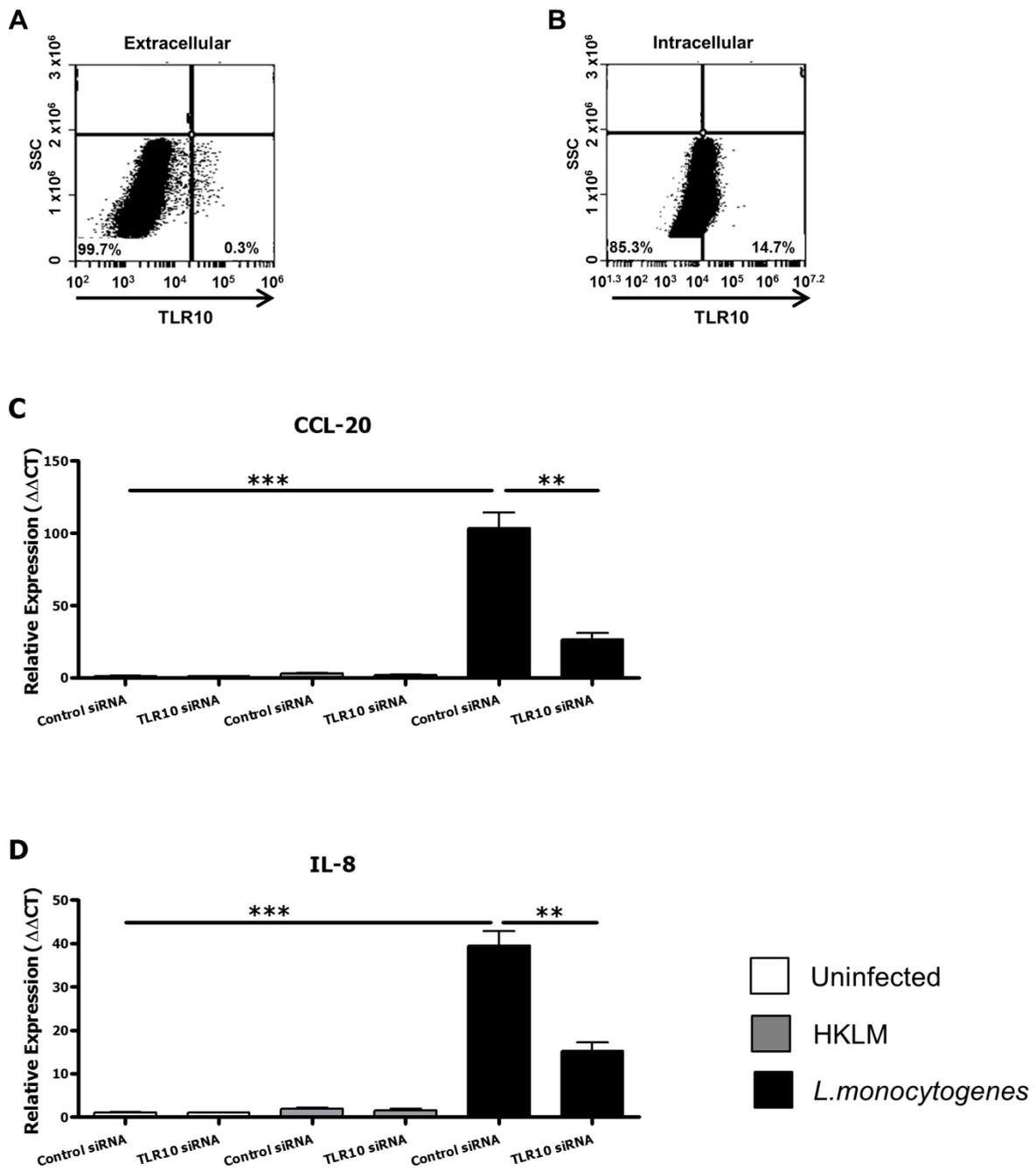


Figure 6.13 TLR10 is expressed intracellularly in HT-29 cells. Flow cytometry was used to analyse expression levels of TLR10 in HT-29 cells extracellularly (A) or intracellularly (B). Following transfection with TLR10 siRNA, HT-29 cells were subject to 3 hour stimulation with either live or heat-killed *L.monocytogenes* (HKLM). qRT-PCR was used to analyse cells for expression levels of CCL-20 (C) or IL-8 (D). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Values are shown as Mean \pm SEM, $n = 3$. Statistics were carried out using t test with Welch's correction.

6.2.7 TLR10 is not involved in recognition of other known TLR2 ligands but is involved in detection of another motile intracellular pathogen, *Salmonella typhimurium*.

We had observed a comparable result between the TLR1/TLR2 dimer and the TLR2/TLR10 dimer in NF- κ B activity following *Listeria* infection when these TLRs were transfected in together. Since TLR10 is most closely related to the more extensively researched TLR1 [9], we wished to compare the TLR1 versus TLR10 mediated response to some known TLR-ligands. Using THP-1 macrophages, IL-8 production was measured in response to a range of TLR2 ligands following siRNA treatment against TLR1 or TLR10 (Fig. 6.14). We also investigated the response to another intracellular pathogen, *Salmonella typhimurium*, in addition to *L.monocytogenes*, based on the intracellular location of TLR10 expression outlined in Fig. 6.13. While a significant reduction in IL-8 production was measured following TLR1 siRNA treatment in response to *L.monocytogenes* infection, this reduction was not as significant as that seen in TLR10 siRNA treated cells. Silencing of TLR1 and TLR10, however, resulted in a similarly significant reduction in IL-8 production in response to *Salmonella* infection. Besides the significant reduction of IL-8 production seen in response to Pam3Cys following TLR1 siRNA treatment, no further statistically significant changes in IL-8 were observed in response to the other TLR2 ligands; Peptidoglycan (PGN), Lipoteichoic Acid (LTA) or fibroblast stimulating ligand-1 (FSL-1), nor by the positive control, LPS.

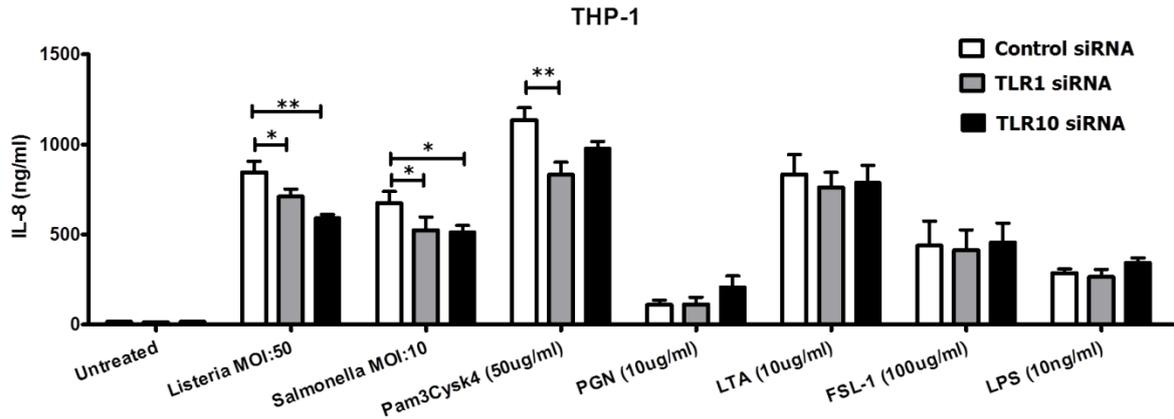


Figure 6.14 Effects of TLR1 versus TLR10 siRNA treatment on IL-8 production in THP-1-macrophages following 24 hour stimulation with TLR2 ligands. THP-1 cells were treated with either non-targeting control, TLR1 or TLR10 siRNA with 5ng/ml PMA for 48 hours. The cells were then subject to stimulation with the ligands indicated. Bacterial infections were performed as described in the methods section 2.14. 24 hours after stimulation, supernatants were analysed by ELISA for IL-8 production. * P<0.05, ** P<0.01 and *** P<0.001, Values are shown as Mean \pm SEM, n=3. Statistics were carried out using t test with Welch's correction.

6.2.8 Hypoxic Inducible Factor-1 α (HIF-1 α) stabilisation increases TLR10 expression and function in intestinal epithelia and macrophages

Hypoxia has been shown to increase bactericidal activity in phagocytes in addition to increasing TLR10 expression levels in THP-1 monocytes [23, 24]. Additionally, the hypoxic gene, HIF-1 α , has been shown to play a role in increasing the barrier function of intestinal epithelial cells, protecting them from an over-active inflammatory response [25]. We wished to investigate the effects on TLR10 expression and function in HT-29 IECs and THP-1 macrophage-like cells when treated with a substance known as Dimethylxaloylglycine (DMOG). DMOG simulates hypoxic conditions by stabilising HIF-1 α expression in cells which were not in a hypoxic environment – simulating the effects of hypoxia within the cell. While TLR10 expression was shown to increase during hypoxia in phagocytic cells such as

THP-1 cells, we hypothesised that a hypoxic environment may induce regulatory features in IECs, such as decreased expression of TLR10 or decreased inflammatory responses following its activation. Thus, the effect of stabilising HIF-1 α on TLR10 induction through the use of DMOG was investigated in HT-29 IECs (Fig. 6.15) and also in THP-1 macrophage-like cells as a positive control (Fig. 6.16). Additionally, the TLR10-mediated inflammatory response was measured in untreated and DMOG treated cells following a 3 hour infection with the TLR10 activating pathogens *L.monocytogenes* or *S.typhimurium*.

The induction of a target gene of HIF-1 α known as Vascular Endothelial Growth Factor (VEGF) was used to confirm the stabilisation of HIF-1 α in DMOG treated cells. In Fig. 6.54A, VEGF expression was increased by 4.5-fold in the DMOG treated cells over the untreated controls. DMOG treatment also increased TLR1 induction levels 1.5-fold (Fig. 6.15 B) and TLR10 was induced 45-fold over untreated controls by DMOG treatment (Fig. 6.15 C). Although TLR2 induction was measured, it was not detectable by qRT-PCR in these cells. CCL-20 induction was decreased from 800-fold in the untreated samples to 50-fold induction in the DMOG-treated samples over uninfected controls in response to *L.monocytogenes* (Fig. 6.15D). Similarly, infection with *S.typhimurium* resulted in a 1,100-fold in the untreated cells compared to a 600-fold induction in the DMOG treated samples. Conversely however, IL-8 induction was found to be increased from 7-fold to 11-fold induction in response to *L.monocytogenes* following DMOG treatment, although this increase was not statistically significant (Fig. 6.15E). Infection with *S.typhimurium* resulted in a statistically significant increase from 6-fold to 11-fold induction in the DMOG treated samples over the uninfected control. Taken together, these data suggested a differential effect of HIF-1 α on the inflammatory response in IEC.

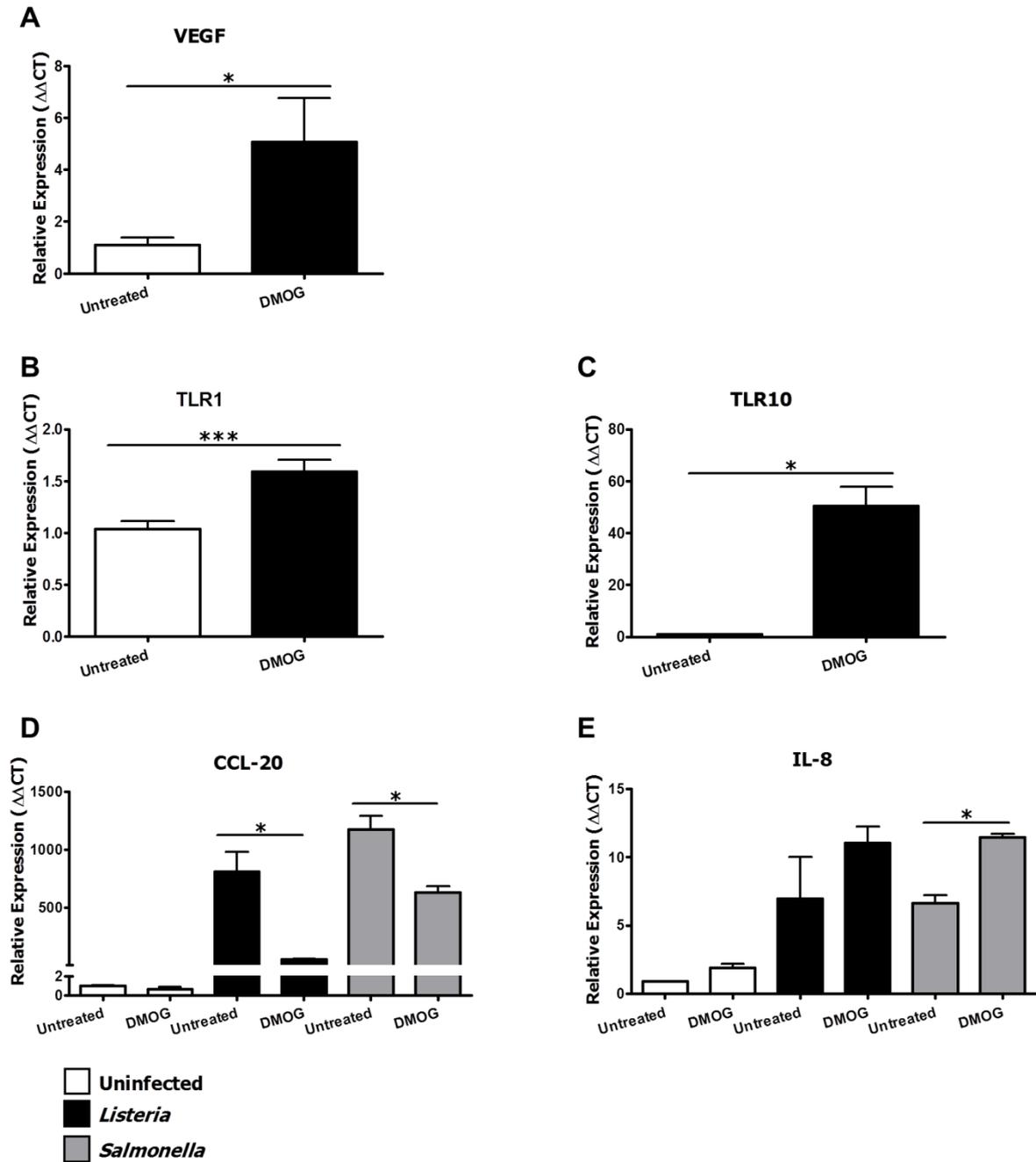


Figure 6.15 Effects of HIF-1 α induction on TLR expression and inflammatory response in HT-29 cells. HT-29 cells were subject to 500 μ M DMOG treatment for 24 hours prior to infection with *L.monocytogenes* MOI 50 or *S.typhimurium* MOI 10 for 3 hours. qRT-PCR was used to analyse cells for expression levels of VEGF (A), TLR1 (B), TLR10 (C), CCL-20 (D) or IL-8 (E). * P<0.05, ** P<0.01 and *** P<0.001, Values are shown as Mean \pm SEM, n=3. Statistics were carried out using t test with Welch's correction.

We subsequently investigated the effects of DMOG on THP-1 macrophage-like cells. Fig. 6.16A displays a 10-fold increase in VEGF induction in DMOG treated samples. DMOG treatment increased the expression of TLR1 3 –fold (Fig. 6.16B), TLR2 4–fold (Fig. 6.16C) and resulted in a 12 –fold increase in TLR10 induction (Fig. 6.16D). In correlation with this, IL-1 β induction was induced 3000-fold compared to the uninfected controls in untreated cells following infection with *L.monocytogenes* compared to a 9000-fold induction in DMOG treated cells in response to the bacteria (Fig. 6.16E). Similarly following infection with *S.typhimurium*, IL-1 β induction increased from 2000-fold in the untreated cells to 13000-fold induction in the DMOG treated samples over the uninfected controls. IL-8 followed a similar pattern with a 1000-fold induction over uninfected cells observed following infection with *L.monocytogenes* compared to a 13000-fold induction in DMOG treated samples (Fig. 6.16F). Infection with *Salmonella* resulted in a 500-fold increase in IL-8 induction in the untreated samples and an 8000-fold induction over uninfected controls seen in DMOG treated samples.

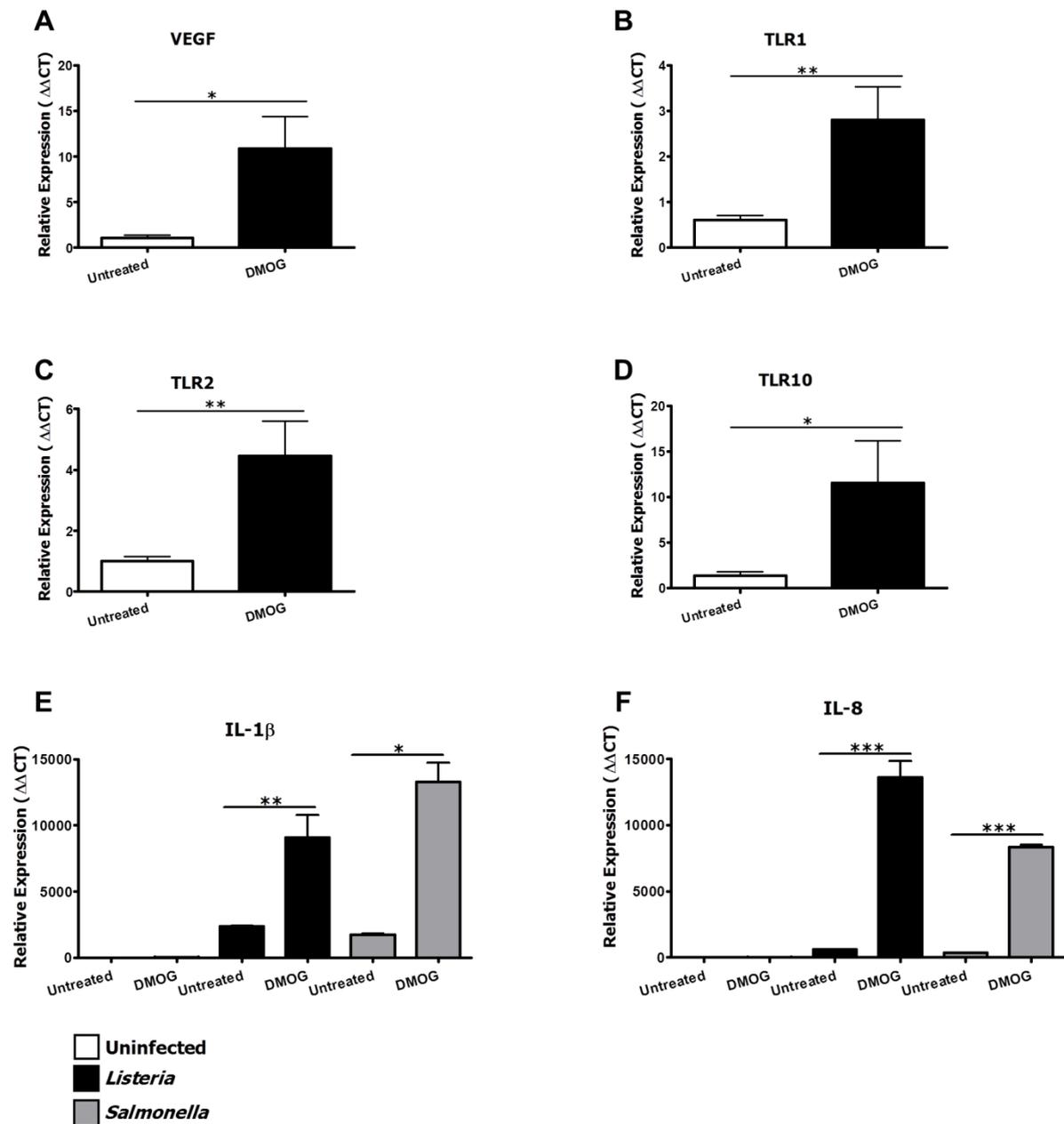


Figure 6.16 Effects of HIF-1 α induction on TLR expression and inflammatory response in THP-1 macrophage-like cells. THP-1 cells were differentiated into macrophage-like cells as described in the methods section 2.1 by PMA treatment. Following this, they were subject to 500 μ M DMOG treatment for 24 hours prior to infection with *L.monocytogenes* MOI 50 or *S.typhimurium* MOI 10 for 3 hours. qRT-PCR was used to analyse cells for expression levels of VEGF (A), TLR1 (B), TLR2 (C), TLR10 (D), IL-1 β (E) or IL-8 (F). * P<0.05, ** P<0.01 and *** P<0.001, Values are shown as Mean \pm SEM, n=3. Statistics were carried out using t test with Welch's correction.

6.2.9 Attempt to generate a stable TLR10 knockdown HT-29 cell line by use of shRNA lentiviral particle transfection

For reasons of practicality while studying TLR10 function in IECs, I attempted to create a stable knockdown of TLR10 in HT-29 cells. In addition to reduced cost and resources, this would also avoid the inconsistencies of siRNA transfection and the level of silencing achieved from each assay. Benefits of creating such a cell line would allow the effects of TLR10's absence to be more easily distinguished in comparative studies between this cell subset, the untreated parental HT-29 cells and over-expression studies using a TLR10 plasmid construct.

Following transfection with either scrambled control, or TLR10 shRNA lentiviral particles as described in the methods section 2.18, HT-29 cells were treated with a selective antibiotic pressure using puromycin. The lentiviral particles carried resistance to this antibiotic, thus ensuring the removal of any non-transfected cells. After three more passages, cells were seeded at a very low density. This allowed for individual cell colonies to develop. A week after allowing these clones to develop, 24 individual colonies were selected. Initially the cells' responsiveness to *L.monocytogenes* infection was measured – given that based on my earlier work, a phenotype of silencing TLR10 in HT-29 cells should be a significantly reduced responsiveness to *L.monocytogenes* infection. 17 of the healthiest looking TLR10 shRNA treated clones were selected together with a scrambled shRNA treated control clone and 2 clones of the parental HT-29 cells. Cells were then seeded and subjected to a 24 hour incubation with *L.monocytogenes* as described in the methods 2.14. Supernatants were analysed for CCL-20 production in response to the bacteria (Fig. 6.17). None of the uninfected samples from any clone produced more than 100 pg/ml of CCL-20 (not shown). In contrast, the parental cells produced an average of 600 pg/ml CCL-20 following infection.

Several of the TLR10 shRNA treated clones produced CCL-20 levels in response to *L.monocytogenes* comparable with CCL-20 production seen in the uninfected samples. Clones S2, L1, L5 and L7 produced less than 100 pg/ml CCL-20 in response to the bacteria; hence these clones were selected for further analysis.

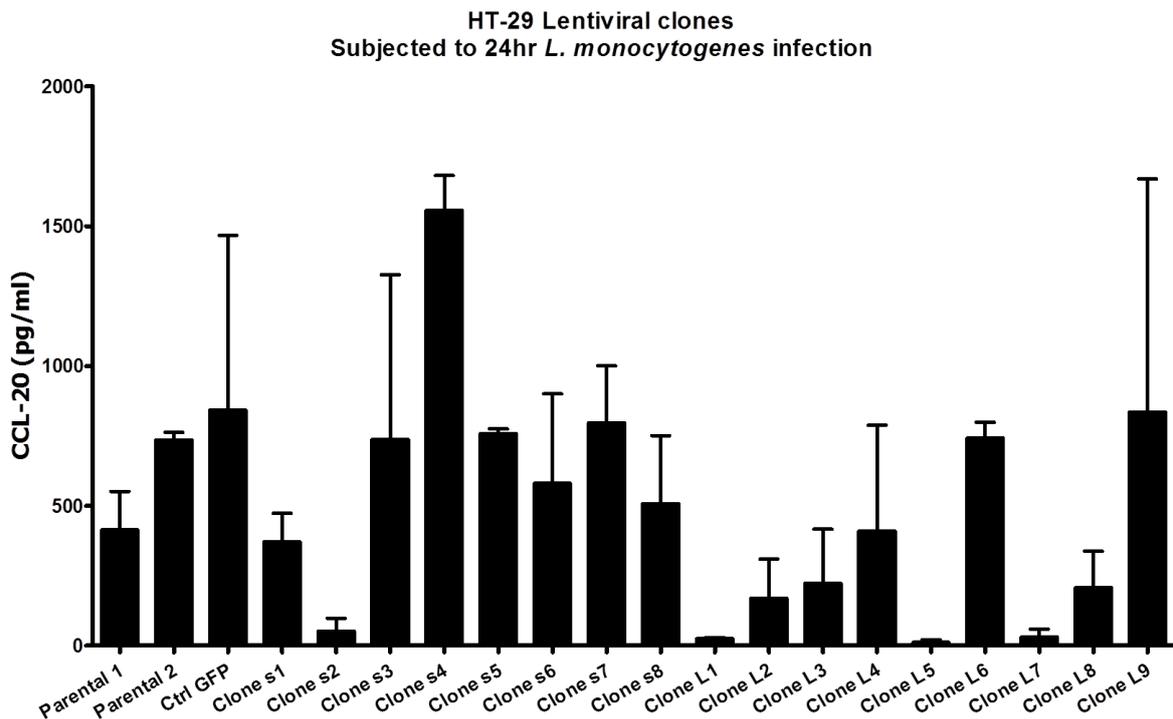


Figure 6.17 CCL-20 production by HT-29 lentiviral clones in response to *L.monocytogenes* infection. Following lentiviral transfection and clonal selection, HT-29 clones were seeded on a 96 well plate and subject to a 24 hour *L.monocytogenes* infection. Supernatants were then analysed for CCL-20 production. Statistics were carried out using t test with Welch’s correction.

To further analyse the phenotypes of TLR10 silencing in the selected clones from Fig. 6.17, qRT-PCR analysis was performed following infection with *L.monocytogenes*. Figure 6.18A displays CCL-20 induction in response to infection with *L.monocytogenes* from the TLR10 shRNA clones S2, L1, L5 and L7 in conjunction with the parental and control clones.

The most visible reduction in CCL-20 production when compared to the parental or control lentiviral clones was observed in TLR10 shRNA clones L5 and L7. These clones were further analysed once again for CCL-20 production in response to *L.monocytogenes* infection (Fig. 6.18B). The most significant reduction in CCL-20 induction when compared to the parental and control shRNA clones was observed in the L5 clone. The L5 clone was selected for further study and this cell line was now referred to as TLR10 Knock Down (KD).

