Manipulation of macrophage programmed cell death pathways by enteropathogenic *Escherichia coli*.

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I hereby declare that the work presented in this thesis is my own, unless referenced appropriately. Any resources or external contributes have been clearly references, and a full bibliography can be found at the end of this thesis.

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Abstract

Enteropathogenic *Escherichia coli* (EPEC) is a human-restricted, clinically significant diarrhoeagenic pathogen that colonises the gut mucosa of hosts via the formation of characteristic attaching and effacing (A/E) lesions. To establish infection and cause disease, EPEC subverts a range of host cell processes through the injection of a suite of bacterial effector proteins into host cells via a Type 3 Secretion System (T3SS). EPEC virulence is critically reliant on the T3SS-delivered Translocated intimin receptor (Tir), which triggers actin polymerisation at the bacterial attachment site in intestinal epithelial cells. However, the manner in which EPEC effector proteins activate and subvert signalling pathways in human immune cells, such as macrophages, remains to be established. During infection the innate immune compartment of the intestinal mucosa plays a pivotal role in antimicrobial immunity, and activation of inflammasome signalling pathways is central to bacterial clearance. This has been established *in vivo* using the model A/E pathogen *Citrobacter rodentium.* Interestingly, although evolutionarily closely related to the enteric pathogens *Citrobacter, Salmonella* and *Shigella*, the EPEC T3SS needle and flagellin proteins evade detection by inflammasomes. Currently EPEC-induced inflammasome activation in human macrophages is poorly understood. Here I show that infection of human primary macrophages with EPEC rapidly induces NLRP3-inflammasome-driven pyroptosis in a strictly Tir-dependent manner. Mechanistically, Tir-induced actin polymerisation, either via Tir tyrosine phosphorylation and the Nck-N-WASP-ARP2/3 actin polymerisation pathway, or through Tir and the Nck-mimicking effector TccP, stimulated rapid caspase-4-dependent inflammasome responses. Notably, in contrast to caspase-4-driven pyroptosis induced by non-pathogenic *E. coli* or cytosolic LPS, both pyroptosis and cytokine processing in response to EPEC infection required NLRP3, ASC and caspase-1, and occurred independently of pannexin-1. This study identifies a surprising and indispensable role for both human caspase-4 and NLRP3 in detecting EPEC, and an essential role for pathological actin polymerisation by a bacterial effector in inflammasome activation.

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Abbreviations

2ME- β-mercaptoethanol

A/E - Attaching and Effacing

AAF - Aggregative Adherence Fimbriae

ADF - Actin Depolymerising Factor

aEPEC - atypical EPEC

Afa - Afimbrial adhesins

AIEC - Adherent-Invasive *E. coli*

AIM2 - Absent in Melanoma 2

AIP1 - Actin Interacting Protein 1

ALRs - AIM2-Like Receptors

ANOVA – Analysis of variance

ARP2/3 - Actin-related protein 2/3

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

ATP - Adenosine triphosphate

BFP - Bundle-Forming Pilus

BI-1 - Bax inhibitor-1

BID - BH3 Interacting- Domain death agonist

BMDM - Bone-Marrow Derived Macrophages

CARD - Caspase Recruitment and Activation Domain

CD14 - Cluster of differentiation 14

CFs - Colonisation Factors

CFU - Colony Forming Unit

cIAP - cellular Inhibitor of Apoptosis

Cif - Cycle inhibiting factor

CLRs - C-type Lectin Receptors

C-ring - Cytoplasmic ring

Cyto-D - Cytochalasin-D

DAEC - Diffusely Adherent *E. coli*

DAF - Decay-Accelerating Factor

DAMPs - Damage-Associated Molecular Patterns

DAPI - 4,6-Diamidino-2-phenylindole

DC - Dendritic Cell

DD - Death Domain

DED - Death effector Domain

DISC - Death Inducing Signalling Complex

DMEM - Dulbecco's Modified Eagle's Medium

DR - Death Receptor

EAEC - Enteroadherent *E. coli*

EAEC - Enteroaggregative *E. coli*

EAST-1 - EAEC heat-stable toxin 1

ECP – *E. coli* common pilus

Efa1 - *E. coli* factor for adherence 1

EhaA - EHEC autotransporter encoding gene A

EHEC - Enterohaemorrhagic *E. coli*

Ehx - EHEC toxin

EIEC - Enteroinvasive *E. coli*

EPEC - Enteropathogenic *E. coli*

ERK - Extracellular Signal-Regulated Kinase

EspFU - *E. coli* secreted protein F in prophage U

ETEC - Enterotoxigenic *E coli*

ETT2 - EHEC Type III secretion system 2

ExPEC - Extraintestinal Pathogenic *E. coli*

FADD - Fas Associated via Death Domain

FAK - Focal Adhesion Kinase

FASL - FAS Ligand

FCS - Foetal Calf Serum

GBP - Guanylate-Binding Protein

GEF - Guanine Nucleotide Exchange Factors

GFP – Green florescent protein

GlrR - Global regulator of LEE Repressor

GrlA - Global Regulator of LEE-Activator

GSDMD - Gasdermin D

HEPES - N-2-Hydroxyethylpiperazine-N-2-Ethane sulfonic acid

HGT - Horizontal Gene Transfer

hNAIP - Human neuronal apoptosis inhibitory protein

HUS - Haemolytic-Uremic Syndrome

IBS - Inflammatory Bowel Disease

IEC - Intestinal Epithelial Cell

IE-DAP - D-glutamylmesodiaminopimelic acid

IFNs – Interferons

IκBα - NF-κB inhibitor alpha

IκK - IκB Kinase

IL - Interleukin

IL1R - Interleukin-1 Receptor

iNOS - inducible Nitric Oxide Synthase

IRAK - IL-1R-Associated Kinase

IRF3 - Interferon regulatory factor 3

IRTKS - Insulin Receptor Tyrosine Kinase Substrate

ISGs - IFN Stimulated Genes

IPTG - Isopropyl β-D-1-thiogalactopyranoside

JNK - Jun N-terminal Kinase

LB - Lysogeny Broth

LBP - LPS-Binding Protein

LDH - Lactate Dehydrogenase

LEE - Locus of Enterocyte Effacement

Ler - LEE encoded regulator

LFP - Long Polar Fimbriae

LPS - Lipopolysaccharide

LRR - Leucine-rich repeat

MACS - Magnetic-Activated Cell Sorting,

Map - Mitochondria- Associated Protein

MAPK - Mitogen Activate Protein Kinase

M-CSF - Macrophage colony-stimulating factor

MDM - Monocyte–Derived Macrophage

MDP - Muramyl Dipeptide

MLKL - Mixed Lineage Kinase domain Like pseudokinase

MOI - Multiplicity Of Infection

MyD88 - Myeloid Differentiation primary response protein 88

NAIP - Neuronal apoptosis inhibitory protein

NACHT - Nucleotide-binding and oligomerisation domain

Nec-1 - Necrostatin-1

NF-κB - Nuclear Factor kappa-light-chain-enhancer of activated B cells

Nle - Non LEE Effector

NLR - Nucleotide-oligomerisation domain-Like Receptor

NO - Nitrous Oxide

NOD - Nucleotide-binding Oligomerisation Domain-containing protein

N-WASP - Neural Wiskott-Aldrich Syndrome Protein

OD - Optical Density

OMV - Outer-Membrane Vesicle

OM – Outer membrane

ORF – Open reading frame

PAI - Pathogenicity Island

PAMP - Pathogen-Associated Molecular Pattern

PBS - Phosphate buffered saline

PCR - Polymerase Chain Reaction

Pen-Strep - Penicillin Streptomycin

Per - Plasmid encoded regulator

Pet - Plasmid-encoded toxin

PFA - Paraformaldehyde

PFD - Pore Forming Domain

PI - Propidium Iodide

PMA - Phorbol Myristate Acetate

PMSF - Phenyl Methyl Sulfonyl Fluoride

PRR - Pattern-Recognition-Receptor

PYD - Pyrin Domain

qRT-PCR - Quantitative Reverse transcription PCR

RDEC - Rabbit Diarrhoeagenic *E. coli*

RIPK - Receptor Interacting serine/threonine-protein kinase

RIPK1 - Receptor-Interacting Kinase 1

RIPK3 - Receptor Interacting Protein Kinase 3

RLRs - RIG-Like Receptors

RNA – Ribonucleic acid

ROS - Reactive Oxygen Species

RPM - Revolutions per minute

RPMI - Roswell Park Memorial Institute medium

RT – Room Temperature

SDS-PAGE - SDS Polyacrylamide Gel Electrophoresis

SEM – Standard error of the mean

SH - Src Homology region

shRNA - short-hairpin RNA

SIEC – Synthetic injector *E. coli*

siRNA - small interfering Ribonucleic Acid

SLIC - Sequence- and Ligation-Independent Cloning

SPATE - Serine Protease Autotransporter

STEAEC - Shiga-Toxin producing Enteroaggregative *E. coli*

Stx - Shiga Toxin

T2SS - Type Two Secretion System

T3SS - Type Three Secretion System

T6SS - Type Six Secretion System

TAB2/3 - TGF-β activated and MAP3K7 binding protein 2/3

TAK1 - Transforming growth factor-β-activated kinase 1

TccP - Tir- cytoskeleton coupling protein

tEPEC - typical EPEC

Th - T helper cell

Th17 - T helper cell expressing IL-1

TIR - Toll-IL-1 Receptor domain

Tir - Translocated Intimin Receptor

TIRAP - TIR-domain-containing Adaptor Protein

TJ - Tight-Junction

TLR - Toll-Like Receptor

TNF - Tumour-Necrosis Factor

TNFR - Tumour Necrosis Factor Receptor

TRADD - TNF Receptor-Associated protein with Death Domain

TRAF6 - TNFR-associated factor 6

TRAILR1 - TNF-Related Apoptosis-Inducing Ligand Receptor 1

TRAM - TRIF-Related Adaptor Molecule

TRIF - TIR-domain-containing adaptor protein Inducing IFN-β

TSS - Transformation and Storage Solution

UTI - Urinary Tract Infection

VASP - Vasodilator-Stimulated Phosphoprotein

VB - Viromer Blue

WT - Wild Type

Introduction

Enteropathogenic *E. coli*

1.1. *Escherichia coli*

In 1885, the German paediatrician Theodor Echerich isolated and characterised a rod-shaped bacterium from infant stool (1). This was the first reported record of *Escherichia coli,* a Gram-negative facultative anaerobe of the Enterobacteriaceae family. Since its identification, it has become one of the most well-studied bacteria in existence, with the first genome sequence analysis of *E. coli* being reported in 1997. *E. coli* became the organism in which many of the fundamental aspects of life, including the genetic code, transcription, translation, and replication, were first resolved (2, 3). The continuous developments of molecular methods for investigating its biology have led to *E. coli* becoming a fundamentally important model organism in biology, with high prominence in academia, commercial genetic engineering, pharmaceutical production, and experimental microbial evolution (4). However, since 1945, *E. coli* has also been recognised as an important agent of disease, capable of causing both diarrhoeal and extraintestinal infections such as urinary tract infections (UTIs), sepsis, meningitis and respiratory infections (5, 6).

1.2. Diarrhoeagenic *E. coli*

Diarrhoeal illnesses are a significant public health risk and a leading cause of morbidity and mortality globally (7-9). Diarrhoeal diseases are associated with an estimated 1.3 million deaths annually, (10) with most occurring in resource-limited countries such as Africa and south-east Asia, where up to 25% of recorded deaths in young children are caused by acute gastroenteritis (11, 12).

E. coli is a principal constituent of the mammalian gut microbiome, and these commensal *E. coli* strains typically reside harmlessly within the host and rarely cause disease, except in immunocompromised hosts or in situations where the normal gastrointestinal barriers are breached. However, several highly adapted *E. coli* strains have acquired distinct virulence attributes and harbour an increased ability to cause diarrhoeal diseases (5). Data from the Global Enteric Multi-Center Study (GEMS), a study investigating the impact of paediatric diarrhoeal disease in sub-Saharan Africa and South Asia (13), illustrates the importance of diarrhoeagenic *E. coli.* Pathogenic *E. coli* is one of the predominant causative agents of moderate to severe diarrhoea among children in these areas, and enteropathogenic *E. coli* infections are associated high mortality rates.

Whilst all *E. coli* strains share a common genetic backbone, significant divergence has occurred between strains due to lineage-specific acquisition of genetic material as a result of Horizontal Gene Transfer (HGT). Pathogenic *E. coli* strains harbour large clusters of virulence genes termed Pathogenicity islands (PAIs) that are either located on plasmids or integrated into their chromosome. Therefore, the genomes of pathogenic strains are typically larger and more diverse than those of commensal *E.coli* isolates (14) (Table 1.1). Diarrhoeagenic *E. coli* isolates are have a core genome of approximately 2,200 genes and an open pan-genome consisting of more than 13,000 genes (15-17), which allows for greater genetic diversity (18). Although pathogenic *E. coli* isolates share many virulence strategies, the variations in acquired virulence determinants confer differences regarding virulence mechanisms and clinical symptoms. Diarrhoeagenic *E. coli* strains are therefore classified into eight well-described categories: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), Shiga-toxin producing enteroaggregative *E. coli* (STEAEC), diffusely adherent *E. coli* (DAEC), adherent-invasive *E. coli* (AIEC) and enteroinvasive *E. coli* (EIEC) (19, 20), with each pathovar having a district mechanisms of attaching to and exploiting host cells (Table 1.2 Reviewed; (20)). Three pathotypes (EHEC, EPEC and EIEC) employ a Type Three Secretion System (T3SS) to translocate bacterial effector proteins directly into the host cell to disrupt host cell processes and facilitate infection, whereas EAEC, DAEC and AIEC pathogenesis occurs independently of the T3SS (20). Although EAEC expresses two T3SS, the role of these in pathogenesis is yet to be established. In addition to grouping into pathotypes, *E. coli* strains are characterised by O (lipopolysaccharide, LPS) and H (flagellar) antigens where the O antigen defines serogroups and O:H defines a serotype (20).

Table 1.1: Genome sizes in pathogenic *E. coli*

Strain	Genome Size	Number of Protein Genes	Plasmids
E. coli K12	4646332	4226	0
Enteropathogenic *E. coli* (EPEC) 0127:H7 E69	4965553	4653	2
Enterohemorrhagic *E. coli* (EHEC) 0127:H7 EDL933	5528445	5449	1
Enterotoxigenic *E. coli* (ETEC) O139:H28	4979619	4997	6
Enteroadherent *E. coli* (EAEC)	5154862	4763	0
Extraintestinal pathogenic *E. coli* (ExPEC)	5132068	4725	0
Uropathogenic *E. coli* (UPEC) O6:K2:H1	5231428	5379	0

Table 1.2: Summary of Diarrhoeagenic *E. coli* pathotypes

Pathotype	Disease	Site of colonisation	Adhesins	Virulence Determining Secretion Systems	Toxins	Ref
EPEC	Infant diarrhoea	Small intestine	Intimin Bundle forming pili (BFP) Porcine attaching- and effacing associated (Paa) Long polar fimbriae (LPF) EspA filaments IrgA homologue adhesion (Iha) EhaA	T2SS T3SS T4SS T5SS		(21-23)
EHEC	Haemorrhagic colitis HUS. Food poisoning	Small intestine	Intimin Paa Toxin B (ToxB) Haemorrhagic coli pilus (HCP) is a type IV pilus *E. coli* factor for adherence (Efa)-1 *E. coli* immunoglobulin-binding protein (EibG) Outer membrane protein A (OmpA) EHEC autotransporter encoding gene A (EhaA)	T3SS T2SS T6SS	Stx1, Stx2, Hemolysin (Hly) EHEC toxin (Ehx)	(24-26)
ETEC	Infant diarrhoea (< 5yo) Travellers' diarrhoea	Small intestine	TibA (glycosylated autotransporter) Toxigenic invasion Loci A (Tia) Colonisation factors (CF) EtpA Porcine A/E associated adhesin (Paa)	T2SS	Heat-labile enterotoxin (LT) Heat-stable enterotoxin (ST) Cytolysin A (ClyA)	(27, 28)
EAEC	Travellers' diarrhoea Infant diarrhoea	Small intestine and/or colon	Aggregative adherence fimbriae (AAF) Flagellin Tia Afimbrial adhesions Heat-resistant agglutinin (Hra1+2) Dispersin	Two T3SS* Three T6SS*	EAEC heat-stable enterotoxin 1 (EAST 1) Shigella enterotoxin (ShET)1 Hemolysin E (HlyE) Plasmid-encoded Toxin (Pet) Pic (protein involved in intestinal colonisation)	(29, 30)
STEAEC	Food poisoning	Distal ileum, colon	AAF Iha		Shiga toxin (Stx2a) Pet Pic ShET	(20, 31)

DAEC	Acute diarrhoea (< 5yo) May contribute to Crohns disease	Small intestine, Urinary tract	Afimbrial (Afa) adhesins Fimbrial (Dr) adhesins		Sat	(14, 32, 33)
AIEC	Associated with Crohn disease- Persistent intestinal inflammation	Small intestine	Type 1 pili (FimH, Binds to CEACAM6, which is upregulated in Crohns patients) LPF	T633	High-temperature requirement A stress protein (HtrA)	(34, 35)
EIEC	Shigellosis-like Watery diarrhoea, Bacillary dysentery	Colon		T3SS	ShET 1/2	(36, 37)

1.2.1 Enteropathogenic *Escherichia coli* (EPEC)

Identified in 1945, EPEC is the first pathotype of *E. coli* (38) and is an important cause of fatal diarrhoea in infants from resource limited countries worldwide (19, 39). EPEC, along with EHEC, the murine pathogen *Citrobacter rodentium* (40), the rabbit diarrhoeagenic *E. coli* (RDEC) (41) and the emerging pathogen *Escherichia albertii* (42, 43) belong to a group of extracellular intestinal pathogens that utilise histopathological attaching and effacing (A/E) lesions to adhere intimately to host cell plasma membranes and induce localised cytoskeletal rearrangements to form distinct actin-rich pedestals beneath the site of attachment (14, 44). The genes necessary to trigger A/E lesions are encoded within a ~35kb chromosomal pathogenicity island (PAI) termed the locus of enterocyte effacement (LEE) (45, 46). The LEE is conserved among, and restricted to, A/E pathogens (47) and encodes a T3SS, a multiprotein complex that assembles across bacterial membranes and provides Gram-negative pathogens the ability to directly deliver a suite of bacterial effector proteins into the cytoplasm of host cells (discussed further in section 1.5).

The EPEC pathovars are typically subdivided into two categories, typical EPEC (tEPEC), which has the plasmid-encoded bundle-forming pilus (BFP), and atypical EPEC (aEPEC), which does not (48). Typical EPEC is a human-restricted pathogen and remains a principal cause of mortality in children from low-income countries, due to life threatening chronic diarrhoea and the cyclical relationship between tEPEC-infections and other conditions such as malnutrition, pneumonia, malaria and measles (49, 50). tEPEC is usually associated with an increased fatality rate in children aged 0-11 months (51). Although primarily associated with infections in low-income countries, tEPEC also remains a significant cause of sporadic infection of infants worldwide, with a recent epidemiological study finding tEPEC to be an important cause of diarrhoeal disease in England (52). However, aEPEC has recently been implicated as a more important cause of diarrhoea in industrialised countries (53, 54). In contrast to tEPEC, both animals and humans can be reservoirs for aEPEC (55). aEPEC are a diverse group of isolates that are often genetically more similar to other *E. coli* pathotypes than they are to tEPEC (56). However, due to the presence of the LEE and the absence of Shiga toxin (Stx) these strains are classified as EPEC. The current genetic definition for EPEC and EHEC is therefore based on molecular detection of the EAF plasmid (bfp+), the LEE PAI (eae+) and Shiga toxin genes (stx+) with typical EPEC defined as bfp+, eae+, stx-, atypical EPEC as bfp-, eae+, stx- and EHEC as bfp-, eae+, stx+. Phylogenetic analysis further categorises tEPEC into EPEC lineages 1-4 (57, 58).

Typical EPEC, with the prototypical isolate being EPEC O127:H6 E2348/69 (EPEC E69), is characterised by the presence of the virulence plasmid EAF (EPEC adherence factor). The EAF plasmid encodes for the type IV BFP which, via filamentous bacteria-bacteria interaction, mediates the formation of characteristic EPEC micro-colonies (5, 59). BFP mediates the initial interaction of EPEC with the apical surface of host enterocytes via binding to the N-acetyl-lactosamine moieties of cell surface glycoproteins (60, 61). Although both adherence and pathogenesis of tEPEC are significantly abrogated in the absence of BFP (62-64), both typical and atypical EPEC adherence is additionally regulated by a number of additional virulence factors. These include the *Escherichia coli* common pilus (62), flagella (65), long polar fimbriae (LFP), EspA filaments (66), and most importantly the LEE-encoded adhesin intimin and its cognate translocated intimin receptor (Tir) (67-69) (Tir and Intimin are discussed further in section 1.8).

Upon intimate attachment, EPEC secretes a broad repertoire of effectors into host cells via the T3SS, which subvert a number of host cell processes, including: inflammatory signalling, phagocytosis, regulation of the actin cytoskeleton, cell death, and protein trafficking (Table 1.3) (70). In conjunction with the T3SS effectors, EPEC also encodes the lymphocyte inhibitory factor (LifA), which enhances epithelial cell adherence *in vitro* (71), and inhibits the activation of lymphocytes (5). Some EPEC strains also encode a toxin from the serine protease autotransporter of Enterobacteriaceae (SPATE) family termed EspC. EspC is a non-LEE-encoded autotransporter that is secreted from the bacteria into the extracellular space by a type V secretion system (T5SS), but is translocated into epithelial cells by the T3SS (72-74). During infection, EspC functions to regulate the translocation pore of the T3SS (75).

The co-ordinated effect of virulence factors enables EPEC to colonise the gut lumen and cause disease. However, the exact mechanism by which EPEC, and its associated effector proteins, causes diarrhoea is not well defined. It has been proposed that disruption of the intestinal microvilli during EPEC infection may contribute to the loss of absorptive surfaces within the intestine resulting in diarrhoea (76). Other effector dependent mechanisms have also been implicated in the diarrhoeal process, including the T3SS effectors Tir, Map, EspF and EspG. These effectors regulate ion channels and disrupt tight junctions, enhancing intestinal permeability, which may lead to diarrhoea (77).

1.2.2 Enterohaemorrhagic *Escherichia coli* (EHEC)

First recognised as a cause of human disease in 1982 (78), EHEC infection is an important health concern in Europe, North America and many other regions of the world. Despite other enteric pathogens such as *Salmonella* or *Campylobacter* spp., having a higher infection rate worldwide than EHEC, EHEC infections have an increased mortality rate (79). Human infection caused by EHEC can have a wide range of symptoms, ranging from virtually asymptomatic cases to life-threatening infections (80). Upon infection, EHEC, like EPEC, forms characteristic A/E lesions on the surface of the gut epithelium, has a chromosomally encoded LEE PAI, and utilises an extensive repertoire of LEE and non-LEE encoded effector proteins that enhance its virulence (81). This suggests that EHEC O157:H7, the prototypical EHEC strain, shares a common ancestor with EPEC E69 (82, 83). However, despite this shared evolutionary background there are a number of fundamental differences between EPEC and EPEC strains.

Unlike tEPEC, which is a human-restricted pathogen, EHEC is a zoonotic pathogen, with the principal reservoir being the bovine intestinal tract (84). In cattle, EHEC colonisation is asymptomatic, however, EHEC infections in humans are responsible for outbreaks of bloody diarrhoea and haemolytic-uremic syndrome (HUS) worldwide (85-87). Genetically, while both EPEC and EHEC encode the LEE, the EHEC pathotype diverges from that of EPEC due to the absence of the EAF plasmid and the acquisition of the genes which encode Shiga toxin 1 and 2 (Stx1/2; also known as verocytotoxin. EHEC strains are therefore categorised as bfp-, eae+, stx+. Stx is an AB_5 toxin, which induces rapid host cell death during infection. In the absence of a designated secretory system, the release of Stx during infection is mediated by bacterial lysis in response to DNA damage and the resulting SOS response (88). As conventional antimicrobials that interfere with DNA replication, cell wall synthesis and cell viability typically trigger an SOS response in bacteria (89, 90), the use of these antibiotics during EHEC infections can promote the release of the Shiga toxin and exacerbate EHEC infections (91).

Clinical isolates of the prototypical EHEC strain *E. coli* O157:H7 also contain a putative virulence plasmid called pO157, which harbours 100 open reading frames (ORFs) (92). Some of the proteins encoded on pO157 include the EHEC toxin (Ehx) a pore-forming haemolysin and pro-apoptotic toxin (26, 93), a catalase-peroxidase (*katP*) (94), a type II secretion system (T2SS; *etp*) (95), a serine protease (*espP*) (96), a putative adhesin (*toxB*) (97), and a zinc metalloprotease (s*tcE*) (98). Currently, however, the role of pO157 in EHEC pathogenesis has not been established.

As EHEC lacks the EAF plasmid, the mechanism of its initial attachment to host cells during infection is not fully understood. EHEC possesses 16 potential fimbria-like operons (99), a type IV pilus, called the haemorrhagic coli pilus (100), the LifA-like Toxin B (ToxB), the *E. coli* factor for adherence 1 (Efa1), the outer membrane protein A (OmpA), and the autotransporter *E. coli* immunoglobulin binding protein G (EibG), all of which are thought to contribute to initial attachment. However, these have not been extensively studied (101, 102).

Unlike initial attachment, the mechanism involved in the intimate attachment of EHEC to host cells has been extensively characterised. Similarly to EPEC, intimate attachment occurs through interactions between intimin and Tir, although the mechanism of pedestal formation by EHEC is mechanistically distinct from that of EPEC (See section 1.9). EHEC intimate attachment is also enhanced by the interaction of intimin with nucleolin, a host cell encoded surface-localised intimin receptor, which is upregulated in response to Stx2 (103).

Although there is a high degree of conservation between the LEE-encoded effector proteins of EPEC and EHEC pathotypes, typically the repertoire of T3SS-effector proteins found in EHEC strains is greater than that of EPEC strains, with EHEC expressing approximately twice as many effectors compared to the 21 commonly found in EPEC (104, 105).

1.2.3 Citrobacter rodentium

Citrobacter rodentium is the causative agent of transmissible murine colonic hyperplasia, a disease that is typically associated with infantile sucking mice (106, 107). Infections caused by *C. rodentium* infections in mice can present a broad clinical spectrum ranging from mild diarrhoea to rectal prolapse and host mortality (108), with the outcome of infection usually depending upon the mouse sub-strain being infected (109). This range of symptoms has been attributed to the different compositions of the intestinal microbiota between strains, which is thought to alter the immune response to *C. rodentium* infection, increasing the susceptibility of some mouse strains to infection (110-113).

The ability to study EPEC and EHEC pathogenesis *in vivo* is limited by the fact that mice are inherently resistant to infection (109, 114). Therefore, as a murine gastrointestinal pathogen, *C. rodentium* is commonly used as a model to investigate the *in vivo* mechanisms of pathogenesis of the human pathogens EPEC and EHEC (115, 116). *C. rodentium* is a member of the A/E family of bacterial pathogens, which utilise characteristic A/E lesions to intimately adhere to host intestinal

epithelial cells during infection. A/E lesion formation results in the effacement of the gut microvilli and reorganisation of the actin cytoskeleton to form pedestal-like extensions (110, 117).

Within A/E pathogens, all the virulence genes responsible for lesion formation are encoded by the LEE pathogenicity island. Although the LEE of *C. rodentium* differs in gene sequence and organisation from that of EPEC and EHEC (118), the 41 open reading frames (ORFs) within the LEE are highly conserved (118). These encode a number of virulence factors, including the T3SS and secreted effector proteins, that play a vital role in A/E pathogenesis. The functional conservation of virulence strategies between A/E pathogens, therefore, make *C. rodentium* a physiologically relevant model to study pathogen–host interactions *in vivo* (108, 116, 119-121).

C. rodentium deletion mutants have been instrumental in identifying and characterising the role of various LEE encoded and non-LEE encoded effector proteins in enteropathogen pathogenesis (122). Furthermore, *C. rodentium* infection of mice has become an excellent tool for the analysis of host responses to A/E pathogens, which is an important step in the development of novel treatment strategies. The *C. rodentium* mouse model has been at the heart of many fundamental discoveries including the function of the noncanonical inflammasome pathway in response to enteric pathogens (123-126), the role of CD4(+) T-helper cells (127, 128), and the action of proinflammatory cytokines, which provide critical signals for the initiation of innate and adaptive immunity during enteropathogenic infection (129-133). More recently, enteric bacterial infections have also been linked to Inflammatory Bowel Diseases (IBD) (120, 134-136), with *C. rodentium* infections being used to model several important human intestinal disorders, including Crohn's disease, ulcerative colitis and, colon tumorigenesis (110, 135, 137-139).

1.3. The Locus of Enterocyte Effacement (LEE)

In A/E pathogens a number of virulence factors, including the T3SS, that are critical for colonisation and pathogenicity are encoded by a 35 kb chromosomally located PAI termed the LEE (140) (Figure 1.1). The LEE contains the genes required to form attaching and effacing lesions, which enable intimate attachment of bacteria to host enterocytes by inducing a signalling cascade culminating in microvilli destruction, and localised actin polymerisation. McDaniel and colleagues first defined the LEE in EPEC E2348/69 (47). It contains 41 genes organised in five operons (LEE1 to LEE5), a bicistronic operon (*grlAB*) and four monocistronic units (46, 141). LEE1, LEE2, and LEE3 harbour the *esc* and *sep* genes that encode the major structural components of T3SS. LEE1 also encodes

the LEE-encoded regulator (*ler*) gene, this encodes Ler, a DNA binding protein which functions as the central transcriptional regulator of both the LEE region and non-LEE pathogenicity islands (142). The LEE5 operon contains intimin (*eae*) and *tir* genes, and the Tir chaperone CesT. The LEE4 operon encodes the effector proteins EspB, EspD, EspF, and the T3SS needle and filament proteins EscF and EspA (143-145). Multiple *in vivo* infection studies have demonstrated the essential nature of both the T3SS and LEE-encoded effector proteins during infection of A/E pathogens (122, 146-148).

The comparatively lower G+C content of the LEE (38.3%), in contrast to that of the *E. coli* chromosome overall (50.8%), suggests that it was originally acquired by horizontal gene transfer during the evolution of pathogens from their non-pathogenic ancestors (44, 118, 149). The core LEEs of EPEC and EHEC share 93.9% nucleotide identity, although the EHEC LEE has 13 additional ORFs at the 5′ end of the locus (150). Furthermore, comparisons of the EPEC and EHEC LEEs reveal that the rate of sequence divergence is inconsistent along the locus, with most genes having greater than 95% sequence conservation, whereas some genes have a high degree of variance. For example, the *tir* gene only has 33.52% identity between EPEC and EHEC (150). Consistent with this variation in Tir, studies have previously demonstrated that the introduction of Tir from EPEC to a non-pathogenic *E. coli* K12 strain is sufficient to induce Tir-mediated actin polymerisation, whereas Tir from EHEC is not (151, 152). The LEE is fundamental to EPEC virulence, and therefore the expression of the five LEE operons is highly regulated, with numerous transcription factors regulating expression in response to a variety of intra- and extracellular stimuli.

1.4. Regulation of EPEC Locus of enterocyte effacement

Expression of the LEE is a highly regulated process that is dependent on several diverse factors such as; quorum sensing, environmental changes, and both transcriptional and post-transcriptional regulation. The LEE encodes its own transcriptional regulators, including; Ler, GrlA (Global Regulator of LEE-Activator), and GrlR (Global Regulator of LEE-Repressor) (153). The principal regulator responsible for controlling the expression of LEE genes is the Ler, a 15 kDa protein encoded at the start of the LEE1 operon. Below 37°C, the global regulator histone-like nucleoid structuring protein (H-NS) inhibits the transcription of the LEE virulence locus by binding to A-T rich regions and creating loops in the DNA structure, suppressing expression. However, at temperatures above 37°C, Ler functions as an HNS-like regulatory protein, and directly suppresses

the activity of H-NS resulting in its release from the DNA strand enabling gene expression of LEE2-5 (142). Ler expression is essential for LEE-gene expression, and thus EPEC virulence (122, 142). Interestingly, Ler can neutralise the H-NS located on LEE promoters (154, 155), but not on its own promoter that is also repressed by H-NS (156). Ler, in fact, acts as a negative regulator of its own expression (157, 158).

Two additional regulators are therefore required for Ler expression, GrlA and GlrR, which are encoded in the LEE bicistronic *grlAB* operon, and work antagonistically to regulate Ler expression (122, 159). GrlA is a positive regulator of the LEE and can activate LEE gene expression by directly binding to the LEE1 promoter (160, 161). GrlR represses LEE gene transcription by binding to and sequestering GrlA, preventing its binding to the LEE1 promoter and limiting downstream *ler* expression (159). GlrA activity is further regulated by external cues from host cells during the infection process (162), these autoregulatory mechanisms facilitate the dynamic expression of the LEE during infection. This mechanism may reflect the need for EPEC to regulate gene expression within different environments during infection, thus maximising virulence gene expression to promote colonisation, but suppressing expression of these potentially immunostimulatory factors until required.

Regulation of the LEE is essential for EPEC pathogenesis, therefore the expression of its genes is under the control of a myriad of additional transcriptional regulators encoded throughout the chromosome. More than 40 additional regulators of LEE transcription have already been identified (163). The external environmental signals that activate these regulators include carbon sources such as butyrate and fucose (164, 165), quorum sensing (166-168), and host hormones (169). In addition to external cues, internal metabolic status and the translocation of effector proteins by the T3SS further regulates EPEC virulence gene expression (162, 170, 171). Collectively these factors work to repress virulence gene expression whilst in the lumen/mucus layer, but induce transcription as the bacterium moves through the mucus layer and closer to the epithelium. Considering the many different regulatory mechanisms controlling expression of the LEE, it is unsurprising that its regulation is central to virulence (166, 170, 172).

As the expression of EPEC virulence factors is highly regulated and dependent on multiple host-derived signals, it is important to consider this regulation during *in vitro* infection models. It has previously been demonstrated that EPEC virulence gene expression and secretion takes place in conditions similar to those found in the gastrointestinal tract. Therefore, expression is maximal at

37˚C, pH 7, and physiological osmolarity (173-175). Furthermore, the acidic nature of the stomach contents is rapidly neutralised upon its entry into the small intestine by high levels of sodium bicarbonate which are present in the pancreatic juices and released by the intestinal mucosa (176). Therefore, during EPEC infection, sodium bicarbonate may function as a signal to indicate passage from the stomach into the small intestine, the site of EPEC host cell interactions. Consistent with this, the presence of bicarbonate ions (HCO_3^-) in culture medium has previously been shown to significantly increase EPEC LEE-encoded gene expression (147). In addition, maximal EPEC effector secretion requires the presence of calcium and $Fe(NO_3)_3$, which enhance the expression of the LEE-encoded genes via activation of the Ler master regulator (175-177).

