A molecular analysis of the interactions of *Escherichia coli* and macrophages

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Declaration

I declare that the research presented in this thesis is my own work and that it has not been submitted for any other degree, either at University College Cork, or elsewhere. Wherever contributions of others are involved, every effort has been made to indicate this clearly, by reference to the literature and by acknowledgement of collaborative research.

This work was completed under the guidance of Dr. David Clarke at the School of Microbiology, University College Cork, Ireland.

Ian O'Neill

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List of abbreviations

2CST	Two component signal transduction
AIEC	Adherent invasive *E. Coli*
AMP	Antimicrobial peptides
ATG	Autophagy related genes
C-section	Aaesarean section
CD	Crohn's disease
CFs	Colonisation factors
CLRs	C-type lectin receptors
DAMPS	Danger associated molecular patterns
E. coli	Escherichia coli
EAEC	Enteroaggregative *E. Coli*
ED	Entner-doudoroff
EHEC	Enterohaemorrhagic *E. Coli*
EIEC	Enteroinvasive *E. Coli*
EIEC	Enteroinvasive *E. Coli*
EMP	Embden-meyerhof-parnas
EPEC	Enteropathogenic *E. Coli*
ETEC	Enterotoxigenic *E. Coli*
ExPEC	Extraintestinal *E. Coli*
FISH	Fluorescent in situ hybridization
HlyA	Alpha-hemolysin
HPLC	High performance liquid chromatography
IDB	Inflammatory bowel diseases
IEC	Intestinal epithelial cells
IL-1β	Interleukin beta
INF-α	Interferon beta
IRAK	IL-1 receptor associated kinase
IRAK4	IL-1 receptor associated kinase 4
LAP	LC3 associated phagocytosis
LEE	Locus of enterocyte effacement
LPS	Lipopolysaccharide

LRR	Leucine-rich repeat
Mtb	Mycobacterium tuberculosis
MUC2	Mucin 2
MyD88	Myeloid differentiation primary response gene 88
NLRs	NOD-like receptors
NMEC	Neonatal meningitis *E. Coli*
NOD	Nucleotide-oligomerisation domain
NOS	Reactive nitrogen species
NOS	Nitric oxide synthase
NOX	NADPH oxidase
PAMPs	Pathogen-associated molecular patterns
PDHC	Pyruvate dehydrogenase complex
PE	Phosphatidylethanolamine
PP	Pentose-phosphate
PRR	Pattern recognition receptor
PYHIN	Pyrin or HIN domain-containing
RLRs	RIG-I-like receptors
ROS	Reactive oxygen species
SCFA	Short chain fatty acids
SCV	Salmonella containing vacuoles
T3SS	Type 3 secretion system
T3SS	Type 3 secretion system
TCA	Tricarboxylic acid
TIR	Toll-interleukin-1 receptor
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
TRAF6	TNFR-associated factor 6
UC	Ulcerative colitis
UPEC	Uropathogenic *E. Coli*
vita-PAMPs	Viability-associated-pamps

Abstract

Escherichia coli (*E.coli*) is a diverse bacterial species that primarily forms a beneficial symbiotic relationship with the host in the human lower gastrointestinal track (GIT), however it can also be pathogenic in this environment. Furthermore, some strains can diverge from the GIT and occupy niches such as the urinary tract. In all these environments, *E. coli* interacts with the immune system and macrophages represent the front line of the innate immune system. In this study we characterise the immune response by macrophages to *E. coli* infection. It was shown that *E. coli* broadly provoke a similar cytokine response during macrophages infection and furthermore are degraded primarily by the phagocytosis pathway. Recently a new group of *E. coli* called Adherent Invasive *Escherichia coli* (AIEC) has been described. AIEC are present in the guts of Crohn's disease (CD) patients at a higher frequency than in healthy patients. AIEC can replicate in macrophages but the mechanism for this is not fully understood. The processing of AIEC by macrophages was investigated and it was shown that AIEC only replicated in permissive macrophages. Furthermore, even in a permissive macrophages AIEC are trafficked through macrophages in a similar manner to commensal *E. coli*. This supports the hypothesis that AIEC are highly similar to commensal *E. coli* and only cause pathogenicity when present in the permissive environment of the gut of CD patients. Replication in macrophages requires functioning metabolic pathways and it was identified that glycolysis is important for AIEC survival in macrophages. AIEC mutants without a fully functioning glycolysis pathway induced less IL-1β cytokine release from macrophages than wild type strain suggesting that metabolism plays a role in inflammasome activation. Furthermore, AIEC mutants that could not produce the glycolytic end product acetate induced significantly reduced IL-1β release during infection. This suggest that the acetate molecule or a phenotypic effect of its production may be a driver of IL-1β release from AIEC infected macrophages. The interaction of uropathogenic *E. coli* (UPEC) with macrophages was also investigated. UPEC induced very high levels of cytotoxicity in human macrophages which was shown to be dependent on the production of the pore forming toxin α-hemolysin. However, UPEC did not induced high levels of cytotoxicity in murine macrophages suggesting there are species specific sensitivity to α-hemolysin that should be considered when studying UPEC pathogenicity in murine models.

Chapter 1 Introduction

1.1 The human gut microbiome

The human gut contains an estimated 10^{14} bacteria from up to 1000 different species and it provides an excellent environment for the growth of bacteria (Orel, 2014). The collective community of microbes in the gut is known as the gut microbiome and will be referred to herein as the microbiome or microbiota. The vast majority of bacteria in the gut are comprised of the phyla Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Guinane & Cotter, 2013). The microbiota confer a number of health benefits to the host including immune modulation, food digestion and protection from pathogens (Jandhyala et al., 2015). In the colon where there is the largest concentration of bacteria, most bacteria are fermenting anaerobes of the phyla Firmicutes and Bacteroidetes but the colon also contains the facultative anaerobes. Bacteria in the colon ferment carbohydrates to produce the short chain fatty acids (SCFA) butyrate, acetate and propionate which function as an energy source for colonocytes (McNeil, 1984). Aside from energy production, SCFA also function to modulate the immune response through regulation of T-cell homeostasis and also have anti-inflammatory properties (Smith *et al*., 2013b; Vinolo *et al*., 2011).

The profile of bacterial phyla changes with age, disease and external factors such as diet, smoking, and antibiotic use (Guinane & Cotter, 2013; Jandhyala *et al*., 2015). In new-born babies, a major factor in shaping the first microbiota is the mode of delivery i.e. vaginal or caesarean section (C-section) birth. Unsurprisingly the gut microbiome of vaginal birth babies is comprised of organisms associated with the birth canal such as *Lactobacillus* and *Prevotella* (Mackie et al., 1999). The gut microbiome of C-section birth babies is more similar to that found on the skin of the mother with genera such as *Streptococcus*, *Corynebacterium,* and *Propionibacterium* (Dominguez-Bello *et al*., 2010; Mackie *et al*., 1999). The microbiota of the developing child has an adult-like signature from somewhere between 1-4 years but has a higher levels of Bifidobacterium than adults and this appears to be maintained into adolescence (Agans *et al*., 2011; Ringel-Kulka *et al*., 2013). In later life the microbiota stabilises

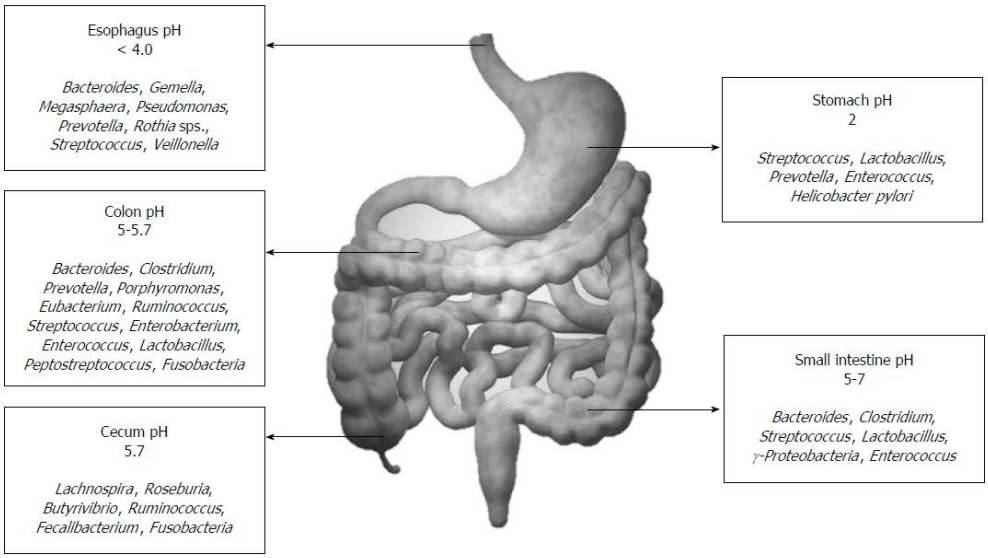

Figure 1. 1 Distribution of the normal human gut flora.

Adapted from (Jandhyala et al., 2015).

but as people age there is a decrease in *Bifidobacteria*, Firmicutes, and *Fecalibacterium prausnitzii* and an increase in Proteobacteria such as *Eschericia coli* and Firmicutes such as *Staphylococcus* (Jandhyala et al., 2015).

1.1.1 Effect disease on microbiota composition

Many disease states result in a change in the composition of the gut microbiota and these include the inflammatory bowel diseases (IDB) i.e. Crohn's Disease (CD) and Ulcerative colitis (UC) (Guinane & Cotter, 2013). Although CD and UC are both classified as IBDs they do have distinct pathologies. CD is characterised by segmented inflammation of the gut which can occur anywhere from the mouth to the anus whereas UC is characterised by inflammation and ulceration of the colon (Kaser et al., 2010). The role of the microbiota in both these diseases is under investigation and it has been shown that there are significant shifts in microbiota profile in patients with IBD compared to healthy controls. In IBD there is a reduction in Firmicutes and Bacteroidetes and an expansion of Actinobacteria and Proteobacteria (Sartor & Mazmanian, 2012). There is also a reduction in the diversity of the microbiota in IBD patients (Orel, 2014).

1.2 The epithelial barrier

With such a large volume of bacteria existing within the gut it is essential that there is constant surveillance of the contents of the microbiota and that there is a barrier between the luminal contents of the gut and the sterile internal environment. The intestinal epithelial cells (IECs) provide a spatial barrier between the microbiota and the host through a web of tight junctions (Zhang et al., 2015). The microbiota helps to shape the integrity of the epithelial barrier. For example, toll-like receptor (TLR) 2 (a pattern recognition receptor (PRR)) is activated by bacterial cell wall peptidoglycan and enhances tight junctions and TLR2 null mice have reduced epithelial barrier integrity (Cario et al., 2007).

The intestinal epithelium is highly organised and contains cells that are specialised in sampling of luminal contents (Mabbott et al., 2013). Microfold (M) cells are specialised enterocytes that belong to the follicle associated epithelium overlaying the Peyer's patches and deliver antigens including commensal and pathogenic bacteria to the underlying immune cells including macrophages (Zhang et al., 2015). Other specialised IECs such as goblet and Paneth cells provide different roles in maintaining the homeostasis of the intestines. Goblet cells secrete an inner and outer apical mucus layer comprised of heavily modified glycoprotein mucin 2 (MUC2) that helps protect the underlying epithelial layer from bacteria (Zhang et al., 2015). Mice with *Muc2*-deficiencies have a reduced mucus layer and these animals produce increased pro-inflammatory cytokine levels and develop spontaneous colitis (Van der Sluis et al., 2006). The outer layer of mucus is fluid and is colonised by bacteria whereas the inner layer is rigid and generally though to be absent of any bacteria (Johansson et al., 2008). Moreover, the mucus layer is constantly shedding thus preventing interactions between the colonised bacteria and the underlying epithelium (Ermund et al., 2013). Paneth cells produce a raft of antimicrobial peptides (AMP) that are active against a wide range of Gram-positive and Gram-negative bacteria (Bevins & Salzman, 2011). Production of these AMPs by Paneth cells is achieved through the direct sensing of bacteria by TLRs in a Myeloid differentiation primary response gene 88 (MyD88)-dependent manner (Vaishnava et al., 2008). Host epithelium expresses a number of pattern recognition receptors (PRRs) such as TLR 1, TLR2, TLR3, TLR4, TLR5 and

TLR9 as well as nucleotide-oligomerisation domain (NOD) 1 and 2 (Zhang et al., 2015). Ligands for TLRs and NOD1/2 are bacterial products such as lipopolysaccharide (ligand for TLR4), flagella (ligand for TLR5) and muramyl dipeptide (MDP, ligand for NOD2) (Takeda & Akira, 2004). When PRRs are activated, epithelial cells release pro-inflammatory cytokines, however, it is thought that these PRRs are expressed at low levels on epithelial cells to avoid over-activation by commensal bacteria (Buttó et al., 2015). Epithelial cells can also be bactericidal and can kill invading bacteria by containment within a double-membrane vehicle in a process known as autophagy and, furthermore, mice deficient in autophagy display increased bacterial invasion across the epithelial barrier (Benjamin et al., 2013).

1.3 Macrophages

Bacteria that manage to traverse the epithelial barrier will encounter macrophages in the lamina propria (Weber et al., 2009). Macrophages are professional immune cells that play a key role in mediating the host response to infection. These cells are found in all tissues in the body where they continuously monitor the body for the presence of bacteria and they are in high abundance in the gut (Kühl et al., 2015). Once a bacterium is detected, the macrophages respond by initiating regulatory pathways resulting in the production of soluble cytokines and chemokines that orchestrate the appropriate local (and systemic) immune response to the invading bacterium. The macrophages also control infection by phagocytosing and destroying the invading bacteria.

In order to prevent excessive activation of the immune system by commensal bacteria, intestinal macrophages are 'inflammation anergic' as they do not express a large number of innate immune response receptors including the receptor for the Gram-negative bacterial outer membrane component, lipopolysaccharide (LPS), CD14. However, intestinal macrophages phagocytose and kill bacteria normally (Smythies et al., 2005). TLRs expressed on macrophages are receptors for many bacterial ligands and their activation usually results in the production of pro-inflammatory cytokines. However, it appears that whilst intestinal macrophages do express most TLRs normally, they do not respond to stimulation as the downstream NF-κB signalling

pathway is inactivated through the increased expression of IκBα, a negative regulator of NF-κB (Smythies et al., 2010). This study also showed a decrease expression of key proteins of the MyD88-dependent and –independent signalling pathway (detailed in section 1.3.2).

1.3.1 Bacterial recognition by macrophages

Macrophages sense bacteria and other potential pathogens such as viruses through PRR. Currently there are 4 classes of PRRs: The TLRs; the cytosolic NOD-like receptors (NLRs); the RIG-I-like receptors (RLRs); and the cell-surface C-type lectin receptors (CLRs) (Blander & Sander, 2012). These receptors recognise exogenous pathogen-associated molecular patterns (PAMPs) such as LPS and endogenous damage-associated molecular patterns (DAMPS) such as cell membrane components of damaged cells. The PRRs can be membrane bound, such as TLRs and CLRs, or cytosolic, such as NLRs and RLRs (Takeuchi & Akira, 2010). In general, TLRs recognise bacterial and viral PAMPs, RLRs recognise viral PAMPs, CLRs recognise fungal PAMPs, and NLRs recognise cytosolic bacterial PAMPs (Takeuchi & Akira, 2010).

PRRs have diverse ligands but converge on conserved signalling pathways that control the immune response of the cell. Furthermore, many PAMPs such as LPS are ubiquitous across bacteria, however, not all bacteria elicit the same response from macrophages. Virulence factors such as type 3 secretion systems (T3SS) in *Salmonella* and *E. coli* result in enhance immune response compared to non-pathogenic *E. coli* (Blander & Sander, 2012). Live attenuated pathogens are better vaccines than dead counterparts and commensal bacteria do not elicit a strong immune response whereas bacterial invasion does (Blander & Sander, 2012). Indeed, it has been suggested that the macrophages can detect a number of different microbial threat levels and respond accordingly (Figure 1. 2). This involves different check points in the immune response to test for the level of microbial threat. These checkpoints begin with TLR signalling and continue through phagocytosis and internalisation constantly measuring the threat posed by the microbe (Blander & Sander, 2012). Following internalisation, immune

cells are able to detect, using inflammasomes, whether a microbe is alive or dead and if it is a pathogen and mount an appropriate response to each scenario.

1.3.2 TLR signalling pathways.

Macrophages express an array of TLRs on their plasma membrane that recognise different PRRs from bacteria including: TLR2 that recognises Lipoteichoic Acids from Gram-positive bacteria; TLR4 that recognises LPS; and TLR5 that recognises flagellin (Jiménez-Dalmaroni et al., 2016). Ligand binding to TLRs results in activation of conserved signalling pathways (Sanjuan et al., 2009). TLR4 has been extensively studied and is the best characterised of the plasma membrane TLRs. Activation of TLR4 by LPS leads to a signalling cascade that results in the secretion of pro-inflammatory cytokines and in some cases type I interferons (Chow *et al*., 1999; Poltorak *et al*., 1998). TLR4 signalling occurs via two separate pathways: the MyD88-dependent pathway and the MyD88-independent pathway (Figure 1. 3) (Lu et al., 2008; O'Neill et al., 2013). The MyD88 pathway is activated by external stimulus such as soluble LPS whereas the MyD88-independent pathway requires internalisation of TLR4 for signalling.

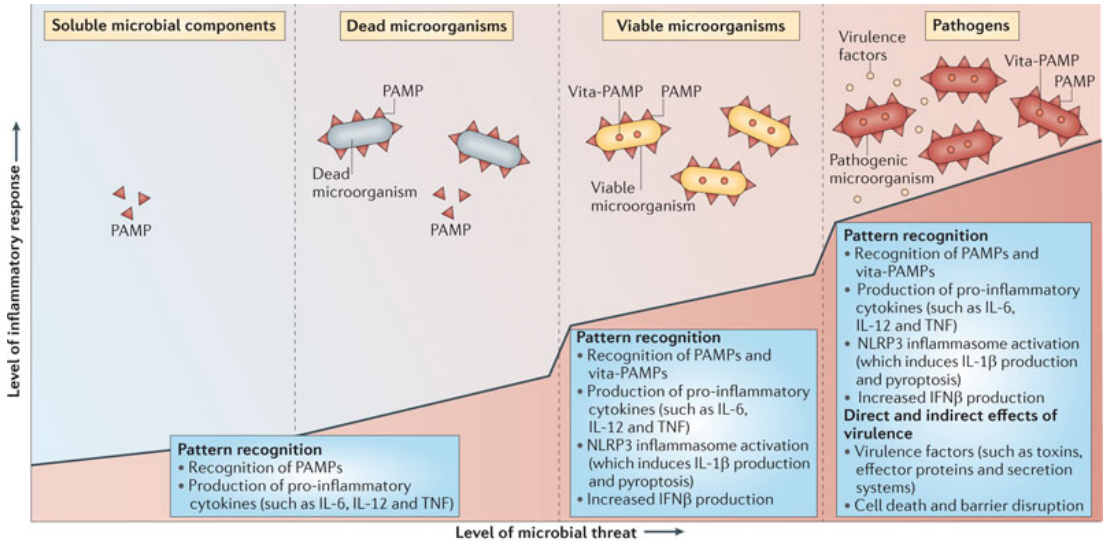

Figure 1. 2 Different microbial threat levels lead to a controlled immune response

Cells can recognise different levels of microbial threat and respond accordingly. Soluble microbial compounds pose the least threat while whole bacteria are a greater threat. Once internalised viable bacteria produce vita-PAMPs and pathogenic bacteria have virulence factors such as T3SS which can both be detected by inflammasomes

Adapted from (Blander & Sander, 2012).

1.3.2.1 The MyD88-depdendent pathway

LPS interacts with TLR4 resulting in a signalling cascade that drives the expression of genes for pro-inflammatory cytokines (Figure 1. 3). Upon activation, TLR4 oligomerises and recruits downstream adaptors through interactions with the toll-interleukin-1 receptor (TIR) domain. MyD88 directly couples to the TIR of TLR4 dimers via the bridging adaptor MAL. MyD88 is essential for TLR4-mediated signalling and MyD88 knockout macrophages do not produce pro-inflammatory cytokines following LPS stimulation (Kawai et al., 1999). MyD88 contains a death domain (DD) in the N-terminus that interacts with the IL-1 receptor associated kinase 4 (IRAK4). Interactions between MyD88 and IRAK4 results in IRAK4 autophosphorlyation and recruitment of IRAK1 or IRAK2 (Lye et al., 2004). Another adaptor protein, the E3 ubiquitin ligase TNFR-associated factor 6 (TRAF6) is recruited to the MyD88/IRAK4/IRAK1 complex where it is activated and released into the cytosol. Free TRAF6 forms a complex with TAK1, TAB1 and TAB2/3 and

this activates the IKK complex of NEMO, IKK α and IKK β, resulting in the phosphorylation (and subsequent degradation) of I**κ**B and the release of NF-**κ**B (Lu et al., 2008). The TAK1 signalling also leads to the activation of the transcription factor AP-1 via the MAP kinase pathway. The TRAF6 complex also activates a third transcription factor IRF5 and, together with NF-**κ**B and AP-1, these transcription factors drive pro-inflammatory cytokine gene expression in response to the LPS found in the outer membrane wall of Gram negative bacteria.

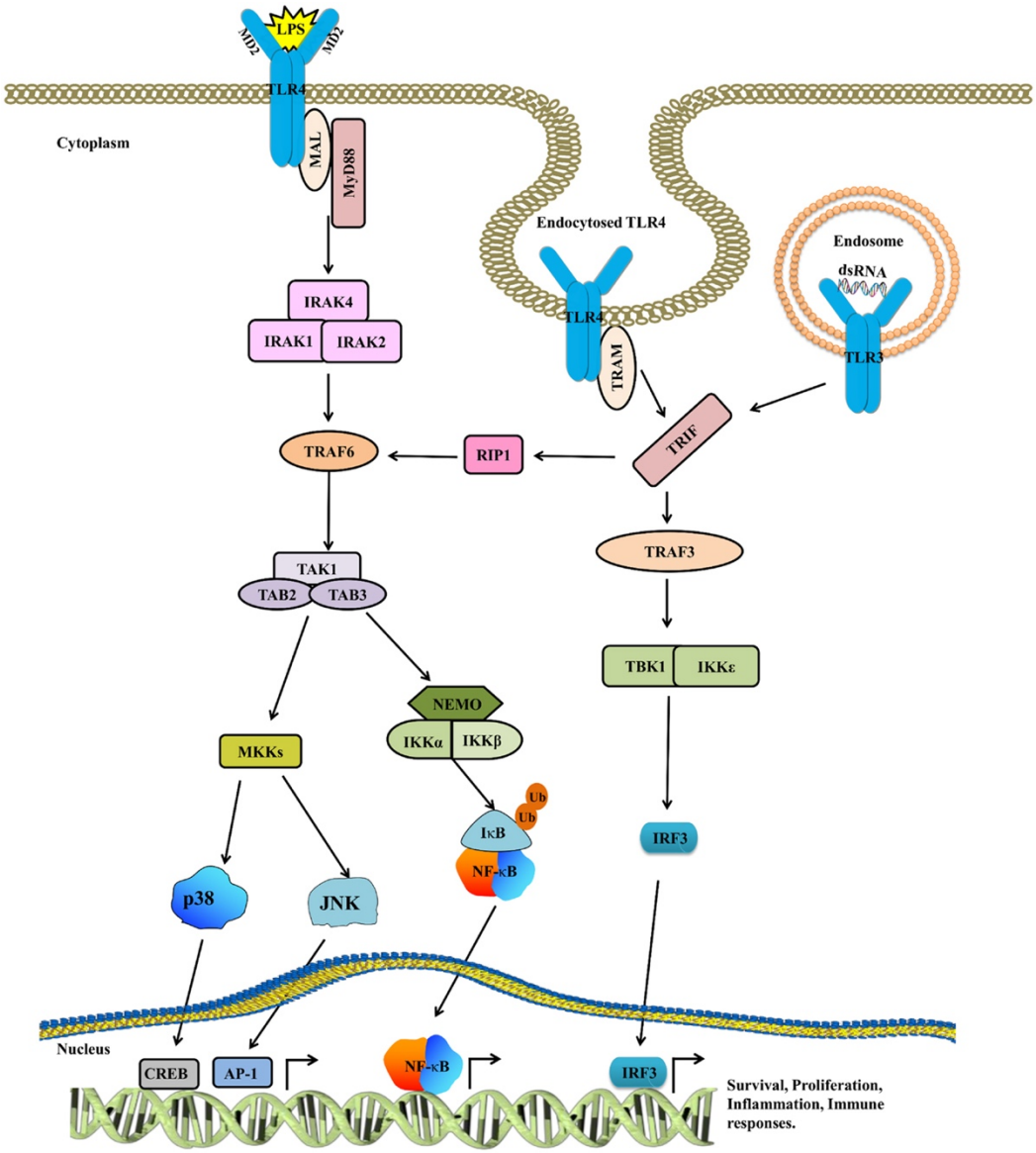

Figure 1. 3 TLR4 signalling pathway

The MyD88 dependent and independent pathways control proinflammatory cytokine and interferon release following signalling from TLRs

Reproduced from Yesudhas, D. et al., 2014

1.3.2.2 The MyD88-independent pathway

TLR4 also induces the MyD88-independent pathway that leads to the expression of type I interferons in a process that is dependent on TLR4 endocytosis (Kagan et al., 2008). Following activation of TLR4 and its endocytosis, the adaptor proteins TRAM interacts with TRIF allowing for its association with TRAF6 and TRAF3 (Figure 1. 3). This leads to TRAF3 association with TBK1, TANK and IKKi which is important for the dimerization and nuclear translocation of the transcription factor IRF3. NF-κB is also activated in a TRIF-dependent manner through TRAF6 recruitment triggering RIP1 kinase. Together IRF3 and NF-κB activate transcription of type I interferons (Underhill & Goodridge, 2012).

Thus, the MyD88-depedendent pathway is activated in response to extracellular signalling whereas the MyD88-indepedendent pathway requires the endocytosis of TLR4 for signalling to occur. This is an example of how macrophages can distinguish between different levels of microbial threat (see Figure 1. 2). A low level threat such as soluble LPS would lead to MyD88-dependent signalling only resulting in the release of pro-inflammatory cytokines. A higher level threat such as that exhibited by a LPS sensed in the context of a whole bacterium would trigger endocytosis and thus would result in both the MyD88-dependent and –independent pathway and the production of pro-inflammatroy cytokines and type I interferons.

1.3.3 Phagocytosis

Phagocytes plays a vital role in innate immunity and cytokine secretion. Furthermore, dendritic cells and macrophages function as a bridge between innate and adaptive immune system and phagocytosis is essential for antigen presentation (Underhill & Goodridge, 2012).

Macrophages phagocytosis relies on the recognition of PAMPs, such as LPS. The opsonin, LPS binding protein (LBP) (Schumann et al., 1990), which is produced by hepatocytes and intestinal epithelial cells is present in the blood at high levels (Su *et al*., 1994; Vreugdenhil *et al*., 2000; Wan *et al*., 1995). LBP recognises LPS with a low

specificity but high affinity (Stuart & Ezekowitz, 2005) and whole *E. coli* are opsonised by LBP (Schiff et al., 1997). The LBP-LPS complex is recognised by CD14 found on the surface of the macrophages (Wright et al., 1990). LPS is then transferred to the TLR4/MD2 complex leading to downstream signalling (da Silva Correia et al., 2001; Miyake et al., 2000). LPS stimulation results in the formation of CD14-TLR4 clusters in the plasma membrane and the signalling molecules MyD88 and JNK/SAPK are recruited to these rafts (Triantafilou et al., 2002). The activated receptor is then internalised and actin remodelling is essential for the engulfment of the receptor bound ligand through formation of pseudopodia that envelop the target. The so called phagocytic cup forms by the polymerisation of actin at the leading edge of the pseudopodia in concert with depolymerisation at the base of the 'cup' leading to engulfment of the bound target (Flannagan *et al*., 2015; Scott *et al*., 2005). Once the target is internalised it exists within a membrane bound vesicle called the phagosome that can be identified by the presence of the small GTPase, Rab5 (see Figure 1. 4) (Fratti et al., 2001). Rab5 recruits a number of effector proteins, including the type III phosphatidylinositol 3-kinase hVps34, which generates phosphatidylinositol 3-phosphate [PtdIns(3)P] on the cytosolic leaflet of early phagosomes (Kinchen et al., 2008). As the phagosome matures, it loses Rab5 and acquires another GTPase, Rab7, that is essential for the fusion of phagosomes with lysosomes (Harrison et al., 2003). Mature phagosomes also acquire lysosome-associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2) which, until recently, were thought to be derived from lysosomal fusion. However, it appears that phagosomes acquire these proteins before fusion and they are required for fusion of phagosomes with lysosomes (Huynh et al., 2007).

Fusion with lysosomes marks the end of the maturation process (see **Figure 1. 4**) with the formation of phagolysosomes, an acidic vacuole containing degrading enzymes such as proteases, nucleases, lipases, phosphatases and glycosidases (Schröder et al., 2010). Acidification of the mature phagolysosome is very important in degrading the phagosomal contents. The acidic environment of pH 5.0 or less is antimicrobial and also provides the optimal environment for activity of enzymes such as cathepsins (Turk et al., 1993). Acidification is achieved by an ATPase H^+ pump that uses energy to pump H^+ ions into the lumen of the phagosome (Lukacs et al., 1990). Reactive

oxygen and nitrogen species (ROS and NOS), generated by NADPH oxidase (NOX) and nitric oxide synthase 1/2 (NOS1/2) are also targeted to the phagosomes and have potent bacteriocidal activity (Underhill & Ozinsky, 2002). The phagolysosomal environment contains a mixture of antimicrobial enzymes, ROS, NOS, and H^+ that together results in the destruction of the phagolysosome contents.

1.3.4 Autophagy

Whilst the predominant pathway for bacterial degradation in macrophages is the phagocytic pathway there has been much research in recent years into the clearance of bacteria through the autophagy pathway. The autophagic response of cells to bacterial infection is sometimes termed xenophagy because autophagy is also a normal cellular catabolic process that degrades large protein complexes. Autophagy in response to cytosolic bacteria results in the formation of a double membrane vesicle, called an autophagosome, around targeted bacteria which is then delivered to lysosomes. This results in the formation of an autophagolysosome that degrades the contained cargo. Formation of autophagosomes proceeds in 4 main steps: (1) initiation, (2) elongation, (3) closure, and (4) fusion with lysosomes (Vural & Kehrl, 2014). The main source of the membrane for autophagosome formation is the ER and mitochondrial-associated ER membranes (MEM) but it can also come from plasma membrane and endosomes (Deretic & Levine, 2009; Lamb *et al*., 2013). Initiation begins with the formation of an isolation membrane from ER membrane and MEM which expands with the help of autophagy related genes (ATG) proteins. Mammalian target of Rapamycin (mTOR) controls autophagy by inhibiting the formation of an isolation membrane so inhibition of mTOR is essential for the formation of autophagosomes (Ganley et al., 2009). Inhibition of mTOR allows translocation of a protein complex consisting of Unc-51-like autophagy activating kinase (ULK)1/2, ATG13, FIP200

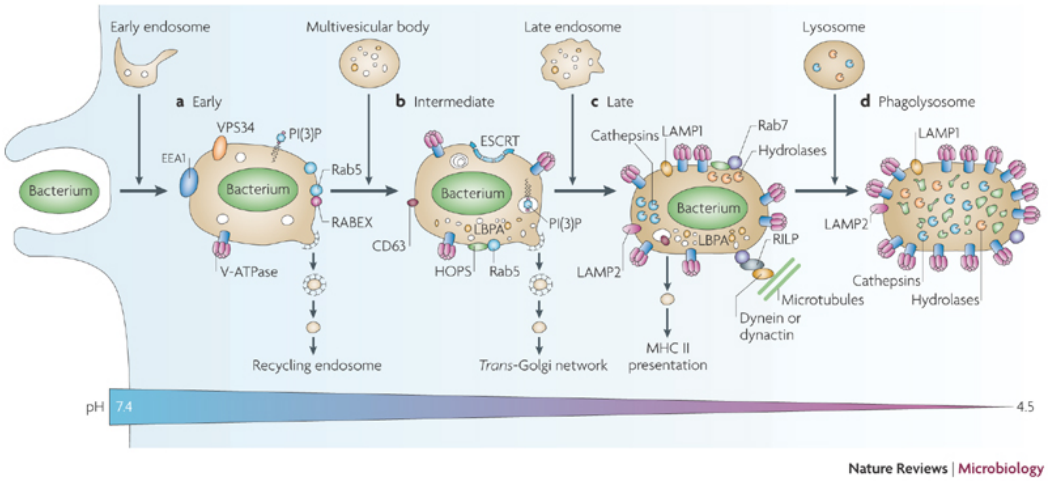

Figure 1. 4 Stages of phagosomal maturation

Shortly after pathogen uptake, the phagosome undergoes a series of transformations that result from its sequential interaction with subcompartments of the endocytic pathway. As the phagosome matures, it acquires and loses different Rab proteins and eventually fuses with lysososomes to degrade its contents. Lamp proteins are key markers of phagolysosomes

Adapted from: Flannagan & Grinstein, 2009.

and ATG101 to the ER and is dependent on ULK1. Another complex containing the type III phosphatidylinositol-3-kinase, VPS34, beclin 1 and ATG14 is then recruited to the developing autophagosome (Vural & Kehrl, 2014). This complex integrates downstream signals with the ATG5/ATG12/ATG16L1 complex that adds phosphatidylethanolamine group to the carboxyl terminus of LC3, which is incorporated into the autophagosome membrane as LC3-II, a commonly used marker of autophagy (Ma et al., 2013). Together with other factors LC3 promotes the elongation and closure of the autophagosome. Subsequent attachment of the SNARE protein syntaxin 17 to the autophagosome mediates fusion with lysosomes (Deretic & Levine, 2009).

1.3.4.1 Phagocytosis and autophagy

There is crosstalk between phagocytosis and autophagy and TLR4 signalling from bacteria results in the recruitment of autophagy protein LC3 to the phagosomes in a process termed LC3 associated phagocytosis (LAP) (Sanjuan et al., 2007) (see **Figure 1. 5**). LC3 is recruited single membrane phagosomes (unlike double membrane

autophagosomes) and conjugated with PE. It is distinct from autophagy in that it does not require ULK1 although ATG5 and ATG7 are required (Martinez et al., 2011). Recruitment of LC3 to the phagosomes requires generation of ROS and by NOX2 by NADPH oxidase (Huang et al., 2009). Differentiating LAP from autophagy requires electron microscopy to distinguish single from double membrane vesicles thus many early autophagy studies may have actually been reporting LAP (Gomes & Dikic, 2014).

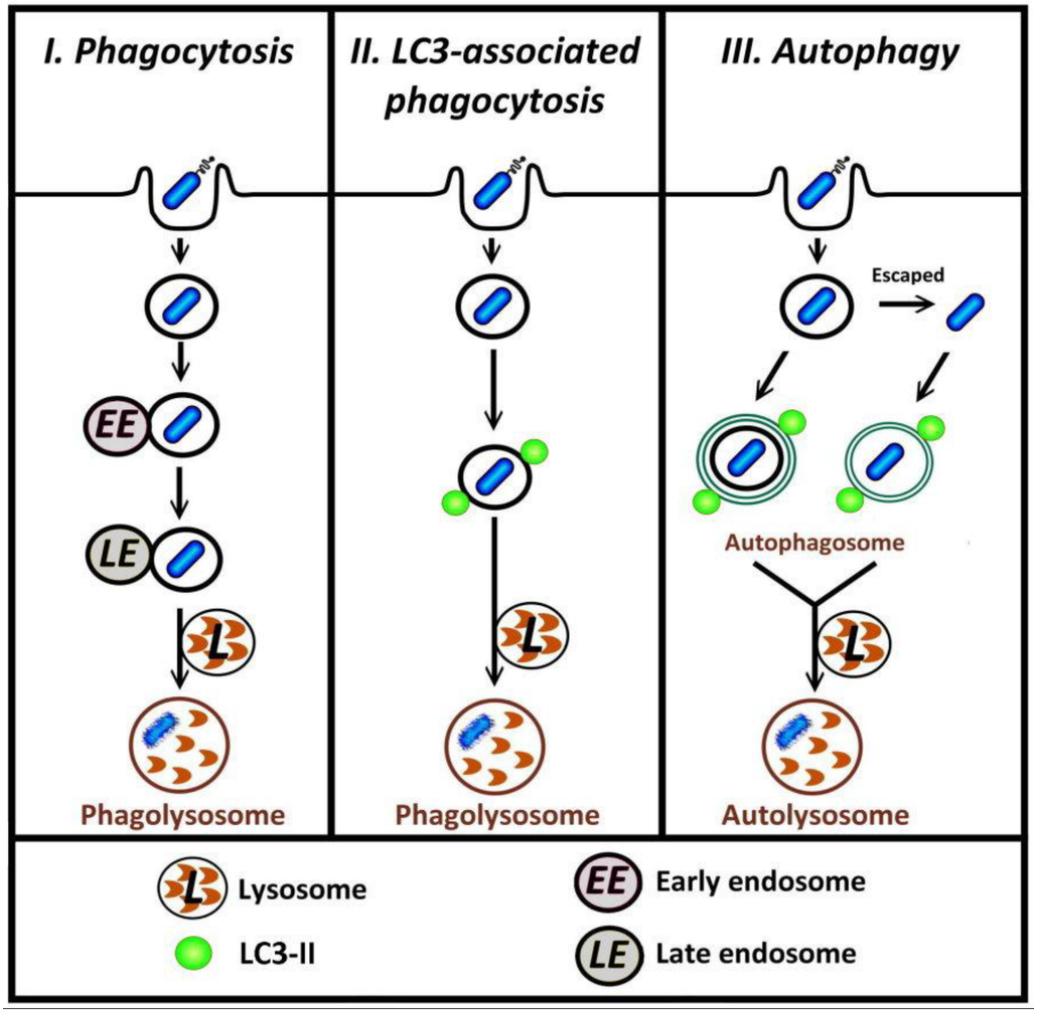

Figure 1. 5 Different pathways by which bacteria in phagosomes can be degraded

I, in typical phagocytosis of bacteria, the phagosome may undergo fusion with endosome and lysosome (phagosome maturation) such that the bacterium is contained within a phagolysosome, the degradative compartment. II, in LC3-associated phagocytosis, autophagy proteins including LC3-II are recruited to the surface of phagosomal membranes; these vesicles subsequently fuse with lysosome. III, bacteria that are retained in, or escape from, phagosome can be targeted by autophagy.

Adapted from: Lai & Devenish, 2012.

1.4 The inflammasomes

The inflammasomes are multi-protein complexes found in the cytosol of macrophages that convert pro-IL-1β and pro-IL-18 into their active, secreted forms. In addition, inflammasomes trigger pyroptosis, a form of programmed inflammatory cell death (Schroder & Tschopp, 2010). Inflammasomes are made up of a sensor protein and an adaptor protein (in most cases) that links the sensor protein to the inflammasome effector, caspase-1. There are two families of sensor protein, the NOD-like receptor (NLR) family comprised of NLRC4, NLRP1b, NLRP3, NLRP6 and NLRP12 and the pyrin or HIN domain-containing (PYHIN) family comprised of AIM2 (de Zoete et al., 2014). The NLR family proteins contain a central NACHT domain which mediates oligomerisation of NLRs and also has a leucine-rich repeat (LRR) that is believed to act as a ligand sensor. NLRs also have a CARD or PYRIN domain that mediates downstream signalling. AIM2 does not have a LRR domain but instead interacts with its ligand, dsDNA, via a HIN200 domain. Following ligand sensing, NLRs oligomerise through the NACHT domain while AIM oligomerises around dsDNA. Oligomerisation provides a platform for the clustering, and subsequent autoproteolysis, of caspase-1 via the adaptor protein ASC. Activated caspase-1 can then cleave the pro-forms of IL-1β and IL-18 into active cytokines (Chen & Schroder, 2013). The inflammasome is generally considered to be protective as ablating this immune complex often increases the susceptibility of animals to infection (de Zoete et al., 2014). Different NLR inflammasomes are activated by different ligands, for example NLRC4 is activated by flagellin or bacterial T3SS rod and needle proteins; NLRP1b is activated by anthrax toxin from *Bacillus anthracis;* NLRP3, the best studied inflammasome, is activated by PAMPs including LPS, ATP, prokaryotic mRNA and pore-forming toxins such as α-hemolysin; NLRP6 is activated by the intestinal microbiota and NLRP12 is activated by *Yersinia pestis* (Chen & Schroder, 2013; de Zoete *et al*., 2014; Schroder & Tschopp, 2010; Storek & Monack, 2015).

Activation of the NLRP3 inflammasome requires two separate signals (see Figure 1. 6). The first signal is through interaction of PAMPs and DAMPs with TLRs which leads to the NF-**κ**B dependent transcription of *IL1b* and *NLRP3*. A secondary signal such as extracellular ATP or intracellular prokaryotic mRNA, is required for NLRP3

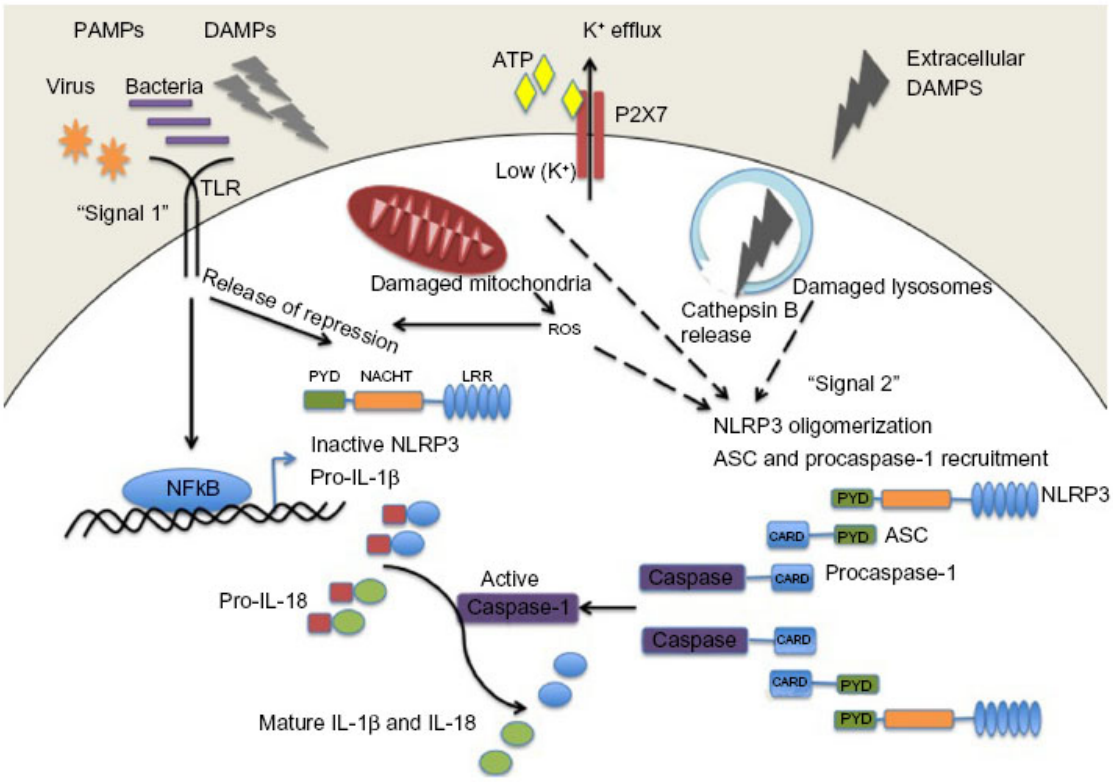

Figure 1. 6 NLRP3 inflammasome activation.

The NLRP3 inflammasomes is activated by 2 sequental signals. Signal 1 requires singallign from TLR4 to induce *Il1b* and *Il18* transcription and release of repression of the NLRP3 inflammasomes. Signal 2 results in oligomerisation of NLRP3, ASC and pro-caspase-1 recruitment and caspase-1 activation. Pro-IL-1β and pro-IL-18 are then cleaved and activation leading to their release. NLRP3 activation also leads to pyroptosis.

Adapted from: Ozaki *et al*, 2015.

oligomerisation and activation (Mariathasan *et al*., 2006; Sander *et al*., 2011). This leads to recruitment and actvaiton of caspase-1 and subsequent cleavage or pro-IL-1β and pro-IL-18 into their active forms. NLRP3-dependent caspase-1 activation also leads to pyroptosis (Haneklaus & O'Neill, 2015).

Furthermore, there is a non-canonical inflammasome that is controlled by murine caspase-11 or its human homologue caspase-4. The non-canonical inflammasome is activated by cytosolic LPS, independent of TLR4, and can stimulate pyroptosis directly and IL-1β release via the NLRP3 inflammasome (Kayagaki *et al*., 2013; Rühl & Broz, 2015; Schmid Burgk *et al*., 2015; Shi *et al*., 2014). The mechanism for activation of the NLRP3 inflammasomes by caspase-11/4 has recently been elucidated. Caspase-11/-4 appears to drive K^+ efflux from cells and this activates the NLRP3 inflammasome leading to caspase-11/4 mediated IL-1β secretion (Rühl & Broz, 2015; Schmid Burgk *et al*., 2015).

The inflammasomes act as another checkpoint for determining the threat level of an engulfed microbe. For example, Sander *et al* showed that phagocytosis of live *E. coli* results in a different immune response to phagocytosis of the same but dead *E. coli* (Sander et al., 2011). Phagocytosis of dead *E. coli* results in release of the pro-inflammatory cytokines IL-6 and TNF-α but phagocytosis of the same but live *E. coli* results in release of IL-6, TNF-α and also IL-1β. Therefore, the cell is able to recognise the presence of live bacteria and responds by releasing additional cytokines. The authors identified that prokaryotic mRNA activates the NLRP3 inflammasomes and thus induces IL-1β release. Since mRNA is unstable and degrades quickly, only live bacteria produce enough for it to be sensed by the NLRP3 inflammasomes, therefore this type of signal was called a viability associated PAMP (vita-PAMP) (Sander et al., 2011). Inflammasomes can also distinguish if an engulfed microbe is a pathogen or not by detecting virulence factors. For example, the NLRC4 is activated by T3SS apparatus that is a virulence factor on some *Salmonella* and some *E. coli* (Miao et al, 2010) .

In summary, macrophages can sense microbes, engulf and degrade them. During this process macrophages also release pro-inflammatory cytokines in order to co-ordinate

the immune response. Furthermore, macrophages can distinguish between different levels of microbial threat and respond accordingly.

1.5 Escherichia coli

Escherichia coli (*E. coli*) is a facultative anaerobic, rod shaped bacterium that resides in the intestinal track of mammals, reptiles, birds and fish (Conway & Cohen, 2015; Tenaillon *et al*., 2010). *E. coli* is the main facultative microorganism in the colon and is one of the earliest colonisers of the infant gut (Adlerberth & Wold, 2009). Following colonisation, *E. coli* metabolism contributes to creation of a reduced environment in the colon that is required for the colonisation of obligate anaerobes such as *Bacteroides* thus making *E. coli* an important member of the microbiota (Adlerberth & Wold, 2009) (Palmer *et al*., 2007; Rotimi & Duerden, 1981). *E. coli* reside in the mucus lining of the intestine and are frequently shed into the gut lumen and eliminated in host faeces (Conway & Cohen, 2015; Kaper *et al*., 2004; Tenaillon *et al*., 2010). Thus, *E. coli* are often used as an indicator of faecal contamination of water. *E. coli* exists as a commensal in the intestines of over 90% of humans but many strains have evolved or acquired virulence mechanisms that results in pathogenicity in this environment (Croxen & Finlay, 2010). These diarrhoeagenic *E. coli* consist of the subgroups: enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); enteroaggregative *E. coli* (EAEC); enterohaemorrhagic *E. coli* (EHEC); and enteroinvasive *E. coli* (EIEC). Another subset of *E. coli,* classified as extraintestinal *E. coli* (ExPEC), have virulence factors that allow them to colonise niches outside of the gastrointestinal tract (GIT) including the urinary tract (uropathogenic *E. coli* (UPEC)) and the brain (neonatal meningitis *E. coli* (NMEC)) (Croxen & Finlay, 2010; Kaper *et al*., 2004; Wiles *et al*., 2008b).

E. coli preferentially grows on mono- or disaccharide sugars. In the gut, these sugars arise from either the diet, host secreted mucus or as by-products of bacterial metabolism (Conway & Cohen, 2015). When colonising and maintaining numbers in the gut, *E. coli* MG1655 primarily uses glycolysis for growth and mutants in glycolysis have severe colonisation defects (Chang et al., 2004; Miranda et al., 2004). Mutants in the Entner-Doudoroff (ED) pathway are also severely defective in colonisation and

gluconate, a sugar metabolised by the ED pathway, is catabolised by *E. coli* in the mouse intestine (Sweeney et al., 1996). Other strains such as EHEC EDL933 also use glycolysis to colonise the gut but when they are in competition with other *E. coli* they switch to both glycolytic and gluconeogenic substrates, such as amino acids, for maintenance (Miranda et al., 2004). An interesting hypothesis put forward by Conway and Cohen, called the 'restaurant hypothesis', suggests a model for how a *E. coli* can exist in the colon, an environment that is rich in dietary fibre but low in mono- and disaccharides that are required for *E. coli* growth (Conway & Cohen, 2015). It is suggested that the obligate anaerobes such as *Bifidobacterium* and *Bacteroides* provide *E. coli* with the sugars it needs to survive in a location specific manner. Thus, these biofilms act as 'restaurants' where the *E. coli* can survive (Conway & Cohen, 2015). Different commensal *E. coli* 'dine' at different 'restaurants' and thus can occupy different niches within the intestine. Indeed, fluorescent in situ hybridization (FISH) microscopy has shown the colocalisation of *E. coli* with anaerobes in streptomycin treated mice. This hypothesis is in contrast to the nutrient niche hypothesis that suggests that there is a collective pool of nutrients for all bacteria to exploit for colonisation. The restaurant hypothesis is intriguing and suggests a level of co-operation between distinct groups of bacteria in the gut.

Sequenced *E. coli* genomes are characterised by having a core genome of approximately 2, 200 genes that is shared by all strains and a pan-genome that varies between strains and cumulatively contains approximately 13, 000 genes (Rasko et al., 2008). Pathogenic *E. coli* often possess large clusters of virulence genes on pathogenicity island (PAIs) which can be found on plasmids, such as pO157 in EHEC (Nataro & Kaper, 1998), or integrated into the chromosome. These virulence factors distinguish pathogenic from commensal *E. coli* and are discussed in detail below. Due to the presence of these PAIs, pathogenic *E. coli* are better characterised than commensal *E. coli*.

E. coli are classified into 4 main phylogenetic groups based on the ECOR reference library: Group A, B1, B2 and D. More recently these groups have been expanded to included 4 new phylogroups C, E, F and clade I (Clermont et al., 2012). In the human microbiota, the main phylogroups are A and B2 but there is now a general trend

towards increased levels of B2 *E. coli* in humans (Chaudhuri & Henderson, 2012; Tenaillon *et al*., 2010; White *et al*., 2011). Group A contains K12 lab strains of *E.coli* such as MG1655 and the commensal HS. Group B2 contains many commensal *E. coli* including Nissle 1917 as well as the UPECs CFT073 and UTI89 and the AIECs HM605 and LF82 (Chaudhuri & Henderson, 2012; la Fuente *et al*., 2014). Furthermore, another ExPEC, the NMEC, are predominantly from the B2 phylogroup (Wijetunge et al., 2015). Therefore, B2 group *E. coli* are thought to be 'aggressive commensals' as they are harmless in the gut but carry the most virulence genes of all groups and also contain many ExPEC strains (Smith *et al*., 2013a; White *et al*., 2011).