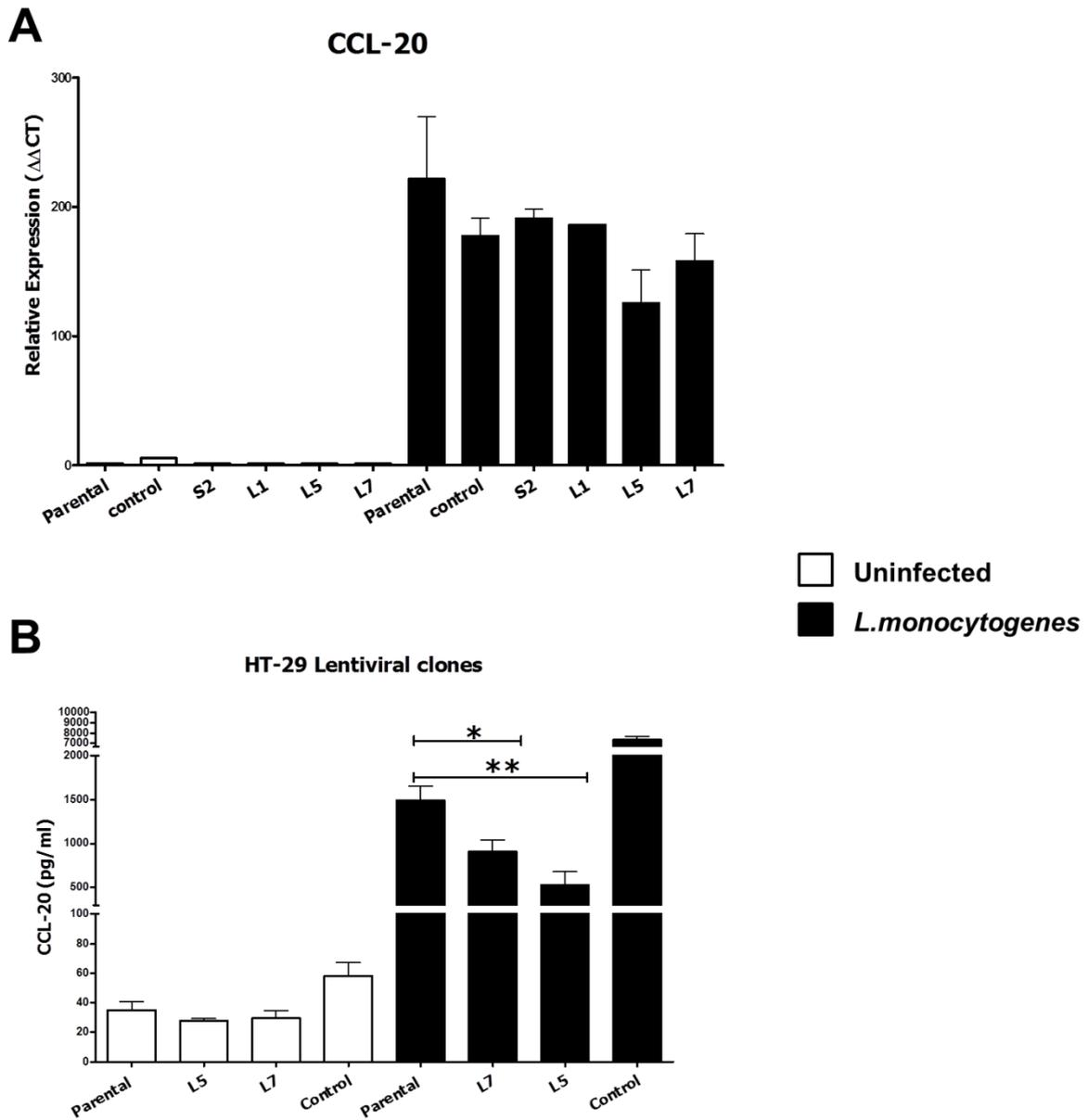


Figure 6.18 CCL-20 induced by lentiviral clones in response to *L.monocytogenes* infection. HT-29 lentiviral clones indicated were seeded in a 12-well plate and were subject to infection with *L.monocytogenes* for 3 hours. Relative CCL-20 induction was then measured by qRT-PCR (B). Selected HT-29 clones were seeded on a 96-well plate and infected with *L.monocytogenes* for 24 hours. Supernatants were then analysed for CCL-20 production (C). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Values are shown as Mean \pm SEM, $n = 3$. Statistics were carried out using t test with Welch's correction.

In order to confirm silencing of TLR10 protein expression in the selected TLR10 KD clone, L5 and lentiviral clone L7, further analysis by Western blotting was performed. The left hand panel of Figure 6.19 displays a TLR10 Western blot analysis of parental and control clones in addition to the TLR10 shRNA clones and L7, indicated in lanes A-D. β -actin expression on the right hand panel verified equal loading of the lysates. The Western blot revealed that the TLR10 KD L5 clone expressed almost no detectable TLR10. The L7 clone, however, expressed TLR10 levels more similar to that of the parental.

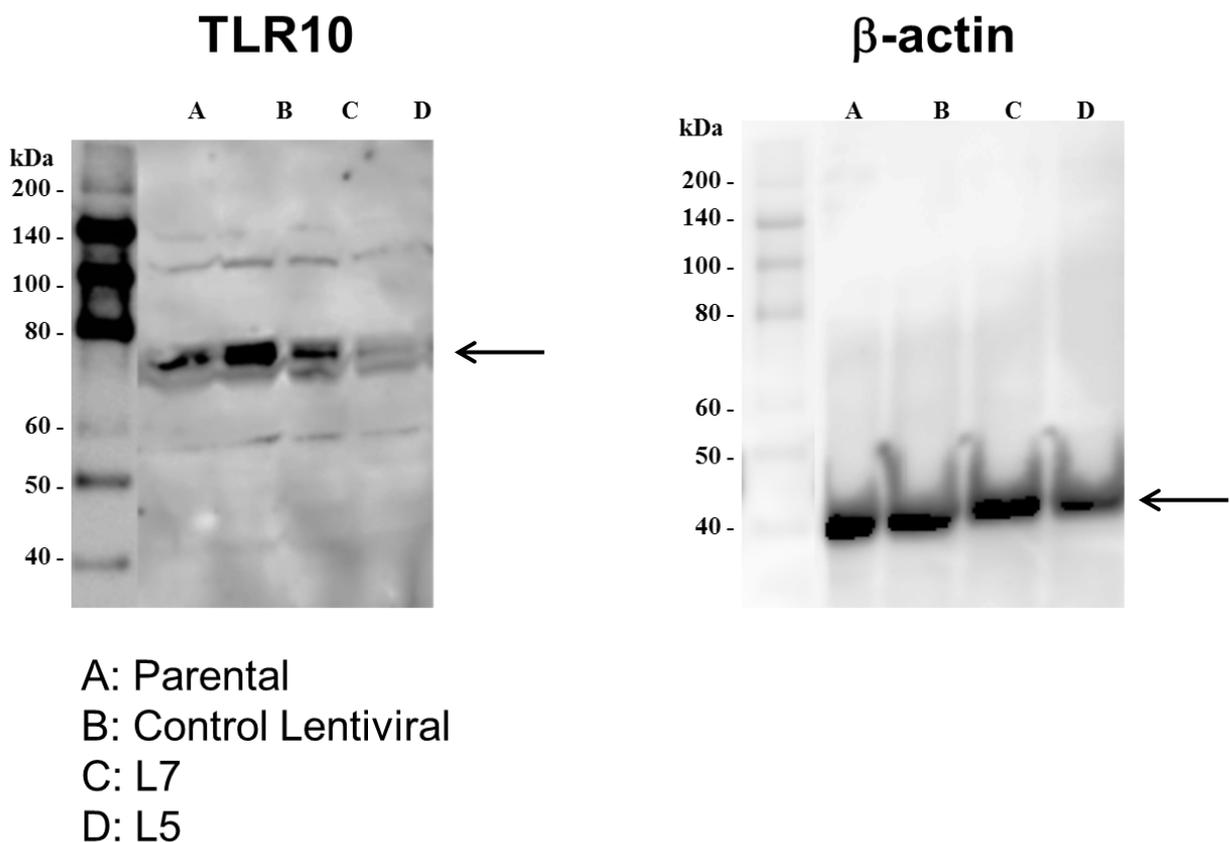


Figure 6.19 TLR10 protein expression in HT-29 TLR10 KD lentiviral clones. Lysates of Parental, Control shRNA and TLR10 shRNA HT-29 lentiviral clones (L5 and L7) were prepared and analysed by Western blot for TLR10 expression (left-hand panel). Housekeeper gene, β -actin, levels were also measured to ensure equal amounts of protein were loaded in each lane (right-hand panel).

The TLR10 KD (L5), Parental and Control lentiviral clones were further analysed for CCL-20 and IL-8 mRNA induction in response to *L.monocytogenes* infection in Fig. 6.20. The control and parental clones yielded a robust response to the bacteria; 130- and 170-fold induction of CCL-20 and IL-8 was increased to 40 and 55-fold over uninfected cells in the parental and control samples respectively. Both CCL-20 (A) and IL-8 levels (B) were significantly reduced by over 50% in the TLR10 KD clone relative to levels induced by the control and parental HT-29 clones in response to infection with *L.monocytogenes*.

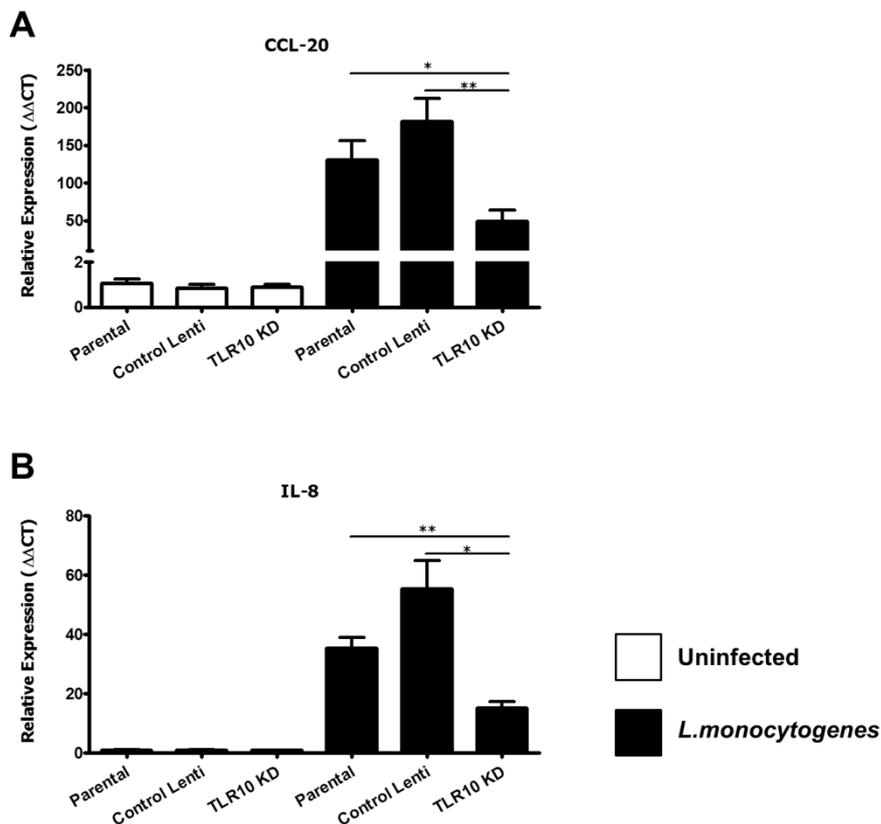


Figure 6.20 Inflammatory response to *L.monocytogenes* infection in HT-29 lentiviral clones. Selected HT-29 lentiviral clones were seeded overnight and subject to infection with *L.monocytogenes* the following day for 3 hours. qRT-PCR was used to analyse relative mRNA expression levels of CCL-20 (A) and IL-8 (B). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Values are shown as Mean \pm SEM, $n=3$. Statistics were carried out using t test with Welch's correction.

Frozen stocks were made of the TLR10 KD (L5 clone). Upon thawing these stocks to grow more cells, an inflammatory response to *L.monocytogenes* infection was examined (Fig. 6.21). The control and parental clones responded to the infection similarly to the response seen in Fig. 6.19 with a 120-fold increase in CCL-20 and 40-fold increase in IL-8 mRNA induction over uninfected controls. The TLR10KD clones, however, responded with similar levels of induction, with no statistical difference between any of the 3 clone types.

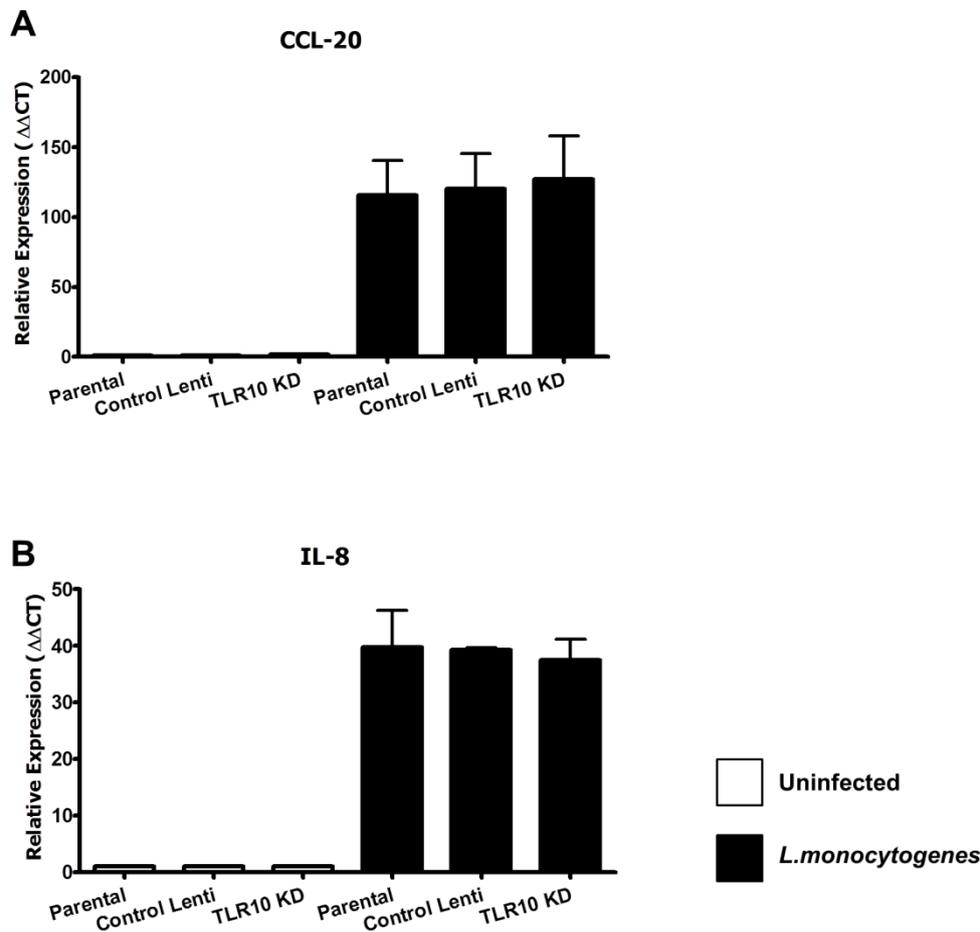


Figure 6.21 Inflammatory response to *L.monocytogenes* infection in HT-29 lentiviral clones. Selected HT-29 lentiviral clones were seeded overnight and subject to infection with *L.monocytogenes* the following day for 3 hours. qRT-PCR was used to analyse relative mRNA expression levels of CCL-20 (A) and IL-8 (B). * P<0.05, ** P<0.01 and *** P<0.001, Values are shown as Mean ± SEM, n=3. Statistics were carried out using t test with Welch’s correction.

This was a surprising result, since reduced TLR10 expression should have resulted in a reduced inflammatory response from HT-29 cells to *Listeria* infection. I next investigated by Western blot the TLR10 expression levels in each of the 3 clone types; parental, control and TLR10 KD (Fig. 6.22). The parental and control clones display similar levels of TLR10 protein in each lysate (Fig. 6.22 lanes A, B). The TLR10 KD (L5) clones displayed slightly lower levels of TLR10 expression (lane C) than either of the other 2 clones. However, this difference in expression levels is not comparable to that seen in Fig. 6.19. Taken with the results from Fig. 6.21, we concluded that the TLR10 KD (L5) clone had regained TLR10 expression levels upon growing them back from frozen stocks.

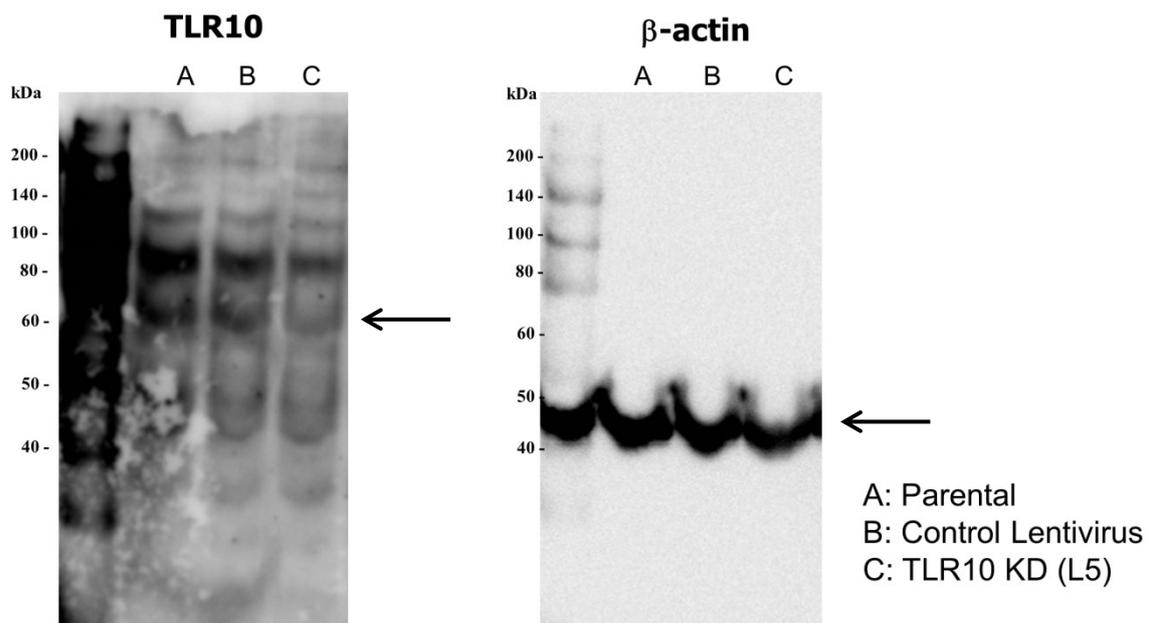


Figure 6.22 TLR10 expression in HT-29 lentiviral clones. Lysates of Parental, Control shRNA and TLR10 shRNA HT-29 lentiviral clones were prepared and analysed by Western blot for TLR10 expression (left-hand panel). Housekeeper gene, β -actin, levels were also measured to ensure equal amounts of protein were loaded in each lane (right-hand panel).

6.3 Discussion

Here, for the first time, we have shown recognition of a pathogen by TLR10. Additionally, we have demonstrated that in addition to macrophages, TLR10 plays a functional role in intestinal epithelial cells. In humans, TLR10 expression has been reported to be somewhat restricted. It was initially characterised to be primarily expressed on immune cell subtypes with a predominance found in spleen, lymph nodes, thymus and tonsils [4]. It is worth noting that in this study by Chuang *et al*, expression of TLR10 in colon tissue was not investigated. However, a study by Otte *et al*. in 2004 reported no detectable expression of TLR10 in intestinal epithelia [26]. Problems with detection have been encountered in the past, for example, its mRNA had been detected in gingival epithelial cell cultures by qRT-PCR, but the same group and others failed to detect its presence in tissue samples immunohistochemically [27, 28]. Furthermore, unlike TLR1 and TLR6, TLR10 is expressed in a highly restricted fashion as a highly N-glycosylated protein [6]. This heavy glycosylation may account for some of the difficulty encountered in detecting its presence in the past. The development of better detection methods in recent years has allowed us to more accurately explore the extent of its expression in human cells. More recently, two separate groups have reported its expression in human monocytes and the monocytic THP-1 cell line [4, 23]. Its presence has also been detected in human PBMCs, trophoblasts, airway epithelium, renal cells in addition to the SW480 colonic adeno-carcinoma cell line [26, 29-32]. Human regulatory T cells (Treg cells) have also been shown to express TLR10, and this expression is believed to be regulated through a cooperative complex of immune-regulating transcription factors Forkhead box P3 (FOXP3) [33]. Following results from our study, the list of cell types that express TLR10 can now be extended to include colonic adeno-carcinoma HT-29 and HCA-7 intestinal epithelial cell lines.

To date, no specific ligands for TLR10 have been shown, nor has TLR10 activation been shown outside of this study to elicit an inflammatory immune response. Recently, TLR10 has been indicated to be involved in Peptidoglycan (PGN) mediated TLR signalling [30]. This study demonstrated using a dominant negative TLR10 that trophoblast TLR10 plays a role in promoting apoptosis triggered by gram-positive bacterial components and suggests that TLR10 may play a role in determining trophoblast survival and cell death, but not in any inflammatory response [30]. A possible involvement of TLR10 has been implicated in response to specific pathogens, such as malaria *Plasmodium falciparum* [34] and a study on TLR10 polymorphism with asthmatic patients suggested that TLR10 may be involved in the recognition of airborne pathogens or airborne allergens [15]. Until my own studies however, TLR10 had not been linked with inducing any innate immune activation in response to purified ligands or pathogens. In fact, several groups have suggested an immune-regulatory role for TLR10 in preventing an over-active inflammatory response [9, 30, 33]. We have since demonstrated TLR10 to be involved in eliciting an immune response to at least two different intracellular pathogens.

Previous studies suggested that similar to TLR1 and TLR6, TLR10 signals with TLR2 but does not activate NF- κ B following stimulation [9]. Since we had reported an inflammatory immune response from NF- κ B activated genes such as IL-8 and CCL-20, we wished to test this hypothesis regarding the requirement for TLR2 to mediate TLR10 signalling using an NF- κ B reporter cell line. These HEK reporter cells also stably expressed the adaptor molecules CD14 and MD-2, both believed to be involved in TLR signalling [35, 36]. Although NF- κ B activation was not recorded when TLR10 was transfected alone or with TLR1 or TLR6, we confirmed NF- κ B activation in response to *L.monocytogenes* after transfecting TLR10 with TLR2. This level of activation appeared to be similar to that which was observed when the TLR1 and TLR2 receptors were transfected together. This data

suggested that TLR10 could mediate an NF- κ B response following infection with *L.monocytogenes* but, similar to TLR1 and TLR6, required TLR2 for this activation. Interestingly, NF- κ B activation was observed following infection with *L.monocytogenes* when TLR2 was transfected alone. Further analysis on the HEK cells through western blotting revealed an endogenous expression of TLR10. While this data was previously unpublished, it was reported in the data sheets from several companies pertaining to the HEK cells they provide. HEK 293 cells are not reported to express endogenous levels of any other TLRs besides small levels of TLR3 and TLR5 [37] so this endogenous TLR10 expression may account for the NF- κ B activation observed in response to *L.monocytogenes* when TLR2 is transfected alone. The results from this study also highlight the advantages of using siRNA techniques instead of over-expression studies using plasmids; while one may over-express a protein of interest in cells such as HEKs, it is impossible to know every adaptor molecule which this protein may require interaction with in order to fully mediate signalling, and these adaptor molecules might be absent in HEKs. In this manner, observing the effects in a cell type when a particular gene is switched off rules out this potential problem. In our case, it may be that the CD14 receptor is required to fully mediate NF- κ B activation by the TLR2/TLR10 signalling dimer since previous studies reported that this signalling dimer was unable to activate NF- κ B in response to ligand activation [9].

In addition to sharing strong homology with one another, TLR1 and TLR10 have also been hypothesised to detect similar ligands [8, 9]. In our own studies, we observed similar levels of NF- κ B activation observed in response to *L.monocytogenes* infection between the TLR1/2 and TLR2/10 signalling dimers. The direct effects of TLR1 versus TLR10 mediated signalling were investigated in this study using various TLR2 ligands. Using Pam3Cysk4, the well characterised TLR1/TLR2 ligand, also suggested as a potential ligand for TLR10 [8] we indeed observed a decrease in IL-8 production following TLR10 siRNA treatment, although

this decrease was not statistically significant, as it was in the case of TLR1 siRNA treated cells. This suggests that, contrary to predictions [8], TLR10 does not sense Pam3Cysk4 but may be involved in sensing structurally similar ligands. Additionally, IL-8 production in response to the TLR2 ligand PGN was higher in the TLR10 siRNA treated samples although TLR1 siRNA treatment yielded no significant effects. While this increase was also not found to be statistically significant, it is worth noting since the only instance in which TLR10 was directly linked to PGN mediated-signalling, mentioned earlier, implicated an immunoregulatory role for TLR10 in trophoblasts [30]. While a similarly significant decrease in IL-8 production was reported in response to infection with the intracellular gram negative pathogen *S.typhimurium* following siRNA treatment against TLR1 and TLR10, TLR10 siRNA treatment resulted in a more significant decrease in IL-8 production in response to *L.monocytogenes* infection. Taken together, these data imply a differential role for TLR1 and TLR10 in innate immune signal activation and ligand binding, contrary to the reports of Guan *et al.* and Govindaraj *et al.* [8, 9]. While their ligand binding may be similar, it certainly appears to be distinct from one another also. Moreover, this study provided the first evidence that TLR10 may be involved in the detection of other intracellular pathogens. It should also be noted that while both *L.monocytogenes* and *S.typhimurium* are intracellular bacteria, the former is Gram-positive and the latter is Gram-negative. Furthermore, both bacteria are motile. Therefore, it may be worthwhile investigating potential ligands from the flagellae or pili of these bacteria also in determining the specific ligand for TLR10.

Investigation into the sub-cellular localisation of TLR10 by intracellular and surface flow cytometry staining revealed a predominantly intracellular expression of TLR10 in HT-29 intestinal epithelial cells. It should be pointed out, however, that the antibody may preferentially bind the intracellular region of surface bound TLR10. Confocal microscopy may yield a clearer answer concerning TLR10 subcellular localisation although few efficient

antibodies for this gene exist. The lack of inflammatory gene induction from these cells following stimulation with non-viable HKLM in comparison to the strong induction of inflammatory genes in response to live *L.monocytogenes* suggested that the intracellular expression of TLR10 in the intestinal epithelium may help regulate the innate inflammatory signalling against microbial antigens, highly abundant in the gastro-intestinal tract. However, it should be noted that heat-treatment of the bacteria may denature the ligand for TLR10. That is, the ligand may be heat labile. For this reason, a strain of *Listeria innocua* or *L.monocytogenes* mutants with inactive InlA and InlB virulence factors may give a clearer indication as to whether TLR10 may recognise the bacteria extracellularly as these do not invade host cells. Such subcellular compartmentalisation of TLRs has been well documented to regulate homeostasis in IECs. For example, TLR2 and TLR4, which detect bacterial lipoproteins and lipopolysaccharides respectively, are known to translocate from apical to basolateral surfaces upon stimulation by commensal bacterial associated molecular patterns [38]. TLR5 has been shown to only be expressed intracellularly or basolaterally in the colon [39], thereby avoiding an inappropriate inflammatory response to flagellated commensal bacteria present in the gut lumen. Our data showing TLR10 as the dominant TLR involved in mediating the immune response to *L.monocytogenes* in the intestine open up intriguing possibilities concerning the balance of pro-inflammatory versus homeostatic TLR responses to infection in epithelial cells. It is possible that a TLR2/10 dimer may be pro-inflammatory in epithelial responses whilst TLR2 on its own or in combination with 1 and 6 may mediate a more homeostatic effect. Additionally, it could be that TLR10, reported elsewhere to be present on the cell surface [6, 23] might localise to the cytoplasm of IEC in order to avoid inappropriate inflammatory activation. Indeed as several polymorphisms in TLR10 are associated with Crohn's disease [12-14], a possible line of investigation would be to determine if these polymorphisms affect the subcellular localisation of TLR10 in the

intestinal epithelium. Inappropriate subcellular expression forms may then lead to inappropriate inflammatory activation from, for example, increased luminal expression.