Therefore, culturing EPEC in Dulbecco's Modified Eagle Medium (DMEM) which contains sodium bicarbonate, calcium and $Fe(NO_3)_3$, can stimulate physiological effector secretions during *in vitro* infection models (174). During *in vitro* infections, optimum expression of LEE virulence genes is observed when EPEC is grown to mid-log phase in DMEM, at 37˚C, 5% $C0_2$ (175), and this has previously been shown to induce a fivefold increase in LEE transcription compared with growth in rich medium, such as Lysogeny Broth (LB) (178).

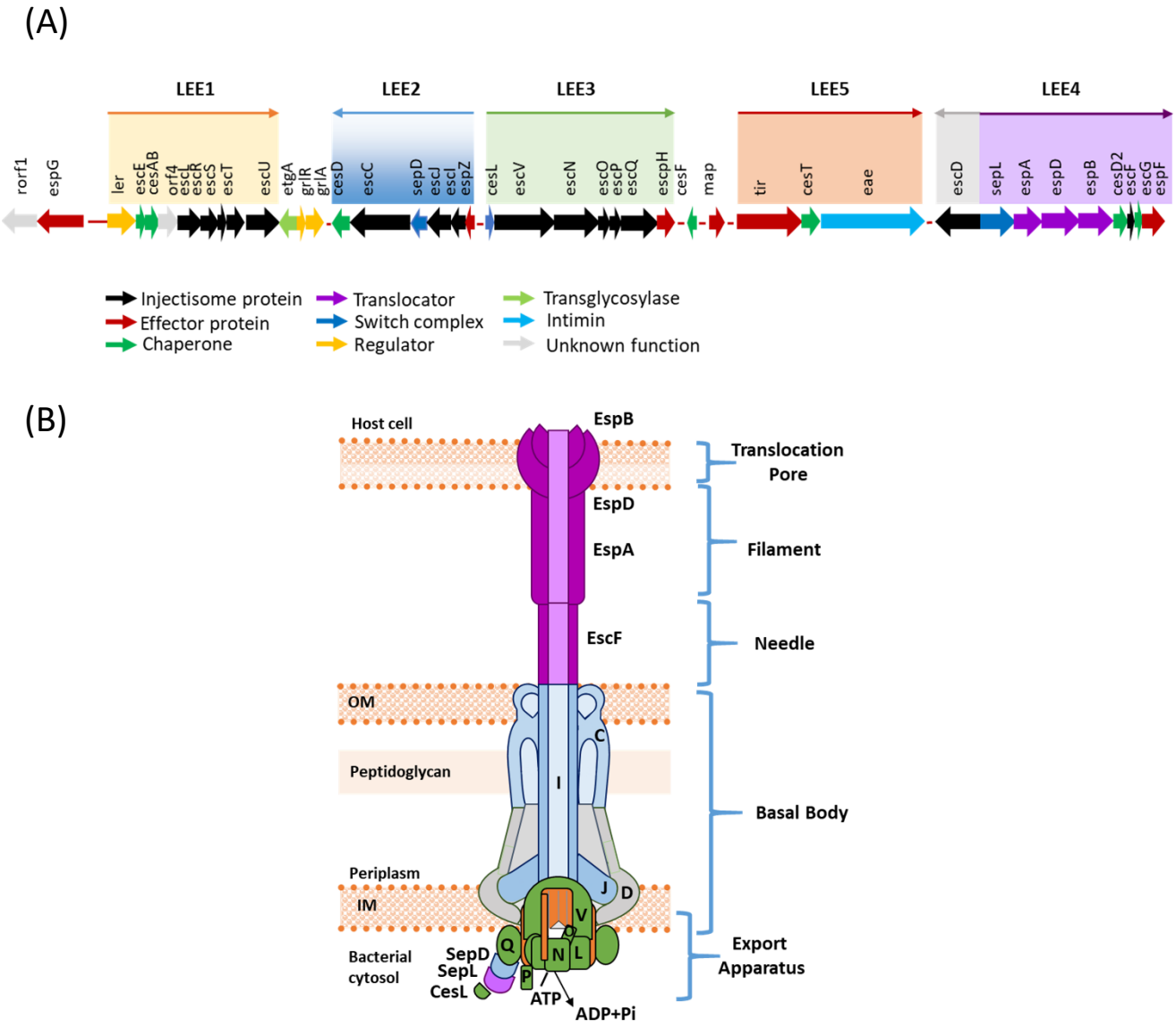

Figure 1.1. The LEE and T3SS of EPEC

(A) Schematic representation of LEE operons (LEE1-LEE5) of EPEC E2348/69 0127:H6 strain Genes are coloured according to their function.
(B) Structural organisation of the EPEC T3SS. The T3SS consists of: A basal body made up of EscD, EscJ, EscI and EscC; Export apparatus consisting of EscR, EscS, EscT, EscU, and EscV, a needle structure EscF, a filament comprised of polymerised EspA subunits, a translocation pore made of EspB and EspD, and the cytoplasmic EscN ATPase complex which provides the energy for effector translocation.

1.5. The Type III Secretion System (T3SS)

Protein secretion has a pivotal role in bacterial infection, as bacteria must translocate a number of effector proteins from the bacterial cytosol into the infected cells to modify numerous host cell responses. However, transporting proteins across cellular membranes and into host cells is a challenging biochemical feat. To achieve this, bacteria have evolved seven dedicated secretion systems (type I to type VII, reviewed; (179, 180)). T3SS are protein transport nanomachines that are found in many Gram-negative bacterial pathogens including; A/E pathogens, *Salmonella* spp., *Yersinia* spp., *Pseudomonas* spp., and *Chlamydia* spp., among others. The ability of the T3SS to translocate effector proteins into target eukaryotic cells is essential to EPEC virulence. These effectors moderate multiple diverse cellular functions that contribute to bacterial survival and colonisation (Table 1.3).

The EPEC injectosome consists of more than 20 different proteins that oligomerise in a coordinated manner to form a complex nanomolecular machine of about 3.5 MDa (181, 182). The injectisome spans the bacterial inner and outer membranes and host cell membrane, and is comprised of several distinct substructures, including a cytosolic ATPase complex, a cytoplasmic ring (C-ring), an inner membrane export apparatus, a basal body and a translocation pore (Figure 1.1).

The basal body consists of three membrane ring structures that are embedded in the bacterial inner and outer membranes creating a channel through the periplasm (183). Situated at the base of the basal body is the export apparatus, which is assembled from five membrane proteins, namely EscR, EscS, EscT, EscU, and EscV (122, 184). This membrane-embedded complex is critical to T3SS function and contributes to the recruitment and regulation of substrates into the injectisome (185, 186). Directly beneath the export apparatus is the ATPase complex, which consists of the ATPase EscN, EscL and the stalk protein EscO, all of which are important for T3SS function. EscN, which is located at the base of the needle complex, energises the secretion process by releasing substrates from their cognate chaperone and unfolding them for secretion. Collectively, these components constitute the sorting platform for substrate secretion (187, 188).

The inner rod is formed by oligomerisation of the EscI protein, which forms a short structure that lines the inside of the basal body rings (189). The rod protein anchors the needle to the

basal body, where it extends to the external milieu. The needle complex is comprised of multiple copies of the EscF protein that, upon oligomerisation, form a hollow helical tube (145), which is required for the secretion of all T3SS substrates and hence for virulence (122). EscF associates with the filament protein EspA (145) creating a channel between the bacterial cytoplasm and the host cell. The EPEC T3SS differs from that of other Gram-negative pathogens, due to the presence of this proteinaceous EspA filament that extends the length of the injectosome from 40-70 nm to approximately 90 nm, although in can extend up to 600 nm in length (190). The tip of the EspA filament interacts with two hydrophobic translocators, EspB and EspD to form the translocon (191, 192). EspB and EspD are inserted into the host cell plasma membrane, where they oligomerise to form the translocator pore. This establishes a conduit between the bacterial cytosol and host cell cytoplasm, enabling the direct delivery of effector proteins (192-194).

The LEE-encoded T3SS apparatus is highly conserved between A/E pathogens. However, although EPEC only encodes a single T3SS, EHEC encodes an additional T3SS termed the EHEC Type III secretion system 2 (ETT2). Although frameshift mutations within the ETT2 inhibit its functionality, the accessory regulatory factors associated with the ETT2 may impact the expression and regulation of the EHEC T3SS (195).

1.6. EPEC effector proteins

A/E pathogens encode a number of secreted effector proteins that promote virulence. Seven effector proteins are located on the LEE-PAI and are conserved between A/E pathogens. However, A/E pathogens also encode a repertoire of addition effectors located outside of the LEE, termed non-LEE-encoded effectors (Nle), which are encoded on prophages or other integrative elements (44, 81, 122). The prototypical EPEC E2348/69 strain encodes 21 putative LEE and non-LEE effector genes (104), compared to 28 in EPEC B171-8, 40 in EPEC E22 (rabbit-EPE), and more than 50 in EHEC O157:H7 (105). Table 1.3 contains a summary of all currently recognised functional T3SS effector proteins from EPEC E2348/69 including their PAI, host targets and biochemical activity.

EPEC effectors are hierarchically secreted and translocated into host cells, with Tir being the initial effector injected in to host cells, followed by EspZ, EspF, EspH, EspG, and Map (196, 197). The precise order enables different effectors to function at different stages of infection (197-199). This secretion hierarchy is regulated by a number of factors, including the differential expression of effectors and the relative abundance of their designated effector chaperones. Most T3SS

secreted proteins require cognate chaperones for secretion (200, 201). Chaperone–substrate complexes are targeted to the sorting platform at the base of the T3SS where the chaperone is cleaved, and the substrate is unfolded by the EscN ATPase before secretion (202). A prominent example of this is the Tir chaperone CesT, which binds to the T3SS ATPase EscN, bringing Tir into proximity of the T3SS to facilitate secretion (203).

Table 1.3: Summary of Effector Proteins of EPEC O127:H6 E2348/69

Effector	PAI	Cellular Location	Targeted Host Pathway	Cellular function	Host Binding Partners	Biochemical Activity/ Functional Motif	Homologue	Ref
Tir	**LEE**	Plasma membrane	Actin Cytoskeleton	Actin rearrangement and intimate attachment.	NcK, IRSp53 α-actinin, cortactin Cytokeratin-18	SH3-Binding		(204-207)
		Cytoplasm	Inflammatory Signalling	Targets TRAF2 for proteasome degradation; inhibits TRAF6-mediated NF-κB activation in an ITIM-dependent manner	SHP-1 SHP-2 SHIP2 PI3K,CK18, Talin, Vinculin,, 14-3-3tau			
Map	**LEE**	Mitochondria	Cell death	Tight-Junction (TJ) Disruption, Mitochondria Dysfunction.	EBP50 (NHERF1) NHERF2 Claudin-1	Rho GTPase modulation PDZ-binding domain MTS WxxxE	IpgB2 SopE	(208, 209)
		Plasma-membrane	Actin-Cytoskeleton	Filopodia formation, Microvilli effacement, Invasion of non-polar cells.	Cdc42			
EspF	**LEE**	Mitochondria Cytoplasm	Cell Death	Mitochondrial dysfunction	ABCF2	PRR SH3 Binding N-WASP and SNX9 binding domains MTS		(210-212)
		Apical and lateral membranes	Actin Cytoskeleton	TJ Disruption, Microvilli elongation, SGLT- inactivation, Pedestal maturation Inhibition of NHE3 activity, Membrane remodelling, N-WASP activation.	ZO-1/ZO-2 Profilin Arp2/3 Cytokeratin18 Sorting nexin 9 N-WASP 14-3-3 Mito protein			
EspB	**LEE**	Cytoplasm, Plasma-membrane	Actin Cytoskeleton Phagocytosis	Translocation Pore Formation, Anti-phagocytosis, Microvilli effacement, Actin disruption.	Antitrypsin A-Catenin Myosin-1c		IpaC YopD	(213-216)

EspG	**LEE**	Cytosol Golgi	Actin Cytoskeleton Microtubules Protein Trafficking	Disruption of microtubules, TJ disruption, Paracellular permeability, Stress Fibre formation, Inhibiting ARF GTPase signalling, Inhibition of endomembrane trafficking, Inhibition of protein secretion, Ion channel disruption.	Tubulin, Arf1/6, PAK1/2/3, Rab35	TBC-like Rab GAP	VirA	(217-220)
EspH	**LEE**	Plasma-membrane – Localises to Pedestals	Actin Cytoskeleton Phagocytosis	Rho-GTPase modulation, Inhibition of FcγR-mediated phagocytosis, Regulates intimate attachment and pedestal formation, Facilitates caspase-3 activation and apoptosis.	DH-PH domain of RhoGEFs			(221, 222)
EspZ	**LEE**	Plasma-membrane	Cell Death	Enhances β1-integrin and FAK signalling, Inhibition of apoptosis, Maintenance of mitochondrial membrane potential, Maintenance of focal adhesion. Regulation of T3SS secretion	CD98 TIM17b			(223, 224)
NleA (EspI)	**PP6**	Golgi Plasma-membrane	Protein Trafficking	TJ disruption, Disruption of protein trafficking. Inhibition of COPII-dependent protein export from the ER.	Syntrophin, Sec24, MALS3, PDZK11, SNX27, TCOF, NHERF1/2 MAGI-3, SAP97, SAP102, PSD-95	PDZ1		(225, 226)
		Cytosol	Inflammatory Signalling	Interrupts de-ubiquitination and activation of NLRP3; Reduces the maturation and secretion of IL-1β,	NLRP3			

NleF	**PP6**	Mitochondria Cytosol	Cell Death Inflammatory Signalling	Inhibition of intrinsic and extrinsic apoptosis, Inhibition of caspase-4 and the non-canonical inflammasome pathway, Activation of NF-κB signalling.	Tmp21 Caspase-4,8 and 9			(227-230)
NleH2	**PP6**	Cytosol	Cell Death Immune Signalling	Counterbalances the effect of NleH1; regulates MAPK activity by enhancing AP-1-dependent transcription.	RPS3	Serine/Threonine kinase	**OspG**	(231-234)
NleH1	**PP2**	Cytosol	Cell Death Immune Signalling	Enhancement of RPS2 nuclear translocation, Suppresses expression of the RPS3-dependent subset of NF-κB and MAPK induced genes, Inhibits caspase-3 activation and intrinsic apoptosis, Suppresses ERK1/2 and p38 activation.	RPS3 V-Crk sarcoma virus CT10 oncogene-like protein Bax Inhibitor-1	Serine/Threonine kinase	OspG	(231-233, 235, 236)
EspJ	**PP2**	Cytosol Mitochondria	Phagocytosis	Inhibition of FCγR-mediated and CR3-mediated trans-phagocytosis Regulates non-receptor tyrosine kinases	Non-receptor tyrosine kinases	Amidation and ADP-ribosylation	SboC HopF	(237-239)
NleD	**PP4**	Cytosol	Immune Signalling Cell Death	Cleaves JNK, p38 as a metalloprotease, Inhibits IL-8 production, Cleaves and inactivates the p65 subunit of NF-κB, Suppresses death receptor-mediated apoptosis.	p38, p65 JNK	Zinc Metalloprotease	HopAP1, HopH1	(240, 241)
NleC	**PP4**	Cytosol Nucleus	Immune Signalling	Cleaves the NF-κB subunits p65, p50, c-Rel and p300, Reduces activity of p300 and RPS3, Inhibits p38 phosphorylation, Inhibits MAPK activation.	p65, p50, c-Rel Iκβ p300	Zinc metalloprotease	AIP56	(240, 242-245)

NleG	**PP4**	Cytosol		Unknown cellular function	UBE2D2 E2 ligase	U-box E3 ubiquitin ligase	STY1076	(246)
NleI	**PP4**	Cytosol		Unknown cellular function				(247)
NleB1/2	**IE6**	Cytosol	Immune Signalling, Cell Death	NF-κB inhibition, Glycosylation of FADD and TRADD death domains, Prevents death receptor signalling, Inhibits TRAF2 polyubiquitination, Inhibits caspase-8 activation and extrinsic apoptosis.	GAPDH, FADD, TRADD, RIPK1	Glycosyltransferase activity	SseK2	(248-250)
EspL	**IE6**	Pedestal	Cell Death Inflammatory Signalling	Intimate attachment and pedestal formation, Enhances F-actin bundling activity of Annexin 2, Cleaves RHIM proteins, Inhibits necroptosis and inflammation.	Annexin 2 RIPK1, RIP3, TRIF, ZBP1/DAI	Cysteine Protease	OspD	(251, 252)
NleE1	**IE6**	Nucleus, Cytosol	Inflammatory Signalling	Inhibits polymorphonuclear transepithelial migration, Inhibition of p65 and c-Rel to inhibit NF-κB signalling, Reduced IL-8 expression, Modifying the NZF domain of Tab2/3.	TAB2/3, ZRANB3	S-adenosyl-l-methionine (SAM)-dependent Cysteine methytransferase	OspZ	(253-256)
NleE2	**IE2**	Not translocated into cells						(256)

Effector manipulation of the actin cytoskeleton

1.7. The actin cytoskeleton

Actin is one of the most ubiquitous proteins in eukaryotic cells, and many critical cellular activities are reliant on the dynamic remodelling of the actin cytoskeleton. Maintenance of cell polarity and the formation and stability of surface extensions including lamellipodia, microvilli, or filopodia critically depend on modification to actin networks. Mammalian actin exists in two principal states of organisation: the monomeric, globular G-actin or the polymerised, filamentous form (F-actin). The transition between these two forms of actin is highly regulated by a number of signalling, scaffolding and actin-binding proteins (ABPs). Recently, various components associated with the actin cytoskeleton have been shown to play an important role in innate immunity and host defence (257). Due to its conserved nature, relative abundance and anti-microbial properties, actin and its regulating factors are preferred targets of bacterial effectors, which subvert the host actin cytoskeleton to facilitate infection (258, 259). A number of EPEC effector proteins target the host cytoskeleton, either directly or through modification of host small GTPases (Table 1.3).

One hallmark of EPEC and EHEC infections is the induction of histopathological A/E lesions on host cells, characterised by intimate attachment of the bacterium to the host epithelium and localised effacement of the microvilli (143). While remaining extracellular, both EPEC and EHEC secrete T3SS effector proteins into host cells that subvert the cytoskeletal actin network inducing localised actin assembly at the site of attachment. In cultured cells, this actin accumulation is visualised as raised pedestal-like structures underneath adherent bacteria (260). EPEC has derived an efficient way to ensure that this critical step in colonisation occurs by secreting its own receptor Tir into host cells, where it localises to the host cell plasma membrane and functions as a receptor for the EPEC outer membrane protein intimin (261).

1.8. Translocated intimin receptor (Tir)

In 1992 Rosenshine *et al*. first demonstrated that during infection EPEC triggered the tyrosine phosphorylation of a 90 kDa protein that functioned as a receptor, which enabled the intimate attachment of EPEC to host cells (262). This protein was initially termed Hp90. However, subsequent research revealed that Hp90 was not a host-derived receptor, but the tyrosine

phosphorylated form of the 78 kDa EPEC LEE-encoded secreted effector protein Tir (263). This concept, by which EPEC actively secretes its own receptor into host cells was later found to be conserved among all A/E pathogens and is essential for their virulence (181, 264-267).

Following translocation by the T3SS, cytosolic Tir spontaneously inserts into the host cell plasma membrane (268), adopting a distinct topology composed of a extracellular loop containing an intimin-binding domain (IBD) (residues 255–364), and two intracellular domains; the N-terminal domain (residues 1–233) and C-terminal domain (residues 385–550). These domain are linked by two transmembrane helices (residues 234–254 and 365–384) (268, 269). In this conformation, the extracellular domain is available to bind the EPEC outer-membrane protein intimin (270), facilitating intimate attachment. The N- and C-terminal domains remain within the host cell cytoplasm and can interact with a diverse array of host proteins.

Intimin is a 94 kDa outer membrane protein encoded by all A/E pathogen. Intimin, similarly to Tir, is encoded by the LEE PAI (*eae*), and its expression is highly regulated. After transcription, intimin is exported to the periplasm and is inserted into the bacterial outer membrane. The C-terminus of intimin extends from the bacterium and directly interacts with Tir. More recently the cocrystal structure of this intimin-Tir interaction (271), and mutagenesis analysis of the binding interface (272), have provided a more detailed insight into the molecular mechanisms of this interaction.

1.9. Tir-induced actin polymerisation

The T3SS effector Tir not only acts as the receptor for intimin, but upon intimin binding mediates protein signalling within host cells, and induces localised actin polymerisation. Tir-induced actin polymerisation requires intimin mediated Tir clustering to initiate the downstream signalling cascade. Intimin is formed of three structural domains; a flexible N-terminal domain, a central membrane-integrated domain and a Tir-binding C-terminal domain. The central beta-barrel membrane-anchoring domain is responsible for intimin dimerisation, and this intimin dimerisation is believed to influence Tir interactions by altering the binding stoichiometry and facilitating a 2:1 binding model, in which intimin binding intrinsically generates Tir clustering within the host cell membrane (273).

More recently, the mechanism by which Tir signalling facilitates the remodelling of the host cytoskeleton and the molecular architecture of the pedestals has been investigated. EPEC Tir driven

actin polymerisation relies on two conserved tyrosine residues within the intercellular C-terminal domain, Tyrosine 474 and Tyrosine 454 (Figure 1.2). The essential role of these tyrosine residues in actin pedestal formation was demonstrated by point mutations which abrogated actin polymerisation (260, 274). These tyrosines differentially contribute to actin polymerisation and utilise distinct signalling cascades to recruit the actin nucleation machinery.

1.9.1 EPEC Tir Tyrosine-474-dependent actin polymerisation

Actin polymerisation downstream of tyrosine (474) is the principle pathway utilised by the prototypical EPEC 2348/69 strain. Upon intimin-induced clustering EPEC-Tir is phosphorylated on Y474 by redundant host non-receptor tyrosine kinases (275-278). This tyrosine phosphorylation is critical for EPEC pedestal formation but does not play a role in either the membrane insertion of Tir or intimin binding (263). The identity of the host tyrosine kinases responsible for this phosphorylation remains unclear. Initially, the Src family kinase c-Fyn was reported to be necessary for EPEC pedestal induction (275), however subsequently roles for Abl family kinases were identified in infections which replicated physiological conditions (276). This observed redundancy in the repertoire of kinases able to phosphorylate Tir is reinforced by the fact that only non-specific tyrosine kinase inhibitors can inhibit pedestal formation during EPEC infections effectively (277). Recently, the EPEC effector proteins EspJ was also shown to ADP-ribosylate and inhibit multiple Src, Abl, Csk, Tec, and Syk non-receptor tyrosine kinase families (279). As Tir-induced actin polymerisation is dependent on the activity of these non-receptor kinases, EspJ has also been reported to inhibit Tir-mediated actin pedestal formation (237).

Phosphorylation of Y474 recruits the cellular SH2-SH3 (Src homology-2/3) adaptor Nck via its SH2 domain (260, 280), in turn Nck recruits and activates the Neural Wiskott-Aldrich syndrome protein (N-WASP), which is released from its autoinhibitory bond (281). Active N-WASP is able to activate the actin-nucleating factor Arp2/3 (actin-related-protein-2/3) (282), which nucleates actin polymerisation and ultimately results in pedestal formation at the site of bacterial attachment (Figure 1.2).

1.9.2 EPEC Tir Tyrosine-454-dependent actin polymerisation

Typically, EPEC Tir relies on the phosphorylation of Y474 to recruit the host adaptor protein Nck to induce actin polymerisation. The vital role of Nck in EPEC-induced actin polymerisation has been confirmed by research showing that EPEC E2348/69 is unable to initial normal actin polymerisation

in Nck-deficient host cells (283). However, it has since been shown that Tir can trigger actin polymerisation in cultured cells via an Nck-independent mechanism, although efficiency is much reduced when compared with the Nck pathway (274). This highlighted the existence of a secondary, although much weaker, Tir-dependent actin polymerisation pathway. This actin pathway was attributed to phosphorylation of a second tyrosine residue Y454 (274), as the double mutant EPEC—Tir Y454F/Y474F is unable to induce any actin pedestal formation (274, 283). It has been suggested that phosphorylation of this residue recruits other proteins with SH2 domains to mediate N-WASP recruitment and Arp2/3 activation. However, the adaptors responsible for this have not yet been identified.

In the C-terminus Y454 is found within a conserved asparagine-proline-tyrosine (NPY) motif that is also present in EHEC Tir (NPY458), and this motif has been shown to be essential for EHEC-induced actin polymerisation (357, 358). However, the mechanism utilised to induce actin polymerisation in EHEC occurs independently of tyrosine phosphorylation and requires an additional T3SS effector protein which is absent from typical EPEC. Therefore, the role of this secondary actin polymerisation pathway in EPEC remains unclear. Recently, phosphorylated Y454 has been shown to recruit the regulatory subunit of phosphoinositide-3 kinase (PI3K) and the SH2-containing tyrosine phosphatase (SHP-2) to the EPEC pedestal (284, 285). Recent work with EPEC has revealed membrane phosphoinositide signals function to regulate actin assembly within the pedestal (206, 285, 286). The disruption of phosphoinositide (PI) signalling within the host cell plasma membrane by bacterial effectors can destabilise actin dynamics and alter membrane integrity. Tir residue Y454 may therefore play a functional role in regulating actin polymerisation. Interestingly, the functional significance of this event, although unclear, may be associated with the ability of EPEC to modulate membrane morphology, cell death and innate immunity (284).

1.9.3 EHEC Tir and the Tir-cytoskeleton coupling protein (TccP)

In contrast to EPEC Tir, Tir from EHEC strains of serotype O157:H7 induces actin polymerisation independently of tyrosine phosphorylation of and Nck signalling. Additionally, EHEC Tir is not able to induce actin pedestal formation when expressed in EPEC strains (280, 287, 288). Despite a relatively high level of conservation between EPEC and EHEC Tir effectors, EHEC Tir lacks an equivalent tyrosine 474 residue within its C-terminus and is unable to generate pedestals via the Nck-N-WASP-Arp2/3 recruitment cascade. However, EHEC Tir can induce robust actin polymerisation independently of Nck (260). This actin polymerisation is dependent on the

conserved NPY motif found in both EPEC (NPY454) and EHEC (NPY458) Tir molecules. However, unlike in EPEC where phosphorylation of Y454 accounts only for low levels of actin polymerisation, the EHEC NPY motif induces phosphorylation-independent actin polymerisation at a level equivalent to that induced via the EPEC Tir-Nck pathway. This is dependent on an additional EHEC non-LEE encoded T3SS effector protein termed TccP (Tir-cytoskeleton coupling protein) (289), sometimes referred to as $EspF_U$ (*E. coli* secreted protein F in prophage U) (290). TccP is indispensable for effective pedestal formation during EHEC infections (291, 292) (Figure 1.2).

TccP is an intrinsically disordered protein that is formed of an N-terminal sequence that promotes translocation, followed by multiple short tandem repeats of 47 amino acids (289, 290, 293). The 20 residues located at the N-terminus of the tandem repeat bind to the autoinhibitory GTPase binding domain (GBD) of N-WASP, releasing it from the activity-bearing VCA domain, leading to the activation N-WASP. This in turn activates the Arp2/3 actin nucleator complex (294, 295). TccP, however, cannot bind to Tir directly, and therefore requires an additional adaptor protein to effectively localise to the site of infection. The 22 C-terminal residues of the TccP repeat consist of a proline-rich sequence that can interact with the SH3 domains of the host proteins IRTKS (insulin receptor tyrosine kinase substrate) and the related IRSp53 (insulin receptor phosphotyrosine 53 kDa substrate) (205, 296, 297). IRTKS and IRSp53 bind directly with the conserved NPY458 tripeptide motif within the EHEC Tir C-terminus, and therefore function as a bridge between TccP and Tir, effectively recruiting the TccP:N-WASP:Arp2/3 complex to the Tir C-terminus and enabling localised actin assembly.

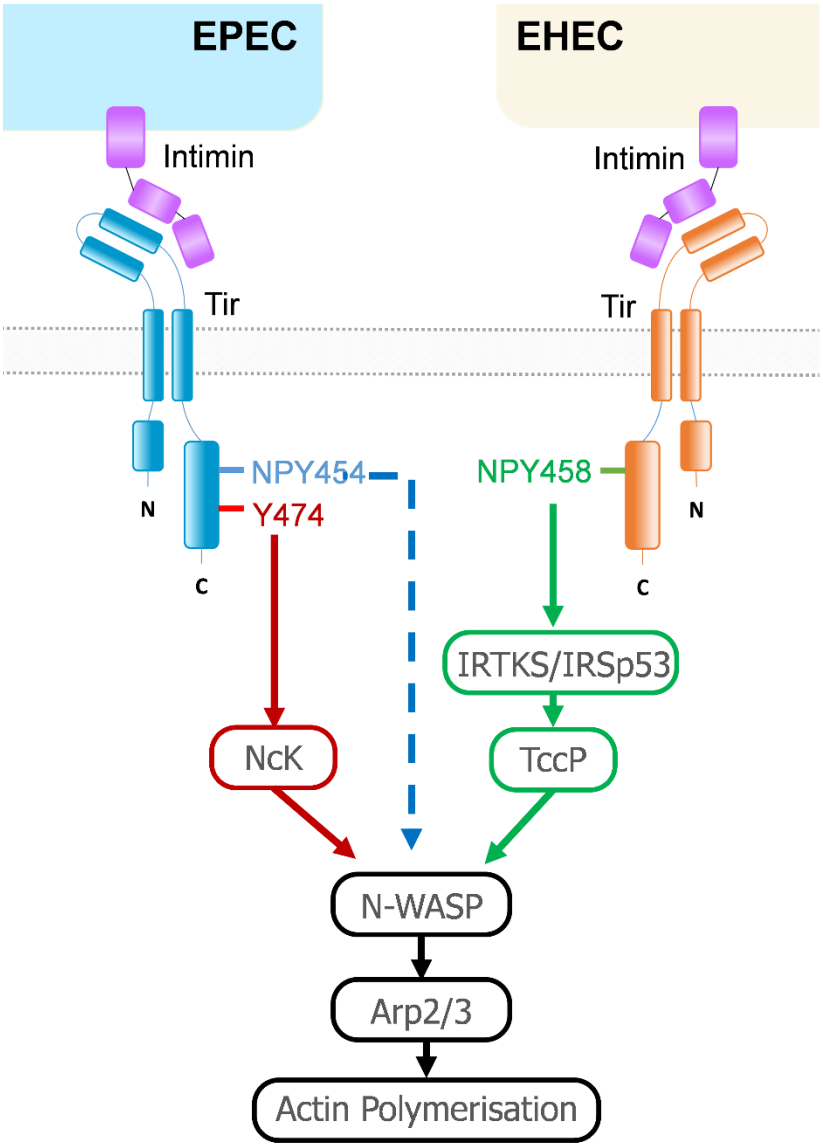

Figure 1.2. Tir-induced actin polymerisation pathway.

During infection, EPEC Tir is translocated into the host cell, via the T3SS, and inserts itself into the host-cell plasma membrane. Within the host cell plasma membrane Tir functions as a receptor for the EPEC surface protein intimin, the binding of Tir and intimin anchors the bacterium to the host cell. Intimin-mediated clustering induces Tir phosphorylation at tyrosine residue Y474 by numerous redundant host tyrosine kinases. The phosphorylation of Tir recruits the host adaptor protein Nck to the site of bacterial attachment, which in turn activates N-WASP and the ARP2/3 complex to mediate actin polymerisation. EPEC Tir can also promote an additional weak Nck-independent actin polymerisation, through the C-terminal tyrosine residue Y454, which is found within a conserved NPY motif.
During EHEC infection, however, Nck is not recruited to Tir as it lacks a corresponding Y474 residue; instead, an additional bacterial effector, TccP (also known as EspFu), functions to recruit N-WASP directly. TccP interacts with host the adaptor proteins IRTKS and IRSp53 to localise to the EHEC-Tir NYP motif to enable actin polymerisation and pedestal formation at the site of bacterial attachment.

1.9.4 EPEC lineage 2 strains and TccP2

Most of the research completed on Tir-induced actin polymerisation focused on prototypical EPEC 2348/69 O127:H7 or EHEC O157:H7 strains. However, recent screens of clinical and environmental strains have identified surprising variations between different lineages. Firstly, *tccP* was also found in some typical and atypical EPEC isolates, including strains belonging to serogroups O26 (EHEC), O119 (tEPEC), and O55 (aEPEC) (298). Additionally, the recent identification of a second *tccP2* gene ($EspF_M$), in a number of EPEC strains has revealed that a subset of EPEC strains can utilise both the Nck and TccP2 actin polymerisation pathways simultaneously during infection. TccP2 shares a high level of sequence similarity with TccP, with the N-terminus having 69.5% sequence identity and the short tandem repeats being almost identical between the two proteins. Indeed, TccP2 is functionally interchangeable with TccP in the context of EHEC O157:H7 infections (299).

Interestingly, although *tccP2* is missing from the prototype EPEC strain E2348/69 (O127:H7), a *tccP2* screen of EPEC strains identified an interesting division between two distinct evolutionary lineages of EPEC (EPEC 1 and EPEC 2). In contrast to EPEC 1 strains, which do not encode TccP2, most strains belonging to EPEC 2 were found to be *tccP2* gene positive, so were able to utilise both the Nck and TccP2 pathways to promote actin recruitment at the bacterial attachment site (300). *tccp2* was also identified and conserved in 95% of non-O157 EHEC strains, with over 90% of these strains also expressing a Tir molecule capable of signalling through the Nck pathway. As such, the presence of both pathways now seems to be the norm in contrast to the segregation of signalling between EPEC O127:H6 E2348/69 and EHEC O157:H7. The conservation of both these seemingly redundant pathways suggests they must confer some competitive advantage to the pathogen, however it is currently not clear what this might be. Further work is needed to establish the role of these complementary pathways in the context of infection.

1.10. EPEC-induced actin polymerisation – Is Tir enough?

The principle pathway for actin pedestal formation by EPEC appears simple after Y474 phosphorylation. However, identification of the exact repertoire of proteins required for this actin polymerisation during infection has been complicated by a number of factors. It has previously been proposed that other EPEC proteins must be co-delivered with Tir to promote its tyrosine phosphorylation and signalling within the host (301). Characterisation of the minimal components required to mediate actin polymerisation is limited by the fact that ectopically expressed full-length

Tir in mammalian cells localises to the perinuclear regions and inefficiently binds to intimin at the host membrane. However, this limitation has been circumvented by the expression of a functional membrane-targeted EPEC Tir (TirMC) within transfected mammalian cells (283).

Studies utilising ectopically expressed TirMC demonstrate that Tir and intimin are the only EPEC derived elements required to trigger actin pedestal formation at the site of infection, and that actin polymerisation occurs independently of any other secreted EPEC proteins. Further, all of the information required for EPEC-mediated actin assembly lies within the C-terminal cytoplasmic region of Tir, as a version of Tir lacking its N-terminal cytoplasmic domain is sufficient to trigger actin pedestal formation (283). However, this study utilised modified membrane-targeted Tir derivatives, so could not rule out a role for other EPEC virulence factors in targeting Tir to the plasma membrane during infection.