1.6 Commensal *E. coli*

Commensal *E. coli* provide the host with many benefits. *E. coli* ferments sugars to short chain fatty acids (SCFA) in the colon and contributes to the overall SCFA pool that is used as an energy source for colonocytes (Macfarlane & Macfarlane, 2003), Furthermore, SCFA such as acetate have been shown to be anti-inflammatory with acetate, butyrate and propionate decreasing IL-8 release from cultured macrophages and decreasing NF-κB activity in cultured colonocytes (Tedelind et al., 2007). Commensal *E. coli* also provide protection from invading pathogens. Pre-colonisation of mice with the commensal E coli HS and Nissle 1917 prevented colonisation with the pathogenic *E. coli* EDL933 (Maltby et al., 2013). Moreover, Nissle 1917 is marketed as a treatment for travellers diarrhoea and also used in the treatment of UC (Maltby et al., 2013; Orel, 2014). In a clinical trial comparing treatment of UC patients with Nissle 1917 and mesalazine 500, an anti-inflammatory drug used to to maintain remission of UC, both Nissle and mesalazine 500 had similar outcomes after 1 year of treatment. This led the authors to conclude that Nissle 1917 has equivalent efficacy and safety in maintaining remission in UC patients as mesalazine 500 (Kruis et al., 2004)

1.7 Diarrhoeagenic *E. coli*

1.7.1 Enteropathogenic *E. coli* (EPEC)

EPEC cause potentially fatal diarrhoea in infants in the developing world and are part of a family of pathogens that cause attaching and effacing (A/E) lesions on intestinal epithelium (Figure 1. 7, (Croxen & Finlay, 2010). The attached bacteria efface the microvilli and polymerise host actin molecules to form pedestal-like structures below the site of attachment (Wong et al., 2011). A large chromosomal PAI known as the locus of enterocyte effacement (LEE) confers the attaching effacing phenotype to EPEC (McDaniel et al., 1995). A plasmid, the EPEC adherence factor (EAF) plasmid encodes bundle forming pili (BFP) that mediate the initial attachment of EPEC to the epithelium and formation of microcolonies on the epithelial cell surface (Giron et al., 1991). Using the LEE-encoded T3SS, EPEC inject translocated intimin receptor (Tir) into the host cell allowing the outer membrane protein intimin to bind to Tir and form an intimate attachment with the cell (Kenny et al., 1997). Binding of intimin to Tir leads to clustering of Tir and subsequent host tyrosine kinase mediated phosphorylation of Tir (Kenny, 1999). EPEC use a repertoire of effectors, translocated into the host cell by the T3SS, to subvert normal cellular processes such as phagocytosis and inflammatory responses (Wong et al., 2011). Uptake of EPEC by macrophages is inhibited by the LEE-encoded effectors EspF (Quitard et al., 2006) and EspB (Iizumi et al., 2007) through inhibition of phosphatidylinositol-3 (PI-3) kinase-dependent uptake and myosins, respectively. More recently, EspH has been shown to inhibit phagocytosis by inhibiting Rho guanine nucleotide-exchange factors (GEFs) that control cytoskeleton remodelling during phagocytosis (Dong et al., 2010). EPEC effectors NleE, NleB, NleC, NleD and NleH inhibit NF-**κ**B activation and thus pro-inflammatory cytokine production by various mechanisms (Wong et al., 2011). For example, NleE prevents I**κ**B degradation, a negative regulator of NF-**κ**B, in response to TNF-α and IL-1β stimulation thus stopping the translocation of NF-**κ**B into the nucleus (Nadler et al., 2010) and NleC is a protease that cleaves and inactivates the p65 subunit of NF-**κ**B again inhibiting NF-**κ**B signalling (Yen et al., 2010). NleB interacts with host glyceraldehyde 3-phosphate dehydrogenase (GADPH) resulting in its modification and inhibition of NF-**κ**B dependent immune response (Gao et al., 2013). NleB also targets death receptor signalling and inhibits TNF-α induction of the canonical death-inducing signalling complex (DISC) thereby inhibiting apoptosis, a host response to limit the spread of the infection (Pearson et al., 2013).

1.7.2 Enterohaemorrhagic *E. coli* (EHEC)

EHEC, another A/E pathogen, causes diarrhoea, occasionally bloody diarrhoea (also known as haemorrhagic colitis) and haemolytic uremic syndrome (HUS), the leading cause of renal failure in children (Croxen & Finlay, 2010; Ho *et al*., 2013). Cattle are a reservoir for EHEC and the source is usually contaminated food or water (Wong et al., 2011). Most outbreaks in Europe are due to the O157:H7 serotype and all EHEC O157:H7 harbour a 92 kb virulence plasmid known as pO157. EHEC possess the same LEE as EPEC and thus injects a number of similar effectors into the cell and attach to epithelium through pedestal formation in a similar manner to EPEC (Figure 1. 7, (Wong et al., 2011). An important additional virulence factor in EHEC is Shiga toxin (Stx), an AB_5 toxin that is not secreted from EHEC but instead relies on lambdoid phage-mediated lysis in response to DNA damage and SOS response (Toshima et al., 2007). AB_5 toxins typically are made up of an A and five B subunits. The StxB subunit binds to globotriaosylceramid (Gb3) receptors on intestinal mucosa and kidney epithelia and is endocytosed by the cell thus internalising the StxA subunit. The StxA subunit is activated by cleavage in the golgi and the active toxin targets the 28S rRNA this blocking protein translation, resulting in necrosis and cell death (Pacheco & Sperandio, 2012). There are different subtypes of Stx circulating in the EHEC population, Shiga toxin type 1 (Stx1) and Shiga toxin type 2 (Stx2). Stx1 is almost identical to Shiga toxin from *Shigella* but Stx2 displays approximately 56% sequence similarity with Stx1 and *Shigella* Stx (Lee et al., 2013). Stx2 is more commonly associated with haemorrhagic colitis and HUS in humans (Croxen & Finlay, 2010). Cell death caused by Stx give the toxin access to the bloodstream where it travels to the kidneys and can bind to kidney epithelium and cause HUS.

EHEC also possess another virulence factor encoded on pO157, Enterohemoylsin (Ehx), a repeats in toxin (RTX) toxin that is similar in structure to the well-characterised α-hemolysin toxin found in many UPEC strains (Kaper et al., 2004). Like α-hemolysin, Ehx is located in an operon with the genes predicted to encode proteins required for its export: *ehxC*, an acyltranferase; *ehxA*, the hemolysin; *ehxB*, an ABC transporter; and *ehxD*, a transmembrane protein (Schmidt et al., 1996). Ehx is proposed to be transported out of the cell using a type I secretion system

(Bielaszewska et al., 2014). Recently, Ehx was reported to induce cytotoxicity and IL-1β release from human macrophages in an NLRP3 inflammasome dependent manner (Zhang et al., 2012). Interestingly, murine macrophages did not undergo similar cytotoxicity due to an apparent difference in sensitivities of human and murine NLRP3 to Ehx (Cheng et al., 2015).

1.7.3 Enteroaggregatative *E. coli* (EAEC)

EAEC are a leading cause of diarrhoea and symptoms include abdominal pain, nausea, vomiting, fever, water or bloody/mucoid stool. EAEC are able to adhere to intestinal cells and produce a variety of enterotoxins and cytotoxins (Kong et al., 2015). Adherence is mediated through binding of aggregative adhesion fimbria (AAF) to host proteins such as fibronectin in the extracellular matrix (Berry et al., 2014). There are four variants of AAF all encoded by the pAA virulence plasmid (Croxen & Finlay, 2010). EAEC are heterogenous in their genetic composition and some strains also express non-pilus adhesins including outer membrane porin (OMP), dispersin and Hda (Boisen *et al*., 2008; Kong *et al*., 2015; Monteiro Neto *et al*., 2003). Dispersin allows for dispersal of EAEC throughout the mucosa by neutralising the negative charge created by LPS and creating a positive charge supporting binding of AAF to host receptors and outward diffusion (Sheikh et al., 2002). EAEC form biofilms *in vitro* and *in vivo,* and biofilms in the mucus can block absorption of nutrients and lead to malnutrition (Kong et al., 2015). EAEC also secrete toxins, the best characterised of which is the EAEC heat-stable entertoxin 1 that is encoded on the pAA plasmid and causes watery diarrhoea (Chaudhuri et al., 2010). EAEC induce mucosal inflammation and infection is associated with increased pro-inflammatory cytokine markers such as IL-1β and IL-8 (Huang et al., 2004; Jiang et al., 2002). The prototypical strain 042 has also been shown to increase release of IL-6, TNF-α, granulocyte macrophages colony-stimulating factor (GM-CSF), intercellular adhesion molecule (ICAM)-1 and IL-1β from intestinal tissue (Kong et al., 2015). Secretion of these cytokines has been linked

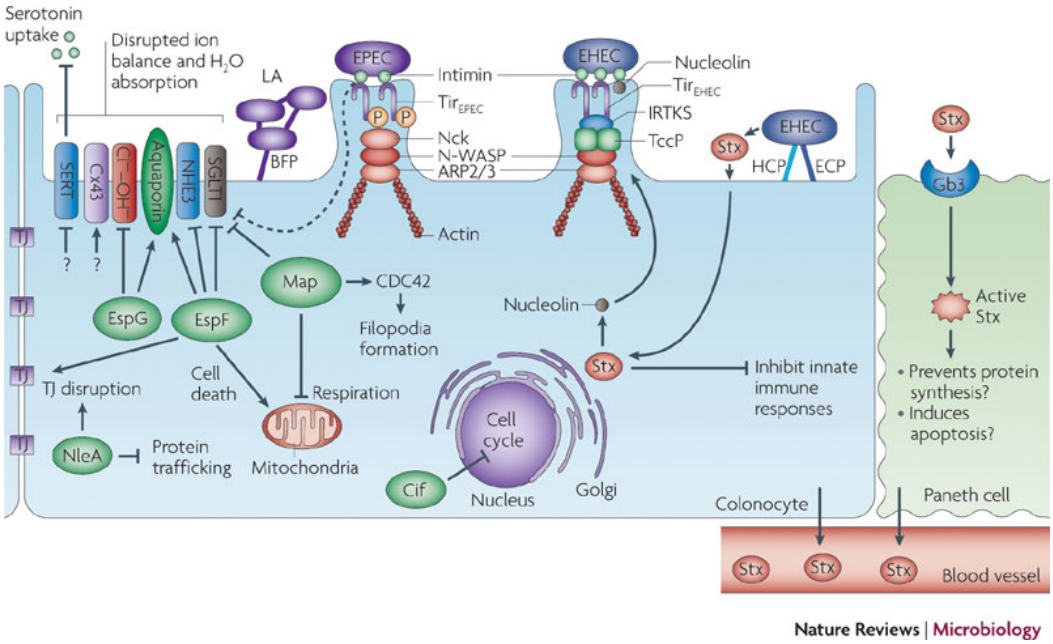

Figure 1. 7 Pathogenic mechanisms of enteropathogenic and enterohaemorrhagic *E. coli.*

EPEC and EHEC are both attaching and effacing pathogenic *E. coli* that use PAI encoded pathogenicity factors to attach to epithelial cells and inject effectors using a T3SS. These effectors modulate many immune responses including inhibiting innate immune responses. EPEC use bundle forming pili (BFP) enabling formation fo microcolonies on the epithelial surface. EHEC alone secretes Shiga toxin (Stx)which is taken up into Paneth cells and can enter the blood stream.

Adapted from From: Croxen & Finlay, 2009.

to signalling from TLR5 after its interaction with EAEC flagellin surface protein (Harrington et al., 2005).

1.7.4 Enterotoxigenic *E. coli* (ETEC)

ETEC adhere to intestinal epithelium through plasmid encoded colonisation factors (CFs) (Turner et al., 2006) and secrete enterotoxins that cause efflux of water into the gut lumen and result in diarrhoea (Croxen & Finlay, 2010). ETEC like EAEC is a common cause of traveller's diarrhoea and also diarrhoea in piglets. A large number of CFs have been identified with the most common being colonisation factor antigen I (CFA/I). Adhesion moieties interact with cognate receptors such as carbohydrate moieties of non-acid glycosphingolipids and glycoporteins in the case of CFA/I (Jansson et al., 2006) and acid glycophingolipid sulphatide as is the case for CFA/IV (Jansson et al., 2009). ETEC also possess chromosomally encoded adhesins, Tia and TibA, that allow for the adhesion of ETEC to epithelial cells. Tia, a 25 kDa outer membrane protein that interacts with host glycolproteins promoting adherence and invasion of epithelial cells *in vitro* (Fleckenstein et al., 2002) and TibA is an autotransporter protein that allows for intimate attachment to host cells (Sherlock et al., 2005). EtpA, a secreted glycoprotein that is thought to bind both host cells and ETEC flagella allows for interactions between the host and bacteria (Roy et al., 2009). Once attached to the cell, ETEC produce heat-stable enterotoxin (ST) or heat-labile enterotoxin (LT) which induce fluid secretion or inhibit fluid absorption in the gut (Turner et al., 2006). ST can be further classified into STa (typically associated with human ETEC) and STb (typically associated with porcine strains). STs secreted by ETEC bind the extracellular domain of guanylyl cyclase C (GC-C) on the brush border of intestinal epithelium which induces internalisation of the receptor and an increase in cyclic GMP (Fleckenstein et al., 2010). An increase in cGMP results in opening of the cystic fibrosis transmembrane receptor (CFTR), Cl^- efflux, inhibition of NaCl absorption and a loss of water through osmotic diarrhoea (Chao et al., 1994). Heat labile toxin (LT) is closely related to cholera toxin and is a AB_5 toxin that binds to intestinal epithelium receptor monosialogangliosides GM1 via its pentameric B subunit. Following binding, the toxin is internalized on lipid rafts (Kesty et al., 2004) and trafficked via the endoplasmic reticulum (ER) to the cytosol where it has ADP-

ribosyltransferase activity. The stimulatory guanine-nucleotide-binding (G) protein α-subunit is ADP-ribosylated which activates adenyl cyclase and leads to an increase in cAMP which in turn activates CFTR resulting in secretion of electrolytes and water and ultimately diarrhea (Nataro & Kaper, 1998).

1.7.5 *Shigella* spp./ Enteroinvasive *E. coli* (EIEC)

EIEC and *Shigella* have the same mechanisms of pathogenicity and its is widely accepted that they should form a single pathovar (Sims & Kim, 2011; Strockbine & Maurelli, 2015). *Shigella* are the cause of bacillary dysentery and bloody diarrhoea; *Shigella* also invade and replicate within epithelial cells. *Shigella* do not express flagella, form biofilms or express any known adhesion but instead expresses a surface protein, IcsA, that facilitates intracellular motility. *Shigella* traverse Peyer's patches through M-cells and are phagocytosed by macrophages. Macrophages NLRC4 inflammasomes detect the *Shigella* T3SS leading to caspase-1 activation, pyroptosis and IL-1β and IL-18 release resulting in the recruitment of polymorphonuclear cells and inflammation of the epithelium (Miao et al., 2010; Suzuki et al., 2007). The T3SS effector IpaB also induces caspase-1 dependent cell death (Chen *et al*., 1996; Hilbi *et al*., 1998) which is likely to be caused by the spontaneous formation of ion channels in the plasma membrane by IpaB (Senerovic et al., 2012). *Shigella*-induced inflammasome activation leads to damage to tight junctions and this allows for further access of *Shigella* to the submucosa where *Shigella* can invade epithelial cells from the basolateral side (Carayol & Van Nhieu, 2013). Once *Shigella* has invaded the cells, it lyses the phagosomal vacuoles in which is it contained in a process that is dependent on the T3SS effectors IpaB, IpaC, IpaD and IpaH (Croxen & Finlay, 2010). Ruptured membrane remnants are polyubiquinated and recruit the autophagic markers LC3 and p62 and are targeted for degradation (Dupont et al., 2011). Septins entrap *Shigella* and inhibit their motility targeting them for autophagy (Mostowy et al., 2010) but interestingly *Shigella* secretes the IcsB T3SS effector that prevents the recognition of IcsA by the autophagy adaptor protein ATG5 and thus inhibits bacteria degradation (Ogawa et al., 2005). When free in the cytosol, *Shigella* secrete a number of other proteins that inhibit host process such as epithelial cell turnover and detachment, and apoptosis: cell turnover is inhibited by IpaB (Iwai et al., 2007); cell detachment is

inhibited by OspE (Kim et al., 2009); and IpgD can activate Akt proteins that regulate cell survival and thus inhibit apoptosis (Pendaries et al., 2006). *Shigella* also silences inflammation through a number of effectors. OspG prevents degradation of IκBα and OspF translocates to the nucleus where it dephosphorylates MAP kinases required for transcription of NF-κB regulated genes (Kim et al., 2005) (Arbibe et al., 2007; Li et al., 2007). *Shigella* spread from cell to cell by hijacking actin filaments for motility and formation of protrusion at cadherin-based cell junction in the donor cell and endocytosis by the recipient cell (Carayol & Van Nhieu, 2013). IcsA is necessary for *Shigella* motility and binds to N-WASP which activates the Arp2/3 actin nucleator complex and the growth of actin filaments propels the bacteria through the cell (Egile et al., 1999).

1.8 Extraintestinal Pathogenic *E. coli* (ExPEC)

1.8.1 Neonatal Meningitis *E. coli* (NMEC)

NMEC is the most common cause of neonatal meningitis and has mortality rates approaching 40% (Dale & Woodford, 2015). NMEC is a common part of the gut microbiota and the source of NMEC is usually maternal. NMEC gain access to the bloodstream by transitioning through enterocytes and eventually NMEC crosses the blood brain barrier to cause inflammation, oedema and neural damage. High level bacteraemia (> 10^3 CFU per ml blood) is required for progression of the disease (Wang & Kim, 2013). NMEC is able to survive in the blood through the formation of an anti-phagocytic capsule and subversion of the complement system by the outer membrane protein OmpA (Wooster et al., 2006). NMEC is also able to prevent apoptosis and cytokine release following phagocytosis by macrophages and monocytes thus providing the bacteria with a replication niche (Selvaraj & Prasadarao, 2005; Sukumaran *et al*., 2004). Attachment to the microvascular endothelial cells of the blood brain barrier is mediated through OmpA binding to ECGP965 (Prasadarao, 2002) and type I pili FimH adhesion to CD48 (Khan et al., 2007). Invasion is dependent on OmpA, FimH, invasion of brain endothelium (Ibe) proteins and cytotoxic necrotising factor 1 (CNF1), a Rho GTPase involved in myosin rearrangement (Kim, 2008).

1.9 Uropathogenic *E. coli* (UPEC)

UPEC accounts for 75% of all uncomplicated urinary tract infections (UTIs) and 65% of complicated UTIs (Flores-Mireles et al., 2015). The majority of UPEC belong to the B2 phylogroup of *E. coli* and the sequence similarity of the UPEC CFT073 to the commensal Nissle 1917 suggest that they are closely related (Welch *et al*., 2002; Wiles *et al*., 2008b). UPEC ascend the urethra and preferentially colonise the bladder causing cystitis but some strains can ascend the ureters to infect the kidneys causing pyelonephritis (Flores-Mireles *et al*., 2015; Ulett *et al*., 2013; Wiles *et al*., 2008b). In the bladder, UPEC adhere to, and invade, bladder epithelial cells (BECs) and are contained within acidic compartments with similar features to late endosomes or lysosomes (Eto et al., 2006). UPEC invade the large, terminally differentiated superficial umbrella cells that line the lumen of the bladder and the bacteria are able to escape into the cytosol where they multiply and form biofilm like colonies of many thousands of bacteria called intracellular bacterial communities (IBCs) (Anderson *et al*., 2003; Mulvey *et al*., 2001; Rosen *et al*., 2007). Bladder cells respond by shedding and are removed with the flow of urine but this leaves the immature underlying cells exposed and susceptible to infection (Mulvey *et al*., 2001; Wiles *et al*., 2008a). UPEC are common members of the normal human gut microbiota but have acquired, by horizontal gene transfer, a number of colonisation factors that are located on PAIs (Chen *et al*., 2006; Welch *et al*., 2002). These colonisation factors, described below, allow UPEC to colonise the bladder, obtain nutrients from urine, attack infiltrating phagocytes and modify host responses to infection.

1.9.1 Survival in urine

Urine is a nutritionally poor environment and UPEC has established ways to acquire nutrients and adapt their metabolism to survive in this environment (Alteri & Mobley, 2015; Wiles *et al*., 2008b). Urine contains amino acids and small peptides and UPEC uses these substrates for gluconeogenesis and conversion into pyruvate and oxaloacetate for entry into the TCA cycle to generate ATP. Mutations in both these processes results in fitness defects (Alteri et al., 2009). Genes in d*e novo* nucleotide

synthesis of pyrimidines (*pyrD*) and guanine (*guaA*) have also been identified in mutants screens as necessary for fitness in urine (Bahrani Mougeot *et al*., 2002; Russo *et al*., 1996). UPEC are able to utilise D-serine produced by mammals and present at high levels in the urine. Prolonged asymptomatic UTI results in the increase in expression of genes for D-serine deaminase and amino acid transport highlighting the importance of these pathways in pathogenesis (Roesch et al., 2003).

Urine is an iron poor environment and UPEC also have to compete for the available iron with host iron-chelating proteins such as transferrin (Wiles *et al*., 2008b). UPEC have multiple iron-binding siderophores that have higher affinity for free iron than host proteins. The most common siderophore found in UPEC is enterobactin and this has a K_d of ~10^{-49} for iron. However the host protein lipocalin 2 can bind enterobactin and prevent it from binding iron (Raymond et al., 2003). Therefore, UPEC possess multiple iron siderophores including the enterobactin-variant salmochelin, yersiniabactin and aerobactin (Alteri & Mobley, 2015). Interestingly, salmochelin is modified by glucosylation by IroB encoded within the salmochelin gene cluster which prevents its binding by lipocalin 2 (Smith, 2007). The redundancy of siderophores in UPEC is an indication of the importance of iron acquisition for fitness in urine.

1.9.2 Colonisation factors

UPEC express a number of pili, the most common of which are type 1, P, S, and 1FC pili encoded by the *fim, pap, sfa* and *foc* operons respectively. Pili are expressed in a hierarchical manner due to cross-talk amongst operons leading to changes in expression depending on the environment in a process called phase variation (Holden *et al*., 2007; 2006; Lindberg *et al*., 2008). For example, type 1 fimbriae expression is repressed by P pili expression through PapB inhibition of FimB recombination maintaining the *fim* switch in the 'off' orientation (Holden et al., 2006). Type 1 and P pili are the most common expressed by UPEC and are amongst the most important virulence factors for the establishment of UTIs in mice and humans (Bahrani Mougeot et al., 2002; Connell et al., 1996; Mulvey et al., 2001). Using the FimH adhesin located at the fimbrial tip, UPEC bind to urothelial mannosylated glycoproteins uroplakin Ia and IIIa and $\alpha_1\beta_3$ integrins to initiate invasion into umbrella cells (Eto *et al*., 2007;

Mulvey *et al*., 1998). FimH binding activates Rho GTPases causing actin rearrangement and internalisation of bacteria (Martinez & Hultgren, 2002). Interestingly, internalization triggers TLR4 signalling, increased cAMP levels and exocytosis of bacteria from bladder epithelial cells (BECs) (Song et al., 2009) but UEPC can subvert this process and form IBC as described above (Anderson *et al*., 2003; Hannan *et al*., 2010). P pili, encoded by pyelonephritis associated pili (*pap*) operon, are often associated with pyelonephritis but cystitis isolates strains also carry this operon. PapG, an adhesin on the tip of P pili, binds to glibosides on host cells (Strömberg et al., 1990). In addition to attaching to host cells, PapG binding to glycosphingolipids results on the release of ceramide, a TLR4 agonist, leading to release of IL-8 and local inflammation and reduction of polymeric immunoglobulin receptor (PlGR) expression and inhibition of release of immunoglobulin A into the kidney lumen (Fischer et al., 2007; Rice et al., 2005).

1.9.3 Secreted toxins

UPEC encode a number of secreted toxins that cause tissue damage and may lead to an inflammatory response. These toxins included secreted autotransporter toxin (SAT), vacuolating autotransporter protein (VAT), cytotoxic necrotizing factor 1 (CNF1) and α-hemolysin (HlyA) (Bien *et al*., 2012; Flores-Mireles *et al*., 2015; Restieri *et al*., 2007; Wiles *et al*., 2008a).

The prototypical pyelonephritis isolate CFT073 expresses both SAT and VAT while the prototypical cystitis isolate UTI89 only expresses VAT, however distribution of these autotransporter proteins across the UPEC group is varied. The role for VAT in UTI pathogenesis is not studied thoroughly but it is involved in virulence in avian pathogenic *E. coli* (APEC), a close relative of UPEC (Parreira & Gyles, 2003). SAT on the other hand has been shown to induce loss of BECs and vacuolisation of both bladder and kidney cell lines (Guyer et al., 2000). SAT also induces actin rearrangement and autophagy in BECs leading to a block in autophagic flux and the accumulation of autophagy protein LC3-positive vacuoles (Liévin-Le Moal et al., 2011). CNF1, another secreted toxin, is encoded by a third of all UPEC strains and

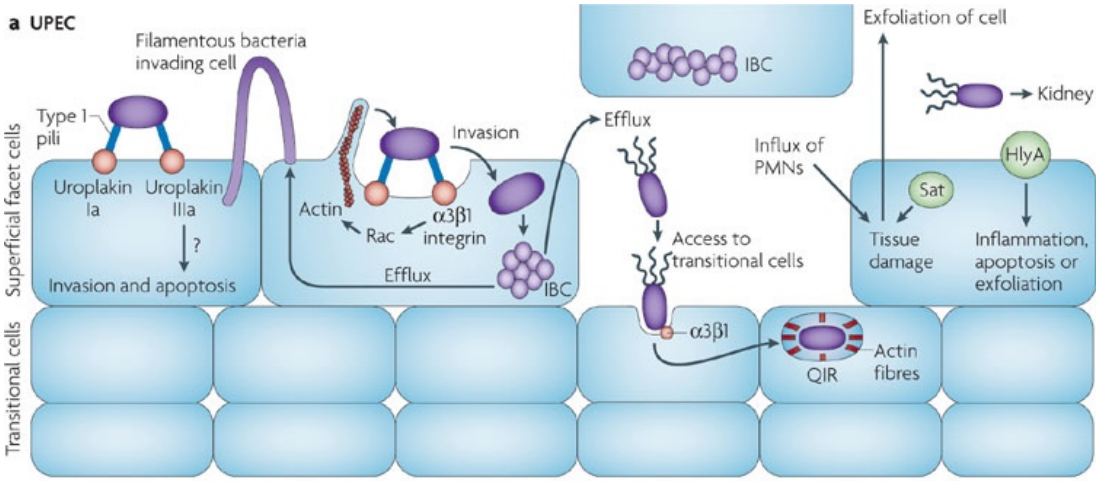

Figure 1. 8 The different stages of UEPC infections.

UPEC attaches to the uroepithelium through type 1 pili, which bind the receptors uroplakin Ia and IIIa; this binding stimulates unknown signalling pathways (indicated by the question mark) that mediate invasion and apoptosis. Binding of type 1 pili to α 3β1 integrins also mediates internalization of the bacteria into superficial facet cells to form intracellular bacterial communities (IBCs) or pods. Sublytic concentrations of the pore-forming haemolysin A (HlyA) toxin can inhibit the activation of Akt proteins and leads to host cell apoptosis and exfoliation. Exfoliation of the uroepithelium exposes the underlying transition cells for further UPEC invasion, and the bacteria can reside in these cells as quiescent intracellular reservoirs (QIRs) that may be involved in recurrent infections.

Adapted from Croxen & Finlay, 2009.

constitutively activates Rho GTPases RhoA, Rac and/or Cdc43 (Lemonnier et al., 2007). CNF1 exerts its influence in the cytosol and is internalized by triggered endocytosis following binding to lamelin receptor precursor (Chung et al., 2003). Activation of Rho GTPases results in the formation of actin stress fibers, lamellipodia, filopodia and membrane ruffling (Bower et al., 2005). CNF1 has been implicated in increased invasion of epithelial cells (Doye et al., 2002) and induction of apoptosis of BECs *in vitro* (Mills et al., 2000) but its exact role in UPEC pathogenicity *in vivo* is still unknown.

1.9.3.1 α-hemolysin

There is a strong correlation between the expression of HlyA and severity of UTIs. 31-48% of cystitis isolates and 78% of pyelonephritis isolates express HlyA (Ristow & Welch, 2015). Hemolysin is a member of the repeats in toxin (RTX) family of toxins and is a pore forming hemolysin and potent cytolysin known to target a wide range of cell type including epithelial cells, monocytes, and neutrophils (Gadeberg *et al*., 1983; Russo, 2005; Schaale *et al*., 2015; Wiles & Mulvey, 2013). Hemolysin is encoded by an operon that contains the genes required for the production of the toxin, its activation and secretion from the cell in the arrangement *hlyCADB* (Welch & Pellett, 1988). Interestingly different strains of UPEC express different quantities of hemolysin and cloning of the *hlyCADB* promoter region from a virulent strain into a less virulent strain increases the virulence of the less virulent strain (Welch & Falkow, 1984). Expression of *hlyCADB* is under the control of the transcriptional anti-terminator *rfaH* that also control expression of the LPS core and *cnf1* (Bailey et al., 1997; Landraud et al., 2003)

Hemolysin is not required for colonization of the gut and the requirement of HlyA for colonization of the bladder is strain dependent (Ristow & Welch, 2015). Hemolysin negative mutants of the prototypic pyelonephritis and cystitis isolates CFT073 and UTI89 can colonise the bladder like wild type strains but another UPEC, CP9, cannot.

HlyA pore formation *in vivo* has never been directly shown by crystallography but studies linking hemolysin to pore formation have shown insertion of HlyA into

membranes affects their permeability (Ristow & Welch, 2015) Experiments have shown that an amphipathic region at amino acids 272-301 is required for pore formation in artificial membranes (Valeva et al., 2008). Incorporation into host membranes occurs independently of acylation by HlyC (Sánchez-Magraner et al., 2006) but is dependent on Ca^{2+} binding (Boehm et al., 1990). Ca^{2+} binding results in a structural and conformational change in HlyA that increases hydrophobicity (Bakás et al., 1998). Acylation, however, is required for cell lysis (Stanley et al., 1994).

Binding of hemolysin to non-erythrocytes is mediated through the integrin heterodimer CD11a/CD18 (also known as LFA-1) that is expressed on neutrophils and monocytes and expression of LFA-1 on cells not normally sensitive to HlyA resulted in increased sensitivity (Lally et al., 1997; 1999). Contrary to the above evidence another group has suggested that binding to target cells is receptor-independent (Valeva et al., 2005). Moreover LPS may be involved in chaperoning hemolysin to epithelial cells by binding to CD14 but the lytic effects of HlyA occur independently of the LPS/CD14 interaction (Månsson et al., 2007). Therefore the exact mechanism of HlyA binding to host cells remains to be elucidated.

1.10 Adherent-invasive *E. coli*

Crohn's Disease is characterised by the transmural inflammation of any point of the gastrointestinal tract that results in chronic pain, diarrhoea, ulcerations and poor absorption of nutrients. This most commonly occurs in the terminal ileum of the small intestine or the large intestine. The exact aetiology of IBD is not completely understood but it is thought to be a mixture of host genetic factors and dysbiosis of the gut microbiota. There are 30 gene loci classified as being associated specifically with CD indicating a stong genetic component to this disease (Jostins et al., 2013). There is a change in the overall composition of the microbiota in patients with IBD with an increase in Actinobacteria and Proteobacteria including *E. coli* compared to healthy controls (Guinane & Cotter, 2013).

E. coli are more commonly isolated from patients with CD than healthy controls (Darfeuille-Michaud *et al*., 1998; Elliott *et al*., 2013; la Fuente *et al*., 2014; Martin *et*

al., 2004; Martinez-Medina *et al*., 2009) and the mucosa of CD patients tends to harbour more *E. coli* than healthy controls (Darfeuille-Michaud *et al*., 1998; 2004). Moreover, *E. coli* have been identified in the granulomas, the histological hallmark of CD pathogenesis, and can induce granuloma formation *in vitro* (Meconi et al., 2007). Over the past 15 years mounting evidence suggests that CD-associated *E. coli* have the ability to adhere to, and invade, epithelial cells and can replicate in macrophages *in vitro* (Darfeuille-Michaud *et al*., 1998; Glasser *et al*., 2001; Martin *et al*., 2004; Subramanian *et al*., 2008b). *E. coli* that possess these abilities have been proposed to be part of a new pathotype called Adherent-Invasive *E. coli* (AIEC) (Darfeuille-Michaud, 2002). A recent genome comparison study suggests that *E. coli* from CD patients possess 3 common characteristics: 1) classified as members of the B2 phylogenetic group, 2) possess type 6 secretion system genes, and 3) are highly related to ExPEC (Desilets et al., 2015). Indeed, the comparison to ExPEC is not unusual as ExPEC also have a greater tendency to be from the B2 phylogroup (Johnson et al., 2001). Despite these potential common characteristics, there has been little evidence to identify a common set of virulence factors in AIEC (Martinez-Medina & Garcia-Gil, 2014). Nonetheless the current model suggests that AIEC are opportunistic pathogens or pathobionts that capitalise on an environmental niche present in the inflamed gut of CD patients (Smith *et al*., 2013a).

1.10.1 AIEC and the intestinal epithelium

In vitro, AIEC have been shown to adhere to and invade many epithelial cell lines (Darfeuille-Michaud et al., 1998; Martin et al., 2004) and intracellular *E. coli* have been identified in 29% of colonic mucosa biopsies from CD patients compared to 9% in healthy controls (Martin et al., 2004). A more recent study reported that 90% of CD mucosal biopsies of a cohort of ileal, ileaocolonic and colonic disease phenotypes contained intracellular *E. coli* compared to 0% of heathy controls (Elliott et al., 2013). Whist there are large differences in these two studies, they both suggest that *E. coli* can exist within epithelial cells *in vivo* in CD patients.

Adhesion to epithelial cells by AIEC is dependent, in part, on the expression of type I pili which interact with carcinoembryonic antigen-related cell adhesion molecule-6

(CEACAM6) in a mannose dependent manner (Barnich et al., 2007; Carvalho et al., 2009). CEACAM6 is overexpressed in CD ileal epithelium possibly making CD patients more susceptible to AIEC infection. Variants of the adhesin FimH are expressed in AIEC that allow them more efficient binding of epithelial cells *in vitro* (Dreux et al., 2013). Flagella are also important in adhesion to epithelium and mutants in *fliC* also have decreased expression of type I pili suggesting that they are co-regulated (Barnich et al., 2003; Eaves-Pyles et al., 2008). Furthermore, mutants in *fliC* induce less IL-8 production from polarized epithelial cells than their wild type parents which resulted in less migration of dendritic cells and neutrophils (Eaves-Pyles *et al*., 2008; Subramanian *et al*., 2008b). Moreover, binding of type I pili to CEACAM6 and flagella to TLR5 results in HIF-1α production and induction of the NF-**κ**B pathway resulting in secretion of VEGF and IL-8 indicating that type I pili and flagella cooperatively regulate IL-8 production by the epithelium.

Outer membrane vesicles (OMV) containing OmpA appear to be able to interact with endoplasmic reticulum stress response glyocoprotein 96 receptor that is overexpressed in the apical surface of ileal epithelial cells in CD (Rolhion et al., 2010). LF82 *ompA* mutants are defective for invasion, a phenotype that can be restored through pre-treatment with OMV from wild type LF82 indicting a role for OmpA in invasion. A secreted protease dubbed Vat-AIEC that degrades mucin was suggested to be important for AIEC to cross the mucosal barrier and gain access to the underlying epithelium (Gibold et al., 2015). Vat-AIEC mutants do not colonise the mouse gut as well as their wild type parent and an *in vitro* model showed that they were not able to penetrate mucus to the same degree as the parental strain. Furthermore, Vat-AIEC expression was increased in the presence of bile salts and mucin *in vitro* both of which are present in the GIT suggesting that Vat-AIEC may be expressed *in vivo*.

One of the early sites of inflammation in CD is the follicle associated epithelium (FAE) overlying ileal Peyer's Patches and colonic lymphoid follicles (Tawfik et al., 2014). AIEC strains, including LF82 and HM605, were able to translocate M cells, which account for 5% of the cells in the FAE, better than Caco2 cells and these AIEC strains also translocated human explant FAE (Roberts et al., 2010). Adhesion of LF82 to M cells is dependent on the production of long polar fimbriae (Lpf) which can

interact with the M cell surface protein GP2 (Chassaing et al., 2014). The adhesive tip of LF82 Lpf is encoded by *lpfD* which binds specifically to murine intestinal mucosa and submucosa. IbeA (Invasion of the brain endothelium) has also recently been proposed to be an AIEC invasin (Cieza et al., 2015). IbeA is reported to contribute to the pathology of NMEC and with traversal of the blood brain barrier. Mutants in *ibeA* in the AIEC strain NRG857c have significantly reduced invasion, but not adhesion, of epithelial cells and M cells and also have reduced translocation across M cells *in vitro* (Cieza et al., 2015).

Following entry into cells it has been shown that AIEC LF82 cells are localized to various different compartments. LF82 has been identified in single membrane vesicles both individually and in groups and also within $LC3^+$ autophagosomes suggesting that autophagy is activated in infected epithelial cells (Lapaquette *et al*., 2010; Martinez-Medina & Garcia-Gil, 2014). Defects in autophagy led to increased bacterial numbers *in vitro* suggesting that autophagy is important in restricting AIEC expansion (Lapaquette et al., 2010). Interestingly mutations in the autophagy genes *IGRM* and *ATG16L1* are associated with CD in humans (Hampe *et al*., 2007; Parkes *et al*., 2007; Rioux *et al*., 2007). Expression of microRNAs (MIR) MIR30C and MIR130A is increased in LF82-infected epithelial cells which causes a decease in the expression of the autophagy genes *ATG5* and *ATG16L1* and reduced autophagy (Nguyen et al., 2014). Another MIR, MIR106B, is present at higher levels in intestinal epithelia from patients with active CD. MIR106B targets *ATG16L1* mRNA and leads to reduced levels of autophagy *in vitro*. ATL16L1 levels were also lower in CD patients compared to controls. Furthermore, knockdown of HIF-1α also leads to increased LF82 survival in epithelial cells possibly through impaired autophagic flux (Mimouna et al., 2014). Together these findings suggest that autophagy is employed by the epithelium to restrict AIEC survival and defects in the process, such as in CD, may lead to increased susceptibility to AIEC.

1.10.2 AIEC replication in macrophages

Intestinal macrophages in healthy individuals are inflammation anergic but this does not seem to be the case in IBD. There is an increase in macrophages number in the

inflamed intestinal mucosa of IBD patients and these macrophages have a pro-inflammatory phenotype (Weber et al., 2009). Macrophages present in inflamed mucosa have increased expression of TLR2, TLR4, and TLR5 and respond more severely to stimulation by LPS (Hausmann et al., 2002). Macrophages that infiltrate intestinal tissue have increased expression of CD14 and TREM-1 and this results in the increased expression of pro-inflammatory cytokines TNF-α, IL-6, IL-8, MCP-1 and IL-1 (Grimm et al., 1995; Schenk et al., 2007). Moreover, the increased level of pro-inflammatory macrophages in the lamina propria of CD patients likely leads to decreased epithelial barrier function (Lissner et al., 2015). Normal intestinal macrophages have enhanced phagocytic and bactericidal activity but it appears that macrophages from CD patients have an impaired ability to clear bacteria highlighted by the presence of intracellular *E. coli* in isolated CD patient macrophages but not in macrophages from healthy controls (Elliott *et al*., 2015a; Smythies *et al*., 2005).

Once AIEC transverse the epithelial barrier and gain access to the lamina propria, they are engulfed by macrophages which are in high abundance in this tissue. One of the characteristics of AIEC is their ability to survive and replicate within macrophages *in vitro* (Bringer *et al*., 2006; Subramanian *et al*., 2008b). Other bacteria including *Salmonella* and *Shigella* are also known to replicate in macrophages and these bacteria manipulate key host defence mechanisms in order to establish infection. Bacteria need to be able to survive within the low nutrient, low pH, highly oxidative and nitrosative environment of the phagosome. Some, like *Shigella* escape the phagosomes and replicate in the cytoplasm while others, like *Salmonella*, prevent lysosome-phagosome fusion (Deretic & Levine, 2009). AIEC, however, do not appear to escape the phagosome or prevent its acidification but instead are able to survive in the phagosome and some reports suggest that acidification is required for replication in macrophages *in vitro* (Bringer et al., 2006). Studies with LF82 have identified that the genes *htrA*, *dsbA* and *hfq* are important in replication in macrophages (Bringer *et al*., 2005b; 2007; Simonsen *et al*., 2011). The *htrA* gene encodes a high temperature stress protein, *dsbA* encodes a oxidoreductase and *hfr* encodes a RNA chaperone important in response to chemical stress. Theses environmental stress resistance genes are ubiquitous in *E. coli* and do not represent a set of AIEC specific virulence factors.

Infection of macrophages with AIEC LF82 was reported to result in secretion of high levels of TNF-α but does not induce host cell death (Glasser et al., 2001). Release of TNF-α can result in decreased permeability of epithelial cells suggesting that TNF-α release from macrophages may assist AIEC in traversing the epithelial barrier (Mankertz et al., 2009). LF82 was shown to replicate better in macrophages deficient in autophagy suggesting that, similar to what has been described in epithelial cell, autophagy is a key mechanism for destruction of AIEC (Lapaquette *et al*., 2012b). This is clinically relevant since defects in autophagy gene *ATG16L1* are associated with CD. A study using monocyte-derived macrophages (MDM) from CD patients demonstrated that these cells were unable to restrict AIEC replication and resulted in increased TNF-α and IL-6 release compared to non-pathogenic *E. coli* (Vazeille et al., 2015). However, this effect may be strain specific as another study using CD MDM but different AIEC strains showed that all CD MDMs had reduced TNF-α release compared to healthy controls whether infected with AIEC or non-adherent-invasive *E. coli* (Elliott et al., 2015a). Furthermore, the amount of TNF-α released did not depend on the presence of CD associated polymorphisms in either *NOD2, IL-23R, ATG16L1* or *IGRM* (Elliott et al., 2015a). This study also showed that both AIEC and non-AIEC survived well in CD MDM suggesting that it is a host deficiency in handling *E. coli* that leads to increased survival in macrophages. Interestingly, the dysregulation of bacterial handling in monocytes from patients homozygous for the ATG16L1 CD risk allele was only evident upon stimulation with phorbol 12-myristate 13-acetate (PMA) or IL-1β suggesting that inflammation is important in the AIEC phenotype (Sadaghian Sadabad et al., 2015).

Other CD associated genes include the PRR nucleotide-binding oligomerisation-domain-containing protein 2 (*NOD2*/*CARD15*), Immunity-related GTPase M (IGRM) and interleukin 23 receptor (*IL-23R*) (Tawfik et al., 2014). NOD2 recognises muramyl dipeptide a component of bacterial cell wall peptidoglycan that upon activation stimulates the NFκB pathway leading to cytokine release. Polymorphisms in NOD2 account for approximately 15% of CD in the Western World (Schreiber et al., 2005). NOD2 mutations in CD are considered to be loss of function mutations with reduced IL-8 production following stimulation of mononuclear cells with MDP (van Heel et al., 2005). Moreover, dendritic cells from CD patients with *NOD2* risk variants are

defective in MDP-mediated autophagy activation and diminished in AIEC clearance (Cooney et al., 2010). *IL-23R* is expressed by activated macrophages and dendritic cells and activation can induce pro-inflammatory cytokine release contributing to inflammation in the CD gut (McGovern & Powrie, 2007). *IGRM* knockdowns leads to defective clearance of AIEC from fibroblasts and macrophages and increased cytokine release from macrophages (Lapaquette *et al*., 2010; 2012b).

It is clear that CD patients have decreased ability to clear *E. coli* infection and this may contribute to the ability of AIEC to replicate in macrophages. It is also apparent that some strains of *E. coli*, including AIEC, are better able to replicate in the macrophages niche than others but the molecular mechanisms underlying replication are not clear.

1.11 *E. coli* interactions with the innate immune effectors of macrophages

E. coli are the main facultative anaerobe in the colon and can exist as pathogens, pathobionts or commensals. Thus *E. coli* will interact with the innate immune cells of the gut either as a result of intestinal disease such as Crohn's Disease are through normal sampling of the gut lumen contents by the innate immune system. When *E. coli* interact with innate immune cells such as macrophages they come into contact with effectors such as inflammasomes and in some cases with the autophagy machinery that can deliver *E. coli* to lysosomes for degradation.

1.11.1 Inflammasomes

Whilst the NLRC4 inflammasome can be activated by EPEC T3SS apparatus (Miao et al., 2010), it appears that a wide variety of pathogenic and non-pathogenic *E. coli* can activate the NRLP3 inflammasomes through different mechanisms (Cheng *et al*., 2015; la Fuente *et al*., 2014; Sander *et al*., 2011; Schaale *et al*., 2015; Zhang *et al*., 2012). The NLRP3 inflammasome requires two separate signals for activation the first of which is activation of TLR signalling (Ozaki et al., 2015). LPS, a TLR4 ligand, is ubiquitous on *E. coli* and thus interactions of macrophages with *E. coli* LPS should

prime cells for NLRP3 activation. NLRP3 oligermisation requires a second signal and many studies have shown that *E. coli* derived factors can function as this second signal (Cheng *et al*., 2015; Sander *et al*., 2011; Zhang *et al*., 2012). Studies that identified vita-PAMPs were carried out in macrophages infected with *E. coli* indicating the *E. coli* activates the NLRP3 inflammasomes through mRNA (Sander et al. 2011). Both UPEC and enterohaemorrhagic *E. coli* strains produce RTX toxins that have been shown to activate the NLRP3 inflammasome (Cheng *et al*., 2015; Schaale *et al*., 2015; Zhang *et al*., 2012). Although the exact mechanism of NLRP3 activation by pore-forming toxins has not been fully described it is likely the result in K^+ efflux from the cell which is an activator of the NLRP3 inflammasome (Muñoz-Planillo et al., 2013). *E. coli* isolates from CD patients have been shown to activate the NLRP3 inflammasome but commensal *E. coli* also did this suggesting a general response to non-pathogenic *E. coli* infection most likely mediated through NLRP3 sensing of mRNA (la Fuente et al., 2014). These data suggest that the NLRP3 is the primary inflammasome used for detection of *E. coli*

1.11.2 Autophagy

Early evidence for the activation of autophagy by bacteria infection was seen following the infection of the HeLa cells line with group A *Streptococcus* (GAS) showing co-localisation of GAS with LC3. Moreover, $ATG5^{-/-}$ cells infected with GAS resulted in increase bacteria viability (Nakagawa et al., 2004). In macrophages, infection with *Mycobacterium tuberculosis* (Mtb) showed colocalisation of Mtb with LC3 (Gutierrez et al., 2004). The activation of autophagy inhibited survival of Mtb in macrophages linking bacteria killing with autophagy. *E. coli* have also been shown to interact with autophagy (Amer et al., 2005a). The infection of macrophages with Uropathogenic *E. coli* (UPEC) resulted in the transfer of *E. coli* from lipid rafts to vacuoles decorated with the ATG7 initially, then ATG8 and LAMP1 suggesting that macrophages target UPEC to autophagosomes (Amer et al., 2005a). Moreover, a recent study has shown that both epithelial cells and macrophages deficient in autophagy resist infection by UPEC and the authors suggests that the autophagosomes may act as a protective niche for UPEC (Symington et al., 2015; Wang et al., 2012a). Homozygous polymorphism in NOD2 is associated with CD and dendritic cells with

the NOD2 mutation have impaired autophagy and have impaired *E. coli* clearance (Cooney et al., 2010). Furthermore, AIEC have also been implicated with activating autophagy and defects in autophagy appear to confer an advantage to AIEC allowing them to replicate in macrophages (Lapaquette *et al*., 2012b). In addition, treatment of RAW264.7 macrophages cell line with non-pathogenic *E. coli* resulted in accumulation of LC3-II, a marker of autophagy (Fujita et al., 2011). However, LPS stimulation of RAW264.7 murine macrophages-like cells also resulted in the accumulation of LC3 in cells (Xu et al., 2007). Therefore, it is possible that the activation of autophagy by *E. coli* may be due to the interaction of LPS and TLR4 rather than a specific response to *E. coli*.

While there is limited evidence that *E. coli* can interact with the autophagy machinery, it is not clear if autophagy is a general response to all *E. coli* infections.

1.12 Objectives of this study

The overall objective of this work is to further understand the molecular mechanisms of *E. coli*-macrophages interactions. As there is no clear consensus on the role of autophagy in *E. coli* clearance, a panel of *E. coli* will be screened for their interaction with the autophagy machinery in macrophages. Furthermore, the cytokine immune response to *E. coli* will also be characterised.

AIEC can replicate in macrophages but the molecular mechanisms are not fully understood and will be investigated here. Furthermore, AIEC have been proposed to induce higher levels of cytokine release during macrophages infection contributing to inflammation in CD. Previous evidence suggests that pro-inflammatory cytokine production by AIEC infected macrophages depends on a functioning glycolysis pathway and this will be further investigated.

UPEC are the main cause of urinary tract infections and recent evidence suggests α-hemolysin produced by UPEC may be toxic to phagocytes such as macrophages. The molecular mechanisms underpinning this proposed cytotoxicity will be characterised.

Chapter 2 Materials and Methods

2.1 Bacterial strains and plasmids

2.1.1 Bacterial culture, growth conditions and storage

E. coli were routinely cultured in Luria-Bertani (LB) broth (5 g L^{-1} Yeast Extract (Merck), 10 g L^{-1} NaCl (Sigma), 10 g L^{-1} Tryptone (Merck)) and for solid media 1.5% (w/v) Agar (Merck) was added. Each week bacteria from -80°C stocks were plated onto solid media containing appropriate antibiotics and grown overnight at 37°C and stored at 4°C until needed.

Liquid cultures were inoculated with a single colony from solid agar and grown overnight in LB plus required antibiotics at 37°C shaking at 200 r.p.m unless otherwise stated.

In order to assess swimming motility, 0.25% (w/v) LB agar was freshly prepared on the day of the assay. An overnight culture was diluted to an OD_{600} = 1.0 and 5 µl inoculated onto the surface of the agar.

For growth in Dulbecco Modified Eagle Media (DMEM) cells were grown overnight in LB, washed twice in 1X PBS and diluted to an OD_{600nm} = 0.05 in DMEM (Gibco) clear 96-well plate (Corning) and covered with mineral oil. Cells were grown in a MWG Sirius HT plate reader at 37°C for required time taking OD_{600} every 15 min.

Antibiotics were added in the following concentrations where applicable: Ampicillin (Amp) 100 µg ml^{-1}; Chloramphenicol (Cm), 20 µg ml^{-1}; Kanamycin (Km), 50 µg ml^{-1}; Gentamicin (Gm), 50 µg ml^{-1}; Tetracycline, 12.5 µg ml^{-1}. For long term storage, overnight cultures were mixed 1:1 with 40% (v/v) glycerol in cryovials (Nunc) and stored at -80°C.

Strains used in this study are listed in Table 2. 4 and plasmids are listed in Table 2. 5.