Subcellular location of TLR2 is known to change upon stimulation with certain ligands [40] and the type of signal induced by TLR2 activation is sometimes dependent on the location of TLR2 at the time of stimulation. As predicted in published studies and, indeed, demonstrated in my own data, TLR10 is believed to require TLR2 for signalling, similar to TLR1 and TLR6 [6, 8, 9]. *L.monocytogenes* appears to activate an inflammatory innate immune response via TLR10. Therefore, if TLR10 utilises TLR2 for signal transduction, subcellular location of TLR2 may be different in the absence of TLR10 following infection with *L.monocytogenes*. For this reason, confocal microscopy would be an interesting tool to investigate subcellular localisation of TLR2 in parental or a stable TLR10 silenced HT-29 IEC line upon stimulation with various TLR2 ligands or intracellular microbes such as *L.monocytogenes*. Additionally, measuring the inflammatory response after stimulating the TLR10 silenced or parental HT-29 cells using *L.monocytogenes* with lipoprotein mutations might also help determine the specific ligand for TLR10. In order to follow this line of investigation, I attempted to create a stable TLR10 Knock Down (KD) cell line in HT-29 cells by lentiviral transfection. However, upon thawing frozen stocks of the TLR10 KD cell line I had generated, the associated phenotype (unresponsiveness to *L.monocytogenes* infection) had been lost. Furthermore, Western blotting analysis revealed that the selected TLR10 KD clone had regained TLR10 expression to a level comparable with the control and parental HT-29 clones. The control scrambled lentiviral particle shRNA clones all carried a GFP protein as an additional marker. None of the TLR10 shRNA clones displayed any presence of GFP, even after the TLR10 KD clone was grown back from frozen stocks. This ruled out the possibility of contamination of the TLR10 KD clone with the control cells. Furthermore, since the selective antibiotic, puromycin, was used on the control and TLR10

KD cells, a contamination from the parental HT-29 cells was also ruled out. It is possible, however, that during initial clonal selection, the single colony selected may have originated from more than one single cell. As colonies grew from these cells, they may have merged and appeared as a colony from one cell. The result of this would be that different integration of the TLR10 shRNA particles may have occurred throughout this mixed population. The cells exhibiting the TLR10 silenced phenotype may have initially predominated. Upon freezing the cells to make stocks and re-growing them, much stress would have been placed on the cells. This stress may have allowed any remaining cells from the other population present, which may not have had correct integration of the TLR10 shRNA particles, to out-compete the cells which had achieved TLR10 silencing.

In addition to THP-1 macrophage-like cells, we have also shown that depletion of TLR10 increases *Listeria* survival in HT-29 epithelial cells. Whilst the mechanism by which TLR10 might act to reduce bacterial survivability in intestinal epithelial cells such as HT-29s is unclear although our observation that TLR10 may regulate expression of chemokines such as CCL-20 may be a key factor. Indeed observations by Yang *et al.* [41] and Starner *et al.* [42] have demonstrated the antimicrobial effect of chemokines and cytokines such as CCL-20. These studies demonstrate that some chemokines, in addition to their role as a signalling messenger molecules, may also function similar to Human Beta Defensins (HBDs) which have been well characterised for their antimicrobial properties [43]. CCL20 was shown to rapidly permeabilise bacterial membranes over a time course comparable to HBDs [42]. Structural analysis of CCL20 revealed that most of its positively charged residues are concentrated to one area of its topological surface, a characteristic considered to be important for the antimicrobial activity of defensins [41]. Yang *et al.* also discovered that 17 out of a total of 30 chemokines which they had investigated exhibited antimicrobial properties *in vitro*. We have shown that TLR10 activation leads to chemokine production in response to

L.monocytogenes infection. Thus, in this manner, TLR10 activation may result in increased bacterial killing through antimicrobial chemokine production. Specific mutagenesis studies of both TLR10 and bacterial strains may also further elaborate this question.

We have shown that simulating hypoxic conditions through stabilisation of HIF-1 α using DMOG not only had the effect of greatly increasing TLR10 expression in THP-1 macrophage-like cells but in HT-29 IECs also. Hypoxia had already been shown to increase TLR10 expression in THP-1 monocytes [23] and is also believed to increase bactericidal activity in these cells [44, 45]. Areas of inflamed, infected, and diseased tissues are often hypoxic due to multiple factors: metabolically active inflamed tissue results in elevated oxygen demand; local blood supply is occluded; local vessel growth cannot keep pace with growth and/or infiltration of cells in affected tissue [45]. Reactive Oxygen Species (ROS), primarily superoxide and hydrogen peroxide (O_2^- and H_2O_2), are used by phagocytic cells in bacterial killing, with their ability to damage biomolecules and the integrity of DNA [46]. In hypoxic conditions, macrophages not only increase intracellular levels of bactericidal ROS but also up-regulate the expression of genes required for macrophage survival, angiogenesis, and recruitment and activation of macrophages and/or other inflammatory cells increasing bacterial killing [47]. The intestinal epithelium, on the other hand, exists in a unique environment with a hypoxic gradient across the cell from the oxygen rich vascular mucosa across to the apical surface facing the low oxygen environment of the lumen. Levels of hypoxia are known to increase for these cells during IBDs such as Crohn's disease when the intestinal epithelial barrier is disrupted. This increase in hypoxia leads to increased levels of HIF-1 α which is believed to mediate a protective effect for the intestinal barrier [25]. We wished to investigate if this protective effect had any role to play in the modulation of TLR10 expression levels or in the IEC response to TLR10 activating microbes, *L.monocytogenes* and *S.typhimurium*. Our results revealed that an increase was observed in TLR1 and TLR10

induction following HIF-1 α induction. This increase in TLR mRNA correlated with an increase in IL-8 induction in response to infection with both bacterial species. However, a significant decrease in CCL-20 was observed in response to the bacteria when cells were treated with HIF-1 α inducing DMOG. Interestingly, while HCA-7 cells were found to express more TLR10 mRNA than HT-29 cells, IL-8 induction was lower in HCA-7 cells than in HT-29 cells following infection with *L.monocytogenes*. Conversely, there was increased CCL-20 induction in HCA-7 following infection compared HT-29 cells. Silencing TLR10 in both cell types resulted in a decrease in both CCL-20 and IL-8 induction in response to *L.monocytogenes*. Although, despite HCA-7 cells and DMOG treated HT-29 cells both displaying greater expression of TLR10 than untreated HT-29 cells, their differences in IL-8 and CCL-20 induction levels do not align. Not only does this result highlight the caveats associated with different phenotypes across different cell lines in culture, but it also demonstrates that despite increased TLR expression during hypoxia, much further complex regulation must be taking place to curtail the inflammatory response from intestinal epithelia. This is likely to be a specific protection mechanism against over-responsiveness to the vast array of microbial ligands the intestinal lumen is exposed to, especially during IBD when the intestinal epithelial barrier's integrity is decreased. It is also possible therefore that the polymorphisms in TLR10 associated with Crohn's disease mentioned earlier may lead to over-activation in response to gastro-intestinal antigens when TLR10 induction is increased in the hypoxic environment of the gut mucosa during IBD. This may be due to the mutations in TLR10 preventing appropriate transduction of the regulatory features deployed during hypoxia.

Evidence supports the claim that the increase in TLR10 expression during hypoxic conditions in THP-1 cells is directly due to the subsequent increase in ROS [23]. This is reflected in our own studies with DMOG treated THP-1 macrophage-like cells; these

displayed much higher expression of TLR1, TLR2 but especially TLR10. Following this, we observed a significant increase in IL-1 β and IL-8 induction following infection with *L.monocytogenes* and *S.typhimurium*. Both of these pathogens have been shown to mediate an inflammatory response via TLR10 in our studies. As discussed earlier, we revealed that decreased TLR10 expression in THP-1 macrophage-like cells resulted in increased survival of *L.monocytogenes*. Taken together, these data suggest that TLR10 may play a role in ROS mediated clearance of bacteria in a positive feedback loop i.e. TLR10 activation from detection of intracellular microbes causes inflammation; this inflammation may increase hypoxic conditions in the area, due to increased oxygen consumption by the proliferation of bacteria, the accumulation of phagocytes at the infected site, and/or the vasoconstriction of vessels in the area that could impede delivery of oxygen to the site of inflammation. This is followed by an increase in ROS which in turn may up-regulate TLR10 expression resulting in further inflammatory activation (see Fig. 6.23). It should also be noted that in the absence of hypoxia, mouse and rat macrophage cell lines have been shown to rapidly up-regulate HIF-1 α levels when exposed to Gram-positive or Gram-negative bacteria [47] or even in response to the presence of LPS alone [48].

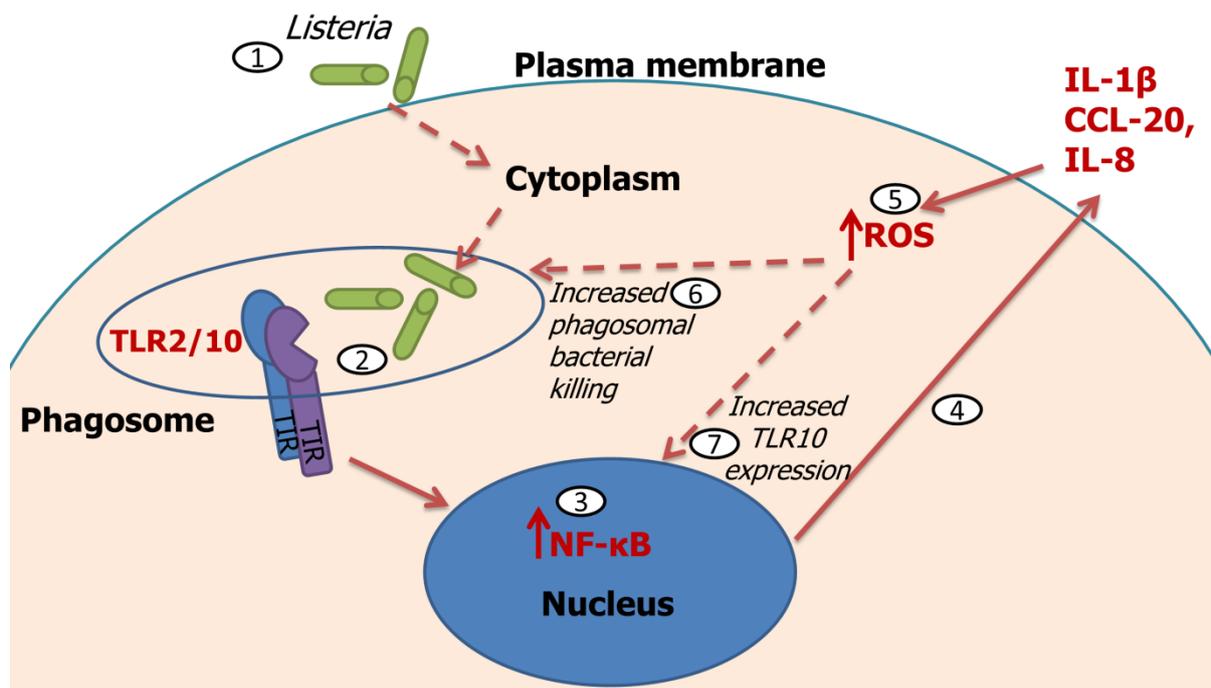


Figure 6.23 *L.monocytogenes* mediated TLR10 activation in macrophages

1. *L.monocytogenes* invades the cytoplasm of the cell. 2. Bacterial particles are detected in the phagosome by TLR10 sensing. 3. TLR10 mediates an inflammatory signal through its TIR domain. 4. Chemokines induced by TLR10 activation increase inflammation and attract more macrophages to the site of inflammation. 5. This increase in inflammation and oxygen consumption leads to hypoxia which increases in ROS within the cell. 6. ROS production increases bacterial killing within the phagosome. 7. ROS also increases TLR10 expression, believed to be mediated through NF-κB activation.

Despite recent advances in gene detection, I encountered several difficulties in my own efforts over the duration of this study with regard to TLR detection, TLR10 in particular. We initially wished to confirm the effect of TLR10 silencing seen in the PRR siRNA screen; this involved verifying the specificity of the siRNA constructs used and excluding the possibility of off-target effects. As TLRs 1, 2, 6 and 10 are all closely related [6] and siRNA against TLR1, TLR2 and TLR10 were used in our studies, the specific targeted silencing of each gene was verified by qRT-PCR and flow cytometry. Although the screen was performed on HT-29 cells, it was not possible to detect TLR10 by qRT-PCR in these cells using the primers we had initially obtained. The consistent effect of reduced immune response following infection seen in the TLR10 siRNA treated cells prompted the acquisition of more

suitable primers in order to detect its presence in HT-29 cells. Having accomplished this, mRNA levels detected were still relatively low compared to the THP-1 cell line. Hence, we confirmed the siRNA specificity using THP-1 macrophage-like cells which express high levels of TLRs allowing more accurate results. Detection of TLR10 through Western blot proved difficult also, with a high degree of variation in blot quality encountered relative to detection of other proteins. Detection of TLR protein using flow cytometry produced the most consistent results. However, intra-assay discrepancies in TLR quantification were encountered using this method also. Inconsistencies in levels of detection were also met between cell types using different assays. An example of this may be seen in the flow cytometry data versus qRT-PCR data displaying TLR10 expression in THP-1 macrophage-like cells compared to HT-29 cells. The HT-29 cells were shown to have 14.7% staining compared to 1.3% in the THP-1 macrophage-like cells despite THP-1 cells having more detectable TLR10 mRNA than HT-29 cells. Indeed a recent study has found that the circadian rhythm of intestinal epithelial cells governs the signalling of TLR mediated homeostasis [49] which may in part explain the intra-assay variation associated with TLR10 detection over the course of this study. The difficulties I encountered in detection and quantification of TLR10 during the course of my work might also explain why previous studies have reported a lack of TLR10 expression in certain cell types [26].

Much previously unknown information regarding TLR10 functionality, expression and signalling has been uncovered in this study. It is clear, however, that we are only just beginning to understand its role and much further analysis is required to fully reveal its physiological functions. A particularly useful tool would be clear visualisation of the protein at an endogenous level within the cell. Over-expression studies are useful for visualising the protein if it has a fluorescent tag although over-expression may prove to obstruct a clear view

of the natural role TLR10 is playing within the cell. Trafficking of TLR10 upon ligand recognition in addition to differences in sub-cellular localisation throughout different cell types have yet to be investigated thoroughly. With particular regard to the recent information surrounding the expression of TLR10 throughout the body [4, 23, 26, 29-32] in addition to the information revealed here regarding TLR10 expression, it is clear that advancements in antibody specificity and detection formats will aid in this endeavour. It should also be mentioned here that throughout all western blotting analysis, it was the ~75 kDa isoform of TLR10 that was detected rather than the more commonly referred to 94 kDa isoform. I have found this to be a common phenomenon through discussions with other researches investigating TLR10. More study in this area could reveal functionally distinct isoforms of TLR10, similar to those of TLR7 and TLR9 where cleavage of the full-length protein is required for efficient signal transduction [50].

6.4 References

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Chapter 7

General Discussion

7.1 The intestinal epithelium is increasingly recognised as an important site for innate immune regulation.

Throughout the course of this thesis, several novel aspects of innate immune regulation of inflammation and signalling have been uncovered within the setting of the intestinal epithelium. As discussed throughout each chapter, the intestinal epithelial interface is a unique area for inflammatory regulation in the maintenance of homeostasis. Furthermore, in addition to their role in maintaining homeostasis, it is only recently that intestinal epithelial cells have also been identified as important mediators of the inflammatory response. Many PRRs, previously believed not to be expressed in the epithelium are now known to function as innate immune activators in these cells, involved either in initiating an inflammatory response or in the maintenance of homeostasis. In this thesis, several PRRs have been revealed to be involved in the detection of *L.monocytogenes* in IECs. These novel data not only suggested a role for PRRs believed not to be expressed in IECs, but also revealed several PRRs to function as regulators of the inflammatory response in IECs.

In recent years, we have begun to understand the true function of our immune system is not to eliminate every non-self organism in our body; rather it is to maintain a homeostatic balance between our own bodily functions and those of the various microbes in our environment, including our resident commensal bacteria. The intestinal epithelium is a key region in regulating this balance. The last decade has revealed several mechanisms for regulating PRR activation in IECs. For instance, Single Immunoglobulin (Ig) Interleukin-1R-Related molecule (SIGIRR) is an Ig-like membrane protein found in IECs [1]. It acts as a critical negative regulator of TLR4-mediated signalling. Furthermore, as discussed throughout this thesis, the expression patterns of PRRs in IECs are known to modulate to avoid inappropriate activation [2-6]. Several mutations in such genes are known to lead to autoimmune disorders such as IBD [7-12]. Novel data presented in this thesis have revealed

new information pertaining to PRRs involved in curtailing an inflammatory response to the invasive pathogen *L.monocytogenes* in IECs. For instance, NLRC3-5, several NLRP receptors and four members of the CLR family were all found to be involved in mediating this abrogation of inflammatory gene induction. It is quite possible that several of these PRRs may be involved in the maintenance of homeostasis in the unique environment of the gastro-intestinal tract through the detection of microbial antigens from the bacteria filled lumen. Moreover, the involvement of PRRs previously not known to be expressed in IECs requires further attention, in particular the CLR family of PRRs, about which we know relatively little compared to other PRR families such as the much studied TLRs. Studies have demonstrated induction of the bactericidal C-type lectin RegIII γ in response to MyD88 and TLR signalling during *L.monocytogenes* infection in IECs [13]. However, research has not yet focussed on potential regulatory roles for CLRs in maintaining homeostasis in the gastro-intestinal tract. Given the function and setting of IECs, we should begin to speculate further about the function of these PRRs in the context of maintaining homeostasis and, indeed, their roles in autoimmune disorders such as IBD. It is clear that previous detection methods are lacking in efficiency and further data are required for a better understanding of innate immune signalling in the context of IECs.

7.2 A more comprehensive knowledge of innate immune signalling mechanisms in the gut could deliver safe and efficient treatments against autoimmune and pathogenic diseases.

Therapeutic targets in treating IBD require specifically directed treatments to avoid undesired side-effects affecting the immune system which may lead to infection or an inappropriately increased inflammatory response. Through direct targeting of novel PRRs involved in regulating inflammatory responses in IECs, we open possibilities to more

precisely treat IBD, thus reducing over-active inflammation in this region. Furthermore, the gut is a unique region through which our body may become tolerised to certain antigens [14]. In addition to the treatment of over-active inflammatory disorders in the gut, a greater knowledge of the innate immune mechanisms through which this mucosal region is regulated could also lead us to discover novel mechanisms of inducing systemic tolerance to self-antigens. Using a broader understanding of innate immune functioning in IECs, for example, we may induce tolerance to self-antigens which the body may be inappropriately recognising, as in the case of other autoimmune disorders. This may be due to mutations in innate immune detection systems which respond inappropriately to self or commensal antigens. If a systemic response may be achieved through correct and specific targeting of the innate immune system through the gut in this manner, it opens up the possibility of generating vaccines for a whole range of auto-immune disorders, such as rheumatoid arthritis or lupus erythematosus. Naturally, the ease of vaccine delivery through oral ingestion is an enormous advantage.

Further to the use of oral vaccines to induce tolerance, there is also an obvious advantage to the convenience of cost and administration of oral immune vaccines over the currently more commonly used intradermal application. In addition to regulating homeostasis and avoiding inappropriate inflammatory responses, we are also becoming increasingly more aware of innate immune mechanisms by which a response is initiated in IECs against pathogens. These cells, forming a monolayer as an integral part of the intestinal barrier, have now also been established to express several intracellular PRRs that detect bacteria and mediate an inflammatory immune response [15, 16]. The importance of this physical barrier has been highlighted throughout the thesis but cannot be overlooked. While retaining homeostasis is crucial in this region with the presence of so many transient and resident antigens in the luminal space, this barrier is also a frontline defence against any potentially harmful pathogens that may try to break through. Therefore, when confronted with an

invasive pathogen, it is important that the IECs offer the earliest response in order to ready the leukocytes of the underlying mucosa and prime a systemic immune response if required. A complete understanding of the unique innate signalling mechanisms in the intestinal epithelia will offer invaluable assistance in generating efficient adjuvants for vaccine delivery through this route [17]. The importance of basic science in this regard is crucial for the generation of vaccines directed at initiating an overall systemic cellular response to offer protection against pathogenic attack while retaining a balanced response.

If we are to gain insight into new therapeutic deliveries for existing inflammatory disorders, or indeed the development of oral vaccines, further basic scientific research is required if we wish to unravel the intricacies of innate immune signalling. In particular, there is a clear requirement for an update on expression patterns of PRRs. For example, TLR10 was previously believed not be expressed in IECs [18]. Several groups have since reported a more widespread expression pattern for TLR10 than was originally suggested [18-24]. However, this highlights that despite advances in technology, there exist many shortcomings in conventional methods of determining expression patterns, such as the use of antibodies, which may not always be specific or sensitive enough, for example. This again emphasises the importance of other PRRs in these cells, the presence or function of which may have been overlooked until now. Moreover, it also illustrates the advantage of siRNA screening techniques, such as that used in this thesis, in the contribution of knowledge towards the function of genes not known to be expressed in various cell types. The potential issue of adequately efficient detection formats not providing us with complete knowledge of expression patterns may be avoided by measuring the effects of silencing particular genes in cells when challenged with pathogenic attack for example. Novel information on PRR expression and function will be fundamental in the advancement of available therapy and preventative medicine.

Attention has been growing towards the direct targeting of PRRs in treating autoimmune disorders [25]. Recent success has been reported having used an IgG antibody against TLR2 for the prevention of reperfusion injury following transplantation. Thus far, it has proved effective in treatment against myocardial ischemia/reperfusion injury [26] and clinical trials have shown positive results in the prevention of reperfusion injury following renal transplantation [27]. Targeting TLR2 has also shown promise in reducing inappropriate cytokine signalling in rheumatoid arthritis [28]. Genetic analysis studies have shown a correlation between certain mutations in TLR10, or in the TLR1-6-10 gene cluster, such as nasopharyngeal carcinoma, prostate cancer, non-Hodgkin's lymphoma, asthma, sarcoidosis and Crohn's disease [29-37]. It may be possible that specifically targeting TLR10 may result in a more directed response against these diseases such as the autoimmune disorder, Crohn's disease, in the gut. Furthermore, we and other groups have shown that TLR10 is likely to use TLR2 in signal transduction. This leaves open the possibility that a more specifically targeted therapy directed at blocking TLR2 may be achieved through blocking TLR10 in other disorders also. The identification of the specific ligand for TLR10 and each of its signalling dimers is essential for these potential treatments.

7.3 It is evident that there exists a high level of complexity involving multi-functional PRRs and innate immune cross-talk.

The evidence of interplay between different PRRs has been growing in recent years. Furthermore, it is not acceptable to distinguish PRR functions according to their separate families, but rather to examine their role in the broader sense of innate immunology with a more open mind. As previously mentioned, TLR activation during *L.monocytogenes* infection in IECs is believed to be responsible for the induction of the bactericidal C-type lectin RegIII γ [13]. Furthermore, as discussed in the Introduction section of this thesis, clear

synergy exists between CLR member Dectin-1 and TLR2 [38]. In addition to activation synergy, there also exists a level of regulation between PRR families, such as between TLR and NLR families. NOD2 activation has been demonstrated to inhibit TLR mediated signalling [8] while TLR signalling has also been suggested to reduce NOD1 and NOD2 responses [39]. Clear examples of how TLR functions may vary between different cell types have also been given throughout this thesis [4-7, 40]. Thus, it is also important to recognise the setting in which the PRRs are expressed in order to fully understand the context of their function. Our finding that TLR10 performs a pro-inflammatory role may not be true for all cell types. Our finding that CLEC1B somehow acts to reduce inflammatory responses against *L.monocytogenes* infection in IECs, while it is known to have a pro-inflammatory role in phagocytic cells [41], is a specific example of such inter-cellular multi-functionality. It could also be possible that TLR10 acts to regulate the activation of an inflammatory response or to regulate signalling from other TLRs when various regions of the body are confronted with different challenges.

The multi-faceted role of several PRRs has also by now been well established. Particularly in IECs, a regulatory role has been described for several PRRs often associated with an inflammatory response. For example, TLR2, TLR9 and NOD2 have all been well defined in maintaining a homeostatic environment in IECs through direct regulation of inflammatory responses [4-6, 8-12, 42, 43]. Additionally, MyD88 did not appear to be required for mediating an immune response against *L.monocytogenes* infection in Chapter 5. Whilst TLR1, TLR2 and TLR10 were all believed to be involved in mediating an inflammatory response during this infection, all are believed to require MyD88 for signal transduction [44]. This suggests that in this setting MyD88 may be mediating more homeostatic and less pro-inflammatory responses through the immuno-suppressive effects associated with TLR activation in IECs, for example [7]. It could also be the case that TLR10 somehow bypasses

the requirement for MyD88-mediated signal transduction for an inflammatory response upon ligand recognition, a possibility which charges us with the question of what alternative signal transduction pathways TLR10 may be utilising. TLR4 is known to use the TRIF pathway for intracellular signalling and the MyD88 pathway for signalling from the plasma membrane [45, 46]. TRIF has also recently been implicated in TLR2 signalling [47]. It may be interesting to examine more closely potential involvement of TRIF in TLR10-mediated signalling. Several NLR proteins other than NOD2, such as NLRP3 and NLRP6, have been associated with maintaining a homeostatic environment in this region through maintaining an efficient barrier and controlling epithelial cell proliferation [48-53]. The CLR, LY75, has previously been implicated in cell adhesion and migration in microvilli in addition to phagocytosis in macrophage cells [54] but has been outlined in Chapter 5 to have a potential role in mediating an inflammatory response against *L.monocytogenes* in IECs. Conversely, CLRs DC-SIGN and CLEC1B have been associated with the activation of phagocytosis in macrophages and PBMCs [41, 55] but have been demonstrated in our data to reduce inflammatory responses towards *L.monocytogenes* in IECs. The novel discovery of the TLR10 mediated inflammatory response to intracellular pathogens in IECs should be regarded as a potentially important jig-saw piece we have yet to find a place for in the grand puzzle of understanding innate immune regulation in this unique environment. TLR10 has also recently been identified as mediating PGN-induced apoptosis of trophoblasts [22]. Results from research presented in Chapter 6 would suggest that the inflammatory response from *L.monocytogenes* mediated TLR10 activation occurs through the TLR2/TLR10 receptor pair, with the possible requirement of CD14. However, future studies need to address the function of the TLR1/TLR10 heterodimer in addition to the TLR10/TLR10 homodimer of which we are also aware. It is likely that these dimers may be responsible for the detection of different specific ligands and, indeed also possible, that these dimers may be involved in the

initiation of differential responses, such as apoptosis in the case of trophoblasts [22] or potentially initiating a regulatory rather than inflammatory immune response. Such potentially differential signalling outcomes are also likely to operate using alternate signalling intermediates, another facet of investigation required to elucidate the full extent of TLR10 functionality. Furthermore, the potential requirement of CD14 requires further examination and draws attention to the importance of these co-receptors in mediating efficient and potentially alternate responses from PRR activation. However, elucidation of these potential complex differential interplays requires an open mind and careful observations. Perhaps a starting point for such studies might involve siRNA screening techniques such as the PRR siRNA screen used in Chapter 5. Moreover, several diseases are associated with PRR mutations. Examining the functional consequences of these mutations and how they lead to disease symptoms and characteristics might reveal novel interplays or functionalities of these PRRs.

7.4 Summary and future directions.

Together with the novel discoveries made throughout the course of this thesis, it has been illustrated that there exists a level of inflammatory regulation in the intestinal epithelium, perhaps far more complex than we might have previously imagined, which we know very little about. We have known for some time now that IECs protect us from invasive attack by providing a valuable barrier function and anti-microbial defensin production. We have also known that IECs are crucial in the maintenance of homeostasis in the environment of the commensal microbe-filled gastro-intestinal tract. However, as more data in this field is generated we are learning more and more the concept that, in addition to their barrier function, they are also involved in initiating an innate inflammatory immune response against pathogenic attack. We are not only discovering that the repertoire of PRRs extends beyond

that which we previously believed, but that PRRs also display multi-faceted functioning [40]. In other words, the same PRRs involved in pathogen recognition may be involved in activation or abrogation of an inflammatory response depending on the context in which they themselves are activated. Additionally, factors such as hypoxic gradient may alter the expression patterns and functionality of some of these PRRs, but this is an area that has only recently been investigated in the context of the intestinal barrier [56]. Moreover, aside from internal regulation of innate immune inflammatory responses in the gut, it is clear that we also have much to learn concerning potential mechanisms of biomimicry utilised by microbes in the subversion of our immune surveillance system. This has been seen in the recent discoveries of TIR domain containing proteins in bacteria being involved in hi-jacking our TLR signalling mechanisms to aid their own survival. While this has been demonstrated in several pathogenic strains of bacteria [57] it has yet to be investigated in the context of commensal microbes. Some of these themes concerning IEC interactions in this broader context of the gastro-intestinal tract at a molecular level are illustrated in Figure 7.1 below. It outlines the differential functionality of PRRs such as CLEC1B when expressed in different cell types. Additionally, it displays an example of differential functionality of PRRs such as TLR9 depending on their subcellular localisation. A greater understanding of these signalling mechanisms will undoubtedly yield a more directed and systemic control over our bodies' immune functionality.