The recent construction of synthetic *E. coli* K12 injector strains (SIEC) has helped to resolve this pathway further. SIEC expresses all the structural components of EPEC T3SS in the absence of any additional effectors, promoters and transcriptional regulators (302). This *E. coli* K-12 strain has five engineered LEE operons (eLEEs Figure 1.3) integrated into specific chromosomal sites. The resulting strain, named SIEC, assembles a functional T3SS comparable to that expressed by EPEC, but does not express any other EPEC virulence factors (302). Importantly, the addition of engineered operon eLEE5 to this strain which encodes Tir, its chaperon CesT and intimin (*eae*) (Figure 1.3), was sufficient to induce actin pedestal assembly at the site of infection. This strain can efficiently translocate Tir into host cell membranes and trigger intimin-dependent actin pedestals comparable to that seen in EPEC infections (302). This confirmed that Tir secreted via the T3SS and intimin are the only EPEC derived virulence factors required for actin pedestal formation.

1.11. Composition of the EPEC actin pedestal

The composition of the EPEC-induced actin pedestal is highly complex, and other of constituents besides Nck, N-WASP and Arp2/3 might play important roles during infection. Pedestals effectively function as a molecular niche to recruit a diverse range of host proteins to the sight of infection. These include proteins typically associated with focal adhesions, a number of cell cortex proteins and various signalling or adaptor proteins such as CT10 regulator of kinases (Crk), among others (23, 303-306). Although the role of these proteins within the pedestal remains to be

elucidated, it is possible that the interactions of these proteins with Tir may play an important role in regulating pedestal formation.

Important accessory constituents of the actin polymerisation machinery also localise to the pedestal in a Tir-dependent manner. These include: cortactin, the actin depolymerising factor cofilin, the actin-filament-severing protein gelsolin, and the polymerisation rate regulator VASP (vasodilator-stimulated phosphoprotein). Although the function of these regulators in regards to pedestal formation remains to be fully established, cortactin has the ability to stimulate Arp2/3 in a manner similar to N-WASP (307) and is necessary for F-actin accumulation in EPEC pedestals (308). Furthermore, although no evidence directly implicates cofilin and gelsolin directly in actin pedestal formation, these molecules may function to provide free actin subunits for incorporation into the growing end of the pedestal. Interestingly, cofilin plays an important role in the actin-based motility of *Shigella* spp. (309), and a number of pathogenic bacteria have evolved mechanisms to manipulate the activity of cofilin to promote infection and dampen immune responses (310). By sequestering these important actin polymerisation components EPEC may subvert other important host pathways, including phagocytosis and inflammatory signalling, however this remains to be experimentally verified.

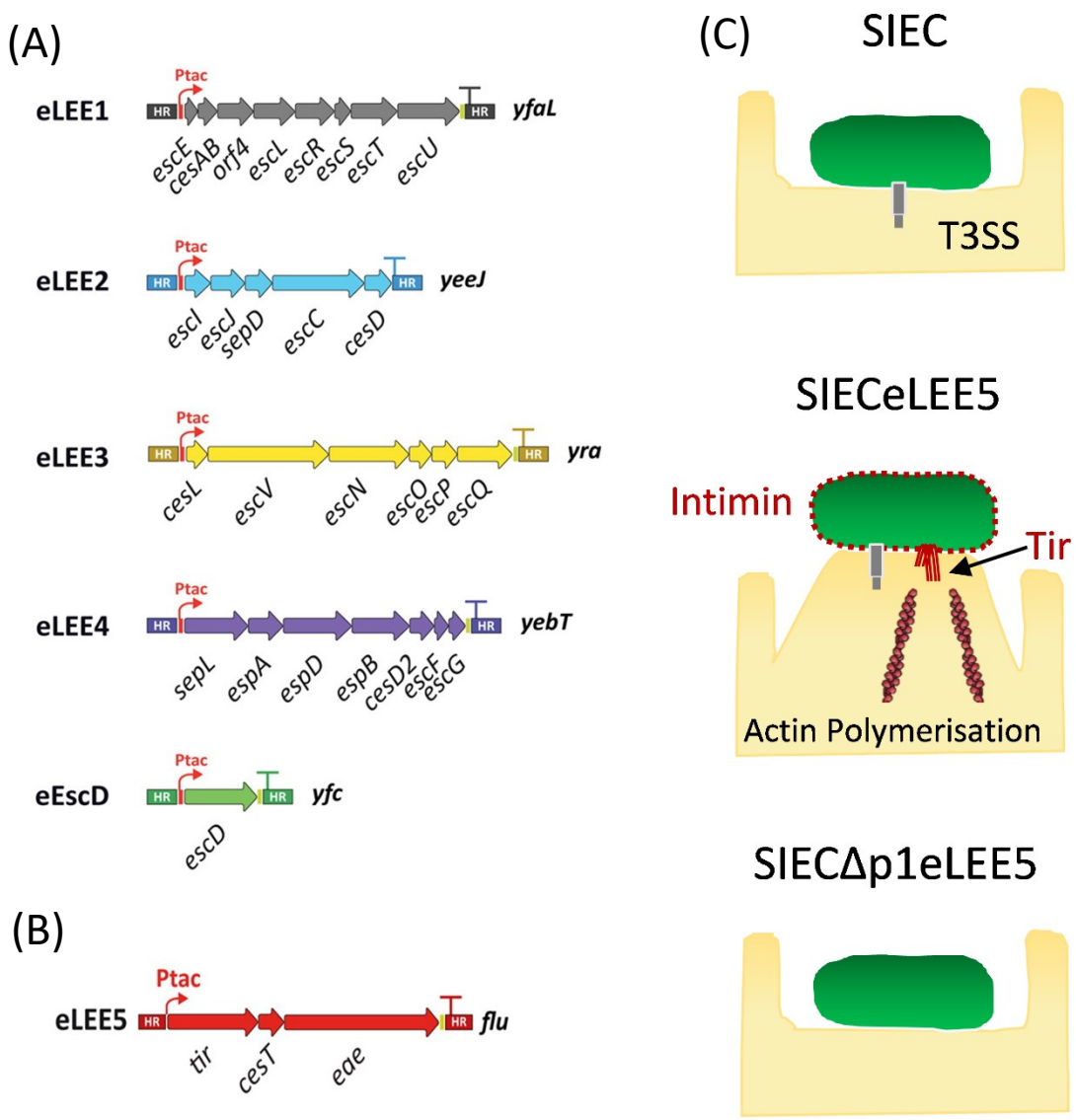

Figure 1.3. Genetic configuration of the synthetic *E. coli* K12 injector strains (SIEC)

(A) Schematic representation of the engineered LEE operons integrated into the chromosome of K-12 *E. coli* to encode a functional T3SS.
(B) Scheme of the engineered LEE5 operon (eLEE5) encoding *tir, cesT* and *eae* under that regulation of an IPTG inducible Ptac promotor. eLEE5 is integrated into the chromosomes of SIEC and SIECΔp1eLEE5.
(C) SIEC strains; SIEC encodes the structural components of the T3SS under the control of an IPTG inducible promotor Ptac. SIEC is able to assemble a functional T3SS in a similar manner EPEC, can form pores in the host cell plasma membrane and can translocate substrate proteins. SIECeLEE5 encodes a functional T3SS and an additional eLEE5 operon that encodes Tir, its chaperone CesT, and intimin (eae). SIECeLEE5 is able to trigger actin polymerisation at the site of infection, similar to actin pedestals triggered by EPEC. SIECΔp1eLEE5 has the eLEE5 operon integrated into the chromosome, but cannot assemble the T3SS as it lacks the promoter for eLEE1.
(Modified from; 302)

1.12. Role of Tir signalling *in vivo*

It has been well established that Tir has an essential role in intestinal colonisation and the formation of A/E lesions during *in vivo* infection models (122, 266, 311, 312). Surprisingly however, none of the Tir-driven actin polymerisation pathways are necessary for either colonisation or A/E lesion formation at the mucosal surfaces (265, 266, 312). Since mice are resistant to EPEC infection, *in vivo* studies are commonly carried out using the model murine A/E pathogen *C. rodentium*. Similarly to EPEC lineage 1 strains, *C. rodentium* has the ability to trigger actin polymerisation via both the Nck-dependent and the Nck-independent pathways. Using *C. rodentium* to investigate these different Tir signalling pathways *in vivo*, it has been demonstrated that despite their crucial role in actin polymerisation in cultured cells, both the Nck-dependent and Nck-independent actin polymerisation pathways are not essential for A/E lesion formation (312). In addition, infection of human *in vitro* organ cultures (IVOC) with EPEC expressing Tir^{Y474F} or $Tir^{Y454F/Y474F}$ resulted in A/E lesion formation (265). These results genetically uncoupled the processes of A/E lesion formation from Tir-induced actin polymerisation in the context of infection. Furthermore, A/E lesion formation is severely attenuated in the absence of non-LEE effector proteins, indicating that at least one or more of these effectors contribute to this process, unlike actin polymerisation that occurs independently of all other effector proteins (68).

Importantly, although the Tir-dependent actin polymerisation pathways appear to be under intense selective pressure, the functional relevance of pedestal formation during EPEC infection remains to be established. Interestingly, recent work by Crepin *et al.* has identified an important role for *Citrobacter* Tir residues Y451 (Equivalent to EPEC Y454) and Y471 (Equivalent to EPEC Y454) in activating host innate immune responses *in vivo* (313). Infection with *C. rodentium* strains expressing $Tir^{Y451A/Y471A}$ induced significantly lower concentrations of the chemokines CXCL1 (KC) and CXCL2 (MIP2-alpha) compared to Wild-Type (WT) infections. CXCL1 and CXCL2 signal via CXCR2 to promote the influx of neutrophils (314). Consistent with this, infections with $Tir^{Y451A/Y471A}$ mutants recruited significantly fewer neutrophils to the mucosa. Notably, actin staining in enterocytes was markedly increased under attached *C. rodentium* expressing WT Tir than the $Tir^{Y451A/Y471A}$ mutant, demonstrating that although not responsible for A/E lesion formation, Tir retains its ability to induce actin polymerisation *in vivo.* In this scenario, perhaps Tir-mediated actin assembly functions to activate innate immune signalling pathways. This is not a novel concept, as

subversion of the actin cytoskeleton by bacterial virulence factors has previously been shown to be an important mediator of immune signalling (315-318).

1.13. Effector manipulation of host small GTPase activity

Tir is one of many bacterial effectors that modulate the host cells actin cytoskeleton during infection. A number of EPEC and EHEC bacterial effectors activate cytoskeletal remodelling by activating Rho GTPase signalling. Rho GTPases regulate nucleation of actin and formation of stress fibres, filopodia, and lamellipodia. There are three major classes of Rho GTPases: Rho, Rac, and Cdc42, which regulate the formation of these actin-structures in host cells. Recently, a group WxxxE-Effectors including EspM, EspT and Map from EPEC/EHEC, were reported to induce a plethora of actin structures generally associated with activated Rho GTPases.

The EPEC effector Map exhibits GEF activity towards Cdc42 (319) leading to the formation of actin-rich filopodia clusters (209, 320). Mechanistically, Map interacts with the EBP50–ezrin and nucleates Cdc42 at the cell membrane leading to localised actin polymerisation and sustained Cdc42 activity at the adhesion site (321). Interestingly, Tir has been shown to regulate Map-induced filopodia formation (209, 320), and together Tir and Map have been implicated in both the effacement of microvilli during infection and the rapid loss of function of the host sodium-glucose transporter SGLT-1 (322). The onset of watery diarrhoea induced by EPEC infection has therefore partly been attributed to the co-ordinated action of these two effectors (322).

EspT alters the dynamics of the actin cytoskeleton through activation of both Rac-1 and Cdc42, triggering membrane ruffles and lamellipodia (208, 323, 324). EspT-carrying strains are also capable of invading non-phagocytic cells and forming intracellular actin pedestals (325). EspT-induced membrane ruffles facilitate entry into host cells via a mechanism termed “trigger entry” that is utilised by a number of intracellular pathogens including *Shigella flexneri* and *Salmonella* (326). Internalised EPEC mobilises Tir to the vacuolar membrane where it forms intracellular actin pedestals that promote intracellular bacterial survival (325).

EspM regulates actin dynamics during infection through the targeted activation of the RhoA signalling pathway. While the homologous EspM1 and EspM2 effectors both induce the formation of actin stress fibres within infected cells (323, 327), the phenotypes displayed by the EspM effectors are subtly different. For example, while EspM1 induces the formation of parallel stress

fibres that are restricted to the site of bacterial attachment, EspM2 induces phosphorylation of cofilin and parallel stress fibre formation throughout the entire infected cells, which are linked to the plasma membrane through focal adhesions (323).

While expression of Map is conserved among all known EPEC and EHEC strains, analysis of over 1000 clinical isolates demonstrated that EspM was encoded by 69% of EPEC and EHEC strains, while EspT was found in less than 2% of EPEC strains and none of the EHEC strains examined (328).

The Host Innate Immune Response

1.14. Innate immune response and programmed cell death pathways

Multicellular organisms possess sophisticated defence mechanisms that effectively counteract microbial infections. The mammalian immune system consists of both innate and acquired immune pathways. The innate immune system functions as the initial defence mechanism against pathogens and is primarily mediated by professional phagocytes including macrophages and dendritic cells (DCs), although a number of non-professional cells including epithelial cells also contribute to this.

Programmed cell death has recently emerged as an important immune mechanism, that plays a key role in fighting bacterial, parasitic, fungal and viral infections (329). Upon infection, innate immune cells induce several suicide programmes to prevent microbial replication and expose pathogens to immune attack (329, 330). The repertoire of known cell death pathways is continuously expanding, with the best characterised being apoptotic, pyroptotic and necroptotic cell death (Table 1.4). In addition to their critical role during infection, aberrant regulation of these programmed cell death pathways and the associated release of pro-inflammatory cytokines, underlies a variety of human diseases, including autoimmune diseases and neurodegeneration (331, 332).

The innate immune response, although paramount to host defence against pathogens, needs to be tightly regulated. This ensure that it is optimally activated in response to pathogenic insult, but remains dormant in the absence of infection (333). This is particularly important within the intestinal tract, as the intestinal mucosa is a dynamic environment in which the host continually interacts with a diverse array of between 10–100 trillion commensal microorganisms, known as the microbiota, in a symbiotic relationship (334, 335). However, some microorganisms have evolved specific virulence strategies and are able to breach the mucosal barrier to establish infection. The first line of host defence against these invading enteric pathogens is the innate immune system. Initiation of innate immune responses depends on the activation of host cell receptors that sense conserved microbial motifs, termed Pattern-Recognition-Receptors (PRRs) (336).

The innate immune compartment of the intestinal mucosa has a pivotal role in mucosal homeostasis and antimicrobial immunity during infections with A/E pathogens. Using the mouse pathogen *C. rodentium* as a model, a number of key innate immune responses have been shown to be paramount to effective pathogen clearance. Toll-like receptor (TLR) and interleukin-1 receptor (IL1R) signalling, both of which regulate the transcriptional activation of several immune-related genes, control *C. rodentium* infection via several mechanisms. This includes the recruitment of neutrophils, macrophages and dendritic cells to the mucosa, the expression of iNOS (inducible nitric oxide synthase), and the induction of epithelial cells proliferation (110, 337-340).

Infiltration of immune cells to the site of infection is crucial for host defence against pathogens, with the migration of macrophages and neutrophils to the mucosa playing a critical role in bacterial clearance during *C. rodentium* infections (110, 341, 342). Macrophages and neutrophils also play important roles in activating the adaptive mucosal immune response. Both B cells and $CD4^+$ T are central to the development of sterilising immunity and are required for effective clearance and subsequent resistance to *C. rodentium* infection (343, 344). T_H17 cells, a specific type of $CD4^+$ T helper cell capable of producing IL-17, comprise a distinct branch of mucosal immune defences and have a particularly important role in *C. rodentium* infection control (345). The development of the T_H17 cell response is linked to *C. rodentium*-induced host cell death, as blocking cell death pathways using the pan-caspase inhibitor Q-VD-OPH during infection impairs the T_H17 cell responses in the lamina propria (346) which are essential for protection against *C. rodentium* infections (127, 346, 347). This established that the immunological consequences of cell death during infection have an important role, in combination with TLR engagement, in mediating tailored immunity to intestinal bacterial infection through the activation of adaptive immune responses.

1.15. Pattern Recognition Receptors (PRRs)

An absolute prerequisite in innate immunity against pathogens is the recognition of microbes by a group of germ-line encoded PRRs (348). PRRs function through the detection of conserved patterns expressed by microbes termed pathogen-associated molecular patterns (PAMPs) (349). These molecules are produced by pathogens, and as such are recognised as non-self by the innate immune system. PRRs recognise ligands of all origins, ranging from bacterial proteins and lipids to fungal sugars, as well as unique nucleic acids structures or sequences. PRRs can also recognise immunostimulatory products that are derived from damaged host tissue or necrotic cells, termed

damage-associated molecular patterns (DAMPs). This recognition is crucial for host defence and tissue remodelling. Upon recognition of these PAMPs/DAMPs, PRRs trigger downstream intracellular signal transduction cascades that induce the expression of various genes involved in inflammatory and immune responses and the activation of programmed cell death pathways.

PRRs can be classified into five distinct families based on their protein domain homology. These five families include: Toll-like receptors (TLRs) (350), nucleotide-binding domain, leucine-rich repeat (LRR)-containing (or NOD-like) receptors (NLRs) (351), RIG-I-like receptors (RLRs) (352), AIM2-like receptors (ALRs) (353) and C-type lectin receptors (CLRs) (354). These families can be further differentiated according to their cellular location. Membrane-bound receptors consist of the TLRs and CLRs and are found at the cell plasma membrane or on endocytic compartments (355, 356). These receptors respond to the presence of pathogenic ligands in the extracellular milieu or within endosomes. Intracellular PRRs include the NLRs, RLRs, and ALRs and are located in the host cell cytoplasm, where they detect intracellular PAMPs (353, 357-359). A number of PRRs respond to a diverse array of different PAMPs to induce transcriptional activation of inflammatory mediators. However, NLRs and ALRs harbouring a pyrin domain or a BIR domain in their N-terminus are not involved in the transcriptional regulation of pro-inflammatory genes, but are instead components of the inflammasome and regulate caspase-1 activation. These are discussed in more detail in Section 1.19.

1.16. Recognition of Gram-negative bacterial by Toll-like receptors

The best-characterised of the PPR are the TLRs. There are 10 TLRs in humans, 13 in mice (360) and 222 in sea urchins (361), which have evolved to recognise conserved constituents of pathogenic bacteria. Innate immunity relies on signalling by these TLRs to activate the immune system in response to pathogenic bacteria.

One of the most prominent activators of TLR signalling during Gram-negative bacterial infection is the outer membrane component lipopolysaccharide (LPS) (362), that activates TLR4. LPS is a complex glycolipid that consists of a hydrophobic lipid A domain, an oligosaccharide core, and a highly variable distal polysaccharide (O-antigen) (363). However, the exact structure of LPS molecules can vary slightly between different bacterial species. In contrast to the LPS surface exposed O-antigens, the structure of lipid A is highly conserved, and it is this lipid A portion that is recognised by TLR4. The extracellular domain of TLR4 forms a stable heterodimer with MD-2 (Also

known as LY96) (364), and the resulting complex induces a conformational change in MD2 and is responsible for lipid A recognition (365, 366). However, TLR4 signalling is also dependent on two additional accessory proteins; LPS-binding protein (LBP) (367), and Cluster of differentiation 14 (CD14) (368). LBP functions as a transport protein that binds to LPS within the outer membrane of Gam-negative bacteria and transfers it to membrane-bound CD14 (368). CD14 splits LPS aggregates into monomeric molecules and transfers LPS-monomers onto preformed heterodimers of MD2–TLR4. LPS binding then triggers multiple downstream signalling pathways including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Interferon regulatory factor 3 (IRF3), and the subsequent transcription of pro-inflammatory genes (350, 369-371).

In addition to LPS responses by TLR4, other TLRs play an important role in the recognition of pathogenic bacteria; TLR2 is activated through the detection of lipoproteins, a group of ubiquitous bacterial proteins that are located within the cell membranes of bacteria (372-374). TLR5 detects a conserved domain on flagellin monomers to induce inflammatory signalling (375), and intracellular TLR9 can recognise bacterial genomic DNA within endosomal compartments (376, 377). TLR9 senses the presence of unmethylated CpG dinucleotides (CpG-DNA), which are found throughout the bacterial genomes, but are suppressed and often methylated in mammalian genomes (378).

1.17. Inflammatory signalling pathways during bacterial infections

TLRs are type I transmembrane receptors formed of an extracellular ligand-binding domains, a central transmembrane domain and a cytosolic Toll/IL-1R (TIR) domain (350). The biological activity of TLRs is dependent on ligand binding induced conformational changes within the transmembrane domain of the receptor, which leads to the dimerisation of the cytosolic TIR domains. This results in the formation of an intracellular receptor interface that function as a scaffold for the recruitment of various host derived adaptor proteins. These adaptor proteins contain a structurally conserved TIR domain and associate specifically with TLRs through TIR–TIR interactions. Five TLR adaptor molecules have been identified (379), these include: Myeloid differentiation primary response protein 88 (MyD88) (380), TIR-domain-containing adaptor protein (TIRAP: also termed MAL) (381, 382), TIR-domain-containing adaptor protein inducing IFN-β (TRIF; also known as TICAM1) (383, 384), TRIF-related adaptor molecule (TRAM; also known as TICAM2) (385, 386), and sterile α- and armadillo-motif-containing protein (SARM) (387). The signalling

pathways activated downstream of TLRs are complex, as TLR adaptor molecules display pleiotropic functions that are dependent on both the TLR that is activated and specific type of host cell. Collectively, these TLR signalling pathways modulate a range of innate immune responses such as cytokine signalling and secretion, phagosome maturation, NOD-like receptor (NLR) activation, inflammasome activation, autophagy and programmed cell death (388).

1.17.1 NF-κB and MAPKs pathways

During bacterial infections, the detection of microbial molecules through the TLRs results in NF-κB-dependent and mitogen-activate protein kinase (MAPK), transcriptional upregulation of proinflammatory cytokines and key inflammasome components, such as interleukin-1β (Il-1β), and NLRP3 (389). The TLRs and IL-1 receptor-1 (IL1R1) share sequence similarity in their intracellular region called the TIR-domain, and signalling by these receptor families is similar (Figure 1.4) (390, 391). After binding with their respective ligands, both TLRs and IL1R1 undergo conformational changes, leading to the recruitment of TIR-domain-containing adaptor molecules.

Activation of inflammatory signalling downstream of TLRs requires the recruitment of MyD88 via TIR-TIR domain interactions, with the exception of TLR3 which signals only via TRIF. TLRs signalling through the adaptor MyD88, requires the bridging adaptor TIRAP (381, 382). TIRAP engages directly with TLRs and mediates the recruitment of MyD88 (392). The C-terminus of MyD88 contains a TIR domain, and the N-terminus of MyD88 contains a death domain (DD) (393, 394). Upon receptor binding MyD88 recruits the IL-1R-associated kinase 4 (IRAK-4). Receptor mediated clustering of IRAK-4 results in its autophosphorylation and activation. Active IRAK-4 then recruits IRAK-1 and IRAK2 via homophilic death-domain interactions (395). Subsequent to its association with MyD88, IRAK-1 is phosphorylated by active IRAK-4. This nucleates the formation of a larger complex which includes the E3 ubiquitin ligase TNFR-associated factor 6 (TRAF6) (396), and in the case of TLR4, cellular inhibitors of apoptosis (cIAP1 and cIAP2). TRAF6, together with a ubiquitination E2 enzyme complex consisting of UBC13 and UEV1A, then catalyses the formation of K63-linked polyubiquitin chains on lysine residues within TRAF6 itself and IRAK1. These polyubiquitin chains form a platform for the recruitment of TAB2 and TAB3, which exist in a complex with TGF-β-activated kinase 1 (TAK1). TAK1 is a central regulator of NF-κB and MAPK signalling. Activation of NF-κB via TAK1 is termed canonical NF-κB signalling, (390, 397, 398). Briefly, in this pathway, K63-linked polyubiquitin chains synthesised by TRAF6 activate TAK1 by inducing its autophosphorylation (399). Active TAK1 phosphorylates and activates IκB kinases (IkKs). IκKβ

phosphorylates the NF-κB inhibitor alpha (IκBα), targeting it for proteasomal degradation. Under resting conditions, IκBα is in a complex with the NFκB subunits p65 and p50, preventing nuclear translocation. Upon IκBα degradation, p65 is released and is translocated into the nucleus where it promotes the expression of various inflammatory and cell survival factors. Alternatively, active TAK1 can phosphorylate and activate MAPK family members such as Jun N-terminal Kinase (JNK), Extracellular Signal-Regulated Kinase (ERK) and p38 MAPK ERK1/2, which mediates activation of AP-1 family transcription factors to regulate inflammatory responses (400-402).

At the point of TAK1 activation, the NF-κB and MAPK signalling pathways converge with the Tumour necrosis factor receptor (TNFR) inflammatory signalling pathway (Figure 1.4). TNFRs are typically divided into two groups: inflammatory activating receptors and death receptors (DRs). The majority of TNFRs are activating receptors and function by activating NF-κB and MAPK pathways. However, eight TNFR including TNFR1 and Fas, possess an intracellular death-domain which, upon activation, stimulates programmed cell death signalling pathways. These will be discussed in section 1.29. Interestingly, TNFR1 is a pleiotropic receptor and has the capacity to induce both transcriptional and death signalling pathways (403). Despite the presence of a cytoplasmic death domain, TNFR1 activation in response to TNFα binding primarily activates inflammatory responses (Figure 1.4), whereas cell death is induced only under certain conditions. Activation of NF-κB and MAPK downstream of TNF signalling occurs via an different receptor-mediated signalling pathway than TLR driven activation (124, 125). Stimulation of the canonical TNFR results in receptor aggregation and the subsequent recruitment of Tumour necrosis factor receptor type 1 associated death domain protein (TRADD) and Receptor-interacting protein kinase 1 (RIPK1), through the homotypic interactions of their death domains (DD). Ubiquitylation of RIPK1, by TRAF E3 ligases (TRAF2/5), recruits TAK1 via TAB2/TAB3, at this point TNFR signalling converges with the TLR pathways to activate TAK1 and stimulate the induction of NF-κB and MAPK.

In the case of TLR3, which is unable to recruit MyD88, recruitment and activation of the adaptor protein pair TRAM-TRIF can also activate NF-kB signalling (402) . TRIF binds to TRAF6 and RIPK1 to initiate the NF-κB activation cascade. TRIF can also participate in the delayed induction of inflammatory cytokines in the TLR4 signalling pathway (404, 405). Interestingly, unlike MyD88 signalling, the TRIF-dependent pathway does not lead to MAPK activation, however, the mechanistic differences conferring this specificity have yet to be established (406).

1.17.2 Type I IFN pathway

Upon detection of microbial products some TLRs, such as TLR4 and TLR3 induce production of interferon (INF). IFN signalling occurs independently of MyD88 and relies on the adaptor protein TRIF. TRIF-dependent signalling can activate type 1 interferon signalling via the transcription factor IRF3 (404). TLR3 can directly bind TRIF whereas TLR4 requires the protein adapter TRAM (407).

Downstream of TRIF activation, two TRAF proteins, TRAF3 and TRAF6, are required for type I IFN production (408, 409). TRIF, in co-ordination with TRAF3/6, activates (TANK-)binding kinase 1 (TBK1). TBK1 then directly phosphorylates IRF-3 and IRF-7 (410, 411). Phosphorylated IRF-3/7 forms homodimers which translocate into the nucleus and upregulate the transcription of type 1 interferon genes (IFNs) (412). IFNs play essential roles in establishing and modulating host defence against bacterial infections, by triggering transcription of a group of genes termed IFN stimulated genes (ISGs). Proteins encoded by ISGs contribute to multiple innate immune responses, including inflammasome activation pathways through several different mechanisms (413). However, while some of the ISG genes enhance the host defence against bacterial infections, a number of ISG dysregulate inflammatory responses and can exacerbate infections (414).

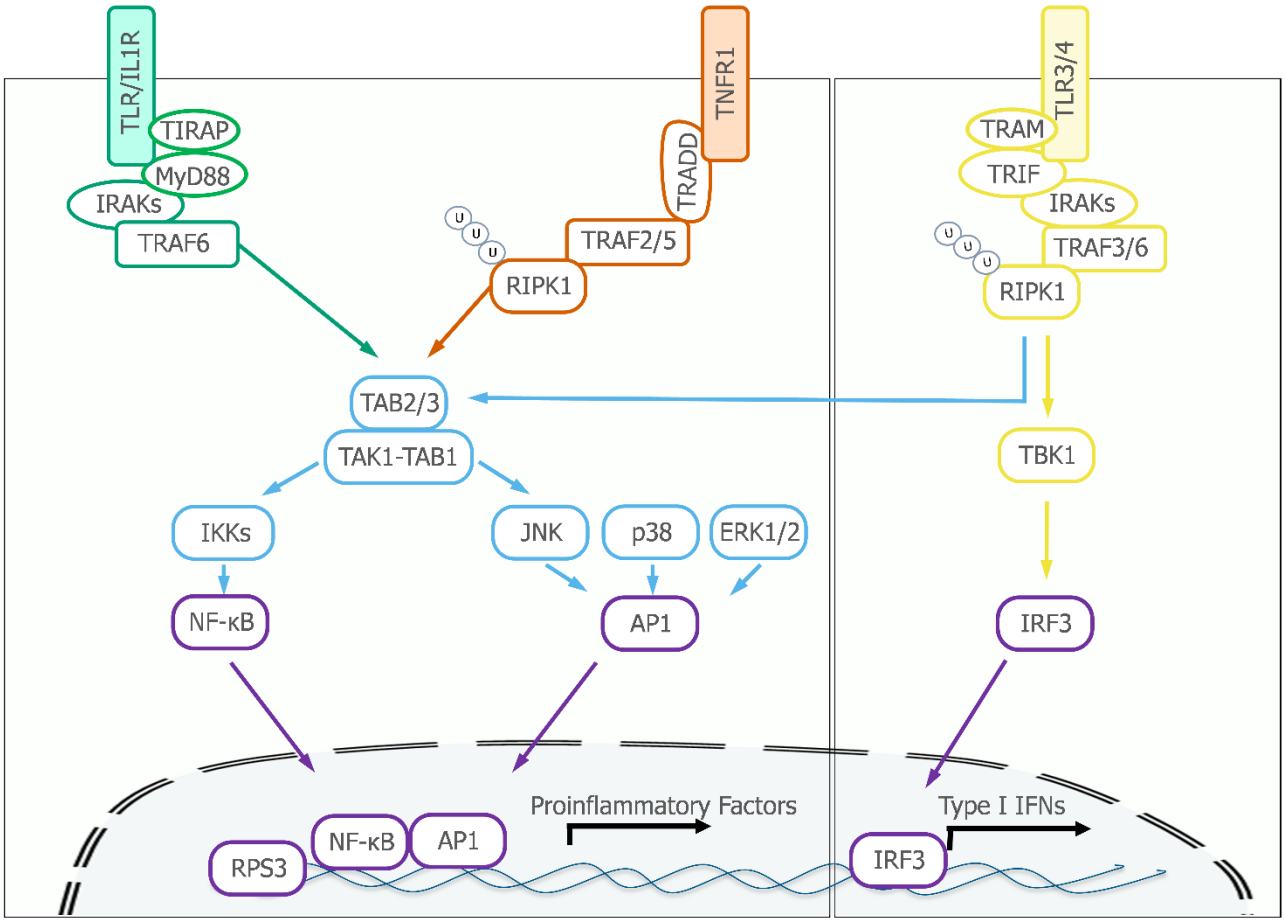

Figure 1.4. Inflammatory signalling pathways induced by membrane TLR, IL1R and TNFR1

TLRs transduce their signals through two different adapter protein pairs, TRIAP-MyD88 and TRAM-TRIF. Virtually all TLRs signal via MyD88, except for TLR3 which signals via TRIF. In addition, TLR4 can use both mediators. IL1R receptors also signal via MyD88. MyD88-driven signalling mediates a rapid and acute pro-inflammatory response through the activation of NF-κB and MAPK-dependent kinases; JNK, p38 and ERK1/2 to induce the transcription factor AP-1. In contrast, TRIF triggers a delayed pro-inflammatory response mediated by NF-κB as well as IRF-3-dependent type I IFN expression. TNFR1 can also induce NF-κB via TRADD and RIPK1. Ubiquitylation of RIPK1, by TRAF2/5 recruits TAK1 via TAB2/TAB3, here TNFR signalling converges with the TLR/IL1R signalling. Modified from: (422)

1.18. EPEC effector-mediated manipulation of inflammatory signalling pathways

EPEC is a flagellated Gram-negative, predominantly extracellular pathogen, and therefore EPEC infections trigger potent inflammation by activating TLR2/4/5-pathways during infection of professional immune cells such as macrophages. Additionally, during infection of polarised epithelial cells, in which membrane-associated TLRs (TLR2,4,5) are typically localised to the basolateral plasma membrane (415), activation of NF-κB signalling relies on the T3SS (416). EPEC also secretes an array of effector proteins that have been reported to have immunostimulatory effects, such as NleF which induces NF-κB activation early during infection (227).

In vivo work with the mouse A/E pathogen *C. rodentium* has demonstrated the importance of NF-κB and innate immune signalling in the clearance of infection. Critical roles for NF-κB (417), MyD88 (337, 338), and TLR2 (339) in the defence against this pathogen have previously been identified. p38-dependent MAPK signalling also plays a major role in eliciting the inflammatory response to *C. rodentium* infection by inducing the recruitment of CD4+ cells, which are involved in the host immune defence against enteric pathogens (418, 419). However, A/E pathogens, including EPEC, utilise various virulence mechanism to effectively evade, impede or down-regulate these innate immune responses. A large body of work exists on the redundancy of effectors used by EPEC to block NF-κB activation (Reviewed; (420-422) (Table 1.3, Figure1.5). This has provided credence to the idea that EPEC actively subverts innate immune detection and restricts intestinal inflammation to cause disease.