Table 2. 4 Strains used in this study

Strain	Characteristics	Source
Adherent Invasive (AIEC), HM605	Clinical isolate from colonic mucosal biopsy. AmpR	Dr. Barry J. Campbell, Division of Gastroenterology, University School of Clinical Science, Liverpool, UK
MG1655	F- λ- ilvG- rfb-50 rph-1	Prof. Ian Henderson, University of Birmingham, UK
Enteroaggregative *E. coli* (EAEC) O44:H18 strain 042	Isolated from a child with diarrhoea in the course of an epidemiologic study in Lima, Peru, in 1983	Prof. Ian Henderson, University of Birmingham, UK
Enteropathogenic *E. coli* (EPEC) serotype O127:H6 strain E2348/69	Isolated in Taunton, United Kingdom, in 1969 during an outbreak of infantile diarrhoea	Prof. Ian Henderson, University of Birmingham, UK
Enterohemorrhagic *E. coli* (EHEC) O157:H7 Sakai Δ*stx*	Isolated from a typical patient during an outbreak in Sakai City, Japan in 1996; - attenuated to not produce shiga toxin	Prof. Ian Henderson, University of Birmingham, UK
Enterotoxigenix *E. coli* (ETEC) O78:H11:K80 strain H10407	Isolated from an adult with cholera-like symptoms in the course of an epidemiologic study in Dacca, Bangladesh	Prof. Ian Henderson, University of Birmingham, UK
E. coli LF82	AIEC. Isolated from a chronic ileal lesion of a patient with Crohn's Disease	Prof. Arlette Darfeuille-Michaud, Pathogénie Bactérienne Intestinale, Laboratoire de Bactériologie, Faculté de Pharmacie, Clermont-Ferrand, France
E. coli A0 34/86 serotype O83 : K24 : H31	Commensal porcine isolate	Carmen Buchrieser, Institut Pasteur, France
Uropathogenic *E. coli* (UPEC) UTI89	Clinical cystitis isolate	Prof. Matt Mulvey, University of Utah, USA
Uropathogenic *E. coli* CFT073	Clinical pyelonephritis isolate from University of Maryland Hospital	Prof. Ian Henderson, University of Birmingham, UK
Nissle 1917	Commensal. Intestinal *E. coli* isolate from Freiberg, Germany, 1917	Institut Pasteur, France
HM605 Δ*aceE*:Km	Tn5 insertion in *aceE*	Lab stocks
HM605 Δ*pfkAB*:Km	P1: BW25113 ΔpfkAB → HM605	Lab stocks
AJW2294	Isogenic Δpta strain	Gift from Prof. Alan Wolfe
AJW2283	Isogenic ΔackA strain	Gift from Prof. Alan Wolfe
AJW2781	Δ*ackA-pta* strain	Gift from Prof. Alan Wolfe
HM605 Δ*pta*:Km	P1:AJW2294 → HM605	This study
HM605 Δ*ackA*::Km	P1: AJW2283 → HM605	This study

HM605 Δ*ackA-pta*::Cm	P1: AJW2781 → HM605	This study
HM605 Δ*adhE*::Km	P1: BW25113 Δ*adhE* → HM605	This study
HM605 Δ*icd*::Km	P1: BW25113 Δ*icd* → HM605	Lab stocks
HM605 Δ*yfiQ*::Km	P1: BW25113 Δ*yfiQ* → HM605	This study
Salmonella enterica subsp. enterica (ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium ATCC14028		Gift from Dr. Craig Altier, Cornell University
Salmonella ATCC14028 Δ*ackA*::Cm		Gift from Dr. Craig Altier, Cornell University
Salmonella ATCC14028 Δ*pta*::Cm		Gift from Dr. Craig Altier, Cornell University
Salmonella ATCC14028 Δ*pta ackA*::Km		Gift from Dr. Craig Altier, Cornell University
BW25113 ΔaceE::Km		Keio Collection (Baba et al., 2006)
BW25113 Δ*aceE*	BW25113 Δ*aceE*::Km with KmR cassette removed by FLP recombinase	This study
UTI89 ΔUTI89_C3844::Km (5:A5)	Putative ADP heptose synthase Tn5 transposon mutant	This study
UTI89 Δ*rfaH*::Km (6:C6)	ΔrfaH::Km Tn5 transposon mutant	This study
UTI89 Δ*tolC*::Km (27:H5)	ΔtolC::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyC*::Km 40:H3	Δ*hlyC*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyA*::Km (40:E4)	Δ*hlyA*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyA*::Km (6:C3)	Δ*hlyA*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyA*::Km (6:C9)	Δ*hlyA*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyA*::Km (5:F3)	Δ*hlyA*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyA*::Km (40:F12)	Δ*hlyA*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyA*::Km (29:H12)	Δ*hlyA*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyA*::Km (6:F12)	Δ*hlyA*::Km Tn5 transposon mutant	This study

UTI89 Δ*hlyA*::Km (9:E3)	Δ*hlyA*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyA*::Km (26:F12)	Δ*hlyA*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyA*::Km (41:H7)	Δ*hlyA*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyA*::Km (3:E2)	Δ*hlyA*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyA*::Km (10:D10)	Δ*hlyA*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyA*::Km (18:F4)	Δ*hlyA*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyA*::Km (10:D12)	Δ*hlyA*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyD*::Km (27:F6)	Δ*hlyD*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyD*::Km (40:E5)	Δ*hlyD*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyD*::Km (36:A9)	Δ*hlyD*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyB*::Km (20:B2)	Δ*hlyB*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyB*::Km (21:C8)	Δ*hlyB*::Km Tn5 transposon mutant	This study
UTI89 Δ*hlyB*::Km (3:H3)	Δ*hlyB*::Km Tn5 transposon mutant	This study
UTI89 Δ*cpxRA*	UTI89 double knockout for cpxRA	Gift from Prof. Matt Mulvey.

Table 2. 5 Plasmids used in this study

Plasmid	**Relevant characteristics**	**Source**	**Comments**
pFLP3	AmpR, TetR, source of Flp recombinase	(Choi et al., 2005)	Used to excise the kanamycin cassette from P1 transductions
pCA24N *cobB*	CmR, *lacI*q, pCA24N pT5-lac::cobB	ASKA library. (Kitagawa et al., 2006)	Used to over produce *cobB* in HM605
pPROBE-gfp[LVA]	KmR	pPROBE-gfp[LVA] (Addgene plasmid # 40170) (Miller et al., 2000)	Promoter GFP fusion vector with tagged GFP for rapid degradation
pProbe-ackA	KmR	This study	Promoter region of *ackA* cloned from pUA66-ackA with primers pUA66_fw_*Sal*I and pUA66_rv_*Eco*RI
pUA66	KmR	(Zaslaver et al., 2006)	Promoter-less pUA66 plasmid
pUA66-ackA	KmR	Lab stock	Promoter region of *ackA* in pUA66 vector (Zaslaver et al., 2006)
pUA66-iraP	KmR	Lab stock	Promoter region of *yaiB* (*iraP*) in pUA66 vector(Zaslaver et al., 2006)
pDiGc	AmpR	Gift from Prof. David Holden	Constitutive GFP vector with inducible DsRed

phlyCADB	CmR	Gift from Prof. Scott Hultgren, Washington University	Constitutive expression of *hlyCADB* operon

2.2 Measurement of gfp expression during growth *in vitro*

HM605 containing fluorescent transcriptional reporters (Zaslaver et al., 2006) were grown overnight, washed with 1X PBS and diluted to an OD_{600} = 0.05 in high glucose DMEM and grown in 96-well black walled optical bottom plates (Nunc) at 37°C in a MWG Sirius HT plate reader for required time. OD_{600} and GFP fluorescence readings were measured every 15 min. For GFP fluorescence, samples were excited with light passed through a 485/20 band pass filter and emitted light captured through a 528/20 band pass filter. A non-fluorescent HM605 strain was used to subtract background fluorescence and promoter activity was calculated as previously described (Zaslaver et al., 2006).

For analysis of expression in acid media, cells were grown in M9 Minimal Media (Bacto) + 0.4% Glucose (Sigma) or the same media adjusted to pH 5 with 2-(*N*-morpholinoethanesulfonic acid (MES) before autoclaving.

2.3 Kits and Enzymes

Plasmids were purified using a GeneJet Plasmid MiniPrep Kit (Thermo Fisher) according to manufacturers recommendations. PCR products and single enzyme restriction digests were purified using a GeneJet PCR purification Kit (Thermo Fisher) according to manufacturers recommendations. Products of double restriction enzyme digests were purified using a GeneJet Gel Extraction Kit (Thermo Fisher) according to manufacturers recommendations. Genomic DNA was extracted from overnight cultures with a Genomic DNA extraction kit (Sigma-Aldrich) according to manufacturers recommendations. All restriction enzymes, T4 DNA polymerase, T4 DNA ligase and Calf intestinal phosphatase were from New England Biolabs (NEB).

2.4 DNA gel electrophoresis

DNA electrophoresis was carried out on 1% (w/v) agarose gels in Tris-Acetate-EDTA (TAE) buffer (4.84 g Tris, 1.14 ml glacial acetic acid, 2 ml 0.5 M EDTA pH 8, per litre of H_2O). Ethidium bromide (Sigma) was added fresh to each gel at a concentration of 500 ng ml^{-1}. Loading dye (Thermo Fisher) was added to a final concentration of 1X to samples. GeneRuler 1 kb plus (Thermo Fisher) DNA ladder was added to each gel. Gels were routinely run at 100 V, 400 mA for 30 min.

2.5 Primers

Primers were purchased from MWG-Biotech (Germany). Lyophilised primers were re-suspended in sterile MilliQ H_2O to a final concentration of 100 pmol μl^{-1} and stored at -20°C. Primers used in this study are listed in **Table 2. 6**.

Table 2. 6 Primers used in this study

Primer	Sequence (5'-3')	Tm (°C)	Description
ARB1	GGCCACGCGTCGACTAGTTACNNNNNNNNNGATAT	72.9	Arbitrarily primed PCR
ARB2	GGCCACGCGTCGACTTAGTTAC	64	Arbitrarily primed PCR
ARB6	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC	>75.0	Arbitrarily primed PCR
Tn5Ext	CCTGATTGCCCGACATTATCGCG	64.2	Arbitrarily primed PCR
Tn5Int	GAATATGGCTCATAACACCC	55.3	Arbitrarily primed PCR
KD13_K1	CAGTCATAGCCGAATAGCCT	57.3	Kanamycin Cassette
KD13_K2_2	CGGTGCCCTGAATGAACTGC	61.4	Kanamycin Cassette
HM605_adhE_L	CGCGGTTTCTGATTTTTCTT	53.2	Confirmation of *adhE* P1 transduction in HM605. Used in conjunction with KD13_K1
HM605_ackA	GCACGTTCGCACAGTTTAGA	57.3	Confirmation of *ackA* P1 transduction in HM605. Used in conjunction with KD13_K2_2

HM605_pta 2	GTTCGCCTGCTTCGTTA GTC	59.3	Confirmation of *pta* P1 transduction in HM605. Used in conjunction with KD13_K2_2
HM605_ack A_R	GCATGGGTAAACTTAAG GCG	57.3	Confirmation of *ackA pta* P1 transduction in HM605. Used in conjunction with HM605_pta2
HM605_yfi Q	TCGTGCTGATGCAGGTA AAC	57.3	Confirmation of *yfiQ* P1 transduction in HM605. Used in conjunction with KD13_K2_2
hlyA_fw	ATCATCCCCGTCTCCCA GGT	61.4	Testing for presence of *hlyA*
hlyA_rv	GCTGCTGCCGGTTTAAT TGC	59.3	Testing for presence of *hlyA*
pUA66_fw_ SalI	GCC**GTCGAC**AAAAATA GGCGTATCACGAGG	55.9	Amplifying promoter region of pUA66-*ackA* for cloning into pProbe-gfp[LVA]. Restriction site in bold: *Sal*I
pUA66_rv_ EcoRI	TGTATATCTCCTTCTTAA ATCTAGA**GAATTC**GGC	54.8	Amplifying promoter region of pUA66-*ackA* for cloning into pProbe-gfp[LVA]. Restriction site in bold: *Eco*RI

2.6 Colony PCR

Single colonies were picked using sterile 200 µl tips, and resuspended in 100 µl sterile MilliQ H_2O and vortexed. 1 µl of each resuspended colony was then used as a DNA template for the colony PCR. Colony PCR was carried out using Taq DNA polymerase (Qiagen) and a dNTP mix containing 10 mM of each dNTP (Promega). Each PCR reaction contained 0.5 µl template DNA, 2.5 µl of 10X CoralLoad PCR Buffer, 0.2 mM of each dNTP, 100 pmol of each primer, 0.125 µl Taq DNA polymerase and sterile milliQ H_2O to a final volume of 25 µl. PCR conditions were as follows: initial denaturation at 95°C for 5 min and 35 cycles of 95°C for 30 s, primer specific annealing temperature for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. 5 µl of each PCR reaction was run on a 1% (w/v) agarose gel.

2.7 Cloning

The region of pUA66-ackA containing the promoter region of *E. coli ackA pta* operon was amplified using primers pUA66_fw_SalI and pUA66_rv_EcoRI and digested with restriction enzymes *Sal*I and *Eco*RI (New England Biolabs) as indicated and gel

purified with a GeneJet Gel extraction kit as per manufacturers instructions. The vector pProbe-gfp[LVA] (Miller et al., 2000) was digested with *Sal*I and *Eco*RI and the digested plasmid was treated with Calf Intestinal Phosphatase (NEB) according to manufacturers recommendations and agarose gel purified. Concentrations of DNA were obtained by nanodrop and a ligation mixture at a 1:3 vector to insert ratio was calculated using NEBioCalculator (http://nebiocalculator.neb.com/#!/ligation) and ligated with T4 DNA ligase (NEB) according to manufacturers recommendations. The ligation product was transformed into TransforMax™ EC100™ Electrocompetent *E. coli* (Epicentre). Colonies were screened for positive inserts by restreaking onto LB agar with Km. Colonies were grown overnight in LB broth with Km, the plasmid was isolated with a GeneJet Plasmid Mini Prep Kit (Thermo Fisher) and digested with *Bam*HI and *Sal*I to confirm the presence of an insert. One colony with the correct size insert was then re-streaked onto LB agar with Km and the plasmid was sequenced to confirm the promoter sequence.

2.8 Construction of deletion mutants in *E. coli* by P1 phage transduction

The Keio library of single gene knockouts in *E. coli* K12 BW25113 (Baba et al., 2006) was used as donor strains for all P1 transductions unless otherwise stated. All steps were carried out in L medium (5g L^{-1} Yeast Extract (Merck), 5g L^{-1} NaCl (Sigma), 10g L^{-1} Tryptone (Merck), (Silhavy, 1985)) and for solid media 1.5% (w/v) Agar (Merck) was added. For donor stains with a Km insert, a P1cml lysate was used to create a lysogen while for donor strains with a Cm insert, a P1kml lysate was used to create a lysogen.

2.8.1 Preparation of lysogens

Three colonies of the donor strain were suspended in 50 µl of L broth to which 50 µl of a phage lysate (induced from *E. coli* ZK2686 containing either P1cml or P1kml) and 5 µl 1 M $CaCl_2$ was added and incubated statically for 30 min at 30°C. The entire mixture was then plated on L agar supplemented with Km and Cm to select for lysogens and incubated overnight.

2.8.2 Preparation of lysates

A high titre lysate was prepared as follows: 2 ml of L broth was inoculated with a single colony from the prepared lysogen and incubated overnight at 30°C with shaking. 5 ml of L broth was then inoculated with the overnight culture to an OD_{600} = 0.02 and grown at 30°C until the culture reached an OD_{600} = 0.1-0.2. The culture was heat shocked at 42°C with shaking at 140 r.p.m for 20 min followed by incubation at 37°C, shaking at 200 r.p.m, until complete lysis was observed. 100 µl of chloroform was added to ensure sterility of the lysate and the sample was vortexed and centrifuged at 3,000 x *g* for 10 min at 4°C to remove cells debris. The supernatant containing the phage particles, was transferred to a new tube containing 100 µl chloroform and stored at 4°C.

2.8.3 P1-mediated transduction

5 ml of L broth was inoculated with a single colony of the recipient strain and incubated overnight at 37°C with shaking. Cells were pelleted at 3,000 x *g* for 10 min and suspended in 2.5 ml transduction buffer (10 mM $MgSO_4$, 5 mM $CaCl_2$). 10 µl, 100 µl or 200 µl of the phage lysate was added to 100 µl of recipient cells and the mixture was incubated at 37°C for 30 min. 200 µl of 1 M Sodium Citrate was added and the mixture was incubated at 37°C for 1 h. Cells were centrifuged at 12, 000 r.p.m on an Eppendorf 5418 tabletop centrifuge for 1 min and the supernatant was removed. Cells were then washed in L medium containing 20 mM sodium citrate and resuspended in 100 µl of L medium containing 20 mM sodium citrate. The mixture was plated on L agar containing 20 mM sodium citrate and the appropriate antibiotic and incubated at 37°C overnight. Colonies were purified by restreaking on L agar containing appropriate antibiotics and 20 mM sodium citrate. Each mutant was confirmed by colony PCR using either KD13_K1 or KD13_K2_2 and one of the following: HM605_adhE_L; HM605_ackA; HM605_pta2; HM605_ackA_R; HM605_yfiQ. A negative control using the wild type strain was always used for comparison.

2.8.4 Flp recombinase-mediated excision of antibiotic resistance gene

To create unmarked bacterial mutants constructed using P1 transduction from the KEIO library, the antibiotic resistance gene can be excised using a helper plasmid which expresses the FLP recombinase. FLP recombinase acts on the **F**LP **R**ecognition **T**arget sites (FRT sites) which flank the antibiotic resistance gene, thus "cutting out" and eliminating the marker gene. The plasmid pFLP3 (TetR *sacB*) was used for the excision of the Km-resistance cassette in this study. pFLP3 was transformed into the KmR HM605 mutant strains and Tet-resistant colonies were selected after incubation at 37°C. Tet-resistant colonies were re-streaked onto a plain LB agar plate, an LB agar plate containing Tet (to confirm the presence of the plasmid) and an LB agar plate containing Km (to confirm elimination of the KmR cassette). Growth on both the plain LB agar plate and the LB agar (+ Tet) plate, but not on the LB agar (+ Km) plate indicated that cells had lost the Km-resistance cassette. KmS TetR cells were re-streaked onto LB agar plates containing 5% (w/v) sucrose to eliminate the pFLP3 plasmid. Excision of the KmR cassette was finally confirmed by performing a colony PCR with the KmR cassette specific primers, KD13_K1 and KD13_K2_2 (See ***Table 2. 6***).

2.9 Identification of Tn5 insertion site

Transposon insertion sites were determined by amplifying the region within the genome surrounding the transposon using two rounds of arbitrarily primed PCR. In the first PCR (PCR 1), the random primers ARB1 and ARB6 were paired with an external Tn5 specific primer, Tn5ext (see ***Table 2. 6***). The products from PCR 1 were purified and used as a template for the second PCR (PCR 2), using the internal Tn5 specific primer, Tn5int and the random primer ARB2 (see ***Table 2. 6***). ARB2 was designed to match the 5' (non-random) sequence of ARB1 and ARB6. Arbitrary PCR was carried out using the VelocityTM PCR kit (Bioline). PCR reaction conditions were as follows: 98°C for 5 min followed by 30 cycles of: 98°C for 30 s, 56°C (PCR 1) or 50°C (PCR 2) for 30 s and 72°C for 1 min. A final extension at 72°C for 5 min was performed before samples were kept at 4°C until analysis by gel electrophoresis. Amplicons were sequenced using PCR primer Tn5int by GATC Biotech. Sequences

of PCR product were then compared to UTI89 genome using BLASTN at NCBI (http://blast.ncbi.nlm.nih.gov) to determine insert location.

2.10 Preparation of electrocompetent *E. coli*

Electrocompetent *E. coli* were prepared to allow for transformation with plasmids by electroporation. A single colony of *E. coli* was suspended in 5 ml of LB broth and grown overnight at 37°C. Overnight cultures were diluted 1:500 into fresh LB broth and grown until at an OD_{600} = 0.5-0.7 with shaking. 100 ml of bacteria was centrifuged at 3,000 x *g* for 10 min at 4°C and supernatants discarded. Pellets were resuspended in 100 ml of ice-cold, sterile, MilliQ H_2O and then centrifuged again at 3,000 x *g*. Supernatant was discarded and pellets resuspended in 50 ml of ice-cold sterile MilliQ H_2O. Bacteria were centrifuged again, supernatant removed and the cell pellet was resuspended in 1.6 ml ice-cold sterile 20% (v/v) glycerol. Bacteria were centrifuged for a final time, supernatants removed and the cell pellet was resuspended in 160 µl ice-cold sterile 20% (v/v) glycerol, aliquoted into 50 µl volumes and and stored at -80°C until needed.

2.11 SDS-PAGE and Western immunoblot

Unless otherwise stated, samples were loaded onto a 12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel and run in 1X running buffer [25 mM Tris base, 190 mM glycine, 1% (w/v) SDS; pH 8.3] at 180 V for approximately 1 h. The gel and the PDVF membrane were rinsed in water and then equilibrated for 15 min in transfer buffer containing 48 mM Tris base, 39 mM glycine, 20% (v/v) methanol. Proteins were transferred onto the PVDF membrane for 1 h at 100 V, the membrane was then washed and blocked with 5% (w/v) skimmed milk prepared in 1X TBS + 0.05% Tween-20 (TBST) for 2 h at room temperature.

For detection of lysine acetylated proteins, *E. coli* were grown and then lysed in 2X final sample buffer [3% (w/v) SDS, 20% (v/v) glycerol, 123.75 mM Tris-HCl pH 6.8,

0.005% (w/v) bromophenol blue, 4% (v/v) β- mercaptoethanol]. Cells lysates were then diluted in 9.2 M Urea (Sigma) to give a final concentration of 4.6 M urea/1X sample buffer. Cells lysates were then incubated for 10 min at 100°C and separated by SDS-PAGE as above. Each well was loaded with the 20 µl of lysate from cells at a density of approx. 0.02 OD_{600nm}. Proteins were transferred to membranes and blocked as above. The blot was washed with TBST two times for 5 min each. A rabbit polyclonal antibody raised against an acetylated lysine-containing peptide (9441; Cell Signaling) was used at a 1:500 (v/v) dilution in 5% (w/v) BSA in TBST at 4°C overnight with gentle agitation. The blot was washed 3 x 5 min in TBST and then 1 x 10 min in TBST. A secondary HRP-conjugated antibody of goat anti-rabbit IgG (7074S; Cell Signalling) was added to the blot at 1:2000 (v/v) dilution in 5% (w/v) BSA in TBST and incubated for 1 h at room temperature. The blot was washed x 4 with TBST and antibody was detected by enhanced chemiluminescence, using the SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific), following the manufacturers recommendations.

For detection of LC3B in cell lysates, the membrane was probed with a mouse anti-rabbit LC3B antibody (Sigma; L7543) diluted to 1:2000 (v/v) in 1% normal goat serum in TBST overnight at 4°C. The membrane was subjected to 3 x 10 min washes in TBST to remove any unbound antibody. A goat anti-rabbit horse-radish peroxidase (HRP) conjugated secondary antibody (Sigma: A5420) was diluted to 1:20000 (v/v) in 5% (w/v) BSA/TBST and incubated with the membrane for 1 h at room temperature. The membrane was subjected to a further 3 x 10 min washes in TBST. Bound antibody was detected by enhanced chemiluminescence, using the SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific), following the manufacturers recommendations. Membranes were stripped of bound antibodies by incubation in stripping buffer [100 mM β-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl pH 6.8] at 50°C for 30 min, with rotation. Stripped membranes were subjected to 3 x 10 min washes in TBST, re-blocked and re-probed with an anti-β-actin antibody (Santa Cruz Biotechnology), as a loading control.

2.12 LPS SDS-PAGE with Silver Stain

Bacteria were grown to mid exponential phase and pelleted by centrifugation at 3000 x g for 5 min. Bacteria were resuspended in lysis solution (0.1M Tris-HCL pH6.8, 5% (v/v) β-mercaptoethanol, 2% SDS and 20% glycerol) to an OD_{600} of 2 and then heated for 5 min at 95°C. Samples were then treated with 1 mg ml^{-1} Proteinase K and incubated at 65°C for 1 h (Chart, 1994). Samples were adjusted to an OD of 1.0 in 1X sample buffer and were separated on a 9% SDS-PAGE. 20 µl was loaded per well. LPS was detected by silver staining with Thermo scientific silver staining kit with the following adaption - following fixation, gels were oxidised by treatment with 0.7% periodic acid for 10 min (Tsai & Frasch, 1982).

2.13 Preparation of heat killed bacteria and bacterial supernatants

Heat-killed bacteria were prepared by growing the bacteria overnight statically at 37°C. The bacterial culture was diluted to a cell density of $1x10^7$ CFU ml^{-1} in DMEM + 10% FBS and then heated at 65°C for 1 h. A sample was then plated on LB agar and incubated overnight to confirm that the bacteria had been killed.

Bacterial supernatants were prepared from bacterial cultures that were grown statically overnight at 37°C in DMEM + 10% FBS and then centrifuged at 3,000 x *g* for 5min. Supernatants were then sterilised by passing through a 0.22 µm filter.

2.14 Eukaryotic cell cultures

J774A.1 and RAW264.7 murine macrophages cell lines were obtained from the ATCC, ATCC TIB-67 and ATCC TIB-71 respectively. Both cell lines were maintained in tissue culture flasks (Sarstedt) in DMEM containing high glucose, GlutaMAX and sodium pyruvate (Gibco:31966-021) + 10% (v/v) Foetal Bovine Serum (FBS: Gibco) and 100 U ml^{-1} Penicillin/Streptomycin (Gibco) at 37°C/5% CO_2 in a humidified incubator. When cells reached 70-90% confluence they were gently scraped using a cell scraper and diluted into fresh medium in a new flask. Cell were maintained for a maximum of 30 passages.

THP-1 human monocyte cell line was obtained from ATCC (ATCC TIB-202) and maintained in RPMI-1640 medium supplemented with L-glutamine, 10% (v/v) FBS and 100 U ml^{-1} Penicillin/Streptomycin at 37°C/5% CO_2 in a humidified incubator. Cells were maintained at a density of 2 x 10^5 – 8 x 10^5 cells ml^{-1}.

THP1-defASC and THP1-defCASP1 were obtained from Invivogen. These cell lines are stable shRNA knockdowns of the inflammasome components ASC and caspase-1 and are kept under positive selection using the antibiotic hygromycin B (Invivogen). Cells are cultured in the same manner as wild type THP-1 but with supplementation of media with 100 μg ml^{-1} hygromycin B every other passage.

Murine immortalised bone marrow macrophages (iBMM) containing a plasmid expressing LC3-GFP were a gift from Dr. Ed Lavelle in Trinity College Dublin (Harris et al., 2011) and maintained in RPMI-1640 medium supplemented with L-glutamine, 10% (v/v) Newborn Calf Serum and 100 U ml^{-1} Penicillin/Streptomycin 37°C/5% CO_2 in a humidified incubator. 1 μg ml^{-1} Puromycin (Sigma) was added at all culturing steps to ensure plasmid retention. When cells reached 70-90% confluence they were gently scraped using a cell scraper and diluted into fresh medium in a new flask.

For long term storage cells were stored in full culture medium supplemented with 5% (v/v) DMSO (Sigma) in cryovials in liquid nitrogen.

2.15 Mouse DSS colitis model

All animal experiments were performed under license from the Irish Dept of Health (B100/4108) and approved by University College Cork Animal Experimentation Ethics Committee (2011/023). Male C5BL/6OlaHsD age 8-15 weeks were purchased from Harlan and were housed under barrier-maintained conditions at University College Cork. 24 h pre-treatment, mice were supplied with streptomycin (5 mg ml^{-1} final concentration) followed by 3 days of recovery. Mice were orally infected with HM605, HM605 Δ*ackA-pta* or PBS control at 10^9 CFU ml^{-1}. Three days later, DSS-treated mice were given 2.5% (v/v) DSS in their drinking water and this was maintained for the duration of experiment. Mice were culled and 2 separate cecum

samples were taken and weighted for bacterial enumeration and SFCA analysis by High Performance Liquid Chromatography (HPLC).

For bacterial enumeration, the contents of the cecum were re-suspended in sterile 1X PBS at a 1:100 (w/v) dilution and vortexed with sterile 3-4 mm glass beads (Sigma) for 1 min. Samples were then serially diluted and plated on LB agar with Amp plates to enumerate AIEC HM605 (HM605 is naturally resistant to Amp) or LB with Amp and Cm to enumerate HM605 Δ*ackA-pta* mutants.

For HPLC analysis, the contents of the cecum were re-suspended in sterile MilliQ water at 1:10 (w/v) dilution (e.g. 100 mg cecum in 1 ml H_2O). Samples were vortexed for 1 min with 3-4 mm glass beads (Sigma). Homogenates were centrifuged at 10,000 x *g* for 10 mim and then filtered though a 0.22 μm filter (to remove bacterial cells) and analysed for SCFA content as per Section 2.25.

Blood was recovered by cardiac puncture and held in EDTA treated tubes and kept on ice until centrifugation. The plasma fraction was removed and flash frozen in dry ice and kept at -80°C until analysis for IL-1β and TNF-α by using a V-Plex multiplex ELISA kit (Meso-scale Discovery) according to manufacturers instructions.

Animal husbandry and experimental procedures were performed by the Biological Services Unit and approved by the University College Cork Animal Experimentation Ethics Committee. Weighted cecum samples were provided to Mr. I. O'Neill and all subsequent steps carried out by Mr. O'Neill

2.16 Gentamicin protection assay

Gentamicin protection assays were used to infect macrophages with bacteria. One colony of bacteria was suspended in 3 ml of LB broth plus appropriate antibiotics and grown statically overnight at 37°C. Bacteria were washed once with 1X PBS and diluted to the desired cell density in appropriate cell culture medium. J774A.1 were seeded in antibiotic free DMEM + 10% (v/v) FBS in a 96-well microtitre plates (Corning) at a density of $1x10^5$ cells per well and incubated for 20-24 h at 37°C/5% CO_2 in a humidified incubator. For THP-1, 1 x 10^5 cells were added to each well of a

96-well microtitre plate and incubated for 20-24 h at 37°C/5% CO_2 in a humidified incubator in antibiotic free RPMI media containing 50 ng ml^{-1} phorbol 12-myristate 13-acetate (PMA) to differentiate the THP-1 monocytes into macrophages. Cells were then washed twice with warm 1X PBS and the media was replaced with antibiotic-free, PMA-free culture media and incubated for 20-24 h at 37°C/5% CO_2 in a humidified incubator. Before infection the macrophages were washed twice with warm 1X PBS. Bacteria were added at the appropriate multiplicity of infection (MOI), generally 10 (unless otherwise stated). The bacteria and macrophages were incubated at 37°C/5% CO_2. After the appropriate infection time the wells were washed twice with warm 1X PBS (to remove excess bacteria) and the media was replaced with DMEM + 50 μg ml^{-1} Gm (to kill bacteria not phagocytosed by the macrophages). For bacterial enumeration, infected macrophages were, at the appropriate time, washed twice with ice cold 1X PBS and lysed with ice cold 1% (v/v) Triton X-100 in 1X PBS. Lysates were then serially diluted, plated on LB agar containing appropriate antibiotics and incubated at 37°C until the following day. If macrophages were being analysed for cytokine production, they were incubated for a further 24 h before supernatants were removed and stored at -80°C until analysed.

When indicated macrophages were pre-incubated for 1 h with following compounds at the indicated concentrations:

Z-WEDH-FMK (R&D Systems) – 40 μM
Q-VS-Oph (R&D Systems)– 40 μM
Bay 11-7072 (Invivogen) – 30 μM
Glybenclamide (Invivogen) – 25 μg ml^{-1}
Parthenolide (Invivogen) – 5 μM
Potassium Chloride (KCl, Sigma) -5 mM
Wortmannin (Invivogen)- 100 nM
Cytochalasin D (Sigma)– 2 μM
LPS from *E. coli* 0111:B4 (Sigma) - 1 μg ml^{-1}

2.17 Measurement of cytokine production by ELISA.

THP-1 cell culture supernatants were analysed for IL-1β TNF-α, IL-6 and IL12p70 using a human V-PLEX Validated Assay Kits (Meso Scale Discovery) according to manufacturers instructions. J774A.1 cell culture supernatants were analysed using a U-PLEX Mouse IL-1β Assay Kit (Meso Scale Discovery) according to manufacturers instructions. Briefly, each sample was diluted 1:2 in Diluent 43. 25 µl of each diluted sample or calibrator solution was dispensed into a separate well of the MSD plate. Calibrators were prepared by serially diluting a stock calibrator solution in Diluent 43, generating a calibration curve. The plate was sealed and incubated for 2 h at room temperature with vigorous shaking (1,000 r.p.m). 25 µl of a 1X detection antibody solution was dispensed into each well and the plate was incubated for a further 2 h at room temperature with vigorous shaking (1,000 r.p.m). The plate was washed three times with 1X PBS containing 0.05% (v/v) Tween-20 and 150 µl of 2X Read Buffer T was added to each well. The plate was analysed on a SECTOR®Imager (Meso Scale Discovery).

2.18 Cytotoxicity (LDH) assay

Supernatants from infected macrophages were centrifuged to remove cell debris and bacteria and were then analysed for release of lactate dehydrogenase (LDH) using a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturers instructions. Briefly, a maximum LDH release control was obtained by adding 10 µl of Lysis Solution (10X) to cells 45 min prior to harvesting supernatants and uninfected cells were used as a spontaneous LDH release control. A maximum LDH release and spontaneous release control was included on each plate. All samples were diluted 1:2 in tissue culture medium without FBS. 50 µl of diluted sample was added to to 50 µl CytoTox 96® Reagent and incubated at room temperature for 30 min in the dark. 50 µl of stop solution was then added to each well. Absorbance at 490 nm was read on a MWG Sirius HT plate reader. Percentage cytotoxicity was calculated using the following formula:

$$\frac{Sample\ LDH\ relase - Spontaneous\ LDH\ release}{Maximum\ LDH\ release - Spontaneous\ LDH\ release} \times 100$$

2.19 Activation of autophagy in iBMM.

iBMM were seeded on sterile 13 mm diameter No 1.5 coverslips (VWR) at a density of 1 x 10^5 cells per well in 24-well microtitre plates (Corning) in RPMI-1640 + 10% (v/v) FCS and incubated for 20-24 h at 37°C/5% CO_2 in a humidified incubator. Cells were infected with the appropriate bacteria at an MOI = 100. Plates were then centrifuged at 300 x *g* for 10 min followed by an incubation at 37°C/5% CO_2 in a humidified incubator for 20 min. Cells were then washed 2 x with warm 1X PBS. Gentamicin-containing medium was then added to each well and plates incubated for 30 min; this is the basal time point, T_0. At T0 and 3 h later (T_3), cells were washed twice 1X PBS followed by fixation with 4% (w/v) paraformaldehye (PFA)/PBS pH 7.4 for 15 min at room temperature. Coverslips were washed three times with 1X PBS and treated with 300 nM 4', 6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature in dark followed by 3 further washes in 1X PBS and one final wash in dH_2O. Coverslips were then mounted on microscope slides using Dako mounting medium (DAKO) and incubated overnight at room temperature in the dark. Cells were analysed using a Zeiss LSM 5 Laser scanning confocal microscope running Zen 2008 SP2 software. Slides that were not analysed the next day were stored at 4°C in the dark and used within one week. All experiments were performed in technical triplicate on three separate occasions.

Control experiments to determine the bacterial load in iBMM at T_0 and T_3 were performed in a similar manner but at each time point cells were lysed using 1% (v/v) Triton X-100 and the bacteria enumerated.

Co-localisation of bacteria with iBMM-LC3-GFP experiments were carried out as above but bacteria were stained with 1 μM BacLight Red (Thermo-Fisher) for 30 min in dark at room temperature before infection. Bacteria were then washed with 1X PBS and diluted for infection as above.

2.20 Analysis of LC3B activation

THP-1 macrophages were seeded in 6-well tissue culture plates (Corning) at a density of 1 x 10^6 and infected with *E. coli* as previously described (see 2.16). At T_0 and T_3, macrophages were washed twice with ice-cold 1X PBS and lysed with radioimmunoprecipitation assay (RIPA) buffer containing a 1X protease and phosphatase inhibitor cocktail (Thermo Scientific). Whole cell lysates were clarified by centrifugation at 13,000 r.p.m. in a Jouan C4 centrifuge for 10 min at 4°C. Supernatants were transferred to new tubes and stored at -80°C until needed. The protein concentration in each sample was determined using Bradford Reagent (Sigma), following the manufacturer's protocol. 20 μg of each protein sample was mixed with 2X final sample buffer (giving a final concentration of 1X) and heated to 90°C for 5 min. Samples were separated by SDS-PAGE and transferred to PVDF membrane as per section 2.11.

2.21 Immunofluorescence

J774A.1 were seeded on sterile No. 1.5 coverslips (VWR) at a density of 1 x 10^5 cells $well^{-1}$ in antibiotic free media in a 24-well microtitre plate and incubated for 20-24 h at 37°C/5% CO_2 in a humidified incubator. Cells were infected at an MOI of 10 with either HM605/pDiGc or MG1655/pDiGc. The pDiGc plasmid constitutively expresses a stable derivative of GFP (Helaine et al., 2010). Plates were centrifuged for 10 min at 300 x *g* followed by 20 min incubation at 37°C/5% CO_2 (T_{-30}). Cell were then washed (x 2) with 1X PBS and either incubated for a further 30 min in gentamicin supplemented media (T_0). At T_{-30} and T_0, cells were washed with 1X PBS and fixed using 4% (w/v) PFA/PBS for 15 min at room temperature in the dark. Following fixing, cells were blocked using 5% (v/v) Goat Serum (Sigma)/0.05% (v/v) Tween-20/1X PBS for 30 min at room temperature in the dark. Cells were then washed three times with 1X PBS. Primary rabbit anti-mouse EEA1 (Santa-Cruz Biotechnology) was added at 4 μg ml^{-1} in 1X PBS/1% (w/v) BSA(Fisher Scientific)/0.05% (v/v) Tween-20 for 1 h at room temperature followed by 3 washes with 1X PBS. Anti-mouse IgG(H+L) AlexaFluor 633 conjugate was added at 4 μg ml^{-1} in 1X PBS/1% (w/v) BSA /0.05% (v/v) Tween-20 for 1 h at room temperature in the dark. Coverslips were then mounted on microscope slides with Dako mounting medium and incubated at room temperature in the dark overnight. Slides were visualised using a Zeiss LSM 5 laser scanning confocal microscope with a Plan-Apochromat 63x/1.40 Oil DIC M27

lens. Slides were excited with laser light at 488 nm and emission light acquired using a bandpass emission filter of 505-550 nm for GFP. For Alexafluor-633 staining, cells were excited with a laser light at 633 nm and emission light acquired using a longpass emission filter of 650 nm. Samples were acquired using Zen 2008 SP2 software. Cells were scored as positive for colocalisation if bacteria (GREEN)-containing vacuoles directly colocalised with EEA1 vesicles (RED) or if there was a distinct EEA1 staining circling surrounding the bacteria (as EEA1 is localised in the phagosomal membrane). Slides were stored at 4°C in the dark until analysed and all analysis was performed within one week. All experiments were carried out in triplicate.

2.22 Lysotracker staining

Cells were infected in the same manner as for immunofluorescence staining. Following incubation with gentamicin supplemented media for 30 min a sample was taken (T_0) and another sample was taken 6 h later (T_6). 30 min prior to each time point, cells were washed in 1X PBS and 250 nM Lysotracker Red-DND99 (Molecular Probes), diluted in gentamicin-supplemented culture medium, was added to sample wells. After sample collection the cells were washed and fixed and mounted as per section 2.21. Slides were visualised with a Zeiss LSM 5 laser scanning confocal microscope with a Plan-Apochromat 63x/1.40 Oil DIC M27 lens. For Lysotracker visualisation, samples were excited with laser light at 543 nm and emission light filtered through a bandpass filter at 560-615 nm. Samples were acquired with Zen 2008 SP2 software. Cells were scored as positive for colocalisation if green bacteria containing vacuoles overlapped with red Lysotracker vesicles. Slides were stored at 4°C in the dark until analysed and all analysis was performed within one week. All experiments were carried out in triplicate.

2.23 Promoter activity during growth in the macrophages

J774A.1 were seeded on coverslips as per section 2.21 and infected with HM605/pProbe-ackA as per 2.16. At T0, T3 and T6 coverslips were washed twice with cold 1X PBS and fixed with 4% (w/v) PFA for 10 min at room temperature in the dark. Coverslips were extensively washed and mounted on microscope slide with

DAKO (DAKO) mounting medium and left to dry overnight at room temperature in the dark. Cells were analysed for by fluorescence microscopy as described in 2.21.

2.24 Detection of mitochondrial reactive oxygen species (mtROS) using MitoSox Red.

J774A.1 were seeded at a density of 4 x 10^5 cells per well in 24 well microtitre plates and infected as before. 30 min before each time point cell culture media was replaced with media containing 5 μM MitoSox Red (Molecular Probes) and incubated at 37°C/5% CO_2. Cells were then washed with 1X PBS and resuspended in Live Cell Imaging Solution (Gibco). Control cells were treated with 20 μM antimycin-A (Sigma) to stimulate mtROS production at this point and this remained in the sample until analysis by flow cytometry. Cells were scraped and transferred into 1.5 ml tubes and kept on ice in the dark until analysis. Cells were sampled using a FACSCalibur flow cytometer (Becton Dickinson) excited with laser light at 488 nm and emission light obtained in the FL2 and FL3 channel. Median fluorescent intensity of the FL2 channel was calculated by CellQuest software (Becton Dickinson) and representative histogram plots are from the FL2 channel.

2.25 Hexose and Organic Acid detection by High Performance Liquid Chromatography

Cell-free culture supernatants were collected and analysed for glucose, lactic acid, acetic acid, formic acid and ethanol using high performance liquid chromatography (HPLC) with a refractive index detector (Agilent 1200 HPLC system). An Agilent Hi-Plex H 300 x 7.7 mm column was used with 0.01 N H_2SO_4 as the elution fluid, at a flow rate of 0.6 ml min^{-1}. The temperature of the column is maintained at 65°C and 20 μl of each sample was injected for analysis.

2.26 UTI89 Tn5 random mutant insertion library screen for reduced cytotoxicity mutants

A Tn5 random transposon mutant library of UTI89 was previously prepared by Dr. Emma Smith using an EZ-Tn5TM <KAN-2> Tnp Transposome kit (Epicentre). Differentiated THP-1 macrophages were infected as before at an MOI of 10 in 96-well plates with each well containing a different mutant. After 2.5 h of infection, each well was inspected for cytotoxicity using a bright field microscope for evidence of cytotoxicity (see Figure 2.1). Wells with reduced cytotoxicity were noted and screened a second time to eliminate false positives. A total of 25 mutants were identified as having reduced cytotoxicity and each was subjected to a cytotoxicity assay (section 2.18) in triplicate to quantify this reduction. Genomic DNA from each mutant was extracted using GenElute Bacterial Genomic DNA kit (Sigma) as per manufacturer's instructions.

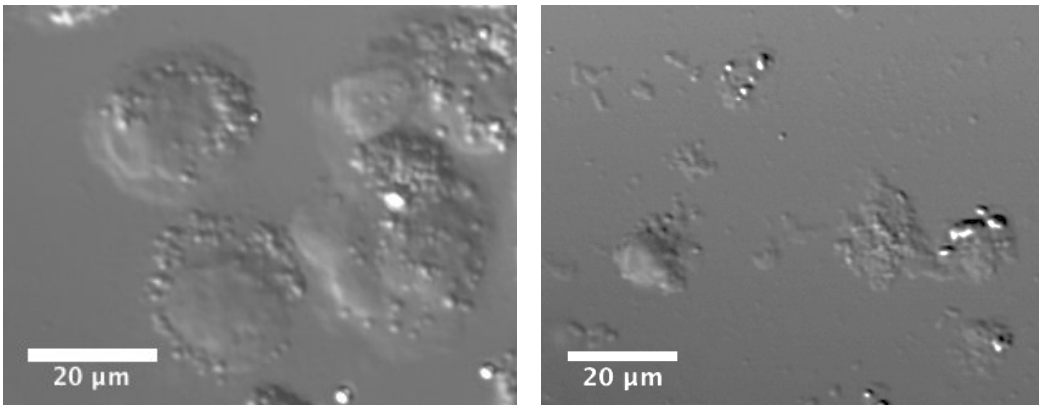

Figure 2. 1 Infection of THP-1 macrophages with UTI89 results in cytotoxicity that is visible using bright field microscopy

THP-1 cells seeded on No. 1.5 coverslips were incubated with UTI89 for 2.5 h at an MOI = 10. Cells were washed with PBS and fixed using 4% Paraformaldehyde and imaged using a Zeiss Axiovision Z1 confocal microscope and images acquired with a Zen 2008 SP2 software (Zeiss). Left panel shows a representative image of an uninfected THP-1 macrophages. Right panel shows representative image of a THP-1 macrophages infected with UTI89.

2.27 Hemolysis assay

To test for hemolysin activity a liquid blood hemolysis assay was modified from (Warawa et al., 1999). In brief, *E. coli* were grown overnight, diluted into phenol red-free DMEM and grown to OD_{600} = 0.7 at 37°C/5% CO_2 in a humidified incubator in 24-well microtitre plates. MG1655/pHlyCADB was grown to OD_{600} = 0.1 due to the slow growth of MG1655 in DMEM. 0.5 ml of bacteria was added to a 4% (v/v) sheep red blood cell-DMEM solution and centrifuged at 1000 x g for 1 min to initiate bacteria-cell contact. This mixture was incubated for 30 min at 37°C/5% CO_2 in a humidified incubator. Cells were then centrifuged at 1000 x g for 5 min and the supernatant was monitored for the presence of released hemoglobin at the optical wavelength of 543 nm. Spontaneous lysis was measured using uninfected red blood cells (RBCs) and maximum lysis was measured by resuspending RBCs in MilliQ water. Percent hemolysis was determined as follows:

$$\frac{Sample\ hemoglobin\ release - Spontaneous\ hemoglobin\ release}{Maximum\ hemoglobin\ release - Spontaneous\ hemoglobin\ release} \times 100$$

2.28 Statistical analysis

All statistical analysis was performed using the GraphPad Prism 6.0 software. All experiments were performed in triplicate, unless otherwise stated. The students T- Test was used to compare two groups of data. When three of more groups were compared a one-way ANOVA (or Kruskal-Wallis test for non-parametric data) was used with a Dunnetts post test (or non-parametric equivalent) to compare all conditions to a control. Differences were considered to be significant if $P \leq 0.05$.

Chapter 3 Molecular and cellular analysis of *E. coli* infected macrophages

3.1 Introduction

Macrophages are professional phagocytes that engulf and degrade bacteria. These important innate immune cells present antigens to B and T cells as well as releasing a variety of pro-inflammatory cytokines to mount an appropriate local and systemic immune response to the invading pathogen. In the context of the human gastrointestinal tract, macrophages present in the intestinal lamina propria remove bacteria that have passed the epithelial barrier of the gut, thus limiting bacterial growth to the gut lumen whilst also maintaining homeostasis (Kayama & Takeda, 2012). In inflammatory bowel diseases such as ulcerative colitis (UC) and Crohn's disease (CD), this homeostasis is perturbed (by a combination of host and environmental factors) resulting in an increase in inflammation. Moreover in CD, there is excessive inflammation in the intestines that leads to an increase in the number of pro-inflammatory macrophages in the tissues of the gut (Lissner et al., 2015). Over the past 15 years a large number of studies have uncovered host genetic loci associated with CD (Barrett *et al*., 2008; Jostins *et al*., 2013). These include mutations in genes encoding proteins involved in the recognition of bacteria and autophagy. Simultaneously, a group of *E. coli*, named adherent-invasive *E. coli* (AIEC), have been repeatedly isolated from biopsies taken from CD patients. AIEC can be distinguished from commensal *E. coli* as they are able to invade epithelial cells and replicate within macrophages *in vitro* (Darfeuille-Michaud *et al*., 1998; Martin *et al*., 2004; Subramanian *et al*., 2008b). Replication in macrophages by AIEC is believed to cause a persistent intestinal bacterial infection in CD patients allowing constant activation of the immune response and hence chronic inflammation (Smith *et al*., 2013a).

Macrophages can resolve bacterial infections using phagocytosis and autophagy. Phagocytosis involves the initial detection of bacteria by cell membrane receptors; in the case of *E. coli*, TLR4 and TLR5. Attachment is followed by engulfment, isolation in a membrane bound compartment (the phagosome) which then fuses with lysosomes that contain proteases and other hydrolytic enzymes (Underhill & Goodridge, 2012). Autophagy is a cytosolic process that involves the recognition of cytosolic bacteria by, amongst others, nucleotide-binding oligomerization domain (NOD) proteins such as NOD2 (Levine et al., 2011). Following recognition, a double membrane, the

autophagosome, is formed around the bacteria. The autophagosome then fuses with lysosomes leading to degradation of the contents of the autophagolysosome. Interestingly, TLR4 has been shown to activate autophagy in macrophages as indicated by the accumulation of the autophagy protein LC3 (seen as puncta microscopically) and the formation of double membrane vesicles in macrophages (Xu et al., 2007). There is also a process where LPS coated beads stimulate TLR4 leading to the recruitment of LC3 to the the phagosome (Sanjuan et al., 2007). These beads were then contained in a LC3+ single membrane vacuole distinct from the double membrane seen in autophagy leading to this processing be termed LC3-associated phagocytosis. Therefore, there appears to be significant regulatory crosstalk between autophagy and phagocytosis.

Genome wide association studies have uncovered autophagy genes that are linked to CD including *NOD2*, *ATG16L1* and *IGRM*. NOD2 recognises cytoplasmic bacteria and activates autophagy (Cooney et al., 2010), ATG16L1 is required for the development of the autophagosome (Deretic & Levine, 2009) and IGRM is involved in the regulation of autophagic bacterial clearance (Singh et al., 2006). Dendritic cells from individuals expressing CD-associated *NOD2* or *ATG16L1* variants show impaired autophagy induction and AIEC clearance was also reduced (Cooney et al., 2010; Lapaquette et al., 2010). A recent study showed that macrophages infected with AIEC strain LF82 rapidly activate autophagy (Lapaquette *et al*., 2012a). This study also showed that siRNA-mediated knockdown of *ATG16L1*, *NOD2* or *IGRM* expression led to increased replication of AIEC in macrophages. This suggests that autophagy plays an important role in the clearance of AIEC and the defects in autophagy associated with CD may contribute to the pathology of this disease by reducing the clearance of AIEC. However, it is not clear whether impaired autophagy has a specific effect on AIEC replication or whether it has a more general effect on the clearance of *E. coli*. A previous study, undertaken in fibroblasts, showed that AIEC replication, but not that of other *E. coli*, was restricted by autophagy (Lapaquette et al., 2010). However, another study showed the direct interaction of a non-pathogenic *E. coli* with the autophagy activator, NOD2 in epithelial cells (Perez et al., 2010). Similarly, *E. coli* activates TLR4 and the activation of TLR4 has been shown to activate autophagy and is also associated with phagocytosis (Sanjuan *et al*., 2009; Shi

& Kehrl, 2008; Xu *et al*., 2007). LPS or *E. coli* stimulated macrophages induce $LC3^+$ dot formation that occurs independent of the classical autophagy machinery (Fujita et al., 2011). While the above evidence shows that both pathogenic and non-pathogenic *E. coli* may be interacting with the some components of the autophagy machinery, there is no comprehensive study that describes the role of autophagy in the control of different strains of *E. coli* by macrophages. Therefore, in this study, a range of pathogenic and non-pathogenic *E coli* will be tested for their interactions with autophagy to test whether it may be a crucial pathway for *E. coli* degradation. Furthermore the processing of AIEC and commensal bacteria by macrophages will be compared in an attempt to identify the molecular mechanisms that enable AIEC to replicate in macrophages.

3.2 Results

Recent studies have reported that AIEC are taken up into autophagolysosomes in the macrophages and, thus, the compromised autophagy response in many CD patients would allow AIEC to proliferate in this niche. However, whether macrophages handled AIEC in a manner that was different from non-AIEC strains of *E. coli* is not known. Therefore, a group of *E. coli* that represented pathogenic, non-pathogenic and commensal strains was selected and their interaction with human THP-1 macrophages was analysed. This group contained the following bacteria: two AIEC HM605 and LF82; EAEC strain 042; EIEC-like *Shigella sonnei*; ETEC H10407; the EPEC E2348/69; EHEC O157 Δ*stx*; 2 UPEC, CFT073 and UTI89; 2 commensal *E. coli* A0 34/86 and Nissle 1917 and the *E. coli* K-12 strain MG1655 (Figure 3.1a).

3.2.1 Growth of *E. coli* in vitro

In the first instance bacterial replication *in vitro* was assessed to test if any differences in growth exists between these strains. After 18 h growth in DMEM, UTI89 and O157 Δ*stx* had grown to a higher density than other *E. coli* strains. All other strains grew to a similar final density (Figure 3. 1). MG1655 and LF82 had a different growth profile to the other strains and grew to the lowest density of all strains. In MG1655, this is likely to be due to a frame-shift mutation leading to low levels of *pyrE* expression thus reducing the *de novo* synthesis of pyrimidines (Jensen, 1993), however, this has not been previously reported in LF82.