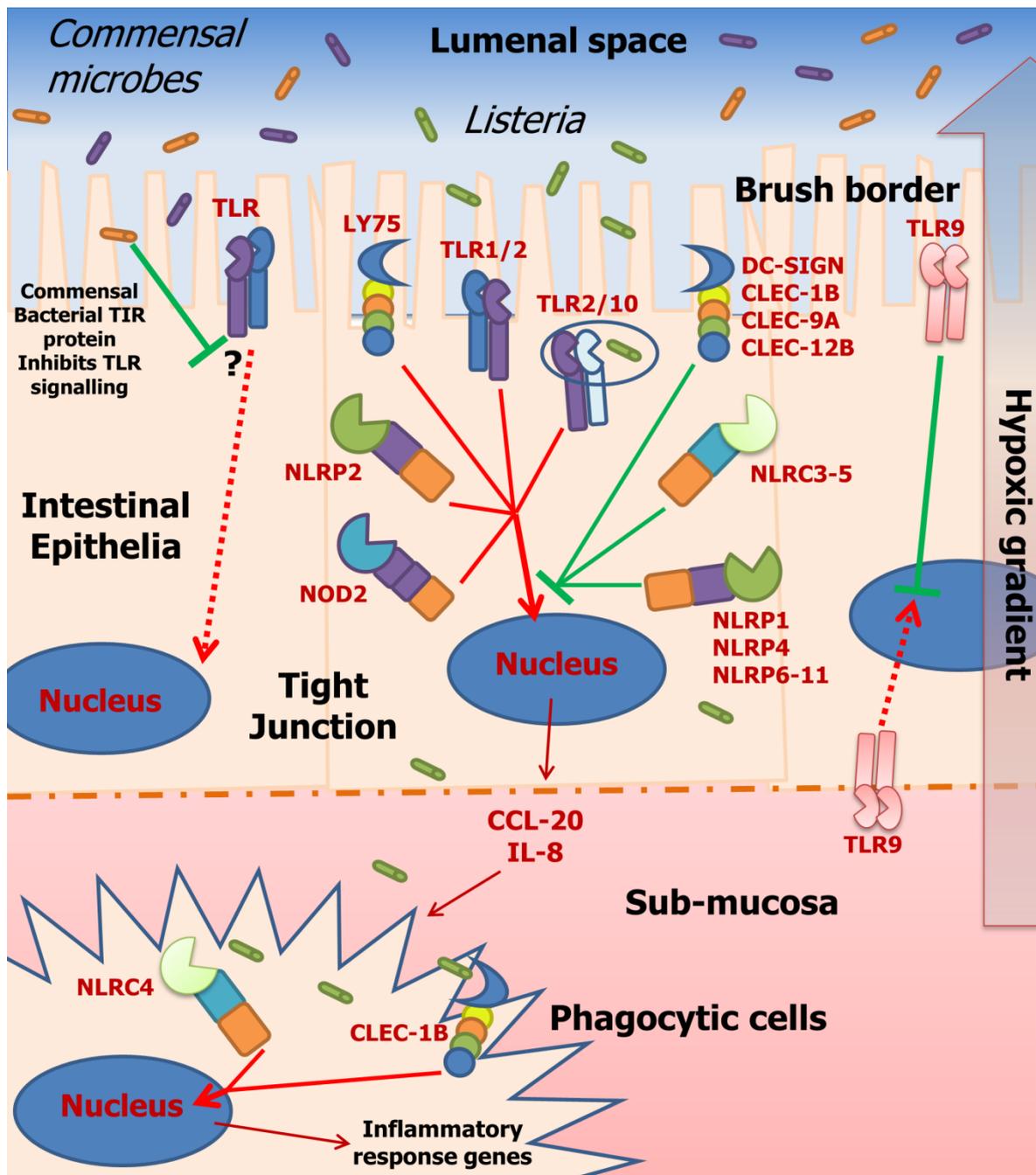


Figure 7.1 Molecular mechanisms of regulating innate immune inflammatory responses in the gastro-intestinal tract. Depicted above are commensal bacteria potentially utilising TIR domain containing proteins to modulate inflammatory TLR signalling in IECs. PRRs involved in the activation and abrogation of an inflammatory response when met with *L.monocytogenes* in IECs infection are also illustrated. This also offers an example of the differential response of PRRs such as NLRC4 and CLEC-1B depending on which cell they are expressed in. This is also true of the subcellular localisation of PRRs; apical TLR9 sensing of microbial ligands inhibits inflammatory signalling induced by basolateral TLRs. This apical/basolateral polarity is strengthened by the steep hypoxic gradient between the lumen and the vascular rich mucosa. It is known to promote barrier integrity and may regulate PRR expression and function.

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8. Appendix

Table 1 PRR siRNA library sequences

Gene Symbol	GENE ID	Gene Accession	GI Number	Sequence
TLR1	7096	NM_003263	41350336	GGCAAUAUGUCUCAACUAA
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TLR1	7096	NM_003263	41350336	GUUGAGCACCACACACUUA
TLR2	7097	NM_003264	68160956	AAAUCUGAGAGCUGCGAUA
TLR2	7097	NM_003264	68160956	AGGUAAAGUGGAAACGUUA
TLR2	7097	NM_003264	68160956	UGUUUGGAACUGCGAGUA
TLR2	7097	NM_003264	68160956	AGUAGGAAUGCAAUAACUA
TLR3	7098	NM_003265	19718735	GAACUAAAGAUCAUCGAUU
TLR3	7098	NM_003265	19718735	CAGCAUCUGUCUUUAAUAA
TLR3	7098	NM_003265	19718735	AGACCAAUCUCUCAAAUUU
TLR3	7098	NM_003265	19718735	UCACGCAAUUGGAAGAUUA
TLR4	7099	NM_138554	88758616	UGGUGGAAGUUGAACGAAU
TLR4	7099	NM_138554	88758616	GUUUAGAAGUCCAUCGUUU
TLR4	7099	NM_138554	88758616	CAUUGAAGAAUUCGGAUUA
TLR4	7099	NM_138554	88758616	GGAAAUGGUGUAGCCGUU
TLR5	7100	NM_003268	19718736	AGACAUUAUUGUGUGUAC
TLR5	7100	NM_003268	19718736	CACGGAAGGUUGUGAUGAA
TLR5	7100	NM_003268	19718736	CUUCUUGAGUGGCAAUAAA
TLR5	7100	NM_003268	19718736	CACAGAACCUGAUUUGUAC
TLR6	10333	NM_006068	20143970	GGUGAAAAGUGAAUUGGUA
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TLR6	10333	NM_006068	20143970	GCACCAAGCACAUUCAAGU
TLR6	10333	NM_006068	20143970	CCUGUGGAAUAUCUCAUA
TLR7	51284	NM_016562	67944638	CAACAACCGGCUUGAUUUA
TLR7	51284	NM_016562	67944638	GGAAAUUGCCCUCGUUGUU
TLR7	51284	NM_016562	67944638	GAAUCUAUCACAAGCAUUU
TLR7	51284	NM_016562	67944638	GGAAUUACUCAUAUGCUAA
TLR8	51311	NM_016610	20302165	GAACGGAAAUCCGGUAUA
TLR8	51311	NM_016610	20302165	CAGAAUAGCAGGCGUAACA
TLR8	51311	NM_016610	20302165	GUGCAGCAAUCGUCGACUA
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TLR9	54106	NM_138688	20302170	CAGACUGGGUGUACAACGA
TLR9	54106	NM_138688	20302170	GCAAUGCACUGGGCCAUAU
TLR9	54106	NM_138688	20302170	CGGCAACUGUUAUACAAG
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TLR10	81793	NM_001017388	62865620	CGAAUUAUCUUGCAACACA
TLR10	81793	NM_001017388	62865620	CAACAAGGAGUUAAGAUAU
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MYD88	4615	NM_002468	19923143	GCUAGUGAGCUCAUCGAAA
MYD88	4615	NM_002468	19923143	GCAUAUGCCUGAGCGUUUC
MYD88	4615	NM_002468	19923143	GCACCUGUGUCUGGUCUAU

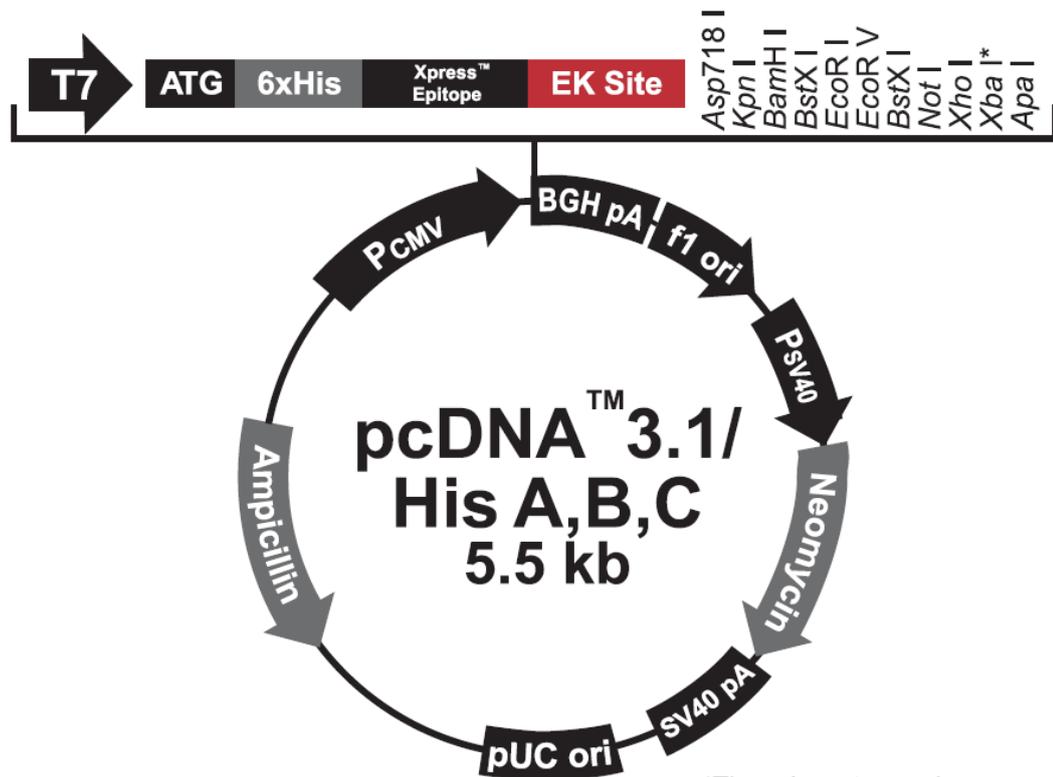
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MHC2TA	4261	NM_000246	73747680	GAAGUGAUCGGUGAGAGUA
MHC2TA	4261	NM_000246	73747680	CAAGGCCGACGCCCUAUUU
BIRC1	4671	NM_004536	4758751	GCAUCGAACUCCAUUUAAA
BIRC1	4671	NM_004536	4758751	GAACAUUAUCCAGCAGUUA
BIRC1	4671	NM_004536	4758751	GAGCGAAAUUUAGCUGAAA
BIRC1	4671	NM_004536	4758751	GUACAAGAUUCCCUGUCUA
CARD4	10392	NM_006092	5174616	CAAUAUCUUCUCUGAGGUU
CARD4	10392	NM_006092	5174616	AGAAGCGGAUUUAUCUGUUU
CARD4	10392	NM_006092	5174616	GCAAAGGCCUCACGCAUCU
CARD4	10392	NM_006092	5174616	GGGUAAGGUGCUAAGCGA
CARD15	64127	NM_022162	11545911	GGAAUUACCAGUCCCAUUG
CARD15	64127	NM_022162	11545911	GCUCUGUAUUUGCGCGAUA
CARD15	64127	NM_022162	11545911	GGGAAUAACUACAUCACUG
CARD15	64127	NM_022162	11545911	GUAUGAAUGUGAUGAAAUC
NOD3	197358	NM_178844	30524927	GCGGAGAACCAGAUCAGUA
NOD3	197358	NM_178844	30524927	GCUUAGAUUUACAGGAGAA
NOD3	197358	NM_178844	30524927	GAGCUCAUUGUUCUCCAGUA
NOD3	197358	NM_178844	30524927	GCAACACCGUUAGGGAUGA
CARD12	58484	NM_021209	40788014	GACAUUACAUCCACUUAUA
CARD12	58484	NM_021209	40788014	UUAAAGGACUUGUACCAUA
CARD12	58484	NM_021209	40788014	GCACAUACAUCUGUAACA
CARD12	58484	NM_021209	40788014	GUACACAGCAGGACGAAGA
NLRC5	84166	NM_032206	28951070	GAGAUCAGCUUGGCGGAAA
NLRC5	84166	NM_032206	28951070	GAGCAGAGCUUCCGGAUUC
NLRC5	84166	NM_032206	28951070	AGCAGAAGCUUGACGCUCA
NLRC5	84166	NM_032206	28951070	GGGUUAGCAGGAAGUAAA
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NALP1	22861	NM_001033053	74271813	CCAAUUGGCCACUUUAAA
NALP1	22861	NM_001033053	74271813	GAUGAAAGGCAAGCAAUUA
NALP1	22861	NM_001033053	74271813	GAGCUGGACCUAAGUGGAA
NALP2	55655	NM_017852	8923472	GAAUGUAACCUGCGUAUUC
NALP2	55655	NM_017852	8923472	GCAAAGAGGUCCAGGUUAU
NALP2	55655	NM_017852	8923472	GCACUAAAUGCAGUAGAC
NALP2	55655	NM_017852	8923472	GCGUAAACCUCUCCGACAA
CIAS1	114548	NM_183395	34878689	GGAUCAAAACUACUCUGUGA
CIAS1	114548	NM_183395	34878689	UGCAAGAUCUCUCAGCAAA
CIAS1	114548	NM_183395	34878689	GAAGUGGGGUUCAGAUAAU
CIAS1	114548	NM_183395	34878689	GCAAGACCAAGACGUGUGA
NALP4	147945	NM_134444	54792746	GCUCUGACGCAUACGGAUU
NALP4	147945	NM_134444	54792746	CUGAAAUGCUUCUGCGUAA
NALP4	147945	NM_134444	54792746	ACAACAAGAAGCUGACGUA
NALP4	147945	NM_134444	54792746	CCUAUCAAGCUCACGCAAA

Gene Symbol	GENE ID	Gene Accession	GI Number	Sequence
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NALP5	126206	NM_153447	32481210	AAGUGUGGCUUCCGAUUA
NALP5	126206	NM_153447	32481210	GCGUGAAGCUUCUGUGCGA
NALP5	126206	NM_153447	32481210	CGGUGGUUCUGCACGGAAA
NALP6	171389	NM_138329	21264319	GCGCCUACCGCUUCGUGAA
NALP6	171389	NM_138329	21264319	GGGCGCAGUUUGCCGAGAA
NALP6	171389	NM_138329	21264319	UCUCCGUGUCCGAGUACAA
NALP6	171389	NM_138329	21264319	GUGCAGACGGUCAGGGUAC
NALP7	199713	NM_206828	75709195	UGGGAUCGCUCUACGAAUU
NALP7	199713	NM_206828	75709195	CGACCUGAUUCAAGUAGGA
NALP7	199713	NM_206828	75709195	CUGUCGAGAGGUAGGAUUU
NALP7	199713	NM_206828	75709195	GAGAAACAGUCUUUGGUCU
NALP8	126205	NM_176811	33667039	UGGAGAGGCUGUCGAUAGA
NALP8	126205	NM_176811	33667039	GCAAGGUGUCUUUCGGUAA
NALP8	126205	NM_176811	33667039	GGAAAUGGGUGUUAGGUAA
NALP8	126205	NM_176811	33667039	UCUAAAGACUCUCAUACUA
NALP9	338321	NM_176820	33519449	GAACAGAGCCCGACUAAAA
NALP9	338321	NM_176820	33519449	GAACAUUUGGUAUAGCUU
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NALP9	338321	NM_176820	33519449	GAAGUCGUUUUCUCCUAC
NALP10	338322	NM_176821	78191799	CGCAGGAUCUGAAGCAUUU
NALP10	338322	NM_176821	78191799	GCUCCUAUUUCACGGAUGA
NALP10	338322	NM_176821	78191799	GAGAGGCAAAGUUGUCUUA
NALP10	338322	NM_176821	78191799	GGAGGGCAAAGAUAAUUA
NALP11	204801	NM_145007	21450724	AGAAGUAUCUGGCACGCAA
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NALP12	91662	NM_033297	15193291	CCAAUAAGAAUUUGACAAG
NALP12	91662	NM_033297	15193291	GGAUGGACCUGAAUAAAAU
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NALP13	126204	NM_176810	28827788	CCACGUUAUAUCCGUAAU
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NALP14	338323	NM_176822	31543282	GAAUAUGGACUAUGACUUA
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NALP14	338323	NM_176822	31543282	GAAUGAAGAUUCGAGUAAAA
NALP14	338323	NM_176822	31543282	GAAUUGGACCUGUACCAUA
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Gene Symbol	GENE ID	Gene Accession	GI Number	Sequence
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CLEC5A	23601	NM_013252	31747574	AGGAUCAGUUUUACGGAAA
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MICL	160364	NM_201623	189181661	CUCUACAACUGAUGAGUAA
MICL	160364	NM_201623	189181661	GGUAUGAGAGUGGAUAAUA
MICL	160364	NM_201623	189181661	CCACCAAUUUAUGUCGUGA
CLEC2	51266	NM_016509	56711305	UAGCAAAGCGCUUCUGUCA
CLEC2	51266	NM_016509	56711305	GGGAGGAUGGCUCGGUUUAU
CLEC2	51266	NM_016509	56711305	AGCCAGGACUCAUUUAAUU
CLEC2	51266	NM_016509	56711305	CGGAUUUAUCUCGCCAGAAG
UNQ9341	283420	NM_207345	141803213	GAGUUAAGAACAACGCAA
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Gene Symbol	GENE ID	Gene Accession	GI Number	Sequence
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CLEC4C	170482	NM_203503	45580691	GGGAUGCAAUCUUGGACUA
CLEC4C	170482	NM_203503	45580691	CCUGCGUCAUGGAAGGAAA
CLEC4E	26253	NM_014358	90577173	ACGAGAAAUUUGUGCGCUG
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CLEC4E	26253	NM_014358	90577173	CAAUUUAUUCUAAGCGAAC
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MRC1	4360	NM_002438	4505244	GAAGCAAAGUGGAUUACGU
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LY75	4065	NM_002349	4505052	UUUCAUAGGCCAUGGCGAA
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Figure 8.1 (a) Plasmid Map for pcDNA 3.1 A Myc/His (adapted from Invitrogen)

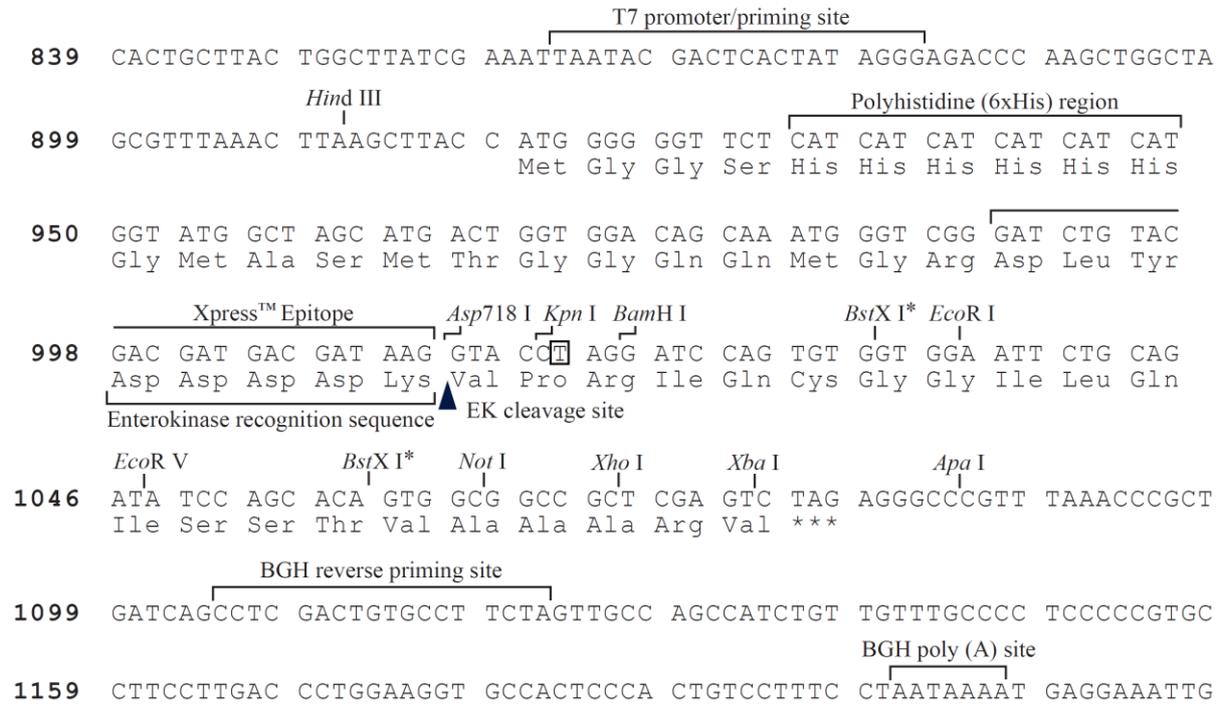


**Comments for pcDNA™ 3.1/His A
5514 nucleotides**

*There is a stop codon following the Xba I site in version A.

- CMV promoter: bases 209-863
- T7 promoter/priming site: bases 863-882
- ATG initiation codon: bases 920-922
- Polyhistidine region: bases 932-949
- Xpress™ epitope: bases 989-1012
- Enterokinase recognition site: bases 998-1012
- Multiple cloning site: bases 1012-1085
- BGH reverse priming site: bases 1105-1122
- BGH polyadenylation signal: bases 1104-1318
- f1 origin: bases 1381-1794
- SV40 early promoter and origin: bases 1859-2183
- Neomycin resistance gene: bases 2219-3013
- SV40 polyadenylation signal: bases 3029-3268
- pUC origin: bases 3700-4373 (Complementary strand)
- Ampicillin resistance gene: bases 4518-5379 (Complementary strand)

Figure 8.1 (b) Multiple Cloning Site (MCS) for pcDNA 3.1 A Myc/His (adapted from Invitrogen)



*Note that there are two *Bst*X I sites in the polylinker.

Table 8.2 TIR domain containing bacterial proteins

1	<i>Abiotrophia defectiva</i> ATCC 49176 C4G0E7_ABIDE
2	<i>Acaryochloris marina</i> (strain MBIC 11017) A8ZQA4_ACAM1
3	<i>Acaryochloris marina</i> (strain MBIC 11017) A8ZQX8_ACAM1
4	<i>Acaryochloris marina</i> (strain MBIC 11017) B0BZW8_ACAM1
5	<i>Acaryochloris marina</i> (strain MBIC 11017) B0C5D8_ACAM1
6	<i>Acaryochloris marina</i> (strain MBIC 11017) B0C8G4_ACAM1
7	<i>Acaryochloris marina</i> (strain MBIC 11017) B0C978_ACAM1
8	<i>Acaryochloris marina</i> (strain MBIC 11017) B0CEC7_ACAM1
9	<i>Acaryochloris marina</i> (strain MBIC 11017) B0CFH1_ACAM1
10	<i>Acidithiobacillus ferrooxidans</i> (strain ATCC 53993) B5EKQ0_ACIF5
11	<i>Acidobacteria bacterium</i> (strain Ellin345) Q1IIP0_ACIBL
12	<i>Acidobacteria bacterium</i> (strain Ellin345) Q1IKX5_ACIBL
13	<i>Acidovorax</i> sp. (strain JS42) A1W808_ACISJ
14	<i>Acidovorax</i> sp. (strain JS42) A1W966_ACISJ
15	<i>Actinosynnema mirum</i> DSM 43827 C0T5V7_9PSEU
16	<i>Actinosynnema mirum</i> DSM 43827 C0TBX5_9PSEU
17	<i>Agrobacterium tumefaciens</i> O52292_9RHIZ
18	<i>Algoriphagus</i> sp. PR1 A3I187_9SPHI
19	<i>Algoriphagus</i> sp. PR1 A3I1A7_9SPHI
20	<i>Algoriphagus</i> sp. PR1 A3I2H7_9SPHI
21	<i>Alteromonadales bacterium</i> TW-7 A0Y5N5_9GAMM
22	<i>Alteromonadales bacterium</i> TW-7 A0Y5W7_9GAMM
23	<i>Anabaena</i> sp. (strain PCC 7120) Q8YWN0_ANASP
24	<i>Anabaena variabilis</i> (strain ATCC 29413 / PCC 7937) Q3M5F3_ANAVT
25	<i>Anabaena variabilis</i> (strain ATCC 29413 / PCC 7937) Q3M6G5_ANAVT
26	<i>Anabaena variabilis</i> (strain ATCC 29413 / PCC 7937) Q3M6G6_ANAVT
27	<i>Anaeromyxobacter</i> sp. (strain Fw109-5) A7HHI0_ANADF
28	<i>Anaeromyxobacter</i> sp. (strain Fw109-5) A7HHI1_ANADF
29	<i>Arthrobacter chlorophenolicus</i> (strain A6 / ATCC 700700 / DSM 12829 / JCM 12360) B8HJ58_ARTCA
30	<i>Arthrobacter nitroguajacolicus</i> A4V8K8_9MICC
31	<i>Azoarcus</i> sp. (strain BH72) A1K4X6_AZOSB
32	<i>Azoarcus</i> sp. (strain BH72) A1K8M8_AZOSB
33	<i>Azoarcus</i> sp. (strain BH72) A1K8Q9_AZOSB
34	<i>Bacillus anthracis</i> Q81R77_BACAN
35	<i>Bacillus anthracis</i> (strain CDC 684 / NRRL 3495) C3LKC8_BACAC
36	<i>Bacillus cereus</i> 172560W C2P7Y4_BACCE
37	<i>Bacillus cereus</i> 95/8201 C2TFW9_BACCE
38	<i>Bacillus cereus</i> A1BYI4_BACCE
39	<i>Bacillus cereus</i> A1BZT6_BACCE
40	<i>Bacillus cereus</i> AH621 C2PY00_BACCE
41	<i>Bacillus cereus</i> AH676 C2YJM0_BACCE
42	<i>Bacillus cereus</i> ATCC 10876 C2N145_BACCE
43	<i>Bacillus cereus</i> BDRD-Cer4 C2T6L0_BACCE
44	<i>Bacillus cereus</i> BDRD-ST26 C2SCP6_BACCE
45	<i>Bacillus cereus</i> F65185 C2XMK4_BACCE
46	<i>Bacillus cereus</i> H3081.97 B5Z5J6_BACCE
47	<i>Bacillus cereus</i> NVH0597-99 B3Z968_BACCE
48	<i>Bacillus cereus</i> R309803 C2QHL7_BACCE
49	<i>Bacillus cereus</i> Rock3-42 C2VT33_BACCE
50	<i>Bacillus cereus</i> (strain AH187) B7I119_BACC7