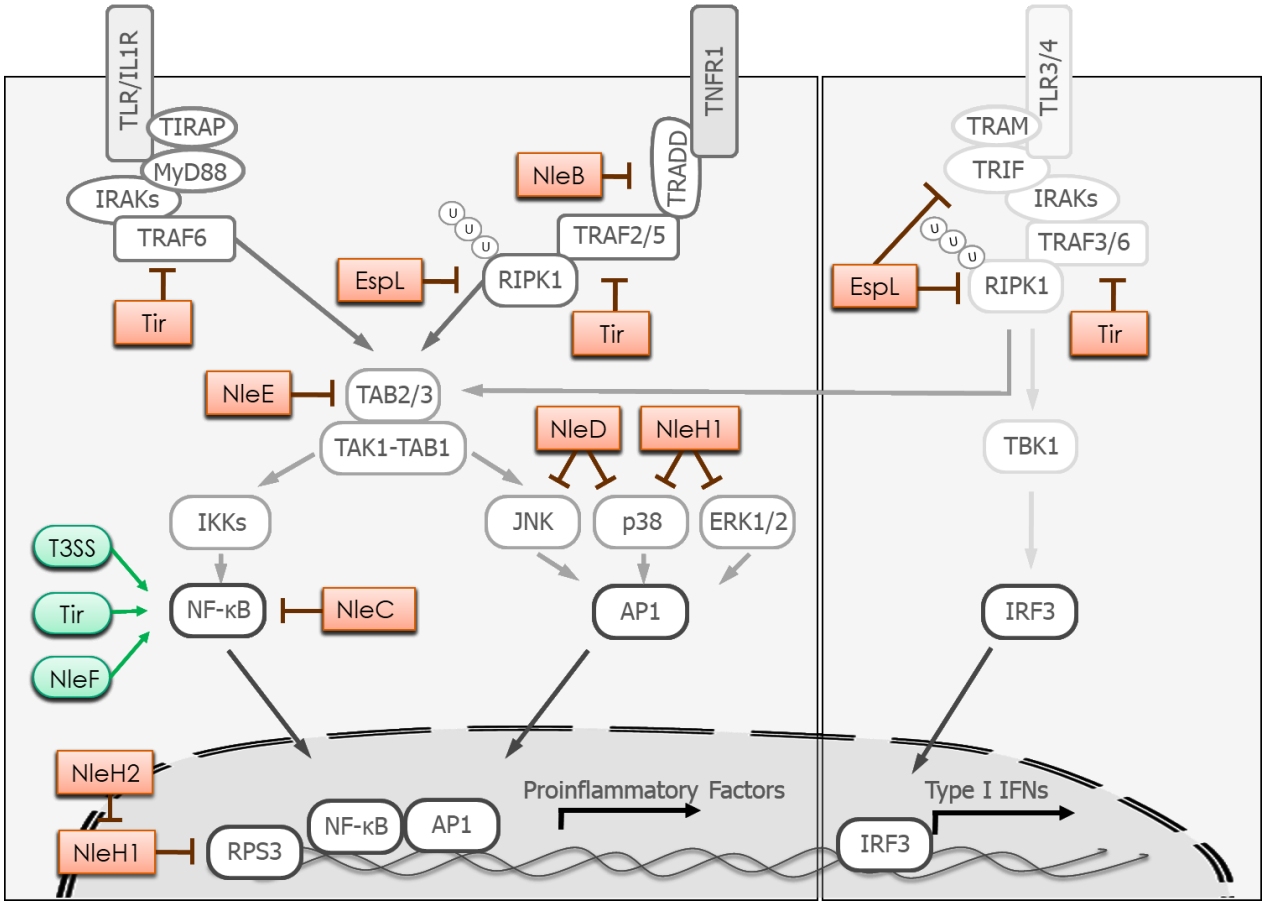

Figure 1.5. EPEC manipulation of inflammatory signalling pathways

Schematic showing signalling via TLR, IL1R and TNFR1 to induced NF-κB, MAPK and INF transcriptional upregulation. EPEC inhibitory effectors are shown in red, EPEC effectors that have been shown to activate NF-κB pathways are shown in green.

Programmed Cell Death Pathways

Receptor stimulation leads to a cascade of signalling events that ultimately results in the production of inflammatory cytokines, infiltration of inflammatory immune cells and the subsequent activation of programmed cell death pathways. Multiple forms of cell death have been described and these include (but are not limited to) apoptosis, pyroptosis and necroptosis (Table 1.4). In host defence, programmed cell death can inhibit microbial infections through the removal of invading bacteria and the priming of the hosts innate and adaptive immune responses.

Both apoptosis and pyroptosis utilise caspases to mediate the morphological changes required to induce cell death (423). Caspases are broadly grouped into initiator caspases (caspase-2, -8, and -9), executioner caspases (caspase-3, -6, and -7), and inflammatory caspases (human caspase-1, -4, and -5; mouse caspase-1 and -11) (424). Caspases gain activity through dimerisation, resulting in the autocatalytic cleavage and stabilisation of a dimer capable of cleaving numerous downstream target proteins that contain conserved caspase cleavage motifs (425, 426). Two distinct pathways trigger apoptosis in response to bacterial infection, the intrinsic mitochondrial pathway and the extrinsic receptor-mediated pathway (427). Pyroptosis can be triggered by either canonical caspase-1-dependent inflammasome activation (428) or by a non-canonical caspase-4/5/11-mediated pathway (123). Host cells may also induce caspase-independent programmed cell death, termed necroptosis, which depends on RIPK1, RIPK3 and the Mixed Lineage Kinase domain Like pseudokinase (MLKL) (429).

These distinct types of cell death induce different immune responses. Typically, apoptosis is considered immunologically silent as upon apoptosis, cells shrink and are fragmented into apoptotic bodies that are engulfed by surrounding macrophages, leading to the non-inflammatory nature of the cell death. Necroptosis and pyroptosis, however, both result in cell rupture, releasing intracellular immunostimulatory contents, and elicit a robust inflammatory immune response. These inflammatory responses can help to enhance pathogen clearance during infection, but dysregulation of these cell death pathways can also lead to autoinflammatory disorders (424, 430). The type of cell death induced during bacterial infections is dependent on the activating PAMP, the formation of distinct receptor-proximal complexes and the caspase activation status of the infected cell. These are highly regulated processes and have been extensively reviewed (431).

Table 1.4: Summary of programmed cell death pathways

Pathway	Activators	Biochemical Features	Executioners	Chemical Inhibitions	Inflammatory Features	Ref
Intrinsic Apoptosis	Various stimuli including DNA damage and cytotoxic insults.	Mitochondrial outer membrane permeabilisation. The release of cytochrome c and Smac/DIABLO. Formation of an Apoptosome complex (containing cytochrome c, Apaf-1 and pro-caspase-9) Activation of Caspase-9	Caspase-3, Caspase-7	Delayed but not entirely prevented by Pan-caspase inhibitors (e.g., z-VAD-Fmk)	Non-inflammatory. Apoptotic cell death actively suppresses inflammation. Exposure of phosphatidylserine during the formation of apoptotic bodies induces the generation of anti-inflammatory mediators such as TGF-β1.	(432-435)
Extrinsic Apoptosis	Death receptor signalling	Formation of a death-inducing signalling complex (DISC) Recruitment of caspase-8 and/or -10 by FADD containing receptors (TNFR, Fas or TRAIL-R). Activation of Caspase-8/10	Caspase-3, Caspase-6, Caspase-7	Pan-caspase Inhibitor (z-VAD-Fmk)	As Above	(435, 436)
Necroptosis	TNF and TLR signalling in the absence of caspase-8 activity.	Assembly of the necrosome RIPK1-RIPK3 complex. RIPK1 and RIPK3 phosphorylation. Recruitment and phosphorylation of MLKL. MLKL-induced alternations in ion influx, and osmotic pressure.	MLKL	Active Caspase-8. RIPK1 Inhibitor (Nec1) MLKL inhibitor (NSA)	The release of endogenous danger signals (e.g. HMGB1) Paracrine inflammasome activation in neighbouring cells.	(437-442)
Pyroptosis	Multiple stimuli including; LPS, nucleic acids, Gram-ve bacteria secretion apparatuses, flagellins and sterile danger signals.	Activation of Inflammatory caspases: Caspase-1 via NLR/ALR/pyrin induced inflammasome assembly. Caspase-4 (Caspase-11 in the mouse) and Caspase-5 activation via cytosolic LPS. Cleavage of GSDMD by caspase-1,4,5, and the release of its N-terminal pore-forming fragment (p30).	GSDMD	Pan-caspase inhibitor, (zVAD-Fmk) Caspase-1 Inhibitor, (YVAD-Fmk)	Caspase-1-induced IL-1β and IL-18 maturation and secretion. ASC speck assembly and secretion. Paracrine activation	(443-447)

Inflammasomes and Pyroptosis

Inflammasomes play a fundamental role in regulating the mucosal immune homeostasis and have recently emerged as a central defence mechanism against bacterial pathogens (448). Inflammasomes are macromolecular scaffolds found primarily in the cytosol of immune cells and are responsible for proteolytic maturation of caspase-1 (443, 449), which in turn is responsible for the processing of inflammatory cytokines and inducing a form a lytic cell death termed pyroptosis. These caspase-1-mediated mucosal responses have been shown to be crucial for host resistance to the model A/E pathogen *C. rodentium*, as caspase-1-deficient mice have increased bacterial loads, abnormal inflammatory responses, severe immunopathology and rapid weight loss (126). It is, therefore, not surprising that inflammasomes have recently been demonstrated as a target for EPEC-mediated immune subversion.

1.19. Inflammasome signalling pathways

Several families of intracellular PRRs are essential components in the inflammasome signalling pathway. These receptors are grouped according to their structural features into nucleotide-binding domain-like receptors (NLRs), absent in melanoma 2–like receptors (ALRs), and the recently identified PYRIN. Members of the NLR family typically have a modular structure formed of three parts; a C-terminal Leucine-rich repeat (LRR) domain, a central Nucleotide-binding and oligomerisation domain (NACHT) domain, and a N-terminal caspase recruitment and activation domain (CARD) or pyrin domain (PYD) (450). Despite NLRs being categorised as PRRs, and inducing inflammasome activation in response to diverse stimuli, there is currently no evidence that NLRs interact directly with microbial PAMPs. In contrast, members of the ALR family have a PYD and HIN-200 domain which can directly bind to dsDNA, leading to their activation (451). Pyrin contains an N-terminal PYD domain and is activated in response to host Rho GTPase modifying toxins from pathogenic bacteria (452).

Upon activation inflammasome receptors oligomerise and form a single large multimolecular inflammasome complex within the host cell (453). Caspase-1 contains an N-terminal CARD domain and is recruited to the inflammasome complex via CARD-CARD interactions. To recruit caspase-1, receptors that contain only a PYD domain, such as NLRP3, therefore require the adapter apoptosis-associated speck-like protein containing a CARD (ASC; Also termed Pycard). ASC contains both a

CARD and PYD domain and functions as a bridge between PYD containing receptors and caspase-1. The binding of ASC to an inflammasome sensor protein results in its oligomerisation into prion-like filaments which are characteristically observed as a single ASC “speck” in immunofluorescence analysis (454). Interestingly, while CARD-containing NLRs such as NLRC4 can directly recruit caspase-1 in the absence of ASC through homotypic CARD-CARD interactions to promote pyroptosis, there is evidence that the ASC adaptor is still required for optimal IL-1β and IL-18 processing (455). However, this concept has recently been challenged as a similar mechanism for caspase-1 recruitment via CARD-CARD interactions by either ASC and NLRC4 has been proposed (456, 457).

Despite their cytosolic location, inflammasome receptors are able to initiate an effective immune response against both intracellular and extracellular bacteria. A variety of bacterial ligands can elicit robust inflammasome activation, and distinct inflammasomes assemble depending on the activation signal and the specific sensor (Table 1.5, Figure 1.6, Reviewed (443)),

Recently the NLRC4, canonical NLRP3 and non-canonical NLRP3 inflammasomes have all been implicated in the host immune response to a diverse array of Gram-negative bacteria, including the A/E pathogens *C. rodentium,* EPEC and EHEC (126, 458-461)

Table 1.5: Summary of *in vivo* inflammasome responses to bacterial pathogens

Bacteria	PRR	PAMP	Co-activator	*In vivo* Inflammasome Function	Ref
Citrobacter rodentium	Nlrc4	Flagellin, T3SS	Naip1/2/5/6?	Bacterial clearance	(124, 126)
	Nlrp3		K^+ efflux	Bacterial clearance	
	Caspase-11	LPS			
Salmonella enterica	Nlrc4	Flagellin, (FliC) T3SS Rod (PrgJ), T3SS Needle (PrgI)	Naip5/6 Naip2 Naip1	Host Survival, Bacterial clearance	(462-465)
	Nlrp3		K^+ efflux	Bacterial clearance,	
	Caspase-11	LPS		Bacterial clearance IECs,	
Legionella pneumophila	Nlrc4	Flagellin (FlaA)	Naip5/6	Bacterial growth restriction Bacterial clearance	(466, 467)
Mycobacterium tuberculosis	Nlrp3	ESAT-6,	K^+ efflux	Unknown	(468, 469)
	Aim2	DNA	Type I IFN	Bacterial clearance	
Listeria monocytogenes	Nlrp3	LLO	K+ efflux, GBP5	Bacterial clearance	(470-473)
	Aim2	DNA	Type I IFN	Bacterial clearance	
	Nlrp6			Impedes bacterial clearance	
Francisella tularensis	Aim2	DNA	Type I IFN	Host survival, Bacterial clearance	(474, 475)
Yersinia pestis	Nlrp12			Host survival, Bacteria clearance	(476)
	Nlrp3		K^+ efflux	Host survival	
Bacillus anthracis	Nlrp1b	Anthrax Lethal Toxin,		Host survival, Bacterial clearance	(477, 478)
Burkholderia cenocepacia	Pyrin	TecA		Bacterial growth restriction, Host survival	(452, 479)

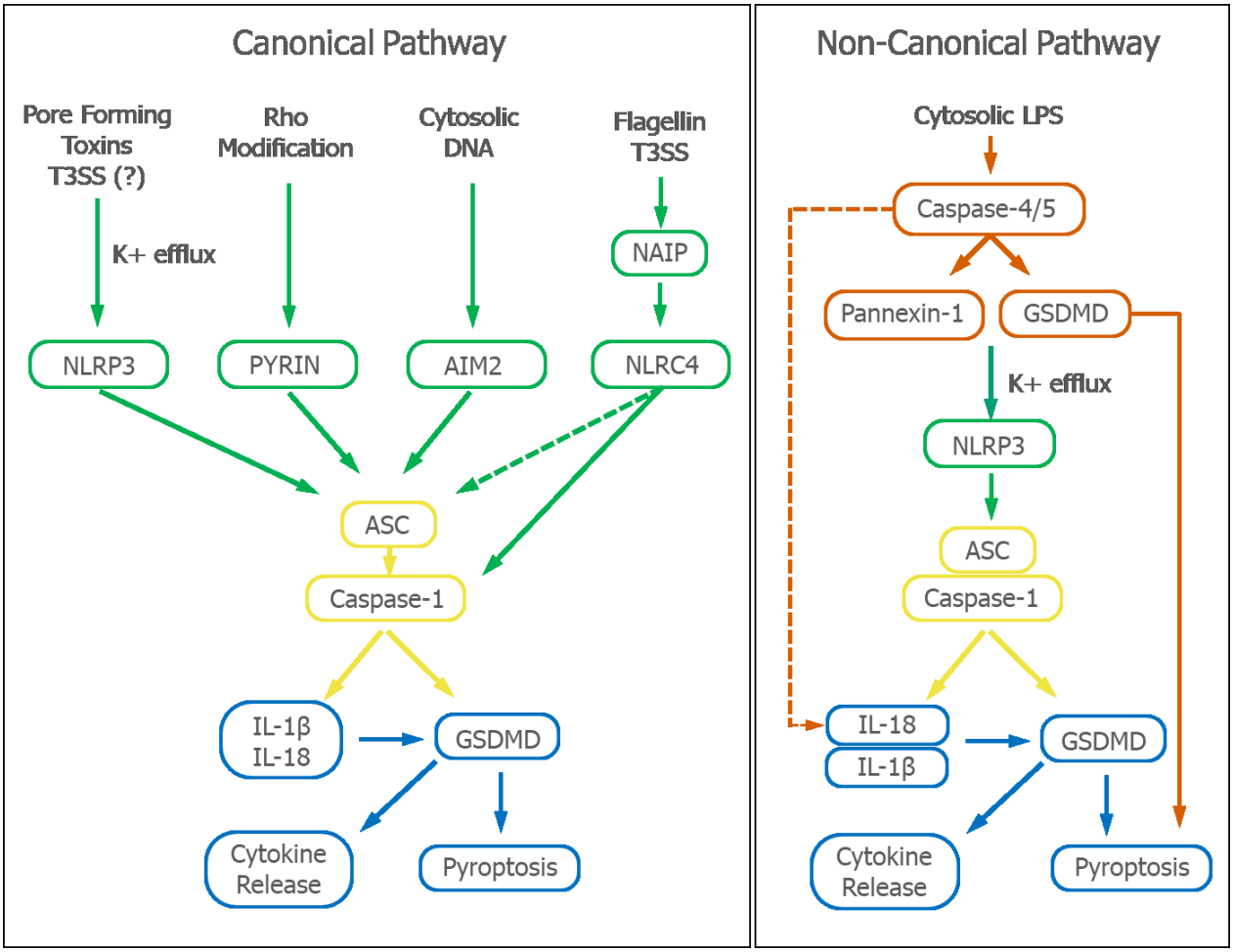

Figure 1.6. Activation of canonical and non-canonical inflammasome pathways by bacterial PAMPs

Various bacterial PAMPs activate canonical inflammasome signalling pathways via sensors belonging to the NLR, ALR or Pyrin family. In human macrophages NLRP3 is activated by bacterial pore-forming cytotoxins which induce potassium (K^+) efflux, AIM2 is activated by cytosolic dsDNA, PYRIN senses pathogen-induced modification of Rho GTPases and the resulting changes to host cell morphology, T3SS proteins and flagellin detected by are NAIP, which functions upstream of NLRC4. Upon activation receptors form an inflammasome complex with the adaptor protein, ASC, which in turn mediates the recruitment of pro-caspase-1. Within the inflammasome complex caspase-1 undergoes auto-proteolysis and activation. Active caspase-1 cleaves IL-1β and IL-18 into their bioactive forms and induces GSDMD cleavage which results in pyroptosis and mediates the secretion of proinflammatory cytokines. In the non-canonical pathway, caspase-4 controls GSDMD cleavage and pyroptosis independently of NLRP3, ASC and caspase-1. Cytoplasmic LPS that enters the cytoplasm during infections with Gram-negative bacteria binds to and activates human caspase-4. Caspase-4 activation also activates the NLRP3 inflammasome via a currently undefined mechanism. Modified from: (443)

1.19.1 The NLRC4 inflammasome

The NLRC4 inflammasome is activated by multiple bacterially derived PAMPs, including flagellin and the rod and needle protein components of the Gram-negative bacterial T3SS (480-483). Notably, NLRC4 is not the receptor for these ligands, instead these are detected by NBD-domain-containing inhibitor of apoptosis proteins (NAIPs). Ligand binding induces conformational changes in the NAIP receptors which exposes a single nucleation surface that binds to NLRC4. Upon NAIP binding, NLRC4 undergoes similar structural rearrangements, causing the sequential recruitment of additional NLRC4 proteins into a ring-like inflammasome complex (484). Pro-caspase-1 is then recruited to the NLRC4-inflammasome via CARD-CARD interactions, in an ASC-independent manner. Humans encode a single NAIP (hNAIP) that is capable of binding the T3SS needle and rod proteins as well as flagellin. In contrast, in the mouse, *Naip* genes have diversified, and different Naips are sensitive to specific ligands; Naip1 binds the T3SS needle proteins, Naip2 binds the T3SS rod proteins, and Naip5 and Naip6 bind flagellins (480).

1.19.2 The canonical NLRP3 inflammasome

The canonical NLRP3 inflammasome is activated by a diverse array of microbial, endogenous, and pollution-associated triggers. Rather than interacting with NLRP3 directly, these activators induce downstream cellular events that lead to its activation. However, the precise stimulus responsible for NLRP3 activation is still under some debate, and proposed activators include; ionic flux (485), lysosomal rupture and cathepsin release (486), generation of mitochondrial-derived reactive oxygen species (ROS) (487), the release of oxidised mitochondrial DNA (488), high extracellular Ca^{2+} (489) and a decrease in cellular and NLRP3-associated cyclic AMP (489). Although, the most regularly accepted trigger for NLRP3 activation is potassium (K^+) efflux (490), which may be a universal downstream consequence of diverse activation pathways, and could explain the multiple mechanisms identified to induce NLRP3 activation. Indeed, activation of NLRP3 by nigericin, pore-forming toxins or Ca^{2+} mobilisation, can all induce K^+ efflux to activate NLRP3 and can be blocked by the addition of extracellular potassium (490-493).

In order to prevent excessive activation in the absence of infection, the NLRP3 inflammasome is regulated on both transcriptional and posttranscriptional levels. The limited expression of *NLRP3* in resting cells limits receptor availability and therefore prevents unwanted NLRP3 activation. Induction of NF-κB pathways induces *NLRP3* transcription, thus NLRP3 activation requires an initial

"priming" signal prior to activation. During bacterial infection, this signal is typically the binding of LPS to TLR4. This activates NF-κB signalling (Figure 1.4) and induces transcription of both NLRP3, and the proinflammatory cytokine pro-IL-1β (494). Deubiquitination of NLRP3 by BRCC3 is also essential for its activation (495). After transcription and deubiquitination, NLRP3 can respond to its stimuli and assemble with ASC to form the NLRP3 inflammasome. ASC recruits pro-caspase-1 to the inflammasome complex through CARD-CARD interactions, where it undergoes proximity induced autoproteolytic maturation.

1.19.3 Caspase-4/5/11 and the non-canonical NLRP3-inflammasome

Canonical inflammasomes of activate caspase-1, downstream of PRR family members, culminates in both pyroptotic cell death and the induction of inflammation through the cleavage and release of mature IL-1β and IL-18. Recently, a non-canonical inflammasome pathway was discovered that involves inflammatory caspases-4/5 in humans and caspase-11 in mice (123, 446, 496, 497). Interestingly, caspase-4/5/11 activation induces pyroptosis through the direct cleavage and activation of the executioner of pyroptosis gasdermin-D (GSDMD) independently of the ASC adaptor or other inflammasome components (123, 445, 498). In contrast to caspase-1 activation of IL-1β and IL-18, cell death appears to be the principal physiological function following activation of caspase-4/5/11, as these caspases are unable to directly cleave and activate inflammatory cytokines, instead they induces proteolytic maturation of caspase-1, in an ASC- and NLRP3-dependent manner to promote cytokine processing (123, 499-503).

The non-canonical inflammasome is activated by Gram-negative bacteria including, but not limited to, *E. coli, Legionella pneumophila, C. rodentium, S. flexneri*, and *Burkholderia* spp. (123, 446, 504). Interestingly, activation of caspase-11 was only evident 15–20 h post-infection with extracellular bacteria, whereas intracellular Gram-negative bacteria such as *L. pneumophila* or genetically modified *Salmonella.* Typhimurium that escaped the vacuole, activated caspase-11 within 2 h (123, 504-506). These observations supported the notion that caspase-11 specifically detected cytosolic Gram-negative bacteria. This concept was further verified by the identification of cytosolic LPS as the bacterial agonist responsible for activating caspase-11 (497, 507). It was initially proposed that additional unknown CARD-containing proteins functioned as intracellular LPS sensors to activate caspase-11 through CARD-CARD interaction. However, more recently, it was discovered that pro-caspase-11 was, in fact, a direct sensor for cytosolic LPS (446). This interaction is accomplished by binding of the CARD motif of pro-caspase-11 to the lipid A tail of LPS (446). Caspase-11 activation

by LPS is dependent on the acylation state of lipid A, with hexa-acylated lipid A inducing optimal activation, penta-acylated lipid-A weakly activating caspase-11 and tetra-acylated lipid A failing to induce any notable caspase-11 activation (507). Infections with intracellular Gram-negative bacteria expressing hexa acylated LPS, therefore induce rapid activation of caspase-11 and subsequent inflammasome responses. Interestingly, some pathogenic bacteria have evolved the ability to selectively modify the acylation state of their lipid A to limit caspase-11 activation during infection and minimise their inflammatory potential (507, 508).

In contrast to mice, humans do not encode caspase-11 but instead encode the genes for both caspase-4 and caspase-5 within their genome. Caspase-4 and caspase-5 are homologues of caspase-11, and are thought to be duplicates derived from a single ancestral caspase-11 gene that have since diverged (509). Similarly to caspase-11, human caspase-4 and caspase-5 were both found to interact directly with intracellular LPS to activate the non-canonical inflammasome in human myeloid cells (446). However, emerging evidence suggests human caspase-4 is the functional orthologue to mouse caspase-11, due to their functional similarities and the fact that caspase-4 and caspase-11 can functionally substitute for each other in the corresponding mouse or human cells. In fact, caspase-11 has recently been renamed mouse caspase-4 (mCasp4), however, for clarity, it will be referred to as caspase-11 throughout this project. Several reports have shown a requirement for caspase-4 in LPS-induced non-canonical inflammasome activation, with both siRNA knock-down and CRISPR/Cas9 deletion of caspase-4 in HeLa cells, THP1 cells, and primary macrophages attenuating the pyroptotic and IL-1β responses to transfected LPS (496, 498, 501). These results suggest that caspase-4 plays the principal role in LPS sensing and non-canonical inflammasome activation within human cells. This was supported by an independent study, in which caspase-4 and caspase-5 were genetically deleted from human monocytic cells. This work demonstrated that deletion of caspase-4 inhibited both cell death and IL-1β cleavage in response to either LPS transfection or *Salmonella* infection. In contrast, deletion of caspase-5 did not confer protection against transfected LPS (503). Although, cell death and IL-1β production were marginally reduced after infection with *Salmonella* in caspase-5 knock-down cells (503).

Mouse caspase-11 and its human counterpart, caspase-4, are oligomerised by direct interaction with intracellular LPS (590). LPS-induced oligomerisation results in the proteolytic cleavage and catalytical activation of these inflammatory caspases. The exact mechanism in which LPS is recognised by caspase-4/11 is currently unclear due to the absence of structural data, however, mutagenesis analysis has identified several basic residues in the CARD of caspase-11 which are

essential for LPS binding (446). Mutations of these residues disrupted LPS binding, inhibited oligomerisation, and prevented caspase-11-induced macrophage pyroptosis (446). Although the precise stoichiometry of LPS and caspase-4/11 has not been fully established, it is thought that LPS binding may induce conformational changes within the caspases that facilitate oligomerisation. Unlike caspase-1 in which activation requires autoprocessing into the p20 and p10 fragments, caspase-4 or caspase-11 activation is often accompanied by the appearance of p32 and p30 fragment respectively, and there is strong evidence suggesting that caspase-4/11 are activated in the absence of complete autoprocessing (510).

As caspase-11 was first identified as a critical modulator in the activation of the non-canonical inflammasome in mouse macrophages, most previous studies have focused on investigating the role of murine caspase-11 during inflammatory responses to infection. However, there are some key differences between the non-canonical inflammasomes in mice and humans. For example, a prerequisite for the activation of the murine non-canonical inflammasome is the transcriptional induction of caspase-11, which is mediated by TLR4-TRIF, type I interferon, and complement signalling (504, 511). In contrast, caspase-4 is constitutively expressed in human cells (446), so does not require an initial priming step to induce activation. Although the mechanism regulating the transcription of caspase-5 in human cells has not been fully established, similarly to caspase-11 in the mouse, caspase-5 expression is upregulated by both type I and II interferons signalling (512). There is also accumulating evidence that the repertoire of proteins capable of interacting with human caspase-4 and murine caspase-11 might not be the same. For example, caspase-4 is able to detect cytosolic tetra-acylated LPS that is not detected by caspase-11 (513) and the T3SS effector OspC3 from *S. flexneri* binds to cleaved caspase-4, thereby preventing its activation, but is incapable of interacting with caspase-11 (514).

In addition to LPS binding, caspase-11 can also be activated by endogenous DAMPs. In response to pathogenic insult or tissue damage cells can enter a state of oxidative stress which results in an increase of reactive oxygen species (ROS) in the tissue. The accumulation of ROS can result in oxidation of host compounds such as plasma membrane phospholipids, and induce the formation of oxidized phospholipids such as 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (oxPAPC). It was recently discovered that oxPAPC, can bind to and activate both caspase-4 and caspase-11. In dendritic cells this binding induces IL-1β secretion independently of caspase-11 catalytic activity, but does not induce pyroptosis (515). Conversely, in macrophages oxPAPC inhibits the non-canonical inflammasome by competing with LPS binding to caspase-11 and

consequently inhibiting LPS-induced pyroptosis (516). Notably, unlike LPS which binds to the CARD domain of caspase-11, oxPAPC binds to the catalytic domain of caspase-11. This provides evidence that different activation states of caspase-4/11 can induce diverse downstream cell-type specific responses.

1.20. Gasdermin-D and pyroptosis

Pyroptosis was initially characterised as caspase-1-mediated proinflammatory cell death (428, 517). However, more recently, advances in understanding have revolutionised the concept of pyroptosis, and a number of inflammatory caspases have been implicated in pyroptotic cell death, including human caspase-1, caspase-4 and caspase-5. The identification of GSDMD as the caspase-substrate responsible for inducing membrane rupture and cell death has prompted a new definition of pyroptosis as gasdermin-mediated programmed cell death (445, 518).

The role for GSDMD as the executioner of pyroptosis was first identified simultaneously by two independent research groups, who reported that GSDMD was required for both caspase-1-mediated IL-1β secretion, and caspase-1 and caspase-11-mediated pyroptosis in mouse macrophages (445, 498). GSDMD is a highly conserved protein in mammals. Human GSDMD is 484 amino acids long with two main domains: an N-terminal GSDMD-N domain (p30) and a C-terminal GSDMD-C domain connected by a linker loop, that contains a caspase cleavage motif ($_{272}$FLTD$_{275}$) (445). GSDMD can be cleaved by the inflammatory caspases; caspase-1/4/5/11, but is insensitive to apoptotic caspases, making it an inflammasome specific substrate. Interestingly, inhibiting GSDMD cleavage by mutating the cleavage site rescued cells from pyroptosis induced by any of the known inflammasomes, suggesting that interdomain cleavage of GSDMD is sufficient for pyroptosis. This concept was supported by the discovery that expression of the N-terminal domain of GSDMD in mammalian cells is sufficient to induce pyroptosis (445, 498, 519). Structural analysis of GSDMD found that full-length GSDMD is inactive due to autoinhibition by its gasdermin-C domain. Upon caspase-mediated cleavage, the N-domain is released and localises to cell membranes where lipid binding induces oligomerisation and the formation of GSDMD-pores within the membrane (519-521). These pores are thought to disrupt the osmotic potential of the cell, causing cell swelling and eventually pyroptosis.

1.21. Non-canonical inflammasome pathways and pyroptosis

A number of studies have investigated the molecular mechanisms by which activation of caspase-4/11 induces pyroptosis and downstream non-canonical NLRP3-inflammasome activation in macrophages. However, the precise mechanism is still under debate, and two models have been proposed (Figure 1.7). In the first model, GSDMD has been identified as the critical player in these events, as GSDMD-deficient mice have defects in both pyroptosis and IL-1β secretion in response to intracellular LPS or Gram-negative bacterial infection and exhibit resistance to LPS-induced septic shock (123). Upon activation caspase-4/11 induce the proteolytic cleavage and activation of GSDMD and subsequent membrane pore formation, and this is proposed to directly induce pyroptosis, as well as K^+ efflux to activate the NLRP3-ASC-caspase-1 pathway (502). This model is supported by the observation that GSDMD is required for both cell death and the processing of pro-caspase-1 and pro-IL-1β in response to cytoplasmic LPS, placing GSDMD downstream of caspase-4/11, but upstream of NLRP3 and caspase-1 within this activation cascade (498).

However, more recently a second pathway was proposed, in which activated caspase-4/11 cleaves the cytosolic C-terminus inhibitory portion of the cell surface pannexin-1 channel to cause ATP release and K^+ efflux (500). Pannexin-1 (Panx1) is a plasma membrane channel ubiquitously expressed in both animal and mammalian tissues. Previously, cleavage of pannexin- 1 by caspase-3 and caspase-7 has been shown to induce apoptosis (522). Interestingly, caspase-11 cleaves the same site as these apoptotic caspases (500). Mechanistically, the cleavage of pannexin-1 by caspase-11 opens its pore and promotes the of release ATP. Extracellular ATP activates the ATP-sensitive purinergic receptor P2X7, which undergoes conformational changes and forms a gated channel facilitating rapid K^+ efflux (523). In addition, depending on the concentration of ATP, P2X7 can form larger membrane pores that disrupt the membrane integrity and induce pyroptosis (500). This research demonstrated that in the absence of either P2X7 or pannexin-1, cytosolic LPS failed to induced pyroptotic cell death. Consist with this observation, *Casp11*$^{-/-}$, *Panx1*$^{-/-}$, or *P2x7*$^{-/-}$ mice, were protected from LPS-induced sepsis *in vivo* (500). Whether GSDMD cleavage plays a role in inducing cell death downstream of P2X7 is not clear, however, as the GSDMD-deficient macrophages are protected from pyroptosis induced by cytosolic LPS it is likely that GSDMD functions within this pathway.

In addition to pyroptosis, pannexin-1 mediated K^+ efflux was shown to trigger NLRP3-caspase-1 activation independently of P2X7 and pyroptosis (500). This was demonstrated by the fact that

LPS-induced IL-1β secretion was inhibited by either chemical inhibitors of the pannexin-1 channel (probenecid) or in *Panx1* knock-out cells, but was not prevented in *P2x7* knock-out cells (500). Interestingly, the requirement for pannexin-1 within this pathway was shown to be time dependent, with pannexin-1 being required for IL-1β secretion induced by cytosolic LPS delivery at 2 h and 8 h post-stimulation, but was dispensable for IL-1β release at 16 h post-stimulation (123, 500). Additional work is required to clarify whether caspase-4/11 cleavage of GSDMD or pannexin-1 is the principle stimulus for downstream NLRP3 activation.

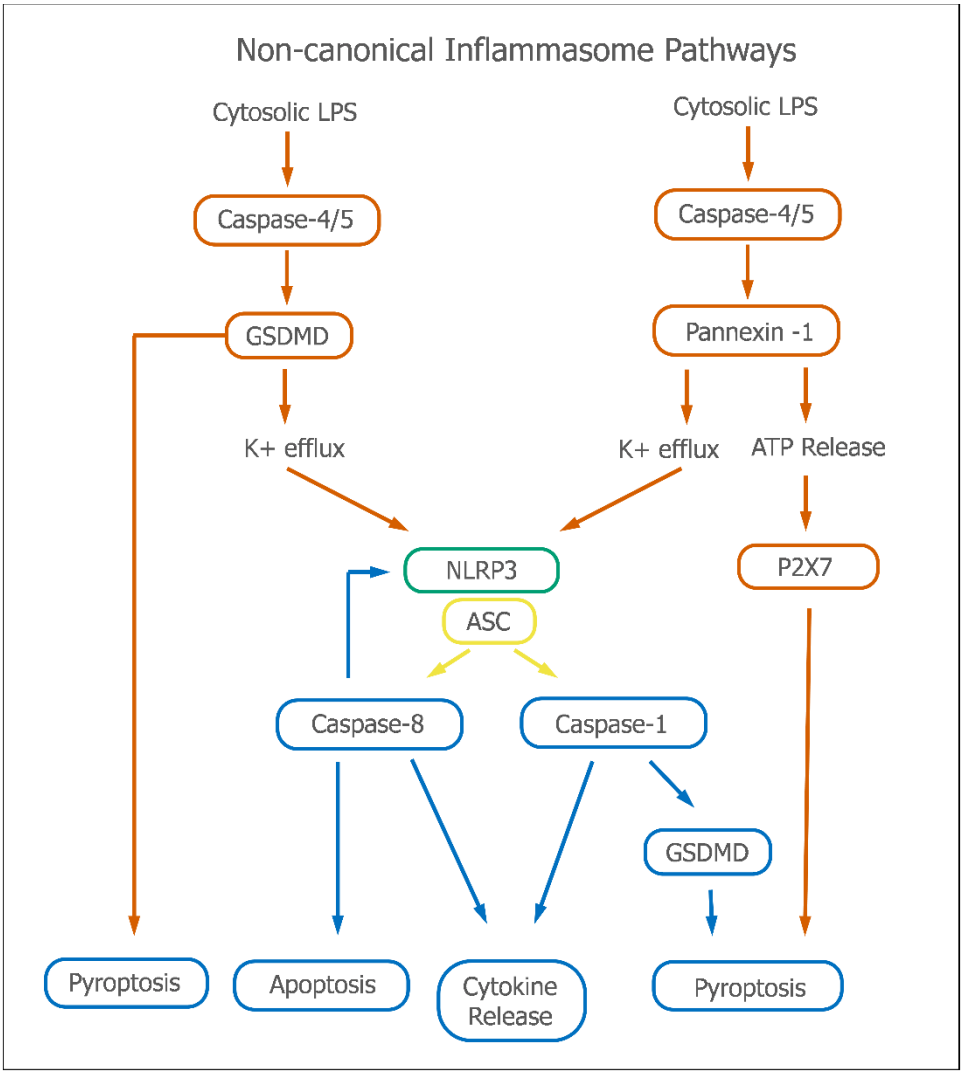

Figure 1.7. Schematic representation of the non-canonical-NLRP3 inflammasome pathway

Caspase-4 activation by cytosolic LPS induces pyroptosis and NLRP3 activation. Two distinct mechanisms have been proposed for this activation. In the first, caspase-4-mediated cleavage of GSDMD is thought to directly induce pyroptosis, and potassium (K^+) efflux to activate NLRP3. Alternatively, caspase-4-induced pyroptosis has been proposed to result from caspase-4 cleavage of pannexin-1 which triggers K^+ efflux to promote NLRP3 activation independently of pyroptosis. Pannexin-1 cleavage also induces ATP release from host cells, which can induce P2X7-dependent pyroptosis. After K^+ efflux induced activation NLRP3 oligomerises with the adaptor protein ASC to recruit caspase-1, which in turn cleaves and activates GSDMD to induce pyroptosis and processing of the proinflammatory cytokines IL-18 and IL-1β. In the absence of caspase-1 the NLRP3-ASC complex can bind and activate caspase-8 to induce apoptotic cell death and IL-1β processing.