3.2.2 Replication of *E. coli* in THP-1 macrophages

AIEC have been shown to replicate in cultured macrophages however there is little evidence on whether non-AIEC can do so (Subramanian *et al*., 2008a). Therefore, replication in differentiated THP-1 macrophages was assessed using the gentamicin protection assay (Glasser et al., 2001). PMA differentiated THP-1 were infected at an

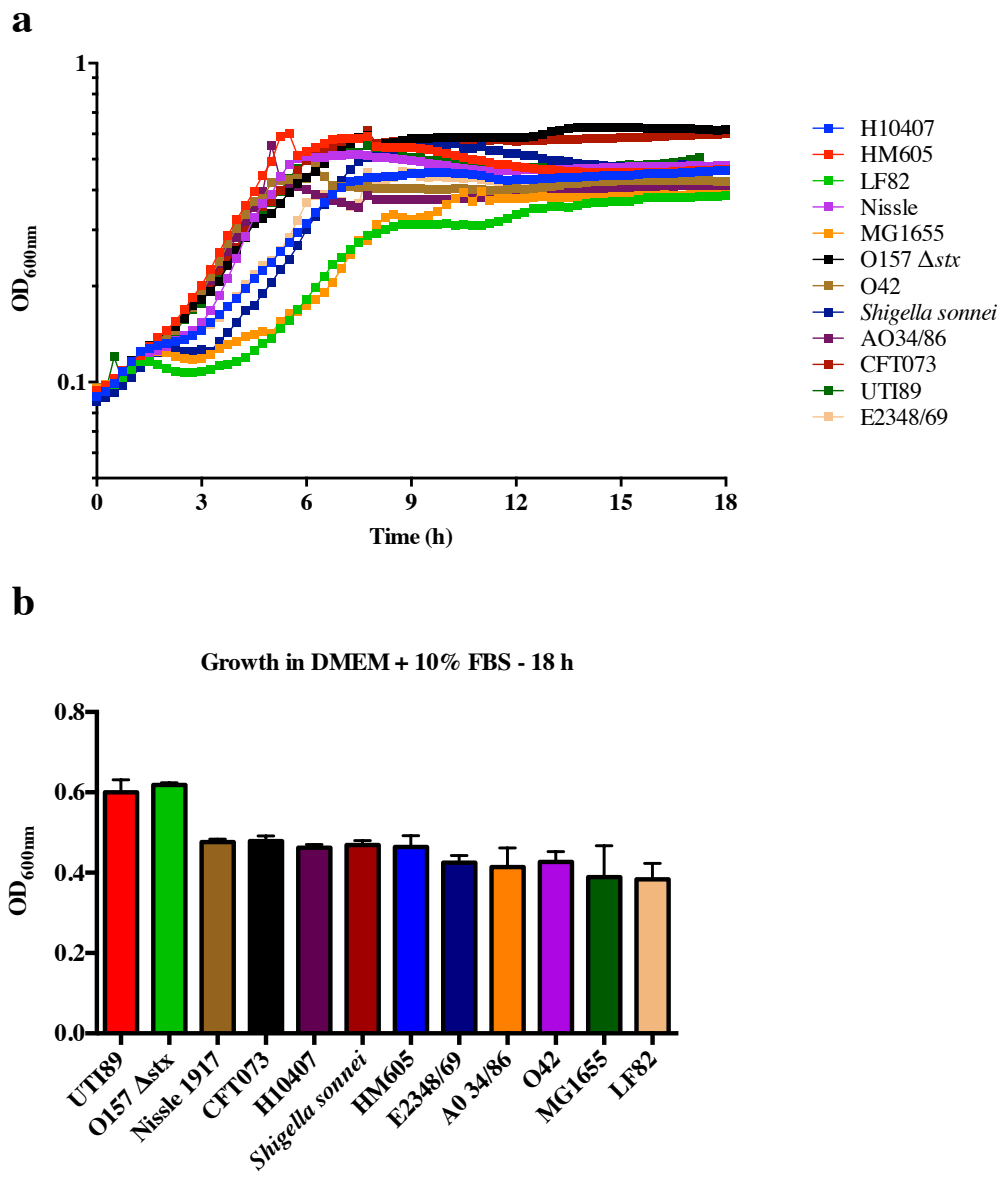

Figure 3. 1 Growth of *E. coli* in cell culture media.

a) *E. coli* diluted to about 0.05 OD_{600nm} in high glucose DMEM and were grown in 96-well microtitre plates for 18hrs in a plate reader at 37°C taking optical density readings at 600 nm every 15min. Data shown is a mean ±S.E.M. of 3 independent experiments. **b)** End point OD_{600nm} showing differences in growth of *E. coli*

MOI of 100 and bacteria were centrifuged onto macrophages and incubated for 20 min followed by 30 min of treatment with gentamicin to kill extracellular bacteria. Intracellular bacteria were then enumerated to determine the initial bacterial load; this is referred to a T_0. Intracellular bacteria were again enumerated 3 h later by plate counting (T_3) and replication was determined by comparing bacterial load at T_0 to T_3 (Figure 3. 2a-b). No *E. coli* strain was able to replicate in THP-1 cells indicating that the replication phenotype is cell-line specific. However, the intracellular load of some strains including the AIEC LF82 and HM605 as well as pathogenic strains H10407, 042 and O157 Δ*stx* did not decrease indicating that these bacteria were able to persist in the macrophages and this may indicate a rate of bacterial growth that equals the rate of bacterial cell death. In contrast the total number of cells of many other strains including E2348/69, MG1655, Nissle 1917, *S. sonnei,* A0 34/86 and CFT073 had reduced by nearly half in 3 h suggesting that THP-1 macrophages are able to resolve infections by these strains.

3.2.3 Macrophages cytokine response to *E. coli* infection

AIEC have been reported to induce high levels of TNF-α and IL-6 release from THP-1 macrophages, however, this was compared to uninfected cells (Lapaquette *et al.*, 2012b). Therefore, the pro-inflammatory cytokine response of macrophages to infection by a panel of *E. coli* was determined. PMA differentiated THP-1 macrophages were infected with MG1655, Nissle 1917, H10407, *S. sonnei*, HM605, LF82, O157 Δ*stx*, 042, E2348/69, UTI89, CFT073 and A0 34/86 for 24 h and the quantity of IL-1β, IL-6, IL12p70 and TNF-α released into the culture supernatant was measured by ELISA. The very low levels of cytokine production induced by UTI89, CFTO73 and A0 34/86 is due to the extreme cytotoxicity of these bacteria to the THP-1 macrophages (for molecular details of this toxicity see Chapter 5). Indeed, all THP-1 macrophages are killed in less than 3 h by these strains of *E. coli* (Figure 3. 3e), therefore cytokine data for infection with these strains are shown but excluded from this analysis as they are not representative. This cytotoxicity also explains the low level of intramacrophagic replication observed with these bacteria (see Figure 3. 2).

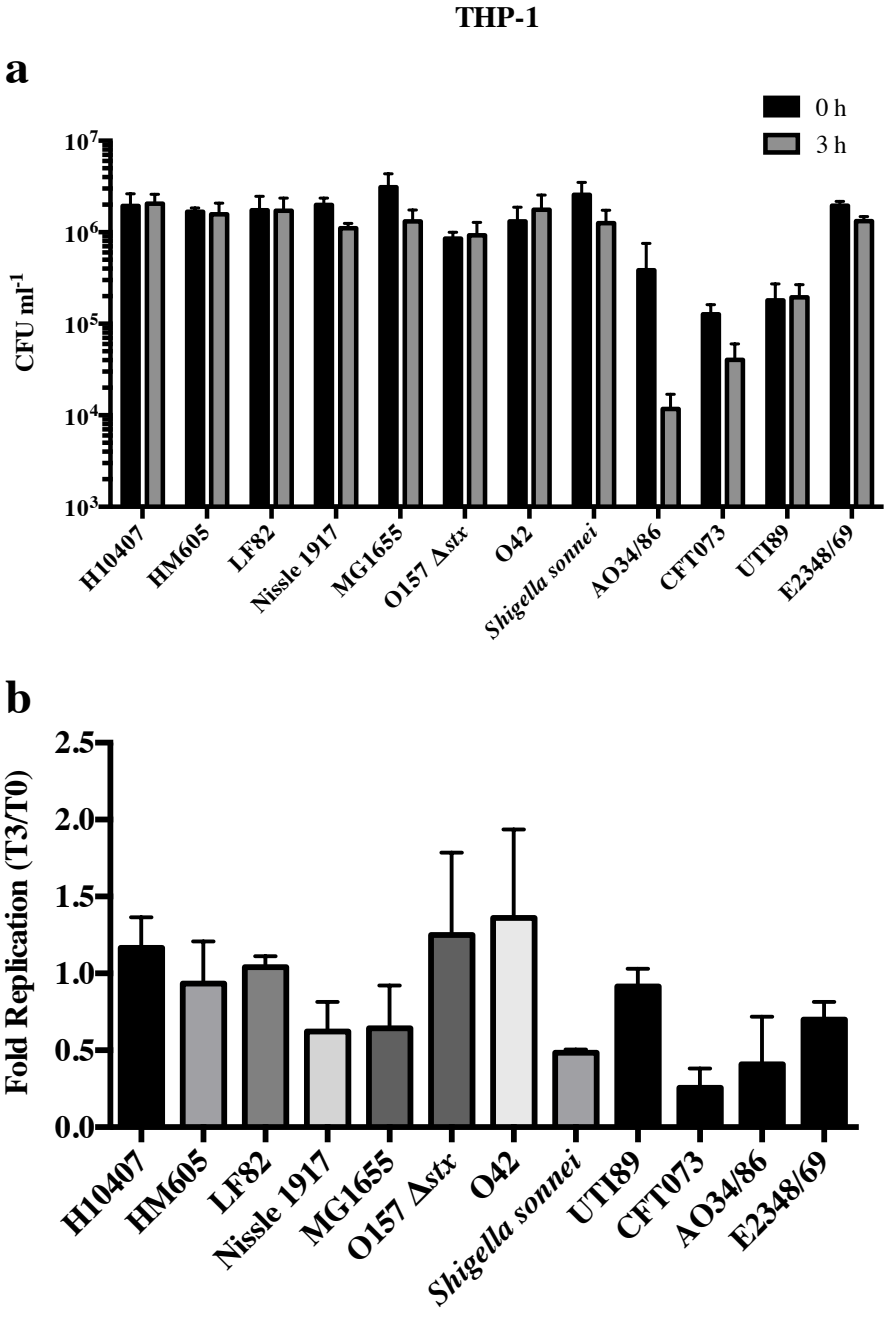

Figure 3. 2 *E. coli* uptake and replication in differentiated human THP-1 macrophages.

THP-1 macrophages were infected at an MOI of 100. Infection was synchronised by centrifugation at 300 x g for 10mins followed by 20 min incubation at 37°C/5% CO_2. Bacteria was removed, cells washed and incubated for another 30 min with gentamicin supplemented media at which time cells were washed, lysed and bacteria enumerated - this is referred to as 0 h/T_0. Bacteria were again enumerated 3 h later (3 h/T_3). **a)** Bacterial load at 0 h and 3 h. **b)** Fold replication of bacteria over 3 h. Data shown is mean±SEM of 3 independent experiments performed on 3 separate occasions

Of the other strains tested for cytotoxicity, only O157 Δ*stx* was significantly more cytotoxic than MG1655 and it has been previously reported that O157 produces a hemolysin, enterohemoylin (Ehx), that induces cytotoxicity in THP-1 cells (Zhang et al., 2012).

Using the non-pathogenic strain MG1655 as a baseline, the cytokine response of the macrophages to all other strains was compared (Figure 3. 3). Unsurprisingly the EPEC E2348/69 induced a significantly lower response for all cytokines tested compared to MG1655 as EPEC have a number of effector protein such as NleB, NleC and NleE that are translocated into the cell via type 3 secretion systems and inhibit the cytokine response of the host cell (Wong et al., 2011). O157 Δ*stx* also possess the same effector proteins and induced lower cytokine release than MG1655 but the effect was not as profound as for EPEC. One possible reason could be that E2348/69 possess two copies of NleE, an effector that inhibits I**κ**B degradation and therefore blocks NF-**κ**B activation, while O157 Δ*stx* only possess one copy. Also, EHEC has been shown to induce IL-1β release from THP-1 macrophages in an inflammasome dependent manner through the production of Ehx and thus EHEC may be more immunostimulatory than EPEC (Cheng et al., 2015).

IL-1β is a key indicator of inflammasome activation and its release requires two different signals, a priming signal such as LPS (to induce production of pro-IL-1β) and a variety of secondary signals which activate one of a number of inflammasomes leading to caspase-1 activation and cleavage of pro-IL-1β into IL-1β. EHEC E2348/69 and O157 Δ*stx* induced significantly lower levels of IL-1β in THP-1 macrophages when compared to MG1655 (Figure 3. 3a). E2348/69 and O157 stx both inhibit NF-**κ**B signalling thereby supressing the effect of LPS/TLR4 signalling on *IL1b* transcription and ultimately IL-1β release. Surprisingly, no difference in IL-1β response was observed when comparing MG1655 and HM605 although it was previously observed that HM605 induces significantly higher levels of IL-1β in murine J774A.1 macrophages cell line suggesting that there are cell-specific differences in IL-1β release induced by *E. coli*. All other strains tested showed no significant difference in IL-1β compared to MG1655 indicating that most *E. coli*

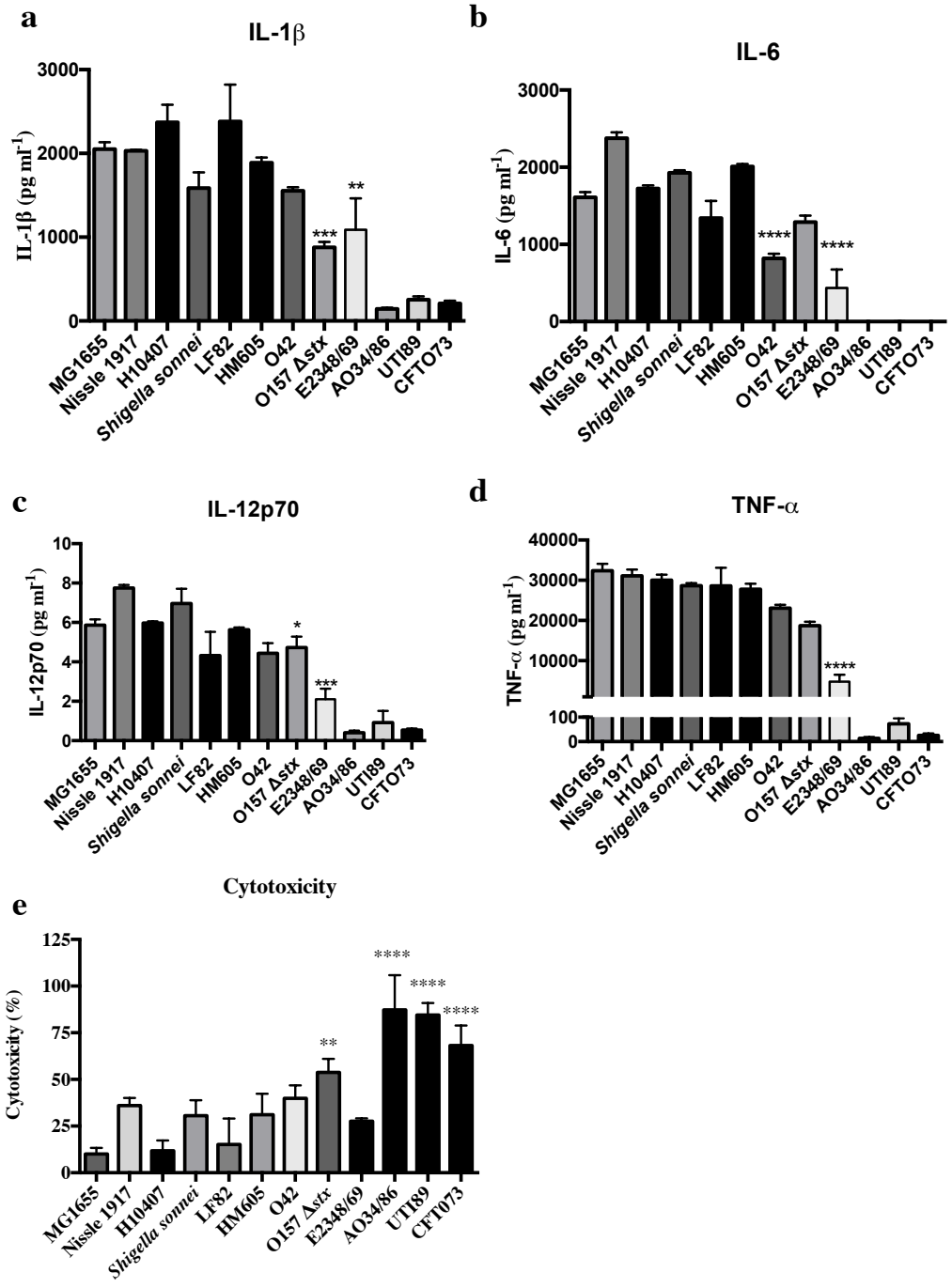

Figure 3. 3 Cytokine release by THP-1 macrophages infected with *E coli*:

Macrophages were infected with *E. coli* using a gentamicin protection assay and 24 h later cell culture supernatants were tested for IL-1β **(a)**, IL-6 **(b)**, IL-12 **(c)** and TNF-a **(d)**. Data is mean of 3 ± independent experiments. * $P< 0.05$, *** $P< 0.001$, **** $P< 0.0001$ determined by on way ANOVA. **e)** Cytotoxicity of strain to THP-1 during 3 h incubation with bacteria. Data shown is mean of 3±SEM of 3 independent experiments. ** $P< 0.01$, **** $P< 0.0001$ determined by on way ANOVA comparing all strains to MG1655 with a Dunnett's post test.

induced similar levels of IL-1β in THP-1 macrophages to that of a non-pathogenic strain.

IL-6 is a potent pro-inflammatory cytokine and its secretion is stimulated by LPS stimulation of macrophages via CD14/TLR4 signalling leading to NF-kB directed transcription of *IL6* and ultimately IL-6 secretion (Matsusaka et al., 1993). IL-6 secretion was similar to that of MG1655 in all strains tested except 042 and E2348/69 (Figure 3. 3b). As previously mentioned E2348/69 inhibits NF-**κ**B and thereby would inhibit IL-6 secretion. However, 042, is reported to induce *IL6* expression in epithelial cells during aggregation but there is no such evidence in macrophages (Harrington et al., 2005). Interestingly the commensal Nissle 1917 induced a significantly greater IL-6 release from macrophages compared to MG1655.

IL-12 (also referred to as IL-12p70) is a pro-inflammatory cytokine that is produced by macrophages following *E. coli* or LPS challenge and influences the development of Th1 cells (Nau *et al*., 2002; Trinchieri, 1997). LPS activates *IL12* transcription through TLR4 signaling and the NF-**κ**B pathway, therefore, it was not unusual that only E2348/69 and O157, which blocks NF-**κ**B signaling, had decreased levels of IL-12 release compared to MG1655 (Figure 3. 3c). Some strains had higher levels of IL-12 than MG1655 including the commensal Nissle 1917 and *S. sonnei* but these levels were not significantly higher. These results are in agreement with another study that tested IL-12 levels in infected macrophages with some of the same strains (Christoffersen et al., 2013) and indicate that apart from strains with known immune evasion virulence factors, most enteric *E. coli* induce similar levels of IL-12, probably via LPS/TLR4 signaling.

TNF-α is a key pro-inflammatory cytokine produced by macrophages in response to infection and anti-TNF-α drugs are a widely used therapy to treat autoimmune diseases such as CD and rheumatoid arthritis (Parameswaran & Patial, 2010). TNF-α is released from macrophages following signaling from TLR4 via NF-**κ**B, thus, LPS is a major stimulus for TNF-α release. Two strains tested, MG1655 and LF82, lack an O-antigen as part of their LPS however this did not significantly affect the amount of TNF-α secreted from cells infected with these strains. In contrast, MG1655 induced

the highest amount of TNF-α secretion (Figure 3. 3d). E2348/69 had significantly reduced TNF-α secretion when compared to MG1655 (Figure 3. 3d). O157 also exhibited reduced TNF- α release compared to MG1655 but this reduction was not statistically significant. As previously mentioned E2348/69 and O157 have immunosuppressant virulence factors that inhibit NF-κB signaling and this reduces the secretion of TNF-α.

Together the data presented above shows that many different pathotypes of *E. coli*, with the exception of EPEC and EHEC (strains with known anti-inflammatory virulence factors) are able to induce a similar inflammatory response in human macrophages. Additionally, over when compared to other *E. coli* AIEC like LF82 and HM605 induce similar levels of TNF-α and IL-6 release.

3.2.4 Activation of autophagy by *E. coli*.

Autophagy is a normal cellular process whereby large protein complexes are degraded and recycled (Levine et al., 2011). More recently autophagy has also been shown to trap bacteria that have either escaped into the cytosol or reside within damaged vacuoles (Deretic & Levine, 2009). In some instances the autophagic machinery is recruited to the phagosome in a process referred to LC3-associated phagocytosis (LAP) (Ma et al., 2013). LAP is distinguished from autophagy in that vacuoles have single membranes compared to the classical double membrane seen in autophagy. Recent studies using AIEC LF82 have reported that this strain activates autophagy in the macrophages and defects in autophagy favour LF82 intracellular replication suggesting that autophagy has a direct role in the clearance of AIEC (Lapaquette *et al*., 2010; 2012b). However, it was not clear in these studies whether the autophagy response is specific for AIEC or whether all *E. coli* activate autophagy in macrophages. Therefore activation of autophagy in macrophages infected by a group of *E. coli* that represent pathogenic and non-pathogenic strains was tested.

LC3B is an autophagy protein that is converted to LC3B-I by ATG4 soon after it is synthesised. LC3B-I is the conjugated to phosphatidylethanolamine (PE) by

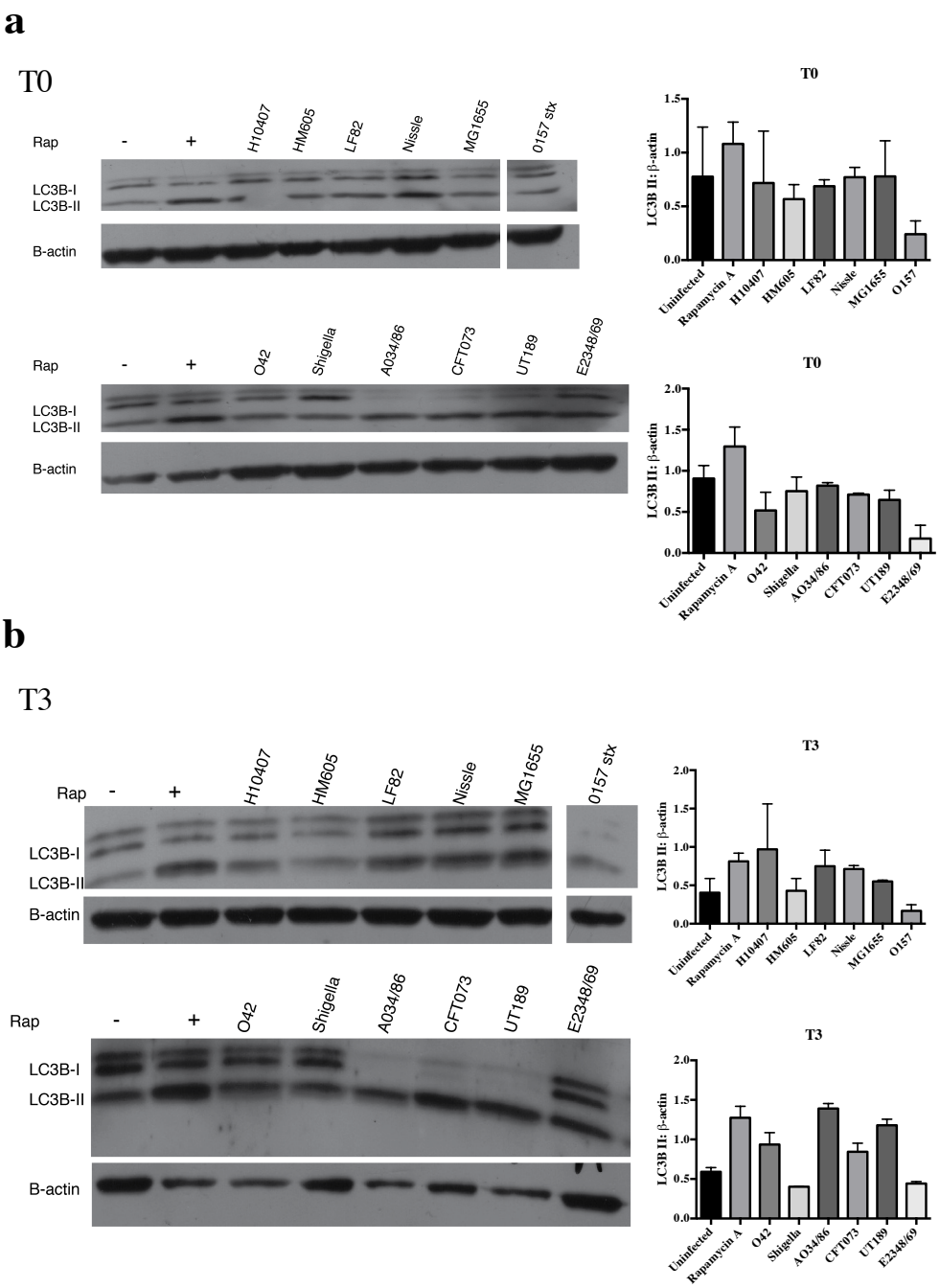

Figure 3. 4 LCB-II levels in THP-1 macrophages infected with *E. coli*

LC3-II concentration, measured by immunoblot, of whole cell lysates of THP-1 macrophages infected with *E. coli* and samples taken at T_0 **(a)** and T_3 **(b)** (see text for details). Each membrane was stripped and probed for β-actin as a loading control. Right panels show quantification, measured by densitometry, of ratio of LC3B-II to actin. Data shown is mean ±S.E.M. of 2 independent experiments.

ATG3/7/4 complex to become LC3B-II and this is incorporated into the membranes of autophagic vacuoles (Deretic, 2010). The conversion of LC3-I to LC3B-II is an indicator of autophagy activation. Cells with active autophagy show increased levels of LC3B-II and this can be visualised by immunoblotting. Therefore, immunoblotting was employed to identify the amount of autophagy activation in THP-1 macrophages infected with different strains of *E. coli*. It has been reported that autophagy is activated rapidly (within 1 h) in THP-1 infected with AIEC (Lapaquette *et al*., 2012b) and decreases within 4 h. Therefore, activation of autophagy was analysed at similar time points here. Bacteria were added to cells, centrifuged to increase bacteria/macrophages contact and incubated for 20 min to allow phagocytosis of bacteria. Following extensive washing and incubation with gentamicin-supplemented media for 30 min, cells were washed and whole cell lysate probed for LC3B-II; this time point as is referred to as T_0. This step was repeated 3 h later to identify if there was a reduction of autophagy as has been seen with AIEC (Lapaquette *et al*., 2012b); this time point is referred to as T_3. Using densitometry, the LC3B-II/ β-Actin ratio was quantified for each *E. coli* infection (Figure 3. 4). At T_0 all strains showed similar, or lower, amounts of LC3B-II:β-Actin compared to the uninfected control (Figure 3. 4a) whereas at T3 there was more variability (Figure 3. 4b). The 3 strains that cause high cytotoxicity in THP-1, A0 34/86, UTI89 and CFT073 all had increased LC3B-II compared to uninfected cells and both A0 34/86 and UTI89 had similar levels of LC3B-II compared to the positive control i.e. rapamycin-treated cells (Figure 3. 4b). This may be an artefact of cytotoxicity although both UTI89 and CFT073 have recently been shown to activate autophagy in macrophages and defects in autophagy allow faster clearance of infection with UPEC. Since A0 34/86 is genetically very similar to UPEC, it is not unsurprising that it also engaged with the autophagy machinery. AIEC strain LF82 was not seen to increase autophagy over background levels at either time point contrary to other reports and another AIEC strain, HM605, did not activate autophagy either. In general, most *E. coli* tested by immunoblotting, apart from UPECs, did not appear to show significant activation of autophagy in THP-1 macrophages.

Autophagy activation can also be studied by microscopy. LC3 is a key autophagy protein that is normally diffusely distributed in the cytoplasm but, upon activation of

autophagy, LC3 (through its conversion to LC3B-II) is concentrated in the membrane of autophagosomes. Therefore, activation of autophagy can be studied in cell lines expressing LC3-GFP as an increase by the formation of LC3-GFP puncta (Figure 3. 5). To this end a GFP-LC3 expressing immortalised murine bone marrow macrophages (iBMM) cell line was obtained from Dr Ed Lavelle, Trinity College Dublin (Harris et al., 2011). As this cell line expresses LC3-GFP it is does not require antibody staining and thus is conducive to screening a large panel of *E. coli* for formation of LC3 puncta in macrophages. iBMM were infected in the same manner as THP-1 (see Material and Methods (section 2.16)). At T_0 and T_3 cells were washed, paraformaldehyde (PFA) fixed, mounted on slides and visually inspected for LC3 puncta using fluorescent confocal microscopy (Figure 3. 5 LC3-GFP puncta in iBMM infected with *E. coli*.). The autophagy activator rapamycin was used as a positive control to identify LC3 puncta in conjunction with an uninfected control to identify LC3 puncta formation in uninfected cells. An artefact of plasmid based LC3-GFP

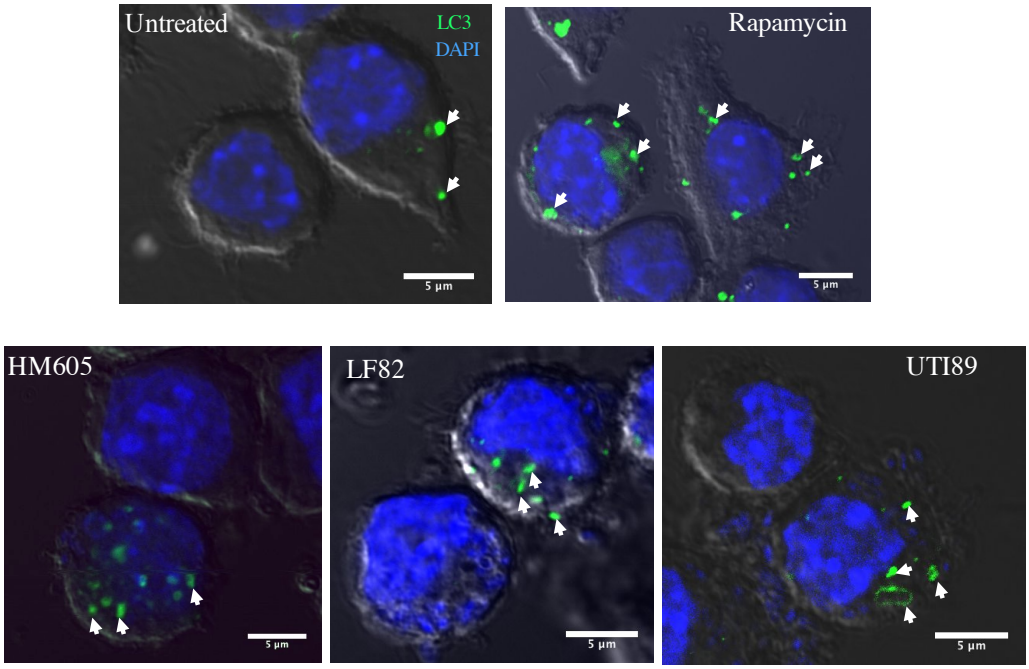

Figure 3. 5 LC3-GFP puncta in iBMM infected with *E. coli*.

Sample images of immortalised bone marrow macrophages expressing GFP tagged LC3 infected with *E. coli* strains indicated using a gentamicin protection assay. Following infection cells were paraformaldehyde fixed and mounted for confocal microscopy. Arrows indicate LC3-GFP puncta (green). Cells were counterstained with DAPI (blue).

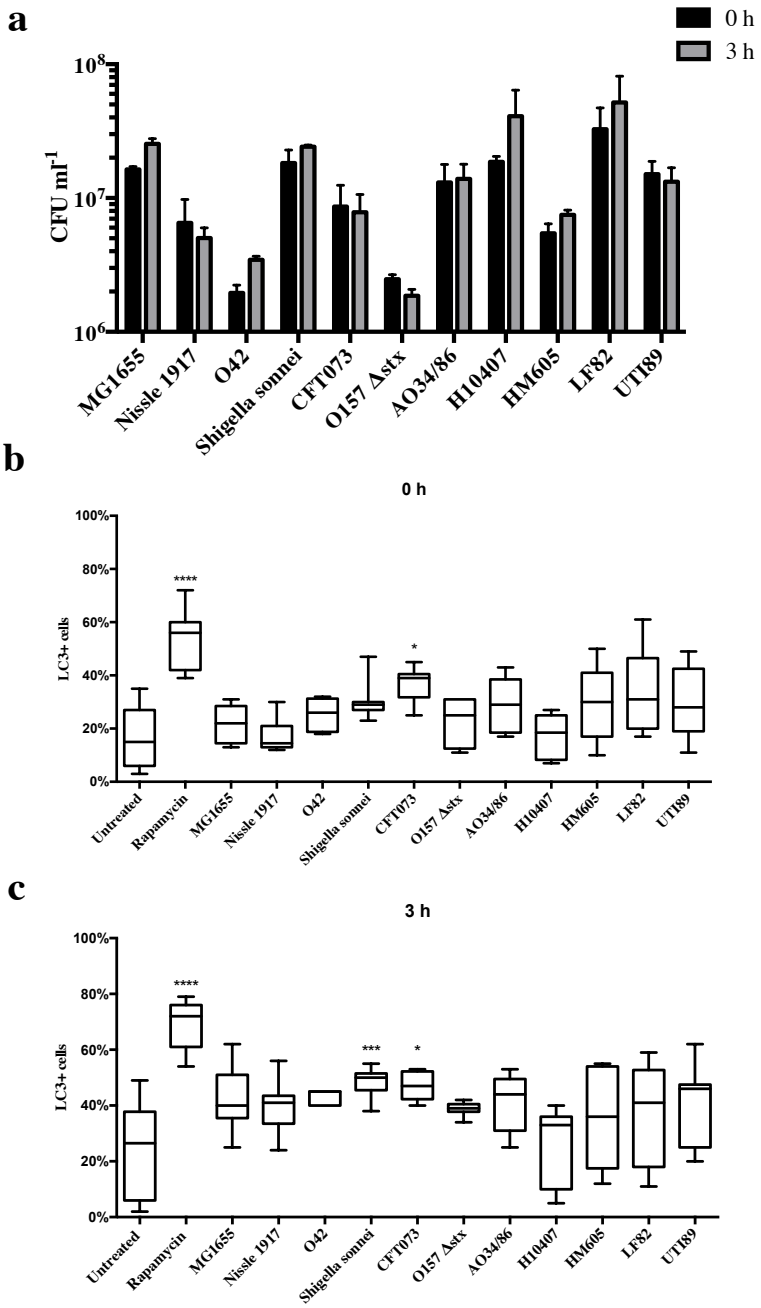

Figure 3. 6 Activation of autophagy by *E. coli* in iBMM:

Immortalised bone marrow macrophages expressing GFP tagged LC3 were infected with *E. coli* strains indicated using a gentamicin protection assay. Cells were paraformaldehyde fixed and mounted for confocal microscopy at time points indicated. **a)** Bacterial load in macrophages at both time points from control experiments. **b-c)** Quantification of number of $LC3^+$ cells as percentage of total cells counted at T_0 and T_3. All data is mean±SEM of 3 independent experiments. * $P< 0.05$, *** $P< 0.001$, determined by Kruskall-Wallis test with Dunn's post test comparing all samples to uninfected.

expression is the formation of puncta in non-activated cells, therefore all results need to be compared to an uninfected control. In total 300 macrophages were counted (100 per replicate) and scored for the presence or absence of LC3 puncta ($LC3^+$). The amount of bacteria present in iBMM was also monitored by counting CFUs in control experiments at both time points to ensure that the *E. coli* are successfully phagocytosed by the iBMM and to monitor any change in bacterial load over time (Figure 3. 6a). At T_0 all strains except CFT073, a reported activator of autophagy in macrophages (Amer et al., 2005b) , had no significant increase in $LC3^+$ cells above that of uninfected cells (Figure 3. 6b). At T3 CFT073 infected cells continued to have significantly higher percentage of LC3+ cells than uninfected cells (Figure 3. 6c) as did *S. sonnei* (another known activator of autophagy (Ogawa et al. 2005)). Interestingly, the UPEC strains that showed high LC3B-II levels in THP-1 cells at T3 did not have significantly higher levels of LC3+ cells in this screen. However this may due be the high cytotoxicity of these strains to THP-1 but not murine macrophages (See Chapter 5). No *E. coli* strain tested induced similar levels of LC3+ puncta as the rapamycin treated positive control cells. This suggests that while some autophagy activation may occur following infection of cells with *E. coli*, it is occurring at low levels and is not likely to be a main mechanism for resolving infection. TLR4 signalling is known to activate autophagy even in the absence of intracellular bacteria (Harris et al. 2011) and this may account for the low levels of induction seen here.

To determine whether *E. coli* exist in LC3 decorated vesicles we stained MG1655, Nissle 1917 and HM605 with BacLight Red prior to infection in order to visualise the bacteria and determine the level of LC3-GFP colocalisation at 0 and 3 h. Whilst control experiments were carried out to identify the optimal concentration of BacLight Red need to efficiently stain all bacteria in a sample we found that there was low intensity BacLight Red signal from bacteria that were infected in iBMM. This may be due to the effects of PFA fixing and/or loss of signal intensity due to bacterial degradation by macrophages. As a result of this low intensity bacterial staining, we were unable to fully identify all individual bacteria and thus cells were scored as positive for colocalisation if they contained at least 1 bacterium in a LC3-associated vacuole and negative if all bacteria were in LC3 negative vacuoles. At T0, single *E. coli* were evident in non-LC3 decorated vacuoles for all strains, however, at T3,

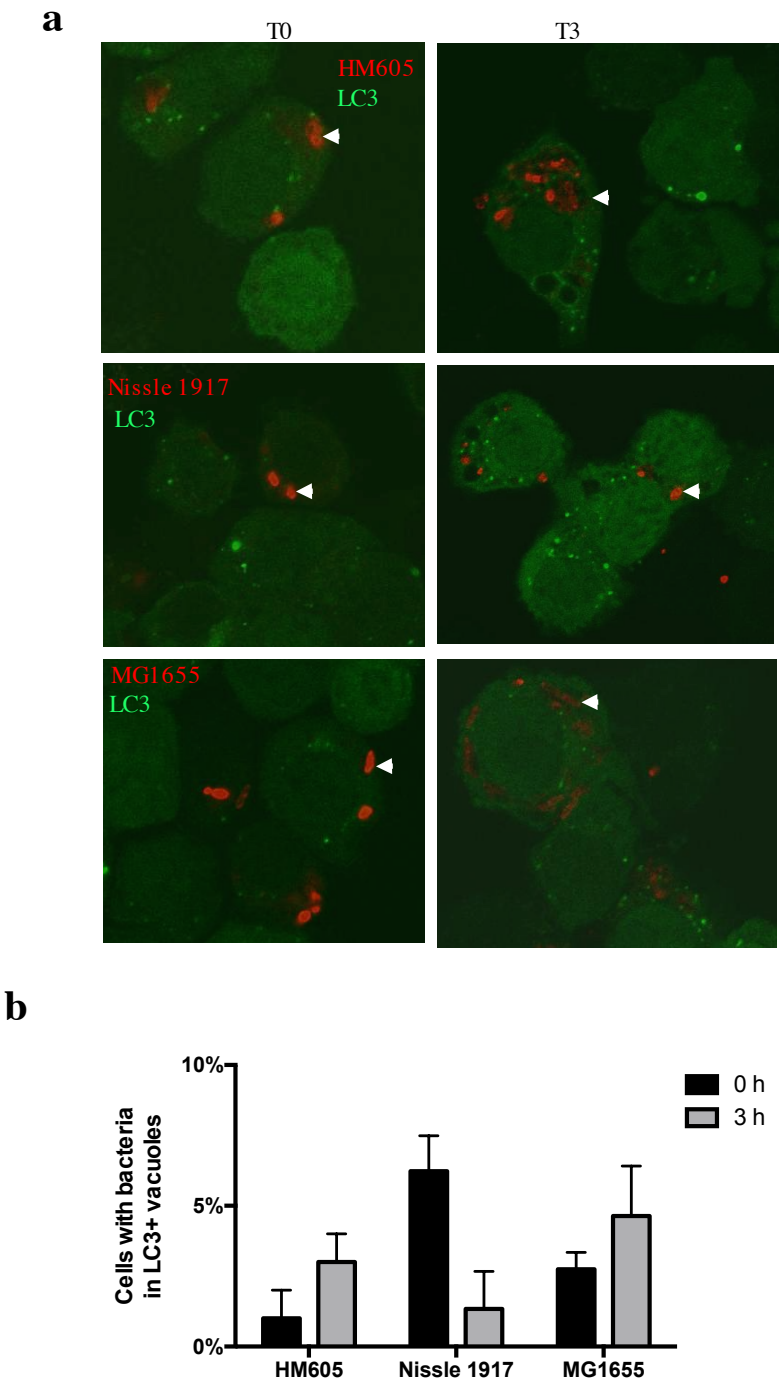

Figure 3. 7 Colocalisation of *E. coli* with LC3 in iBMM

Immortalised bone marrow macrophages expressing GFP tagged LC3 were infected with BacLight Red stained *E. coli* strains indicated using a gentamicin protection assay and cells were paraformaldehyde fixed and mounted for confocal microscopy at time points indicated. **a)** Sample confocal micrographs of infected cells showing bacteria in $LC3^-$ vacuoles (arrows). **b)** Quantification of cells with bacteria in LC3+ vacuoles. All data shown is mean ± SEM of 3 independent experiments.

vacuoles appeared large and seemed to contain more than one bacterium (Figure 3. 7a). Analysis indicated that there appears to be little colocalisation (less than 7%) of HM605, Nissle 1917 and MG1655 in LC3+ vesicles (Figure 3. 7b) suggesting that the autophagy machinery is not recruited en masse to *E. coli* vacuoles. There was a small proportion of bacteria in LC3+ vesicles and this may be attributed to either LC3 associated phagocytosis at early time points or damaged phagosomes being targeted for degradation by autophagy – both well reported phenomena (Lai & Devenish 2012; Mansilla Pareja & Colombo 2013).

1.1.1 Inhibition of autophagy does not alter *E. coli* load in macrophages

Previously, AIEC strain LF82 has been shown to have a higher bacterial load in macrophages that are deficient in autophagy (Lapaquette, Bringer, et al. 2012). Therefore, the effect of the pharmacological inhibition of autophagy on the bacterial load was tested. The same cell line, iBMM GFP-LC3, was infected and the number of intracellular bacteria was enumerated at 0, 3 and 6 h in the presence or absence of wortmannin, a non-specific inhibitor of autophagy. In order to test the efficacy of wortmannin the number of LC3+ cells in untreated cells, wortmannin treated cells and rapamycin-treated cells was counted by microscopy and it was found that the wortmannin significantly decreased the number of LC3+ puncta cells compared to untreated cells (Figure 3. 8d-e). Furthermore, there was no significant difference in bacterial load between wortmannin and control cells at 0 h indicating that wortmannin does not affect phagocytosis. In contrast to results seen by other groups using the AIEC strain LF82, there was no significant difference in bacterial load with the treatment of cells with wortmannin at any time points. There was a non-significant increase in bacterial load with wortmannin-treatment cells infected with the AIEC HM605 at 3 h and 6 h but not at 0 h (Figure 3. 8d). At all time points both MG1655 and Nissle 1917 exhibited non-significant decreases in bacterial load with wortmannin treatment (Figure 3. 8a-b). These data indicate that inhibition of autophagy does not significantly alter *E. coli* load in infected iBMM.

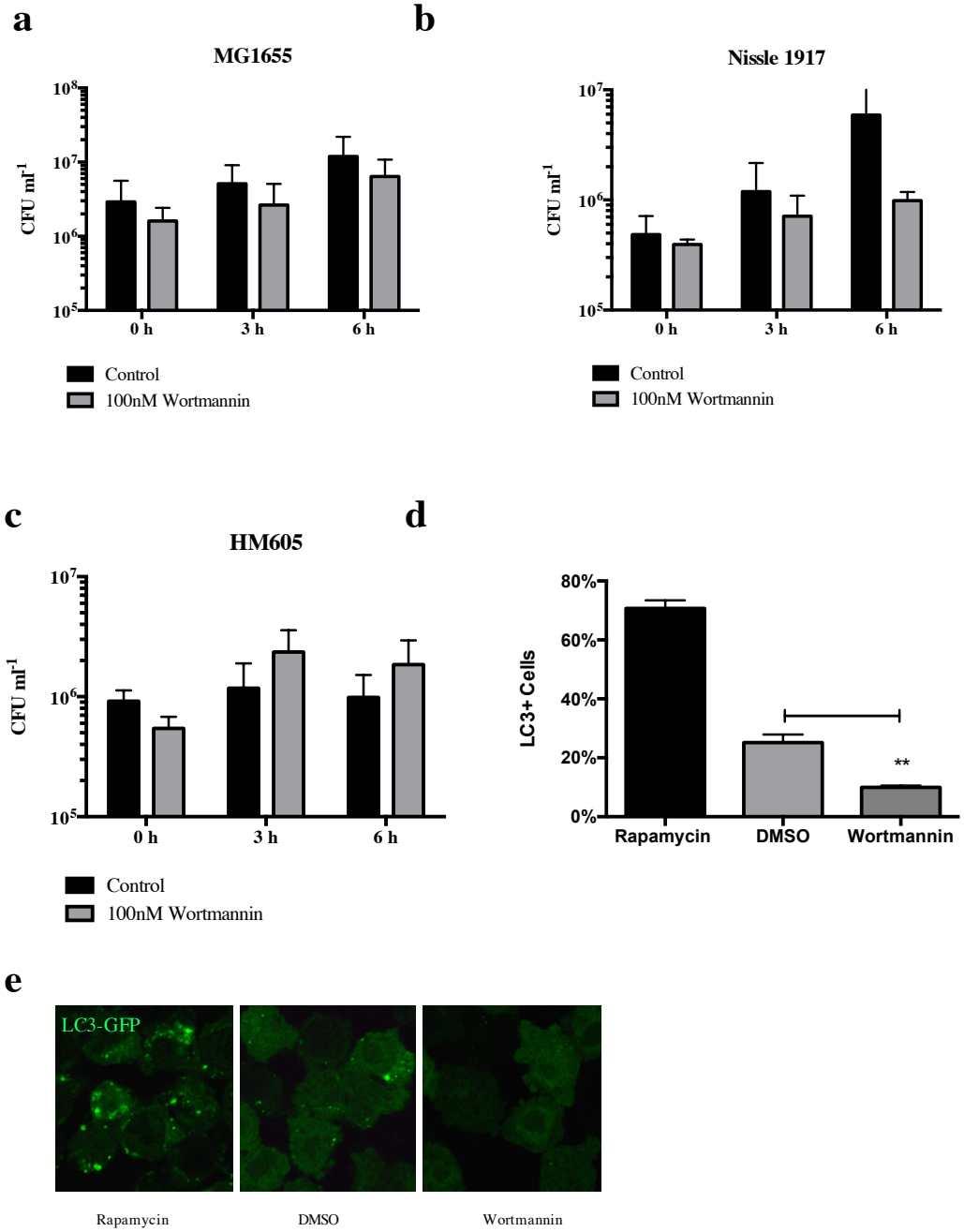

Figure 3. 8 Inhibition of autophagy does not affect bacterial load in iBMM

Immortalised bone marrow macrophages expressing GFP tagged LC3 were infected with *E. coli* strains indicated using a gentamicin protection assay in the presence or absence of autophagy inhibitor wortmannin. **a-c)** Samples of intracellular bacteria were taken, plated and counted at times indicated. **d)** Control experiments were carried out to ensure efficacy of wortmannin by counting $LC3^{+}$ cells and comparing wortmannin treated samples to the autophagy activator rapamycin and a vehicle only control. **e)** Representative micrographs of iBMM-LC3-GFP treated with rapamycin, DMSO or wortmannin. All data shown is mean±SEM of 3 independent experiments. ** $P< 0.01$ determined by Kruskal-Wallis test.

Together the results above indicate that while some strains of *E. coli* interact with the autophagy machinery to a minor degree, there is no significant activation of this cellular process. Experiments whereby autophagy was inhibited did not result in slower bacterial clearance suggesting that autophagy is not the main method employed by macrophages to resolve *E. coli* infection.

3.2.5 *E. coli* replication in J774A.1 macrophages

Our group has reported that AIEC replicates in murine J774A.1 macrophages. Therefore, the ability of pathogenic and non pathogenic *E. coli* to replicate in J774A.1 macrophages was tested. J774A.1 were infected and replication over 6 h measured. Contrary to results obtained with THP-1 macrophages there were considerable strain-dependent differences in both the number of intracellular bacteria at 0 h and replication (Figure 3. 9). At 0 h, HM605, 042 and A0 34/86 are present in the macrophages in the greatest numbers. 042 is a strain of enteroaggregative *E. coli* that adheres to colonic epithelium using a number of adhesins. The presence of these adhesins may lead to increased macrophages interaction and could explain the relatively high phagocytosis rate of this *E. coli*. Like 042, A0 34/86 possess a number of adhesin genes which may account for the higher levels of phagocytosis by these strains (Hejnova, 2005). Both EPEC and EHEC have genes that inhibit phagocytosis by macrophages explaining why these strains are present at lower levels at 0 h than HM605 (Goosney et al., 1999; Wong et al., 2011). Non-opsonised phagocytosis by macrophages is driven by the detection of bacterial membrane components such as LPS. Interestingly both *S. sonnei* and MG1655 do not produce an O-antigen and thus have rough LPS which may account for why these strains are phagocytosed to a lesser degree than HM605.

Following 6 h of infection only HM605 showed a significant increase in bacterial load whilst numbers of *S. sonnei*, EAEC 042 and the *E. coli* commensal strain A0 34/86 did not change during the 6 h (Figure 3. 9b). In contrast the numbers of CFT073, MG1655 and EHEC O157 Δstx all decreased by approx. 50% over 6 h. These data highlight the unique ability of AIEC strains to replicate in J774A.1 macrophages.

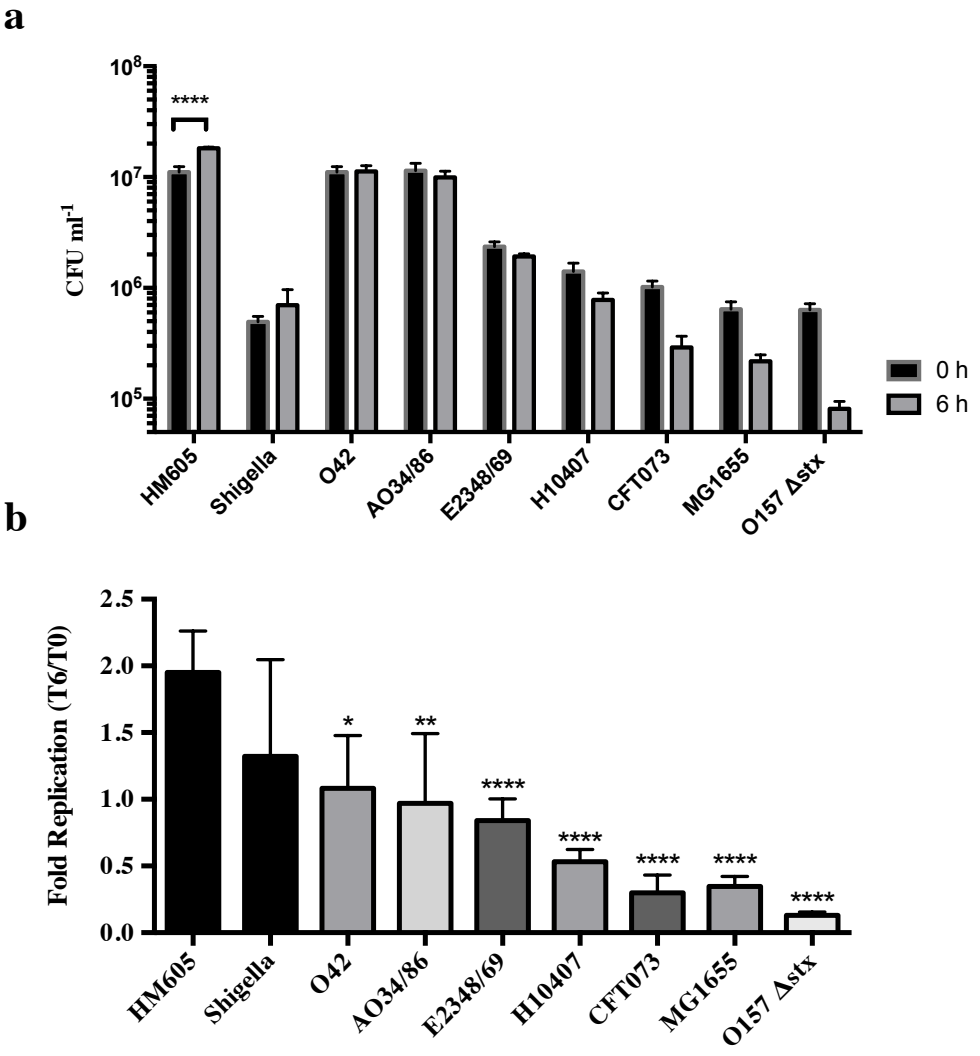

Figure 3. 9 Replication of *E. coli* in J774A.1 macrophages

Macrophages were infected with *E. coli* using a gentamicin protection assay with samples taken and intracellular bacteria plated at 1hr post gentamicin (0 h) and again 6 h later. Fold change was calculated as number of bacteria at 6 h divided by number of bacteria at 0hrs. **a)** Colony forming units (CFU) of all strains tested at 0 h and 6 h. Dashed line indicated number of bacteria initially added to macrophages. **b)** Fold change in bacteria over 6 h. Values shown are mean±S.E.M. of 3 independent experiments. *P< 0.05, **P< 0.01, ****P< 0.0001 determined by students t-test in **(a)** and one-way ANOVA with Dunnett's post test comparing all samples to HM605 in **(b)**.

Moreover, this data also confirms our previous observations that the *E. coli* genus exhibits a strain-dependent ability to replicate in the J774A.1 macrophages. Since the intracellular environment is highly stressful, some *E. coli* may have evolved different mechanisms to survive this stressful environment.