51	Bacillus cereus (strain AH820) B7JLM7_BACC0
52	Bacillus cereus (strain AH820) B7JU61_BACC0
53	Bacillus cereus (strain ATCC 14579 / DSM 31) Q817S7_BACCR
54	Bacillus cereus (strain G9842) B7ITS9_BACC2
55	Bacillus cereus (strain ZK / E33L) Q63BZ9_BACCZ
56	Bacillus cereus W B3YTA2_BACCE
57	Bacillus mycoides Rock3-17 C3B0W1_BACMY
58	Bacillus subtilis YDDK_BACSU
59	Bacillus thuringiensis serovar andalousiensis BGSC 4AW1 C3G2D4_BACTU
60	Bacillus thuringiensis serovar andalousiensis BGSC 4AW1 C3GCI3_BACTU
61	Bacillus thuringiensis serovar monterrey BGSC 4AJ1 C3F103_BACTU
62	Bacillus thuringiensis serovar pondicheriensis BGSC 4BA1 C3GI95_BACTU
63	Bacillus thuringiensis serovar pulsiensis BGSC 4CC1 C3HH54_BACTU
64	bacterium Ellin514 B9XJV3_9BACT
65	bacterium Ellin514 B9XT12_9BACT
66	Bacteroides dorei DSM 17855 B6VWV8_9BACE
67	Bacteroides ovatus ATCC 8483 A7LTE8_BACOV
68	Bacteroides ovatus ATCC 8483 A7M4L8_BACOV
69	Bacteroides ovatus ATCC 8483 A7M4L9_BACOV
70	Bacteroides sp. 2 2 4 C3QSH1_9BACE
71	Bacteroides sp. 2 2 4 C3R0H4_9BACE
72	Bacteroides sp. D1 C3QAR9_9BACE
73	Bacteroides sp. D4 C3RB07_9BACE
74	Bacteroides thetaiotaomicron Q8A3F1_BACTN
75	Bacteroides uniformis ATCC 8492 A7UZX0_BACUN
76	Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / NCTC 11154) A6L6F9_BACV8
77	Beggiatoa sp. PS A7BQY8_9GAMM
78	Beggiatoa sp. PS A7BWJ5_9GAMM
79	Beijerinckia indica subsp. indica (strain ATCC 9039 / DSM 1715 / NCIB 8712) B2ILB7_BEII9
80	Bifidobacterium longum subsp. infantis CCUG 52486 C5ECT5_BIFLO
81	Blautia hansenii DSM 20583 B7XY17_RUMHA
82	Bordetella bronchiseptica Q7WQ48_BORBR
83	Bradyrhizobium sp. (strain BTAi1 / ATCC BAA-1182) A5EGM8_BRASB
84	Bradyrhizobium sp. (strain BTAi1 / ATCC BAA-1182) A5EU74_BRASB
85	Bradyrhizobium sp. (strain ORS278) A4YL05_BRASO
86	Bradyrhizobium sp. (strain ORS278) A4YQ51_BRASO
87	Brevibacillus brevis (strain 47 / JCM 6285 / NBRC 100599) C0ZIP1_BREBN
88	Brucella abortus Q57E10_BRUAB
89	Brucella abortus Q57FA5_BRUAB
90	Brucella abortus str. 2308 A C4IP75_BRUAB
91	Brucella abortus str. 2308 A C4IQ27_BRUAB
92	Brucella abortus (strain 2308) Q2YN91_BRUA2
93	Brucella abortus (strain 2308) Q2YPC4_BRUA2
94	Brucella abortus (strain S19) B2S4Y7_BRUA1
95	Brucella abortus (strain S19) B2S8Z8_BRUA1
96	Brucella canis (strain ATCC 23365 / NCTC 10854) A9MAB8_BRUC2
97	Brucella ceti str. Cudo C0G5K2_9RHIZ
98	Brucella melitensis biotype 2 (strain ATCC 23457) C0RGW8_BRUMB
99	Brucella melitensis Q8YF53_BRUME
100	Brucella ovis (strain ATCC 25840 / 63/290 / NCTC 10512) A5VNI7_BRUO2

101	<i>Brucella ovis</i> (strain ATCC 25840 / 63/290 / NCTC 10512) A5VPS1_BRUO2
102	<i>Brucella suis</i> Q8G1I0_BRUSU
103	<i>Brucella suis</i> (strain ATCC 23445 / NCTC 10510) B0CJM4_BRUSI
104	<i>Brucella suis</i> (strain ATCC 23445 / NCTC 10510) B0CL60_BRUSI
105	<i>Burkholderia ambifaria</i> (strain MC40-6) B1YX30_BURA4
106	<i>Burkholderia glumae</i> BGR1_C5ADB1_BURGL
107	<i>Burkholderia multivorans</i> (strain ATCC 17616 / 249) A9AIH1_BURM1
108	<i>Burkholderia thailandensis</i> (strain E264 / ATCC 700388 / DSM 13276 / CIP 106301) Q2STL3_BURTA
109	<i>Burkholderia xenovorans</i> (strain LB400) Q13FM1_BURXL
110	<i>Campylobacter coli</i> RM2228_Q4HDI4_CAMCO
111	<i>Capnocytophaga ochracea</i> DSM 7271_C1QKW6_CAPOC
112	<i>Catenulispora acidiphila</i> DSM 44928_C1QPT7_9ACTO
113	<i>Catenulispora acidiphila</i> DSM 44928_C1QPT9_9ACTO
114	<i>Catenulispora acidiphila</i> DSM 44928_C1QVU3_9ACTO
115	<i>Caulobacter crescentus</i> Q9A8E1_CAUCR
116	<i>Caulobacter crescentus</i> (strain NA1000 / CB15N) B8H592_CAUCN
117	<i>Caulobacter</i> sp. (strain K31) B0SV40_CAUSK
118	<i>Chlorobaculum parvum</i> (strain NCIB 8327) B3QMC2_CHLP8
119	<i>Chlorobaculum parvum</i> (strain NCIB 8327) B3QNJ9_CHLP8
120	<i>Chlorobaculum parvum</i> (strain NCIB 8327) B3QPF1_CHLP8
121	<i>Chlorobium ferrooxidans</i> DSM 13031_Q0YTA6_9CHLB
122	<i>Chlorobium limicola</i> (strain DSM 245 / NBRC 103803) B3EDA8_CHLL2
123	<i>Chlorobium phaeobacteroides</i> (strain BS1) B3EQ75_CHLPB
124	<i>Chlorobium phaeobacteroides</i> (strain DSM 266) A1BCV0_CHLPD
125	<i>Chlorobium phaeobacteroides</i> (strain DSM 266) A1BD62_CHLPD
126	<i>Chlorobium phaeobacteroides</i> (strain DSM 266) A1BER4_CHLPD
127	<i>Chlorobium phaeobacteroides</i> (strain DSM 266) A1BEW4_CHLPD
128	<i>Chlorobium phaeobacteroides</i> (strain DSM 266) A1BEX3_CHLPD
129	<i>Chlorobium phaeobacteroides</i> (strain DSM 266) A1BG03_CHLPD
130	<i>Chlorobium tepidum</i> Q8KCF6_CHLTE
131	<i>Chlorobium tepidum</i> Q8KDK2_CHLTE
132	<i>Chloroflexus aurantiacus</i> (strain ATCC 29364 / DSM 637 / Y-400-fl) B9LJZ6_CHLSY
133	<i>Chloroflexus aurantiacus</i> (strain ATCC 29366 / DSM 635 / J-10-fl) A9WHX9_CHLAA
134	<i>Chloroherpeton thalassium</i> (strain ATCC 35110 / GB-78) B3QW13_CHLT3
135	<i>Chloroherpeton thalassium</i> (strain ATCC 35110 / GB-78) B3QZ81_CHLT3
136	<i>Chthoniobacter flavus</i> Ellin428_B4CWT1_9BACT
137	<i>Chthoniobacter flavus</i> Ellin428_B4CYW5_9BACT
138	<i>Clostridium beijerinckii</i> (strain ATCC 51743 / NCIMB 8052) A6LX75_CLOB8
139	<i>Clostridium beijerinckii</i> (strain ATCC 51743 / NCIMB 8052) A6LX76_CLOB8
140	<i>Clostridium bolteae</i> ATCC BAA-613_A8RK35_9CLOT
141	<i>Clostridium bolteae</i> ATCC BAA-613_A8RZZ9_9CLOT
142	<i>Clostridium botulinum</i> (strain Eklund 17B / Type B) B2TJC8_CLOBB
143	<i>Clostridium butyricum</i> 5521_B1QWU9_CLOBU
144	<i>Clostridium butyricum</i> 5521_B1QWV1_CLOBU
145	<i>Clostridium butyricum</i> E4 str. BoNT E BL5262_C4IHA9_CLOBU
146	<i>Clostridium butyricum</i> E4 str. BoNT E BL5262_C4IHB1_CLOBU
147	<i>Clostridium hylemonae</i> DSM 15053_C0BZ05_9CLOT
148	<i>Clostridium perfringens</i> B str. ATCC 3626_B1R789_CLOPE
149	<i>Clostridium</i> sp. M62/1_C0FML8_9CLOT
150	<i>Clostridium thermocellum</i> DSM 4150_B4BDH0_CLOTM

151	<i>Clostridium thermocellum</i> (strain ATCC 27405 / DSM 1237) A3DEI8_CLOTH
152	<i>Clostridium thermocellum</i> (strain ATCC 27405 / DSM 1237) A3DFE7_CLOTH
153	<i>Colwellia psychrerythraea</i> (strain 34H / ATCC BAA-681) Q484I8_COLP3
154	<i>Colwellia psychrerythraea</i> (strain 34H / ATCC BAA-681) Q488J9_COLP3
155	<i>Congregibacter litoralis</i> KT71 A4ACG4_9GAMM
156	<i>Coprococcus eutactus</i> ATCC 27759 A8SY38_9FIRM
157	<i>Coprococcus eutactus</i> ATCC 27759 A8SY52_9FIRM
158	<i>Cyanothece</i> sp. PCC 7822 B4B2I6_9CHRO
159	<i>Cyanothece</i> sp. PCC 8802 B4BUR4_9CHRO
160	<i>Cyanothece</i> sp. PCC 8802 B4BVU7_9CHRO
161	<i>Cyanothece</i> sp. PCC 8802 B4BYU0_9CHRO
162	<i>Cyanothece</i> sp. PCC 8802 B4BZP7_9CHRO
163	<i>Cyanothece</i> sp. PCC 8802 B4C3A5_9CHRO
164	<i>Cyanothece</i> sp. PCC 8802 B4C520_9CHRO
165	<i>Cyanothece</i> sp. (strain ATCC 51142) B1WZ79_CYAA5
166	<i>Cyanothece</i> sp. (strain PCC 7424) B7K6V7_CYAP7
167	<i>Cyanothece</i> sp. (strain PCC 7424) B7K6V8_CYAP7
168	<i>Cyanothece</i> sp. (strain PCC 7424) B7K751_CYAP7
169	<i>Cyanothece</i> sp. (strain PCC 7424) B7K9I0_CYAP7
170	<i>Cyanothece</i> sp. (strain PCC 7424) B7K9I1_CYAP7
171	<i>Cyanothece</i> sp. (strain PCC 7424) B7K9N6_CYAP7
172	<i>Cyanothece</i> sp. (strain PCC 7424) B7K9Q8_CYAP7
173	<i>Cyanothece</i> sp. (strain PCC 7424) B7KBC3_CYAP7
174	<i>Cyanothece</i> sp. (strain PCC 7424) B7KBK1_CYAP7
175	<i>Cyanothece</i> sp. (strain PCC 7424) B7KCU4_CYAP7
176	<i>Cyanothece</i> sp. (strain PCC 7424) B7KD29_CYAP7
177	<i>Cyanothece</i> sp. (strain PCC 7424) B7KEE8_CYAP7
178	<i>Cyanothece</i> sp. (strain PCC 7424) B7KFY0_CYAP7
179	<i>Cyanothece</i> sp. (strain PCC 7424) B7KJH4_CYAP7
180	<i>Cyanothece</i> sp. (strain PCC 7424) B7KJY5_CYAP7
181	<i>Cyanothece</i> sp. (strain PCC 7424) B7KMW7_CYAP7
182	<i>Cyanothece</i> sp. (strain PCC 7424) B7KMW9_CYAP7
183	<i>Cyanothece</i> sp. (strain PCC 7425 / ATCC 29141) B8HMW8_CYAP4
184	<i>Cyanothece</i> sp. (strain PCC 7425 / ATCC 29141) B8HPE5_CYAP4
185	<i>Cyanothece</i> sp. (strain PCC 7425 / ATCC 29141) B8HVB7_CYAP4
186	<i>Cyanothece</i> sp. (strain PCC 8801) B7JVD1_CYAP8
187	<i>Cyanothece</i> sp. (strain PCC 8801) B7JVT5_CYAP8
188	<i>Cyanothece</i> sp. (strain PCC 8801) B7JZR8_CYAP8
189	<i>Cyanothece</i> sp. (strain PCC 8801) B7K1R3_CYAP8
190	<i>Cyanothece</i> sp. (strain PCC 8801) B7K2J6_CYAP8
191	<i>Cyanothece</i> sp. (strain PCC 8801) B7K3U9_CYAP8
192	<i>Cyanothece</i> sp. (strain PCC 8801) B7K4M2_CYAP8
193	<i>Cyanothece</i> sp. (strain PCC 8801) B7K5H1_CYAP8
194	<i>Dechloromonas aromatica</i> (strain RCB) Q47A02_DECAR
195	<i>Dechloromonas aromatica</i> (strain RCB) Q47BW0_DECAR
196	<i>Dechloromonas aromatica</i> (strain RCB) Q47DY5_DECAR
197	<i>Desulfatibacillum alkenivorans</i> (strain AK-01) B8F9F4_DESAA
198	<i>Desulfatibacillum alkenivorans</i> (strain AK-01) B8FEW8_DESAA
199	<i>Desulfitobacterium hafniense</i> (strain Y51) Q24NL9_DESHY
200	<i>Desulfitobacterium hafniense</i> (strain Y51) Q24TJ9_DESHY

201	Desulfobacterium autotrophicum (strain ATCC 43914 / DSM 3382 / HRM2) C0QC05_DESAH
202	Desulfococcus oleovorans (strain DSM 6200 / Hxd3) A8ZZH8_DESOH
203	Desulfococcus oleovorans (strain DSM 6200 / Hxd3) A9A029_DESOH
204	Desulfonatronospira thiodismutans ASO3-1 C0GPH8_9DELT
205	Desulfuromonas acetoxidans DSM 684 Q1JX43_DESAC
206	Diaphorobacter sp. (strain TPSY) B9MGR6_DIAST
207	Dorea formicigenerans ATCC 27755 B0G6J3_9FIRM
208	Dorea longicatena DSM 13814 A6BKR7_9FIRM
209	Erythrobacter litoralis (strain HTCC2594) Q2NDU5_ERYLH
210	Erythrobacter sp. NAP1 A3WA93_9SPHN
211	Erythrobacter sp. NAP1 A3WB86_9SPHN
212	Erythrobacter sp. NAP1 A3WHL2_9SPHN
213	Erythrobacter sp. SD-21 A5P909_9SPHN
214	Erythrobacter sp. SD-21 A5PAK2_9SPHN
215	<i>Escherichia coli</i> O6:K15:H31 (strain 536 / UPEC) Q0TGL3_EC0L5
216	<i>Escherichia coli</i> O6 Q8FGH9_EC0L6
217	<i>Escherichia coli</i> O7:K1 (strain IA139 / ExPEC) B7NL44_EC07I
218	<i>Escherichia sp. 3 2 53FAA C1HM08 9ESCH</i>
219	Eubacterium eligens (strain ATCC 27750 / VPI C15-48) C4Z355_9FIRM
220	Eubacterium hallii DSM 3353 C0ESW3_9FIRM
221	Eubacterium siraeum DSM 15702 B0MKZ3_9FIRM
222	Eubacterium siraeum DSM 15702 B0MMI9_9FIRM
223	<i>Faecalibacterium prausnitzii</i> M21/2 A8S996_9FIRM
224	Finexgoldia magna (strain ATCC 29328) B0S0S4_FINM2
225	Flavobacteria bacterium BBFL7 Q26CI6_9BACT
226	Frankia alni (strain ACN14a) Q0REB5_FRAAA
227	Frankia alni (strain ACN14a) Q0REH0_FRAAA
228	Frankia alni (strain ACN14a) Q0REH8_FRAAA
229	Frankia alni (strain ACN14a) Q0RF07_FRAAA
230	Frankia alni (strain ACN14a) Q0RFT3_FRAAA
231	Frankia alni (strain ACN14a) Q0RFV3_FRAAA
232	Frankia alni (strain ACN14a) Q0RGM6_FRAAA
233	Frankia alni (strain ACN14a) Q0RI85_FRAAA
234	Frankia alni (strain ACN14a) Q0RIN9_FRAAA
235	Frankia alni (strain ACN14a) Q0RIR9_FRAAA
236	Frankia alni (strain ACN14a) Q0RIT4_FRAAA
237	Frankia alni (strain ACN14a) Q0RIX3_FRAAA
238	Frankia alni (strain ACN14a) Q0RKI0_FRAAA
239	Frankia alni (strain ACN14a) Q0RNC0_FRAAA
240	Frankia alni (strain ACN14a) Q0RPP6_FRAAA
241	Frankia alni (strain ACN14a) Q0RPX3_FRAAA
242	Frankia alni (strain ACN14a) Q0RPX4_FRAAA
243	Frankia alni (strain ACN14a) Q0RRH7_FRAAA
244	Frankia alni (strain ACN14a) Q0RSJ2_FRAAA
245	Frankia alni (strain ACN14a) Q0RSZ9_FRAAA
246	Frankia alni (strain ACN14a) Q0RT62_FRAAA
247	Frankia alni (strain ACN14a) Q0RTF6_FRAAA
248	Frankia sp. (strain CcI3) Q2J5A8_FRASC
249	Frankia sp. (strain CcI3) Q2J7D9_FRASC
250	Frankia sp. (strain CcI3) Q2J7E9_FRASC

251	Frankia sp. (strain CcI3) Q2J8P0_FRASC
252	Frankia sp. (strain CcI3) Q2JBL6_FRASC
253	Frankia sp. (strain CcI3) Q2JCV5_FRASC
254	Frankia sp. (strain CcI3) Q2JEN2_FRASC
255	Frankia sp. (strain CcI3) Q2JGC9_FRASC
256	Frankia sp. (strain EAN1pec) A8KXQ9_FRASN
257	Frankia sp. (strain EAN1pec) A8KZ03_FRASN
258	Frankia sp. (strain EAN1pec) A8L0X3_FRASN
259	Frankia sp. (strain EAN1pec) A8L102_FRASN
260	Frankia sp. (strain EAN1pec) A8L106_FRASN
261	Frankia sp. (strain EAN1pec) A8L235_FRASN
262	Frankia sp. (strain EAN1pec) A8L2D0_FRASN
263	Frankia sp. (strain EAN1pec) A8L2Q4_FRASN
264	Frankia sp. (strain EAN1pec) A8L472_FRASN
265	Frankia sp. (strain EAN1pec) A8L7E0_FRASN
266	Frankia sp. (strain EAN1pec) A8L863_FRASN
267	Frankia sp. (strain EAN1pec) A8L8F6_FRASN
268	Frankia sp. (strain EAN1pec) A8L8G1_FRASN
269	Frankia sp. (strain EAN1pec) A8L8G2_FRASN
270	Frankia sp. (strain EAN1pec) A8L8G9_FRASN
271	Frankia sp. (strain EAN1pec) A8LA46_FRASN
272	Frankia sp. (strain EAN1pec) A8LG62_FRASN
273	Frankia sp. (strain EAN1pec) A8LG86_FRASN
274	Frankia sp. (strain EAN1pec) A8LG93_FRASN
275	Frankia sp. (strain EAN1pec) A8LGL3_FRASN
276	Frankia sp. (strain EAN1pec) A8LGX9_FRASN
277	Fusobacterium nucleatum subsp. nucleatum Q8RFH6_FUSNN
278	Fusobacterium nucleatum subsp. polymorphum ATCC 10953 A5TW81_FUSNP
279	Fusobacterium nucleatum subsp. vincentii ATCC 49256 Q7P7J3_FUSNV
280	Fusobacterium sp. 4_1_13 C3WSB4_9FUSO
281	Fusobacterium sp. 7_1 C3WUA1_9FUSO
282	gamma proteobacterium HTCC5015 B5JTY8_9GAMM
283	gamma proteobacterium HTCC5015 B5JV79_9GAMM
284	gamma proteobacterium NOR5-3 B8KL00_9GAMM
285	Gemmatimonas aurantiaca (strain T-27 / DSM 14586 / JCM 11422 / NBRC 100505) C1ADH7_GEMAT
286	Geobacter lovleyi (strain ATCC BAA-1151 / DSM 17278 / SZ) B3E9D8_GEOLS
287	Geobacter lovleyi (strain ATCC BAA-1151 / DSM 17278 / SZ) B3EB23_GEOLS
288	Geobacter metallireducens (strain GS-15 / ATCC 53774 / DSM 7210) Q39WD7_GEOMG
289	Geobacter metallireducens (strain GS-15 / ATCC 53774 / DSM 7210) Q39WE1_GEOMG
290	Geobacter sp. (strain FRC-32) B9M1H6_GEOSF
291	Geobacter sulfurreducens Q749Z9_GEOSL
292	Geobacter uraniireducens (strain Rf4) A5G548_GEOUR
293	Geobacter uraniireducens (strain Rf4) A5GF28_GEOUR
294	Geodermatophilus obscurus DSM 43160 C0U2U1_9ACTO
295	Geodermatophilus obscurus DSM 43160 C0U6E5_9ACTO
296	Geodermatophilus obscurus DSM 43160 C0U6V3_9ACTO
297	Geodermatophilus obscurus DSM 43160 C0U754_9ACTO
298	Geodermatophilus obscurus DSM 43160 C0U991_9ACTO
299	Geodermatophilus obscurus DSM 43160 C0U9P3_9ACTO
300	Gloeobacter violaceus Q7NE51_GLOVI

301	Haemophilus parasuis serovar 5 (strain SH0165) B8F7Y2_HAEPS
302	Haliangium ochraceum DSM 14365 C1URW1_9DELT
303	Helicobacter pullorum MIT 98-5489 C5F1P7_9HELI
304	Hermiinimonas arsenicoxydans A4G5B3_HERAR
305	Hydrogenivirga sp. 128-5-R1-1 A8UX53_9AQUI
306	Hyphomonas neptunium (strain ATCC 15444) Q0C4A7_HYPNA
307	Janthinobacterium sp. (strain Marseille) (Minibacterium massiliensis) A6T1M2_JANMA
308	Kordia algicida OT-1 A9DMA4_9FLAO
309	Leptothrix cholodnii (strain ATCC 51168 / LMG 8142 / SP-6) B1XZU8_LEPCP
310	Leptothrix cholodnii (strain ATCC 51168 / LMG 8142 / SP-6) B1Y6N4_LEPCP
311	Leuconostoc mesenteroides subsp. mesenteroides (strain ATCC 8293 / NCDO 523) Q03X98_LEUMM
312	Limnobacter sp. MED105 A6GUE8_9BURK
313	Limnobacter sp. MED105 A6GUW5_9BURK
314	Magnetospirillum magneticum AMB-1 Q2W1R2_MAGMM
315	Manganese-oxidizing bacterium (strain SI85-9A1) Q1YGV4_MOBAS
316	Maricaulis maris (strain MCS10) Q0ALB9_MARMM
317	marine gamma proteobacterium HTCC2080 A0Z485_9GAMM
318	Marinobacter algicola DG893 A6F385_9ALTE
319	Marinobacter aquaeolei (strain ATCC 700491 / DSM 11845 / VT8) A1U034_MARAV
320	Marinomonas sp. MED121 A3Y9R2_9GAMM
321	Mariprofundus ferrooxydans PV-1 Q0EWQ0_9PROT
322	Mariprofundus ferrooxydans PV-1 Q0F0H5_9PROT
323	Methanococcoides burtonii (strain DSM 6242) Q12US3_METBU
324	Methanococcoides burtonii (strain DSM 6242) Q12YL2_METBU
325	Methanosarcina barkeri (strain Fusaro / DSM 804) Q46EG7_METBF
326	Methanosarcina barkeri (strain Fusaro / DSM 804) Q46F16_METBF
327	Methylobacterium chloromethanicum (strain CM4 / NCIMB 13688) B7L2V6_METC4
328	Methylobacterium chloromethanicum (strain CM4 / NCIMB 13688) B7L2W0_METC4
329	Methylobacterium chloromethanicum (strain CM4 / NCIMB 13688) B7L2Y1_METC4
330	Methylobacterium chloromethanicum (strain CM4 / NCIMB 13688) B7L3I7_METC4
331	Methylobacterium extorquens (strain ATCC 14718 / DSM 1338 / AM1) C5B6R7_METEX
332	Methylobacterium extorquens (strain PA1) A9W413_METEP
333	Methylobacterium nodulans (strain ORS2060 / LMG 21967) B8IV87_METNO
334	Methylobacterium nodulans (strain ORS2060 / LMG 21967) B8IXX4_METNO
335	Methylobacterium populi (strain ATCC BAA-705 / NCIMB 13946 / BJ001) B1ZF35_METPB
336	Methylococcus capsulatus Q602Q0_METCA
337	Methylophaga thiooxidans C0N9D3_9GAMM
338	Microcoleus chthonoplastes PCC 7420 B4VH43_9CYAN
339	Microcoleus chthonoplastes PCC 7420 B4VH44_9CYAN
340	Microcoleus chthonoplastes PCC 7420 B4VIZ2_9CYAN
341	Microcoleus chthonoplastes PCC 7420 B4VJM6_9CYAN
342	Microcoleus chthonoplastes PCC 7420 B4VL88_9CYAN
343	Microcoleus chthonoplastes PCC 7420 B4VPI4_9CYAN
344	Microcoleus chthonoplastes PCC 7420 B4VR58_9CYAN
345	Microcoleus chthonoplastes PCC 7420 B4VS12_9CYAN
346	Microcoleus chthonoplastes PCC 7420 B4VSH8_9CYAN
347	Microcoleus chthonoplastes PCC 7420 B4VTX6_9CYAN
348	Microcoleus chthonoplastes PCC 7420 B4VXA1_9CYAN
349	Microcoleus chthonoplastes PCC 7420 B4VXV2_9CYAN
350	Microcoleus chthonoplastes PCC 7420 B4VY01_9CYAN