1.22. The cytosolic access of LPS

As mentioned earlier, extracellular LPS robustly stimulates transcription through TLR4 signalling. However, cytosolic LPS is required to activate the non-canonical inflammasome. Typically, extracellular LPS molecules are unable to translocate across the plasma membrane of host cells. However, the non-canonical inflammasome is activated in response to both cytosolic, non-cytosolic and extracellular Gram-negative bacterial infections (504), therefore the mechanism by which LPS enters the cytosol during bacterial infection of non-cytosolic or extracellular bacteria to induce caspase-4/11 activation is an important field of study.

Host cell guanylate-binding proteins (GBPs), have been implicated in facilitating the cytosolic entry of both LPS and DNA during bacterial infections (524-527). Type I interferon signalling during Gram-negative bacterial infections upregulates the expression of interferon-inducible GBPs (413), and recently these GBPs have been shown to accumulate on phagosomal membranes to facilitate the release of bacterial products, including LPS, into the cytosol for recognition by caspase-4/11 (528). Macrophages deficient in GBPs also have impaired caspase-11 activation and reduced pyroptosis in response the LPS transfection directly into the host cell cytosol (529), which suggests that GBPs have additional functions within the non-canonical inflammasome pathway. Indeed, GBP5 was shown to be required for both canonical and non-canonical NLRP3 inflammasome activation (470, 526). Other GBPs have since been identified as key regulators of inflammasome responses (527, 530, 531), and additional host-derived proteins, such as the IRG family protein IRGB10, have also been shown to work in co-ordination with GBPs to facilitates the release of ligands from intracellular pathogens for sensing by inflammasomes (524).

One initial observation was that Gram-negative bacteria that activate caspase-4/11 typically possess a T3SS to deliver bacterial effector proteins into the host cell, and it is a possibility that LPS is delivered by the T3SS, however this remains to be experimentally verified. Interestingly, bacterial mutants lacking the T3SS and non-pathogenic strains of *E. coli* that lack virulence factors are able to activate the non-canonical inflammasome, however only when assays were conducted over sufficiently long periods (16–24 hours). This was demonstrated to be dependent on the secretion of LPS-laden outer-membrane vesicles (OMVs) (532), OMVs were shown to enter the macrophages through clathrin-mediated endocytosis and are trafficked to early endosomes where LPS is able to access the cytosol by a currently an unknown mechanism (532). However, recent work has revealed that GBPs may be required to mediate this process. Macrophages lacking GBP2 expression

were protected from OMV induced pyroptotic cell death and proinflammatory IL-1β and IL-18 secretion, and GBPs were shown to control inflammation and sepsis in mice injected with purified OMVs derived from *E. coli.* (526, 531). TRIF mediated expression of GPBs was also shown to be required to facilitate transport of LPS from OMVs into the cytosol to induce inflammasome activation (533).

1.23. Caspase-8 and the inflammasome

Recently several lines of evidence have demonstrated a role for caspase-8 in the cleavage of IL-1β and NLRP3 inflammasome activation (Figure 1.7). Caspase-8 was shown to directly cleave IL-1β in conditions where TLR3 or TLR4 were stimulated to induce transcription of IL-1β, but cellular stressors such as protein synthesis inhibition, ER-stress or chemotherapeutic compounds induced caspase-8 activation (534-537). This resulted in caspase-8 processing of the inactive precursor pro-IL-1β at the same site that is cleaved by caspase-1, although at a significantly less efficient rate than caspase-1 (534).

Furthermore, in the absence of caspase-1, NLRP3-ASC oligomerisation has been linked to apoptotic cell death through the interaction of the ASC-PYD domain with the Death effector domain (DED) of caspase-8. This interaction results in caspase-8-induced apoptosis, as well as IL-1β processing (538-541). Interestingly, unlike caspase-4/11-mediated pyroptosis, NLRP3- and caspase-1-induced death is not completely blocked in GSDMD-deficient cells over extended periods (498, 499). This observation is likely to reflect the ability of NLRP3 to activate caspase-8 to induce apoptosis in the absence of caspase-1-mediated GSDMD cleavage. Currently, attempts to characterise the physiological implications of these ASC-induced caspase-8 responses *in vivo* are limited by the fact that caspase-8 deletion results in RIPK3 necroptotic signalling and causes embryonic lethality in mice (542), and the observation that caspase-8 is required for transcriptional priming of various inflammasome components (543, 544). However, infection models with *S.* Tyhimurium*, Y. pestis* and *L. monocytogenes* have demonstrated that caspase-8 may also have a role in regulating post-translational activation of ASC-dependent inflammasomes (544-546). *Salmonella* infections can induce both the NLRC4 and NLRP3 inflammasomes, and it was recently reported that caspase-8 is recruited to both these inflammasome during infection, in an ASC-dependent manner. Within the NLRC4 inflammasome, caspase-8 is proteolytically activated, independently of caspase-1/11 activity during *Salmonella* infection and contributed to IL-1β processing late in infection (544). *Yersinia* infections activate a RIP1-caspase-8/RIP3-dependent

caspase-1 activation pathway, and *Listeria*-induced caspase-1 activation is also dependent on caspase-8 (545, 546).

1.24. Pro-inflammatory cytokines

The important role of inflammatory signalling, and cytokine production has been well established in the context of host protection against enteric pathogens such as EPEC, and this is underscored by the diverse array of effector proteins that actively subvert these pathways in order to propagate infection. However, whether the inflammatory reaction can be fully elicited is not only dependent on transcriptional control, but also depends on the post-translational modifications that are responsible for the activation and subsequent release of these inflammatory signals. In the case of the proinflammatory cytokines IL-1α, IL-1β and IL-18, this is mediated by inflammasomes, and these cytokines are critical to host defence in terms of enhancing innate and adaptive immunity against invading pathogens.

Pro-IL-1β and pro-IL-18 are biologically inert precursors that require proteolytic maturation by caspase-1 into their bio-active p17 and p18 forms, whereas IL-1α is not a direct substrate of caspase-1. However, all of these cytokines rely on inflammasome activation to mediate their unconventional secretion (547). Mature IL-1β and IL-1α have overlapping immune roles, as they are both agonists for the same IL-1 receptor (548). However, differences in the regulation and expression of these two cytokines exist that impact on their immune function, for example the IL-1α is constitutively expressed in the epithelial cells within the gastrointestinal tract, and upon inflammatory cell death or inflammasome activation is released, initiating a downstream inflammatory cascade. IL-1α is therefore often associated with sterile inflammation (549). In contrast, IL-1β production is limited to a subset of hematopoietic cells such as tissue macrophages in response to TLR signalling and is not expressed in human intestinal epithelial cells. During infection IL-1β plays an important role in inflammatory signalling *in vivo*, and genetic deletion of the IL-1R in mice has revealed the importance of inflammasomes and IL-1 activity for immune responses and protection from a wide range of bacterial pathogens (550). Both IL-1β and IL-1α bind to the IL-1 receptor and induce a signalling cascade which results in the transcriptional upregulation of proinflammatory cytokines and chemokines (Figure 1.4), and includes genes that propagate the recruitment of immune effector cells to the site of infection. While these responses are critical for host protection from many types of bacteria, the dysregulation of IL-1β activity has been implicated in a variety of different autoinflammatory disorders (551). IL-1β is therefore tightly

regulated, requiring transcriptional induction and subsequent activation by caspase-1, and cells express a IL-1 receptor antagonist, a bona fide member of the family, which binds to the IL-1 receptor and reduces IL-1α and IL-1β responses (548).

IL-18 is similar to IL-1β in that both are initially synthesised as inactive precursors and require caspase-1 for cleavage. However, the regulation of IL-18 differs from that of other IL- family members due to the fact that it is constantly expressed. Furthermore, IL-18 does signal via IL-1R family receptors, instead the activity of IL-18 is mediated via a putative IL-18 receptor (IL-18R) complex. IL-18R signalling activates MAPK p38 and the downstream nuclear translocations of AP-1 and NF-κB transcription factors, in a TRAF6-dependent manner, inducing gene expression and synthesis of TNF, IL-1, Fas ligand, and several chemokines (552). During infection, a principle function of IL-18 is to promote the production of type II interferon IFNγ from T and NK cells (552, 553). IFNγ activates and recruits both neutrophils and macrophages for intracellular killing of bacteria. The importance of IFNγ in host defence is demonstrated in human subjects with a deficiency in IFNγ pathways, who have increased risk for bacterial infections (554).

The mouse pathogen *C. rodentium* triggers IL-1 responses in a NLRP3-dependent manner *in vivo (126)*, and *in vitro* studies have shown that *C. rodentium*-mediated NLRP3-dependent caspase-1 maturation and IL-1 responses also require the non-canonical pathway activated by caspase-11 (123). The role of NLRP3-induced IL-1β was demonstrated by infection of *Nlrp3*$^{-/-}$ mice, which developed severe colitis during infection. However, IL-1β treatments reduced the severity of infection in *Nlrp3*$^{-/-}$ mice (461). Conversely, surplus IL-1β during infection of WT mice was shown to have adverse effects, demonstrating the importance of a balanced cytokine response in protection against *C. rodentium* infection.

1.25. Unconventional secretion of pro-inflammatory cytokines

IL-1β and IL-18 are classed as leaderless proteins, because they lack the sorting motif required for entry into the classical ER-Golgi secretory pathway, and are released through a still poorly understood caspase-mediated unconventional secretory pathway. The precise mechanisms behind the release of IL-1β have been highly debated, with a particular focus on whether IL-1β is actively secreted or simply passively released during cell death following membrane rupture. Studies using single-cell imaging technology on monocytic cell lines or macrophages have concluded that cell death is unavoidable following caspase-1 activation, and therefore IL-1β is

only released from dying cells (555). Furthermore, the observation that NLRP3 activation in GSDMD-deficient macrophages does not prevent caspase-1 processing of IL-1β into its mature form, but does prevent IL-1β secretion (445, 499), suggests that GSDMD-induced cellular rupture is required for its release. However, there is a growing body of genetic and biochemical data that suggests that IL-1β secretion and pyroptosis are separable, at least in some cell types. Indeed recent evidence has demonstrated that IL-1β can be actively secreted independently of cellular lysis (500, 515, 556), and osmoprotectants such as glycine can prevent pyroptosis, but not the secretion of mature IL-1β (557). This distinct state in which viable cells are able to release IL-1β, along with other cytokines, in the absence of proptosis has been termed "hyperactivation" (515). Interestingly, in agreement with previous work, GSDMD was shown to be required for IL-1β secretion in this context, but this secretion occurred independently of cell death (558, 559). Rather, GSDMD pores are through to function as conduits for the secretion of inflammatory cytokines during cell hyperactivation.

1.26. Caspase-11 and the actin cytoskeleton

In addition to the well-characterised roles of caspase-11 in inflammasome activation, pyroptosis and inflammatory cytokine maturation, caspase-11 has also been reported to regulate other host innate immune defence mechanisms. Recently, caspase-11 has been shown to play a key role in modifying actin dynamics during infection, by modulating the activity of a number of actin associated proteins. Caspase-11 can regulate cofilin phosphorylation, a key event during actin remodelling. Additionally, the CARD domain of caspase-11 physically interacts with the C-terminal WD40 propeller domain of the actin interacting protein 1 (AIP1) (also known as WDR1) (560). This interaction potentiates cofilin-mediated actin depolymerisation to enhance the remodelling of the cytoskeleton following traumatic events (561). In contrast to its role in pyroptosis, caspase-11-mediated actin depolymerisation occurs independently of its enzymatic activity (560). Thus, caspase-11 expression alone, in the absence of catalytic activation, may be sufficient to direct cell migration to the site of infection as part of the defence against pathogen invasion. The co-ordinated roles of inflammatory caspases and the cytoskeleton are inextricably linked to cell-autonomous host defences. Previous research has shown that caspase-11-dependent phosphorylation of cofilin is essential for the control of *L. pneumophila* replication in macrophages (562) and both caspase-1 and caspase-11 promote bacterial clearance by modulating actin polymerisation via RhoA and Slingshot proteins (563). Flightless-1, a member of the gelsolin

superfamily of actin-remodelling proteins, was also shown to serve as a pseudo-substrate and inhibitor of caspase-1 (564). These studies collectively demonstrate complex interplay between perturbations in the actin cytoskeleton and inflammatory caspases. Interestingly, caspase-11 is not the only inflammasome related component that interacts with the actin cytoskeleton. Perturbation of actin polymerisation by pathogens has previously been shown to activate the pyrin inflammasome (452), and aberrant actin polymerisation can trigger L-18-dependent autoinflammatory disease (565). Both Pyrin, and ASC localise to the leading edge in migrating monocytes, and have been shown to concentrate in dynamically polymerising actin-rich tails generated by *L. monocytogenes (566)*. Furthermore, NLRC4 is required to induce actin dynamics around *S*. Typhimurium during infection of macrophages, which is required to mediate ASC-assembly and the formation of inflammasome complexes, as well as the generation of mitochondrial ROS to restrict intracellular bacterial growth (567).

Caspase-11 has also been implicated in the lysis of phagosomes and the restriction of intracellular pathogen growth, independently of pyroptosis (465, 562, 568). It has been proposed that caspase-11 itself mediates the release of vacuolar bacteria into the cytosol for detection by intracellular PRRS (569). It has also been reported that caspase-11 restricts infections of through altering actin dynamics and promoting the fusion of bacterial containing phagosomes with lysosomes (562). Surprisingly, caspase-11 specifically alters the trafficking of phagosomes containing pathogenic bacteria and not non-pathogenic bacterial strains such as *E. coli* DH5α. This suggests that caspase-11 is able to discriminate between pathogenic and non-pathogenic Gram-negative bacteria in this context, through a currently unknown mechanism. Collectively, this research indicates that in addition to their role in pyroptosis during bacterial infection, inflammatory caspases have additional auxiliary functions within host cells which require further investigation.

1.27. Inflammasome responses to EPEC infection

Inflammasomes play a vital role in the innate immune response to bacterial infections (570). The use of gene knock-out mice has been paramount in determining the specific antimicrobial functions of inflammasomes *in vivo*. In the context of bacterial infection, loss of inflammasomes associated proteins usually causes increased bacterial replication, aberrant inflammatory responses and decreased survival of mice. In an attempt to characterise the inflammatory response to A/E pathogens such as EPEC *in vivo*, *C. rodentium* infections of knock-out C57BL/6 mice have been utilised. These studies demonstrated that caspase-1-mediated responses were crucial for

host resistance to *C. rodentium*, and that loss of inflammasome signalling-related genes (such as *Nlrp3, Nlrc4, Casp1, Casp11, IL1b*, and *IL18*) resulted in a greater bacterial burden at late stages of infection and higher morbidity (125, 126, 571). Collectively, these studies established that caspase-1, caspase-11, NLRP3, NLRC4, IL-1β and IL-18, all play a crucial role in the immune response to *C. rodentium* infection *in vivo*. However, the activating stimulus that induces these inflammasome responses during infection, and whether EPEC infections within human cells elicit the same inflammatory responses still remains to be established.

1.27.1 EPEC and the NLRC4 inflammasome

Due to their presence in diverse pathogenic bacteria, the T3SS and flagellar are a major target for detection by the host immune system. The NLRC4 inflammasome is responsible for recognising these conserved protein agonists, and during *C. rodentium* infection, *Nlrc4*$^{-/-}$ mice have marked weight loss between days 6-12 post-infection and increased pathogen burden (572). This was accompanied by increased intestinal inflammation and pathology, indicating that Nlrc4 is activated in response to infection, and this activation is required to propagate an effective immune response. However, the role of NLRC4 signalling during EPEC infections remains unclear, as EPEC has evolved mechanisms that largely allow it to escape detection by the NAIP-NLRC4 complex. Interestingly, although cytosolic flagellin molecules derived from *S.* Typhimurium (FliC) or *Legionella* (FlaA) interact with Naip5 to induce Nlrc4 activation in mouse macrophages, flagellins from EPEC (FliC) are intrinsically inactive in binding to Naip5 and do not activate the Nlrc4 inflammasome (483), suggesting that tEPEC flagellin has evolved to evade detection by this inflammasome. Consistent with this observation, EPEC FliC was shown to be dispensable for infection-induced caspase-1 activation in mouse macrophages (483). Similarly, the EPEC T3SS needle protein EscF fails to bind to human or mouse NAIP and exemplifies another evasion mechanism (483, 573). The mechanism by which EPEC flagellum and T3SS structures evade detection by NAIP receptors and the NLRC4 inflammasome is currently unclear, however a recent study has identified a critical sequence determinant in flagellin that is essential for ligand–receptor interactions (574). This amino-acid sequence is absent from EPEC flagellin, suggesting that mutations in this region of flagellin may represent a mechanism engaged by some pathogenic bacteria to evade detection by the NLRC4 inflammasome. However, the EPEC T3SS rod protein EscI was shown to be a ligand for mouse Naip2, and transient transfection of EscI into mouse macrophages has been shown to induce Nlrc4-dependent pyroptosis (481) (Figure 1.8). The majority of the characterisation of Nlrc4 responses to EPEC proteins have been completed in the context of mouse infections, where the Naip–Nlrc4

innate immune axis requires specific Naip receptors to respond to these diverse activators (481, 483). In human cells a single hNAIP is responsible for NLRC4 activation (575), and whether this human NAIP receptor can respond to EPEC ligands in a different manner to that of its mouse counterparts remains to be tested.

1.27.2 EPEC and the NLRP3 inflammasome

During bacterial infection the NLRP3 inflammasome can be activated in two ways; by canonical signalling through the loss of cellular K^+, or by LPS-induced non-canonical inflammasome signalling. EHEC, EPEC and *C. rodentium* were among the first Gram-negative pathogens shown to activate the caspase-11-dependent non-canonical inflammasome in mouse macrophages in a TLR4- and TRIF-dependent manner (123, 504, 571). This work demonstrated that caspase-11 and NLRP3 activation occurred independently of the T3SS or other pathogen associated virulence factors, and induced inflammasome activation 16 - 20 h post infection. However, these studies used bacteria that were grown in condition that do not induce T3SS, LEE or non-LEE effector expression. DMEM 'primed' EPEC expressing these virulence factors, have previously been shown to induce significantly more IL-1β secretion than a T3SS-deficient strain (*ΔescF*) during infection of human THP1 cells (226). Notably, this work also demonstrated that EPEC strains expressing the LEE operon induced detectable caspase-1 activation 4 h and 7 h post infection, suggesting that the expression of the LEE during infection may drive rapid inflammasome activation in human macrophages. Therefore, the role of the T3SS and secreted effector proteins during inflammasome activation in human macrophages requires further investigation. This is particularly relevant as a number of secreted EPEC effector proteins have recently been shown to inhibit the canonical and non-canonical NLRP3 inflammasomes (Figure 1.8).

The T3SS pore itself, or T3SS activity has the potential to activate the NLRP3 canonical inflammasome by promoting K^+ efflux. This concept was demonstrated as NLRP3-inflammasome responses were induced by *Yersinia* strains that expressed a functional T3SS in the absence of other translocated virulence factors, which typically inhibit this activation pathway (576). More recently this T3SS-induced activation was attributed to the T3SS translocon constituents YopD (Homologue of EPEC EspB) and YopB (homologue of EPEC EspD) (577). Whether the EPEC translocon also harbours the ability to induce canonical NLRP3 activation has not been established as, similarly to *Yersinia,* this T3SS-induced activation may be masked by the inhibitory functions of secreted effector proteins.

1.27.3 EPEC and the non-canonical inflammasome in intestinal epithelial cells (IECs)

Differential expression of inflammasome sensors and substrates results in distinct outcomes following inflammasome activation in different cell types. Inflammasome signalling is best understood in macrophages and dendritic cells, however, intestinal epithelial cells (IEC) also express several inflammasome-signalling proteins and the pro-IL-18 substrate. Inflammasome activation has an important role in the immune response to infection by A/E pathogens, and recently dissection of the contributions of the different cellular compartments during *in vivo C. rodentium* infection of C57BL/6 mice has revealed inflammasome activation and IL-18 processing by IEC is critical for protection early in infection (228, 464, 572, 578). However, the Nlrp3 response mounted by macrophages plays an essential role in reducing the infection severity and preventing mortality later in infection (520).

1.28. EPEC effector-mediated inhibition of inflammasome pathways

Inflammasome sensors such as NLRP3 and mouse caspase-11 require an initial priming stimulus to induce expression and to enable post-translational licensing. Similarly, pro-IL-1β expression is also upregulated by NF-κB signalling in myeloid cells. As discussed previously, NF-κB inhibition by several EPEC effectors could potentially reduce mature IL-1β production in macrophages and reduce inflammation. In addition to NF-κB-suppressing effectors, functional studies of other EPEC effectors have provided evidence that components of the inflammasome pathways are directly targeted by secreted effector proteins (Figure 1.8).

The EPEC effector NleA has previously been shown to directly target the NLRP3 sensor to inhibit inflammasome formation, caspase-1 activation and IL-1β secretion during EPEC infections (226). NleA (also called EspI) is a non-LEE-encoded T3SS effector that is common to EPEC, EHEC and *C. rodentium,* and has been shown to be an essential virulence factor (579, 580). In EPEC, NleA is encoded on the PP6 PAI, and was initially found to inhibit vesicle trafficking along the host cell ER secretory pathway, by interacting with coatomer protein II (COPII) complex in epithelial cells (581). However, in human THP1 cells NleA was shown to inhibit NLRP3 inflammasome formation, and caspase-1 processing 4 h post infection. This inhibition was mediated by a direct interaction with NLRP3 that interrupted its de-ubiquitination, which is necessary for inflammasome activation. However, the activating stimulus that induces inflammasome

activation in the absence of NleA, and the precise mechanism by which NleA affects NLRP3 ubiquitylation remain to be determined.

During EPEC infection the T3SS translocation pore components EspB and EspD are inserted in the host plasma membrane, whereupon they oligomerise to form the translocator pore. This establishes a conduit between the bacterial cytosol and host cell cytoplasm, enabling the direct delivery of effector proteins. However, this may also have the potential to activate NLRP3 by promoting K^+ efflux from the host cell. Recently, the translocon pore-forming activity of EPEC EspD-EspB was reported to be regulated by EspC. After the insertion of EspD-EspB into the host cell membrane and formation of the translocon pore, EspC degrades EspD to regulate its activity, effectively limiting the cytotoxicity associated with pore formation (75). Therefore, a *ΔespC* EPEC strain has been shown to be more cytotoxic during infection of epithelial cells. However, the mechanisms of cytotoxicity and indeed whether this is NLRP3-inflammasome-dependent remains to be tested.

The EPEC effector NleF is an inhibitor of three caspase family members; caspases-4, 8, and 9 (230). The co-crystal structure of caspase-9-NleF revealed that insertion of four C-terminal residues of NleF into the caspase-9 active site inhibits its proteolytic activity in a manner similar to the inhibitor peptide zEAD-Dcmbk. As a direct caspase inhibitor, ectopic expression of NleF potently blocks cell death. However, in the context of infection NleF cannot inhibit EPEC-induced cell death due to its low abundance (538, 634). The recent identification of the protective role of caspase-4-induced IL-18 secretion during EPEC and *C. rodentium* infections highlighted a potential role of NleF in mediating these inflammatory responses (228, 464). Recombinant NleF from EPEC was shown to bind to and inhibit the catalytic activity of caspase-4 in a dose dependent manner (228). Infections of human IECs demonstrated that EPEC NleF inhibited caspase-4 activation and markedly reduced the secretion of mature IL-18 during infection, pointing to a physiologically relevant subversive role for NleF in inflammasome signalling. Interestingly, *ΔnleF C. rodentium* infections has significantly increased colonic secretion of IL-18 early during *in vivo* infections, and this increase in IL-18 correlated with a significant increase in neutrophil recruitment (228). As caspase-8 has recently been implicated in the non-canonical NLRP3-inflammasome activation pathway, NleF inhibition of caspase-8 may also provide additional negative regulation of the NLRP3 inflammasome. However, this remains to be experimentally validated. Importantly, the mechanism by which the non-canonical inflammasome is activated during EPEC infection still remains elusive.

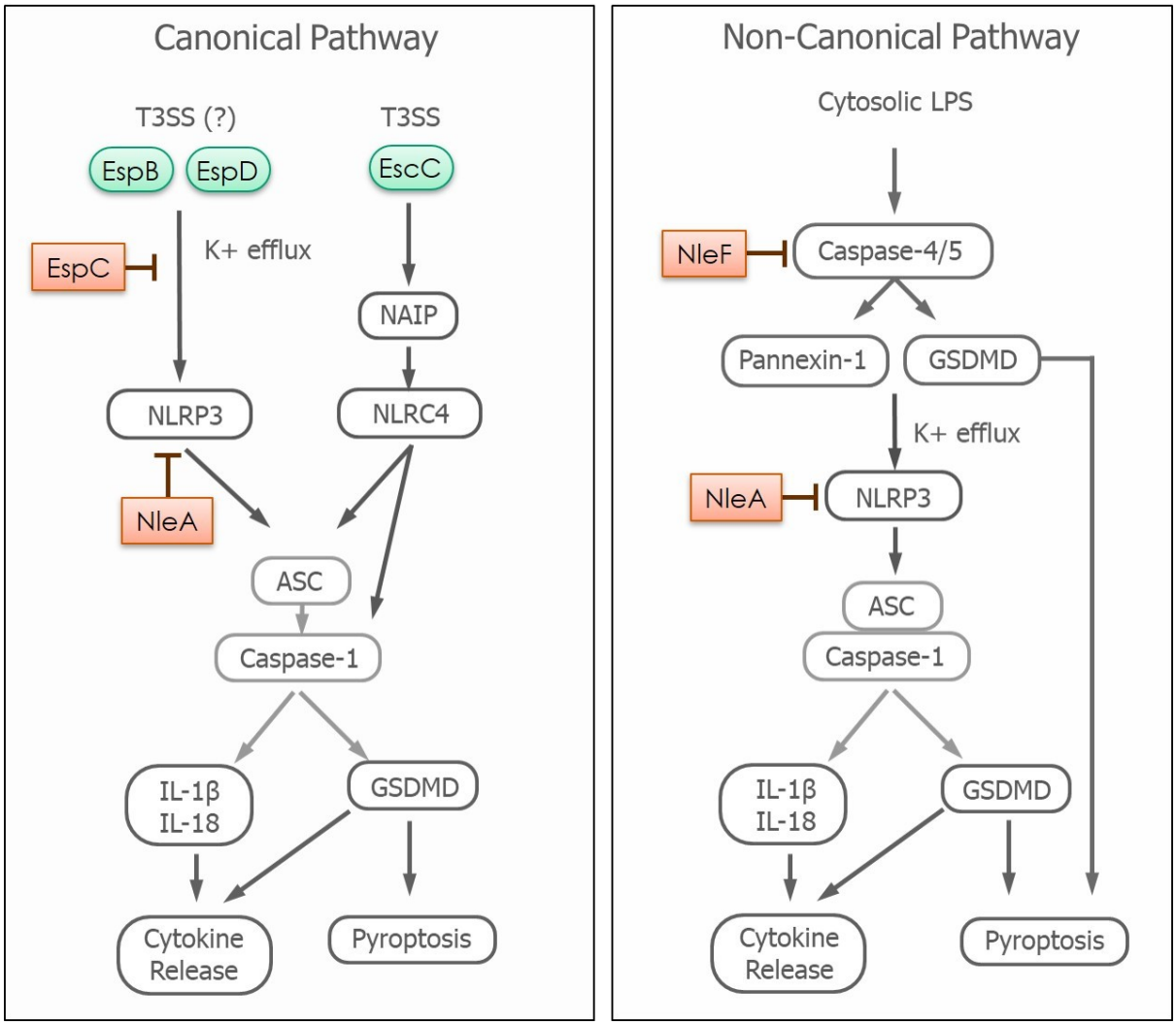

Figure 1.8. EPEC effector manipulation of inflammasome signalling pathways

Schematic showing inflammasome signalling pathways during EPEC infections. EPEC inhibitory effectors are shown in red, EPEC effectors reported to induce inflammasome activation are shown in green. Question marks indicate pathways that have not been experimentally verified.

Apoptosis and Necroptosis

1.29. Extracellular apoptosis and necroptosis

Death receptor signalling plays a critical role in the host response to bacterial gut pathogens. As discussed earlier, the activation of TNF receptors during bacterial infections can induce potent inflammatory responses. Within the TNF receptor family, a subset of receptors known as the death receptors are characterised by the presents a cytoplasmic death domain (DD) sequence of approximately 80 amino acids, which under certain circumstances possesses the ability to activate apoptosis or necroptosis by forming distinct receptor complexes (Figure 1.9) (582).

Death receptors are activated through a direct interaction their cognate ligands. These ligands are a range of complementary cytokines belonging to the TNF protein family. Ligand binding results in receptor aggregation and the subsequent recruitment of numerous adaptor proteins to the receptor, which culminates in the formation of a death inducing signalling complex (DISC) at the receptor cytoplasmic tail. This DISC operates as a molecular platform to regulate the activation and function of caspase-8 (or caspase-10, in some settings) (Figure 1.9, Reviewed; (432, 583, 584)). Catalytic activation of caspase-8 into its p18/p10 processed form leads to apoptotic cell death through two distinct pathways. Caspase-8 can induce the cleavage of the BH3 interacting-domain death agonist (BID) protein activating BCL-2 homologous antagonist/killer (BAK) and the apoptosis regulator BAX, leading to mitochondrial outer-membrane permeabilisation, and the release of cytochrome *c* (585). Following this caspase-9 is cleaved and activates executioner caspases; caspase-3/6/7 inducing apoptosis (586). In some cases however, BID-independent apoptosis proceeds via a mechanism that relies on JNK and ROS to activate the apoptosis executioner caspases-3/7 (587).

Necroptosis can be triggered by both Death Receptor or TLR-signalling when caspases are inhibited (588, 589) (Figure 1.9). Necroptosis is generally defined as programmed cell death mediated through the receptor-interacting protein kinase (RIP)1-RIP3 complex and the pore forming protein MLKL. (590). RIPK1 and RIPK3 have emerged as a critical regulators of programmed necroptosis, and as necroptosis relies of the kinase activity of RIPK1, it can be inhibited by Necrostatin-1 (Nec-1) (590), a allosteric inhibitor of RIPK1 kinase activity (591). The activation of RIPK1 and RIPK3 is strictly regulated by ubiquitination, phosphorylation, and caspase-

mediated cleavage (592). These post-translational modifications regulate assembly the 'necrosome' (592), a signalling complex in which RIPK3 is activated. The necrosome induces the recruitment and phosphorylation of MLKL, which functions as the executioner of necroptosis. Upon phosphorylation, MLKL oligomerises and is translocated to the plasma membrane where it induces membrane rupture and lytic cell death (593, 594).

1.30. Intrinsic apoptosis

The intrinsic apoptosis pathway is triggered by a variety of intracellular stresses such as DNA damage and endoplasmic reticulum (ER) stress which, similarly to the caspase-8 driven cleavage of BID, commonly results in the permeabilisation of the outer mitochondrial membrane and subsequent release of cytochrome *c* into the cytosol (595). Cytosolic cytochrome *c* induces the formation of a signalling complex termed the apoptosome, that includes Apaf-1 and pro-caspase-9 (596). Activation of caspase-9, the initiator caspase for intrinsic apoptosis, then leads to activation of downstream executioner caspases, including caspases-3 and -7 that induce apoptotic cell death (597).