3.2.6 *E. coli* trafficking in J774A.1 macrophages

The AIEC strain HM605 replicates well in the macrophages cell line J774A.1 whereas MG1655 does not replicate. Therefore, it was of interest to identify if there is any difference in the uptake and processing of these two strains that explains this difference in replication ability. Following engulfment, bacteria containing vacuoles possess the early endosomal marker, EEA1, and colocalisation of EEA1 with bacteria is an indicator of early phagosomes (Underhill & Goodridge 2012). Therefore, an immunofluorescent antibody against EEA1 was used to visualise the localisation of *E. coli* during early phagocytosis. Since EEA1 is rapidly acquired and then lost from the early phagosome, staining of this marker was visualised following an initial 20 min incubation (T-30) and again following an additional 30 min incubation with gentamicin supplemented media (T0). At T-30, approximately 20% of both HM605 and MG1655 were co-localised with EEA1-positive vacuoles (Figure 3. 10a-b) indicating rapid phagocytosis and containment of bacteria in EEA1+ phagosomes. However, there was no significant difference in the number of co-localised bacteria between the HM605 and MG1655. There was, on average, significantly more HM605 per cell compared to MG1655 (Figure 3. 10c). At T0, there was a significant increase in MG1655 co-localising with EEA1 and a corresponding significant decrease in HM605/EEA1 colocalisation. There was also significantly more MG1655/EEA1 colocalisation than HM605/EEA1 colocalisation suggesting that phagosomes containing HM605 may mature more rapidly than MG1655-containing phagosomes. The increase in MG1655/EEA1 colocalisation and the corresponding decrease in HM605/EEA1 colocalisation suggested that there may be a difference in the rate of phagocytosis between the 2 strains. Therefore, a control experiment to enumerate, using CFU counts, the number of intracellular bacteria at T0 was conducted. This experiment indicated that there was no significant difference in phagocytosis between

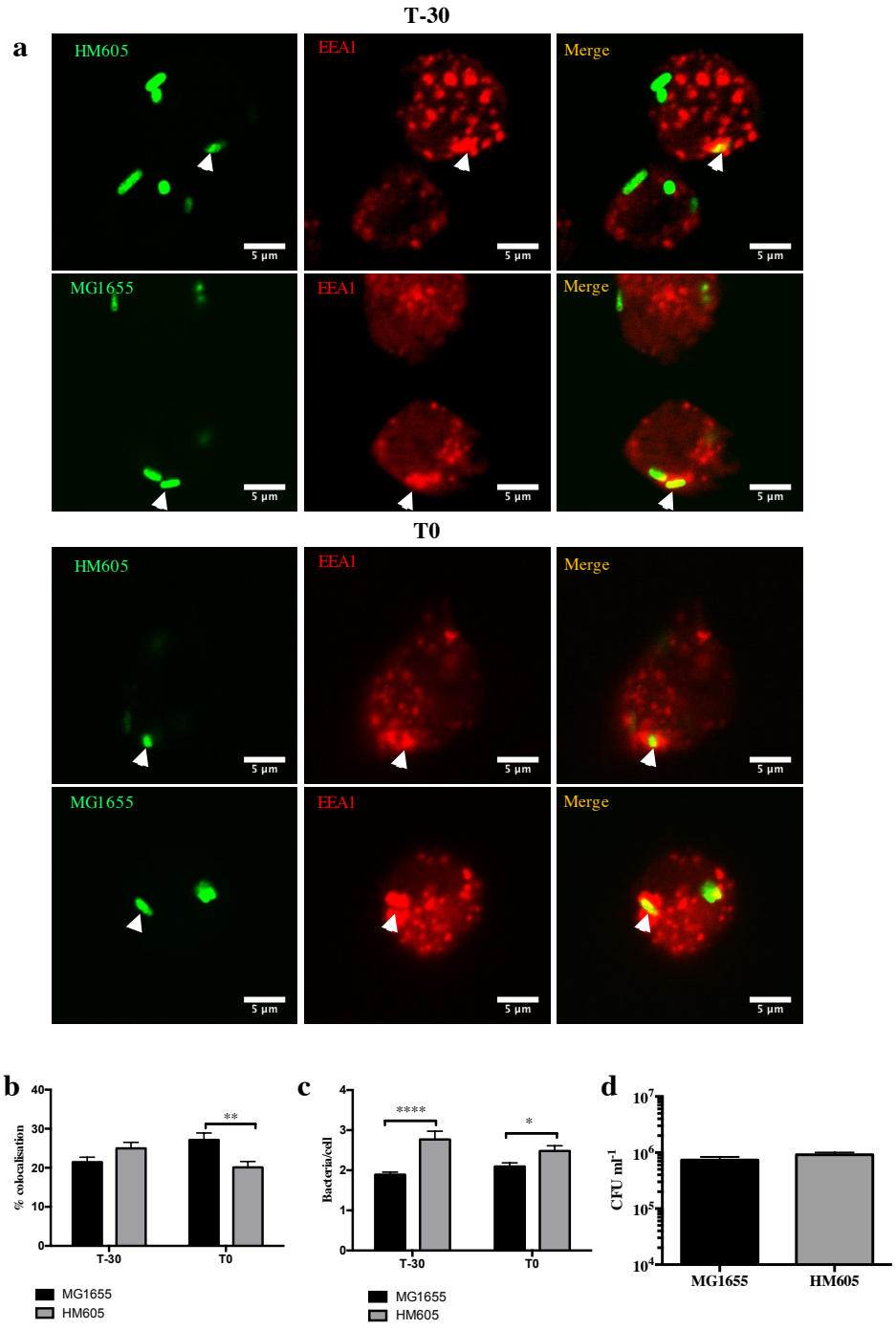

Figure 3. 10 *E. coli* co-localise with early endosomal markers in early infection.

J774 seeded on coverslips were infected with HM605 and MG1655 expressing GFP using a gentamicin protection assay. Cells were fixed and stained for EEA1 at times indicated. Samples were imaged by confocal microscopy **(a)** and intracellular bacteria analysed for co-localisation in EEA1+ vacuoles in at least 100 cells in triplicate **(b)** or total number of bacteria counted and expressed as a percentage of total infected cells **(c)**. **d)** To evaluate phagocytosis, bacteria were also lysed from infected cells, serially diluted and counted at T0. Data shown is mean±SEM of 3 independent experiments. Separate Mann-Whitney tests were used to compare differences between the samples at both time points, * $P< 0.05$, ** $P< 0.01$, **** $P< 0.0001$.

the 2 strains of *E. coli* (Figure 3. 10d). Thus, HM605 and MG1655 are both phagocytosed to a similar degree but HM605 vacuoles lose their EEA1 marker, a marker of early phagosomes, quicker than MG1655 suggesting that HM605 vacuoles may acquire mature phagosomal markers quicker than MG1655.

3.2.7 Both strains of *E. coli* reside in acidified vacuoles

Experiments investigating autophagy-*E. coli* interactions above demonstrated that MG1655 and HM605 do not significantly induce autophagy activation and also that HM605 and MG1655 occupy vacuoles with early endosomal markers at the early stages of infection. The ultimate fate of bacteria containing vacuoles is fusion with lysosomes and this facilitates the degradation of the bacteria. Therefore the co-localisation of GFP expressing MG1655 and HM605 with the acidic vacuole marker Lysotracker Red early (T_0) and late (T_6) in infection was examined (Figure 3. 11). During early infection more MG1655 were contained within acidified vacuoles compared to HM605 (Figure 3. 11b). At T_6 over 60% of all bacteria were contained in an acidified vacuole and there was no significant difference between the strains. Both MG1655 and HM605 appeared as single bacteria contained within single vacuoles rather than large vacuoles containing multiple bacteria (Figure 3. 11a). The number of bacteria per macrophages was compared between the 2 strains and indicated that, at both time points, there was significantly more HM605 per cell than MG1655 (Figure 3. 11c). This is, as expected, consistent with data from CFU counts where a 2.5 fold increase in HM605 per cell was measured, confirming that HM605 can replicate in these cells (Figure 3. 11c).

This study has confirmed, using microscopy, the ability of AIEC strain HM605 to replicate in J774A.1 macrophages. Moreover, subtle differences in the timing of trafficking of HM605 and MG1655 during phagocytosis in J774A.1 macrophages were also observed. However, the ultimate fate of both strains is to reside within acidified vacuoles suggesting that despite these timing differences the strains are processed in a similar manner. Therefore, the macrophages does not appear to respond differently to AIEC compared with other strains of *E. coli*.

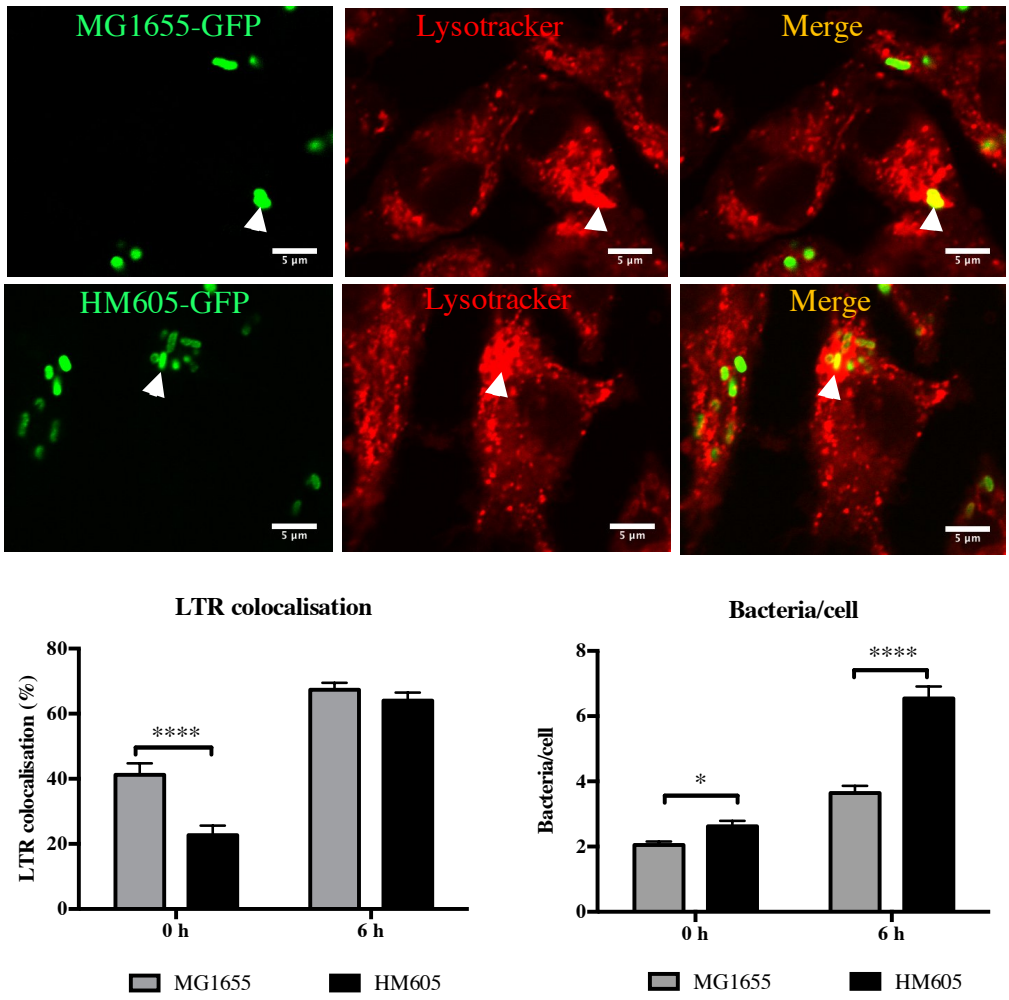

Figure 3. 11 *E. coli* are trafficked to acidic vacuoles

J774 seeded on coverslips were infected with HM605 and MG1655 expressing GFP using a gentamicin protection assay. 30 min prior to fixing LysoTracker Red DND-99 was added to cells. Cell were fixed and mounted and imaged by confocal microscopy. **a)** Sample confocal micrographs at 6 h post infection. **b)** Quantification of bacteria/Lysotracker co-localisation. Bacteria in at least 100 cells in triplicate were analysed for colocalisation at each time point. **c)** Quantification of bacteria in infected cells. Bacteria in at least 100 cells in triplicate were counted. Data shown is mean±SEM of 3 independent experiments. * $P< 0.05$, **** $P< 0.0001$ determined by Kruskall-Wallis test with Dunn's post test comparing all time points to 0 h.

3.3 Discussion

Macrophages can degrade bacteria by two separate but linked mechanisms, phagocytosis and autophagy (Ma *et al*., 2013; Underhill & Goodridge, 2012). Both pathways involve the sensing of PAMPs by PRR resulting in the containment of bacteria in a membrane bound vesicle which matures and fuses with lysosomes resulting in the degradation of the bacteria. In the case of *E. coli*, phagocytosis occurs when extracellular bacteria interact with CD14 and TLR4/MD2 resulting in the internalisation of the bacteria in the phagosome. Furthermore, TLR4 can initiate autophagy through the recruitment of beclin-1 (Shi & Kehrl, 2008). Autophagy occurs in response to cytosolic bacteria sensed by other PRRs such as NOD2 which detects the presence of muramyl dipeptide (MDP), a component of the peptidoglycan cell wall from both Gram-positive and Gram- negative bacteria and initiates the formation of autophagosomes (Homer et al., 2010). *NOD2* polymorphisms are associated with CD and dendritic cells with CD-associated *NOD2* polymorphisms have impaired clearance of *E. coli* (Cooney et al., 2010). AIEC that are implicated in CD have been shown to replicate better in cells with impaired autophagy (Lapaquette *et al*., 2012b). UPEC have also been shown to interact with the autophagy machinery and are transferred to vacuoles decorated with autophagy proteins ATG7 and ATG8 following phagocytosis by macrophages (Amer et al., 2005b). Despite these studies there is no comprehensive data available on whether this is a general mechanism for degrading *E. coli*.

In this study immunoblot analysis of *E. coli* infected THP-1 macrophages indicated that autophagy was not activated above uninfected cells levels for all of the *E. coli* strains tested. Additional fluorescent microscopy analysis of a murine macrophages cell line also indicated that most *E. coli* did not activate autophagy to levels above background. There were two outliers i.e. the UPEC CFT073 and *Shigella sonnei* that had increased autophagy in the LC3-GFP murine cell line. *Shigella* are known activators of autophagy as they can escape from phagosomes into the cytosol (Ma et al., 2013) and CFT073 has been shown to be contained within vacuoles decorated with autophagy related proteins (Amer et al., 2005a). It is possible, however, that these are

not true autophagosomes as a more recent study has shown that UPEC exist in single membrane vesicles in macrophages (Symington et al., 2015).

The AIEC strain LF82 has been shown to co-localise with the key autophagy protein LC3 in macrophages (Lapaquette *et al*., 2012b). This is in contrast to what has been reported here and there is no evidence to indicate that LF82 activates autophagy in this study. Furthermore, another strain of AIEC, HM605, was tested for its interaction with autophagy but again there was no increase in activation above background. Moreover, colocalisation studies did not show comprehensive evidence that HM605 interacts with the autophagy machinery. Autophagy inhibition in macrophages infected with LF82 results in increased bacterial load and reduced clearance suggesting that autophagy is an important mechanism for clearance of AIEC (Lapaquette *et al*., 2012b). In the current study, the rate of clearance of *E. coli* by macrophages with pharmacologically inhibited autophagy was tested. If autophagy was indispensable for resolving *E. coli* infection, then there should a reduced clearance in inhibited cells but there was no difference in the clearance of *E. coli* including HM605 when compared to uninhibited cells. Furthermore, other studies have indicated that deficiencies in autophagy result in increased bacterial uptake in macrophages, however, this was not seen in this study where autophagy is pharmacologically inhibited (Bonilla et al., 2013; Symington et al., 2015). This may be due to differences in the methods for autophagy inhibition. Previous studies have used either ATG protein knockouts or inactive ATG proteins where this study used pharmacological inhibition that has more off target effects. Nonetheless, it is possible to conclude that autophagy is not the main mechanism employed by macrophages to clear *E. coli* infection. Autophagy is a mechanism employed by cells to clear cytosolic bacteria, however, there is little evidence, with the exception of *Shigella*, that either pathogenic or non-pathogenic *E. coli* tested can exist in the cytosol of macrophages, thus, activation of autophagy is unlikely. Whilst there appeared to be a small amount of LC3-*E. coli* co-localisation (less than 7%) it is possible that this could have been LC3-associated phagocytosis that has been previously reported in LPS-coated beads (Sanjuan et al., 2007).

Since autophagy did not appear to be involved in *E. coli* clearance by macrophages, the interaction of AIEC with the phagocytic machinery was investigated. The AIEC

strain HM605 was unique amongst the strains tested in its ability to replicate in the murine J774A.1 macrophages. Interestingly, there was no increase in HM605 intracellular load in the immortalised murine cell line used for visualising autophagy or in the human THP-1 macrophages cell line. It is possible that macrophages cell-line specific differences result in a permissive environment for HM605 replication in J774A.1. Due to this phenotype, J774A.1 were used to compared the trafficking of HM605 and the non-pathogenic (and non-replicating) strain MG1655 with the aim of further uncovering the molecular mechanisms driving this phenotype. Despite a greater number of HM605 per cell at all time points there were only subtle differences in co-localisation of HM605 and MG1655 with early or late phagosomal markers. A higher percentage of MG1655 appeared to reside in acidic vacuoles than HM605 at early time poinst suggesting that MG1655 may be trafficked to these vacuoles at a faster rate. If this is the case, the delay in processing of HM605 to acidic vacuoles may provide HM605 with an opportunity to replicate since replication was clearly seen by the 2.5-fold increase in number of HM605 per cell over 6 hours. Thus it is possible that subtle differences in trafficking results in a permissive environment that allows HM605 to replicate.

Both LF82 and HM605 were isolated as AIEC strains from the sub-mucosa of patients with CD. LF82 was isolated from the ileum whilst HM605 was isolated from the colon (Darfeuille-Michaud *et al*., 1998; Subramanian *et al*., 2008b). It is clear that there are strain specific differences between the intracellular processing of LF82 and HM605. This suggests that there are a range of AIEC mechanisms for replication in the macrophages and these mechanisms may be strain-specific. This is in agreement with recent studies that report that, since the discovery of AIEC, there has been little consensus on any factors that influence their phenotype (Martinez-Medina & Garcia-Gil, 2014). Furthermore, some studies have shown that AIEC, non-AIEC and K-12 *E. coli* all survive longer in macrophages from CD patients compared to those from healthy controls (Elliott et al., 2015a). This is in agreement with research in this thesis suggesting that a permissive environment is important for the ability of AIEC to replicate as replication of HM605 was only seen in J774A.1 but not THP-1 macrophages or immortalised murine bone marrow macrophages. Thus the combination of a permissive environment in conjunction with an opportunistic

pathogen is the most likely model for the role of *E. coli* in CD. What is not disputed, however, is that *E. coli* play a role in the pathology of CD as *E. coli* is more frequently isolated from intestinal mucosa, epithelial cells and macrophages in CD patients compared to healthy controls (Elliott *et al*., 2013, a, b; Martin *et al*., 2004) and an increase in Proteobacteria, of which *E. coli* is a member, is associated with CD (Sartor & Mazmanian, 2012).

In conclusion the current study indicates that autophagy is not engaged as a general response to *E. coli* infection, in both murine and human macrophages. This suggests that classical phagocytosis pathway is the main method of *E. coli* clearance by macrophages. Furthermore, the inflammatory response by macrophages to *E. coli* infection is broadly similar amongst pathogenic and non-pathogenic strains that do not possess any anti-inflammatory cytokine release mechanisms. Finally, the ability of HM605 to replicate in macrophages may be due to alterations in the kinetics of trafficking to acidic vacuoles.

Chapter 4 AIEC metabolism in macrophages

4.1 Introduction

Metabolism is a key factor in the survival of bacteria in the intracellular environment and is also essential for bacteria to replicate. Bacteria that survive in the intracellular environment are heterotrophs and must be able to catabolise a carbon source to generate energy and to also provide intermediate molecules for anabolic processes (Eisenreich et al., 2010). Pathogenic bacteria such as *Salmonella enterica subsp. enterica serovar* Typhimurium *(S.* Typhimurium), *Listeria monocytogenes* and *Shigella flexneri* can occupy distinct niches within the intracellular environment (Beuzón *et al.*, 2000; Ray *et al.*, 2009). Following entry into the cell *Shigella* and *Listeria* both escape the vacuoles in which they are contained and replicate in the cytosol. This gives them access to an array of carbon sources such as pyruvate, lactate, glycerol and glucose (Eisenreich et al., 2010; Ray et al., 2009). *Listeria* growing in macrophages have increased expression of genes involved in gluconeogenesis and a decreased expression of genes involved in glycolysis (Chatterjee et al., 2006; Joseph et al., 2005). Moreover, there is an increased expression of genes involved in the uptake and catabolism of glycerol suggesting that glycerol is a major carbon source for *Listeria* (Chatterjee et al., 2006; Joseph et al., 2005). A recent study in *Shigella* suggested that pyruvate is the preferred carbon source for growth in epithelial cells (Waligora et al., 2014). Moreover, in another study using cultured HeLa cells, it was shown that intracellular *Shigella* primarily used host-derived pyruvate as a carbon source converting it into acetyl-coA and then acetate via the Pta-AckA pathway. This produces ATP and thus supplies *Shigella* with the energy needed for intracellular replication (Kentner et al., 2014).

S. Typhimurium is contained within a *S.* Typhimurium containing vacuole (SCV) in the cell where the access to carbon sources is reduced due to the vacuolar environment (Beuzón et al., 2000). Despite this, *S.* Typhimurium are able to replicate within the SCV. Transcriptional analysis of *S.* Typhimurium growing in cultured macrophages indicate an increase in expression of genes required for glycolysis and the Entner-Doudoroff pathway with a decreased expression of genes in the TCA cycle (Eisenreich et al., 2015). Furthermore, mutations of key glycolysis genes and genes encoding enzymes in the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS)

(used for the uptake of glucose) resulted in a *S*. Typhimurium that is severely attenuated in replication in cultured macrophages (Bowden et al., 2009). In the context of AIEC, *S*. Typhimurium is an interesting example. AIEC is reported to replicate in macrophages in phagosomes (Bringer et al., 2011) that are similar to the intracellular environment encountered by *S*. Typhimurium and also would have limited access to nutrients. Previous studies carried out by members of our laboratory have indicated that AIEC deficient in glycolysis and PTS do not replicate in macrophages like their wild type parent (A. Thompson, unpublished data). This suggests that, similar to S. Typhimurium, AIEC utilise glucose as their primary carbon source during replication in macrophages.

How AIEC and *S*. Typhimurium have access to glucose in phagosomes is not clear. One possibility is the observed 'leakiness' of the phagosome (Sander et al., 2011) that would allow diffusion of nutrients into the phagosome lumen. Interestingly, a recently experiment showed that supplementation of *S*. Typhimurium infected cells with high levels of glucose accelerated their growth (Steeb et al., 2013). *S*. Typhimurium that were not able to import glucose did not show this increased growth suggesting that extracellular glucose can gain access to the SCV and be metabolised by *Salmonella*.

The sensing of bacteria by immune cells is dependent on the recognition of bacterial PAMPs by PRR. The classic example of this is the recognition of bacterial LPS by TLR4 on macrophages resulting in an increase in release of pro-inflammatory cytokines (Kawai & Akira, 2011). More recently, the inflammasome has been shown to be a key sensor of bacteria and a regulator of release of the pro-inflammatory cytokines IL-1β and IL-18 (Schroder & Tschopp, 2010). A recent study has shown that macrophages can distinguish between living and dead *E. coli* (Sander et al., 2011). Therefore, macrophages release different levels of certain pro-inflammatory cytokines dependent on whether the engulfed bacteria is viable or non-viable (Sander et al., 2011). Phagocytosis of viable bacteria results in the release of increased levels of IL-1β compared to phagocytosis of non-viable bacteria. It was found that bacterial mRNA is an activator of the NLRP3 inflammasome and this results in increased IL-1β release. Bacterial mRNA is very unstable and therefore there would not be significant amounts of this molecule in non-viable bacteria. Therefore bacterial mRNA is a viability-

associated PAMP or vita-PAMP. Interestingly, a recent study with *S*. Typhimurium showed that bacteria that accumulate citrate (due to a mutation blocking the TCA cycle) also exhibit an increase in mitochondrial reactive oxygen species (mtROS) which is an activation signal for the NLRP3 inflammasome (Wynosky-Dolfi et al., 2014). Therefore, bacterial derived metabolites, such as citrate, may be another form of vita-PAMP that can stimulate the inflammasome.

In summary, its is clear that pathogenic bacteria utilise specific metabolic pathways during intracellular growth and it is possible by-products of these pathways may be a source of vita-PAMPs for inflammasome activation.

AIEC are implicated in the pathology of Crohn's disease and can replicate in macrophages, however, little is known about the metabolic pathways utilised by these *E. coli* during replication in macrophages. The objective of this study is to expand our knowledge of glycolysis utilised AIEC in macrophages and identify any possible metabolites that may function as vita-PAMPs.

4.2 Results

4.2.1 Glycolysis is required for HM605 induced IL-1β release from macrophages

The AIEC HM605 is able to invade epithelial cells and replicate in macrophages *in vitro* (Subramanian *et al*., 2008a). Previous work in our laboratory has shown that a HM605 mutant in the A and B isoforms of the glycolysis intermediate enzyme phosphofructose kinase (PfkAB) induced significantly reduced IL-1β response in macrophages compared to the wild type strain (Aoife Thompson, unpublished data). It was therefore hypothesised that during growth in macrophages, HM605 metabolises glucose and a consequence of this metabolism is induction of IL-1β release. To confirm a role for bacterial glucose metabolism in IL-1β production it was decided to mutate the PTS system, responsible for the uptake of glucose. Therefore, a mutant in *ptsI* (encoding the enzyme I component of PTS) was constructed and confirmed that the mutant was unable to grow using glucose as a carbon source (A. Thompson, unpublished data). Macrophages were infected with this mutant and it was confirmed that these macrophages secreted less IL-1β than macrophages infected with wild-type HM605. Therefore, it is apparent that glucose metabolism by the bacteria is required for IL-1β production (data not shown).

The PkfAB-catalysed step in glycolysis is near the top of the pathway (see Figure 4. 1) and therefore further investigations of the effects on IL-1β release by macrophages infected with HM605 mutants downstream of PfkAB was performed. When grown on glucose an important metabolite of glycolysis is acetyl-Coenzyme A (AcCoA). Metabolomic analysis has shown that the Δ*pfkAB* mutant produces significantly less AcCoA than the wild type strain (Dr. E. Smith, personal communication). AcCoA is produced from pyruvate through the action of the pyruvate dehydrogenase enzyme complex (PDHC). This complex is encoded by 3 genes *aceE*, *aceF* and *lpd* and together these genes produce proteins that form an enzyme complex that catalyses the oxidative decarboxylation of pyruvate to AcCoA. Initially, pyruvate is decarboxylated

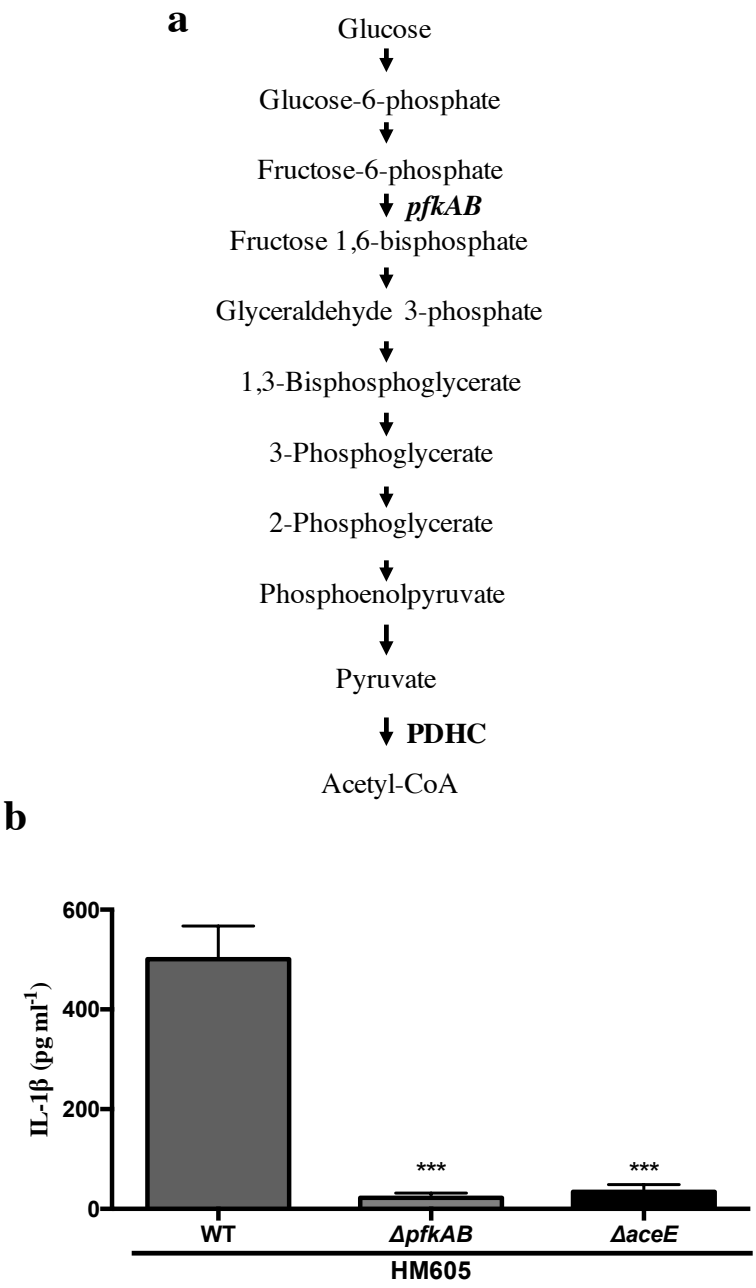

Figure 4. 1 Glycolysis and pyruvate dehydrogenase mutants induce low levels of IL-1β release from macrophages.

a) Conversion of glucose to pyruvate in glycolysis. The pyruvate dehydrogenase complex (PDHC) converts pyruvate to acetyl-CoA. *pfkAB*; genes that encode the A and B isoform of Pfk **b)** IL-1β released from macrophages infected with HM605 mutants in glycolysis (Δ*pfkAB)* and PDH (Δ*aceE*). Data shown is mean ±SEM of 3 independent expriments. *** $P < 0.01$ as determined by one-way ANOVA comparing mutants to wild type with Dunnett's post test.

to 2-hydroxy-ethyl-ThPP by the decarboxylase component E1 (encoded by *aceE*) of the PDHC. The dihydrolipoyl transacetylase component E2 (encoded by *aceF*) and the lipoamide dehydrogenase E3 (encoded by *lpdA*) component then convert 2-hydroxy-ethyl-ThPP to AcCoA (Figure 4. 2). To test for a role for AcCoA in IL-1β production, macrophages were infected with a mutant in AcCoA production, Δ*aceE*, and IL-1β release was measured after 24 h. Similar to the Δ*pfkAB* mutant, IL-1β production was significantly reduced compared to the wild type strain (Figure 4. 1). Therefore, it was confirmed that bacterial glucose metabolism and more specifically the production of AcCoA, is associated with increased IL-1β production from infected macrophages.

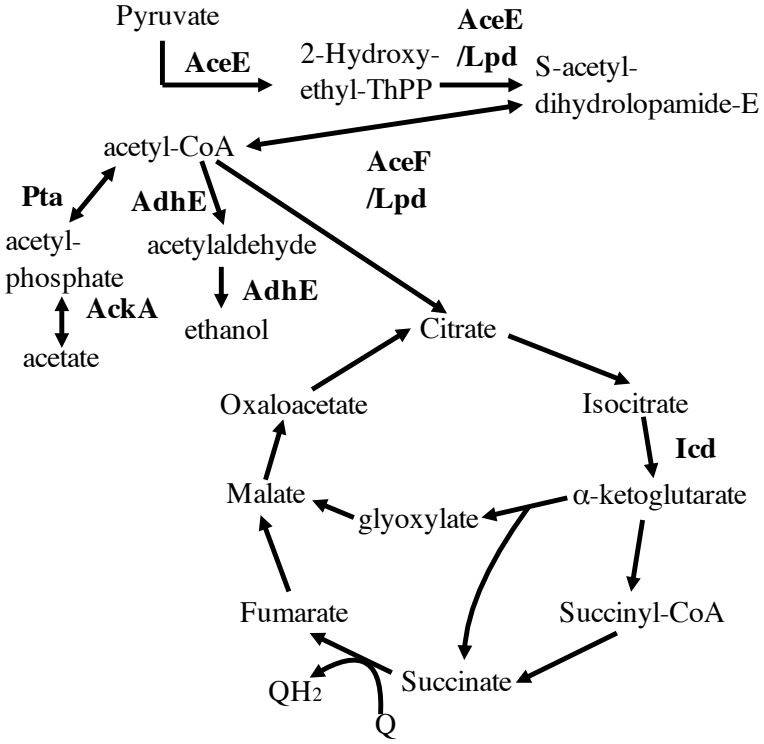

Figure 4. 2 The pyruvate dehydrogenase complex and the citric acid cycle.

Mutants tested for IL-1β release from macrophages in this study are highlighted in bold.

4.2.2 IL-1β release from macrophages infected with AIEC metabolism mutants

AcCoA is a very important metabolite located at a key metabolic node. AcCoA has 3 growth-dependent metabolic fates in *E. coli*: 1) it can be fed into the TCA cycle by reacting with oxaloacetate to form citrate, 2) it can be used to generate ethanol by alcohol dehydrogenase (encoded by *adhE*) and 3) it can be converted to acetate via the high-energy small molecule acetyl-P (AcP) by the action of AckA and Pta (Figure 4. 2). To test the role, if any, of these different fates on IL-1β production, mutants in acetate generation *(*Δ*pta,* Δ*ackA,* and Δ*ackA-pta),* ethanol production (Δ*adhE)* and the TCA cycle (Δ*icd*) were constructed. Macrophages were infected with these mutants and IL-1β levels were measured after 24 h. Interestingly, only mutants in the Pta-AckA pathway had reduced IL-1β release compared to the wild type suggesting that this pathway is important in stimulating a full IL-1β response in macrophages (see Figure 4. 3a).

4.2.3 Replication of metabolism mutants in macrophages

Since a hallmark of AIEC is the ability to replicate in macrophages, intracellular bacterial load after 6 h of infection, a time which previously found to be point of maximum replication by the wild type strain, was also measured (Figure 4. 3b). All mutants tested, except the Δ*icd* mutant, had a significantly reduced intracellular load compared to the wild type including the Δ*adhE* mutant that results in higher IL-1β release than the wild type cell following infection. Therefore, despite significantly reduced bacterial load, *ΔadhE* can still induce a robust IL-1 response suggesting that it is not bacterial load *per se* but rather the metabolic state of HM605 that is important for inducing IL-1β release.

4.2.4 Organic acid production by glycolysis mutants

During growth in glucose, *E. coli* dissimilates acetate forming ATP and the bacteria utilise a branched form of the TCA cycle that does not generate ATP. This aerobic fermentation is called the Crabtree effect. In addition to acetate, other organic acids including formate, lactate, ethanol and succinate are produced to enable the cells to

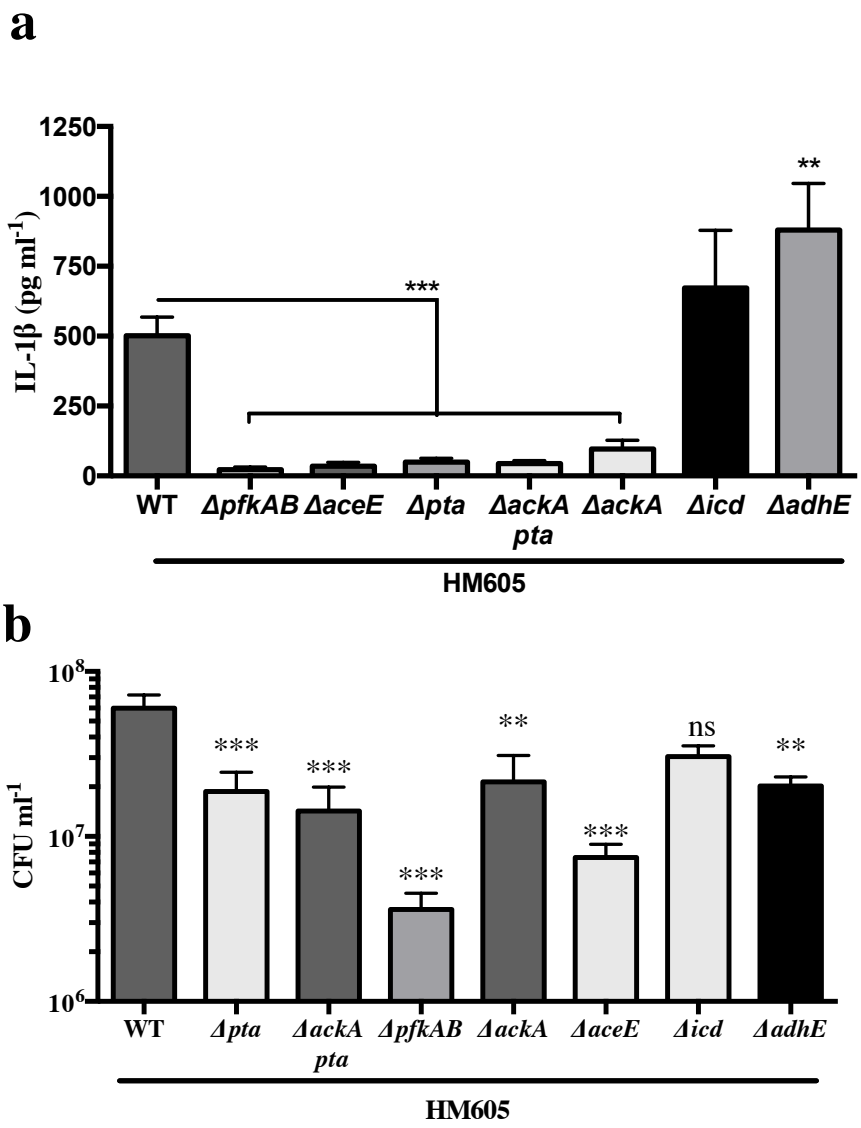

Figure 4. 3 Mutants in acetate production induce low levels of IL-1β release from macrophages

a) IL-1β released from macrophages infected with HM605 mutants in acetate production *** $P < 0.01$ as determined by one-way ANOVA comparing mutants to wild type with Dunnett's post test. **b)** Bacterial load in infected macrophages at 6 h post gentimicin treatment. ** $P < 0.01$, *** $P < 0.001$ determined by one-way ANOVA comparing all mutants to wild type with Dunnett's post test.

recycle NAD^+ that is required during the conversion of glyceraldehyde 3-phosphate to 1, 3,-bisphospho-D-glycerate. Succinate is produced from phosphoenolpyruvate (PEP), lactate and formate are produced from pyruvate whilst ethanol is produced from AcCoA (Figure 4. 4f). Therefore to determine the mode of growth undertaken by the metabolic mutants of HM605, the production of acetate, formate, lactate, succinate and ethanol was measured in the Δ*pfkAB*, Δ*aceE*, Δ*pta*, Δ*ackA*, Δ*ackA-pta*, Δ*adhE* and Δ*icd* mutants following overnight growth in high glucose DMEM using High Performance Liquid Chromatography (HPLC) (Figure 4. 4a-e). Wild type HM605 produced about 17 mM acetate, suggesting that cells are experiencing the Crabtree effect and generating ATP by means of acetate dissimilation (Figure 4. 4). Wild type HM605 produced very low levels of succinate. Succinate production results in one less ATP molecule because PEP is not converted to pyruvate, thus it is unlikely that succinate is used to recycle NAD^+ when all other NAD^+ recycling pathways are functional. Moreover, HM605 produces about 50 mM ethanol, 18 mM lactate and 10 mM formate suggesting that pyruvate and AcCoA are used by HM605 to regenerate NAD+ under these growth conditions (Figure 4. 4).

Mutants in the Pta-AckA pathway (Δ*pta,* Δ*ackA,* Δ*ackA-pta*) all had similar profiles of organic acid production and each mutant, as expected, produced significantly less acetate than the wild type ((Figure 4. 4e). The Δ*ackA* mutant did produce more acetate than both the Δ*pta* and Δ*ackA-pta* mutants but this has previously been reported during high glucose batch fermentation (Wolfe, 2005). Furthermore, the small amounts of acetate produced by the Δ*pta* and Δ*ackA-ackA* mutants may arise through the formation of acetate directly from pyruvate by PoxB. However, a Δ*poxB-aceE* double mutant does not produce less acetate than an *aceE* mutant suggesting that there is an alternative acetate production mechanism that does not use AcCoA (data not shown and (Chang et al., 1999)). The Pta-AckA and AdhE pathways are thought to be linked (Wolfe, 2005) and the production of ethanol by all Pta-AckA pathway mutants is significantly decreased compared to wild type (Figure 4. 4b). Interestingly the Δ*ackA* mutant has significantly reduced ethanol production compared to the Δ*pta* and the Δ*ackA-pta* mutants.

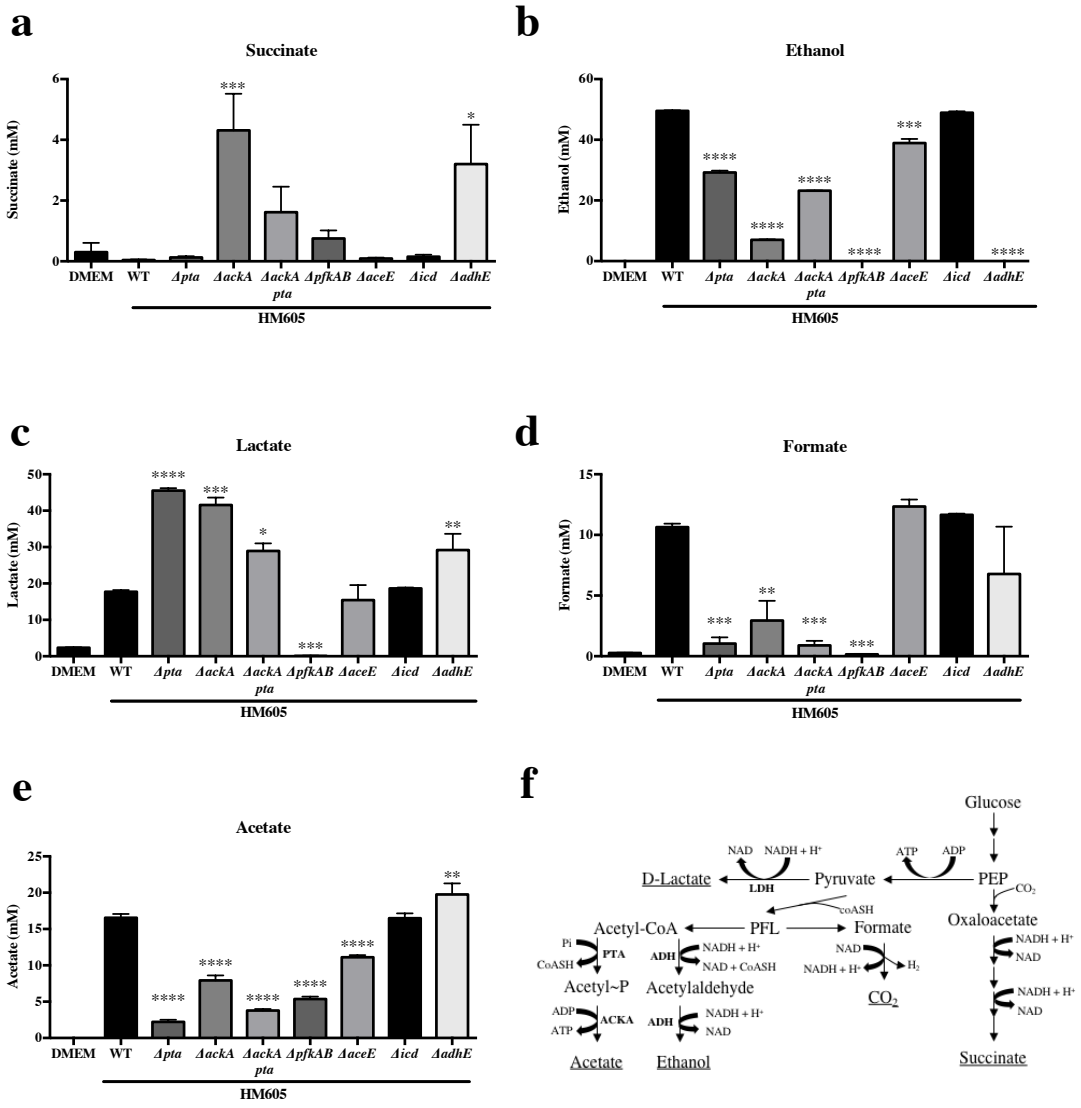

Figure 4. 4 Hexose sugars and organic acid production by HM605 and its metabolism mutants.

a-e) Organic acids indicated, produced by HM605 grown in high glucose DMEM for 24 h at 37°C were measured by HPLC. Data shown is mean±SEM of 3 independent experiments. ** $P < 0.01$, **** $P < 0.0001$ determined by one-way ANOVA comparing mutant strains to wild type HM605 with Dunnett's post test. **(f)** Mixed acid fermentation of glucose in *E. coli*

Formate production is significantly reduced in mutants in the Pta-AckA pathway suggesting that this pathway is not used to regenerate NAD^+ in these mutants (Figure 4.4d). Moreover, there was a significant increase in lactate production in all Pta-AckA pathway mutants compared to wild-type suggesting that, under the growth conditions used here, the production of lactate is the primary driver of NAD^+ regeneration (Figure 4. 4c). Succinate production is significantly increased in the Δ*ackA* mutant compared to the wild type and Δ*pta* mutant suggesting that succinate production may compensate for the decreased ethanol production in this mutant and may be used to recycle NAD^+ (Figure 4. 4a). Importantly, succinate, like ethanol, produces 2 NAD^+ for every one PEP enhancing its NAD^+-recycling capabilities. Interestingly the Δ*adhE* mutant had increased acetate production compared to wild type cells suggesting that excess acetate is produced to recycle CoASH when ethanol production is unavailable (Figure 4. 4e). The Δ*adhE* mutant appears to compensate for the absence of NAD^+ recycling by ethanol production by producing significantly more succinate and lactate than wild type HM605 (Figure 4. 4a).

The Δ*pfkAB* mutant has significantly reduced lactate and formate production compared to wild type most likely due to reduced activity of glycolysis and therefore reduced pyruvate production (Figure 4. 4c-d). Ethanol and acetate production are also significantly reduced in the Δ*pfkAB* mutant, most likely due to the decreased levels of AcCoA production in this strain (Figure 4. 4b and e). The PDHC mutant, Δ*aceE,* had significantly reduced ethanol and acetate production again likely to be due to reduced AcCoA production (Figure 4. 4b and e). However, the Δ*aceE* mutant has similar levels of formate and lactate to wild type HM605 suggesting that the metabolic effects of this mutant are downstream from pyruvate production (Figure 4. 4a, c, d). Finally, the TCA cycle mutant, *Δicd*, has the same organic acid production profile as the wild type and this is consistent with a functioning glycolysis pathway (Figure 4. 4a-e).

The above results show that mutants in the Pta-AckA pathway have reduced ethanol, formate and acetate production and furthermore these mutants also have reduced IL-1β production. This suggests that either ethanol, formate or acetate may be a driver of IL-1β production. However, an Δ*aceE* mutant has normal formate levels but reduced IL-1β indicating formate is unlikely to be responsible for IL-1β induction.

Additionally, the Δ*adhE* mutant does not produce any detectible ethanol but has increased IL-1β level and increased acetate level. These results suggest that acetate is a likely to be a driver of IL-1β release in macrophages.

4.2.5 Acetate production during macrophages infection

Acetate production *in vitro* appears to be associated with increased levels of IL-1β production. Therefore, acetate was measured in the supernatants of macrophages infected with HM605 and the metabolism mutants. Importantly macrophages do not produce acetate and therefore any acetate that is detected in culture supernatants must be derived from bacterial metabolism. As expected no acetate was detected in the culture supernatants of uninfected macrophages (Figure 4. 5a). In addition, similar to what was observed *in vitro*, acetate levels in the culture supernatants were significantly lower in macrophages infected with mutants that are deficient in the acetate production (i.e. *ΔaceE, ΔackA, Δpta* and *ΔackA-pta*) compared to macrophages infected with the wild type (Figure 4. 5a). Supernatants from macrophages infected with strains that induced the highest levels of IL-1β, Δ*adhE* and Δ*icd*, had similar acetate levels as the wild type (Figure 4. 5a). Nonetheless, there is a strong correlation between the amount of acetate detected in the culture supernatants and the level of IL-1β detected in the same supernatants (Figure 4. 5b, $R^2 = 0.8584$, $P = 0.0003$).

These results suggest that the ability to produce acetate may be a contributory factor to the induction of IL-1β release during macrophages infection.

4.2.6 Production of acetate in gentamicin-treated persister cells

The acetate detected in cell culture supernatants must be produced by bacteria as uninfected macrophages do not produce any acetate as they do not possess the appropriate biochemical pathway (Berg *et al*., 2002; O'Neill & Hardie, 2013). Moreover, the macrophages are incubated in culture medium containing gentamicin in order to kill any extracellular bacteria. Nonetheless, it is well established that bacteria can persist in the presence of antibiotics as a non-replicative persister.

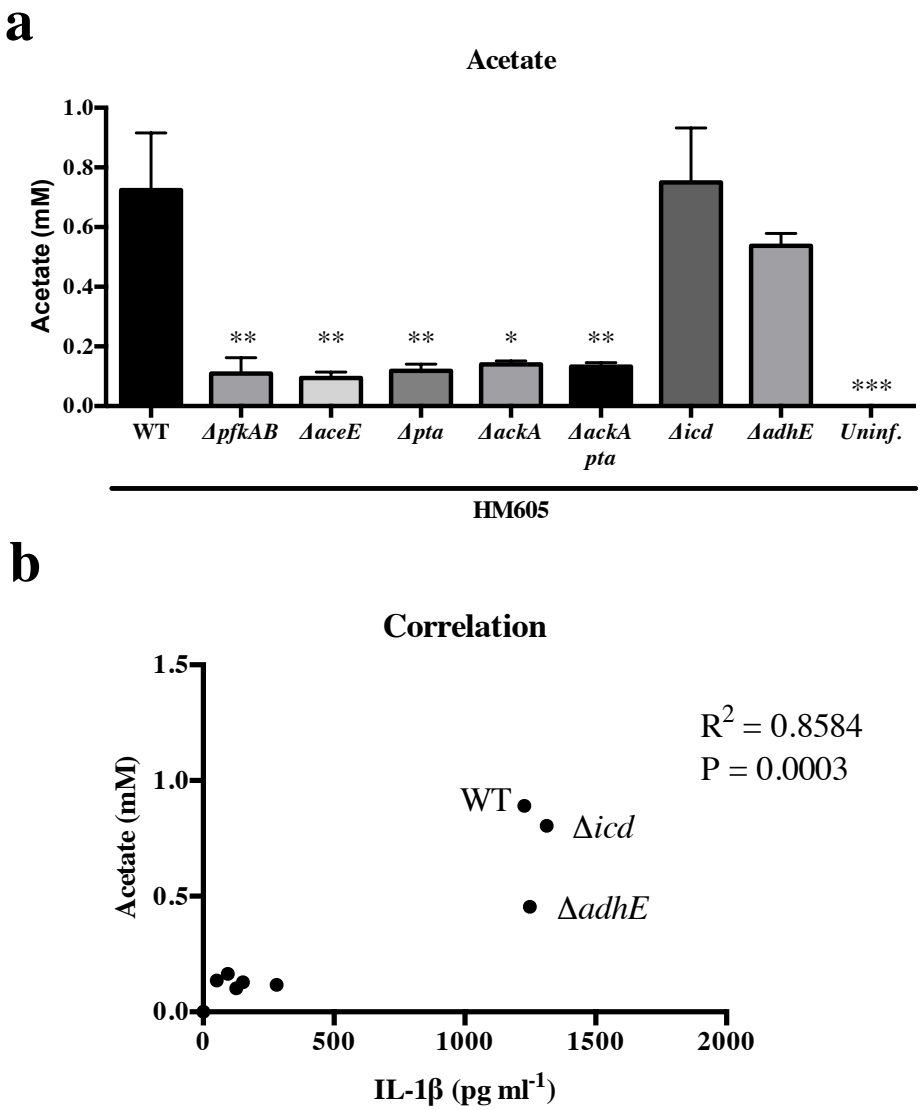

Figure 4. 5 Detection of acetate in cell culture media.

a) Acetate in supernatant of HM605 infected macrophages 24 h post infection were measured by HPLC. Data shown is mean±SEM of 3 independent experiments, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ determined by one-way ANOVA comparing mutant strains to wild type HM605 with Dunnett's post test. **(b)** Pearson correlation coefficient analysis of IL-1β and acetate detected in supernatants of macrophages infected with strains listed in (a).