351	<i>Microcoleus chthonoplastes</i> PCC 7420 B4W0R0_9CYAN
352	<i>Microcoleus chthonoplastes</i> PCC 7420 B4W502_9CYAN
353	<i>Microcystis aeruginosa</i> PCC 7806 A8YCI0_MICAE
354	<i>Microcystis aeruginosa</i> PCC 7806 A8YJP3_MICAE
355	<i>Microcystis aeruginosa</i> PCC 7806 A8YJP4_MICAE
356	<i>Microcystis aeruginosa</i> PCC 7806 A8YKR2_MICAE
357	<i>Microcystis aeruginosa</i> PCC 7806 A8YMR4_MICAE
358	<i>Microcystis aeruginosa</i> (strain NIES-843) B0JFU8_MICAN
359	<i>Microcystis aeruginosa</i> (strain NIES-843) B0JFU9_MICAN
360	<i>Microcystis aeruginosa</i> (strain NIES-843) B0JHN4_MICAN
361	<i>Microcystis aeruginosa</i> (strain NIES-843) B0JKB2_MICAN
362	<i>Microcystis aeruginosa</i> (strain NIES-843) B0JNX3_MICAN
363	<i>Microcystis aeruginosa</i> (strain NIES-843) B0JQF5_MICAN
364	<i>Micromonospora</i> sp. ATCC 39149 C4RNC8_9ACTO
365	<i>Mycobacterium</i> sp. (strain KMS) A1UCZ8_MYCSK
366	<i>Mycobacterium</i> sp. (strain MCS) Q1BC00_MYCSS
367	<i>Nitrobacter winogradskyi</i> (strain Nb-255 / ATCC 25391) Q3SW17_NITWN
368	<i>Nitrococcus mobilis</i> Nb-231 A4BPP6_9GAMM
369	<i>Nitrosococcus oceani</i> AFC27 B6C6I1_9GAMM
370	<i>Nitrosococcus oceani</i> (strain ATCC 19707 / NCIMB 11848) Q3JF60_NITOC
371	<i>Nodularia spumigena</i> CCY 9414 A0ZGX8_NODSP
372	<i>Nodularia spumigena</i> CCY 9414 A0ZJM4_NODSP
373	<i>Nostoc punctiforme</i> (strain ATCC 29133 / PCC 73102) B2IT78_NOSP7
374	<i>Nostoc punctiforme</i> (strain ATCC 29133 / PCC 73102) B2IT96_NOSP7
375	<i>Nostoc punctiforme</i> (strain ATCC 29133 / PCC 73102) B2IUT6_NOSP7
376	<i>Nostoc punctiforme</i> (strain ATCC 29133 / PCC 73102) B2IVL0_NOSP7
377	<i>Nostoc punctiforme</i> (strain ATCC 29133 / PCC 73102) B2IXB5_NOSP7
378	<i>Nostoc punctiforme</i> (strain ATCC 29133 / PCC 73102) B2J0H4_NOSP7
379	<i>Nostoc punctiforme</i> (strain ATCC 29133 / PCC 73102) B2J0I1_NOSP7
380	<i>Nostoc punctiforme</i> (strain ATCC 29133 / PCC 73102) B2J919_NOSP7
381	<i>Nostoc punctiforme</i> (strain ATCC 29133 / PCC 73102) B2J9B9_NOSP7
382	<i>Nostoc punctiforme</i> (strain ATCC 29133 / PCC 73102) B2J9C0_NOSP7
383	<i>Oenococcus oeni</i> ATCC BAA-1163 A0NH77_OENOE
384	<i>Oenococcus oeni</i> (strain BAA-331 / PSU-1) Q04DI5_OENOB
385	<i>Oligotropha carboxidovorans</i> (strain ATCC 49405 / DSM 1227 / OM5) B6JHA2_OLICO
386	<i>Opitutus terrae</i> (strain DSM 11246 / PB90-1) B1ZSG7_OPITP
387	<i>Oribacterium sinus</i> F0268 C2KUX3_9FIRM
388	<i>Oribacterium sinus</i> F0268 C2KYG0_9FIRM
389	<i>Parabacteroides johnsonii</i> DSM 18315 B7BBI1_9PORP
390	<i>Paracoccus denitrificans</i> (strain Pd 1222) A1AY86_PARDP
391	<i>Parvibaculum lavamentivorans</i> (strain DS-1 / DSM 13023 / NCIMB 13966) A7HRR1_PARL1
392	<i>Pelobacter carbinolicus</i> (strain DSM 2380 / Gra Bd 1) Q3A0F4_PELCD
393	<i>Pelobacter carbinolicus</i> (strain DSM 2380 / Gra Bd 1) Q3A0F6_PELCD
394	<i>Pelodictyon phaeoclathratiforme</i> (strain DSM 5477 / BU-1) B4SA08_PELPB
395	<i>Pelodictyon phaeoclathratiforme</i> (strain DSM 5477 / BU-1) B4SBS9_PELPB
396	<i>Pelodictyon phaeoclathratiforme</i> (strain DSM 5477 / BU-1) B4SBT3_PELPB
397	<i>Pelodictyon phaeoclathratiforme</i> (strain DSM 5477 / BU-1) B4SC50_PELPB
398	<i>Pelodictyon phaeoclathratiforme</i> (strain DSM 5477 / BU-1) B4SC53_PELPB
399	<i>Pelodictyon phaeoclathratiforme</i> (strain DSM 5477 / BU-1) B4SEB5_PELPB
400	<i>Pelodictyon phaeoclathratiforme</i> (strain DSM 5477 / BU-1) B4SFR7_PELPB

401	<i>Pelodictyon phaeoclathratiforme</i> (strain DSM 5477 / BU-1) B4SGS9_PELPB
402	<i>Pelodictyon phaeoclathratiforme</i> (strain DSM 5477 / BU-1) B4SGT1_PELPB
403	<i>Pelodictyon phaeoclathratiforme</i> (strain DSM 5477 / BU-1) B4SH53_PELPB
404	<i>Pelodictyon phaeoclathratiforme</i> (strain DSM 5477 / BU-1) B4SHG6_PELPB
405	<i>Pirellula</i> sp. Q7UEH8_RHOBA
406	<i>Pirellula</i> sp. Q7UFB2_RHOBA
407	<i>Planctomyces limnophilus</i> DSM 3776 C1ZL96_PLALI
408	<i>Planctomyces limnophilus</i> DSM 3776 C1ZL97_PLALI
409	<i>Planctomyces limnophilus</i> DSM 3776 C1ZM92_PLALI
410	<i>Planctomyces maris</i> DSM 8797 A6CAG7_9PLAN
411	<i>Planctomyces maris</i> DSM 8797 A6CGQ8_9PLAN
412	<i>Polaromonas</i> sp. (strain JS666 / ATCC BAA-500) Q11ZR2_POLSJ
413	<i>Polaromonas</i> sp. (strain JS666 / ATCC BAA-500) Q122B9_POLSJ
414	<i>Porphyromonas gingivalis</i> Q7MTS7_PORGI
415	<i>Porphyromonas gingivalis</i> Q7MX37_PORGI
416	<i>Porphyromonas gingivalis</i> (strain ATCC 33277 / DSM 20709 / JCM 12257) B2RLS0_PORG3
417	<i>Prevotella copri</i> DSM 18205 B9ALE6_9BACT
418	<i>Prosthecochloris aestuarii</i> (strain DSM 271 / SK 413) B4S3R6_PROA2
419	<i>Prosthecochloris aestuarii</i> (strain DSM 271 / SK 413) B4S588_PROA2
420	<i>Prosthecochloris aestuarii</i> (strain DSM 271 / SK 413) B4S6D3_PROA2
421	<i>Prosthecochloris aestuarii</i> (strain DSM 271 / SK 413) B4S6U5_PROA2
422	<i>Prosthecochloris aestuarii</i> (strain DSM 271 / SK 413) B4S7V1_PROA2
423	<i>Prosthecochloris aestuarii</i> (strain DSM 271 / SK 413) B4S8G9_PROA2
424	<i>Prosthecochloris aestuarii</i> (strain DSM 271 / SK 413) B4S9P4_PROA2
425	<i>Proteus mirabilis</i> ATCC 29906 C2LI69_PROMI
426	<i>Proteus penneri</i> ATCC 35198 C0B384_9ENTR
427	<i>Pseudoalteromonas tunicata</i> D2 A4C8V4_9GAMM
428	<i>Pseudomonas aeruginosa</i> (strain PA7) A6V3V7_PSEA7
429	<i>Pseudomonas paucimobilis</i> A2PZP2_PSEPA
430	<i>Pseudomonas putida</i> (strain W619) B1JB70_PSEPW
431	<i>Pseudomonas syringae</i> pv. <i>syringae</i> (strain B728a) Q500J4_PSEU2
432	<i>Pseudovibrio</i> sp. JE062 B6R0S7_9RHOB
433	<i>Psychroflexus torquis</i> ATCC 700755 Q1VPB0_9FLAO
434	<i>Pyrobaculum aerophilum</i> Q8ZWK8_PYRAE
435	<i>Pyrobaculum arsenaticum</i> (strain DSM 13514 / JCM 11321) A4WIW1_PYRAR
436	<i>Pyrobaculum islandicum</i> (strain DSM 4184 / JCM 9189) A1RV78_PYRIL
437	<i>Reinekea blandensis</i> MED297 A4BGA1_9GAMM
438	<i>Rhizobium loti</i> Q8KGT6_RHILO
439	<i>Rhizobium</i> sp. (strain NGR234) Y4LF_RHISN
440	<i>Rhodobacter sphaeroides</i> (strain ATCC 17023 / 2.4.1 / NCIB 8253 / DSM 158) Q3IWD7_RHOS4
441	<i>Rhodobacter sphaeroides</i> (strain ATCC 17025 / ATH 2.4.3) A4X0P4_RHOS5
442	<i>Rhodococcus</i> sp. (strain RHA1) Q0RX19_RHOSR
443	<i>Rhodococcus</i> sp. (strain RHA1) Q0RX26_RHOSR
444	<i>Rhodoferax ferrireducens</i> (strain DSM 15236 / ATCC BAA-621 / T118) Q21Z38_RHOFD
445	<i>Rhodoferax ferrireducens</i> (strain DSM 15236 / ATCC BAA-621 / T118) Q21ZJ5_RHOFD
446	<i>Rhodopseudomonas palustris</i> Q6N9F4_RHOPA
447	<i>Rhodopseudomonas palustris</i> (strain BisB5) Q132Y4_RHOPS
448	<i>Rhodopseudomonas palustris</i> (strain HaA2) Q21WS6_RHOP2
449	<i>Rhodopseudomonas palustris</i> (strain TIE-1) B3Q7H7_RHOPT
450	<i>Robiginitalea biformata</i> HTCC2501 A4CGS2_9FLAO

451	Roseovarius nubinhibens ISM A3SLC4_9RHOB
452	Ruminococcus lactaris ATCC 29176 B5CS54_9FIRM
453	Saccharophagus degradans (strain 2-40 / ATCC 43961 / DSM 17024) Q21DQ4_SACD2
454	Saccharopolyspora erythraea (strain NRRL 23338) A4FF24_SACEN
455	Salinispora arenicola (strain CNS-205) A8M4S0_SALAI
456	Salinispora arenicola (strain CNS-205) A8M4S1_SALAI
457	Salmonella agona (strain SL483) B5F4B2_SALAA4
458	Salmonella dublin (strain CT_02021853) B5FLV0_SALDC
459	Salmonella enterica subsp. enterica serovar Schwarzengrund str. SL480 B5CAM1_SALET
460	Salmonella enteritidis PT4 (strain P125109) B5QYT9_SALEP
461	Salmonella gallinarum (strain 287/91 / NCTC 13346) B5RBH2_SALG2
462	Salmonella schwarzengrund (strain CVM19633) B4TVM3_SALSV
463	Shewanella baltica (strain OS223) B8EDL9_SHEB2
464	Shewanella baltica (strain OS223) B8EDM1_SHEB2
465	Shewanella denitrificans (strain OS217 / ATCC BAA-1090 / DSM 15013) Q12I73_SHEDO
466	Shewanella denitrificans (strain OS217 / ATCC BAA-1090 / DSM 15013) Q12I75_SHEDO
467	Shewanella pealeana (strain ATCC 700345 / ANG-SQ1) A8H493_SHEPA
468	Shewanella putrefaciens 200 A2UVN5_SHEPU
469	Shewanella woodyi (strain ATCC 51908 / MS32) B1KM00_SHEWM
470	Shuttleworthia satelles DSM 14600 C4GE63_9FIRM
471	Shuttleworthia satelles DSM 14600 C4GE64_9FIRM
472	Slackia heliotrinireducens DSM 20476 C4CEV5_9ACTN
473	Solibacter usitatus (strain Ellin6076) Q01R08_SOLUE
474	Solibacter usitatus (strain Ellin6076) Q01U48_SOLUE
475	Solibacter usitatus (strain Ellin6076) Q01WV2_SOLUE
476	Solibacter usitatus (strain Ellin6076) Q028E9_SOLUE
477	Sorangium cellulosum (strain So ce56) A9EU91_SORC5
478	Sorangium cellulosum (strain So ce56) A9FJE9_SORC5
479	Sorangium cellulosum (strain So ce56) A9G4K5_SORC5
480	Sorangium cellulosum (strain So ce56) A9GJ82_SORC5
481	Sorangium cellulosum (strain So ce56) A9GJ85_SORC5
482	Sorangium cellulosum (strain So ce56) A9GM89_SORC5
483	Sorangium cellulosum (strain So ce56) A9GSK2_SORC5
484	Sorangium cellulosum (strain So ce56) A9GWR0_SORC5
485	Sorangium cellulosum (strain So ce56) A9GWR7_SORC5
486	Spirosoma linguale DSM 74 C4CUS0_9SPHI
487	Spirosoma linguale DSM 74 C4D2A5_9SPHI
488	Spirosoma linguale DSM 74 C4D3U9_9SPHI
489	Staphylococcus aureus (strain MSSA476) Q6GD52_STAAS
490	Staphylococcus hominis Q8VUX6_STAHO
491	Stigmatella aurantiaca DW4/3-1 Q08W08_STIAU
492	Stigmatella aurantiaca DW4/3-1 Q099H8_STIAU
493	Streptococcus infantarius subsp. infantarius ATCC BAA-102 B1SE27_9STRE
494	Streptococcus pneumoniae CDC0288-04 B2DVK7_STRPN
495	Streptococcus pneumoniae CDC1873-00 B1S2R9_STRPN
496	Streptococcus pneumoniae Q97NR7_STRPN
497	Streptococcus pneumoniae serotype 2 (strain D39 / NCTC 7466) Q04IL6_STRP2
498	Streptococcus pneumoniae (strain ATCC 700669 / Spain 23F-1) B8ZNV7_STRPJ
499	Streptococcus pneumoniae (strain ATCC BAA-255 / R6) Q8DNF6_STRR6
500	Streptococcus pneumoniae (strain JJA) C1CGN4_STRZJ

501	<i>Streptococcus thermophilus</i> Q70CB7_STRTR
502	<i>Streptomyces avermitilis</i> Q82BJ2_STRAW
503	<i>Streptomyces avermitilis</i> Q82CN6_STRAW
504	<i>Streptomyces clavuligerus</i> ATCC 27064 B5GMC1_STRCL
505	<i>Streptomyces clavuligerus</i> ATCC 27064 B5GMC2_STRCL
506	<i>Streptomyces clavuligerus</i> ATCC 27064 B5GRK8_STRCL
507	<i>Streptomyces clavuligerus</i> ATCC 27064 B5GY23_STRCL
508	<i>Streptomyces clavuligerus</i> ATCC 27064 B5GY34_STRCL
509	<i>Streptomyces coelicolor</i> O54182_STRCO
510	<i>Streptomyces coelicolor</i> O86757_STRCO
511	<i>Streptomyces coelicolor</i> O86768_STRCO
512	<i>Streptomyces coelicolor</i> Q8CJY8_STRCO
513	<i>Streptomyces coelicolor</i> Q93JD1_STRCO
514	<i>Streptomyces coelicolor</i> Q9L0M8_STRCO
515	<i>Streptomyces coelicolor</i> Q9L1H5_STRCO
516	<i>Streptomyces coelicolor</i> Q9RL12_STRCO
517	<i>Streptomyces griseus</i> subsp. <i>griseus</i> (strain JCM 4626 / NBRC 13350) B1VYB9_STRGG
518	<i>Streptomyces griseus</i> subsp. <i>griseus</i> (strain JCM 4626 / NBRC 13350) B1VZZ4_STRGG
519	<i>Streptomyces griseus</i> subsp. <i>griseus</i> (strain JCM 4626 / NBRC 13350) B1W249_STRGG
520	<i>Streptomyces pristinaespiralis</i> ATCC 25486 B5H9R8_STRPR
521	<i>Streptomyces pristinaespiralis</i> ATCC 25486 B5HBM5_STRPR
522	<i>Streptomyces</i> sp. Mg1 B4VG65_9ACTO
523	<i>Streptosporangium roseum</i> DSM 43021 C4E2W2_STRRS
524	<i>Streptosporangium roseum</i> DSM 43021 C4E2W3_STRRS
525	<i>Streptosporangium roseum</i> DSM 43021 C4E7Z6_STRRS
526	<i>Streptosporangium roseum</i> DSM 43021 C4E912_STRRS
527	<i>Streptosporangium roseum</i> DSM 43021 C4EGT7_STRRS
528	<i>Streptosporangium roseum</i> DSM 43021 C4EH37_STRRS
529	<i>Sulfurovum</i> sp. (strain NBC37-1) A6QC40_SULNB
530	<i>Sulfurovum</i> sp. (strain NBC37-1) A6QC41_SULNB
531	<i>Sulfurovum</i> sp. (strain NBC37-1) A6QC42_SULNB
532	<i>Sulfurovum</i> sp. (strain NBC37-1) A6QC43_SULNB
533	<i>Sulfurovum</i> sp. (strain NBC37-1) A6QC45_SULNB
534	<i>Sulfurovum</i> sp. (strain NBC37-1) A6QC46_SULNB
535	<i>Synechococcus</i> sp. PCC 7335 B4WMX4_9SYNE
536	<i>Synechococcus</i> sp. PCC 7335 B4WW33_9SYNE
537	<i>Synechococcus</i> sp. PCC 7335 B4WW34_9SYNE
538	<i>Synechococcus</i> sp. PCC 7335 B4WW35_9SYNE
539	<i>Synechococcus</i> sp. PCC 7335 B4WW38_9SYNE
540	<i>Synechococcus</i> sp. (strain JA-2-3B'a(2-13)) Q2JHG5_SYNJB
541	<i>Synechococcus</i> sp. (strain JA-3-3Ab) Q2JSZ1_SYNJA
542	<i>Synechococcus</i> sp. WH 5701 A3YXY3_9SYNE
543	<i>Synechococcus</i> sp. WH 5701 A3YYR2_9SYNE
544	<i>Synechococcus</i> sp. WH 5701 A3YYT2_9SYNE
545	<i>Synechocystis</i> sp. (strain PCC 6803) P74570_SYNY3
546	<i>Syntrophobacter fumaroxidans</i> (strain DSM 10017 / MPOB) A0LH80_SYNFM
547	<i>Syntrophomonas wolfei</i> subsp. <i>wolfei</i> (strain Goettingen) Q0AUP4_SYNWW
548	<i>Syntrophus aciditrophicus</i> (strain SB) Q2LRS1_SYNAS
549	<i>Syntrophus aciditrophicus</i> (strain SB) Q2LV14_SYNAS
550	<i>Thaueria</i> sp. MZ1T C4ZIE0_9RHOO

551	Thauera sp. MZ1T C4ZIJ8_9RHOO
552	Thauera sp. MZ1T C4ZL12_9RHOO
553	Thauera sp. MZ1T C4ZM27_9RHOO
554	Thermobifida fusca (strain YX) Q47S01_THEFY
555	Thermococcus gammatolerans (strain DSM 15229 / JCM 11827 / EJ3) C5A5G6_9EURY
556	Thermomonospora curvata DSM 43183 C2AF71_THECU
557	Thermosynechococcus elongatus (strain BP-1) Q8DIX1_THEEB
558	Thermotoga neapolitana (strain ATCC 49049 / DSM 4359 / NS-E) B9K9U6_THENN
559	Thiomicrospira crunogena (strain XCL-2) Q31IR1_THICR
560	Trichodesmium erythraeum (strain IMS101) Q10WW3_TRIEI
561	Verrucomicrobiae bacterium DG1235 B5JE28_9BACT
562	Vibrio angustum S14 Q1ZMN4_9VIBR
563	Vibrio cholerae 12129(1) C2C6Y0_VIBCH
564	Vibrio cholerae 623-39 A6AEZ3_VIBCH
565	Vibrio parahaemolyticus Q87NQ6_VIBPA
566	Vibrio sp. MED222 A3Y2J7_9VIBR
567	Vibrio vulnificus (strain YJ016) Q7MK82_VIBVY
568	Xanthomonas axonopodis pv. citri (Citrus canker) Q8PHG0_XANAC
569	Xanthomonas campestris pv. campestris (strain 8004) Q4V0F1_XANC8
570	Xylanimonas cellulositytica DSM 15894 C0VF34_9MICO
571	Yersinia pestis biovar Antiqua str. B42003004 B0HCF2_YERPE
572	Yersinia pestis biovar Antiqua str. E1979001 B0HXE7_YERPE
573	Yersinia pestis biovar Antiqua str. UG05-0454 B0GIF8_YERPE
574	Yersinia pestis biovar Mediaevalis str. K1973002 B0H7U8_YERPE
575	Yersinia pestis biovar Orientalis str. F1991016 A9ZYS5_YERPE
576	Yersinia pestis biovar Orientalis str. India 195 C4H5E6_YERPE
577	Yersinia pestis biovar Orientalis str. IP275 A9ZFA1_YERPE
578	Yersinia pestis biovar Orientalis str. MG05-1020 B0GS98_YERPE
579	Yersinia pestis biovar Orientalis str. PEXU2 C4HNG7_YERPE
580	Yersinia pestis bv. Antiqua (strain Antiqua) Q1C8J6_YERPA
581	Yersinia pestis bv. Antiqua (strain Nepal516) Q1CHG0_YERPN
582	Yersinia pestis Pestoides A C4HXL1_YERPE
583	Yersinia pestis Q8CL16_YERPE
584	Yersinia pestis (strain Pestoides F) A4TK27_YERPP
585	Yersinia pseudotuberculosis Q6EVT5_YERPS
586	Yersinia pseudotuberculosis serotype IB (strain PB1/+) B2K233_YERPB
587	Yersinia pseudotuberculosis serotype O:1b (strain IP 31758) A7FIS4_YERP3
588	Yersinia pseudotuberculosis serotype O:1b (strain IP 31758) A7FN30_YERP3
589	Yersinia pseudotuberculosis serotype O:3 (strain YPIII) B1JGY8_YERP4
590	Yersinia pseudotuberculosis serotype O:3 (strain YPIII) B1JN39_YERP4
591	Zymomonas mobilis Q5NM47_ZYMMO

Table 8.2 TIR domain containing bacterial proteins. Microbial proteins identified from Chapter 3 found to contain a TIR domain. Pathogenic strains are in red and commensal strains are in green text. Numbers 80 and 218 from this list were selected for study in Chapter 3.

Title: Identification of Toll-Like Receptor 10 as a key mediator of the inflammatory response to *Listeria monocytogenes* in intestinal epithelial cells and macrophages.

Running Title: TLR10 senses *L.monocytogenes*.

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Abstract:

L.monocytogenes is a gram positive bacterium which can cause septicaemia and meningitis. Toll-Like Receptors (TLRs) are central receptors of the innate immune system that drive inflammatory responses to invading microbes such as *L.monocytogenes*. Although intestinal epithelial cells (IECs) represent the initial point of entry utilised by *L.monocytogenes* for infection, the innate immune response to *L.monocytogenes* in these cells has been poorly characterised to-date. The aim of this study was to determine which TLRs are involved in mediating the immune response to *L.monocytogenes* in IECs. We performed an RNAi screen of TLRs 1-10 in the HT-29 IEC cell line and observed the most significant reduction in chemokine output following silencing of TLR10. This effect was also observed in the macrophage cell line, THP-1. The chemokines CCL20, CCL1 and IL-8 were reduced following knockdown of TLR10. Silencing of TLR10 resulted in increased viability of *L.monocytogenes* in both HT-29 and THP-1 cells. TLR10 was found to be predominantly expressed intracellularly in epithelia and activation required viable *L.monocytogenes*. NF- κ B activation was seen to require TLR2 in addition to TLR10. Together these data indicate novel roles for TLR10 in sensing of pathogenic infection in both the epithelium and macrophages and have identified *L.monocytogenes* as a source of ligand for the orphan receptor TLR10.

Introduction

Listeria monocytogenes is a foodborne Gram-positive bacterium that can cause diseases such as septicaemia and meningitis particularly in immune-compromised individuals (1). Following ingestion, *L.monocytogenes* crosses the intestinal barrier by first invading intestinal epithelial cells. From here, infection of macrophages proceeds via phagocytosis followed by escape from the phagosome into the cytosol through the action of the pore-forming toxin Listeriolysin O (LLO) (2, 3). The infecting organisms reach their first target organs; the liver and the spleen via the lymphoid system and the blood, where they are internalized by splenic and hepatic macrophages. Here, they actively multiply until the infection is controlled by a cell-mediated immune response.

Effective immune responses to *L.monocytogenes* infection relies on coordinated innate and adaptive immune responses, with the first line of innate defence being mediated by detection of the invading bacterium by Pattern Recognition Receptors (PRRs). Several families of these receptors have been identified including the transmembrane Toll-Like Receptors (TLRs), the cytosolic NOD-Like Receptors (NLRs) and RIG-I like Receptors (RLRs) and in more recent years C-type Lectin Receptors (CLRs) (4). The activation of innate immunity by PRRs in response to *L.monocytogenes* infection is still not fully understood. *L.monocytogenes* has been shown to be recognised by TLR2 (5, 6), NOD1 (7, 8) and NOD2 (9) resulting in NF- κ B activation and pro-inflammatory gene expression. The role of TLR5 in detection of *L.monocytogenes* remains unclear as although flagellin from *L.monocytogenes* activates TLR5, bacterial mutants deficient in flagellin display an unaltered virulence (10, 11). More recent studies have identified several inflammasome components responsible for recognition of the bacterium such as NLRC4 and AIM2. Recognition of *L.monocytogenes* through these receptors results in activation of caspase-1 and cleavage of IL1- β (12). *L.monocytogenes* infection has also been shown to induce a type I interferon

response, potentially mediated by TLR2 and the adaptor protein TRIF in peritoneal macrophages (13). Most recently the IFN- β response has been shown to be triggered by nucleic acids released from *L.monocytogenes* acting through the intracellular sensor RIG-I (14). Previous *in vitro* mouse studies have predominantly used intraperitoneal *in vivo* infection models where type I IFN responses were shown to be detrimental to bacterial clearance. Recently, however, using an *in vivo* mouse model of oral *L.monocytogenes* infection Kernbauer *et al.* proved that this is only the case when infection occurs through a route other than intra-gastric, highlighting the importance of study required in epithelial mediated innate immunity (15).

Epithelial cells line the enteric mucosal surface providing barrier function against microbial invasion. Similar to immune cells, intestinal epithelial cells express many of these receptors of the innate immune system and are the first site of bacterial recognition in the intestine. Characterisation of the innate immune responses to *L.monocytogenes* infection has been studied in several cells types including endothelial cells, PBMCs, and macrophages (16). However, no extensive screen has been performed of innate mechanisms of detection of *L.monocytogenes* in the intestinal epithelia and little focussed attention has been paid to the role of specific PRRs in detecting *L.monocytogenes* infection at this barrier interface. To date only NOD2 has been directly shown to play a role in detecting *L.monocytogenes* in the intestinal epithelium (9). In this study, we examined the role of TLRs 1-10 in recognition of *L.monocytogenes* infection in epithelial cells. In doing so, we have uncovered a novel role for TLR10 in mediating the inflammatory response to infection by *L.monocytogenes* in IECs and also macrophages. As ligand specificity for TLR10 has to date remained elusive, this is the first report to show an essential role for TLR10 in mediating the inflammatory response to infection by an invasive pathogen.

Materials and Methods

Cell culture

HCT-116, HCA-7 and HEK 293T cells were maintained in DMEM (Thermo Scientific, Leics, UK), HT-29 in McCoys 5A (Thermo Scientific) and THP-1 cells in RPMI (Thermo Scientific), supplemented with 10% FCS (Thermo Scientific) and 10 µg/ml of penicillin and streptomycin (Sigma-Aldrich, Dorset, UK) and cultured in a humidified incubator at 37°C with 5% CO₂. THP-1s were differentiated into macrophages by overnight stimulation with 5 ng/ml PMA (Sigma). The ligands Pam3Cys (50µg/ml); PGN (10µg/ml); LTA (10µg/ml); FSL-1 (5µg/ml); LPS (10ng/ml); Poly(I:C) (20µg/ml); TNFα (10ng/ml) (All Invitrogen, Paisley, UK) were used for cells stimulation assays as were bacteria (below). Each of the ligands were obtained from Invitrogen.