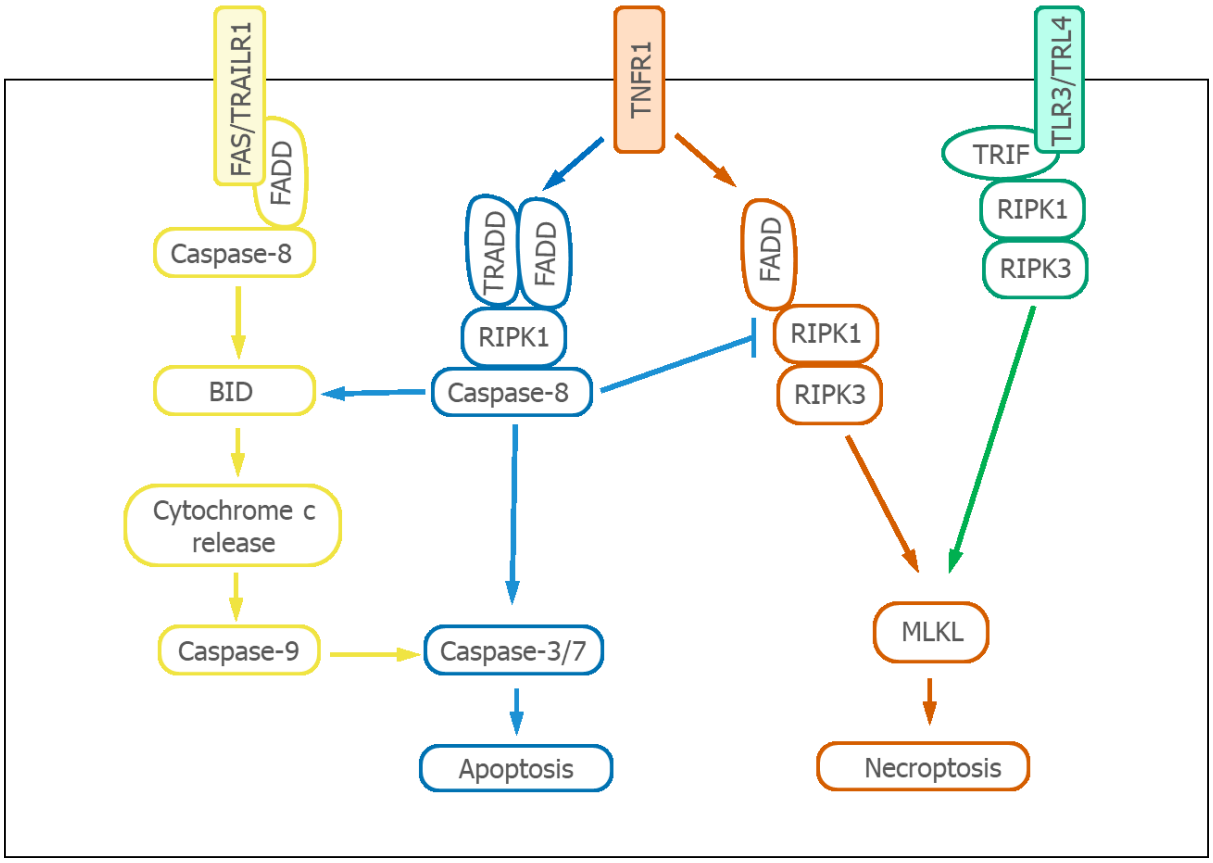

Figure 1.9. Summary of receptor-induced apoptosis and necroptosis cell death pathways

Activation of Fas, or TRAILR1 by their respective cognate ligands FasL, or TRAIL induces apoptosis. The death domain of the receptors recruits FADD and induces the activation of caspase-8. Active caspase-8 either catalyses the activation of downstream executioner caspases (caspase-3/7), or induced BID cleavage which causes mitochondrial permeabilisation, cytochrome *c* release and caspase-9 activation. Activated caspase-9 then functions to activate effector caspases-3/ and -7 to induce apoptosis. TNF-induced apoptosis occurs upon activation of the TNFR1 receptor in the absence of cellular IAPs (c-IAP). C-IAPs prevents the ubiquitination of RIPK1, this enables RIPK1 to associate with caspase-8, FADD and TRADD, through the death domains contained in both molecules, forming a cytoplasmic complex. Within this complex caspase-8 is activated and activates several apoptotic caspases inducing apoptosis. Alternatively, in the presence of CIAPs TNF signalling induces necroptosis. In this context, RIP1 can interact with FADD and RIP3 to form a necrosome, which can induce necroptosis via cleavage and activation of MLKL.TLR3/4 activation can also recruit TRIF, and recruit RIPK3 via a RHIM-dependent interaction to induce necroptosis. Modified from: (422)

1.31. EPEC effector manipulation of apoptosis and necroptosis

EPEC-induced cell death has been extensively characterised during infection of epithelial cells, and a number of studies have shown that EPEC surface properties and some translocated effectors can activate cell death signalling pathways *in vitro* (598-600). However, the features of late stage apoptosis such as cell shrinkage, membrane blebbing or nuclear fragmentation are absent from EPEC infected IECs. This indicates that EPEC has the ability to antagonize epithelial cell death during infection (601). Indeed, multiple studies have identified EPEC effector proteins that function to inhibit apoptosis and necroptosis during EPEC infections of intestinal epithelial cells (Reviewed (598) Table 1.3, Figure 1.10).

The characterisation of EPEC effector-mediated regulation of programmed cell death pathways has mainly focused on infection of IECs. Recent work has provided evidence that EPEC effectors can selectively induce cell death in immune cells. Indeed, the recombinant EPEC effector protein EspB (rEspB) can enter cells autonomously and disrupt the mitochondrial membrane potential, inducing programmed cell death (214). Interesting this cytotoxicity was only observed in macrophage cells, and not epithelial cells (214). Although the role of secreted EspB during infection has not yet been established, this provides evidence that effector-mediated responses may be cell-type specific. Therefore, the potential role of EPEC effector proteins during programmed cell death of innate immune cells requires further investigation.

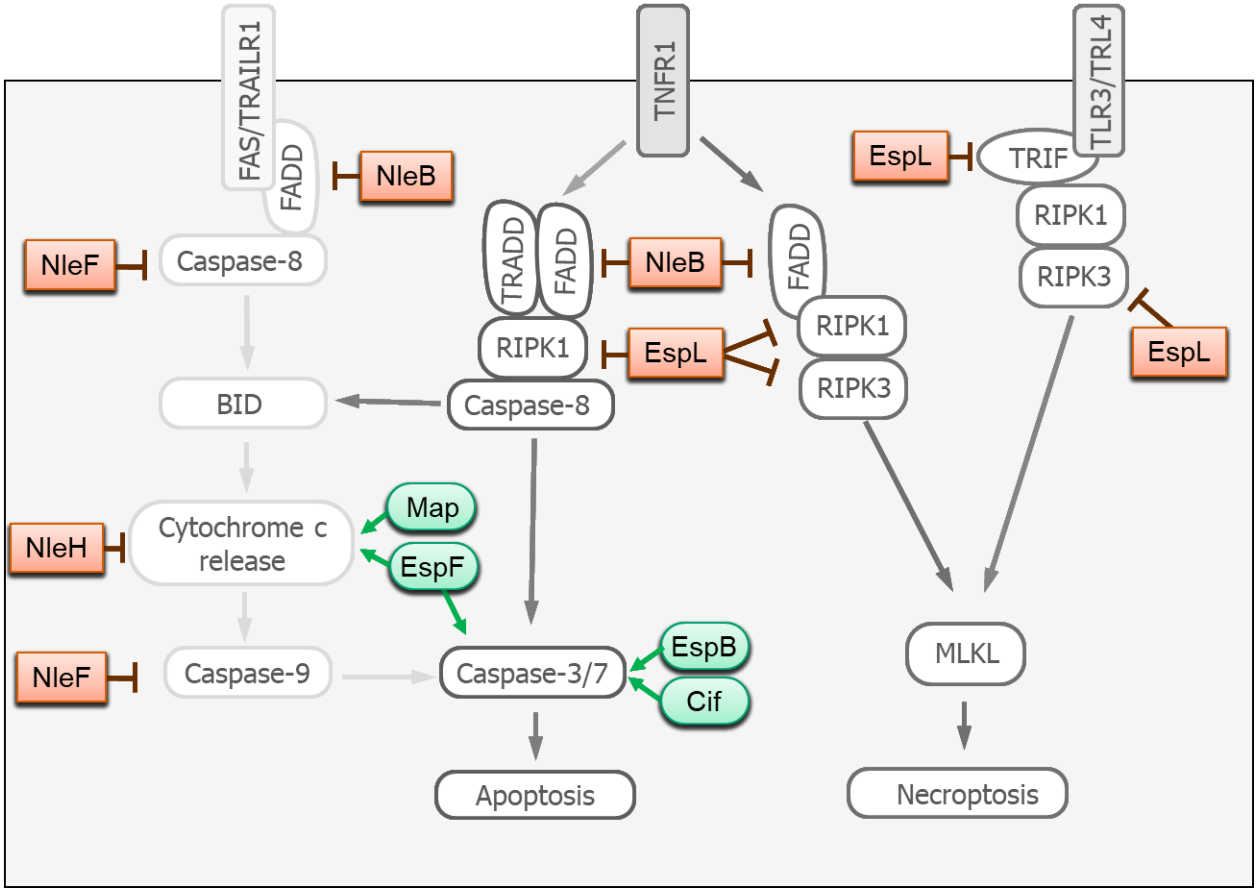

Figure 1.10. EPEC effector manipulation of apoptosis and necroptosis cell death pathways

Schematic showing cell death signalling via death-domain containing receptors (FAS, TRAILR1/TNFR1) and TLR3/4 to induced apoptotic and necroptotic programmed cell death. EPEC inhibitory effectors are shown in red, EPEC effectors that induce cell death pathways are shown in green.

Aims of Project

In light of recent studies that have identified the fundamental role of programmed cell death and inflammasome signalling pathways in the host defence against A/E pathogens, how these pathways are activated in immune cells, such as macrophages, during EPEC infection has become an important field of study. Although recent advances in the characterisation of inflammatory responses to EPEC, EHEC and *C. rodentium* have significantly enhanced our understanding, these studies have primarily focused on inflammasome pathways within mouse models, and often utilise bacterial growth conditions that do not induce T3SS, LEE or non-LEE effector expression. As EPEC is a human restricted pathogen and secreted EPEC effector proteins have distinct biochemical activities that manipulate multiple host cell pathways, the overall aims of this project were therefore to:

1. Characterise the programmed cell death pathways induced by EPEC infection of human macrophages.

2. Define the host cell receptors and downstream signalling pathways responsible for inducing programmed cell death during EPEC infection of human macrophages.

3. Outline the role of secreted EPEC effector proteins in programmed cell death and inflammasome signalling pathways in human macrophages.

4. Identify the specific T3SS effector proteins responsible for inducing macrophage cell death and inflammasome activation during EPEC infection.

Materials and Methods

2.1. Eukaryotic Tissue Culture

2.1.1 Mammalian cell culture

Human THP1 cells were routinely cultured in suspension in Roswell Park Memorial Institute Medium (RPMI 1640; Sigma), supplemented with 10% (w/v) heat-inactivated Foetal Calf Serum (FCS; Sigma), 1 mM sodium pyruvate (Gibco), 10 mM N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES) (Sigma), 100 µg/ml penicillin and 100 µg/ml streptomycin (Pen-Strep) (Complete RPMI). 72 h prior to treatment, THP1 cells were counted using a haemocytometer and seeded onto glass coverslips in 24-well plates (density 5 x 10^5 cells/well) for immunofluorescence, 48-well plates (density 4 x 10^5 cells/well) for western blotting or 96-well black-wall clear-bottom plates (density 1.5 x 10^5 cells/well) for cell death assays. THP1 cells were differentiated into macrophage-like cells using 100 ng/ml phorbol myristate acetate (PMA) for 48 h, and PMA and antibiotics were withdrawn for 24 h before experimental treatment.

HEK293T and HeLa cells were maintained as sub-confluent monolayers in high-glucose (4500 mg/L) Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated FCS, 1 mM sodium pyruvate and 100 µg/ml Pen-Strep (complete DMEM). HEK293T cells and HeLa cells were routinely passaged using 0.1% trypsin/0.02% EDTA (Sigma). HeLa cells were counted and seeded in DMEM minus Pen-Strep in 96-well plates (5 x 10^4 cells/well) or on glass coverslips in 24-well plates (2 x 10^5 cells/well) 24 h prior to treatment. For viral packaging, HEK293 cells were maintained at a sub-confluent level (~70 %) and medium was exchanged every 3 days. Following a minimum of two weeks incubation, 70-80% confluent HEK293T cells were suspended (0.1% trypsin/0.02% EDTA) and seeded in 12-well plates at a density 2 x 10^5 cells/well in 1ml complete DMEM + 20 mM HEPES. Cells were incubated for 20-24 h at 37°C and 5% CO_2 in a humidified incubator prior to viral packaging (See section 2.3.5).

Retro- or Lenti- viral plasmid-transduced cell lines were cultured with puromycin (2 µg/ml), puromycin was withdrawn 24 h prior to experimental treatment. All cell lines were maintained at 37°C and 5% CO_2 and were tested to be mycoplasma-negative (LookOut Mycoplasma PCR Detection Kit, Sigma). Cell lines were validated by short tandem repeat (STR) profiling (Microsynth AG, Switzerland).

2.1.2 Preparation of primary human monocyte derived macrophages (MDMs)

Primary human monocyte–derived macrophages (MDM) were prepared by separating CD14 positive cells (CD14+) from whole-blood buffy coat fractions from Leukocytes cones from anonymous healthy blood-platelet donors, provided by the NHSBT. CD14+ cells were enriched by MACS (Magnetic-activated Cell Sorting, Miltenyi Biotec) from buffy coats prepared using 50 ml LeucoSep tubes (Greiner Bio-One). Briefly, blood from a single donor was mixed with pre-warmed PBS at a ratio of 1:4. 30 ml was processed per LeucoSep tube. LeucoSep tubes were centrifuged at 1000 xg for 20 min at room temperature (RT) with an acceleration of 1 and deceleration of 0 to separate out the buffy coat. The buffy coat layer was gently separated and cells were resuspended in 30 ml fresh RPMI. Cells were washed 3 times with 20 ml of pre-warmed RPMI, centrifuging for 10 min between each wash at 600 xg then 400 xg then 300 xg (acceleration and deceleration of 9). Cells were then washed two times with 20 ml of MACS buffer (50 mg/ml BSA, 2 mM EDTA in PBS) centrifuging at 300xg for 10 min. Cells were resuspended in 5 ml of MACs buffer and counted using a hemocytometer. Approximately 2 x 10^9 cells were prepared per MACS LS column. CD14+ cells were enriched using biotinylated anti-CD14+ antibody and anti-biotin microbeads following the manufacturer's protocol (Miltenyi Biotec). Approximately 1 x 10^5 cells of both the initial follow through and MACS enriched eluted cells were collected and stored on ice for flow cytometry analysis. Flow cytometry analysis was used to determine the purity of the CD14+ population. For flow cytometry, cells were incubated with Fc block for 5 min and stained with antiCD14+ antibodies, or a corresponding isotype control. These cells, along with cells from the flow-through and MACS eluted samples, were resuspended in 100 µl of MACs buffer and incubated with streptavidin-647 on ice for 30 min, washed and resuspended in MACS buffer + 0.8% PFA, before being analyzed using a FACS Calibur (BD bioscience). Enriched cells (found to be ~85-95% CD14+ by flow cytometry) were counted and seeded into plates at a density of 9 x 10^5 per 24-well, 4.5 x 10^5 cells per 48-well and 2 x 10^5 cells per 96-well. Cells were cultured for 7 days in RPMI containing 1 mM sodium pyruvate, 10% heat inactivated foetal bovine serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin, 10 mM HEPES (pH 7.5) (complete RPMI) plus 20 ng/ml human M-CSF to allow differentiation into macrophages. The medium was replenished every 72 h and antibiotics and M-CSF were withdrawn 24 h before experiments.

2.2. Bacterial strains and plasmids

Bacterial strains used in this study are listed in Table 2.1. All EPEC mutants are derivatives of EPEC serotype O127:H6 strain E2348/69 (602), SIEC strains are derivatives of *E. coli* K-12 (MG1655ΔfimA-H) (302). Bacteria were routinely grown in lysogeny broth (LB) at 37°C, 200 RPM, supplemented with appropriate antibiotics where necessary; kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), or ampicillin (100 μg/ml).

The bacterial plasmids used in this study can be found in Table 2.2, eukaryotic plasmids are listed in Table 2.3, and oligonucleotides used for plasmid construction are detailed in Table 2.4.

Table 2.1: Bacterial strains used in this study

Lab Ref	Strain	Description	Resistance	Source
	Stbl2 *E. coli*	Stbl2	n/a	(603)
	Wild Type EPEC E2348/69 0127:69	Wt	n/a	(602)
ICC171	E2348/69 *ΔescF*	*ΔescF*	Kan	(145)
ICC255	E2348/69 *Δtir*	*Δtir*	Kan	(209)
	E2348/69 *Δtir* + pSA10:TirE69	*Δtir* + pTir	Amp/Kan	This Study
	E2348/69 *Δtir* + pACYC:TirEHEC	*Δtir* + $pTir^{EHEC}$	Amp/Cm	This Study
	E2348/69 *Δtir* + pSA10:TccP	*Δtir* + $pTir^{EHEC}$ + TccP	Amp/Kan	This Study
	E2348/69 *Δtir* + pACYC:TirEHEC + pSA10:TccP	*Δtir* + pTccP	Amp/Kan/Cm	This Study
ICC275	E2348/69 *Δeae*	*Δeae*	Kan	(239)
ICC309	E2348/69 Tir^{Y454A}	TirAY	Kan	(604)
ICC310	E2348/69 Tir^{Y474A}	TirYA	Kan	(604)
ICC311	E2348/69 $Tir^{Y454A/Y474A}$	TirAA	Kan	(604)
	E2348/69 $Tir^{Y454A/Y474A}$ + pSA10:Empty	TirAA + pEmpty	Amp	This Study
	E2348/69 $Tir^{Y454A/Y474A}$ + pSA10:MAP(E69)	TirAA + pMAP(E69)	Amp	This Study
	E2348/69 $Tir^{Y454A/Y474A}$ + pSA10:EspT (*C.r.*)	TirAA + pEspT (*C.r*)	Amp	This Study
	E2348/69 $Tir^{Y454A/Y474A}$ + pSA10:EspM2 (Sakai)	TirAA + pEspM2	Amp	This Study
	E2348/69 ΔPP2::Km315 (NleH1 – EspJ – truncated Cif)	ΔPP2	Kan	Frankel Lab
ICC1060	E2348/69 ΔIE6::Km315 (EspL– NleB1– NleE)	ΔIE6	Kan	Frankel Lab
ICC1062	E2348/69 ΔIE2::CmFRT (NleE2 – NleB3- EspL)	ΔIE2	Cm	Frankel Lab

ICC240	E2348/69ΔPP4::CmFRT (NleI/NleG- NleB2 –NleC – NleD)	ΔPP4	Cm	Frankel Lab
	E2348/69 ΔPP6::CmFRT (NleF – NleH2 – NleA/EspI)	ΔPP6	Kan	Frankel Lab
ICC1337	EcM1-Δ*yeeJ*::Ptac-eLEE2 Δ*yra*::Ptac-eLEE3 Δyfc::Ptac-eEscD Δ*yebT*::Ptac-eLEE4 Δ*yfaL*::Ptac-eLEE1	SIEC	n/a	(302)
ICC1338	SIEC Δ*flu*::Ptac-eLEE5	SIEC-eLEE5	n/a	(302)
ICC1339	SIEC Δp1Δ*flu*::Ptac-eLEE5	SIECΔp1eLEE5	n/a	(302)

Table 2.2: Bacteria plasmids used in this study

Plasmid	Description	Insert/Gene Name	Res.	Source
pSA10	pKK177-3 derivative containing *lacI*, pUC-ori, Ptac, AmpR,	N/A	Amp	(605)
pSA10:Tir^{EPEC}	pSA10 expressing IPTG inducible Tir from EPEC strain E2348/69 0127:69	Tir EPEC E69	Amp	(604)
pSA10:TccP	pSA10 expressing IPTG inducible TccP from EHEC 0157:H7 EDL933	TccP EHEC	Amp	(289)
pSA10:Map_E69	pSA10 expressing IPTG inducible Map from EPEC strain E2348/69 0127:69	Map EPEC E69	Amp	(606)
pSA10:EspT_*C.r.*	pSA10 expressing IPTG inducible EspT from Wild-type *Citrobacter rodentium*	EspT *C.r.*	Amp	(324)
pSA10:EspM2	pSA10 expressing IPTG inducible EspM2 from EHEC 0157:H7 Sakai	EspM2 EHEC	Amp	(323)
pSA10:EspJ_E69	pSA10 derivative encoding EspJ from EPEC strain E2348/69 0127:69	EspJ EPEC	Amp	(237)
pACYC184	*E. coli* plasmid cloning vector containing the p15A origin of replication,	N/A	Cm	NEB
pACYC_Tir^{EHEC}	pACYC184 constitutively expressing Tir from EHEC 0157:H7 EDL933	Tir EHEC	Cm	(287)
pFP25.1_GFP	Plasmid expressing GFP via a constitutively active rpsm promotor	GFP	Amp	Clements Lab

Table 2.3: Eukaryotic plasmids used in this study

Plasmid	Backbone	Description	Insert/ Gene	Tag	Selection	Res.	Source
pMX_YFP_CTRL (LacZ)_miR30E	pMX-cmv	pMX-CMV was made from the pMX-IP retroviral plasmid. A CMV promoter and an enhanced YFP form pEYFP-C1 (Clontech) to generate pMXCMV-YFPC1 for increased protein expression. The XhoI-EcoRI sites in pMXCMV-YFPC1 were used to clone the optimised miRNA30E cassette which also introduced a stop codon in YFP protein.	$CTRL^{miR}$	YFP	Puro	Amp	(607)
pMX_YFP_CASP4_ miR30E	pMX-cmv	CASP4 miR30E sequence cloned into pMX_YFP_$CTRL^{miR}$ plasmid at the XhoI-EcoRI sites	$CASP4^{miR}$, YFP	YFP	Puro	Amp	(228)
pMX_YFP_GSDMD_ miR30E	pMX-cmv	GSDMD miR30E sequence cloned into pMX_YFP_$CTRL^{miR}$ plasmid at the XhoI-EcoRI sites	$GSDMD^{miR}$, YFP	YFP	Puro	Amp	(607)
pMX_YFP_ASC_ miR30E	pMX-cmv	ASC miR30E sequence cloned into pMX_YFP_$CTRL^{miR}$ plasmid at the XhoI-EcoRI sites	ASC^{miR}, YFP	YFP	Puro	Amp	This Study (C. Kennedy. Shenoy Group)
pMX_YFP_RIPK1_ miR30E	pMX-cmv	RIP1 miR30E sequence cloned into pMX_YFP_$CTRL^{miR}$ plasmid at the XhoI-EcoRI sites	$RIPk1^{miR}$, YFP	YFP	Puro	Amp	This Study
pMX_YFP_MLKL_ miR30E	pMX-cmv	MLKL miR30E sequence cloned into pMX_YFP_CTRL miR plasmid at the XhoI-EcoRI sites	$MLKL^{miR}$, YFP	YFP	Puro	Amp	This Study
pMX_mAsc_mRFP	pMX-IP	Mouse Asc fused to mRFP via the 5'LTR cloned into the retroviral plasmid pMXsIP	mASC_mRFP	RFP	Puro	Amp	Shenoy Lab
PLPP_mCas11	PLPP	The pLPP-mCas11 plasmid was generated by modifying pLentriCRISPRv2 (Addgene #5293), mouse Caspase-11 was expressed downstream of an EFs-NS promotor at the Afe1-EcoRI sites. The PGK promoter-puromycin cassette (From pRetro-X-Tight-Puro; Clonetech) was cloned downstream	mCas11	n/a	Puro	Amp	This Study (J. Sanchez, Shenoy Group)

PLPP_mCas11-CM (Catalytic Mutant)	PLPP	pLPP-mCas11 plasmid mutated at the catalytic cysteine 254 (Cys-Ala), introducing a silence Xho1 site	$mCas11^{CM}$	n/a	Puro	Amp	This Study
PLPP_mCas11_KKK-EEE (LPS binding mutant)	PLPP	pLPP-mCas11 plasmid mutated at residues Lys62Glu, Lys63Glu, Lys64Glu, introducing a silence SalI site	$mCas11^{KE}$	n/a	Puro	Amp	This Study
pLX_YFP_CTRL_ miR30E	pLX	pLX- plasmid backbone was generated by modifying pLentiCRISPRv2 (Addgene #5293), and the YFP_$CTRL^{miR}$ IRES-puro cassette was cloned from the pMX_YFP_$CTRL^{miR}$ backbone downstream of the EFs-NS promotor.	$CTRL^{miR}$	YFP	Puro	Amp	Shenoy Lab
pLX_YFP_CASP4_ miR30E	pLX	CASP4 miR30E sequence cloned into pMX_YFP_$CTRL^{miR}$ plasmid at the XhoI-EcoRI sites	$CASP4^{miR}$	YFP	Puro	Amp	This Study
pLX_mCas11_CASP4_ miR30E	pLX	Mouse caspase-11 PCR amplified from PLPP_mCas11 and cloned into the pLX_YFP_CASP4_miR30E plasmid at the AfeI and BsrGI sites to replace the YFP cassette	mCas11,	n/a	Puro	Amp	This Study
pLX_mCas11_CM_ CASP4_miR30E	pLX	Mouse caspase-11 catalytic mutant C254A cloned into the pLX_YFP_CASP4_miR30E plasmid at the AfeI and BsrGI sites	$mCas11^{CM}$	n/a	Puro	Amp	This Study
pLX_mCas11_KKK-EEE_CASP4_miR30E	pLX	Mouse caspase-11 catalytic mutant C254A cloned into the pLX_YFP_CASP4_miR30E plasmid at the AfeI and BsrGI sites	$mCas11^{KE}$	n/a	Puro	Amp	This Study
pMX_cmv_FcgRTir_ 2Myc	pMX-cmv	pMX-cmv expressing FcgRIIa from pEGFP-FcγRIIa (608), fused to a double Lys-Lys linker and the C-terminal domain of Tir from EPEC E2348/69 0127:69 (Residues R388-V548), tagged with a double C-terminal Myc	FcgRTir_EPEC_ E69 Chimera	2x Myc	Puro	Amp	This Study
pMX_cmv_FcgRTir_ mCherry	pMX-cmv	pMX-cmv expressing FcgRTir chimera, and a mCherry c-Terminal tag	FcgRTir_EPEC_ E69 Chimera	mCherry	Puro	Amp	This Study

pMX_cmv_FcgRTir_YFYY_mCherry	pMX-cmv	pMX-cmv expressing FcgRTir with mutations in the Tir Tyrosine (Y) residue Y474F , and a mCherry c-Terminal tag	FcgRTir_EPEC Y474F Chimera	mCherry	Puro	Amp	This Study
pMX_cmv_FcgRTir_FFYY_mCherry	pMX-cmv	pMX-cmv expressing FcgRTir with mutations in the Tir Y454F and Y474F, and a mCherry c-Terminal tag	FcgRTir_EPEC Y474F/Y474F Chimera	mCherry	Puro	Amp	This Study
pMX_FcgRTir_EHEC_mCherry (No cmv)	pMX	pMX- with an EFs-NS promotor expressing FcgRIIa fused to Tir from EHEC 0157:H7 EDL933 residues (R384 – V558), and a mCherry c-Terminal tag	FcgRTir_EHEC	mCherry	Puro	Amp	This Study

Table 2.4: Primers used in this study

Primer	Sequence	Description
Primers used for cloning – Uppercase letters are homologous to target sequence, lowercase letters are homologous to plasmid sequence and restriction sites are underlined.		
Primer_miR30E_XhoI-slic-Fwd	tgtacaagtccggactcagatgatcgacttcttaacccaacagaaggcTCGAGAAGGTATATTGCTG	Used to SLIC miRNA sequences into pMX-cmv XhoI and EcoRI cut plasmid
Primer_miR30E_EcoRI-slic-Rev	gcggccgcggcgcgccggccgaattctagcccCTTGAAGTCCGAGGCAGTAGG	Used to SLIC miRNA sequences into pMX-cmv XhoI and EcoRI cut plasmid
Primer_mCas11_KZ-AfeI-Fwd	ggaccggttctagagcgctGCCACCATGGCTGAAAACAAACACCC	Used to amplify mouse-caspase-11 to SLIC into PLPP or pLX plasmids at the AfeI site
Primer_ mCas11-EcoRI-pLPP-Rev	CTCGAGGCCTGCAGGAATTcagttgccaggaaagagg	Used to amplify mouse-caspase-11 to SLIC into PLPP at the EcoRI site
Primer_mCas11_BsrGI_Rev	CCTCTTTCCTGGCAACTGAtgtacaagtccggactcagat	Used to amplify mouse-caspase-11 to SLIC into pLX_YFP_CTRL^{miR} at the BsrGI site
Primer_FcgR_6aa-KZ-AfeI-Fwd	ccgtcagatccgctagcgctagccaccatgactatggagaCCCAAATGTCTCAGAATGT	Used to amplify the FcgR-EPEC Tir chimera adding a Kozak sequence and 6 amino-acid linker. SLICs with the AfeI site in the pMX-cmv plasmid
Primer_Tir_dStop-SLIC-Rev	AACGAAACGTACTGGTCC	Used to amplify the FcgR-Tir chimera removing the stop codon to enable tagging. SLICs with the mCherry or Myc Tag sequences.
Primer_mCherry_Tir_BamHI-Fwd	ggaccagtacgtttcgttggatccATGGTGAGCAAGGGCGAG	Amplifies mCherry to SLIC with the FcgR-Tir C-terminus, introducing a BamHI site
Primer_mCherry_pMX-EcoRI-Rev	ctcgaggcctgcaggaattcaCTTGTACAGCTCGTCCATG	Amplifies mCherry to SLIC with the FcgR-Tir and the EcoRI site within the pMX-cmv plasmid
Primer_Tir-Bam-Myc_pMX-Fwd	ggaccagtacgtttcgttGGATCCgagcagaagctgatctcagaggaggacctgGAACAAAAACTCATCTCAGA	Amplifies 2xMyc to SLIC with the FcgR-Tir C-terminus, introducing a BamHI site
Primer_Tir-Myc-pMX-EcoRI-Rev	CTCGAGGCCTGCAGGAATTCttacagatcctcttctgag	Amplifies 2xMyc to tag the FcgR-Tir and the EcoRI site within the pMX-cmv plasmid
Primer_FcgR_KZ_PacI_noCMV_Fwd	gccggatctagctagttaattaatgccaccATGACTATGGAGA	Used to amplify FcgRIIa to SLIC with pMX- removing the cmv promotor, and introducing a PacI site.

Primer_FcgR_EHECtir_SLIC_Rev	TTGATCTACTGCAGGAAAAAGCGAAAAAATCAGCCGGTAG	Used to amplify FcgRIIa to SLIC with the C-Terminal fragment of EHEC Tir
Primer_EHEC_Tir_CT_Fwd	cgaaaaaatcagccggtag	Used to amplify the C-terminus fragment (Residues 384-559) of EHEC Tir (Strain EDL933)
Primer_EHEC_Tir_CT_BamHI_Rev	CCCTTGCTCACCATggatccgacgaaacgatgggatcc	Used to amplify the C-terminus fragment (Residues 384-559), adding a BamHI site.
Primer_Flag_TccP_pLTet_ClaI-Fwd	aagatgacgatgacaaatcgATGATTAACAATGTTTCTTCAC	Used to amplify EHEC TccP, and SLIC with pLTet-2xFlag plasmid at the ClaI site, adding a Flag tag to the N-terminus of TccP
Primer_TccP_pLTet_XbaI_Rev	TACATCTAAGCGCTCGTGAtctagatggagtcatcctcaattc	Amplifies EHEC TccP to SLIC with the XbaI site in pLTet plasmid
Primer_PGK-BsrGI-pLTrem-Fwd	tgactcggcatggacgagctgtacaagCTACGTAAATTCCTACCGGGT	Used to amplify the PGK promotor, to replace the P-Tight promotor in the pLTet plasmid, reducing the plasmid size and increasing packaging efficiency
Primer_PGK-XcmI-pLTrem-Rev	TCGACCTGCAGCCCAAGCTTgccaccatgtctaggctggacaagagcaaagtc	Used to amplify to PGK promotor
Mutagenic Primers – Mismatch mutations are in uppercase.		
Primer_mCas11_C245A-XhoI-Fwd	tcatcattgtgcaggccGcTCgaggtgggaactctgg	Mutating the catalytic cystine residue in mouse caspase-11, and adding a silent XhoI site
Primer_mCas11_KKK-EEE-SalI-Fwd	gcgttgggtttttgtCgaCgccatgGaaGagGaacacagcaaag	Mutating mouse caspase-11 CARD-residues Lys62, Lys63, Lys64 that are involved in LPS binding to Glu62, Glu63,Glu64 and adding a silent SalI site.
Primer_TirCT_Y454F_Fwd	tccatTtgctgaagttggggg	Mutating Tir tyrosine residue Y454
Primer_TirCT_Y454F_Rev	caacttcagcaAatggattaaccacttc	Mutating Tir tyrosine residue Y454

2.3. Molecular Biology Techniques

2.3.1 Cloning and mutagenesis

Sequence- and Ligation-Independent Cloning (SLIC) previously described (609) was used for routine cloning in this study. Briefly, vectors were digested at 37°C for 2 h using the appropriate restriction enzymes (NEB) in a 50 µl reaction volume with 1 unit (U) of enzyme/µg DNA. Genes of interest were amplified by Polymerase Chain Reactions (PCRs), using primers with ≥ 15 base-pair homology extension to the linearized vector end. PCRs were performed using Phusion® High-Fidelity DNA Polymerises (NEB). PCR amplicons and digested plasmids were purified by gel extraction following the manufacturers protocol (Omega Bio-Tek). For SLIC reactions, linearized vector (150 ng) and PCR insert were combined at a molar ratio of 1:4 for single fragment cloning, or 1:4:4 for multiple fragments cloning, and incubated with 0.6 U T4 DNA polymerase (NEB) and 1% BSA (Sigma). The reaction was incubated at RT for 2.5 min to generate 5' overhangs and then incubated on ice of 10 min before being transformed into chemically competent *E. coli* Stbl2 by heat shock transformation.

Site directed mutagenesis was conducted by overlap PCR using Phusion® High-Fidelity DNA Polymerises, and mismatch primers (Table 2.4). Typical PCR relations included 100 ng of DNA template, 20 pmol of mutagenic primer, 0.2 mM of dNTPs, 0.5 U of Phusion polymerise and 1x Phusion buffer. Typical thermocycling conditions were; Initial denaturation at 98 °C for 1 min, then 20-30 cycles of 98°C for 30 sec, 50-60°C (according to the primer) for 30 sec, 72°C for 30 sec per kb, and final extension at 72°C for 10 min. Mutagenesis PCR products were incubated with DpnI (NEB) at 37°C for 3 h or at RT overnight to digest template and were subsequently transformed into competent Stbl2 *E. coli*. All constructs were confirmed by DNA sequencing (GATC Biotech).

2.3.2 Preparation of chemically competent bacteria and heat shock transformation

Bacterial cultures were grown overnight in LB broth at 37°C and 200 RPM then diluted 1:100 and grown until an OD_{600} of 0.4-0.5. Bacterial cultures were incubated on ice for 20 min and then pelleted at 4°C. Pelleted cells were kept on ice and resuspended in pre-chilled sterile transformation and storage solution (TSS) (10% PEG 4000, 5% DMSO, 5 mM MgCl2 and 2% LB broth). Chemically competent bacteria were either used immediately or 'snap frozen' with liquid nitrogen and stored at -80°C.

For heat shock transformation, 5 µl of SLIC reaction or 1 µl of plasmid DNA was mixed with 50 µl chemically competent bacteria and incubated on ice for 20 min. Bacteria were heat-shocked at 42°C for 45 sec and returned to ice for 2 min. 300 µl of fresh pre-warmed LB was added and cells were allowed to recover at 37°C for 1 h before plating on LB agar with the appropriate selection antibiotic. Colonies were grown in LB with the appropriate antibiotic for plasmid extraction and sequence verification (GATC).

2.3.3 Preparation of electrocompetent bacteria and electroporation

Bacterial strains were cultured overnight at 37°C and 200 RPM then diluted 1:100 into fresh LB and grown until an OD_{600} of 0.6-0.7. Cultures were incubated on ice for 30 min, and maintained at 4°C throughout processing. Bacteria were pelleted by centrifugation at 1500 xg for 15 min before discarding the supernatant and re-suspending the bacterial pellet in ice-cold sterile dH_2O (Half volume of culture). Two subsequent wash steps were completed, resuspending the bacterial pellet in 1/4 and 1/20th volume of ice cold 15% glycerol at each step, and a finally resuspension in 1/200th volume in 10% glycerol. Cells were either used immediately for electroporation, or aliquoted and snap frozen in liquid nitrogen and stored at -80°C. All solutions were chilled to 4°C and kept on ice between each step.