Therefore, in order to determine whether persister cells may be responsible for the acetate produced during the gentamicin-protection assays, DMEM was inoculated with HM605 at the same CFU ml^{-1} normally used to infect macrophages, i.e. 10^6, and also with 10 and 100 fold higher bacterial numbers. Bacteria were then incubated for 24 h in cell culture conditions in the presence of 50 μg ml^{-1} gentamicin. At the normal inoculation levels of 10^6 CFU ml^{-1}, no acetate was detected (Figure 4. 6a). However, when 10^7 and 10^8 CFU ml^{-1} were used as an inoculation there was increasing levels of acetate detected and this corresponded with a decrease in the concentration of glucose in the DMEM (Figure 4. 6b-c). These results suggest that at a high initial inoculum, HM605 does form metabolically-active persister cells in gentamicin-supplemented media. As expected, a mutant defective in acetate production, Δ*aceE*, did not show any acetate production when inoculated at 10^8 CFU ml^{-1}, confirming that the acetate has a bacterial origin (Figure 4. 6c). Therefore, when present at sufficiently high cell densities HM605 can metabolise glucose to acetate even in the presence of gentamicin and preliminary studies suggest that, when present in sufficiently high densities, HM605 can form a very low number of persister cells (H. Byrne, unpublished data). Nonetheless, at the inoculum level used to infect macrophages there is no measureable acetate production. Thus, under the conditions used in these experiments, it is highly likely that the acetate detected in the infected macrophages culture supernatants is originating from intracellular bacteria although this does remain to be confirmed.

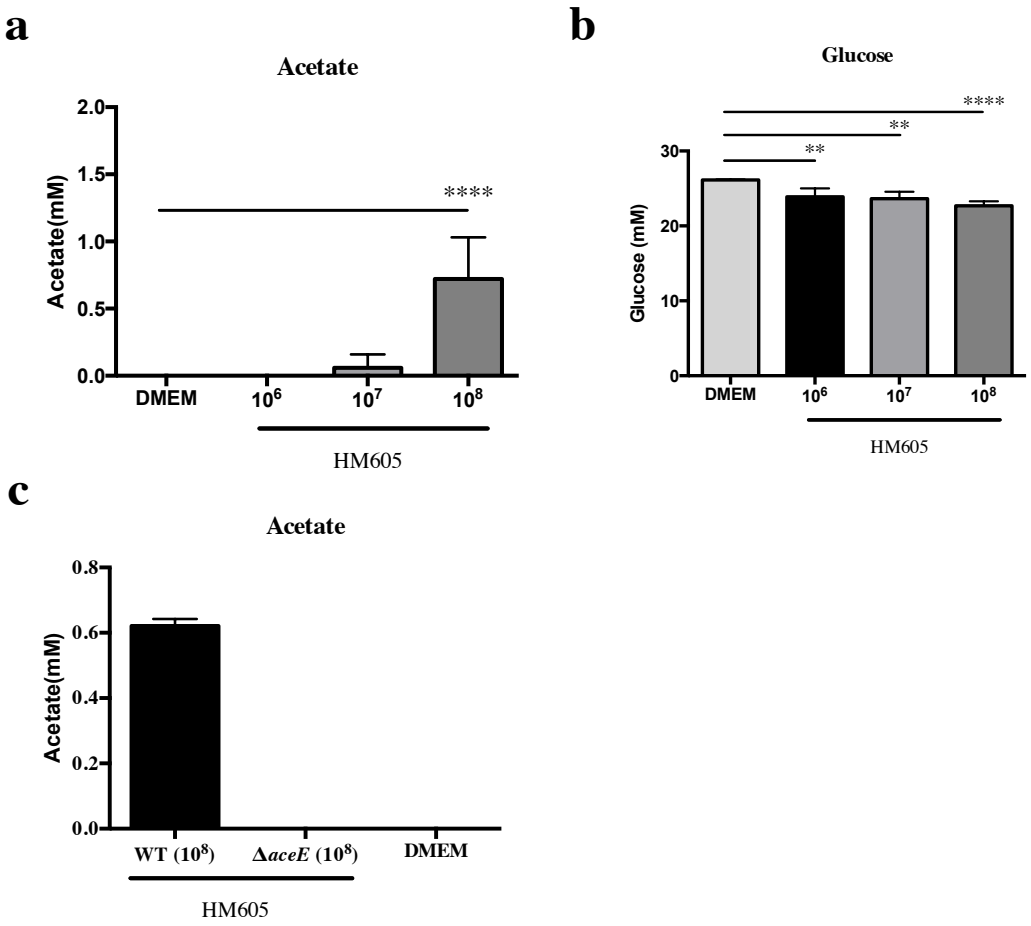

Figure 4. 6 Gentamicin treatment of HM605 results in formation of metabolically active persister cells.

a-b) HM605 was inoculated into gentamicin supplemented high glucose DMEM at CFU ml^{-1} indicated and incubated for 24 h at 37°C/5% CO_2. Organic acids were detected by HLPC. Data shown is mean±SEM of 3 independent experiments, ** $P <$ 0.01, **** $P <$ 0.0001 determined by one-way ANOVA comparing bacterial supernatants to sterile DMEM with Dunnett's post test. **c)** Acetate produced by wild type HM605 and Δ*aceE* mutant inoculated at 10^8 CFU ml^{-1} in high glucose DMEM and incubated for 24hrs at 37°C/5% CO4. Data shown is mean ±SEM of 3 independent experiments. Note: samples in **(c)** acquired by H. Byrne under the supervision of I. O'Neill

4.2.7 Effects of altered Acetyl Co-enzyme A pool in *E. coli*

This study has shown that mutants in acetate production result in decreased production of IL-1β from infected macrophages. However, deletion mutations in key central metabolism enzymes are likely to affect concentrations of other key metabolic molecules such as AcCoA. Growth on glucose media leads to an accumulation of AcCoA (Chohnan et al., 1998) and thus many of the mutants described here would also have altered AcCoA pools. The Δ*pfkAB* mutant does have a reduced level of AcCoA (E Smith, unpublished data) and mutants in acetate (Δ*pta*, Δ*ackA*, Δ*ackA-pta*) production would be expected to alter AcCoA consumption as acetate production is used to regenerate CoASH pools during growth on glucose. AcCoA sits at a crossroads of metabolism and has many metabolic fates (Figure 4. 7). It functions as an intermediate molecule that can transfer acetyl groups between molecules and the

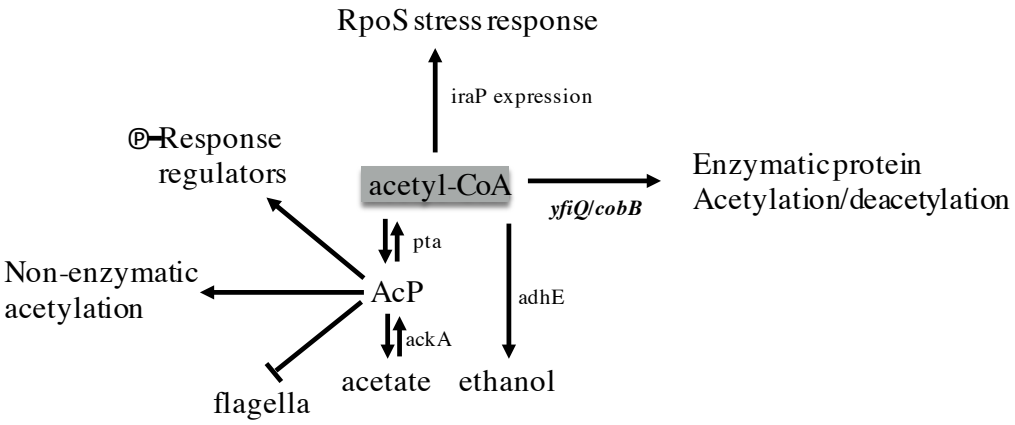

Figure 4. 7 Influence of acetyl-CoA on *E. coli* physiology

Acetyl-CoA is an important metabolite that has multiple phenotypic effects on *E. coli* (see main text for details)

AcCoA pool is tightly regulated (Kuhn *et al*., 2014; Weinert *et al*., 2013; Wolfe, 2005). Recently it has been shown that AcCoA can act as an acetyl donor for the specific enzymatic acetylation of proteins through the action of the lysine acetylase YfiQ (de Diego Puente et al., 2015). AcCoA is also required for the production of AcP and this molecule has been shown to globally acetylate proteins and AcP can also provide phosphoryl groups to response regulators of two component pathways (Wolfe et al., 2008).

4.2.7.1 Acetyl-phosphate dependent flagella biosynthesis

AcP is a key signalling molecule that is 1) able to donate a phosphate group to some response regulators (RR) and 2) non-enzymatically acetylate proteins (Weinert et al., 2013; Wolfe et al., 2008). The response regulator RcsB can use AcP as a phosphate donor and therefore this regulator controls *cps* expression and flagellar biosynthesis in a AcP-dependent manner. Cells that accumulate AcP (Δ*ackA*) have increased activity of the *cps* promoter indicating increased activity of the Rcs phosphorelay. Moreover, Δ*ackA* mutants are non-motile as RcsB activation by AcP leads to inhibition of the master flagellum activator *flhDC* (Fredericks et al., 2006). A number of mutants we have described that induce lower levels of IL-1β production in macrophages are also predicted to have altered AcP pools. The Δ*ackA-pta* mutant cannot make AcP, the Δ*ackA* mutant is predicted to accumulate AcP and the Δ*aceE* mutant is predicted to have reduced AcP. Therefore, these mutants are likely to affect flagellar biosynthesis and, thus, motility. Flagella have been shown to be a trigger for inflammasome activation (Miao et al., 2006) and therefore we wanted to eliminate altered flagella production as an explanation for the reduced level of IL-1β production observed in the mutants outlined in this study. As expected the Δ*ackA* mutant was non-motile whilst the Δ*ackA-pta* double knockout exhibited reduced motility compared to the wild type (although this may be due to the reduced growth seen in this mutant (Figure 4. 8a-b)). Motility in the Δ*ackA* mutant could be fully rescued by complementation with a plasmid expressing *ackA*. Interestingly the Δ*aceE* mutant was slightly more motile than the wild type (Figure 4. 8a-b). Therefore, since the Δ*aceE* mutant has a significantly reduced IL-1β response in macrophages compared to the

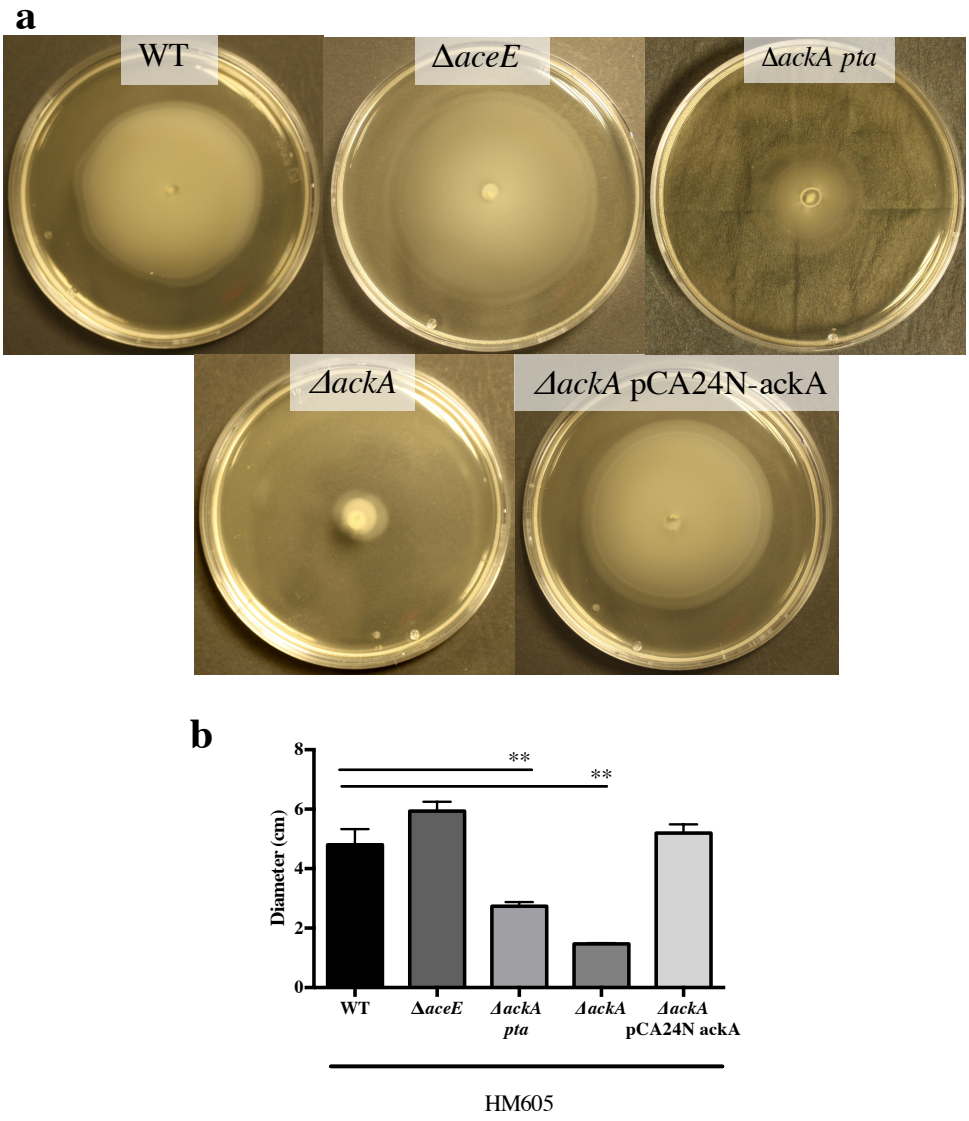

Figure 4. 8 Motility of HM605 and its metabolism mutants.

a) Representative photographs of HM605 and indicated mutants grown on 0.25% LB agar overnight at 37°C. **b)** Quantification of motility by measuring diameter of swarm colony. Data shown is mean±SEM of 3 independent experiments, ** $P < 0.01$ determined by one-way ANOVA comparing all strains to the wild type with Dunnett's post test.

wild type but has similar motility, it is unlikely that flagella biosynthesis is a contributing factor to the observed reduced IL-1β phenotype.

4.2.7.2 RpoS stress response

A reduction in AcCoA results in induction of the σ^S stress response and, an *aceE* mutant in *E. coli* was shown to have increased levels of the σ^S sigma factor (Battesti et al., 2015). This increase in σ^S levels was shown to be dependent on σ^S translation and stabilisation (Battesti et al., 2015). Moreover, mutants in Δ*ackA-pta* and isogenic Δ*ackA* mutants have increased levels of expression of σ^S-dependent genes, *hdeA and hdeB* indicating that mutants in Pta-AckA pathway have increased levels of σ^S (Wolfe et al., 2003). Stabilisation of σ^S is dependent on the increased transcription of the anti-adaptor IraP and Δ*aceE* mutants express significantly more IraP than wild type controls (Battesti et al., 2015). IraP is one of three anti-adaptors (IraP, IraM and IraD) that have increased expression in response to different stress conditions (Bougdour et al., 2008). These anti-adaptors interact with Rss to stabilise σ^S and prevent its degradation (Battesti et al., 2011). Previous studies by our group have shown by metabolomic analysis that when the Δ*pfkAB* mutant is grown in DMEM + glucose it has reduced levels of AcCoA (E. Smith, unpublished data). Moreover, AcCoA accumulates during growth on glucose and it is presumed that a Δ*ackA-pta* mutant would further accumulate AcCoA (since there would be a reduced flux from AcCoA to acetate). Thus, a pUA66-iraP GFP reporter was used to measure changes in activity of the *iraP* promoter in the Δ*ackA-pta* and Δ*pfkAB* mutants. *E. coli* K-12 BW25113 Δ*aceE* (from the KEIO library (Baba et al., 2006)) was used as a positive control. In agreement with previous studies the BW25113 Δ*aceE* mutant had increased *iraP* promoter activity compared to a wild type control (Figure 4. 9a). Expression of *iraP* was turned on during exponential phase and was switched off before entry into stationary phase. In contrast to the BW25113 Δ*aceE* mutant, wild type HM605 had little or no expression at any time during growth (Figure 4. 9a-b). This was as expected as the wild-type HM605 would not be expected to be experiencing any AcCoA-related metabolic stress. However, expression of *iraP* in the HM605 Δ*ackA-pta* double

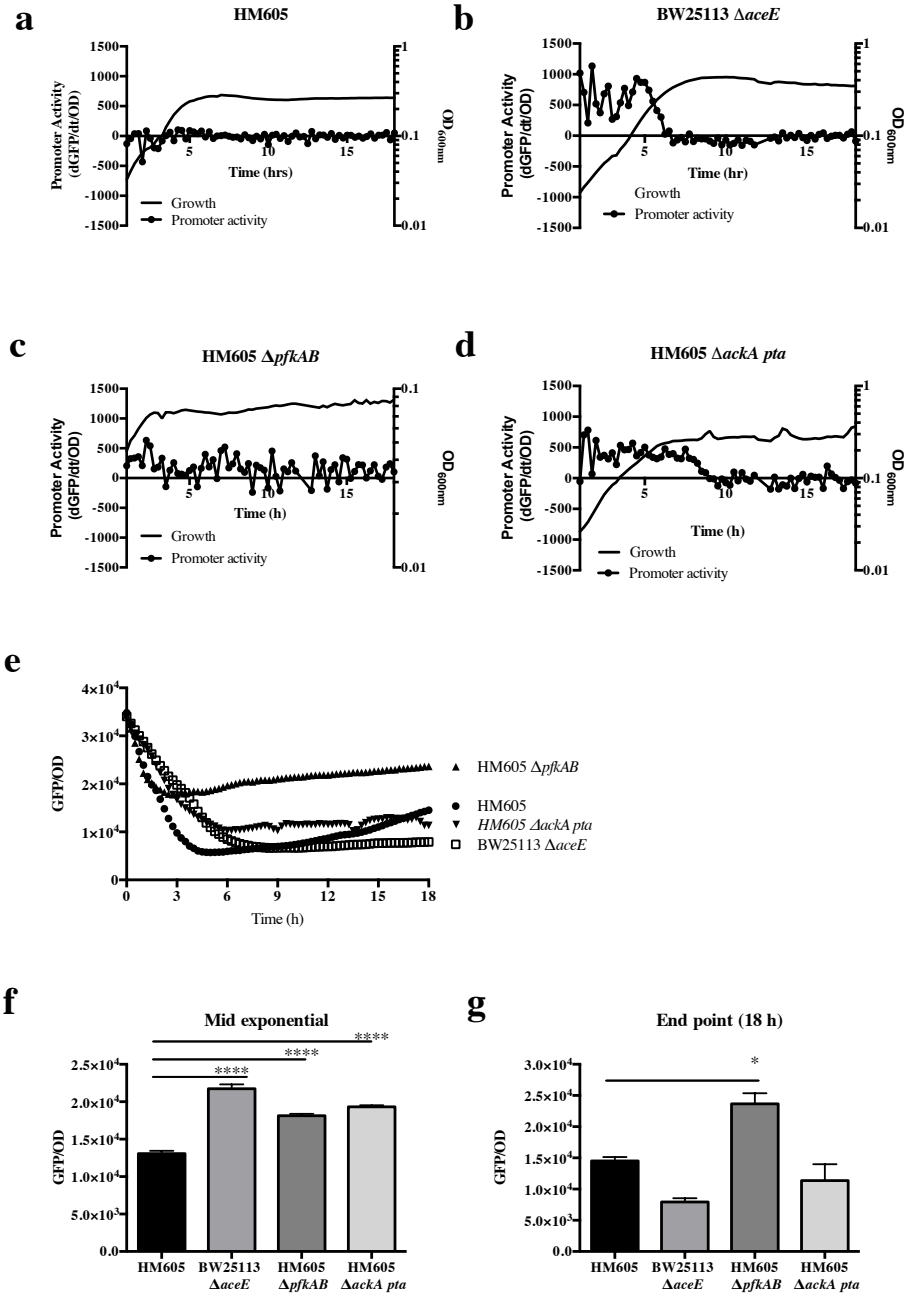

Figure 4. 9 *iraP* expression in acetate and PDH mutants.

(a-d) Promoter activity of *iraP* in strains indicated grown in high glucose DMEM at 37°C. Data shown is mean of 3 independent experiments. **e)** GFP fluorescence of the pUA66-iraP plasmid normalised to growth (GFP/OD). Data shown is mean of 3 independent experiments. **(f)** GFP/OD at mid exponential phase. Data is mean±SEM of expression values from 2-3 h from 3 independent experiments. **g)** GFP/OD at end of experiment. In f and g, data shown is mean ±SEM of 3 independent experiments, * $P< 0.05$, **** $P < 0.0001$ determined by one-way ANOVA comparing all strains to HM605 with Dunnett's post test.

knockout was similar to that observed in the BW25113 Δ*aceE* mutant although *iraP* expression was maintained at high levels into the stationary phase in the ΔackA-pta mutant (Figure 4. 9d). This suggests that the Δ*ackA-pta* mutant is experiencing metabolic stress related to what has been reported in mutants that have reduced levels of AcCoA and is in agreement with previous work showning increase production of members of the σ^s regulon in a Δ*ackA-pta* mutant (Kirkpatrick et al., 2001; Wolfe et al., 2003). The Δ*pfkAB* mutant had a very noisy expression profile throughout growth although the level of expression was clearly higher than what was observed in the HM605 wild-type (Figure 4. 9c). To quantify this difference we analysed GFP fluorescence, normalised to growth, at mid exponential phase (average GFP/OD from 2-3 h). The level of *iraP* expression was clearly significantly elevated in all mutants compared to the wild type (Figure 4. 9e) suggesting that all the Δ*ackA-pta* and Δ*pfkAB* mutants are undergoing a metabolic stress that is resulting in increased *iraP* transcription.

Whist the Δ*pfkAB* mutant and presumably the Δ*aceE* mutant have decreased AcCoA levels, the Δ*ackA-pta* mutant is expected to accumulate AcCoA as this metabolite cannot be dissimilated as acetate. Therefore, cells that cannot make acCoA (Δ*aceE*) have increased *iraP* expression and cells that presumably accumulate acCoA (ΔackA-pta) also have increased *iraP* expression. One possibility to explain this apparent paradox would be that *iraP* is not sensitive to low AcCoA levels *per se* but rather to the AcCoA/CoASH ratio. Wild type *E. coli* growing on glucose have a high AcCoA/CoASH ratio of 3.6 (Chohnan et al., 1998). A Δ*aceE* mutant is likely to have low AcCoA/CoASH ratio due to low levels of AcCoA. Moreover, the Δ*ackA-pta* mutant would also accumulate AcCoA as it cannot make acetate and thus is likely to have a higher AcCoA/CoASH ratio compared to wild type cells. Thus it is possible that an abnormal AcCoA/CoASH ratio may drive *iraP* expression.

Expression of *iraP* can be turned off during stationary phase in the Δ*aceE* mutant suggesting that the stress inducing it can be alleviated through the rerouting of metabolism. This may explain the expression profile of the Δ*pfkAB* mutant which presumably would also have a low AcCoA/CoASH ratio due to severely reduced flux through glycolysis and low levels of AcCoA. It is possible that the Δ*pfkAB* mutant

cannot alleviate the stress inducing *iraP* expression by rerouting metabolism as it is defective in a key step early in glycolysis.

These results indicate that *iraP* expression is increased in mutants with altered AcCoA/CoASH ratio suggesting a stabilisation of σ^S and induction of the σ^S-mediated stress response in these mutants. The consequences, if any, of this on the interaction between HM605 and the macrophages and/or IL-1β production have not been fully explored.

4.2.7.3 Lysine Acetylation

Reversible lysine acetylation of proteins using AcCoA as the acetyl donor has recently been identified as a key post-translational control mechanism employed by *E. coli* and other bacteria (Hu et al., 2010). The enzyme, protein acetyltransferase (YfiQ/Pat (a homologue of *Salmonella* PatZ)), is the only one of the putative 25 GCN5-like acetyltransferases (GNATs) homologues in *E. coli* that has been identified as a lysine acetyltransferase. Similarly the sirtuin-like deacetylase, CobB, is the only known deacetylase (Zhang et al., 2009). These enzyme function to acetylate and deacetylate proteins, respectively, with the effect of controlling their activity. Protein acetylation can also occur non-enzymatically in an AcP-dependent manner (Kuhn et al., 2014) and CobB deacetylates both YfiQ/Pat and AcP-acetylated proteins (AbouElfetouh et al., 2014). The mutants that have been identified to reduce Il-1β production all effect AcCoA and/or AcP levels. Therefore it was decided to specifically test the effect of changes in global acetylation on IL-1β production during macrophages infection. A mutant in *yfiQ* was constructed and *cobB* from a plasmid was overexpressed in wild type HM605 and macrophages were infected with these strains. The Δ*yfiQ* mutant induced macrophages to produce the same amount of IL-1β production as the wild type strain suggesting that YfiQ catalysed acetylation is not important for this phenotype (see Figure 4. 10a). Interestingly, the CobB-overproducing strain induced significantly less IL-1β and this strain also has a profound reduction in the level of global acetylation (Figure 4. 11a). Moreover, there was less acetate detected in the supernatants of cells infected with *cobB* overproducing strain compared to the wild type or Δ*yfiQ* strains. These results suggest that while enzymatic acetylation by YfiQ

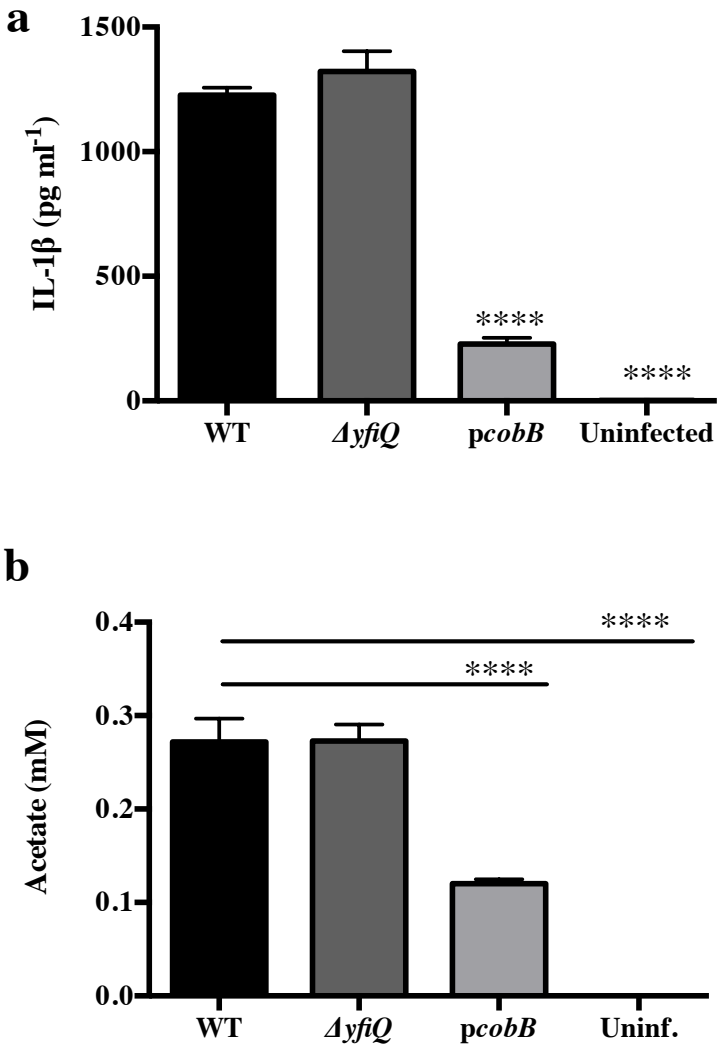

Figure 4. 10 *E. coli* acetylation required to induce full IL-1β response by macrophages.

IL-1β **(a)** and acetate **(b)** detected in supernatant of macrophages infected wirth HM605 (WT), HM605 Δ*yfiQ* or HM605 containing a plasmid expressing *cobB (pcobB)* for 24 h. Data shown is mean±SEM of 3 independent experiments **** $P <$ 0.0001 determined by one-way ANOVA comparing all strains to wild type HM605 with Dunnett's post test.

is dispensable for induction of a complete IL-1β response from macrophages, global acetylation may be important as overproduction of the CobB deacetylase (and reduced acetylation) results in a reduced IL-1β production.

The level of global acetylation in mutant strains that induced lower levels of IL-1β production was measured using immunoblots during growth in high glucose DMEM. The Δ*pfkAB*, Δ*aceE*, Δ*pta* and Δ*ackA-pta* mutant strains all had reduced global acetylation levels most likely due to the reduced levels of AcCoA and/or AcP in these mutants (Figure 4. 11a). The Δ*ackA-pta* mutant showed the most profound reduction in acetylation and this was equivalent to what was observed in the pCobB overexpressing cells. On the other hand, the Δ*ackA* mutant exhibited a much greater level of global acetylation. Increase acetylation in the Δ*ackA* mutant is most likely due to an accumulation of AcP leading to increase AcP-dependent acetylation as reported elsewhere (Kuhn et al., 2014; Weinert et al., 2013). We have shown above that Δ*ackA-pta* double mutant has reduced global acetylation and also has a reduced IL-1β response during macrophages infection. In addition, accumulation of AcP by the Δ*ackA* mutant also leads to increased acetylation but also a reduced IL-1β response. Thus changes in global acetylation levels in the cell, whether an increase or a decrease, may affect the ability of HM605 to elicit a full IL-1β response in macrophages.

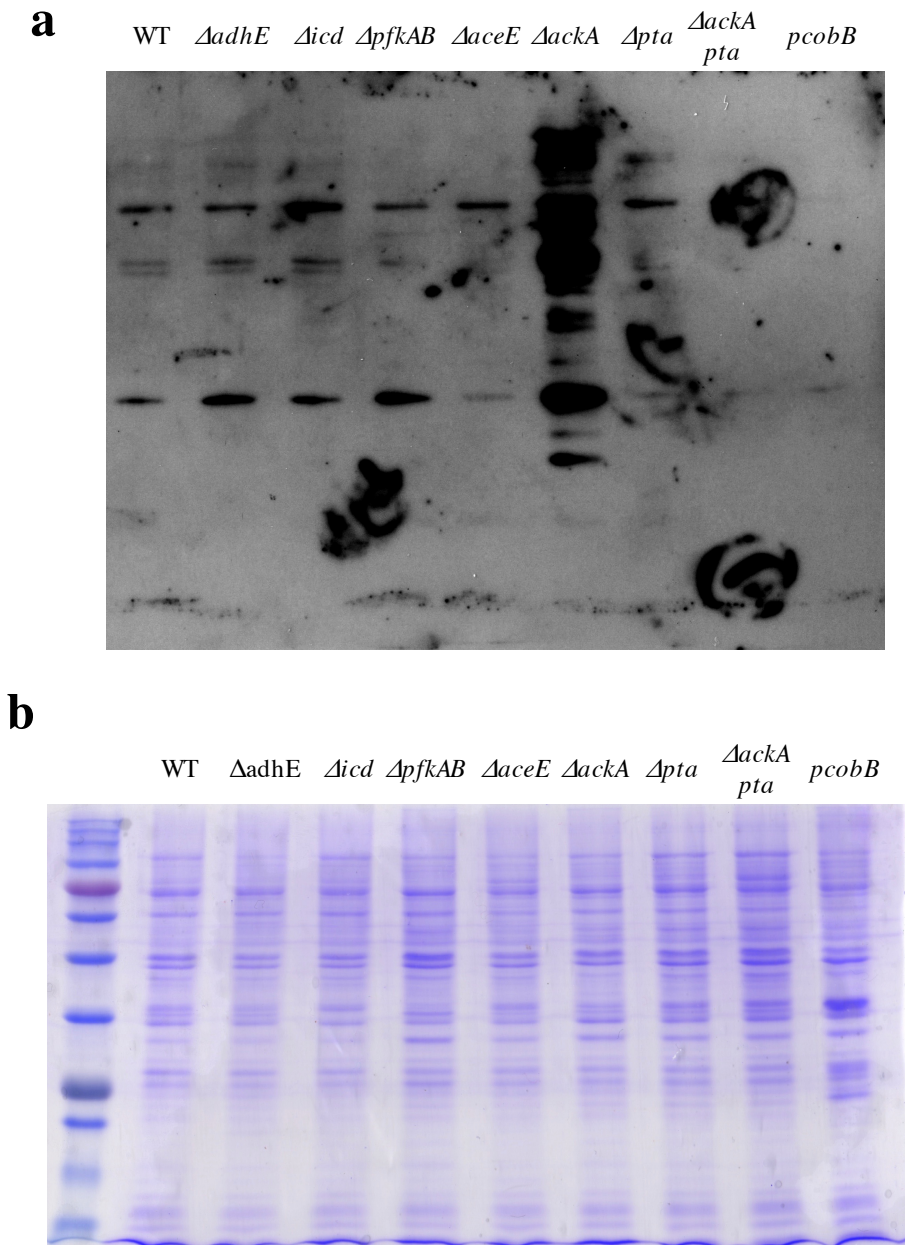

Figure 4. 11 Lysine acetylation in *E. coli*.

a) Anti-acetyllysine western immunoblot of HM605 strains indicated grown for 24 h at 37°C in high glucose DMEM (see Chapter 2 for experimental details). **b)** SDS-PAGE loading control of samples in **(a)**.

4.2.8 Effect of external addition of external acetate on IL-1β production

This work has established that acetate production by HM605 residing in the macrophages is linked to IL-1β production. Therefore, mutants in acetate production induce lower levels of IL-1β production from infected macrophages. One possibility is that the presence of acetate is detected by the macrophages and this increases the production of IL-1β. To test this, acetate was added to the culture medium of macrophages infected with acetate-deficient mutants. To avoid any effects caused by a change in external pH, sodium acetate was added to these cells instead of acetic acid. Sodium acetate has the same pKa as acetic acid and thus dissociates into the acetate anion and Na^+ at neutral pH. As seen in Figure 4. 12a there was no effect of external acetate on IL-1β response by macrophages. Sodium acetate in its dissociated form will not diffuse across membranes thereby it must be actively transported into the cytoplasm. The monocarboxylate transporter (MCT) transporter family actively transport SCFA across cell membranes and macrophages predominantly express MCT 4 that transports lactate out of the cell but there is little evidence that MCTs transport SCFA into macrophages (Adijanto & Philp, 2012; Tan *et al*., 2015). Furthermore, MCT4 has the lowest affinity for acetate of all MCTs (Chang et al., 2014; Moschen et al., 2012). Therefore, in this experiment it seems likely that acetate remained outside of the macrophages. This suggests that intracellular production of acetate by HM605 may be important for driving a complete IL-1β response during macrophages infection.

Acetate production would be expected to result in an increase in $[H^+]$ (and a decrease in pH) and there is evidence that acidification of the external environment can potentiate the inflammasome response (Rajamaki et al., 2013). Therefore, the effect of buffering the external culture medium on IL-1β production by infected macrophages
was tested. As can be seen in Figure 4. 12c infections in HEPES-buffered media showed similar results to standard DMEM and macrophages infected with wild type HM605 produced more IL-1β than infection with mutants in acetate production (Figure 4. 12c).

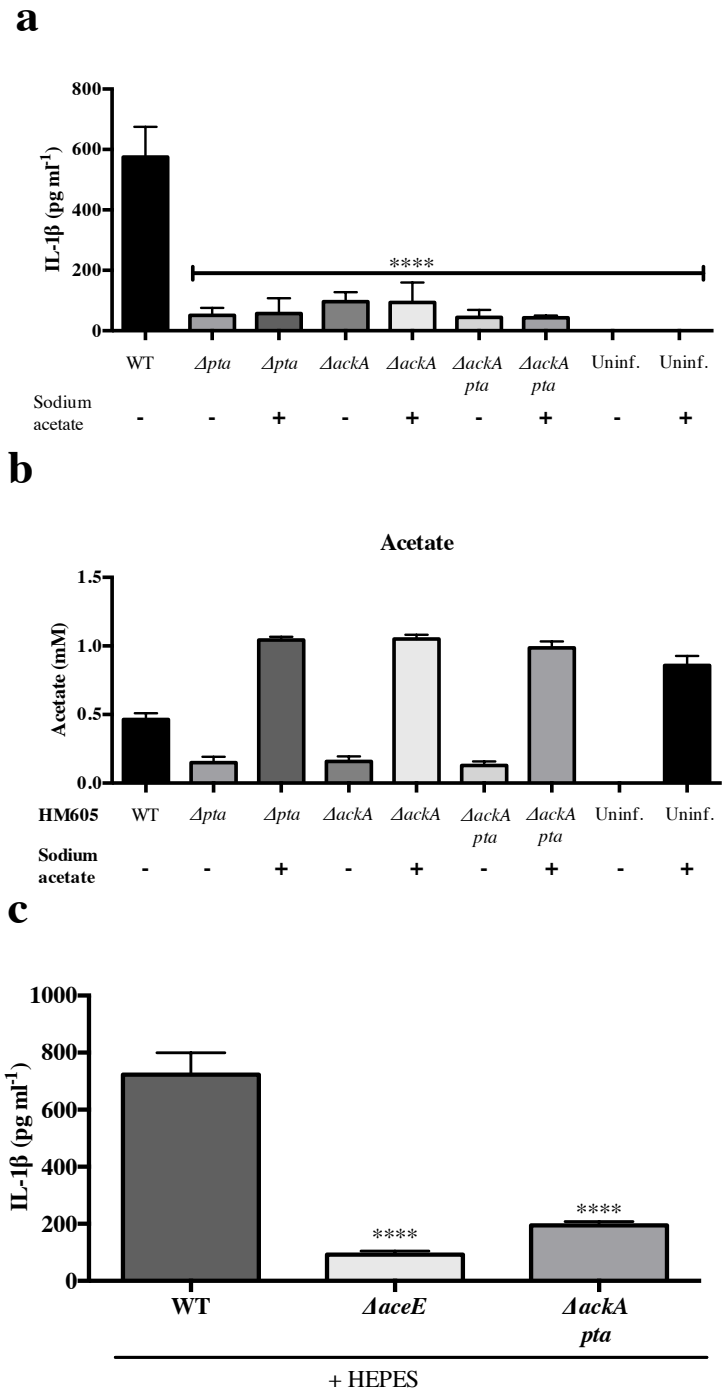

Figure 4. 12 External acetate or buffering does not affect IL-1β induction by HM605.

IL-1β **(a)** and acetate **(b)** detected in supernatants of macrophages infected in the presence or absence of 1 mM sodium acetate for 24 h. Data shown is mean ±SEM of 3 independent experiments, **** $P < 0.0001$ determined by one-way ANOVA comparing all mutants to wild type HM605 with Dunnett's post test. **c)** IL-1β detected in supernatants of macrophages infected HM605 (WT), HM605 Δ*aceE* and HM605 Δ*ackA-pta* in the HEPES buffered medium for 24 h. Data shown is mean ±SEM of 3 independent experiments, **** $P < 0.0001$ determined by one-way ANOVA comparing all mutants to wild type HM605 with Dunnett's post test.

Therefore, acidosis is not responsible for the differential levels of IL-1β production from macrophages infected with wild-type and acetate-deficient HM605.

4.2.9 Expression of the *ackA-pta* operon during intracellular growth

Acetate production in *E. coli* is dependent on the expression of *ackA* and *pta* which exist on a single operon where the genes are transcribed together from a single promoter upstream from *ackA* (Kakuda et al., 1994). Results above suggests that acetate production by HM605 in macrophages contributes to IL-1β release. Therefore, expression of the *ackA-pta* operon in macrophages was investigated as a marker of Pta-AckA activity. Initially, the pUA66-derived *ackA* promoter GFP fusion plasmid (pUA66-ackA) (Zaslaver et al., 2006) was transformed in HM605. Initially bacteria were grown in the same medium used to infect cells, DMEM, and measurements of OD_{600} and GFP fluorescence were taken every 15 min for 24 h. Promoter activity was calculated as previously described (Zaslaver et al., 2006). Clearly there was strong promoter activity during early to mid-exponential growth after which there was a rapid decrease in promoter activity followed by little or no promoter activity during stationary phase (Figure 4. 13a). During exponential phase glucose is consumed and acetate dissimilation is catalysed by AckA and Pta. As glucose concentrations begin to drop (usually in mid-exponential phase), there is a switch from acetate dissimilation to acetate assimilation, the so-called 'acetate switch', which is controlled by the expression of acetyl-CoA synthethase *(acs)* (Wolfe, 2005). The *ackA-pta* promoter activity seen here suggests that the acetate switch occurs during these growth conditions but this remains to be confirmed. Acetate production by *E. coli* is affected by external pH (Wolfe, 2005) with acetate production decreasing as pH decreases. The pH of the phagosome is around pH 5 so the expression of the *ackA-pta* operon was tested at this pH. HM605 was grown in minimal medium with glucose as the sole carbon source with and without buffering to pH 5 with MOPS (Figure 4. 13b). There was no effect of pH on growth in this medium and interestingly there was an increase in promoter activity from *E. coli* growing at pH 5. This suggests that the *ackA-pta* operon is expressed during growth on glucose at pH levels that are equivalent to those expected in the phagosomal environment.

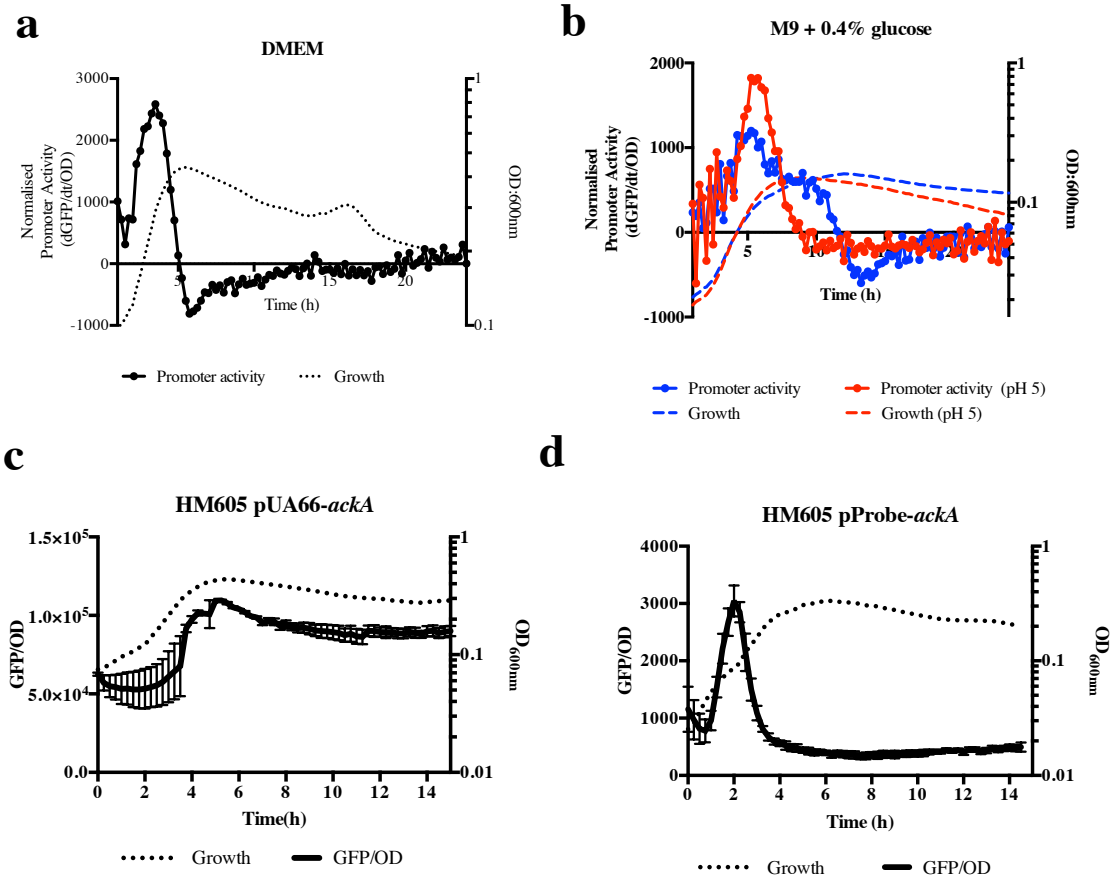

Figure 4. 13 Expression of *ackA-pta* operon *in vitro*.

a) Normalised *ackA-pta* promoter activity of HM605 measured during growth at 37°C in high glucose DMEM and minimal medium + glucose at neutral and low pH. **b)** determined using pUA66-ackA. Data shown is mean of 3 independent experiments. **c-d)** Comparison of GFP fluorescence normalised to OD_{600nm} (GFP/OD) from HM605/pUA66-ackA and HM605/pProbe-ackA grown in DMEM at 37°C. GFP/OD data shown is mean±SEM of 3 independent experiments. Growth measured as absorbance at 600 nm (OD_{600nm}) is shown as mean of 3 independent experiments.

Whilst the pUA66 vector (Zaslaver et al., 2006) is appropriate at investigating *in vitro* promoter activity, it is not as useful for investigating the intracellular expression of genes due to the long lifetime of GFPmut2 (approx. 24 h) (Miller et al., 2000). This becomes problematic when looking at the expression of *ackA-pta* in the macrophages as the infection protocol involves 2 h of incubation in DMEM that contains glucose. Under these conditions it is highly likely that the bacteria will express the *gfp* during the 2 h infection protocol and this *gfp* will persist during intracellular growth (because of the long half-life). Indeed, when GFP fluorescence was normalised to growth *in vitro*, there was an increase in GFP fluorescence that was sustained for over 8 h (Figure 4. 13c). Therefore, the vector pProbe-GFP[LVA] was used as this vector contains an AANDENYALVA tag added to the C-terminal end of wild-type GFP resulting in a GFP variant that has a half life of about 40 min (Miller et al., 2000). This will permit much greater temporal resolution when measuring promoter activity *in vivo*. Therefore the *ackA* promoter region was cloned from pUA66-ackA into pProbe-GFP[LVA] and promoter activity was measured *in vitro* during growth in DMEM. When GFP fluorescence was normalised to growth, the expression profile of *ackA-pta* obtained with pProbe-ackA was almost identical to the normalised promoter activity measured using pUA66-ackA (compare Figure 4. 13a and Figure 4. 13d).

The activity of the ackA promoter was measured in the macrophages using pProbe-ackA. Macrophages were infected with HM605 containing the pProbe-ackA plasmid. Samples were taken and fixed at 0, 3 and 6 h post gentamicin treatment and cells were analysed by confocal fluorescent microscopy (see Figure 4. 13). Fluorescent bacteria were identified within vacuoles in the macrophages at all time points and the fluorescent intensity of these bacteria was heterogeneous, perhaps suggesting differences in the level of *ackA* promoter activity at the single cell level. A control sample with a promoter-less plasmid had no detectable fluorescent bacteria confirming that the signal seen from bacteria with pProbe-ackA was not due to autofluorescence. However, we cannot rule out the possibility that the detected fluorescence is associated with non-replicating cells and several attempts at co-labelling cells with a second DsRed-expressing plasmid were unsuccessful, possibly due to the lack of plasmid selection during infection. Nonetheless, the engineered short half-like of the GFP(LVA) derivative does strongly suggest that expression of *ackA-*

pta occurs within the macrophages and, therefore, the Pta-AckA pathway required for acetate production is turned on in macrophages.

4.2.10 A mutation in acetate production in *Salmonella typhimurium* reduces IL-1β production from infected macrophages.

To test if the intramacrophagic production of acetate was a general pro-inflammatory signal, J774A.1 macrophages were infected with wild type *S. enterica* Typhimurium and isogenic Δ*ackA*, Δ*pta* and Δ*ackA-pta* mutants in the same manner as for *E. coli* and IL-1β production was measured 24 h post infection. As seen in AIEC, the *S.* Tyhphimurium Δ*ackA*, Δ*pta* and Δ*ackA-pta* mutants all had a significantly reduced IL-1β response compared to the wild type strain (Figure 4. 15) suggesting that acetate production is an important intracellular pro-inflammatory signal that is not confined to *E. coli*.

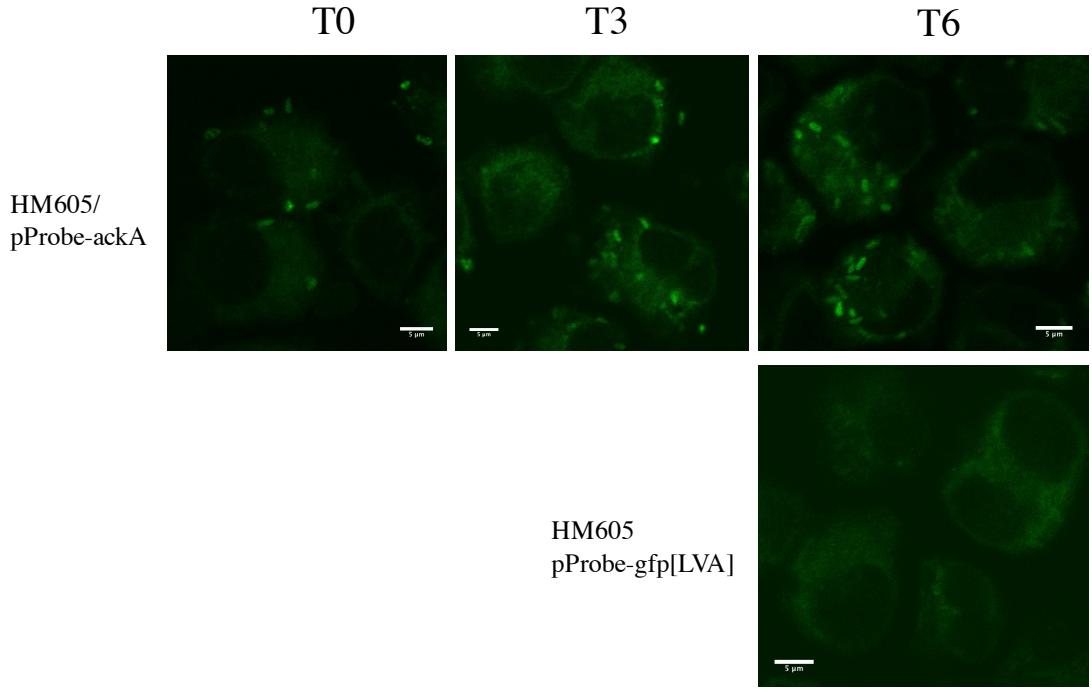

Figure 4. 14 Expression of *ackA-pta* operon *in vivo*.

Micrographs of macrophages infected with HM605/pProbe-ackA or HM605/pProbe-gfp[LVA] for 0, 3 or 6 h (T0, T3, and T6 respectively) were paraformaldehyde fixed and visualised with fluorescent confocal microscopy. Scale bar 5 μm

4.2.11 Effects of *ackA-pta* mutation *in vivo*

Results *in vitro* indicate that the ability of AIEC HM605 to produce acetate may contribute towards production of the pro-inflammatory cytokine, IL-1β, from macrophages. Therefore, it was decided to test whether acetate production may contribute to inflammation *in vivo*. Using a DSS-induced colitis model, mice were treated with either wild type HM605, the Δ*ackA-pta* mutant or PBS. Non-DSS treated mice were used as a control. After 7 days, cecal contents were analysed for bacterial load and short chain fatty acid (SCFA) composition, and plasma TNF-α and IL-1β levels were also measured (Figure 4. 16). There was no difference in the colonisation of non-DSS treated mice with either HM605 or Δ*ackA-pta* mutant suggesting that

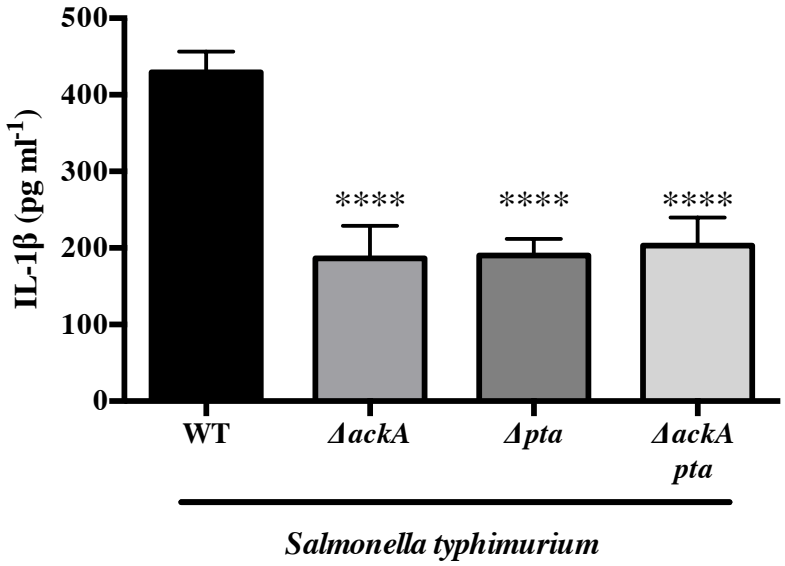

Figure 4. 15 *Salmonella typhimurium* mutants in acetate production induce lower levels of IL-1β release from macrophages.