Bacterial cell culture, infections and survivability assays

The *L.monocytogenes* strain EGD (serotype 1/2a) was a kind gift from Prof. C. Hill, (UCC, Cork, Ireland). Bacteria were grown to the logarithmic growth phase in brain heart infusion (BHI) broth (Sigma) at 37°C shaking at 200 rpm. Bacteria were subsequently washed with PBS by two steps of centrifugation (4000 x g for 5 min) and diluted in PBS for infection at multiplicity of infection (MOI) of 50:1. The cells were washed at 2h post-infection with antibiotic free cell culture medium and supplemented with cell culture medium containing Gentamicin (Invitrogen) (50µg/ml). Cells were then incubated for the times indicated in the results section. For bacterial survival assays, the cells were washed at 8 hours post-infection and treated with 0.2% Triton X-100 PBS. The lysates were then subjected to serial dilution and spotted on a BHI agar plate. Colony forming units (C.F.U.)/lysate of internalised bacteria was then determined. The *Salmonella typhimurium* strain SJW1103 (wild type) was a kind gift from Prof. P. O'Toole (UCC, Cork, Ireland). Bacteria were grown to the logarithmic

growth phase in LB broth (Sigma) at 37°C shaking at 200 rpm. Bacteria were subsequently washed with PBS by two steps of centrifugation (4000 x g for 5 min) and diluted in PBS for infection at multiplicity of infection (MOI) of 10:1.

siRNA transfection

All siRNA transfections were performed with SMARTpool siRNAs (Thermo). Sequences are contained in Supplemental Table 1A. For the TLR siRNA screen, cells were seeded at 1×10^4 /well in a 96 well plate the day before transfection. DharmaFECT 4 (Thermo Scientific) was then used to transfect the cells with siRNA (50nM) according to the manufacturer's instructions 48 hours prior to infection. THP-1 siRNA transfections were performed using the Amaxa (Lonza, Basel, Switzerland) system according to the manufacturer's protocol. Cells were cultured for a further 48 hours prior to infection. The "control siRNA" used in all experiments indicates the non-targeting scrambled siRNA. All analyses on siRNA treatment are of independent triplicate biological replicates. Technical replicates were included in each plate screen to address any intra assay variation.

Gene expression analysis using qRT-PCR

Total RNA was isolated using the RNeasy Kit (Qiagen, West Sussex, UK). cDNA synthesis was performed using Transcriptor RT (Roche, West Sussex, UK) according to manufacturers' instructions. 1µg of total RNA was incubated with 50 pmoles of random hexamers at 25°C for 5 min, 42 °C for 50 min and 4°C for 5 min. Individual PCR primer pairs and probes were designed using the Roche Universal ProbeLibrary Assay Design Centre (<https://www.roche-applied-science.com/sis/rtPCR/upl/adc.jsp>). Primer sequences and probe combinations are provided in Supplemental Table 1B. PCR reactions were performed using Sensi Mix II (Bio Line, London, UK), 900 nM of each primer and 250 nM of probe mix in the LightCycler480 System (Roche). Thermal cycling conditions were as recommended by

the manufacturer (Roche). β -actin was used as a housekeeping gene, the $2^{-\Delta\Delta C_t}$ method (17) was used to calculate relative changes in gene expression. Relative expression in each figure refers to induction levels of the gene of interest relative to a housekeeper control, beta actin, and then compared to an untreated control calibrator sample.

Flow Cytometry

Antibodies against TLR1, TLR2 and TLR6 (IMG-5012, IMG-6720A, IMG-304A) were from Imgenex (San Diego, CA, USA) and the TLR10 antibody was from Santa Cruz Biotechnology (TLR10 H-165). As secondary antibodies, we used Alexa Fluor-488 goat anti-mouse IgG (Invitrogen, Paisley, UK) and PE-conjugated goat anti-rabbit IgG H+L (Imgenex). The respective isotype controls for each antibody were also from Imgenex. On the day of analysis, 1×10^6 cells were harvested using 0.2% EDTA PBS and washed twice in running buffer (PBS 0.5% BSA, 1% FBS 0.1% Sodium Azide). For intracellular staining, cells were fixed in BD Cytotfix Cytoperm (Becton Dickinson, New Jersey, USA) for 30 minutes on ice. For extracellular staining, cells were not fixed. Each primary antibody was used at 0.5 $\mu\text{g/ml}$ for 30 minutes on ice. Each wash step for intracellular staining was performed using BD Perm/Wash buffer (Becton Dickinson) while the running buffer was used for extracellular staining washes. The secondary antibodies were used at a 1:1000 dilution for 20 minutes in the dark on ice and cells were analysed using the Accuri C6 flow cytometer (Becton Dickinson). The gating method employed was designed to include the live cell population only. Population gates were set using negative controls and IgG isotype controls. The results were expressed as the percentage or median fluorescence intensity (MFI) of the total live cell population after subtracting the MFI of control cells stained with the appropriate isotype control antibodies.

Western blot analysis

Cells were lysed in RIPA lysis buffer (50mM Tris-HCL pH 8.0, 50mM, 150mM NaCl, 0.1% SDS, 0.5% Sodium deoxycholate, 1% Triton X-100, 1mM phenylmethylsulfonyl and 10% Protease Inhibitor Cocktail (Sigma). The following antibodies were used: anti-TLR10 (H-165) (sc-30198, Santa Cruz, Heidelberg, Germany) and β -actin antibody (Sigma). Proteins were detected using the Fujifilm LAS-3000 Imager (R&D Systems, Abingdon, UK).

ELISA analysis of supernatants for cytokine production

CCL-20 levels were quantified using the anti-CCL20 kit (Meso Scale Discovery (MSD), Maryland, USA) according to the manufacturer's instructions. Concentrations were determined using the MSD Sector Imager 2400. IL-8 levels were quantified using the Ready-Set-Go!® Human IL-8 ELISA kit (eBiosciences, San Diego, USA) according to the manufacturer's instructions.

Luciferase Assays

HEK 293 cells were seeded at 2.5×10^4 /well in a 96 well plate 24h prior to transfection. Cells were transfected using Turbofect (Fermentas) according to the manufacturer's protocol using 50ng of ISRE luciferase plasmid and 5ng of Thymidine kinase Renilla luciferase along with varying amounts of either TLR1, TLR2, TLR6 or TLR10 (gift from Prof. L. O'Neill, Dublin, Ireland). 24 hours post transfection cells were infected with *L.monocytogenes* as described above for 8 hours. Extracts were prepared by lysing the cells for 15 minutes in 50 μ L Passive Lysis Buffer (Promega, Madison, Wisconsin, USA) and measured for Firefly luciferase and *Renilla luciferase* activity. Firefly luminescence readings were corrected for Renilla activity and expressed as fold stimulation over unstimulated empty vector (EV) control.

NF- κ B Activity

SEAP reporter HEK-Blue-MD2-CD-14 cells (Invivogen, Toulouse, France) were used according to the manufacturer's instructions. Briefly, cells were maintained in selective antibiotics and seeded at 2×10^4 cells/well on a 96 well plate the day before transfection. Cells were then transfected and infected with *L.monocytogenes* as described above. Supernatants were collected 8 hours later and activity measured using QUANTI-Blue™.

Statistics

Results were statistically evaluated using Student's t test. Values of $P < 0.001$ are indicated by three asterisks (***), $P < 0.01$ two asterisks (**), Values of $P < 0.05$ one asterisk (*).

Results

Role of different TLRs in L.monocytogenes induced gene expression

We initially investigated the involvement of all human TLRs in the response of HT-29 IECs to *L.monocytogenes* infection by use of a siRNA screening approach (Supplemental Fig. 1). Inflammatory readouts selected were Interleukin 8 (IL-8) and Early Growth Response protein-1 (EGR-1) as these demonstrate consistently robust expression patterns in response to infection. EGR-1 mRNA was induced 6 fold ($p \leq 0.001$) by *L.monocytogenes* infection. Knockdown of TLRs 1-10 did not affect EGR-1 induction by *L.monocytogenes* indicating that activation of EGR-1 expression is via a TLR independent mechanism (Figure 1A). IL-8 mRNA expression was increased 4.5 fold following *L.monocytogenes* infection and this effect was significantly abrogated following silencing of TLRs 1 and 10 (Figure 1B). Knockdown of TLR2 resulted in reduced IL-8 expression following infection; however this did not reach statistical significance. Of the three genes where any effect was observed following siRNA-mediated knockdown (TLRs 1, 2 and 10), TLR10 silencing had the most significant effect on reduction of IL-8 expression. In addition silencing of the TLRs 1, 2 and 10 were found to be specific and have no off-target effects on the other members of the closely related TLR1, 2, 6 or 10 gene expression by qRT-PCR (Figure 1 C-F) and by flow cytometry (Supplemental Fig. 2).

Role of TLR10 in mediating chemokine production in response to L.monocytogenes infection.

This novel identification of TLR10 as a potential sensor of *L.monocytogenes* infection in an IEC cell line was intriguing given the fact that TLR10 expression is more commonly associated with immune cell subtypes (18). We confirmed both expression and effective silencing by siRNA of TLR10 in the HT-29 cell line by qRT-PCR (Figure 2A) and Western blotting (Figure 2B). In order to fully characterise which cytokines and chemokines were

induced by *L.monocytogenes* in a TLR10 dependent manner we initially examined mRNA expression of an extensive number of TLR induced genes (EGR-1, IL-1 β , IL-2, IL-6, IL-8, IL-9, IL-12, IL-18, IL-23, caspase-1, CCL-1, CCL-2, CCL-5, CCL20, CXCL1, CXCL10) in HT-29 cells following infection. Of these genes only caspase-1, IL-1 β , IL-8, IL-18, IL-23, CCL-1, CCL20 and EGR-1 demonstrated detectable levels of expression by HT-29 cells and of these only EGR-1, CCL-1, CCL20 and IL-8 were significantly induced following infection (Supplemental Figure 3B). As results from the original screen implicated possible roles for TLRs 1 and 2 as well as TLR10 in the *L.monocytogenes* induced immune response, we performed single knockdown with siRNA against TLR1, TLR2 and TLR10 and measured CCL-20 and IL-8 mRNA expression. TLR10 silencing resulted in the most significant decreases in CCL-20 and IL-8 mRNA levels in response to *L.monocytogenes* infection (Figure 2 C and D respectively). TLR 2 silencing reduced IL-8 while TLR1 silencing reduced CCL20 expression. Since CCL-20 was the most strongly induced chemokine gene in response to *L.monocytogenes*, protein production was measured over a longer period of time and a consistent reduction in CCL20 was observed following knockdown of TLR10 by siRNA at all time points (Figure 2E). Given that *L.monocytogenes* is an invasive bacterium we hypothesised that TLR10 might be detecting the pathogen intracellularly. In Figure 2F, intracellular versus extracellular staining for TLR10 by flow cytometric analysis revealed much greater expression levels of intracellular TLR10 than extracellular. Furthermore, Heat-Killed *L.monocytogenes* (HKLM) failed to induce CCL-20 or IL-8 to levels comparable to the induction seen when invasive live bacteria were administered to HT-29 cells (Figures 2G and H). This implies that bacterial invasion must be responsible for such high levels of CCL-20 and IL-8 induction. Together with the data from Figure 2F, it implies that this induction could be mediated by intracellular TLR10.

To ensure that the effect seen was not restricted to one cell line (HT-29) an additional IEC line, HCA-7, was assessed. TLR10 silencing was confirmed by qRT-PCR in HCA-7 cells (Figure 3A). mRNA expression of TLR10 in HCA7 was observed to be much higher than in HT-29 cells. Silencing of TLR10 resulted in reduced CCL-20 and IL-8 mRNA expression in the HCA-7 cell line (Figure 3B and C) as seen in HT-29 cells. CCL-1 induction was, however, not significantly affected unlike TLR10 silenced HT-29s in response to *L.monocytogenes* infection (Supplemental Figure 3 C and D). A consistent reduction in CCL-20 protein production was observed following knockdown of TLR10 by siRNA in HCT-116 cells at all time points (Figure 3D).

As both *L.monocytogenes* infection and TLR10 have been predominantly studied in immune cell subtypes we next chose to examine PMA differentiated THP-1 macrophage-like cells in order to see if this was an IEC specific effect. A consistent reduction in CCL-20 protein production was observed following knockdown of TLR10 by siRNA after 48 hrs (Figure 3E). Confirmation of ~52% TLR10 silencing in THP-1s was confirmed by flow cytometry (Figure 3F and G).

TLR10 effects L.monocytogenes survival in IECs and macrophages

Altering expression of the PRRs NALP3 and Aim2 has been previously shown to influence the ability of *L.monocytogenes* to survive and replicate within cells (12). We wished to identify whether altering expression of TLR10 would have a similar effect. Survival of *L.monocytogenes* was analysed by calculating the intracellular bacterial burden in both HT-29 and THP-1 following TLR10 silencing over an 8 hour period. Increased survival of the bacteria is seen in both cell lines indicating a critical role for the receptor in antagonizing bacterial intracellular viability (Figure 4A and B respectively).

TLR10 requires TLR2 to mediate optimal NF- κ B activation in response to L.monocytogenes.

Recognition of *L.monocytogenes* by different PRRs has been shown to lead to NF- κ B-dependent pro-inflammatory gene expression, inflammasome activation and caspase-1 cleavage and IFN- β responses (19). As both NF- κ B and IFN- β are major downstream signalling outputs of TLRs we investigated activation of these in response to overexpression of TLR constructs with or without stimulation by *L.monocytogenes*. We overexpressed a TLR10 construct in either an NF- κ B reporter cell line or in conjunction with an ISRE (Interferon Stimulated Response Element) - luciferase reporter construct. As TLR10 has been shown to dimerise with TLR2 (20), we overexpressed a variety of combinations of TLRs 1/2/10 in conjunction with the reporter constructs. In Figure 5A it can be seen that overexpression of TLRs 1 or 10 alone did not activate NF- κ B significantly above control level. Stimulation of the overexpressed constructs with *L.monocytogenes*, however, results in a twofold level of activation above control with TLR2 and significantly higher NF- κ B activation following co-transfection of TLR2 and TLR10 (5 fold above control), which was comparable to co-transfection of TLR1 and 2. This data indicates that TLR10 alone is not able to respond to *L.monocytogenes* but that together with TLR2. Endogenous TLR10 levels may explain the response to *Listeria* when TLR2 is transfected alone (see Supplemental Fig 3A). ISRE activation was also measured by luciferase assay in response to *L.monocytogenes* following overexpression of combinations of TLR constructs. Unlike NF- κ B, no increase in the ability of any of the TLR combinations to drive an ISRE-luciferase construct was seen following *L.monocytogenes* stimulation (Figure 5B).

TLR10 is not involved in recognition of other known TLR2 ligands

Since TLR10 is most closely related to TLR1 (20), we wished to compare TLR1 versus TLR10 mediated response to some known TLR2 ligands. Using THP-1 derived macrophages, IL-8 production was measured in response to a range of TLR2 ligands, including 2 strains of intracellular bacteria, following siRNA treatment against TLR1 or TLR10 (Figure 6). While a reduction in IL-8 production was seen following both TLR1 and TLR10 siRNA treatment in response to *Salmonella* infection, it was not as significant as the reduction seen during *L.monocytogenes* infection in TLR10 siRNA treated cells. Besides the significant reduction of IL-8 production seen in response to Pam3Cys following TLR1 siRNA treatment, no further statistically significant changes in IL-8 secretion was observed in response to the other TLR2 ligands or the positive control, LPS.

Discussion

TLRs have been clearly established as the major sensors of the innate immune system. Currently a total of 10 human TLRs (1-10) and 12 mouse TLRs (1-9, 11-13) have been identified. Each TLR recognises a limited repertoire of broadly conserved molecules of microbial origin with ligand specificity having been elucidated for many of the TLRs thus far (4). Importantly, despite being identified over ten years ago as a member of this family, TLR10 has remained the only orphan TLR, having no known ligand (18). The absence of TLR10 in mice has, to date, precluded the identification of synthetic or natural ligands for this receptor. This study is the first identification of an essential role for TLR10 in mediating the inflammatory response to a specific microorganism, *L.monocytogenes*, in both intestinal epithelial cells and macrophages.

The initial aim of this study was to identify which TLRs play a key role in detection of *L.monocytogenes* in the intestinal epithelia, as most prior work has focused predominantly on systemic infections and in doing so we have identified TLR10 as a key mediator of the innate immune response to infection in intestinal epithelial cells. Many human microbial infections are acquired via an oral transmission route and as such the first opportunity the immune system has to encounter and counter the infection occurs in the gastrointestinal tract. Despite this, very few studies have addressed which PRRs are directly involved in detecting *L.monocytogenes* at this interface. Whilst TLR2 (5) and MyD88 (21) have been shown to detect *L.monocytogenes* infection in other cell types only NOD2 has been directly shown to mediate the inflammatory response to *L.monocytogenes* in the intestine (9). Our screen investigating the role of TLRs in *L.monocytogenes* infection has yielded several interesting observations. TLR2 alone does not seem to play as critical a role in response to infection in epithelia as has been observed in immune cell types as shown by the slight but not significant reduction in chemokine expression following knockdown of TLR2. Knockdown of TLR1

seems to have a more significant effect on IL-8 production than knockdown of TLR2 and finally knockdown of TLR10 has the greatest effect on the ability of these cells to mount an immune response to *L.monocytogenes*.

Here we have reported TLR10 to play a functional role in intestinal epithelial cells. In humans, TLR10 expression has been reported to be somewhat restricted. It was initially characterised to be primarily expressed on immune cell subtypes with a predominance found in spleen, lymph nodes, thymus and tonsils (18). It is worth noting that in this study by Chuang *et al*, expression of TLR10 in colon tissue was not investigated. Within immune cell populations the highest levels of TLR10 expression have been reported on T regulatory cells (22), B lymphocytes (23), and plasmacytoid DCs (24) with expression also reported on THP-1 monocytes (25). The initial siRNA screen performed here which identified this novel role for TLR10 was performed in HT-29 intestinal epithelial cells, not in an immune cell type. Expression of TLR10 mRNA has previously been reported in the human SW480 intestinal epithelial cell line (26). In previous studies we have detected TLR10 mRNA in human colon biopsy samples, which comprise approximately 90% intestinal epithelial cells (27). As we have shown expression of TLR10 in two intestinal epithelial cell lines, HT-29 and HCA-7, by both mRNA and protein our data indicate a previously unappreciated role for TLR10 in non-immune cell types in the gut. Moreover we have confirmed our findings in the macrophage cell line THP-1 indicating that the role of TLR10 in the detection and immune response to *L.monocytogenes* is not limited to intestinal epithelial cells.

We have investigated signalling from TLR10 in response to *L.monocytogenes* and have shown that TLR10 alone is not sufficient to mediate *L.monocytogenes* induced NF- κ B activation but that it requires TLR2 to be present. We have also shown that co-expressing TLR2 and TLR10 drives NF- κ B activation significantly more than TLR2 alone. However a

caveat to this observation is that endogenous TLR10 levels may be present at low levels in HEK cells. Many studies involving TLR2 have shown that it can combine with either TLR1 or TLR6 and that this interaction is essential for effective ligand binding by TLR2 and also for discrimination of triacyl and diacyl lipopeptides from bacteria (4). Phylogenetic analysis has shown that TLR10 is most closely related to TLR1 and TLR6 (18). The level of sequence homology between these three suggests the idea of a common 1/6/10 ancestor which evolved into a TLR1/6 precursor and TLR10 (28). Two prior studies have investigated the potential role of TLR10 as a TLR2 co-receptor and signalling via TLR10. Overexpressed TLR10 has been shown to co-immunoprecipitate with TLRs 1 and 2 (29) and also to translocate, co-localise and physically interact with TLR2 in the phagosome (20). Both these studies also show interaction of TLR10 with the adaptor MyD88. Interestingly, the reports differ in their analysis of signalling from TLR10. Hasan *et al* used overexpression of a CD4-TLR10 construct to show that it could activate an NF- κ B luciferase reporter and that this was blocked by a dominant negative form of MyD88, whilst Guan *et al* found no activation with the same construct. Our data indicate that, in response to *L.monocytogenes*, TLR10 is a functional receptor and can induce NF- κ B but that it requires TLR2 to do so. Interestingly, when we examined *L.monocytogenes* induced IL-8 induction following knockdown of TLRs 1, 2 and 10 we saw the greatest effect with TLR10 knockdown possibly indicating that TLR10 is the dominant signalling partner in the 2/10 dimer in response to *L.monocytogenes*. Further studies are required to help identify the signalling components involved in this activation.

Although we have not yet fully investigated the role of TLR10 in detection of other pathogens, it seems unlikely that it will be limited to *L.monocytogenes* detection. In a screen of TLR2 ligands to compare TLR1 against TLR10 induced IL-8 production; we have shown that TLR10 is also involved in detection of the intracellular pathogen *Salmonella*

typhimurium. Indeed the intracellular role of TLR10 detection, in epithelia, appears to be crucial as HKLM did not result in TLR10 activation and subsequent cytokine expression. Further work is required to identify other pathogens detected by TLR10.

We have also shown that depletion of TLR10 increases *Listeria* survival in both HT-29 epithelial and THP-1 macrophage cells. Previous studies have linked TLR10 to ROS activation in THP-1 cells (30), and together with our own data, this suggests that TLR10 may play a role in ROS mediated clearance of *Listeria*. How it acts to reduce bacterial survivability in intestinal epithelial cells such as HT-29s is unclear although reduced expression of antimicrobial factors such as CCL-20 in the absence of TLR10 signalling may be a key factor. Indeed observation by Yang *et al.* (31) and Starner *et al.* (32) have demonstrated the antimicrobial effect of chemokines and cytokines, however, specific mutagenesis studies of both TLR10 and bacterial strains may also further elaborate this question.

The role of TLRs in defence against many pathogenic infections in the intestine remains unclear. Whilst the primary role of TLRs in the intestine is the same as in other tissues -defence against infection- other homeostatic functions have been described. TLR2, for example, is found to be expressed on both apical and basolateral surfaces of intestinal epithelial cells, and activation of TLR2 from the apical surface has been reported to result in cell homeostasis not inflammation (33). Similarly MyD88 has been shown to have a homeostatic role in the epithelium as mice lacking MyD88, are hyper-responsive to DSS-induced colitis (34). Conversely however, another recent study has shown an essential role for MyD88 in inducing the bactericidal lectin RegIII γ in response to *L.monocytogenes* infection in epithelial cells (35). Indeed a recent study has found that the circadian rhythm of intestinal epithelial cells governs the signalling of TLR mediated homeostasis (36). Our data showing TLR10 as the dominant TLR involved in mediating the immune response to

L.monocytogenes in the intestine open up intriguing possibilities concerning the balance of pro-inflammatory versus homeostatic TLR responses to infection in epithelial cells. It is possible that a TLR2/10 dimer may be pro-inflammatory in epithelial responses whilst TLR2 on its own or in combination with 1 and 6 may mediate a more homeostatic effect.

Taken together, our results show that *L.monocytogenes* infected intestinal epithelial cells produce chemokines in a TLR10 dependant manner. Whilst mucosal and systemic immunity to pathogenic infection can be very distinct, our finding of a role for TLR10 in the inflammatory response in macrophages as well as IECs indicates a general role for TLR10 in response to *L.monocytogenes* infection. Although the precise ligand remains to be identified, this report shows the first definitive role for TLR10 in pathogen detection.

Figure legends

FIGURE 1. Inflammatory response of HT-29 epithelial cells to *L.monocytogenes* following silencing of TLR 1-10 expression and siRNA specificity validation. Cells were treated with siRNA to TLRs as indicated, cultured overnight, and infected with *L. monocytogenes* (MOI 50:1) for 3 hours. Gene expression of (A) EGR1 or (B) IL-8 was determined by qRT-PCR. THP-1 cells were treated with control (scrambled) siRNA and TLR1 siRNA, TLR2 siRNA, or TLR10 siRNA as indicated with 5ng/ml PMA for 48 hours. qRT-PCR was used to monitor expression levels of TLR1, TLR2, TLR6 or TLR10 as indicated (C-F). * P<0.05, ** P<0.01 and *** P<0.001, Values are shown as Mean \pm SEM, n=3.

FIGURE 2. TLR10 silencing effects *L.monocytogenes* induced chemokines gene expression. HT-29 cells were treated with TLR10 siRNA and silencing was confirmed by qRT-PCR (A) and Western Blot (B) which contains the lysates from HT-29 which were transfected as follows; Untreated (lane A), TLR10 expressing plasmid (0.5 μ g) (lane B), TLR10 expressing plasmid (1 μ g) (lane C), TLR10 expressing plasmid (2 μ g) (lane D), TLR10 siRNA (lane E) and Control siRNA (Lane F). Upper panel; TLR10 antibody, lower panel Beta-actin antibody. The effects of siRNA for TLR1 and TLR2 on CCL-20 and IL-8 induction were determined following infection with *L.monocytogenes* (C and D). Secretion of CCL20 was determined by ELISA 8, 24 and 48 hours following infection in cells transfected with control or TLR10 siRNA (E). Extracellular versus intracellular expression of TLR10 in HT-29 cells was measured by flow cytometry (F) TLR10 siRNA treated cells were infected with Heat Killed *L.monocytogenes* (HKLM) or live *L.monocytogenes*, (MOI 50:1) as described previously, and the expression of the chemokines IL-8 and CCL20 were measured

by qRT-PCR (**G** and **H** respectively). * P<0.05, ** P<0.01 and *** P<0.001, Values are shown as Mean ± SEM, n=3

FIGURE 3. *L.monocytogenes* induced chemokine production is effected by TLR10 silencing in the epithelia and macrophage-like cell lines. HCA-7 cells were transfected with control or TLR10 siRNA and infected with *L.monocytogenes*, (MOI 50:1). qRT-PCR was used to measure TLR10 (**A**), CCL20 (**B**) and IL-8 expression (**C**). CCL20 secretion, 8, 24 and 48 hours following infection in HCT-116 cells, was determined by ELISA (**D**). PMA differentiated THP1 cells, macrophage-like, were transfected with control of TLR10 siRNA and the cells were then infected with Listeria for 8, 24 and 48 hrs and CCL20 secretion determined (**E**). TLR10 expression was also analysed by flow cytometry following transfection with control siRNA (**F**) or TLR10 siRNA (**G**) in THP-1 macrophage-like cells. * P<0.05, ** P<0.01 and *** P<0.001, Values are shown as Mean ± SEM, n=3.

FIGURE 4. TLR10 silencing increases intracellular *L.monocytogenes* survival in HT-29 epithelial cells. HT-29 and THP1 macrophage like cells were transfected with control or TLR10 siRNA and then infected with *L.monocytogenes*,(MOI 50:1), for 8 hours before intracellular bacterial survival was then determined by plate counting (**A** and **B**). * P<0.05, Values are shown as Mean ± SEM, n=3.

FIGURE 5. TLR10 requires TLR2 for NF-κB activation in response to *L.monocytogenes* and is not required for ISRE activation. (**A**), The NF-κB SEAP reporter HEK293 cell line was transfected with the plasmid combinations indicated, and were then infected with or without *L.monocytogenes* for 8 hours. The cell free supernatants were then collected and used to quantify NF-κB activation by colorimetric QUANTI-blue™ determination (**B**). HEK293 cells were transfected with an ISRE-luciferase reporter, plasmid combinations indicated, and were then infected with or without *L.monocytogenes* for 8 hours. Cell extracts were then

lysed and luciferase activity and corresponding luminescence was determined for ISRE activity ** P<0.01, Values are shown as Mean ± SEM, n=3.

Figure 6. Effects of TLR1 versus TLR10 siRNA treatment on IL-8 production in THP-1-macrophages following 24 hour stimulation with TLR2 ligands. THP-1 cells were treated with non-targeting control, TLR1, or TLR10 siRNA with 5ng/ml PMA for 48 hours. The cells were then subject to stimulation with the ligands indicated. Bacterial infections were performed as described in the methods section. 24 hours after stimulation, supernatants were analysed by ELISA for IL-8 production.