Electrocompetent bacteria (50 µl aliquots) were thawed on ice and incubated on ice with 25-100 ng/µl of plasmid DNA for 5-10 min. Bacteria-DNA suspension was then transferred to a pre-chilled 0.2 cm gap electroporation cuvette and electroporated at 2.5 kV, 200 Ω, 25 mF for 4.5 µs (BioRad MicroPulser™ Program: EC2). Immediately after electroporation, transformed bacteria were resuspended in 500 µl SOC medium (0.5% Yeast Extract, 2% Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ & 20 mM Glucose; NEB), pre-warmed to 37°C and incubated for a minimum of 1 h at 37°C with shaking. Post incubation, 25-50 µl of transformed bacterial suspension was plated onto LB agar plates with appropriate antibiotic(s) for the selection of plasmid(s) and incubated at 37°C overnight (16-18 h). Single colonies were picked and screened for expression of appropriate genes by either PCR or western blot analysis.

2.3.4 Stable silencing of genes using optimised miRNA-based strategy

For stable knock-down, miRNA30E constructs were designed as described previously by Fellmann *et al*, 2013 (610). These utilise short-hairpin RNA (shRNA) technology to enable stable and regulated gene repression, as previously shown by Eldridge *et al,* 2017 (607). Briefly, 22 base oligonucleotides

were converted to miRNA30E by simple PCR amplification using the primers; Primer_miR30E_XhoI-slic-Fwd and Primer_miR30E_EcoRI-slic-Rev (Table 2.4), which also encoded homology overhangs that enable SLIC cloning into the retroviral plasmid pMX_CMV-YFP at the XhoI-EcoRI sites. pMX-CMV-YFP retroviral plasmid for miRNA30E-based silencing (22-mer gene-specific targets or LacZ sequence as non-targeting control) were generated and transduced into THP1 cells. miRNA30E sequences used in this study are listed in Table 2.5.

Table 2.5: miRNA30E sequences used in this study

Target	Sequence
LacZ (Control)	$^{5'}$TCACGACGTTGTAATACGACGT$^{3'}$
hCASP4	$^{5'}$ATATCTTGTCATGGACAGTCGT$^{3'}$
hASC	$^{5'}$CAGCTCTTCAGTTTCACACCAG$^{3'}$
RIPK1	$^{5'}$TTATCCGTCAGACTAGTGGTAT$^{3'}$
MLKL	$^{5'}$TTAAGATTTCATCCACAGAGGG$^{3'}$
GSDMD	$^{5'}$TACACATTCATTGAGGTGCTGG$^{3'}$

2.3.5 Retroviral and lentiviral packaging and transduction

Retro-viral and Lenti-viral plasmids (Table 2.3) were packaged in HEK293T cells using the packaging plasmids pCMV-MMLV-Gag-Pol (for packaging of retroviral plasmids), Lentiviral packaging plasmid 1266 (pHIV, for packaging of lentiviral plasmids) and pseudotyped with pCMV-VSV-G (gifts from Pradeep Uchil and Walther Mothes, Yale University) as described previously (607), with some modifications. Briefly, HEK293T cells were seeded in 1 ml complete DMEM medium supplemented with 20 mM HEPES in 12-well plates at a density 2 x 10^5 cells/well, 24 h prior to packaging. For retro-viral packaging 1 µg DNA at the ratio of 5:4:1 of Plasmid-of-interest:MMLV-Gag-Pol:VSG-G was transfected into HEK293T cells using Lipofectamine-2000^{TM}. Lipofectamine and DNA were diluted in serum-free Opti-MEM at a ratio of 2.5 µl of lipofectamine per 1 µg DNA, and incubated at RT for 15-20 in to allow DNA:lipofectamine complexes to form. Complexes were then gently added to HEK293T cells in a dropwise manner, and incubated for 48 h. For lentiviral transductions, a ratio of 3:2:1 of Plasmid-of-interest:pHIV:VSG-G was used. After 24 h transduction efficiency was determined a Zeiss AxioImager Z1 fluorescence microscope to check for GFP or mCherry expression. After 48 h, virus containing supernatants were collected and filtered through 0.45 µm

low protein binding filters (Pall Life Sciences) and were transferred on pre-seeded target cells. 24 h before receiving virus-complexes undifferentiated THP1 cells were plated at 5 x 10^5 cells/well in 12-well plates in 1 ml of complete-RPMI, and HeLa cells were plated at 1 x 10^5 cells/well in 12-well plates in 1 ml of complete-DMEM and incubated for 24 h at 37°C and 5% CO_2. After 24 h, 300 µl of filtered virus containing supernatant collected from HEK293T cells was added to wells and cells were incubated at 37°C, 5% CO_2 in a humidified incubator. 48 h after viral-transduction, puromycin (2 µg/ml) was added to cells and replenished until stable pools were obtained. Cells were sorted to enrich YFP+ or mCherry+ cells as necessary on a FACS Aria III flow sorter (BD Biosciences) for uniform (>95% +ve) YPF or mCherry expression. Transduction was confirmed by immunofluorescence and/or western blot, as described below.

2.3.6 Transfection of short inhibitory RNA

ON-Target Plus SMARTpool (Dhamacon) small interfering RNA (siRNA) was used to transiently knock-down target genes in MDMs and THP1 cells. siRNA was transfected using the Viromer Blue (VB) transfection reagent following the manufacturer's protocol for suspension cells with slight modifications. Briefly, VB transfection reagent was resuspended in Buffer F at a ratio of 1:90 and siRNA was incubated with diluted VB at a ratio of 35 pmol siRNA:1 µl diluted VB. siRNA mixtures were vortexed for 1 min before being incubated for 15-20 min at RT to allow complexes to form. Complexes were added to pre-seeded cells at a final concentration of 20 nM siRNA per well. For THP1 cells, 2-4 h prior to transfection, cells were plated in 96-well plates at a density of 8 x 10^4 cells/well, or in a 48-well plate at 2 x 10^5 cells/well to achieve 70% confluency. siRNA complexes were added to undifferentiated cells in a dropwise manner, and cells were incubated in suspension for 6 h. Transfected cells were differentiated using 100 ng/ml PMA and used for experiments 72 h post-transfection. The medium was replaced with fresh medium without PMA or antibiotics 24 h before experiments. For primary MDMs, cells were seeded at a density of 2 x 10^5 cells per 96-well, in complete RPMI plus 20 ng/ml human M-CSF, 5 days prior to transfection to enable differentiation into macrophages. Cells were transfected with siRNA using the VB transfection reagent and were used for experiments 72 h post transfection. The medium was replaced with fresh RPMI 18 h prior to experiments. For each siRNA, four additional wells were transfected, and subsequent to each experiment these cell lysates were collected and assayed by immunoblot to determine silencing efficiency. The siRNAs utilised in this study are listed in Table 2.6. All siRNA transfections were completed in RNase free conditions.

Table: 2.6: siRNA sequences used in this study

Target	Gene ID	Gene Accession	Sequence	Reference Number
ON-TARGETplus non-targeting	0	N/A	- UGGUUUACAUGUCGACUAA - - UGGUUUACAUGUUGUGUGA - - UGGUUUACAUGUUUUCUGA - - UGGUUUACAUGUUUUCCUA -	D-001810-10 (D-001810-01) (D-001810-02) (D-001810-03) (D-001810-04)
CASP4	837	NM_001225	- GGACUAUAGUGUAGAUGUA – - CAACGUAUGGCAGGACAAA –- - GAACUGUGCAUGAUGAGAA - - UAACAUAGACCAAAUAUCC -	L-004404-00 (L-004404-05) (L-004404-06) (L-004404-07) (L-004404-08)
ASC (PYCARD)	29108	NM_145183	- GGAAGGUCCUGACGGAUGA - - UCACAAACGUUGAGUGGCU - - GGCCUGCACUUUAUAGACC - - CCACCAACCCAAGCAAGAU -	L-004378-00 (J-004378-06) (J-004378-07) (J-004378-08) (J-004378-09)
CASP8	841	NM_033358	- GGACAAAGUUUACCAAAUG- - GCCCAAACUUCACAGCAUU- - GAUAAUCAACGACUAUGAA- - GUCAUGCUCUAUCAGAUUU-	L-003466-00 (J-003466-13) (J-003466-14) (J-003466-15) (J-003466-16)
Cofilin-1 (CFL1)	1072	NM_005507	- CCUCUAUGAUGCAACCUAU - - CAUGGAAGCAGGACCAGUA - - UAAAUGGAAUGUUGUGGAG - - ACUCUGUGCUUGUCUGUUU -	L-012707-00 (J-012707-05) (J-012707-06) (J-012707-07) (J-012707-08)

2.3.7 Isolation of RNA and RT-qPCR

RNA was prepared from THP1 macrophages infected with EPEC strains for 4 h, in RNase-free conditions. RNA was harvested according to the manufacturers protocol for purifying RNA from animal cells using the PureLink® RNA kit (ThermoFisher). 0.5-1 µl of purified RNA was reverse transcribed with random hexamer primers using the TaqMan Reverse Transcription Reagents (Thermo Fisher Scientific). Quantitative PCR (qPCR) was completed using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories) on a StepOnePlus PCR System (Thermo Fisher Scientific), using the standard cycle conditions and primer pairs shown in Table 2.7. Reactions were performed in duplicate, and changes in gene expression levels were analysed relative to uninfected control samples, using GADPH as a standard and using the ΔΔCT method.

Table 2.7: qRT-PCR primers used in this study

Primer	Sequence
hGAPDH-Fwd	5′TGCCATCAATGACCCCTTC3′
hGAPDH-Rev	5′CTGGAAGATGGTGATGGGATT3′
hIL-1β-Fwd	5′GACAAAATACCTGTGGCCTTG3′
hIL-1β-Rev	5′AGACAAATCGCTTTTCCATCTTC3′
hIL-6-Fwd	5′ CCACTCACCTCTTCAGAACG3′
hIL-6-Rev	5′CATCTTTGGAAGGTTCAGGTTG3′

2.4. Bacterial Infections

2.4.1 Bacterial growth conditions and MOI calculations

EPEC strains were either grown overnight in LB broth prior to infection (termed LB grown in figure legends), or primed to elevate expression of the LEE/non-LEE virulence regulons (termed DMEM-primed in figure legends). To prime bacteria, strains were grown in LB overnight at 37°C with aeration and appropriate antibiotic selection where required. Overnight LB-grown cultures were diluted 1:50 into pre-warmed low-glucose (1000 mg/L) Dulbecco's minimal Eagle medium (DMEM) and grown statically for 3 h at 37°C in a humidified incubator with 5% CO_2, to reach an OD_{600} 0.4-0.6. When required, isopropyl thio-galactopyranoside (IPTG) for pSA10 plasmid-encoded effector expression was added 30 min prior to cultures at 2.5 h post-inoculation into DMEM. For infections with SIEC strains, bacterial strains were grown overnight in LB and diluted 1:50 into fresh LB and grown statically for 3 h at 37°C in a humidified incubator with 5% CO_2. Ptac promoter expression in SIEC strains was induced with IPTG (0.1 mM) 30 min prior to infection.

Prior to infection, the optical density of the bacteria cultures was measured (OD_{600}), and the inoculums were diluted to a uniform OD. The volume of inoculum added to each infection was calculated in accordance to optical density. Bacterial CFU/ml was calculated using the experimentally verified principle that OD_{600} 0.1 = ~$1x10^8$ CFU/ml. Bacteria were infected at the required multiplicity of infection (MOI) which are indicated in Figure legends. Serial dilutions and CFU counts of the inoculum were completed to ensure consistent MOIs were maintained.

2.4.2 Infection of THP1 macrophages and primary MDMs

THP1 cells were seeded 72 h prior to infection in complete-RPMI medium supplemented with 100 ng/ml PMA. MDMs were seeded 7 days prior to infection in complete RPMI medium supplemented with 20 ng/ml human M-CSF. For cell death and cytokine secretion assays, cells were seeded in black walled 96-well plates at a density of 1.5 x 10^5 cells/well for THP1 cells and 2x10^5 cells/well for MDMs. 24 h prior to infection, the medium was replaced with fresh RPMI without phenol red, PMA, M-CSF or antibiotics. For western blot analysis, THP1 cells were seeded in 48-well plates at a density of 4 x10^5 cells/well and MDMs were seeded in 24-well plates at a density of 9x10^5 cells/well. 24 h before infection antibiotics, PMA or M-CSF were removed from the medium, and the next day 1-2 h prior to infection cells were washed in fresh serum-free RPMI to remove serum, and the medium was replaced with Opti-MEM + 1 mM sodium pyruvate. Naïve PMA-differentiated THP1 cells or human MDMs were used for bacterial infections (i.e. without priming with TLR agonists). Bacterial strains were grown and primed as described above and expression of pSA10 plasmids, or Ptac promotors, was induced with the addition of 0.1 mM IPTG at 30 min before infection. Macrophages were infected with bacteria at a MOI of 10 unless otherwise specified, and MOIs were verified by determining viable bacterial counts. Infections were synchronized by centrifugation for 10 min at 750 xg, and incubated at 37°C in a humidified incubator with 5% CO_2. Gentamicin (200 µg/ml) was added 2 h post-infection and incubation continued for 4 h or as indicated in the Figure legends. Where required, inhibitors were added to cells 1 h before infection at the following concentrations: zVAD-fmK (50 µM), zYVAD-cmk (50 µM), DEVD-CHO (100 µM), Necrostatin-1 (25 µM), potassium chloride (KCL; 20 mM), Cytochalasin-D (200 nM), Probenecid (100 µM), MCC950 (5 µM).

2.5. Cell Treatments and Analysis

2.5.1 NLRP3 inflammasome activation

For canonical NLRP3 activation, PMA-differentiated THP1 cells or human MDMs were primed with ultrapure O111:B4 LPS (250 ng/ml) for 3 h. After 3 h cells were washed twice in fresh serum-free RPMI and the medium was replaced with either RPMI-phenol red for cell death assays, or Opti-MEM medium supplemented with sodium pyruvate (1 mM) for western blot analysis. Cells were treated with nigericin (20 µM) for 45 min. After treatment, cell fractions were either processed for western blot analysis, or alternatively the supernatant was extracted for analysis of LDH release;

while cell monolayers were analysed for cell death by propidium iodide (PI) uptake as described in Section 2.5.5.

To stimulate caspase-4/11 non-canonical inflammasome activation, ultrapure O111:B4 LPS was transfected into unprimed cells using Lipofectamine-2000 (1% v/w). Typically, cells were transfected with 5 µg of LPS. Briefly, LPS and Lipofectamine were diluted in Opti-MEM medium supplemented with sodium pyruvate (1 mM) and incubated at RT for 15 min. LPS complexes were then added to cells in a dropwise manner and incubated for 4 h. Lipofectamine in the absence of LPS was added to control wells to ensure Lipofectamine alone did not alter cell viability. After treatment, cell viability was determined by LDH assays or PI uptake analysis, and inflammasome activation was analysed by immunoblots for caspase, or caspase substrate cleavage.

2.5.2 Cell priming assays

THP1 cells were treated with a range of chemical agonists to activate NF-κB signalling pathways, including Pam3CSK4 (PAM 100 ng/ml), ultra-pure *E. coli* O111:B5 LPS (1 µg/ml), R837 (5 µg/ml), IE-DAP (1 µg/ml), MDP (1 µg/ml), IE-DAP+LPS (1 µg/ml + 5 ng/ml respectively) or MDP+LPS (1 µg/ml + 5 ng/ml respectively). Cells were incubated at 37°C with 5% CO_2 for 4 h in the presence of the chemical agonists, and were then washed three times in ice-cold PBS. Cell lysates were collected and analysed for IL-1β expression by immunoblotting, as described in section 2.5.8-2.5.9

2.5.3 Supernatant transfer assays

A two-phased treatment was utilised to establish the toxicity of infection supernatants. THP1 cells were seeded in 48-well plates at a density of 4.5 x 10^5 cells/well, and in black walled flat bottom 96-well plates at a density of 1.5 x 10^5 cells/well. THP1 cells in the 48-well plate were infected with Wild-Type (WT) EPEC at an MOI of 10 for 2 h, as described above. Gentamycin (200 µg/ml) was added to the medium after 2 h and infections continued for a further 2 h. After 2 h the plates were spun at 250 xg for 4 min and supernatants were carefully collected and pooled. Pooled supernatants were centrifuged at 15,000 xg for 5 min to pellet any cell debris. 100 µl of the collected supernatant was plated onto LB agar plates to verify the absence of live bacteria. 2 h after the initial infection, a subset of wells in the 96-well plate were infected with the T3SS EPEC mutant strain *ΔescF* for 2 h and the remaining wells were left untreated. After 2 h the medium was aspirated and replaced with the collected WT infection supernatant (80 µl/well), supplemented with propidium

iodide (5 µg/mL). Inhibitors were added to appropriate wells at the following concentrations: zVAD-fmK (50 µM), zYVAD-cmk (50 µM), DEVD-CHO (100 µM). PI uptake was quantified over 18 h as described in section 2.5.5. A schematic for this is found in Figure 3.6

2.5.4 LDH assays

THP1 cells and MDMs were seeded in 96-well plates at a density of 1.5 x 10^5 and 2 x 10^5 respectively. 24 h before treatment the medium was removed and replaced with RPMI medium – phenol red. The next day macrophages were infected/treated as described, with a minimum of two technical replicates for each treatment condition. After treatment, at specified time points, supernatants were gently collected from cells and assayed for loss of cellular membrane integrity by measuring lactate dehydrogenase (LDH) activity in culture supernatants. LDH activity was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega), following the manufacturer's protocol with slight modifications. Briefly, 40 µl of cell culture supernatant was mixed with assay substrate at a ratio of 1:1 and incubated in the dark for ~ 15–20 min, until the positive control developed (uninfected cell treated with 0.1% Triton-X100 for 15 min). Reactions were stopped by the addition of 80 µL of the provided stop solution. The assay generates a red formazan product that which can be measured at an absorbance of 490 nm. Absorbance was measured at using the FluoStar Omega plate reader. Uninfected controls were used in all experiments and served as blanks, and percentage cell death was calculated as a percentage of a 100% control.

2.5.5 PI time-course assays

Cell membrane permeability was measured using real-time propidium iodide (PI) uptake assays. Cells were seeded in black-walled 96-well plates and were cultured in complete RPMI medium without phenol red. EPEC cultures were primed in DMEM medium without phenol red to minimise any background fluorescence. Three technical replicates were performed for each treatment condition. Immediately prior to infection/treatment, each well was supplemented with 5 µg/ml propidium iodide (PI), and three wells were treated with PI + 0.05% Triton X-100 for a positive control. After treatment, fluorescence was measured every 10 min on a POLARStar Omega plate reader (BMG Labtech). The percentage PI uptake was calculated at each time point by the following equation: % PI uptake=100×(FS−FB)/(FP−FB), where FS is the raw value of the sample well, FB is the raw value of the medium-only blank control and FP is the raw value of the positive control.

2.5.6 Imaging real-time PI uptake

For real-time cell death analysis THP1 cells were plated in 96-well black-walled plates at a density of 1 x 10^5 cells/well. 24 h prior to infection the medium was replaced with RPMI minus phenol red, antibiotics and PMA. The next day, the medium was supplemented with PI (5 μg/ml), and differentiated THP1 cells were infected with DMEM-primed EPEC strains constitutively expressing GFP via the pFP25.1_GPP plasmid at an MOI 10, (confirmed retrospectively by plating). Analysis was performed on the Lionheart™ microplate reader from BioTek which housed the plates in a humidified chamber maintained at 37˚C and 5% CO_2. Images were acquired in 3-5 min intervals for a total time of 120 min to assess the kinetics of cell death. Images and videos were processed using the BioTek Gen5™ image analysis software.

2.5.7 Enzyme-linked Immunosorbent assays

IL-1β in culture supernatants was measured using the human IL-1β ELISA kit (R&D Systems) following the manufacturer's protocol. Samples were measured on a FLUOstar Omega microplate reader (BMG Labtech) at an absorbance of 450 nm, and absorbance at 540 nm was subtracted for well-correction.

2.5.8 Sample preparation for SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

For immunoblot analysis, THP1 cells were seeded in 48-well plates (4 x 10^5 cells/well) and differentiated for 48 h. Primary MDMs were seeded in 24-well plates at (9 x 10^5 cell/well) and differentiated for 7 days. 24 h prior to treatment, medium without PMA or antibiotics was used. 1 h prior to treatment, cells were washed twice in pre-warmed RPMI in the absence of additional supplements, and the medium was replaced with serum-free Opti-MEM + sodium pyruvate (1 mM). All subsequent treatments were completed in serum free-medium. For detection of secreted proteins in cell culture supernatants, Opti-MEM supernatants were collected and proteins were precipitated at -20°C overnight in acetone (1:5 ratio v/v), followed by centrifugation at 15,000xg for 15 min. Air dried pellets were re-suspended in 2X Laemmli loading buffer (4% SDS, 20% Glycerol, 120 mM Tris pH 6.8 and 0.01% bromophenol blue). Cell lysates were prepared in ice-cold RIPA buffer (60 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA; all from Sigma) supplemented with cOmplete protease inhibitor tablets, and 1 mM phenylmethylsulfonyl fluoride (PMSF), followed by the addition of 5% β-mercaptoethanol (2ME) and Laemmli loading buffer. To collect cell lysates, the medium was aspirated from cells and the plate was incubated on

ice. The cells were washed once in ice cold PBS, and RIPA lysis Buffer + inhibitors was added to each well. Plates were agitated to ensure even distribution, and incubated on ice for 5 min. A cell scraper was used to ensure all cells were detached and in solution, and samples were collected and mixed with 5% 2ME + Laemmli loading buffer. Pooled cell extracts, that contained both supernatants and lysates, were prepared by resuspending briefly air-dried (~ 10 min) precipitates of supernatants in cell lysates prepared from respective samples. For gel electrophoresis, samples were boiled at 90 ˚C for 5 mins, then vortexed of 5 minutes. This process was completed two times and then samples were centrifuged at 17,000 xg on a table top centrifuge for 5 min. Proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using Tris-Glycine running buffer (25 mM Tris HCl, 192 mM glycine and 0.1% (w/v) SDS).

2.5.9 Western immunoblotting

For immunoblotting, proteins separated by SDS-PAGE were transferred to Immun-Blot® PVDF membranes (PVDF, 0.2 µm, Bio-Rad) using the BioRad Trans-Blot® Turbo™ Transfer System (1.0 A; 25 V, 30 min). Membranes were washed once in PBS-Tween (PBST, 1x PBS, 0.1% (v/v) Tween-20). Membranes were then blocked in either 10% (w/v) skimmed milk powder in PBST or 5% BSA in PBST at RT for at least 1 h according to the primary antibody specifications. After blocking membranes were washed in PBST and incubated with primary antibodies (Table 2.8). Primary antibodies were diluted as specified by the manufacturer and incubated overnight at 4°C. The following day, membranes were washed 3 x 7 min with PBST and incubated with relevant HRP-conjugated secondary antibodies at RT for 1 h. The membrane was washed 3 x 5 min with PBST and incubated in Clarity Western Enhanced Chemiluminescence (ECL, Bio-Rad) or ECL Prime (GE) for 5 min in the dark, before being developed using BioRad ChemiDoc Imaging System. For HRP-conjugated primary antibodies, after membrane-blocking, antibodies were incubated at RT for 1 h and developed as above. Western blot image processing was completed using the BioRad Image Lab software.

2.5.10 Bacterial adherence assay

THP1 cells (1 x 10^5/well) were seeded in triplicate in 96-well plates and differentiated for 48 h, after which medium without PMA or antibiotics was used. Cells were infected with various strains of EPEC at an MOI of 10 (confirmed retrospectively by plating), by centrifuging bacteria on macrophages for 10 min at 750 xg. After 2 h, cells were washed 5 times in RPMI, lysed in 0.5% Triton-X-100 for 10 min. Samples were vortexed, serially diluted in sterile PBS, and 10 µl drops were

plated in triplicate on LB agar and incubated overnight at 37°C. Colony forming units (CFUs) were enumerated and the mean number of CFUs in each treatment group was calculated.

2.5.11 Immunofluorescence analysis

For immunofluorescence analysis of inflammasome speck formation, cell morphology, and actin pedestal formation, cells were plated on coverslips and were infected with EPEC at an MOI of 10 as described above. At specified time-points cells were washed in PBS and fixed in PBS plus 4% paraformaldehyde (PFA) for 15 min, and were then washed 3x in PBS. Fixed cells were incubated in PBS plus 50 mM NH_4Cl as a quenching agent for 10 min, washed 3 x in PBS and then permeabilised for 4 min in 0.2% Triton X-100, washed 3x in PBS, and blocked in PBS plus 1% bovine serum albumin (BSA) for 30 min before staining with primary antibodies. Primary antibodies listed in Table 2.8 were diluted in 1% BSA-PBS according to the manufacturers specifications and incubated on coverslips for 45 min. Coverslips were washed twice in PBS and incubated with secondary antibodies, Phalloidin-AlexaFluor647 or Phalloidin-AlexaFluor594 and DAPI dye in 0.2% BSA-PBS for 30 min, washed once in PBS and twice in dH_2O before being mounted onto microscope slides using Gold Pro-Long Anti-fade medium. Cover-slips were dried overnight in the dark before being examined by conventional epifluorescence microscopy using a Zeiss AxioImager Z1 fluorescence microscope. Images were processed using Fiji™ and Zen Blue software. For quantifications of ASC aggregates (foci), for each experimental repeat, 5-10 random regions of interest for each condition were imaged at ×40 magnification and the number of ASC aggregate positive cells were quantified. % cells showing events were obtained for each experiment and mean % from 3-5 biologically independent experiments were compared statistically. For representative images, the slides were imaged at ×63 magnification.

2.5.12 Bacteria phagocytosis assays

For phagocytosis assays, THP1 wells were plated onto coverslips in 24-well plates (5 x 10^5cells/well). Cells were infected with EPEC strains at an MOI of 10 as previously described. At various time-points indicated in figure legends, cells were washed 3 times with warm PBS and fixed for 15 min using 4% PFA prior to performing outside-inside staining. For outside-inside staining, fixed THP1 cells were blocked in 1% BSA for 30 min. Cells were incubated in rabbit anti-O127:H6 for 40 min, followed by 3 PBS washes. Cells were incubated with Alexa488 anti-rabbit antibodies diluted in 1% BSA-PBS for 40 min, followed by 3 PBS washes. Cells were then permeabilised with 0.2% Triton X-100 for 3 min,

washed 5 times, and re-blocked for 30 min in 1% BSA-PBS. The same primary antibody was used again for 40 min, but Alexa594 anti-mouse was used as a secondary antibody for 40 min, and cells were also stained with DAPI and Phalloidin-Alexa647 to allow determination of host cell boundaries. After washing, coverslips were mounted in Prolong Gold anti-fade as above. Numbers of internalised EPEC were determined by subtracting values counted for bacteria residing outside from total bacteria. For counts, typically ~50-host cells were counted from at least 5 randomly selected fields. Mean % from 3-4 biologically independent experiments were compared statistically.

2.5.13 FcgRTir-chimera bead activation assays

HeLa cells containing FcγRTir-mCherry plasmid constructs were counted and seeded onto coverslips in 24-well plates at a density of 2 x 10^5 cells/well 24 h prior to treatment. 10 µl per well of BSA-coated SPHERO™ polystyrene bead slurry (Spherotech) were pelleted at 13,500 RPM for 3 min and resuspended 1 ml MES-based labelling buffer (20 mM MES, 8 mM HEPES). 10 µl of mouse anti-BSA antibody was added to beads and the mixture was incubated on a rotating wheel for 1 h at 40 RPM. Beads were centrifuged for 10 min at 13,500 RPM and resuspended in 1 ml labelling buffer. Supernatants were removed and pellets resuspended in serum-free Opti-MEM + sodium pyruvate (1 mM) + HEPES (10 mM). Beads were counted and added to cells at a density of 10 beads/cell. The 24-well plate was subsequently centrifuged at 300 RPM for 5 min and incubated at 4°C for 15 min. Cells were washed twice in serum-free DMEM, and the medium was replaced with fresh Opti-MEM + Sodium Pyruvate (1 mM). Cells were incubated at 37°C 5% CO_2 for 90 min. The medium was removed, and cells were washed on ice in PBS before being fixed in 4% PFA for 15 min at RT. Fixed cells were then processed for immunofluorescence analysis as described in section 2.5.11.

2.6. Statistical Analysis

Experiments were repeated independently at least twice or as indicated in Figure legends. For ELISA, LDH-release, PI-uptake assays and CFU experiments two to three technical replicates were used to estimate experimental mean. Primary MDMs from independent donors were used, and sometimes independent experiments were repeated on cells from the same donor on a different day with different cultures of bacteria, as indicated by donor number and number of experiments in Figure legends. Means from three or more independent experiments, as indicated by the values of *n* in legends, were analysed by statistical methods. Mean ± SEM (Standard Error of the Mean)

are plotted unless indicated otherwise. For dot-plots, all data points are shown as symbols matched by shape and colour and correlating to biological repeats, and the mean is indicated by a horizontal line. For statistical analysis repeat measures one-way ANOVA or paired two-tailed Student's *t*-test were used to compare means, and if more than three comparisons were made from the same dataset, *P* values were corrected by the Dunnett's test or the false-discovery rate (FDR) approach of Benjamini, Krieger and Yekutieli. Log-transformed (for CFU experiments) or original raw data were found to be normally distributed (based on D'Agostino & Pearsons normality test). For immunofluorescence assays, typically >100 host cells were imaged/counted from randomly selected fields. For each experimental repeat the percentage of phenotype positive cells was obtained, and mean percentage values were compared statistically. Data plots and statistics were completed using GraphPad Prism 8 software (Graph Pad Prism®).

2.7. Antibodies and Reagents

Table 2.8: Antibodies used in this study

Antibody	Application	Dilution	Source	Identifier
Primary Antibody				
Monoclonal Anti-beta-Actin-Peroxidase antibody produced in mouse	WB	1:10,000	Sigma-Aldrich	Cat# A3854 RRID:AB_262011
Caspase-4 (4B9) antibody	WB	1:500	Santa Cruz Biotechnology	Cat# sc-56056, RRID:AB_781828
Mouse Anti-Human GSDMDC1 Monoclonal Antibody, Unconjugated, Clone 64-Y	WB	1:500	Santa Cruz Biotechnology	Cat# sc-81868, RRID:AB_2263768
Cleaved Caspase-1 (D7F10) Rabbit Antibody	WB	1:1000	Cell Signalling Technology	Cat# 3866S RRID:AB_2069051
anti-Caspase-1 (p20) (human) mAb (Bally-1) antibody	WB	1:1000	AdipoGen	Cat# AG-20B-0048, RRID:AB_2490257
Human IL-18 Polyclonal Antibody	WB	1:1500	MBL International	Cat# PM014, RRID:AB_592017
Goat Anti-Human Il-1 beta / il-1f2 Polyclonal antibody, Unconjugated	WB	1:1000	R and D Systems	Cat# AF-201-NA, RRID:AB_354387
anti-Asc pAb (AL177) antibody	WB IF	1:1000 1:200	AdipoGen	Cat# AG-25B-0006, RRID:AB_2490440
Rat Anti-Human Caspase-11 Monoclonal Antibody, PE Conjugated, Clone 17D9	WB	1:1000	Thermo Fisher Scientific	Cat# 12-9935-82, RRID:AB_1518784
Anti-NLRP3/NALP3, mAb (Cryo-2)	WB	1:1000	AdipoGen	Cat# AG-20B-0014 RRID:AB_2490202
MLKL (D2I6N)	WB	1:1000	Cell Signalling Technology	Cat# 14993 RRID:AB_2721822
RIP1	WB	1:500	BD Pharmingen	Cat# 551042 RRID:AB_394015
Tir3 CT Rabbit polyclonal sera against Tir	WB	1:500	Frankel Lab (68)	N/A
DnaK (*E. coli*), mAb (8E2/2) antibody	WB	1:10,000	Enzo Life Sciences	Cat# ADI-SPA-880, RRID:AB_10619012
Anti-α0127:H6	IF	1-200	R. La Ragione	N/A
Anti-WASL antibody produced in rabbit	IF	1-100	Sigma-Aldrich	Cat# HPA005750, R RID:AB_1854729

Secondary Antibody				
Donkey Anti-Rabbit IgG, Whole Ab ECL Antibody, HRP Conjugated	WB	1:1000	GE Healthcare	Cat# NA934, RRID:AB_772206
Sheep Anti-Mouse IgG, Whole Ab ECL Antibody, HRP Conjugated	WB	1:1000	GE Healthcare	Cat# NA931, RRID:AB_772210
Donkey anti-goat IgG-HRP Polyclonal, Hrp Conjugated antibody	WB	1:1000	Santa Cruz Biotechnology	Cat# sc-2056, RRID:AB_631730
Donkey anti-Goat IgG (H+L) Secondary Antibody, HRP	WB	1:1000	Thermo Fisher Scientific	Cat# A15999, RRID:AB_2534673
Cy3-AffiniPure Fab Fragment Donkey Anti-Rabbit IgG (H+L) antibody	IF	1:200	Jackson ImmunoResearch Labs	Cat# 711-167-003, RRID:AB_2340606
DyLight 488 AffiniPure Donkey anti Rabbit IgG (H+L) antibody	IF	1:200	Jackson ImmunoResearch Labs	Cat# 711-485-152, RRID:AB_2492289
Alexa Fluor 488 AffiniPure Donkey Anti-Chicken IgY (IgG) (H+L) antibody	IF	1:200	Jackson ImmunoResearch Labs	Cat# 703-545-155, RRID:AB_2340375
Alexa Fluor 647 donkey anti-mouse antibody	IF	1-200	Jackson ImmunoResearch Labs	Cat# 715-606-151, RRID:AB_2340866
Alexa Fluor® 594 Phalloidin antibody	IF	1-100	Thermo Fisher Scientific	Cat# A12381, RRID:AB_2315633
Alexa Fluor® 647 Phalloidin antibody	IF	1-100	Thermo Fisher Scientific	Cat# A22287, RRID:AB_2620155

Table 2.9: Key reagents used in this study

Reagent	Source	Identifier
Chemicals		
LPS-EB (LPS from *E. coli* O111:B4)	Invivogen	Cat# tlrl-3pelps
Nigericin	Sigma-Aldrich	Cat# N7143
Potassium Chloride	Sigma-Aldrich	Cat# P9333
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich	Cat# #I6758
Kanamycin	Sigma-Aldrich	Cat# 60615
Ampicillin	Sigma-Aldrich	Cat# A9518
Chloramphenicol	Sigma-Aldrich	Cat# C0378
Puromycin dihydrochloride for *Streptomyces alboniger*	Sigma-Aldrich	Cat# P8833
Gentamicin	Sigma-Aldrich	Cat # G1272
Propidium iodide (PI)	Sigma-Aldrich	Cat # P4170
zVAD-fmk	R&D	Cat# FMK001
z-YVAD-cmk	R&D	Cat# FMK005
Ac-DEVD-CHO	Enzo Lifescience	Cat# ALX-260-030
Necrostatin-1	Toris Bioscience	Cat# 2324
MCC950	Tocris Bioscience	Cat # 5479
Cytochalasin D	Sigma-Aldrich	Cat# C8273
Mehyl-β-cytodextrin MBCD	Sigma-Aldrich	Cat# 332615
MitMAB	Tocris Bioscience	Cat# 4224
Chlorpromazine	Sigma-Aldrich	Cat# C8138
GDC-0941	Cambridge Bioscience	Cat# CAY11600
AS 19499490	Tocris	Cat# 3718
bpV(HOpic)	Cambridge Bioscience	Cat# CAY15438-5
Probenecid	Sigma-Aldrich	Cat# P8761-25G
Lipofectamine 2000 Transfection Reagent	Life Technologies	Cat# 11668027
DMSO	Sigma	Cat# D2438-50ML
cOmplete protease inhibitor cocktail	Roche	Cat# 04693116001
Pierce™ Phosphatase Inhibitor Mini Tablets	Thermo Fisher Scientific	Cat# A32957
Pierce™ Protease Inhibitor Mini Tablets, EDTA-free	Thermo Fisher Scientific	Cat# A32955
Clarity Western ECL Blotting substrate	Bio-Rad Laboratories	Cat# 1705061
ECL™ Prime Western Blotting Detection Reagent	GE-Healthcare	Cat# RPN2236
DAPI for nucleic acid staining	Sigma-Aldrich	Cat# D9542
Hoechst 33342 dye	Thermo Fisher Scientific	Cat# H1399
ProLong Gold Antifade Mountant	Thermo Fisher Scientific	Cat# P36930
Phenylmethanesulfonyl fluoride	Sigma-Aldrich	Cat# P7626
Phorbol myristate acetate (PMA)	Sigma-Aldrich	Cat# P8139
HEPES solution	Sigma-Aldrich	Cat# H0887
Trypsin-EDTA	Sigma-Aldrich	Cat# T4049
Dulbecco's minimal Eagle media Low Glucose (1000mg/L)	Sigma-Aldrich	Cat# D6046

Dulbecco's minimal Eagle media High Glucose (4500mg/L)	Sigma-Aldrich	Cat# D5796
RPMI 1640	Sigma-Aldrich	Cat# R8758
RPMI 1640 – Phenol Red Free	Gibco	Cat# 11835030
Fetal Bovine Serum	Sigma-Aldrich	Cat# F9665
Sodium pyruvate	Sigma-Aldrich	Cat# S8636
Penicillin-Streptomycin	Sigma-Aldrich	Cat# P4333
Opti-MEM	Gibco	Cat # 31985062
L-Glutamin Solution	Sigma	Cat # G7513
CD14-Biotin	Miltenyi Biotec	Cat# 130-190-485
Anti-Biotin Microbeads	Miltenyi Biotec	Cat# 130-190-485
LS Columns	Miltenyi Biotec	Cat# 130-042-401
LeucoSep Centrifuge Tubes	Greiner Bio-One	Cat# 227288
Critical Commercial Kits		
CytoTox 96® Non-Radioactive Cytotoxicity Assay	Promega	Cat# G1780
Human Il-1 beta/Il-1F2 DuoSet	R and D Systems	Cat# DY201
Viromer Blue	Lipocalyx	Cat# VB-01LB-00

Results

Chapter 3 –

EPEC infections induce rapid caspase-4-dependent NLRP3-inflammasome activation in human macrophages

Introduction

Programmed cell death pathways and inflammatory responses are crucial processes in the innate immune response to bacterial infections. Despite important roles in immunity and disease, the mechanisms by which host cells, specifically human macrophages, activate these pathways in response to A/E pathogens is largely uncharacterised. A number of studies have previously characterised the complex interaction between EPEC and host intestinal epithelial cells (598, 611-613). These identified a range of effector proteins that work in co-ordination to activate and supress various host programmed cell death pathways to help facilitate infection (248, 249, 251, 614). However, the maintenance of tissue homeostasis within the intestine requires the co-ordinated activity of multiple immune effectors, IECs and myeloid cells. The initial aim of this study was therefore to establish whether EPEC infection induced programmed cell death and inflammatory responses in human macrophages.