IL-1β detected in supernatants of macrophages infected with *Salmonella typhimurium* strains indicated for 24 h. Data shown is mean±SEM of 3 independent experiments, **** $P < 0.0001$ determined by one-way ANOVA comparing all strains to wild type with Dunnett's post test.

acetate production is not essential for colonisation (Figure 4. 16a). In DSS-treated mice, however, there was a bloom in HM605 (Figure 4. 16a) which is consistent with published data suggesting that the inflamed gut has a higher presence of nitrate, S-oxides, and N-oxides that *E. coli* can utilise as terminal electron acceptors for anaerobic respiration (Winter et al., 2013). However, this bloom was not observed in DSS-treated mice inoculated with the Δ*ackA-pta* mutant suggesting that this pathway may be important for the observed DSS-associated bloom (Figure 4. 16a). Cecal SCFA were also measured and there was a decrease, though not significant, in acetate in the Δ*ackA-pta* treated mice compared to wild type treated mice in both the DSS-treated and DSS-untreated cohorts (Figure 4. 16b).

Plasma TNF-α was elevated above wild type in mice inoculated with the Δ*ackA-pta* mutant in both the DSS-treated and untreated models (Figure 4. 16c). IL-1β was also increased above wild type in the DSS/Δ*ackA-pta* treated mice (Figure 4. 16d). DSS-treatment is known to result in increased circulating pro-inflammatory cytokines (Alex et al., 2009; Egger et al., 2000) and intestinal SCFA such as butyrate and acetate have been shown to be anti-inflammatory (Chang *et al*., 2014; Tedelind *et al*., 2007; Vinolo *et al*., 2011). Thus it is possible that the lower levels of acetate in the cecum of Δ*ackA-pta* treated mice may contribute to an increase in pro-inflammatory cytokine production after DSS treatment compared to mice treated with wild-type HM605. Since intestinal microbiota derived butyrate is also known to have anti-inflammatory properties, every attempt was made to detect it in this study. However, using the detection conditions that were available, it was not possible to distinguish between butyrate and ethanol. These results, combined with with the differences in IL-1β production observed from infected macrophages, suggest that acetate may have different roles depending on where it is produced. For instance, when acetate is produced in the lumen of the gut (by *E. coli* and other bacteria) it is anti-inflammatory. In contrast when acetate is produced inside a macrophages it appears that this SCFA may be pro-inflammatory.

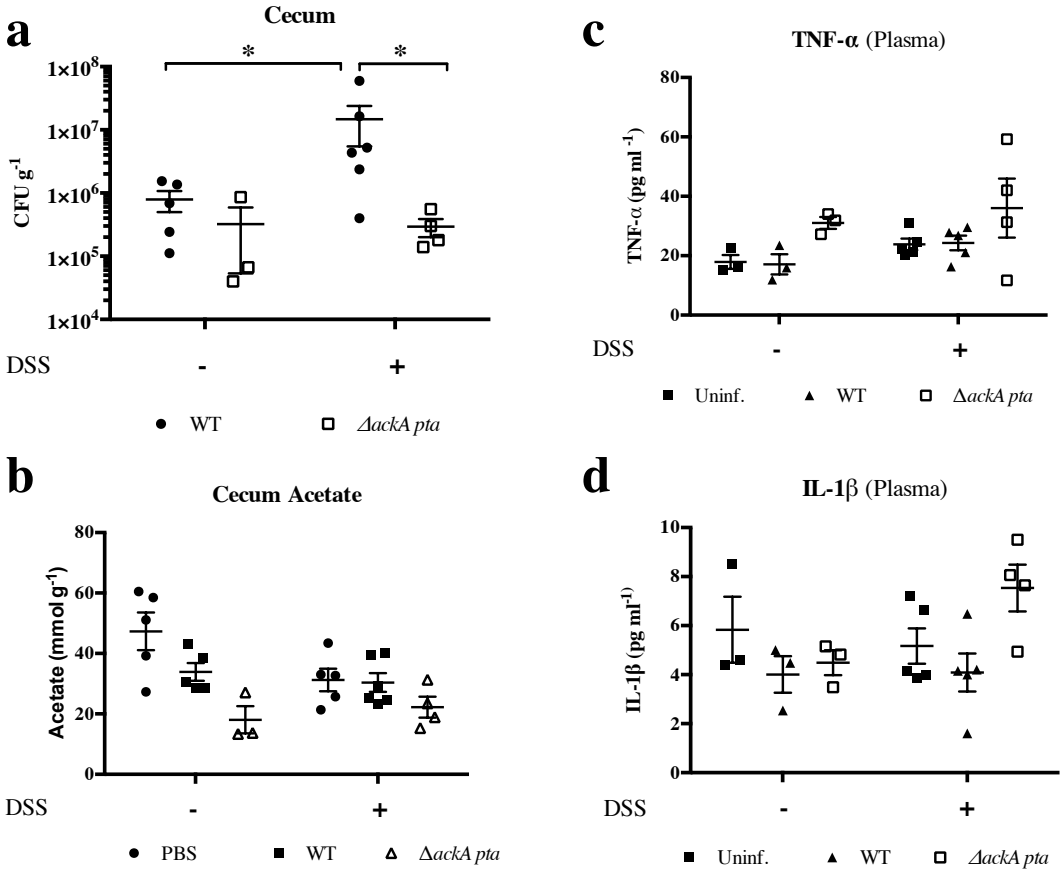

Figure 4. 16 *In vivo* effects of *ackA-pta* mutation.

Mice infected with HM605 or HM605 Δ*ackA-pta* for 7 days without or without DSS treatment. **a)** Cecal bacteria counts at day 7. Data shown is mean±SEM of at least 3 animals. * $P < 0.05$ determined by Kruskal-Wallis test between 2 conditions indicated. **b)** Acetate in cecum sample detected by HPLC. Data shown is mean±SEM of at least 3 animals. **c-d)** Plasma TNF-α and IL-1β detected in ELISA. Data shown is mean ±SEM of at least 3 animals.

4.2.12 Mechanism of AIEC induced inflammatory response

All previous data was obtained through infections of the murine macrophages cell line J774A.1 and this cell line is permissive for HM605 replication (see Chapter 3). Therefore, in order to determine if acetate production affects IL-1β production in other macrophages PMA differentiated human THP-1 macrophages were infected with wild type, Δ*pfkAB* or Δ*ackA-pta* HM605 and IL-1β production was measured after 24 h. Similar to murine macrophages there was a significant decrease in IL-1β released from cells infected with Δ*pfkAB* and Δ*ackA-pta* mutants confirming that the phenotype is not specific to murine macrophages (Figure 4. 17a).

IL-1β release is dependent on the activation of the inflammasome, a large multiprotein complex that activates caspase-1 and thereby stimulates the release of IL-1β and pyroptosis, a form of inflammatory cell death. Previous work carried out by our group found that both wild type and Δ*pfkAB* HM605 elicited an identical macrophages transcriptional response to infection. Importantly, the expression of *Il1b* was increased almost 8.5 fold ($P < 0.0001$) in HM605 infected macrophages and there was no significant difference between HM605 and HM605 Δ*pfkAB* infected cells indicating that both strains induce a similar increase in *Il1b* transcription (J. Catchpole, unpublished data). Therefore, any differences seen in IL-1β release is not due due to a difference in *Il1b* transcription but mostly likely due to differences in the activation of the inflammasome.

To test the role of the inflammasome in our system, PMA differentiated THP-1 macrophages, together with THP-1 cells deficient in CASP1 and ASC, both components of the inflammasome, were infected with HM605 and IL-1β production was measured after 24 h. There was significantly less IL-1β release from inflammasome-deficient macrophages compared to wild type macrophages confirming that HM605 infection of THP-1 macrophages does result in an inflammasome-dependent increase in IL-1β production (Figure 4. 17b). To test if acetate production has an effect on IL-1β production in inflammasome deficient cells, THP-1 deficient in CASP1 or ASC were infected with wild type HM605 and acetate production mutants (i.e. Δ*pfkAB* and Δ*ackA-pta*). Interestingly, in inflammasome

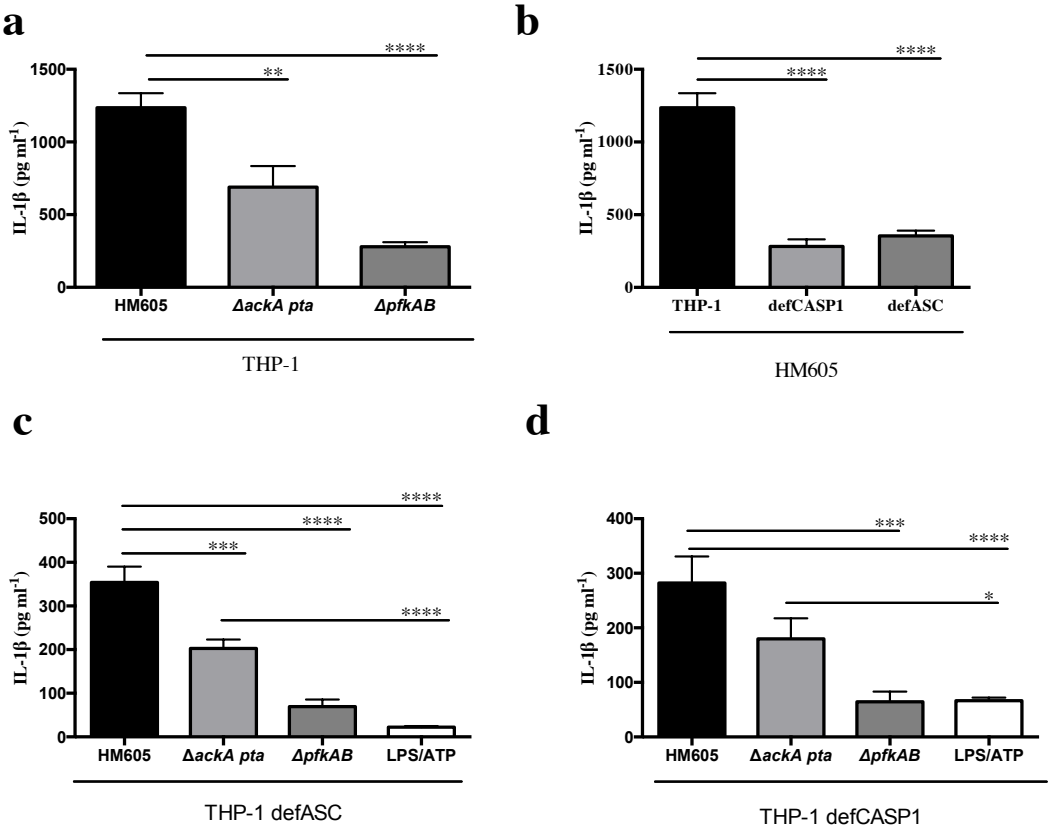

Figure 4. 17 Acetate and glycolysis mutants induced lower IL-1β release from human macrophages.

a) IL-1β detected in supernatants of wild type THP-1 macrophages HM605 strains indicated for 24 h. Data shown is mean±SEM of 3 independent experiments, **** $P < 0.0001$ determined by one-way ANOVA comparing all mutants strains to HM605 with Dunnett's post test. **b)** IL-1β detected in supernatants of THP-1 defective in CASP1 (defCASP) and ASC (defASC) infected with HM605 for 24 h. Data shown is mean±SEM of 3 independent experiments. **** $P<0.0001$ determined by one-way ANOVA comparing THP-1 to defective lines. **c-d)** THP-1 defASC (c) or THP-1 defCASP1 (d) infected with HM605, HM605 Δ*ackA-pta*, HM605 Δ*pfkAB* for 24 h or pre-treated with 1 μg ml^{-1} lipopolysaccharide (LPS) for 1 h followed by incubation with 1 μg ml^{-1} LPS + 5 mM Adenosine trisphosphate (ATP) treated with LPS and ATP for 24hrs. Data shown is mean ±SEM of 3 independent experiments, *** $P < 0.001$ determined by one-way ANOVA comparing mutant strains to wild type or all strains to LPS/ATP treated cells with Dunnett's post test.

deficient cells, there was still a significant decrease in IL-1β production in the Δ*pfkAB* and Δ*ackA-pta* mutants compared to the wild type HM605 (Figure 4. 17c and d). This suggest that in the absence of the inflammasome, acetate production still exerts an effect on IL-1β production as acetate producing HM605 induces higher IL-1β production than non-acetate producing mutants. In the absence of the caspase-1, it is possible that an unknown factor may cause IL-1β activation and release. Indeed, some bacterial derived serine proteases have been shown to mature IL-1β independently of caspase-1 (Netea et al., 2015).

4.2.13 HM605 does not increase mitochondrial ROS production in infected macrophages

Links between bacterial metabolism and the macrophages have been reported in recent years and in particular it has been shown that *Salmonella* mutants blocked in the TCA cycle induce a greater NLRP3-dependent inflammasome response to infection in macrophages (Wynosky-Dolfi et al., 2014). This was shown to be due to the accumulation of bacterially-derived citrate resulting in an increase in the generation of mitochondrial reactive oxygen species (mtROS) and concomitant activation of the NLRP3 inflammasome. Although acetate cannot be used by mitochondria in the same way as citrate it is interesting to determine whether production of acetate by HM605 could drive an increase in mtROS production. Therefore, cells were infected with wild type HM605, the Δ*pfkAB* mutant, and the Δ*ackA-pta* mutant and mtROS production was measured over time using MitoSOX, a mtROS sensitive dye that increases in fluorescence in the presence of mtROS. Live cells were analysed for MitoSox fluorescence intensity using flow cytometry at 3, 6 and 24 h post-infection as previously described (Mukhopadhyay et al., 2007). There was similar levels of mtROS produced in infected and uninfected cells at 3 h and 6 h (Figure 4. 18b). At 24 h, all infected cells had significantly lower levels of mtROS compared to uninfected controls (Figure 4. 18a-b) indicating that infection with HM605 does not result in an increase in mtROS generation.

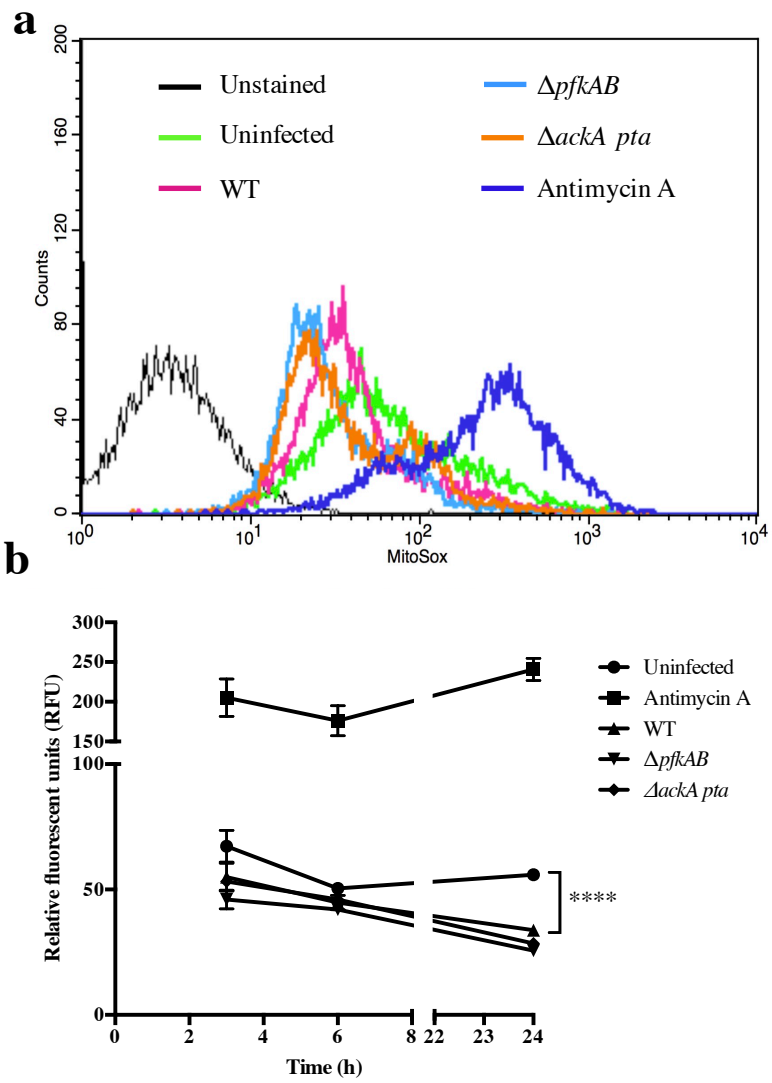

Figure 4. 18 Mitochondrial ROS production by HM605 infected macrophages.

a) Representative flow cytometry graphs from FL2 (annotated as MitoSox) channel of MitoSox stained macrophages 24 h post infection. **b)** Median fluorescent intensity of MitoSox stained macrophages quantified by flow cytometry 3, 6 and 24 h post infection. Data shown is mean±SEM of 3 independent experiments, **** $P < 0.0001$ determined by one-way ANOVA comparing uninfected to infected cells with Dunnett's post test.

4.3 Discussion

Previous experiments in our laboratory have shown that, in the AIEC HM605, mutants in glycolysis and glucose uptake induce the production of less IL-1β from macrophages than their wild type parent. A mechanism for this is not understood but results presented here suggest that a bacterial metabolic by-product is involved.

IL-1β secretion is dependent on the activation of inflammasomes (Schroder & Tschopp, 2010). In the context of *E. coli*, the NLRP3 inflammasome appears to be the most relevant as many studies have shown that both commensal *E. coli* and AIEC can activate this inflammasome (la Fuente *et al*., 2014; Sander *et al*., 2011). Activation of the NLRP3 inflammasome depends on 2 signals: a priming signal, such as the interaction of LPS with TLR4, that initiates *Il1b* and *Nlrp3* transcription and a second signal such as K^+ efflux, bacterial mRNA, mitochondrial ROS or extracellular ATP that results in NLRP3 oligomerisation and subsequent activation (de Zoete et al., 2014). Once active, the NLRP3 inflammasome associates with ASC and stimulates pro-capsase-1 activation. Active caspase-1 then cleaves pro-IL-1β into its active form and this is released from the cell. In this study, it has been confirmed that IL-1β production induced by wild type AIEC was dependent on caspase-1 and ASC.

The inflammasome-deficient cells used in this study were stable shRNA knockdowns of caspase-1 and the adaptor protein ASC. These proteins are essential components of many inflammasome but is not required for the non-canonical inflammasome (Broz & Monack, 2013; de Zoete *et al*., 2014). Caspase-4 (caspase-11 in mice) is the effector protein for the non-canonical inflammasome and this protein directly senses cytosolic LPS leading to pyroptosis, caspase-1 activation and IL-1β release ((Kayagaki et al., 2013; Shi et al., 2014)). IL-1β release induced by caspase-4 activation, however, appears to be dependent on the activation of other inflammasomes and recently both caspase-4 and caspase-11 were shown to indirectly activate the NLRP3 inflammasome by triggering K^+ efflux from the cell (Rühl & Broz, 2015; Schmid Burgk *et al*., 2015). In addition, the direct cleavage of IL-1β by caspase-4/11 has never been reported. Thus, in cells deficient in ASC such as those used in this study, indirect activation of the NLRP3 inflammasome by the non-canonical inflammasome would not be possible

and thus there should be little or no IL-1β released from these cells even if the non-canonical inflammasome was activated. However, IL-1β production was observed in ASC-deficient macrophages infected with HM605 suggesting that there may be an alternative pathway activating IL-1β. Interestingly the level of IL-1β production in the CASP1 and ASC-deficient macrophages was lower in macrophages infected with the Δ*pfkAB* or Δ*ackA*-pta mutant bacteria compared to the same macrophages infected with wild-type bacteria. Therefore the non-inflammasome dependent activation of IL-1β does appear to have a component that is dependent on bacterial metabolism. Serine proteases like proteinase 3 produced endogenously by macrophages have been shown to cleave pro-IL-1β and some microbial proteases have also been shown to have the same activity (Netea et al., 2015). Interestingly acetate has been shown to increase lysosome membrane permeablisation resulting in the leaking of cathepsin D into the cytosol (Marques et al., 2013). Therefore it is possible that acetate production by *E. coli* in the phagosome may facilitate cathepsin leakage into the cytosol resulting in IL-1β maturation.

Recent work in *Salmonella* has identified a bacterial derived metabolite as a potential vita-PAMP (Wynosky-Dolfi et al., 2014). Citrate production by *Salmonella* in macrophages resulted in an increase in mitochondrial ROS (mtROS) which activates the NLRP3 inflammasome by an unknown mechanism (de Zoete et al., 2014). Therefore, the amount of mtROS produced by macrophages infected with wild type AIEC, and mutants in acetate production and glycolysis was tested. Contrary to results seen with *Salmonella*, there was a decrease in mtROS in infected cells compared to uninfected cell at all time points tested. This decrease is probably due to activation of the macrophages which is associated with an increase in glycolysis and a decrease in oxidative phosophorylation (Zhu et al., 2015). Importantly, there was no significant difference in mtROS between macrophages infected with wild type or mutants bacteria. Therefore infection with AIEC does not cause an increase in mtROS although this does not exclude the possibility that acetate or another as yet unidentified metabolite may be acting as a vita-PAMP through another unknown mechanism.

Recent studies have shown that lysine acetylation (acetylation for short) is a dynamic, reversible process that occurs on proteins with various biological functions and

directly affects bacterial physiology (Castaño-Cerezo *et al*., 2011; Kim *et al*., 2006; Kuhn *et al*., 2014; Lima *et al*., 2012; Schilling *et al*., 2015; Thao *et al*., 2010; Weinert *et al*., 2013; Yan *et al*., 2008; Yu *et al*., 2008; Zhang *et al*., 2009). AcetylCoA can function as acetyl donor for lysine acetylation as can acetyl phosphate (AcP), the high energy intermediate of the Pta-AckA pathway (Hu et al., 2010). Thus cells deficient in production of either of these important molecules are likely to have reduced ability to acetylate proteins. Global acetylation studies in *E. coli* have identified over 8000 lysine acetylation sites on 1000 proteins (Weinert et al., 2013). Therefore it appears that protein acetylation is both widespread across the proteome and can occur on multiple sites on a individual protein. In order to investigate the role of acetylation in the bacteria and its affect on IL-1β production in the macrophages, a mutant in Δ*yfiQ* was created along with a CobB overproducing strain. The effect of both of these strains is to reduce total acetylation in *E. coli*. Interestingly the CobB overproducing strain (but not the Δ*yfiQ* knockout) had reduced IL-1β release from infected macrophages suggesting that acetylation is important in producing a complete IL-1β response. However the differences between the Δ*yfiQ* mutant and the cobB-overexpressing strains may be due to differences in their targets. While enzymatic acetylation by YfiQ has been identified on a small number of target proteins, recent studies suggest that it is not essential for acetylation in *E. coli* suggesting that alternative acetylation pathways or acetylases exist (Weinert et al., 2013). Thus a deletion in *yfiQ* may not have a significant effect on the cell. One alternative pathway is the non-enzymatic acetylation of protein by AcP (Kuhn *et al*., 2014; Schilling *et al*., 2015; Weinert *et al*., 2013). During growth on glucose, cells in stationary phase accumulate acetylated proteins in an AcP-dependent manner and this can be reversed by CobB (Weinert et al., 2013). Thus a CobB-overproducing strain would have profound effects on global acetylation patterns (confirmed in this study (see Figure 4. 11a) possibly explaining the reduced IL-1β released during infection with this strain.

The acetylation profile of wild type AIEC and all metabolism mutants tested for IL-1β release was measured during growth in glucose supplemented media *in vitro*. Interestingly, all mutants that induced decreased levels of IL-1β production from macrophages also had altered acetylation profiles. All mutants had a reduced level of global acetylation with the exception of Δ*ackA* which had an increased level of

acetylation. This is likely due to the accumulation of AcP which has been shown in other *E. coli* backgrounds to cause an increase in acetylation (Weinert et al., 2013). Thus it appears that perturbations in global acetylation may be a cause of the reduced IL-1β phenotype reported here. Since the first global acetylation studies in prokaryotes, it was apparent that many acetylated proteins are those involved in central metabolism (Kuhn *et al*., 2014; Schilling *et al*., 2015; Wang *et al*., 2010; Yu *et al*., 2008; Zhang *et al*., 2009). Functional clustering of acetylated proteins has indicated that proteins involved in glycolysis, the PP pathway, pyruvate dehydrogenase complex (PDHC), acetate metabolism and the TCA cycle are all acetylated. In glycolysis, proteins such as phosphofuctose kinase (encoded by Δ*pfkA* and Δ*pfkB*) and pyruvate kinase have been shown to be acetylated (Kuhn *et al*., 2014; Schilling *et al*., 2015; Wang *et al*., 2010; Zhang *et al*., 2009). AceE and AceF, components of the PDHC, were also shown to be acetylated (Kuhn *et al*., 2014; Schilling *et al*., 2015; Zhang *et al*., 2009). In the TCA cycle citrate synthase, isocitrate dehydrogenase and succinate dehydrogenase have also been shown to be to be acetylated (Kuhn *et al*., 2014; Schilling *et al*., 2015; Wang *et al*., 2010; Zhang *et al*., 2009). The level and number of acetylated proteins in these pathways strongly suggests that acetylation is a regulatory mechanism for metabolism. For example, in *Salmonella*, deletion of *cobB* resulted in faster growth on glucose and increased flux through glycolysis suggesting that increased acetylation results in an increase in the activity of glycolytic enzymes (Wang et al., 2010). Furthermore, growth on glucose also increases the number of acetylated proteins compared to growth on amino acids and many of these acetylated proteins are involved in glycolysis, the glyoxylate shunt and the PP pathway (Schilling et al., 2015). In the work reported here it is possible that CobB overproduction resulted in deacetylation of many of the normally acetylated glycolysis proteins and this results in reduced flux through glycolysis. Our results indicate that glycolysis is important for induction of IL-1β release from macrophages and therefore a CobB-overproducing strain may exhibit the same phenotype as a glycolysis mutant because of reduced activity of glycolytic enzymes. In contrast, the Δ*ackA* mutant accumulates AcP and therefore has high levels of protein acetylation. However, it is possible that this results in the over-acetylation of proteins in these important metabolic pathways and leads to changes in metabolic flux that influences IL-1β induction.

In this study it has been shown that the production of acetate and/or a perturbation in the AcCoA/CoASH ratio in the AIEC strain HM605 may be responsible for the induction of IL-1β production from infected macrophages. Whilst protein acetylation levels in the bacteria have been implicated in this phenotype the the exact molecular mechanism(s) that connect these bacterial processes with IL-1β production in the macrophages remains unknown.

Chapter 5: Species specific susceptibility of macrophages to α -hemolysin

5.1 Introduction

Uropathogenic *E. coli* (UPEC) are a group of extraintestinal pathogenic *E. coli* responsible for 80% of all urinary tract infections (UTIs) (Ulett et al., 2013). UPEC can colonise the bladder causing cystitis and some strains can then ascend to the kidneys causing pyelonephritis (Flores-Mireles et al., 2015). Colonisation of both these organs results in tissue damage, pain, cytokine release, infiltration of phagocytes and shedding of the bladder epithelium (Mulvey *et al*., 2001; Wiles *et al*., 2008b). UPEC possess a number of virulence factors that are required for colonisation such as type 1 fimbriae (Bahrani Mougeot et al., 2002). Furthermore UEPC secrete a number of toxins, the best characterised of which is α-hemolysin (HlyA) (Bien et al., 2012) and approximately 40-50% of UPEC secrete this toxin (Ristow & Welch, 2015).

HlyA is a member of the repeats in toxin (RTX) family of toxins. Other RTX toxins include the leukotoxins of *Mannheimia haemolytica* (Lkt), adenylate cyclase toxin (CyaA) of *Bordetella* and the enterohemolysin of EHEC (Ehx) (Linhartová et al., 2010). These toxins are generally pore forming and lyse cells and they can have a diverse range of target cell but many target leukocytes. Some RTX toxins have species-specific targets, for example, Lkt only targets bovine leukocytes (Wiles & Mulvey, 2013). HlyA of *E. coli* has a diverse range of target cells to which is it cytotoxic. It is lytic to erythrocytes from many different species and it has been shown to be cytotoxic to B-cells, granulocytes, leukocytes, monocytes and epithelial cells (Forestier & Welch, 1991; Gadeberg *et al*., 1983; Ristow & Welch, 2015). A study using a zebrafish host infection model indicated that phagocytes such as macrophages and neutrophils may be targets for α-hemolysin *in vivo* (Wiles et al., 2009). HlyA-deficient UPEC have decreased virulence in the zebrafish model however this defect was rescued when neutrophils and macrophages were abolished suggesting that the primary role of HlyA during virulence may be to target host phagocytes. Ehx from EHEC has also been shown to cause cytotoxicity in human macrophages and the RTX toxin from *Kingella kingae* was shown to be cytotoxic to cultured RAW264.7 macrophages cell line (Kehl-Fie & St Geme, 2007; Zhang *et al*., 2012).

While α-hemolysin was initially characterized for its cytotoxic ability, it is also known to modulate a number of inflammatory processes. The first report of this was the reduction of TNF-α, IL-6 and IL-1β release from a cell suspension containing leukocytes, monocyes and basophils stimulated with HlyA-producing *E. coli* compared to a HlyA-negative *E. coli* (König & König, 1993). Inhibition of cytokine production is not limited to circulating cells as another, more recent, study has shown that secretion of IL-6, IL -8 and IL-1β was inhibited by HlyA (Hilbert et al., 2012). α-hemolysin is not always lytic to target cells and sublytic doses can have profound effects of target cell physiology. In bladder epithelial cells sublytic doses of HlyA can inactivate the Akt pathway, a signalling pathway that is involved in processes including cellular metabolism, apoptosis and metabolism (Nicholson & Anderson, 2002; Wiles *et al*., 2008a). Inactivation of Akt by HlyA is capable of blocking strong Akt-activating signals from either EGF or TNF-α (Wiles *et al*., 2008a). Furthermore, sublytic doses of HlyA have also been shown to degrade pallaxin and other proteins involved in cell-cell interactions in a process thought to promote exfoliation (Dhakal & Mulvey, 2012). In the same work, HlyA was shown to inhibit the NF-kB signaling pathway in both macrophages and epithelial cells.

Cell death can occur by a number of different programmed or environmentally influenced methods. Classical programmed cell death is called apoptosis and occurs to maintain tissue homeostasis. Importantly apoptosis is non-inflammatory and the process is tightly regulated and involves the action of cysteine proteases called Caspases (Elmore, 2007). In apoptosis there are initiator caspases (Caspase-2,-8,-9 and -10) and executioner caspases (Caspase-3 and -7). Following activation of apoptosis, cells begin to shrink, chromatin condenses, there is blebbing of the cell membrane and finally the cell breaks apart into small vesicles called apoptotic bodies that are then phagocytosed (Krysko et al., 2008). There is no release of cellular contents and therefore apoptosis does not result in an inflammatory response. Necrosis is a form of passive accidental cell death that occurs in response to environmental conditions (Fink & Cookson, 2005). Necrosis results in swelling of the cell and eventual cell rupture resulting in the release of the cellular contents which induces and inflammatory response through the interaction of danger-associated molecular patterns (DAMPs) with PRR (Sridharan & Upton, 2014). Pyroptosis is another form

of regulated inflammatory cell death. Immune cells such as macrophages engulf pathogens resulting in a signaling cascade than can lead to pyroptosis. It is under the control of multiprotein complexes called inflammasomes of which there are a number of different types with that sense different ligands (Chen & Schroder, 2013). These complexes sense different bacterial ligands resulting in their activation (Schroder & Tschopp, 2010). Activation of the inflammasomes results in the recruitment of caspase-1 which is then activated and stimulates pyroptosis in a mechanism that is not completely understood (Jorgensen & Miao, 2015). Pyroptosis results in cell lysis and release of the cellular contents including the engulfed pathogen. RTX toxins such as Ehx have been shown to stimulate the NLRP3 inflammasome in a mechanism that appears to be driven by K^+ efflux from the cell due to pore formed by Ehx in the plasma membrane (Zhang et al., 2012). K^+ efflux is a known activator of the NLRP3 inflammasome (de Zoete et al., 2014).

While investigating the cytokine response of human macrophages to different strains of *E. coli* it was observed that 3 strains, namely UTI89, CFT074 and A034/86, all induced consistently lower amounts of all cytokines tested compared to all other strains (see Chapter 3, Figure 3.3e). Upon closer inspection, these strains appeared to rapidly induce high levels of cytotoxicity in differentiated THP-1 macrophages (see Chapter 3, Figure 3.3e). Consistent with recent findings, EHEC O157 Δ*stx* also induced high levels of cytotoxicity in this cell line (Zhang et al., 2012) but this level was significantly lower than what was observed for UTI89, CFT073 and A034/86. A high level of cytotoxicity in human macrophages infected by UTI89, CFT074 and A034/86 had not previously been reported and therefore the underlying molecular mechanisms of this cytotoxicity was investigated.

5.2 Results

5.2.1 Cytotoxicity of *E. coli* to human macrophages

Studies focused on the cystitis isolate UTI89 that is extensively studied and has a well-annotated genome. In Chapter 3 it was reported that infection with UTI89, CFT073 and A0 34/86 resulted in high levels of THP-1 cytotoxicity (see Figure 3.3, replicated here as Figure 5.1). In these experiments the THP-1 cells were infected with at an MOI=100 and therefore the effect of infecting macrophages at a lower MOI was tested. THP-1 were infected with UTI89 and MG1655, as a non-cytotoxic control, at a MOI of 12.5, 25, 50 or 100 but there was no significant change in cytotoxicity with a reduction in the MOI (Figure 5. 2). Therefore, UTI89 is highly cytotoxic to THP-1

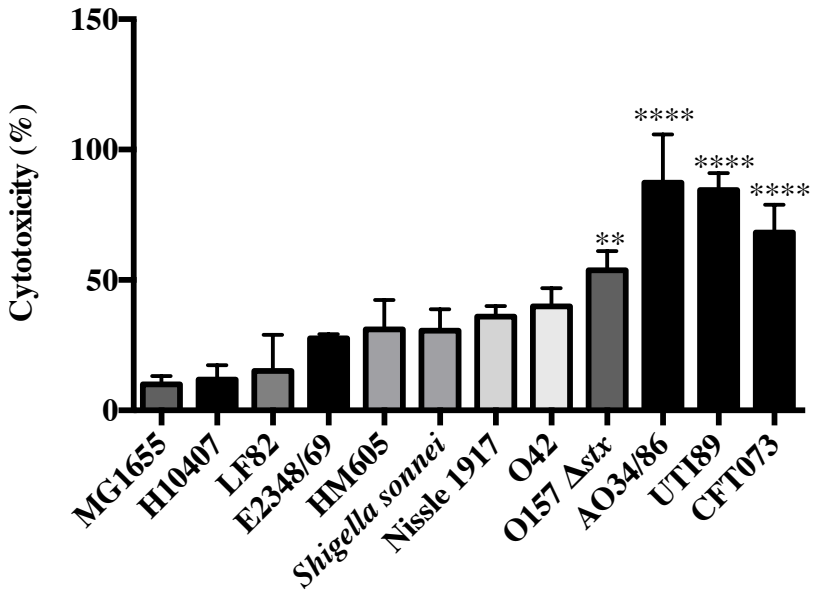

Figure 5. 1 Cytotoxicity of *E. coli* to human macrophages

Cytotoxicity (%LDH release) was measured in PMA differentiated THP-1 infected with *E. coli* for 2.5 h. Data shown is mean ± SEM of 3 biological replicates. ** P< 0.01, **** P< 0.0001 determined by one-way ANOVA comparing all strains to the K-12 MG1655 with Dunnett's post test.

This figure is a reproduction of Figure 3.3e and is shown here for illustration purposes only

even at relatively low MOIs suggesting that bacterial load is not the dominant cause of cytotoxicity. Consequently, all further infections were carried out at a low MOI=10. This data suggested that there was a specific bacterial factor responsible for the high levels of observed cytotoxicity.

As a first step in the characterization of this bacterial factor, cytotoxicity caused by viable and heat killed UTI89 and also filtered UTI89 culture supernatants was investigated (Figure 5. 3). THP-1 cells treated with bacteria sterile culture supernatants were also primed with LPS with no effect suggesting that the cytotoxicity observed is LPS (and therefore TLR4) independent. Surprisingly, there was a negative value seen for cytotoxicity when cells were treated with heat killed (HK) bacteria. This may be an artefact of the short incubation time combined with subtle differences in total cell number in each experimental well meaning that the control wells used to identify spontaneous LDH release may have more spontaneous LDH release than those treated with HK bacteria and this would lead to a negative value. Moreover, almost identical studies carried out by others have yielded similar negative values (Schaale et al., 2015). Nonetheless, it is clear that there is little or no cytotoxicity

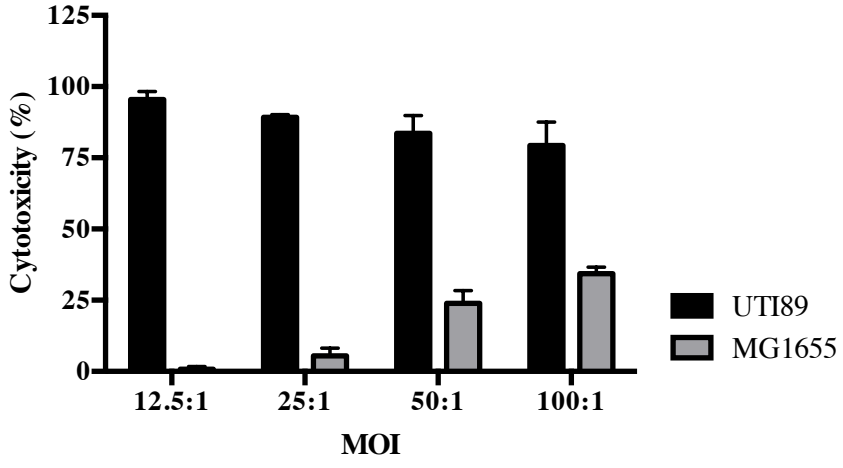

Figure 5. 2 UTI89 is cytotoxic to macrophages at low MOI.

Cytotoxicity (% LDH released) was measured in PMA differentiated THP-1 infected with UTI89 or the non-cytotoxic MG1655 at different MOI for 2.5 h. Data shown is mean ± SEM of 3 independent experiments.

induced by HK bacteria. Therefore, cytotoxicity requires viable bacteria and may involve a secreted factor that is not LPS.

5.2.2 Bacterial internalisation is not required for cytotoxicity

To test whether internalisation of the bacteria is required to induce cytotoxicity, phagocytosis was inhibited by pre-treating macrophages with the actin polymerisation inhibitor cytochalasin D. Using the non-cytotoxic strain MG1655 as a control, internalised bacteria were enumerated to test the efficacy of cytochalasin D on phagocytosis inhibition (Figure 5. 4). For MG1655, cytochalasin D treatment significantly reduced the level of internalization by THP-1 cells indicating that phagocytosis was inhibited. However only very few (as little as 17 CFU ml^{-1}) bacteria

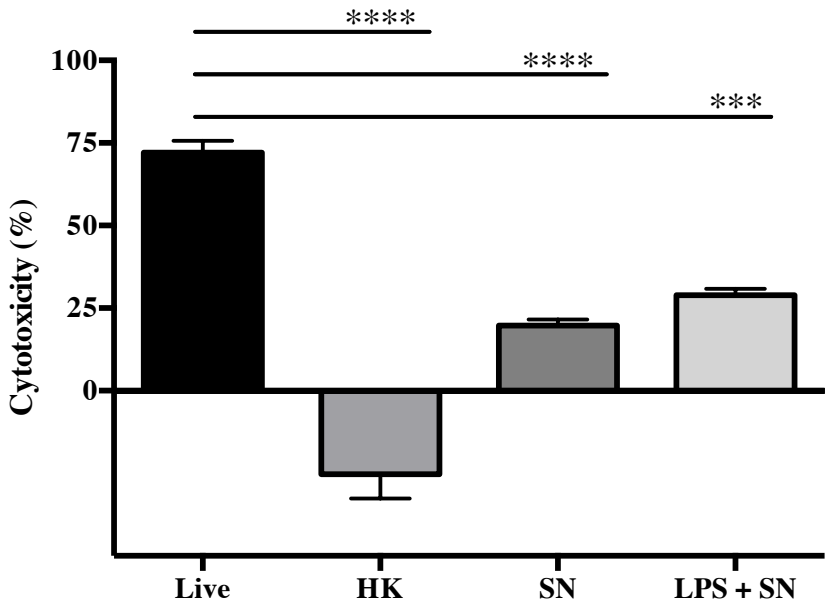

Figure 5. 3 Viable bacteria are required to induce high levels of cytotoxicity in THP-1 macrophages.

Differentiated THP-1 cells were infected with either live of heat killed (HK) UTI89 at MOI=10, or with culture supernatant (SN) from UTI89 grown in DMEM overnight, 0.22 μm filter sterilised and diluted in the same manner as bacteria. Where indicated the THP-1 cells were primed with a pre-treatment of *E. coli* LPS before addition of SN. LDH assays were performed on cell free cell culture supernatants after 2.5 h infection. Data shown is mean ± SEM of 3 independent experiments. *** $P < 0.001$ determined by one way ANOVA comparing each strain to live UTI89 using Dunnett's post test.

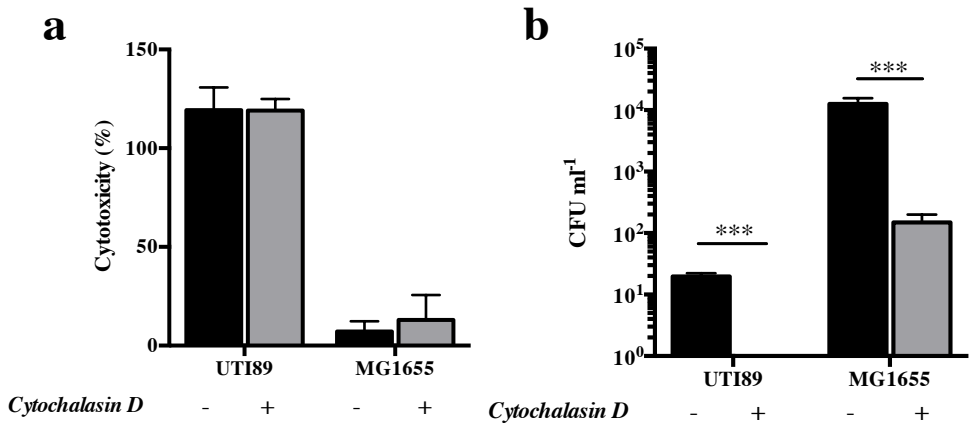

Figure 5. 4 Internalisation of bacteria not required for cytotoxicity in THP-1

Infection of THP-1 with UTI89 or MG1655 in the presence or absence of the phagocytosis inhibitor cytochalasin D. Cells were pre-incubated with Cytochalasin D for 1 h prior to infection and cytochalasin D was maintained in the culture supernatant for the duration of the experiment. **(a)** Cytotoxicity was measured by determining the % LDH release in culture supernatants after 2.5 h infection. **(b)** Cells were treated with gentamicin-supplemented media for a further 1 h after which intracellular bacteria were enumerated. Data is mean±SEM of 3 independent experiments . Statistical significance was determined by a Student T-test (*** P< 0.001).

could be recovered from UTI89-infected macrophages and this is likely to be due to the high cytotoxicity caused by this *E. coli*. Nonetheless there was no difference in cytotoxicity between cytochalasin D treated and untreated UTI89-infected macrophages indicating that phagocytosis and internalisation of bacteria is not required for cytotoxicity.

5.2.3 Cytotoxicity and inflammasome activation

Macrophages cell death (i.e. cytotoxicity) can be a programmed response to specific signals (e.g. apoptosis, pyroptosis) or a non-specific response to the environment (e.g. necrosis, cell lysis). Pyroptosis is associated with the release of LDH and therefore it was decided to determine if the inflammasome was involved in mediating the cytotoxicity observed with UTI89 infections (Further details on different inflammasomes can be found in Chapter 1).

The effector protein that leads to pyroptosis is caspase-1 and to test for a role for caspase-1 in UTI89-mediated cytotoxicity of THP-1 cells, infected macrophages were initially treated with the caspase-1 inhibitor, Z-WEDH-FML, and a pan-caspase inhibitor, Q-VD-Oph. While there was a decrease in cytotoxicity in cells treated with the caspase-1 inhibitor it was not significant ($P = 0.1750$) compared to the vehicle-only treated cells and no such decrease was seen with the pan-caspase inhibitor (Figure 5. 5a). Potassium efflux can also act as a signal for NLRP3 inflammasome activation and this can be prevented if there are high levels of K^+ in the growth medium. Similar to the effects of caspase-1 inhibition, there was a non-significant decrease in cytotoxicity in cells cultured in the presence of high-K^+. The above suggests that the cytotoxicity observed may be, at least partially, dependent on the NLRP3 inflammasome. The fact that no inhibition was observed with the pan-caspase inhibitor may be explained by the increase in non-caspase mediated cell death (e.g. necroptosis) that is observed when caspases are inhibited under some infection conditions (Weng et al., 2014). These results suggested a role for the NLRP3 inflammasome in UTI89-mediated cytotoxicity. To further support this THP-1 cells

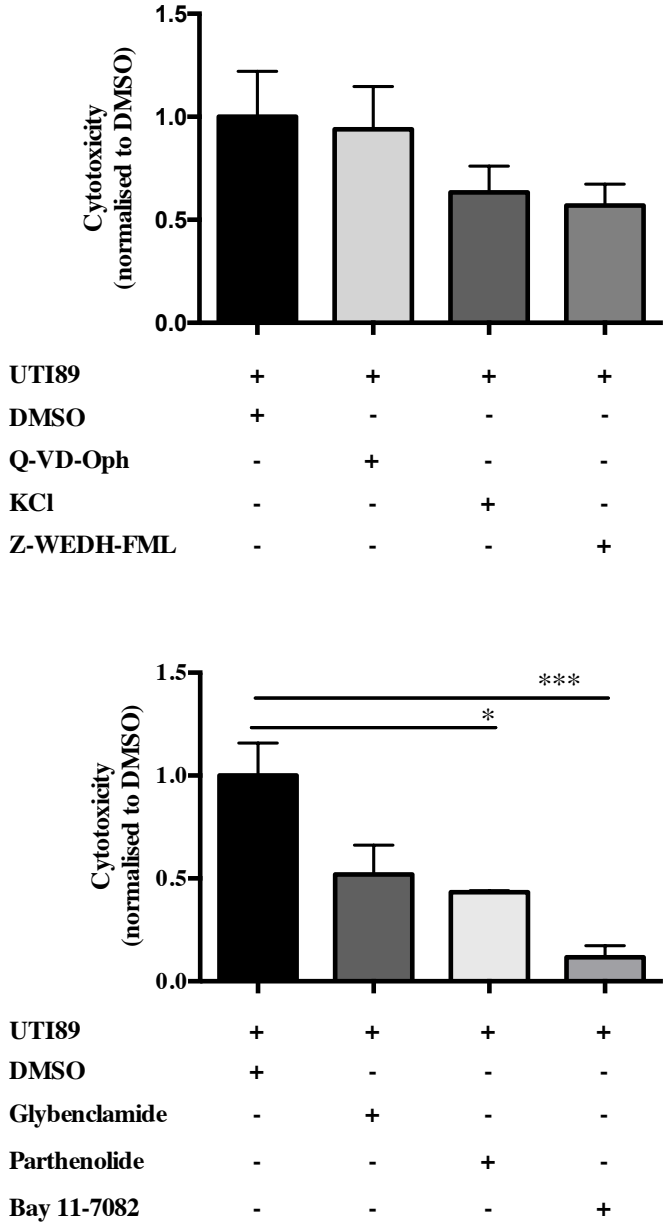

Figure 5. 5 Effects of inflammasome inhibition on UTI89 induced cytotoxicity

(a) and **(b)** LDH assay was performed on UTI89 infected THP-1 cells for 2.5 h in the presence or absence of indicated inhibitors. Cells were pre-treated with each inhibitor for 1 h prior to addition of bacteria and each compound was maintained in the culture medium for the duration of the experiment. Data was normalized to DMSO treated cells where the mean of DMSO was set as 1.0. In **(a)** data shown is the mean±SEM of 6 independent experiments obtained on 2 separate days. Data in **(b)** is mean ± SEM of 3 independent experiments * $P< 0.05$, *** $P< 0.001$ determined by one-way ANOVA comparing each condition to DMSO with Dunnett's post test. DMSO, Dimethyl sulfoxide

were treated with the the NLRP3 inhibitors Parthenolide and Bay 11-7082 (Juliana et al., 2010). K^+ efflux can also activate the NLRP3 inflammasome so a K^+ channel blocker, glybenclamide, which has been shown to delay LPS induced lethality, was also included (Lamkanfi et al., 2009). Similar to the reduction in cytotoxicity observed by increasing the concentration of extracellular K^+, glybenclamide reduced cytotoxicity but in a non-significant manner (P = 0.0886) (Figure 5. 5b). Both inflammasome inhibitors, however, showed a significant decrease in cytotoxicity induced by UTI89 with Bay 11-7082 being the most potent inhibitor (see Figure 5. 5). However, both of these inhibitors also have some off-target effects including the inhibition of the NF-κB signalling pathway that is involved in the priming of the inflammasome.

In order to clearly remove the possibility of off-target effects of inhibitors, THP-1 cell lines that have stable shRNA knockdowns of the NLRP3 inflammasome adaptor protein ASC and the inflammasome effector protein caspase-1 were obtained. These cell lines were infected with UTI89 and cytotoxicity was compared to that of wild type THP-1 cells (Figure 5. 6). It is clear that there was a significant decrease in cytotoxicity in both the ASC- and CASP1-deficient cell lines. Interestingly, even in these cell lines where functional inflammasomes do not form, the level of cytotoxicity still remained at approximately 50% suggesting that UTI89-mediated cytotoxicity has both inflammasome-dependent and –independent components. Nonetheless these results clearly indicate a significant role for the inflammasome (probably involving NLRP3) in UTI89-mediated cytotoxicity of the THP-1 macrophages.

5.2.4 Identification of effector of cytotoxicity

In this study UTI89, CFT073 and A034/86 have been shown to induce high levels of cytotoxicity in the THP-1 macrophages. Both UTI89 and CFT073 are UPEC that possess a number of virulence factors including type 1 pili, vacuolating autotransporter protein (VAT) and the RTX pore-forming toxin α-hemolysin (Tramuta et al., 2011). UTI89 also possess cytotoxic necrotizing factor 1 (CNF1) while CFT073 has secreted autotransporter toxin (SAT) (Wiles *et al*., 2008b). A034/86 is reported to be a commensal strain *E. coli* that is used in treatment of

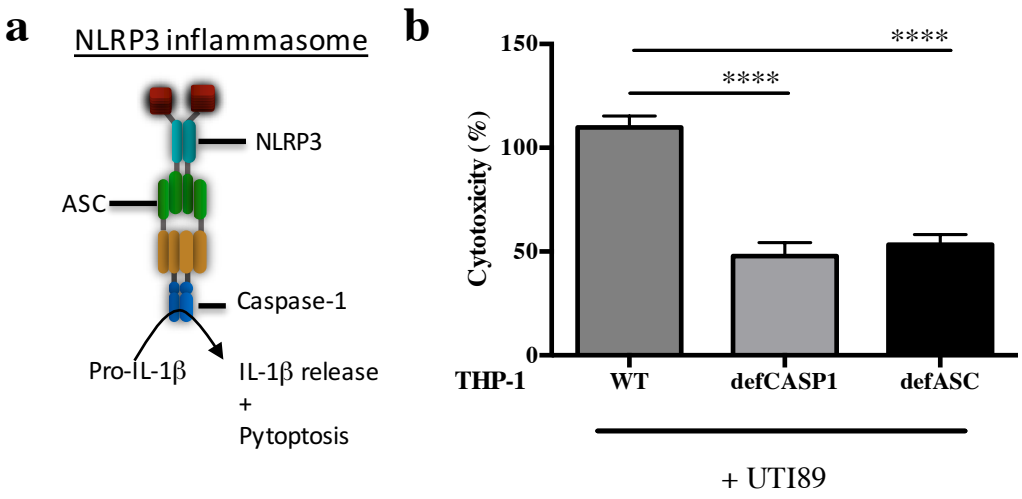

Figure 5. 6 The inflammasome mediates UTI89 induced cytotoxicity.

(a) The NLRP3 inflammasome. **(b)** LDH assay on wild type THP-1 or THP-1 deficient in either ASC (defASC) or caspase-1 (defCASP1) infected with wild type UTI89 for 2.5 h. Data is mean±S.E.M. of 6 independent experiments, **** $P< 0.0001$ determined by one-way ANOVA comparing each cell line to wild type (WT) THP-1 using Dunnett's post test.