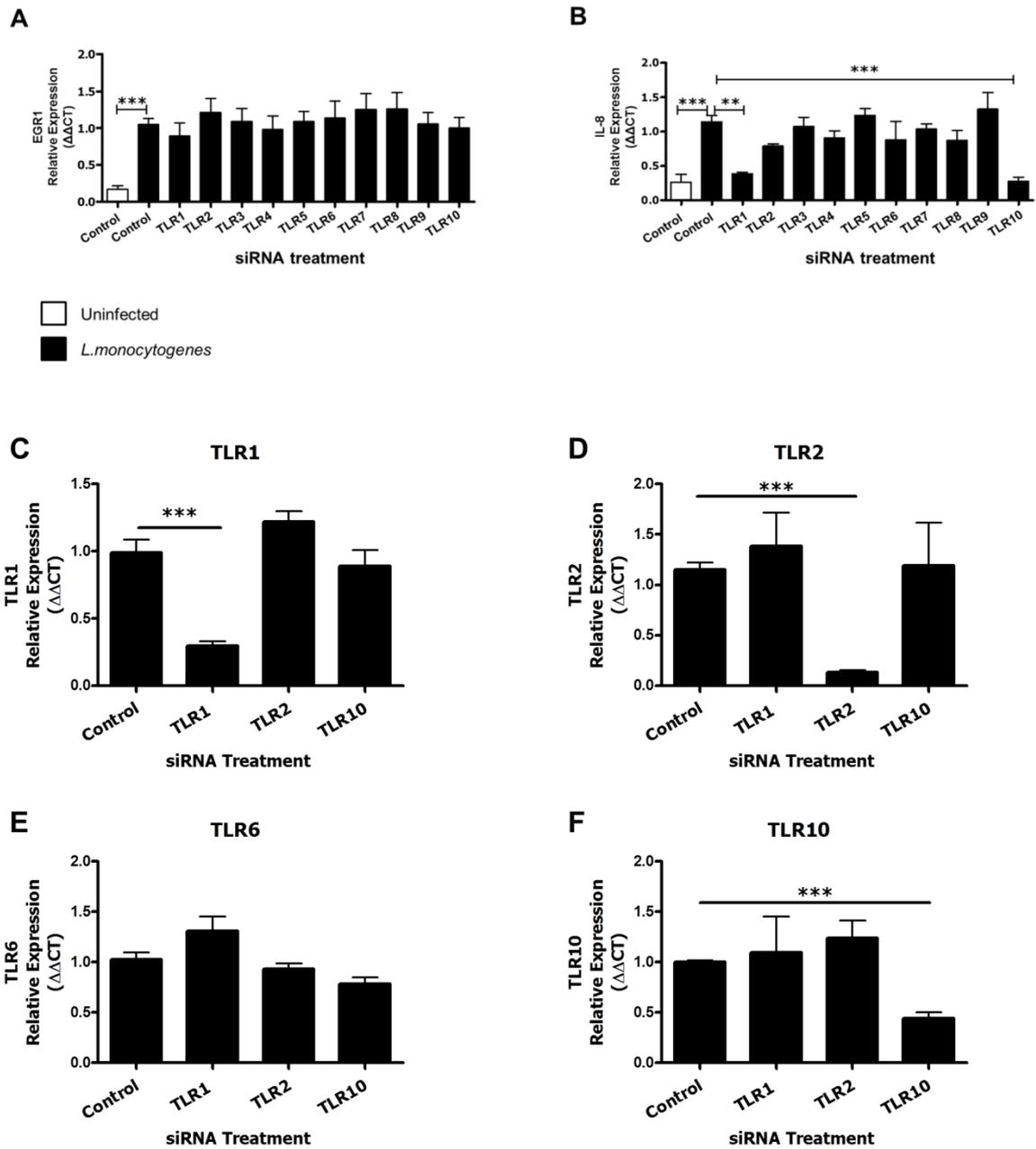


FIGURE 1. Inflammatory response of HT-29 epithelial cells to *L.monocytogenes* following silencing of TLR 1-10 expression and siRNA specificity validation.

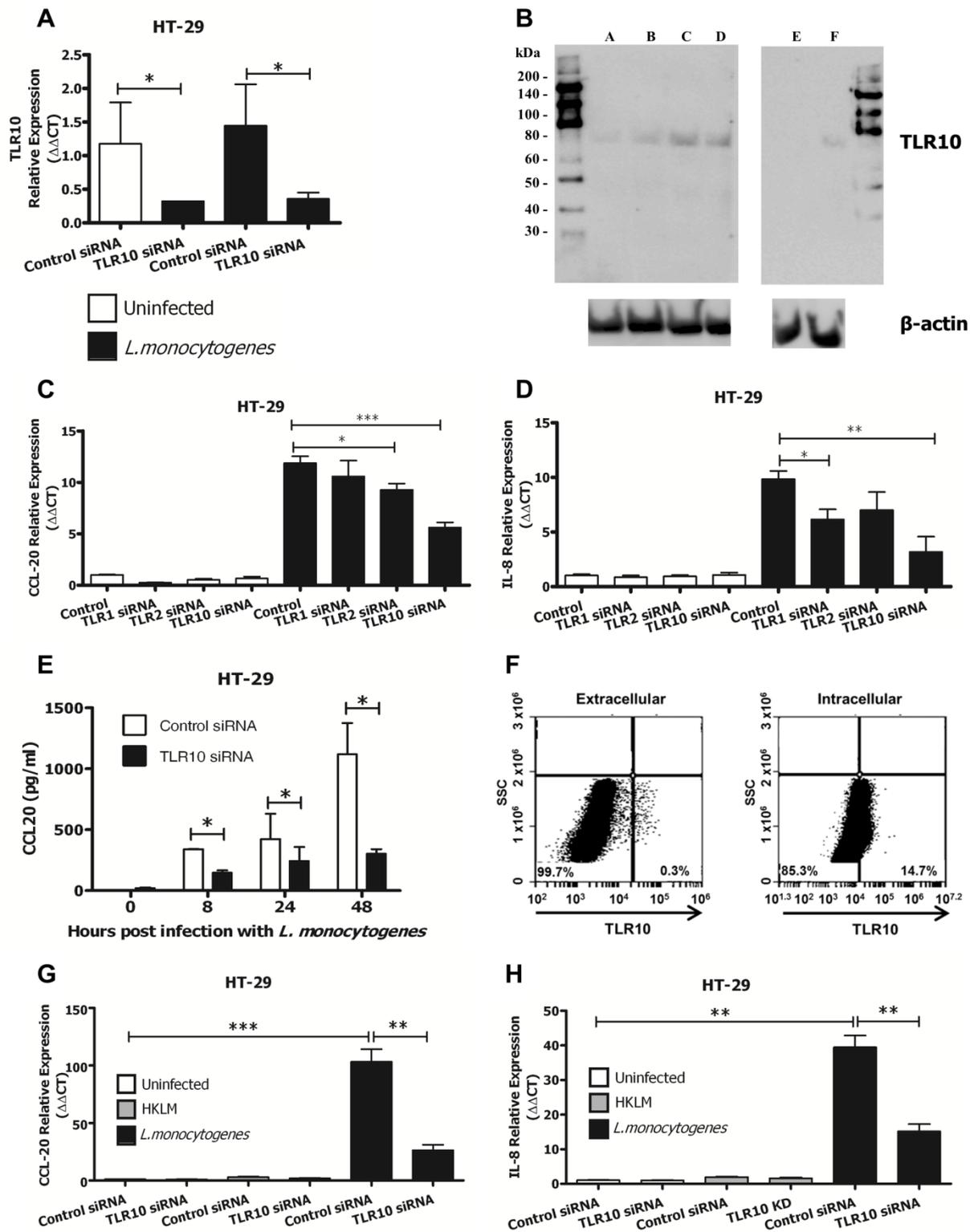


FIGURE 2. TLR10 silencing effects *L.monocytogenes* induced chemokines gene expression.

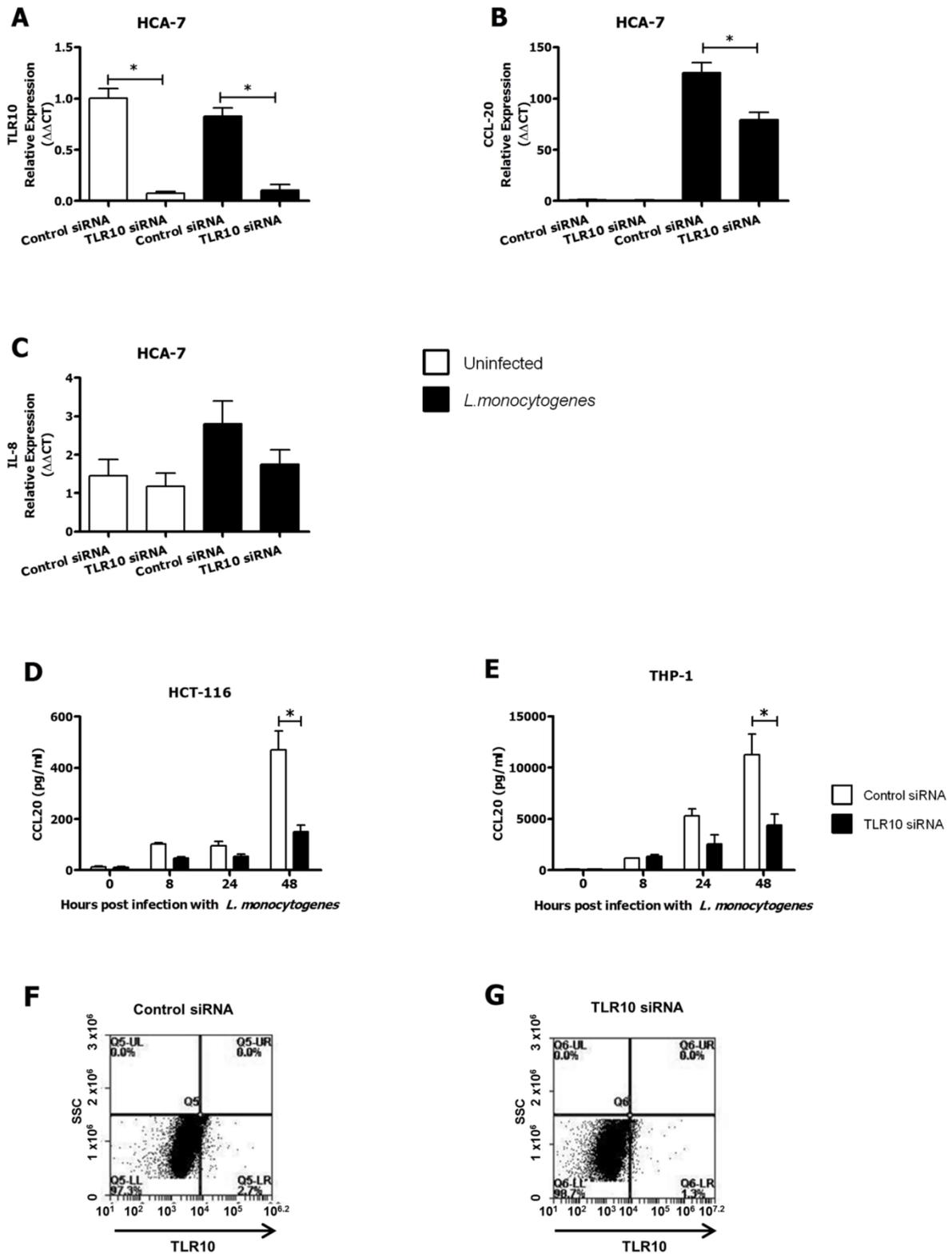


FIGURE 3. *L.monocytogenes* induced chemokine production is effected by TLR10 silencing in the epithelia and macrophage-like cell lines.

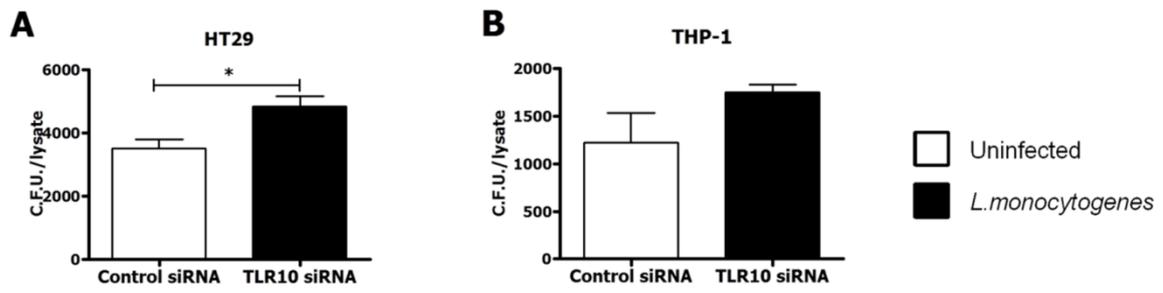


FIGURE 4. TLR10 silencing increases intracellular *L.monocytogenes* survival in HT-29 epithelial cells.

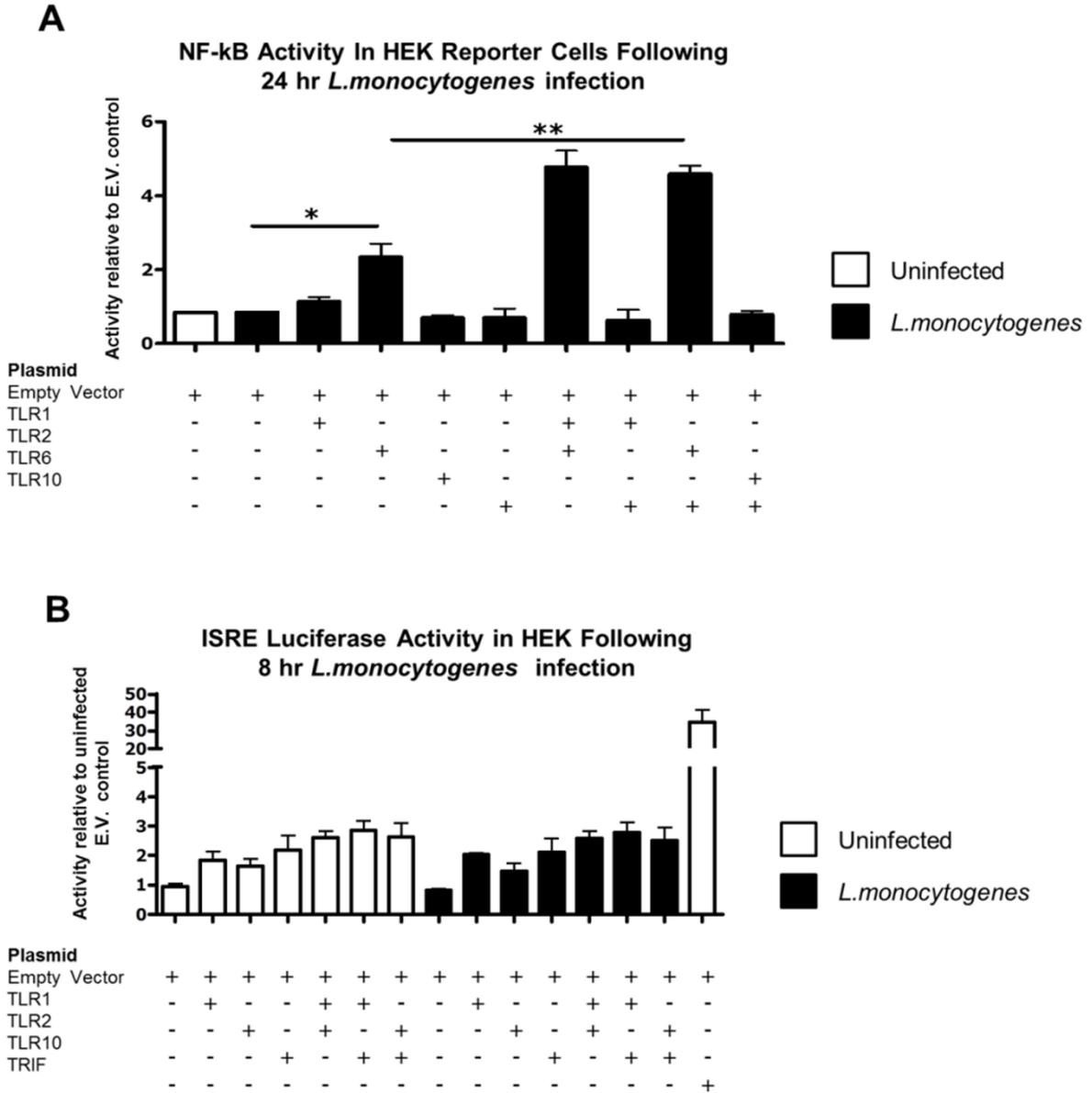


FIGURE 5. TLR10 requires TLR2 for NF-κB activation in response to *L.monocytogenes* and is not required for ISRE activation.

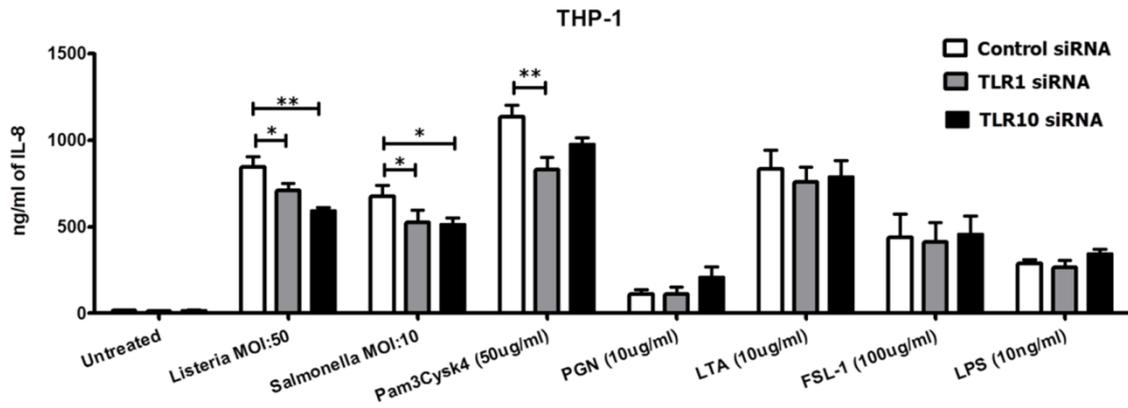
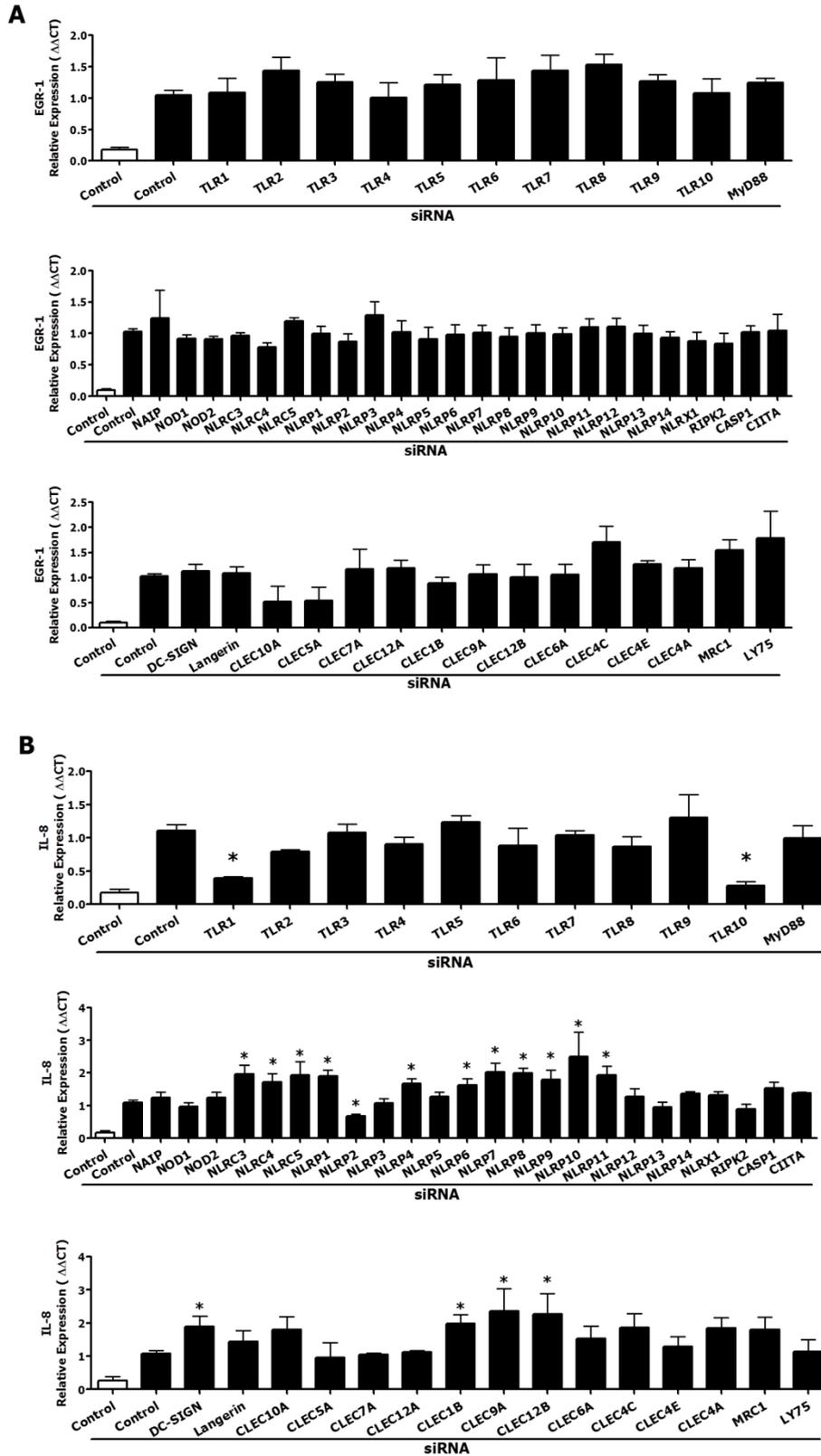


Figure 6. Effects of TLR1 versus TLR10 siRNA treatment on IL-8 production in THP-1-macrophages following 24 hour stimulation with TLR2 ligands.

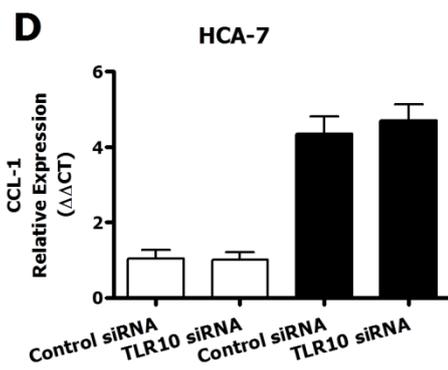
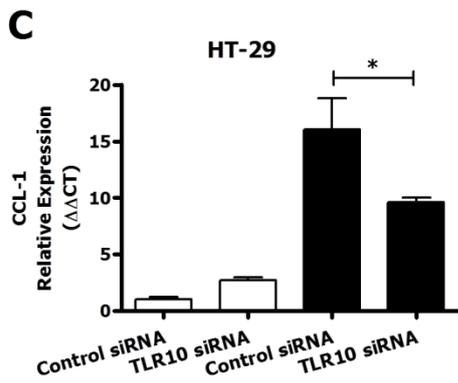
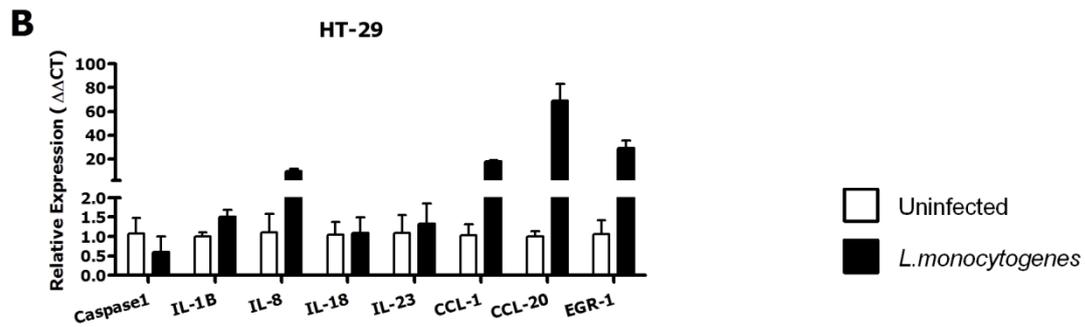
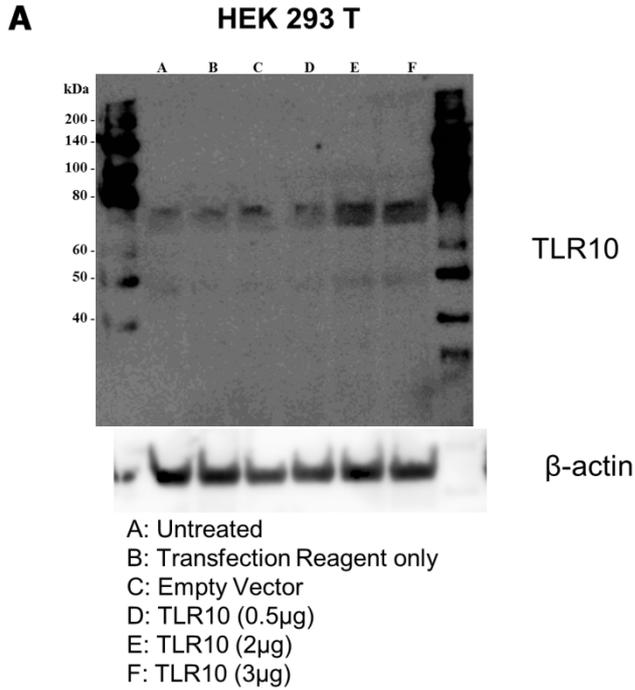
SUPPLEMENTAL FIGURE 1. EGR-1 induction in HT-29 epithelial cells in response to *L.monocytogenes* following silencing of PRR expression. Cells were treated with siRNA to PRRs as indicated for TLRs, NLRs or CLRs. Following siRNA transfection, cells were then infected with *L. monocytogenes* (MOI 50:1) for 3 hours and gene expression of EGR1 (A) or IL-8 (B) was determined by qRT-PCR. * P<0.05, ** P<0.01 and *** P<0.001, Values are shown as Mean ± SEM, n=3.

SUPPLEMENTAL FIGURE 2. Specificity of silencing of siRNA used against TLR1 TLR2 and TLR10. THP-1 cells were treated with control (scrambled) siRNA and TLR1 siRNA (A), TLR2 siRNA (B) or TLR10 siRNA (C) with 5ng/ml PMA for 48 hours. Flow cytometry was used as described in the methods to monitor expression levels of TLR1, TLR2 and TLR10. Gating was performed against the isotype control and all control siRNA samples were measured against the corresponding TLR siRNA at the time of analysis to avoid intra-assay variation in staining efficiency. Representative scatter plots of flow data are shown with relative knockdown (KD) of target TLR.

SUPPLEMENTAL FIGURE 3. Western Blot of TLR10 expression levels in HEK and immune response to *L.monocytogenes* in IEC. Lysates of HEK cells were prepared for Western blotting analysis 48 hours after transfection as described in the methods section. TLR10 detection was confirmed using transfected TLR10 as a positive control. β -actin expression was also examined to ensure equal levels of protein were loaded on the gel (A). HT-29 cells were infected with *L.monocytogenes*, (MOI 50:1), for 3 hours. qRT-PCR was used to measure induction of the cytokine and chemokines indicated (B). Cells were treated with control siRNA or TLR10 siRNA as indicated and infected with *L. monocytogenes* (MOI 50:1) for 3 hours. Gene expression of CCL-1 was determined by qRT-PCR in HT-29 (C) or HCA-7 cells (D). * P<0.05, ** P<0.01 and *** P<0.001, Values are shown as Mean ± SEM, n=3.



SUPPLEMENTAL FIGURE 1. EGR-1 induction in HT-29 epithelial cells in response to *L.monocytogenes* following silencing of PRR expression.



SUPPLEMENTAL FIGURE 3. Western Blot of TLR10 expression levels in HEK and immune response to *L. monocytogenes* in IEC.

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