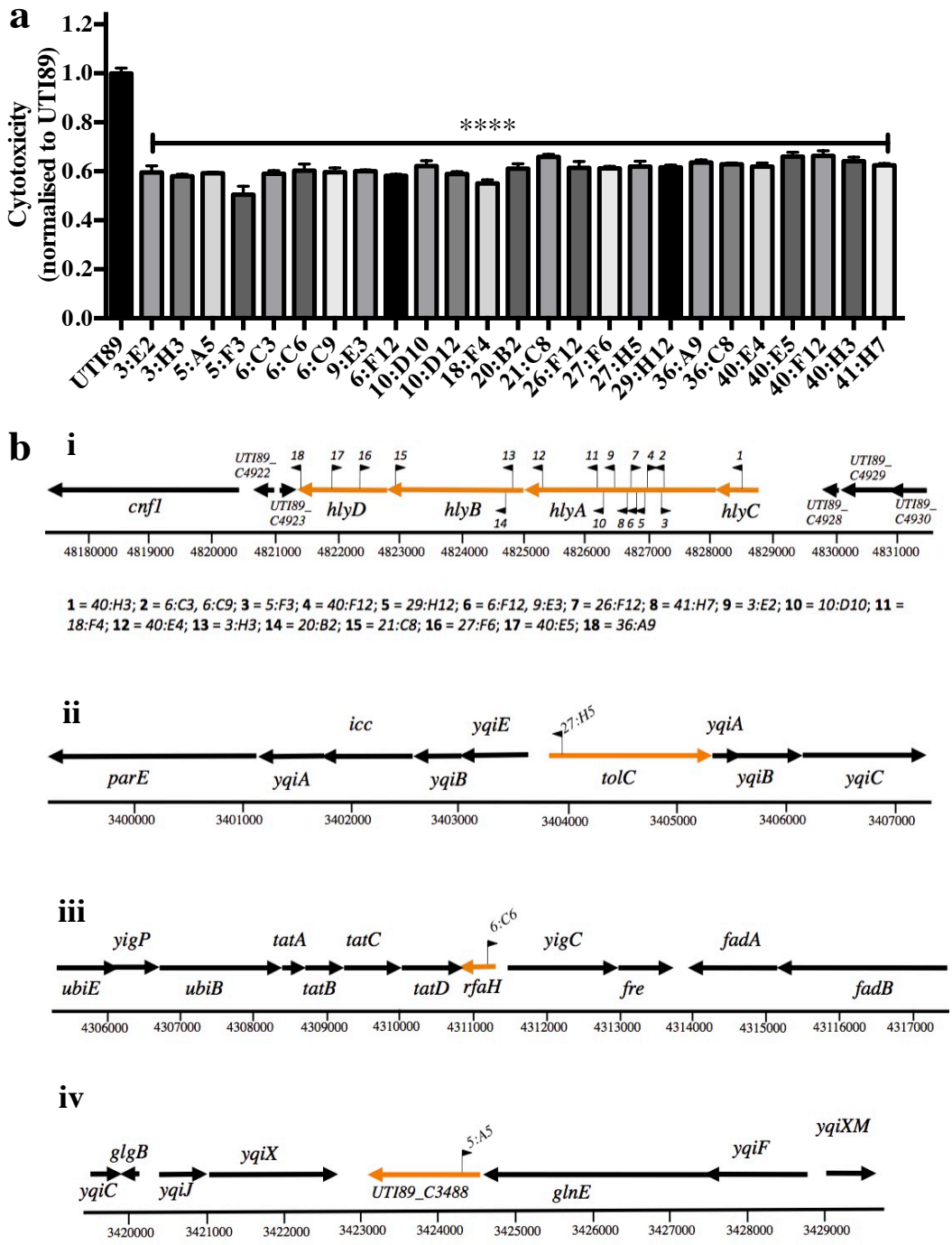

Figure 5. 7 Mutants in UTI89 with reduced cytotoxicity to THP-1.

(a) LDH assay on supernatants from THP-1 infected with UTI89 and its mutants reduced in cytotoxicity along with the non-cytotoxic MG1655 following 2.5 h infection. Cytotoxicity values were normalised to UTI89 by taking the mean of 3 biological UTI89 replicates as 1. **** $P < 0.0001$ determined by one-way ANOVA comparing all samples to UTI89 with Dunnett's post test. **(b)** Chromosomal locations of reduced cytotoxicity mutants. Gene UTI89_C3488 is a homologue of *hldE* in *E. coli* MG1655.

nosocomial infection and diarrhoea in new born infants in the Czech republic (Hejnova, 2005). However, the genome sequence does reveal that this strain contains genes encoding a number of established virulence factors including CNF1, α-hemolysin and enterohemolysin (Hejnova, 2005). These, or indeed other unidentified factors, may be contributing to the observed THP-1 cytotoxicity. Therefore, in an unbiased approach to identifying the cytotoxicity mediators, it was decided to screen a Tn5-based mutant library in UTI89 that was previously constructed in the laboratory (Emma Smith, unpublished data). The level of cytotoxicity observed with UTI89 is high enough for infected macrophages to be screened using a binocular microscope. Macrophages were infected, at an MOI=10, in the wells of 96-well microtitre plates with a single mutant and after 2.5 h cytotoxicity was visually observed. In this way 4,131 mutants were screened and 100 potential candidate mutants with reduced cytotoxicity to THP-1 macrophages were identified. A secondary screen where each of the 100 mutants were tested for cytotoxicity as observed by microscopy consolidated 100 potential mutants down to 25 mutants that were identified to be positive for reduced cytotoxicity on two separate occasions. To quantify the reduction in cytotoxicity LDH release was measured (Figure 5. 7a). In this way it was found that all mutants exhibited a reduction in cytotoxicity of approx. 40%. Arbitrary-primed PCR was used to identify the site of Tn insertion in each of these mutants and 23 of the 25 mutants mapped to the *hlyCADB* operon that encodes the pore-forming toxin α-hemolysin and the proteins required for its export and activation (Figure 5. 7b.i). There was 1 mutant in *hlyC* which encodes a lysine acyltransferase required to acetylate K546 and K690 on HlyA and activate it in the cytosol prior to secretion, 11 mutants in *hlyA,* the gene that encodes the α-hemolysin protein, 3, 5 and 1 mutants in *hlyB*, *hlyD* and *tolC,* respectively, which encode the type I secretion system responsible for secretion of acetylated HlyA. HlyB is an ABC transporter, HlyD is a membrane fusion/channel protein and TolC is an outer membrane protein (Figure 5. 7b.ii). TolC also functions as part of the AcrAB-TolC efflux pump that expels a number of antibacterial agents and TolC is also part of the MacAB-TolC that confers resistance to macrolides (Lin *et al*., 2009; Touzé *et al*., 2004). Therefore, α-hemolysin is clearly a very important effector of THP-1 cytotoxicity. Interestingly there was no mutant identified in the genetically linked *cnf1* gene (encoding the CNF1 toxin) suggesting that α-hemolysin alone is the cause of the observed cytotoxicity.

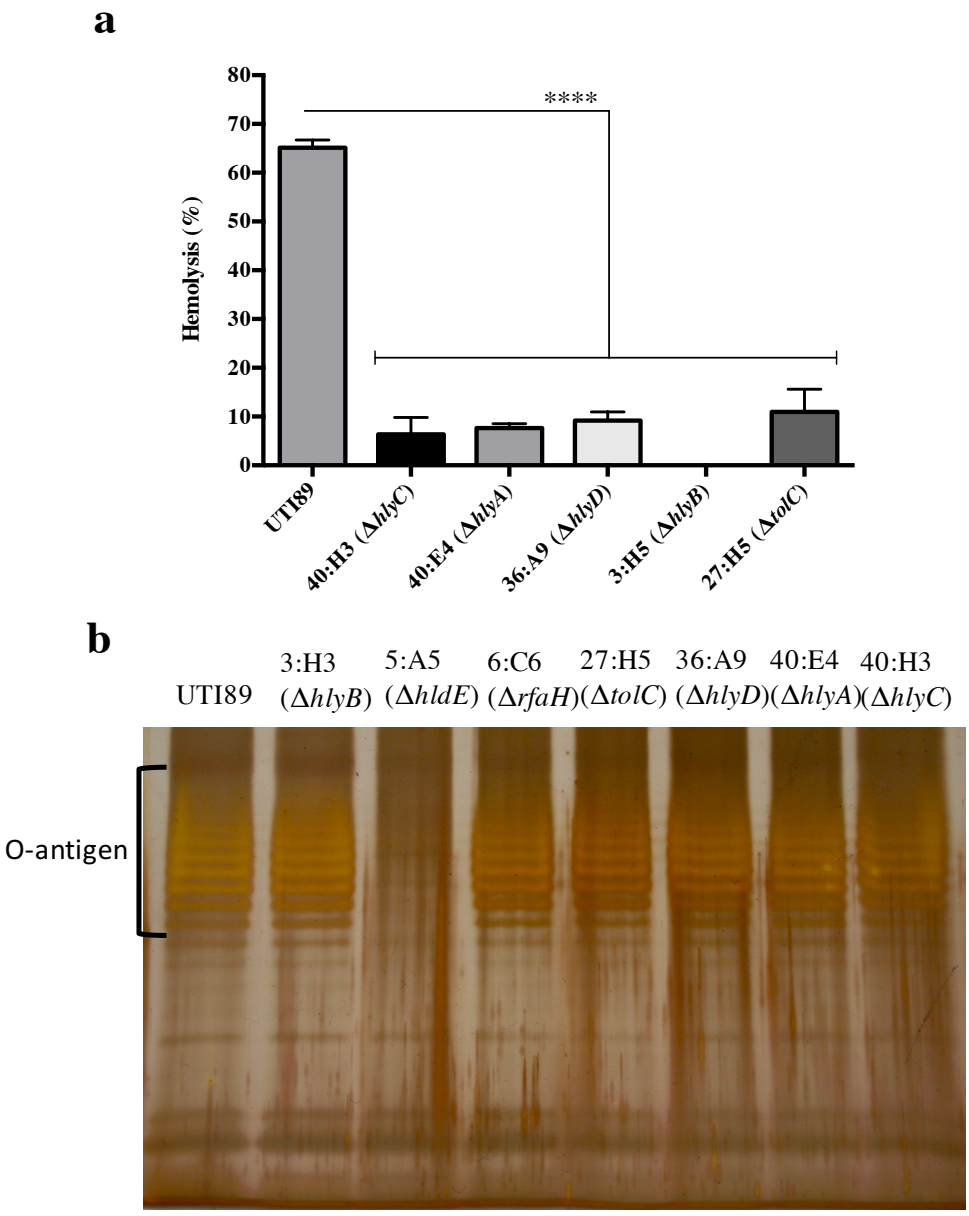

Figure 5. 8 Reduced cytotoxic mutants also have reduced hemolysis.

a) Hemolysis of sheep erythrocytes measured as previously described (Warawa et al, 1999) using UTI89 mutant strains representative of genes illustrated in Figure 5. 7b. Hemolysis is expressed as a percentage of hemolysis induced by addition of sterile H2O to erythrocytes. Data shown is mean±S.E.M. of 3 biological replicates, **** $P<$ 0.0001 determined by one-way ANOVA comparing all mutants to wild type UTI89 with Dunnett's post test. **(b)** LPS PAGE with silver stain of UTI89 mutants in (a). The O-antigen portion of LPS is highlighted

One mutant was also identified in *rfaH* (Figure 5. 7b.iii), a transcriptional anti-terminator. RfaH binds to RNA polymerase (RNAP) that has paused at an operon polarity suppressor (*ops*) sequence and changes the transcription elongation complex

to a termination resistant state thus enhancing elongation (Artsimovitch & Landick, 2002). RfaH controls transcription of a number of operons that are involved in assembly and export of the LPS core, α-hemolysin and CNF1 (Bailey et al., 1997; Landraud et al., 2003). One mutant was found in a gene annotated as UTI89_C3488. A BLASTN search revealed this gene to be 100% identical to *hldE* in multiple *E. coli* strains including the closely related NMEC strain RS218 and 98% identical to the homologue in *E. coli* K-12 MG1655 (Figure 5. 7b.iv). This gene, also referred to as *rfaE*, is predicted to encode a bifunctional ADP-heptose synthase that is involved in the synthesis of the LPS core precursor ADP-L-*glycero*-D-*manno*-heptose (Valvano et al., 2000). HldE has 2 domains with independent functions, Domain I is involved in the synthesis of D-*glycero*-D-*mannose*-heptose 1-phophosphate whereas Domain II enzymatically transfers ADP to form ADP-L-glycero-D-manno-heptose. *E. coli* with heptose-negative LPS has a deep rough phenotype and are more sensitive to antibiotics and detergents as they have a shorter LPS core and less outer membrane structural integrity. The integrity of the LPS on this, and other representative, mutants was determined by SDS-PAGE and silver staining (Figure 5. 8b). This analysis clearly revealed that the *rfaE* mutant in UTI89 lacked an O antigen (Figure 5. 8b).Interestingly it has been shown that mutations in *rfaP,* encoding a protein that catalyses phosphate transfer from ATP to heptoses on the inner core of LPS, secretes a form of α-hemolysin that is inactive indicating that the integrity of the LPS is important in the activation of α-hemolysin (Schnaitman & Klena, 1993). Therefore, it is possible that the *rfaE* mutant identified here is phenocopying the *rfaP* mutant resulting in the secretion of an inactive α-hemolysin and this results in a decrease in cytotoxicity.

As its name implies α-hemolysin lyses red blood cells so all mutants were tested for hemolysis. Hemolysis was expressed as a percentage of total lysis induced by H_2O.

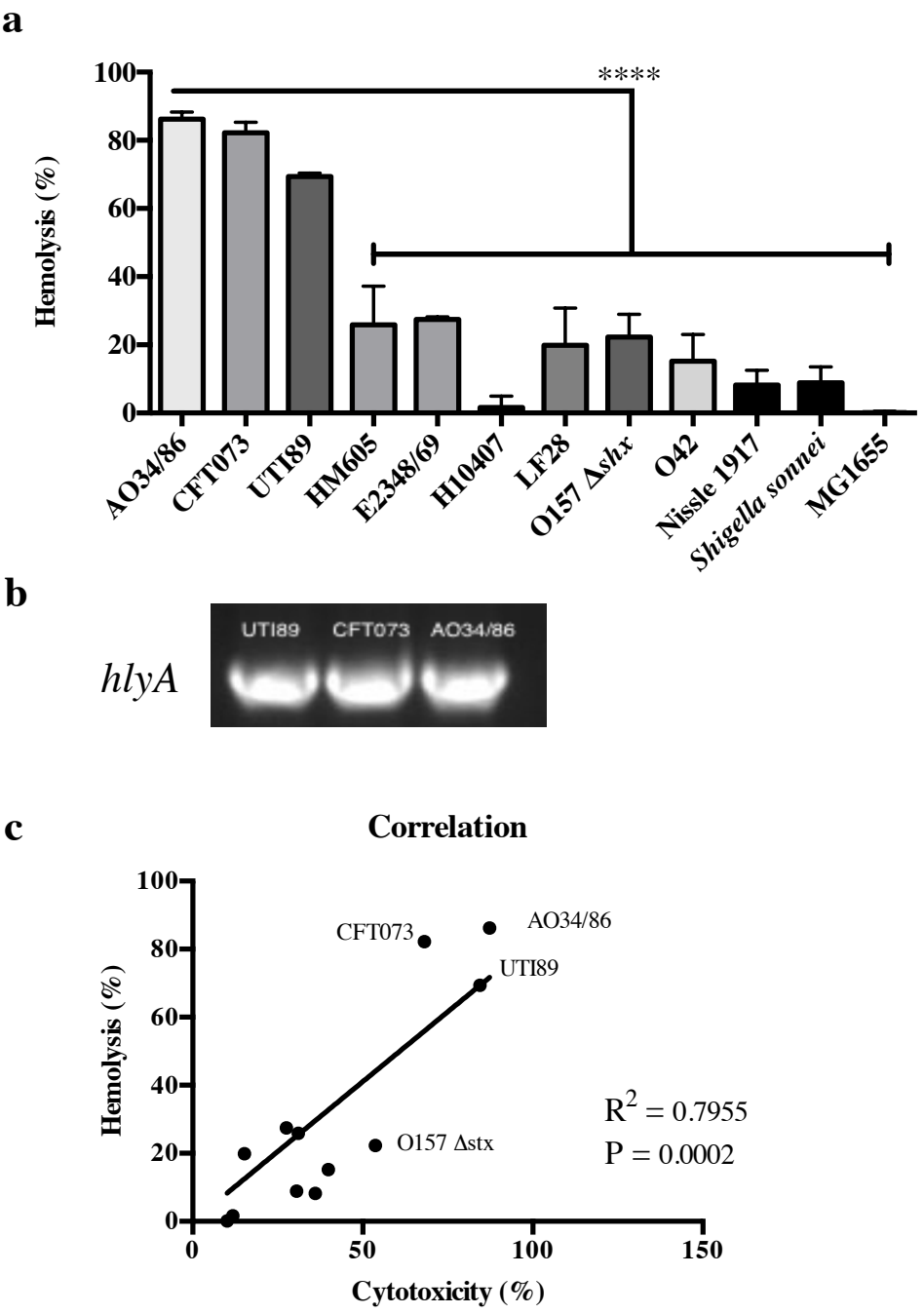

Figure 5. 9 Hemolysis by *E. coli*

(a) Hemolysis of sheep erythrocytes by *E. coli* strains indicated. **** $P< 0.0001$ determined by one-way ANOVA with Dunnett's post test comparing all strain to A0 34/86 **(b)** DNA agarose gel image of PCR to amplify *hlyA* in 3 cytotoxic strains**. (c)** Correlation of cytotoxicity and hemolysis in the panel of *E. coli* tested, only strains that had significantly higher cytotoxicity than MG1655 are indicated.

Wild type strain exhibited 60% hemolysis of sheep's erythrocytes the mutant strains all exhibited <15% hemolysis (Figure 5. 8a). Furthermore, only strains that caused high THP-1 cytotoxicity had high levels of hemolytic activity (Figure 5. 9a). When tested, using PCR, these 3 strains were all positive for the *hlyA* gene (Figure 5. 9b). A0 34/86 was the most haemolytic strain suggesting that it may secrete more α-hemolysin than UTI89 or CFT073 and there was a strong correlation between cytotoxicity and hemolysis (Figure 5. 9c).

Given that all highly cytotoxic strains are also hemolytic and all mutants in UTI89 that have reduced cytotoxicity are in genes involved in either the transcription, synthesis or transport of α-hemolysin, it seems clear that α-hemolysin is a significant effector of cytotoxicity in human THP-1 macrophages.

5.2.5 α-hemolysin mediated cytotoxicity is inflammasome-dependent and –independent

UTI89-mediated cytotoxicity was found to be inflammasome-dependent (see section 5.2.3), however, there was still a substantial amount of cytotoxicity observed in inflammasome deficient cells. To test if α-hemolysin contributes to this inflammasome-independent cytotoxicity, THP-1 defCASP1 and THP-1 defASC cell lines were infected with UTI89 and UTI89 Δ*hlyA* along with a non-cytotoxic control MG1655. There was a further reduction in the inflammasome-independent cytotoxicity in cells infected with UTI89 Δ*hlyA* compared to wild type UTI89 indicating that α-hemolysin contributes to this inflammasome-independent cytotoxicity (Figure 5. 10). It is possible that this is mediated by the non-canonical inflammasome that is dependent on caspase-4. The non canonical inflammasome is activated by cytosolic LPS which leads to pyroptosis even in the absence of the canonical inflammasome (Kayagaki *et al*., 2013; Shi *et al*., 2014). A recent study in human urothelial cells has shown that HlyA induces cell death in a caspase-1/caspase-4 dependent manner so this may be the case in macrophages also (Nagamatsu et al., 2015).

5.2.6 α-Hemolysin concentration-dependent activation of the inflammasome

The CpxAR two-component pathway has been shown to control the expression of α-hemolysin in UTI89 during infection and a mutant in *cpxRA* expresses more *hlyA* than the wild type and was shown to be more cytotoxic to urothelial cells (Nagamatsu et al., 2015). Additionally it was found that constitutive expression of the *hlyCADB* operon from a plasmid (phlyCADB) in UTI89 induced even higher levels of cytotoxicity (Nagamatsu et al., 2015). This study suggests that cytotoxicity is dependent on α-hemolysin concentration. Therefore, the UTI89 Δ*cpxRA* mutant and the phlyCADB plasmid were obtained and the plasmid was transformed into the non-cytotoxic strain MG1655. Hemolysis, as a marker of α-hemolysin secretion by UTI89, UTI89 Δ*cpxRA* and MG1655/pHlyCADB was then measured. The *cpxRA* mutant was 5 times more hemolytic than UTI89 whilst MG1655 pHlyCADB is almost 35 times

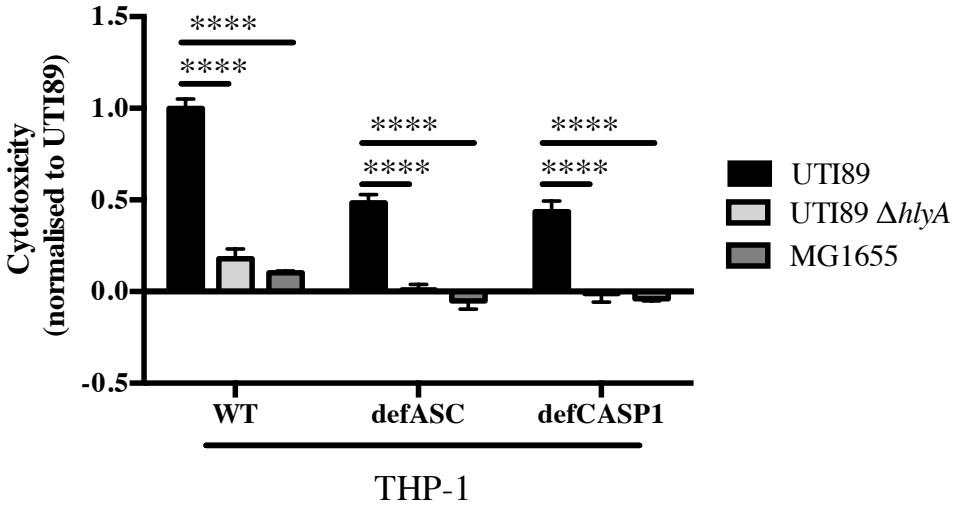

Figure 5. 10 Inflammasome independent cytotoxicity is abolished in UTI89 Δ*hlyA* infected cells

Cytotoxicity of UTI89, UTI89 Δ*hlyA* and MG1655 to wild type and inflammasome deficient THP-1 macrophages after 2.5 h incubation. Data shown is mean±SEM of 3 independent experiments, ** $P< 0.01$, **** $P< 0.0001$ determined by one-way ANOVA comparing each strain to UTI89 with Dunnett's post test.

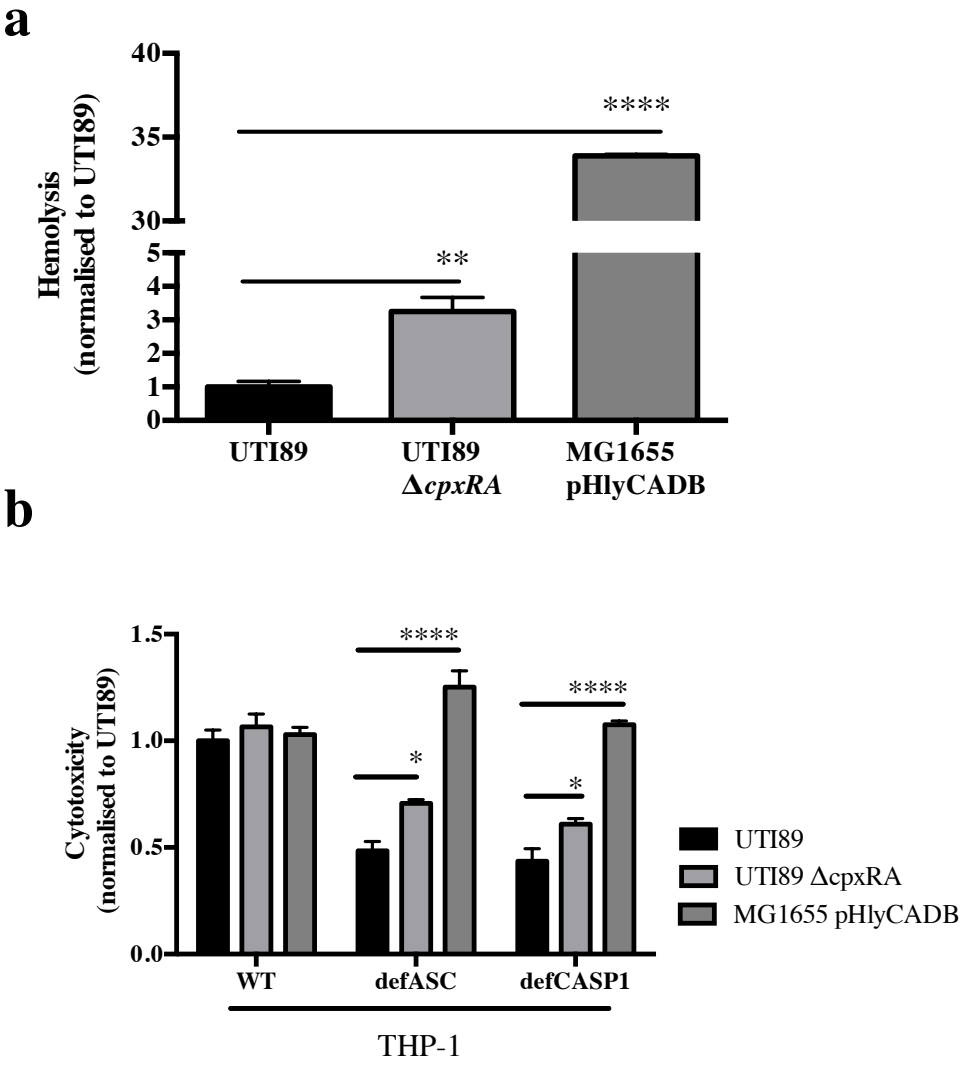

Figure 5. 11 High levels of α-hemolysin abolishes the protective effect of inflammasome deficiency in macrophages.

(a) Hemolysis of sheep's erythrocytes, relevant to UTI89, by UTI89 Δ*cpxRA* and MG1655 expressing pHlyCADB containing the α-hemolysin operon. **(b)** Cytotoxicity of UTI89, of UTI89 Δ*cpxRA* and MG1655 indicated to wild type and inflammasome deficient THP-1 macrophages. Data shown is mean ± SEM of 6 independent experiments. * $P< 0.05$, **** $P< 0.0001$ determined by one-way ANOVA comparing each strain to UTI89 with Dunnett's post test for each cell type.

more hemolytic than UTI89 (Figure 5. 11a). The very high amounts of α-hemolysin produced by MG1655/pHlyCADB led to almost instant hemolysis in a manner similar to adding water indicative of the large amount of α-hemolysin produced by this strain.

The mode of cell death may be determined by the level of α-hemolysin. Therefore, the effect of increased α-hemolysin concentration on cytotoxicity was investigated by infecting THP-1 macrophages with UTI89, UTI89 Δ*cpxRA* and MG1655/pHlyCADB. Since UTI89 is already highly cytotoxic to these cells, there was little difference in cytotoxicity seen with strains that secreted high levels of α-hemolysin (Figure 5. 11b). To investigate any effect of increased α-hemolysin concentration on inflammasome-dependent cytotoxicity, inflammasome deficient THP-1 were also infected with these strains to see if there is any impact on increasing α-hemolysin concentration of inflammasome-dependent cell death. Interestingly, in both THP-1 defCASP1 and THP-1 defASC, infection with UTI89 Δ*cpxRA* mutant or MG1655/pHlyCADB resulted in increased cytotoxicity compared to wild type UTI89. As these cells secrete higher levels of α-hemolysin, the level of α-hemolysin production appears to determine the mode of macrophages cell death. The above data suggest that below a certain threshold of α-hemolysin concentration cytotoxicity is inflammasome dependent and mediated through pyroptosis but with higher concentrations the cell is likely overwhelmed by the damage to plasma membrane caused the pore forming toxin and lysis occurs due to large number of pores in the cell rather than an activated cell death. Furthermore, the data suggests that the cytotoxicity caused by α-hemolysin-secreting strains of *E. coli* has a inflammasome-dependent and inflammasome–independent component.

5.2.7 Species specific macrophages sensitivity to *E. coli*

The very high cytotoxicity exhibited by some strains of *E. coli* on human THP-1 macrophages cells is due to α-hemolysin. To determine if this was cell line-specific, the murine macrophages-like cell line, J774A.1, was infected in the same manner as THP-1. Interestingly the 3 strains that were highly cytotoxic to human macrophages, i.e. UTI89, A034/86 and CFT073, showed significantly less cytotoxicity to murine macrophages (Figure 5. 12a). Cytotoxicity was also tested in another murine

macrophages cell line, RAW264.7, and again it was found that hamolytic strains of *E. coli* did not exhibit very high levels of cytotoxicity, in contrast to what was observed with the THP-1 macrophages (Figure 5. 12b). The commensal bacteria AO34/86 was more cytotoxic to RAW264.7 cells compared to J774A.1 suggesting that some strains may be more cytotoxic depending on the infected cell line background. Both A034/86 and CFT073 were more cytotoxic than MG1655 but levels of cytotoxicity were still much lower than in THP-1. Since the incubation time with the bacteria is relatively short, and there was much lower cytotoxicity in murine cell lines over this time period, the cytotoxicity of these strains to both murine macrophages cell lines was tested over a longer incubation period i.e. 24 h (Figure 5. 12c). Interestingly, at this later time point, AO34/86, CFT073 and UTI89 were all significantly more cytotoxic to RAW264.7 than J774A.1 confirming that macrophages sensitivity to these strains is cell-line specific dependent. Overall J774A.1 macrophages appear to be better protected from the cytotoxic effects of these strains.

It is possible that murine macrophages are less susceptible to the cytotoxicity induced by UTI89, CFT073 and AO34/86 due to reduced phagocytosis compared to THP-1 cell. However, there was no significant difference in the level of phagocytosis of these *E. coli* by the different macrophages cell lines (see Figure 5. 13). Indeed, it appears that UTI89 and CFT073 may be phagocytosed to a greater extent by J774.A1 compared to THP-1 or RAW264.7. Nonetheless AO34/86, the strain that induces the greatest amount of cytotoxicity in THP-1, was phagocytosed to a similar extent by both THP-1 and J774A.1 but to a lesser extent by RAW264.7. Together the data above indicates that the differences in cytotoxicity cannot explained by differences in phagocytosis.

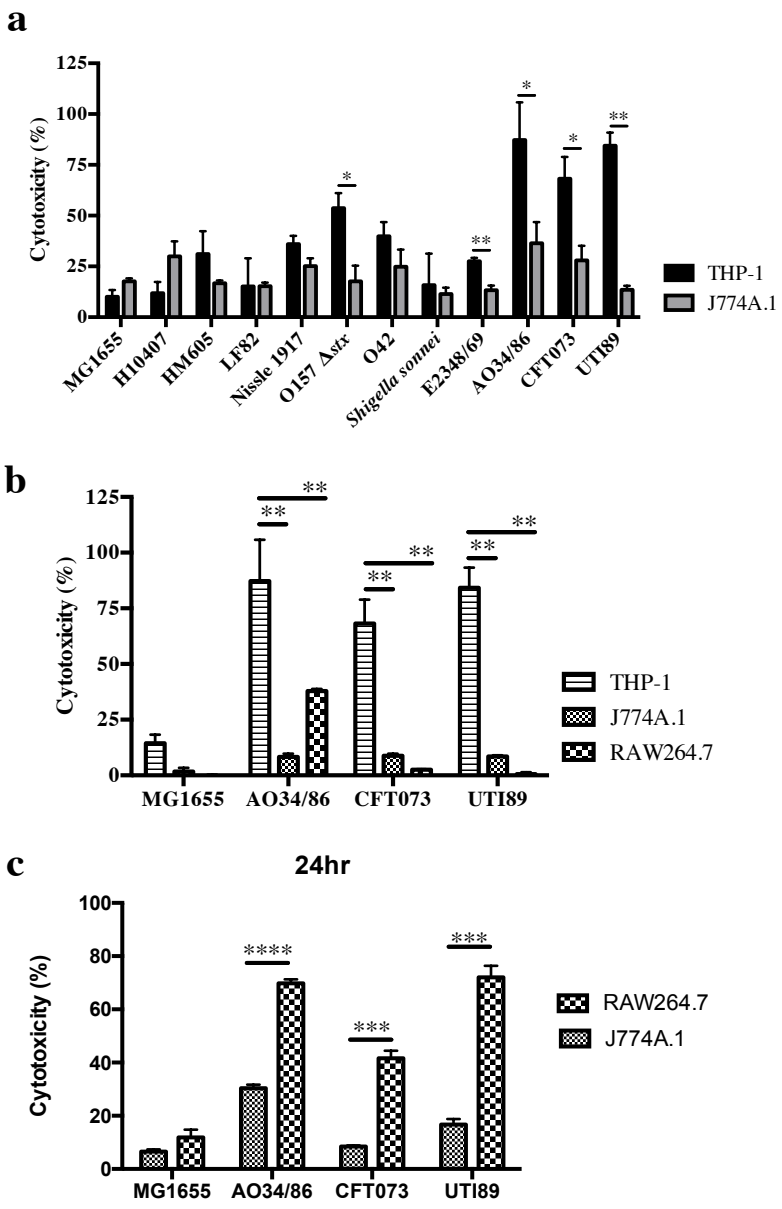

Figure 5. 12 Differential sensitivity of human and murine macrophages to *E. coli* induced cytotoxicity

(a) THP-1 and J774.A1 cells were infected with *E. coli* for 2.5 h and supernatants were assayed for LDH release to measure % cytotoxicity. (b) THP-1, J774.A1 and RAW264.7 were infected with *E. coli* highly cytotoxic to THP-1 for 2.5 h and supernatants were subjected to LDH release assay for measure % cytotoxicity. (c) Cytotoxicity of *E. coli* strains to J774A.1 and RAW264.7 24h post-infection. All data shown is mean ±S.E.M. of 6 independent experiments. * $P< 0.05$, ** $P< 0.01$, *** $P< 0.001$, **** $P< 0.0001$ determined by t-test comparing each strain between the 2 cell types in (a) and (c) and a one-way ANOVA comparing each cell type to THP-1 with Dunnett's post test.

The evidence presented here indicates that some strains of *E. coli* induce high levels of cytotoxicity in THP-1 cells and this is dependent on α-hemolysin production. Moreover, a significant part of this cytotoxicity is caspase-1- and ASC-dependent implying involvement of the inflammasome and increasing the levels of α-hemolysin production induces an inflammasome-independent cytotoxicity. Finally, the sensitivity of different macrophages to α-hemolysin appears to be species-specific with murine macrophages much more resistant than human macrophages. This observation does ask questions of the relevance of murine models in the study of human urinary tract infections.

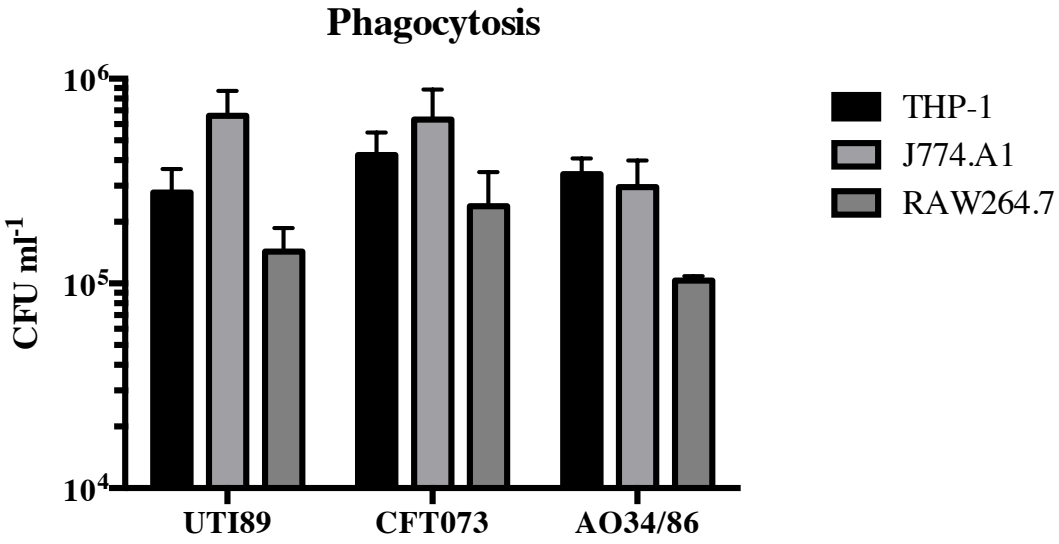

Figure 5. 13 Phagocytosis of cytotoxic strains by human and murine macrophages.

All macrophages cell lines were infected at MOI=10 in DMEM + 10% FBS. Following addition of bacteria, plates were centrifuges at 300 x g for 5 min to synchronise infection. Cells were incubated for 30 min followed by washing to remove extracellular bacteria. Cells were then incubated with culture media + 50 μg ml^{-1} Gm to kill extracellular bacteria. Phagocytosis of *E. coli* by was determined by enumerating intracellular bacteria.

5.3 Discussion

In experiments investigating the inflammatory response of THP-1 macrophages to *E. coli* infection, certain strains were found to rapidly induce very high levels of cytotoxicity to this cell line . These strains included 2 UPEC and a commensal *E. coli*. A study with UPEC infection of cultured epithelial cells also indicated high levels of cytotoxicity (Hilbert et al., 2012). Indeed, similar to what has been reported here, cytotoxicity of epithelial cells was induced at low bacterial concentrations and over 70% cytotoxicity was seen after a relatively short 2 h incubation. Thus, it appears that UPEC can be highly cytotoxic to multiple cell types.

HlyA is an RTX pore forming toxin and recent studies with other RTX toxins indicate these toxins can induce cytotoxicity in macrophages. The Ehx toxin is produced by the enterohaemorrhagic *E. coli* (EHEC) O157:H7, a pathogen that induces hemorrhagic colitis and hemolytic-uremic syndrome (HUS), which can be life-threatening (Croxen & Finlay, 2010). Recently, Ehx has been shown to induce cytotoxicity and IL-1β release from THP-1 macrophages in a process that is dependent on NLRP3, CASP1 and ASC (Zhang et al., 2012). However, the Ehx-induced cytotoxicity seen was not as rapid or as severe as the cytotoxicity induced by UTI89 described here. In the past year, a number of studies with UPEC have also indicated the interaction of UPEC with the NLRP3 inflammasome (Nagamatsu et al., 2015; Schaale et al., 2015; Symington et al., 2015). A study in urothelial cells showed that UTI89-induced cytotoxicity can be inhibited with a caspase-1/4 inhibitor or siRNA knockdown of caspase-1, caspase-4 or NLRP3 (Nagamatsu et al., 2015). Interestingly a caspase-4 knockdown had the most profound effect on reducing UTI89-induced cytotoxic. In this study we found that a cell line with a stable knockdown of caspase-1 had reduced cytotoxicity but this was not reduced to the level of a non-cytotoxic *E. coli* suggesting the presence of a caspase-1 independent mechanism. The non-canonical inflammasome effector caspase-4 can induce pyroptosis without the requirement of canonical inflammasome activation (Kayagaki et al., 2011; 2013; Shi et al., 2014). Therefore, it is entirely possible the the caspase-1 independent cytotoxicity seen in the current study could be through interactions of UTI89 with caspase-4. Furthermore, caspase-4 can directly sense the lipid moiety of LPS in the

cytosol and induce pyroptosis. Here, the caspase-1-independent cytotoxicity was abolished in UTI89 α-hemolysin mutants suggesting a role for α-hemolysin in caspase-1-independent cytotoxicity. Early experiments identified caspase-11 (the murine homologue of human caspase-4) as a mediator of the non canonical inflammasome (Kayagaki et al., 2011). This study identified cholera toxin B (CTB) as an activator of caspase-11 (Kayagaki et al., 2011). However, follow up experiments indicated that the CTB used was contaminated with LPS and showed that CTB mediated cytosolic delivery of LPS (as CTBs normal function is to translocate Cholera toxin A into the cytosol) and that LPS was the true effector on non-canonical inflammasome activation (Hagar et al., 2013). Therefore, it is possible that LPS can gain access to the cytosol through the pore-forming actions of α-hemolysin and activate caspase-4.

Since *E. coli* α-hemolysin has such profound cytotoxic effects on human macrophages, cytotoxicity by the same HlyA-producing strains was tested in other macrophages cell lines. Interestingly, murine macrophages did not exhibit the same level of cytotoxicity as human macrophages suggesting reduced sensitivity to HlyA. Recently studies with Ehx have shown similar results (Cheng et al., 2015). Ehx producing EHEC O157:H7 caused more cytotoxicity and IL-1β release in human than murine macrophages cell lines in an NLRP3-dependent manner. Moreover, this phenomenon was also shown to occur in primary cells (Cheng et al., 2015). The difference appears to be due to differential sensitivities of human and murine NLRP3 to activation by Ehx (Cheng et al., 2015). Thus it is possible that murine and human macrophages have different sensitivities to HlyA. The cytotoxic effect of Ehx on human macrophages could be abolished through limiting K^+ efflux from the cell; K^+ efflux is a known activator of the NLRP3 inflammasome (Muñoz-Planillo et al., 2013). Whist addition of K^+ did reduce the level of UTI89-induced cytotoxicity it did not abolish it suggesting the activation of the non-inflammasome mechanisms of cell death, as discussed earlier. Another recent study has shown that at low MOI, the UPEC strains CFT073 and UTI89 were more cytotoxic to human than murine macrophages and this cytotoxicity occurred over a similar time frame as reported in this thesis (Schaale et al., 2015). Cell death in murine macrophages was seen to be Nlrp3, Asc and Casp1/11-dependent. However, contrary to the current study, cytotoxicity to

human macrophages was not dependent on CASP1 or NLRP3 (Schaale et al., 2015). Furthermore, α-hemolysin-negative UPEC were severely impaired in triggering murine macrophages death but this effect was pronounced in human macrophages. The current study used cell lines whereas the published study used primary cell lines suggesting that there are cell-specific differences in the sensitivity of macrophages to UPEC-induced cytotoxicity that needs to be further characterised.

In conclusion, this study has shown that α-hemolysin-producing strains of *E. coli* induce high levels of cytotoxicity in human but not murine macrophages cell lines. Importantly the differing sensitivities of human and mouse macrophages to α-hemolysin does ask questions of the relevance of murine models in the study of human UTIs. This study has also shown that α-hemolysin production is the major effector of cytotoxicity and the mechanism of cytotoxicity is dependent on the concentration of α-hemolysin and the inflammasome.

General Discussion

Escherichia coli is a diverse bacterial species that can exist within many niches in the humans and the environment. While it primarily forms a beneficial symbiotic relationship with the host in the human lower gastrointestinal track (GIT), it can also be pathogenic in this environment. Furthermore, some strains can spread from the GIT and occupy niches such as the urinary tract. In all of these environments *E. coli* interacts with the local innate immune system and, in particular, macrophages. Therefore, the objective of this thesis was to characterise the molecular relationship between macrophages and different strains of pathogenic and non-pathogenic *E. coli*. Macrophages coordinate the immune response to infection through the release of chemokines and cytokines that have both local and systemic effects. During infection, macrophages release pro-inflammatory cytokines such as TNF-α, IL-6, IL-12 and IL-1β. The release of TNF-α, IL-6, IL-12 is a response to signalling from the interaction of bacterial LPS with TLR4 which results in increased transcription of genes encoding these proteins and their eventual release (Moresco *et al*., 2011; Wu *et al*., 2009). Similarly, IL-1β release is dependent on TLR4 signalling but also requires the activation of inflammasomes adding a layer of complexity to its processing (Chen & Schroder, 2013). Our findings shows that infection with *E. coli* resulted in a similar cytokine release profile with the exception of EPEC and EHEC (which produce and secrete proteins specifically to target host signalling) indicating that THP-1 macrophages respond to different strains of *E. coli* in a similar manner.

Much of the work in this thesis focuses on the adherent-invasive *E. coli* strain HM605. AIEC have been associated with the pathology of Crohn's disease and these bacteria are characterised by their ability to replicate in macrophages (Smith *et al*., 2013a). Indeed some reports have suggested that AIEC stimulate the production of high levels of pro-inflammatory cytokines from infected macrophages and this may contribute to the pathology of CD (Glasser et al., 2001; Lapaquette et al., 2010). While several molecular studies on another strain of AIEC, LF82, have identified genes that are required for the ability to replicate in macrophages (Bringer *et al*., 2005a; 2007), these genes are also present in *E. coli* that cannot replicate in macrophages. Other genomic based studies have attempted to define a set of genes or phenotypes that distinguish AIEC from commensal *E. coli* but these have failed to define any key set of virulence factors (Clarke *et al*., 2011; Desilets *et al*., 2015; Krause *et al*., 2011; Miquel *et al*.,

2010; Nash *et al*., 2010). Thus it appears that AIEC are not pathogens *per se* but opportunistic pathogens (or pathobionts) in the inflamed gut of CD patients. Work in this thesis showed that AIEC elicited a cytokine response from human macrophages that was similar to other *E. coli*. Moreover, the ability of HM605 to replicate in macrophages was cell line-dependent suggesting that permissive environmental conditions within the macrophages are required for AIEC replication. The nature of these conditions is not understood but this study has shown that there were only subtle differences in the post-phagocytic trafficking of HM605 compared to a non-pathogenic strains in the permissive macrophages cell line, J774A.1. Therefore AIEC do not appear to interfere with the normal macrophages phagocytic response to *E. coli*. However, HM605 does induce a higher level of IL-1β production in J774A.1 macrophages than other non-AIEC strains of *E. coli* (A. Thompson, unpublished data). Since AIEC require active metabolism to replicate in the macrophages, it may be that bacterial metabolism drives higher levels of IL-1β production. Indeed, recent studies in *S. enterica* Typhimurium have suggested that bacterial derived metabolites may be stimulators of the inflammasomes and act in a similar manner to vita-PAMPs (Wynosky-Dolfi et al., 2014). Previous work has shown that HM605 relies on glycolysis for intracellular metabolism and mutations in bacterial glycolysis result in lower levels of IL-1β production (A. Thompson, unpublished data). The current work has advanced these initial observations and has shown that microbial derived acetate may act as the signal for inflammasomes activation. Importantly, this phenomenon was also found to occur with another intracellular pathogen, *S. enterica* Typhimurium. Interestingly *Salmonella,* like HM605, metabolise using glycolysis in the macrophages (Bowden et al., 2009). Therefore acetate produced within the macrophages appears to act as a pro-inflammatory signal. Paradoxically, acetate produced by the microbiota has been reported to be anti-inflammatory (this study and (Tedelind et al., 2007)). Thus *E. coli* metabolism may be a double edged sword: in the gut lumen it may be beneficial and anti-inflammatory but when acetate production occurs inside host cells such as macrophages it is a pro-inflammatory. Therefore acetate production can provide spatial information to the host innate immune system.

UPEC can colonise the bladder in humans and are responsible for approximately 80% of all urinary tract infections (Ulett et al., 2013). Infections by UPEC results in considerable tissue damage that is caused by the actions of secreted toxins such as the

RTX toxin α-hemolysin (HlyA). Tissue damage results in the infiltration of phagocytes such as neutrophils and macrophages to the site of infection (Wiles *et al*., 2008b). Indeed some recent studies in the zebrafish infection model have suggested that phagocytes are targets for HlyA-mediated cytotoxicity (Wiles et al., 2009). Findings presented in this thesis show that HlyA-producing *E. coli* are highly cytotoxic to human macrophages. Cell death occurs via a number of different pathways including the inflammatory cell death called pyroptosis. Pyroptosis requires the action of inflammasomes and occurs in response to an invading pathogen. Different strains of *E. coli* have been shown to interact with different inflammasomes. For example, EPEC and ETEC have a type 3 secretion system (T3SS) that injects effectors into target cells and the rod and needle of these T3SS are sensed by inflammasomes containing NLRC4 (de Zoete et al., 2014). Additionally, viable but non-pathogenic *E. coli* are sensed by the NLRP3 containing inflammasome through interactions with bacterial mRNA (Sander et al., 2011). UPEC have recently been shown to activate the NLRP3 inflammasome and trigger cell death in a hemolysin-dependent manner in urothelial cells (Nagamatsu et al., 2015). In this study a similar finding is reported in macrophages although the identity of the inflammasome involved has not yet been determined. However interactions with the NLRP3 inflammasome may be a general feature of *E. coli* RTX toxins. Another RTX toxin, Ehx from EHEC has recently been shown to activate the NLRP3 inflammasomes in human macrophages (Cheng et al., 2015). Findings presented here also suggest that HlyA-induced macrophages cell death is both caspases-1-dependent and independent. The caspases-1-independent mechanisms of cell death have not been characterized in this study but may include CASP4 and/or necrosis/cell lysis.

An important finding reported here, and in other recently published studies, is the differential sensitivity of human and murine macrophages to HlyA. In this study it was shown that that murine macrophages are less sensitive to HlyA-induced cell death than human macrophages and this is in agreement with a previous study (Schaale et al., 2015). Furthermore studies with Ehx have reported different sensitivities of mouse and human NLRP3 inflammasomes to this toxin (Cheng et al., 2015). There are many putative explanation for this difference in sensitivity but primarily one has to consider that the immune systems in the two species are genetically different. For example, the human genome possess 10 TLRs while the mouse genome has 12 (Roach et al., 2005).

Furthermore, there are differences in the number and ligand sensitivity of NLR proteins between humans and mice; there are 22 human *NLR* genes and over 30 *Nlr* genes in mice (Ariffin & Sweet, 2013). Interestingly *Listeria monocytogenes* activates capase-1 in a process involving AIM2, NLRC4 and NLRP3 in murine macrophages (Wu et al., 2010) but only NLRP3 in human peripheral blood mononuclear cells (Meixenberger et al., 2010). Thus extrapolating findings from murine mouse models to humans must be taken in context of differences in the immune systems of the animals.

As professional phagocytic cells, macrophages can kill invading bacteria through phagocytotis (Underhill & Goodridge, 2012). Phagocytosis is a general response employed by macrophages to detect extracellular pathogens, leading to their engulfment and degradation. Furthermore, cytosolic bacteria can be targeted, contained and degraded by autophagy (Gong et al., 2012). The enteroinvasive *E. coli* (EIEC)-like strain *Shigella* is known to interact with and escape autophagy following escape from the phagosome into the cytosol (Ogawa et al., 2005). AIEC have been reported to interact with the autophagic machinery in epithelial cells and macrophages and defects in autophagy have been shown to enhance AIEC intracellular survival (Lapaquette *et al*., 2010; 2012b). UPEC also interact with the autophagy machinery in epithelial cells and macrophages and furthermore, defects in autophagy gene ATG16L1, due to a polymorphism that is associated with CD, enhanced the clearance of UPEC from macrophages and epithelium (Symington et al., 2015; Wang et al., 2012b). This is in stark contrast to the effects of ATG16L1 defects in AIEC clearance by macrophages (Lapaquette *et al*., 2012b; Symington *et al*., 2015). It is interesting that defects in the same gene confers two separate, opposing, phenotypes and suggesting that defects in autophagy may have different effects depending on the mucosal environment i.e. bladder or intestinal mucosa.

In this thesis we have shown that autophagy does not appear to be a key mechanism for the degradation of *E. coli* by macrophages. This is supported by data showing that autophagy is not required for clearance of UPEC (Symington et al., 2015; Wang et al., 2012a). Autophagy is normally a response to cytosolic bacteria and given that, with the exception of EIEC, *E. coli* do not escape into the cytosol, it may be that inflammasome activation plays a more important role in *E. coli* clearance.

Inflammasomes have been proposed to control the immune response to infection by sensing whether a bacteria is live or dead and pathogenic or non-pathogenic and mounting an appropriate immune response to each condition (Blander & Sander, 2012). In wild type murine macrophages infected with UPEC, there is a greater IL-1β release than those infected with non-pathogenic *E. coli* MG1655 suggesting greater inflammasome activation (Symington et al., 2015). Inflammasomes activation leads to pyroptosis which can protect against bacterial infection by exposing the bacteria to killing by secondary phagocytes such as neutrophils (Jorgensen & Miao, 2015). Findings in this thesis show that UPEC infection can result in inflammasome-dependent pyroptosis in human macrophages supporting the hypothesis that inflammasome activation is an important mechanism for bacterial clearance.

In conclusion, findings presented in this thesis support the hypothesis that AIEC are pathobionts that require a permissive niche in order to replicate. Furthermore, evidence is presented showing that inflammasomes are activated differentially depending *E. coli* strain. Metabolising *E. coli* elicit a greater inflammasome response than non metabolising *E. coli* and pathogenic *E. coli* elicit an even greater response resulting pyroptotic cell death. Furthermore, the inflammatory response to *E. coli* may be species dependent. Thus the findings here support the hypothesis that inflammasomes are key regulators of the innate immune response to bacteria.

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