Upon infection, macrophages can induce several suicide programmes to prevent microbial replication and expose them to immune attack (329, 332, 615-618). The repertoire of known cell death pathways is continuously expanding, with the best characterised being apoptotic, pyroptotic and necroptotic cell death (619). Therefore, in order to propagate infections pathogens have evolved several diverse strategies to manipulate and subvert programmed cell death pathways (620-622). This dysregulation of cell death signalling enables pathogen survival and dissemination around the host (427, 623, 624).

Previous work investigating the inflammatory immune responses to infections of EPEC, and other A/E pathogens, has primarily utilised a mouse model of infection to characterise macrophage host cell death pathways (123, 126, 504, 571, 623-625). However, EPEC is a human-restricted pathogen, and there are numerous discrepancies in both innate and adaptive immunity between human and mouse systems (626), including TLR expression and ligand specificity, nitric oxide (NO) production, arginine metabolism, and cytokine expression. Indeed, recent studies have identified key mechanistic differences between human and mouse macrophages in their recognition and response to bacterial factors, which have major implications in understanding and modelling pathogenesis (627-632).

One of the major differences between host species is the expression of pattern recognition receptors (PRRs). PRRs evolved under a high level of selection pressure as host species expressed

mechanisms to deal with an ever-changing repertoire of pathogens. Recent analysis of PRR families in humans and mice have revealed significant gene expansion in PRR subfamilies in the mouse genome (633), with the most dramatic expansions being in the AIM2-like receptor superfamily and the NLR superfamily, that mediate inflammasome responses. Similarly, a number of human inflammasome regulators of ASC, caspase-1 and IL-1β do not have corresponding mouse orthologues (634-638). Inflammasome responses to bacterial infections have also been shown to be species specific (639-641). These immunological differences between mice and humans are exemplified by the altered sensitivity to LPS. With the LPS doses required to induce severe disease in mice being several orders of magnitude higher than in humans (642, 643). Therefore, it cannot be assumed that the host cell responses determined in mouse systems are representative of human macrophage responses. Thus, the initial aim of this work was to clarify EPEC-induced innate immune cell responses during infection of human macrophages, in order to provide a more comprehensive insight into the overall infection strategy of EPEC in human hosts.

Results

3.1 EPEC infections induce rapid cell death in human macrophage cells

To investigate the response of macrophages to EPEC infection, phorbol myristate acetate (PMA)-differentiated THP1 cells were used as a model for human macrophages. THP1 is a human leukaemia monocytic cell line, which has been extensively used to study macrophage functions, mechanisms and signalling pathways (644). THP1 cells can be differentiated into macrophage-like cells using PMA (645). PMA-differentiated THP1 cells mimic native monocyte-derived macrophages and provide a valuable model for studying macrophage responses.

In this study THP1 cells were PMA differentiated for 72 h prior to treatment. Differentiated THP1s were infected with DMEM-primed EPEC and the cytotoxic effect of EPEC infection was measured using Lactate dehydrogenase (LDH) release assays. LDH is a stable cytoplasmic enzyme present in all cells that is rapidly released following necrotic cell death. Infection of THP1 cells with Wild Type (WT) EPEC resulted in rapid host cell lysis within the first 2 h of infection (Figure 3.1). Importantly, the level of EPEC-induced cytotoxicity correlated directly with both the bacterial multiplicity of infection (MOI) (Figure 3.1A) and the time post infection (Figure 3.1B). Host cell death was entirely dependent on the presence of the T3SS. EPEC *escF* encodes the needle structure of the LEE-encoded T3SS, and deletion of *escF* abolishes EspA filament assembly and secretion of effector proteins into host cells (145). The *ΔescF* EPEC mutant strain failed to induce notable cell death in THP1 macrophages up to 8 h post infection (Figure 3.1B).

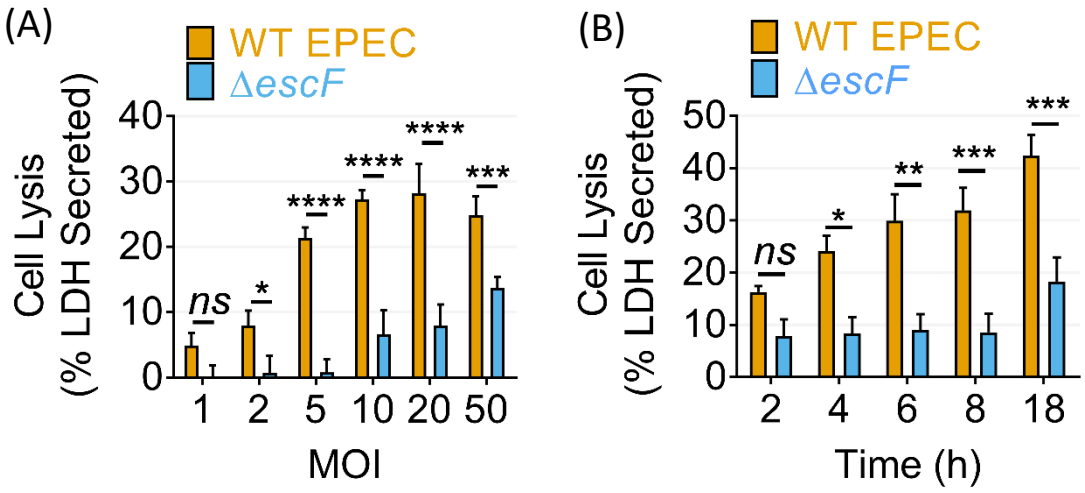

Figure 3.1. EPEC infection induces rapid T3SS-dependent cell death in THP1 macrophages

(A) Cytotoxicity as measured by lactate dehydrogenase (LDH) release from THP1 cells infected with WT and Δ*escF* EPEC at indicated MOIs for 8 h. Culture supernatants were collected and measured for LDH activity. Graph shows mean ± SEM from three independent repeats.

(B) Cytotoxicity as measured by LDH release from THP1 cells infected with WT and Δ*escF* EPEC at an MOI of 10, LDH release was measured at indicated time points. Graph shows mean ± SEM from three independent repeats.

* *P<0.05*, ** *P<0.01*, *** *P<0.001*, *P<0.0001* by two-way ANOVA. *ns*= non-significant.

3.2 EPEC infections induce programmed necrosis in THP1 macrophages

Different types of cell death are often defined by morphological criteria, with the loss of structural integrity of the plasma membrane being a hallmark of programmed necrosis. Propidium iodide (PI) is a membrane impermeant dye that remains outside viable cells. Upon membrane rupture, PI enters cells and binds to double stranded DNA by intercalating between base pairs. Once the dye is bound, its fluorescence is enhanced 20- to 30-fold, and the fluorescent shift can then be quantified or visualised by immunofluorescence microscopy. Using PI as a fluorescent marker to measure membrane integrity, infection of THP1 cells with EPEC caused rapid disruption to the plasma membrane within the first few hours of infection (Figure 3.2A).

EPEC-induced plasma membrane disruption was entirely dependent on the T3SS, as infections with EPEC *ΔescF* failed to induce a significant increase in PI uptake compared with the uninfected control (Figure 3.2A). Importantly, deletion of *escF* did not appear to affect the infection rate as CFU counts of bacteria recovered from the cells 2 h post infection showed no significant difference between levels of initial attachment to the host cells for WT and *ΔescF* EPEC (Figure 3.2B). Therefore, the differences in the cytotoxicity in the infected cells was not due to differences in infection rate by the two strains of EPEC.

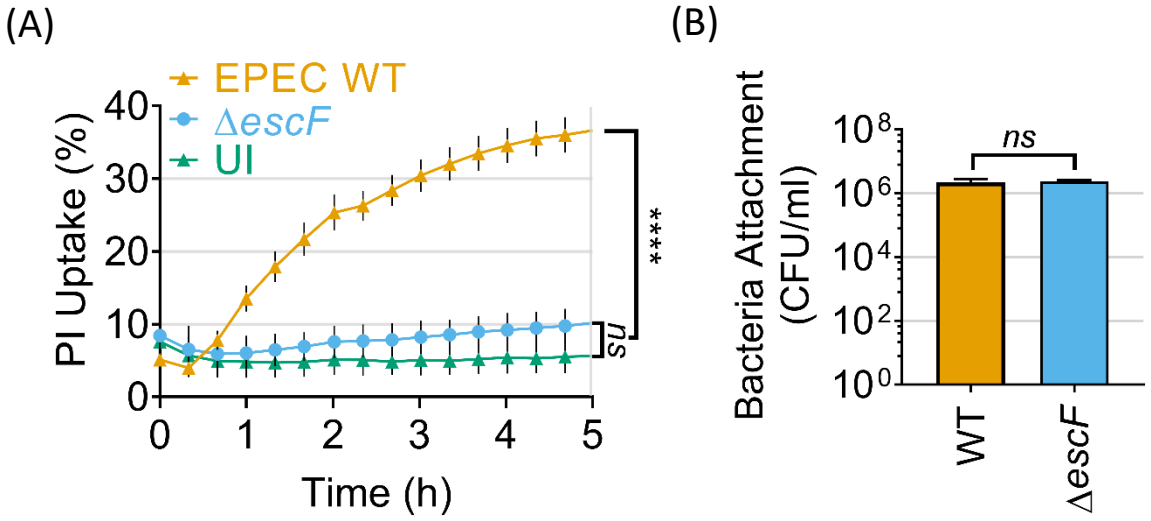

Figure 3.2. EPEC infection induces rapid T3SS-dependent membrane permeabilisation in THP1 macrophages

(A-B) Cytotoxicity as measured by real-time Propidium Iodide uptake (PI).

(A) Real-time PI-uptake from THP1 cells infected with WT and Δ*escF* EPEC at MOI:10 for 5 h. Values are calculated as a percentage of a Triton-X treated positive control. (Mean ± SEM; *n= 6* independent experiments) **** *P<0.0001* by two-way ANOVA for indicated comparisons after FDR-based correction for multiple comparisons; *ns* = non-significant.

(B) Graph showing CFU counts at 2 h from THP1 cells infected with WT and Δ*escF* EPEC at an MOI of 10. Results are presented as mean ± SEM from three independent experiments. Means were compared using Students *t*-test. *ns*= non-significant.

3.3 EPEC infections induce rapid caspase-dependent cell death in THP1 macrophages

Both apoptosis and pyroptosis utilise caspases to mediate the morphological changes and execute cell death. To establish if EPEC induced caspase-dependent cell death, THP1 cells were treated with the pan-caspase inhibitor zVAD-fmk during infection. Inhibition of caspase catalytic activity significantly reduced both PI uptake (Figure 3.3A) and LDH release (Figure 3.3B) in THP1 cells infected with EPEC. Pyroptosis can be triggered by either canonical caspase-1-dependent inflammasome activation, or by a non-canonical caspase-4-mediated pathway. Interestingly, YVAD-cmk, which selectively inhibits both caspase-1 and caspase-4, also significantly reduced cell death (Figure 3.2). Whereas DEVD-CHO, which predominantly inhibits caspase-3, an executioner caspase in the apoptosis pathway, failed to rescue cells from EPEC-induced cytotoxicity. These results suggested EPEC infections induced a rapid form of caspase-1/4-dependent pyroptosis in THP1 macrophages. Uninfected cells did not undergo cell death during experiments (Figure 3.2A, 3.3A-B), and all subsequent experiments were normalised to uninfected controls.

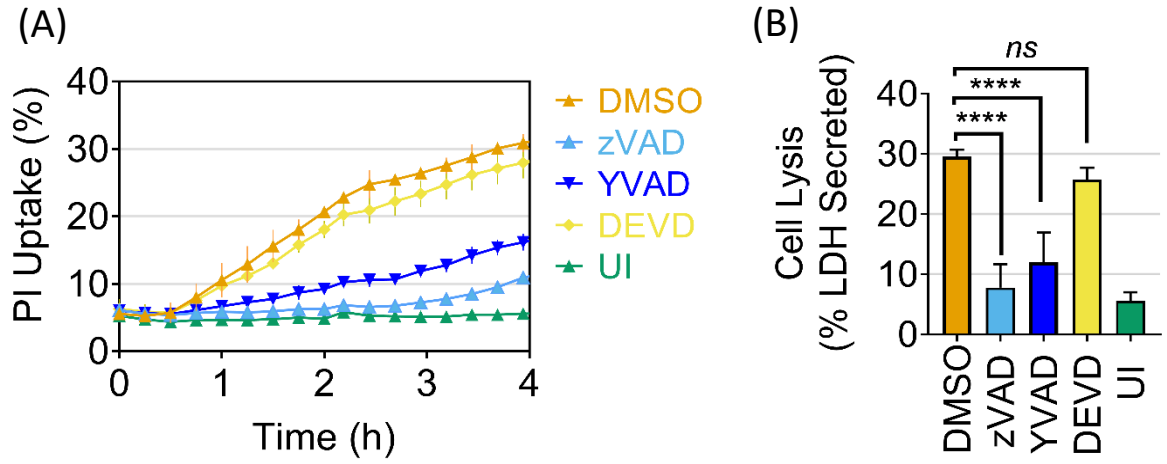

Figure 3.3. EPEC infections induce rapid caspase-dependent cell death in THP1 macrophages
THP1 cells left uninfected (UI) or infected with WT EPEC at MOI:10. Cells were treated with indicated inhibitors or a DMSO control 30 min prior to infection. Inhibitor concentrations: zVAD-fmk (50 μM), YVAD-cmk (50 μM) and DEVD-CHO (100 μM).
(A) Membrane damage measured by real-time PI uptake from THP1 cells treated with indicated inhibitors and infected with WT EPEC at an MOI of 10 for 4 h. Mean ± SEM; *n= 3* independent experiments.
(B) LDH release measured 4 h post infection from THP1 cells treated with indicated inhibitors and infected with WT EPEC at an MOI of 10. Data denotes 3-4 independent repeats.
**** *P<0.0001* by one-way ANOVA, *ns* = non-significant.

3.4 EPEC infections induce a second phase of caspase-independent necroptosis

The results presented above demonstrated that inhibition of the catalytic activity of caspase-1/4 significantly inhibited EPEC-induced cell death of THP1 macrophages within the first 5 h of infection. The next aim of was to establish whether prevention of caspase activity was sufficient to prevent cell death over longer infections. THP1 cells were infected with WT EPEC and cell death was measured over an 18 h infection (Figure 3.4A). Inhibition of caspase-1/4 with YVAD-cmk prevented the initial phase of cell death, however cell death could not be prevented by caspase-1/4 inhibition in infections exceeding 4-5 h (Figure 3.4A). This suggested a second phase of caspase-1/4-independent cell death occurred in response to EPEC infections. Consistent with these findings, the pan-caspase inhibitor zVAD-fmk was able to inhibit EPEC-induced cell death in the initial 4 h of infection, but not the second phase of death, which occurred independently of caspase activity (Figure 3.4A). RIPK1 is an essential upstream signalling molecule in necroptosis. Necrostatin-1 (Nec1) is a small-molecule inhibitor that suppresses necroptosis by specifically inhibiting the kinase activity of RIPK1 (646). Although RIPK1 inhibition alone did not prevent EPEC-induced cytotoxicity, the addition of Nec1, in combination with caspase-inhibition was sufficient to suppress almost all EPEC-induced cell death up to 18 h post infection (Figure 3.4C).

zVAD-fmk has previously been reported to induce necrotic cell death in a subset of host cells, with LPS priming increasing sensitivity (647). Autocrine production of TNF has also been demonstrated to provide a pro-death signal in zVAD-induced necroptosis (648). However, notably *ΔescF* EPEC infections did not induce this second stage of cell death, even in the presence of zVAD-fmk (Figure 3.4B), despite inducing 20-fold higher levels of TNF than WT infections (Figure 3.5). This demonstrated that caspase-independent cell death was not induced by TNF signalling alone, and therefore a mechanism governed by EPEC effector proteins is likely to be responsible.

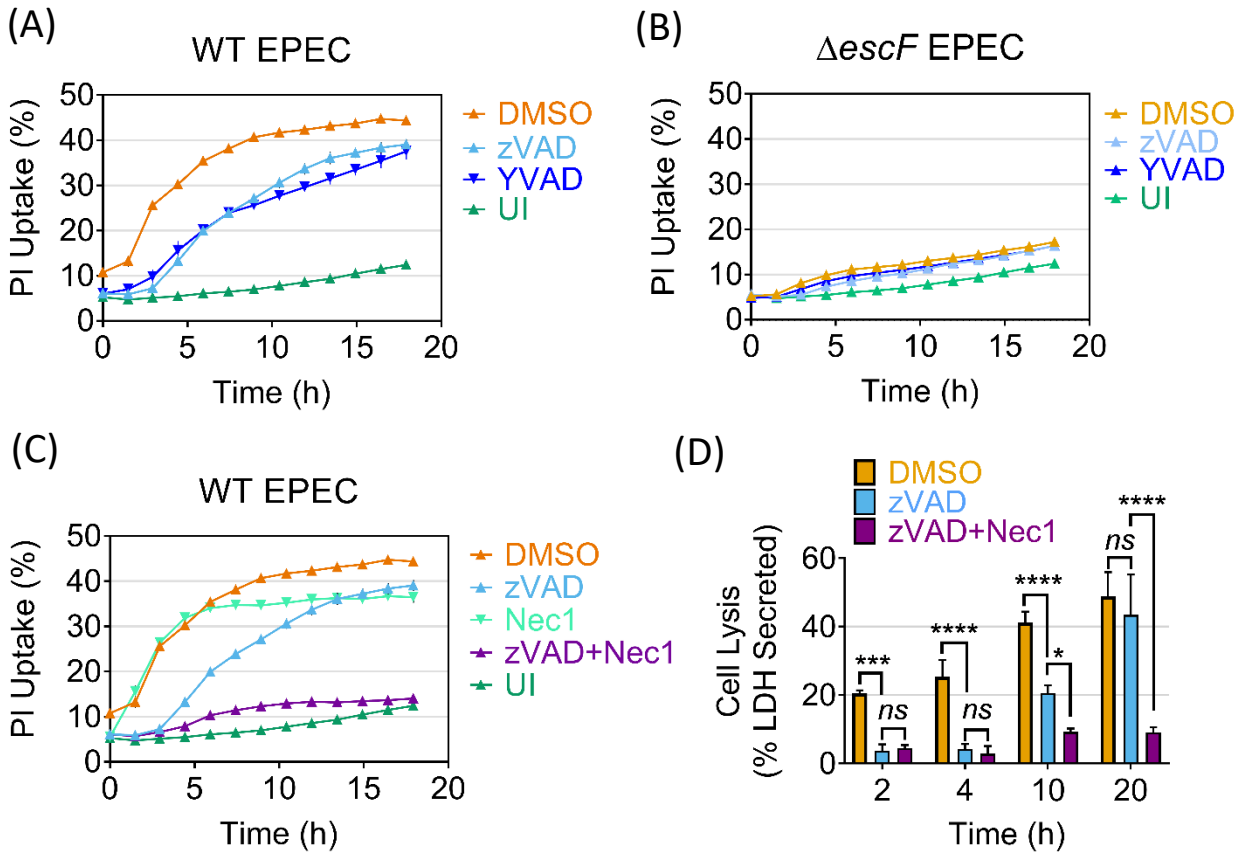

Figure 3.4. EPEC induces RIPK1-dependent necroptosis in prolonged infections

THP1 cells treated with the pan-caspase inhibitor zVAD-fmK (50 μM), caspase-1/4 inhibitor YVAD-cmk (50 μM) or the RIPK1 inhibitor Nec1 (25 μM).

(A-C) Cell lysis measured by real-time PI uptake from THP1 cells left uninfected (UI) or infected with WT EPEC (A and C) or *ΔescF* EPEC (B) at an MOI:10 for 18 h. Cells were treated with indicated inhibitors or DMSO 30 min prior to infection. Graphs display mean ± SEM, Graphs are representative of three independent experiments.

(D) Cell lysis as measured by LDH release from THP1 cells infected with WT EPEC at an MOI:10 at indicated time points. Graph shows mean ± SEM (*n = 3* independent repeats). * *P<0.05,* *** *P<0.001* **** *P<0.0001* by one-way ANOVA. *ns* = non-significant.

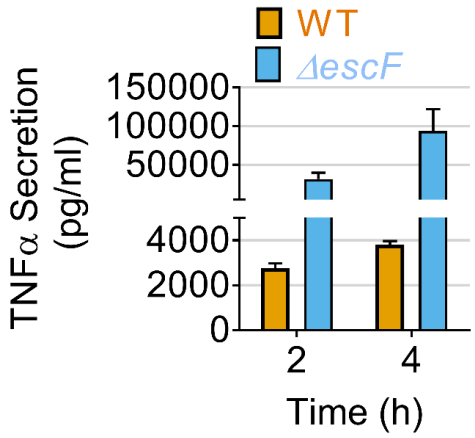

Figure 3.5. EPEC effectors prevent TNF processing during infection
TNF released from THP1 cells infected with WT or *ΔescF* EPEC at MOI:10. Concentration of TNF (pg/ml) in infection supernatants 2 h and 4 h post-infection as measured by ELISA. Graphs display mean ± SEM. *n*= 2 independent experiments.

Another key question was whether this second stage of RIPK1-dependent necroptosis was induced actively by secreted EPEC effectors or occurred in a non-cell-autonomous manner in response to cytokines or other alarmins that may be release from infected cells. To test this theory, cell supernatants from WT EPEC infections were tested for cytotoxicity. Supernatants from THP1 cells infected with WT EPEC were collected 4 h post-infection and transferred onto uninfected THP1 cells and PI uptake was measured over time (Figure 3.6A). The inability of infection supernatants to induce cell death (Figure 3.6B), indicated that the delayed phase of necroptosis induced by EPEC infection was not a result of host proteins released from cells during infection. Furthermore, the observation that infection supernatants failed to induce cell death, even in the presence of caspase inhibitors, confirmed that this RIPK1-mediated cell death was not simply an artefact of caspase-inhibition (Figure 3.6B). Similarly, a secondary *ΔescF* EPEC infection in combination with WT infection supernatant failed to induced necroptosis. This indicated that a functional T3SS and/or secreted effector proteins were required to trigger necroptosis in THP1 cells (Figure 3.5B). Collectively, these results demonstrate that WT EPEC infections induce a delayed phase of cell-autonomous, effector driven, necroptotic cell death in THP1 macrophages.

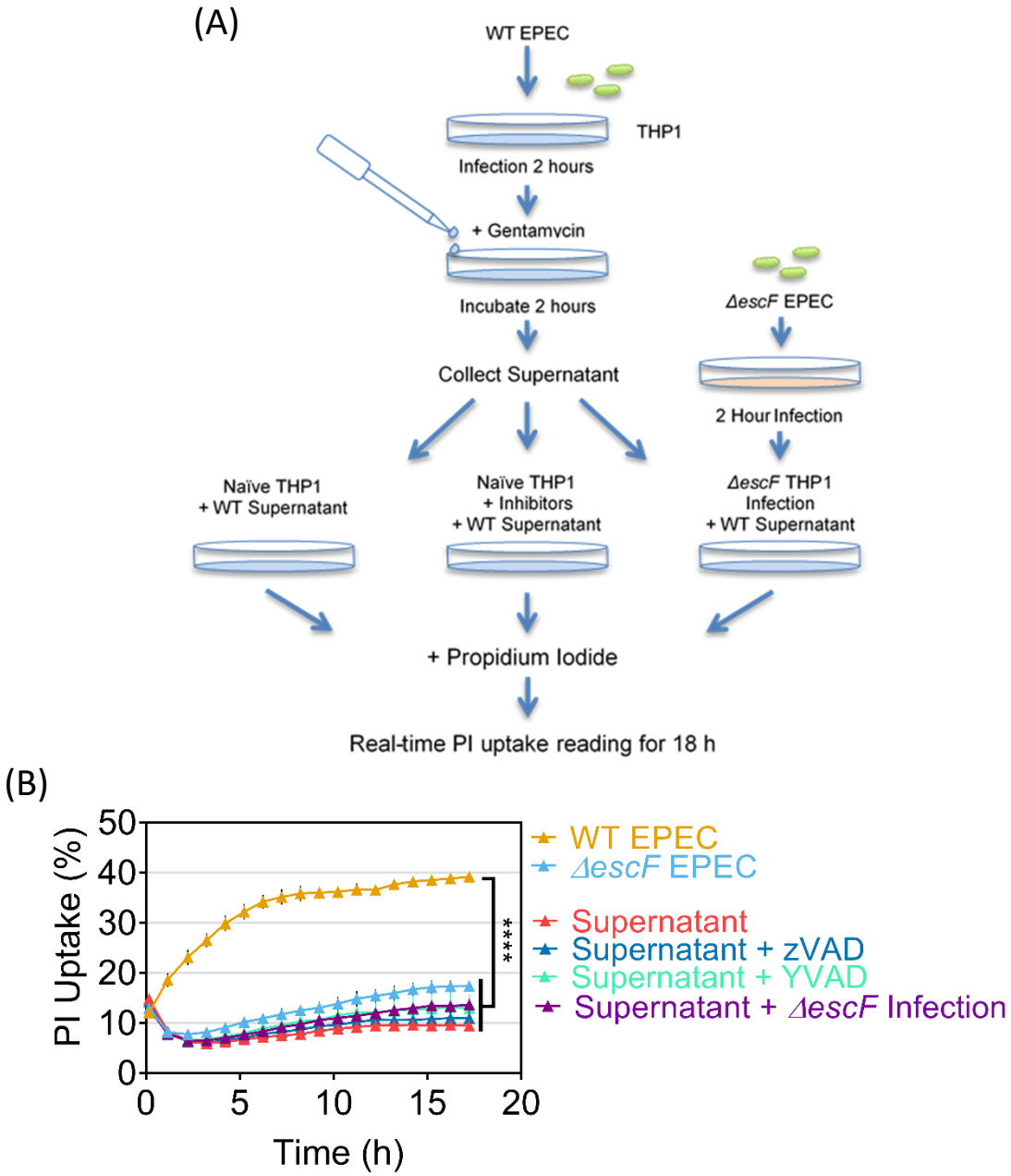

Figure 3.6. EPEC induces cell intrinsic necroptosis in THP1 macrophages

(A) Schematic of supernatant transfer experiments. THP1 cells were infected with WT EPEC for 2 h, gentamycin (200 µg/ml) was added to medium after 2 h and infections continued for a further 2 h. Infection supernatants were collected and transferred onto naïve THP1 cells, THP1 cells in the presence of indicated caspase inhibitors, or THP1 cells pre-infected with *ΔescF* for 2 h. PI was added to the culture medium, and PI uptake was quantified over 18 h.

(B) Real time PI uptake assay from naïve THP1 cells incubated with WT EPEC infection supernatant for 18 h, in the presence of indicated inhibitors. Naïve THP1 cells were also infected with WT or *ΔescF* EPEC to replicate normal infection dynamics. Values represent mean ± SEM and are representative of three independent repeats, **** *P<0.0001* by two-way ANOVA with FDR-based correction for multiple comparisons. Inhibitor concentrations: zVAD-fmk (50 µM) or YVAD-cmk (50 µM).

3.5 EPEC infections induce early inflammasome activation in THP1 macrophages

Two distinct EPEC-driven mechanisms were shown to be responsible for differentially activating programmed cell death pathways in THP1 macrophages. However, the second phase of necroptosis only became apparent in the absence of caspase activity. As the principle form of programmed cell death induced by EPEC infections was pyroptosis, this project focused on characterising the mechanisms driving this response. Pyroptosis is a form of lytic programmed cell death initiated by caspase-1 and/or caspase-4 activation.

Caspase-1 and caspase-4 in the canonical and non-canonical inflammasome pathways, respectively, are crucial for inflammasome-mediated inflammatory responses. Inflammasomes drive activation of caspase-1, which in turn cleaves GSDMD, the executioner of pyroptosis. Similarly, active caspase-4 can cleave and activate GSDMD to induce pyroptosis (445, 499). GSDMD cleavage separates its N-terminal pore-forming domain (PFD) from the C-terminal repressor domain (RD). Upon activation the PFD oligomerises to form large pores in the membrane that causes cell swelling and membrane rupture (649). To test whether EPEC infections activate the inflammasome to induce pyroptosis, naïve THP1 cells (i.e. without priming with TLR agonists) were infected with WT and *ΔescF* EPEC and immunoblots from cell lysates of infected cells were analysed to visualise GSDMD cleavage. Consistent with pyroptosis, EPEC infection induced the proteolysis of GSDMD to release its PFD domain (p30) within 4 h of infection in a T3SS-dependent manner (Figure 3.7A). This is consistent with the release of LDH into the culture supernatant (Figure 3.7B). Infection with the T3SS mutant EPEC strain (*ΔescF*) induced significantly less GSDMD cleavage and pyroptosis than WT EPEC (Figure 3.7). However, there was evidence of low-level GSDMD cleavage in *ΔescF* infected cells when compared to the uninfected control (Figure 3.7A). This suggests that non-pathogenic EPEC can induce some degree of GSDMD cleavage in host cells independently of the T3SS, although at a level that is not sufficient to induce a significant increase in pyroptosis (Figure 3.2A).

Furthermore, immunoblot analysis of supernatants from WT EPEC infected THP1 cells confirmed the presence of active caspase-1 (p20) and caspase-4 (p32), which was also dependent on the T3SS (Figure 3.7A). In addition to pyroptosis, inflammasomes mediate the proteolytic processing of IL-1β and IL-18. IL-1β and IL-18 are produced as cytosolic precursors and require secondary proteolytic cleavage induced by the inflammasome for activation and secretion. Immunoblots for IL-1β and IL-18 confirm EPEC infections induce the proteolytic cleavage and activation of these cytokines (p17 and p18) in a T3SS-dependent manner (Figure 3.7A). Taken together, these results indicate that

EPEC infections induce rapid caspase-1 and caspase-4 activation in human THP1 macrophages, which results in pro-inflammatory pyroptosis within the first 4 h of infection.

Inflammasomes are under the control of multiple regulatory mechanisms, with many inflammasome sensors being transcriptionally and/or post-transcriptionally regulated. For instance, both the NLRP3 inflammasome receptor and the inflammatory cytokine IL-1β have very low basal expression levels in resting macrophages. Optimal activation of the NLRP3 inflammasome and IL-1β processing therefore requires an initial priming step to induce transcription and post transcriptional licencing of NLRP3. This is controlled by transcriptional regulators, such as NF-κB (494, 650, 651). LPS on the outer membrane of *E. coli* activates the TLR4–MD-2 complex in host cells and leads to activation of multiple signalling components, including NF-κB (Figure 1.4 (652, 653)). However, NF-κB signalling is also inhibited by a number of EPEC secreted effector proteins (Figure 1.5 (240, 654-656)). This was evident from the reduced induction of pro-IL-1β in the cell lysates of THP1 cells infected with WT EPEC compared to the *ΔescF* mutant that is unable to secrete NF-κB inhibitory effectors (Figure 3.7A). Despite the inhibitory action of these NF-κB targeting effectors, the results here demonstrate that WT EPEC infections induce all the characteristic features typically associated with non-canonical inflammasome activation during infections of THP1 macrophages, and this occurs in a T3SS-dependent manner.

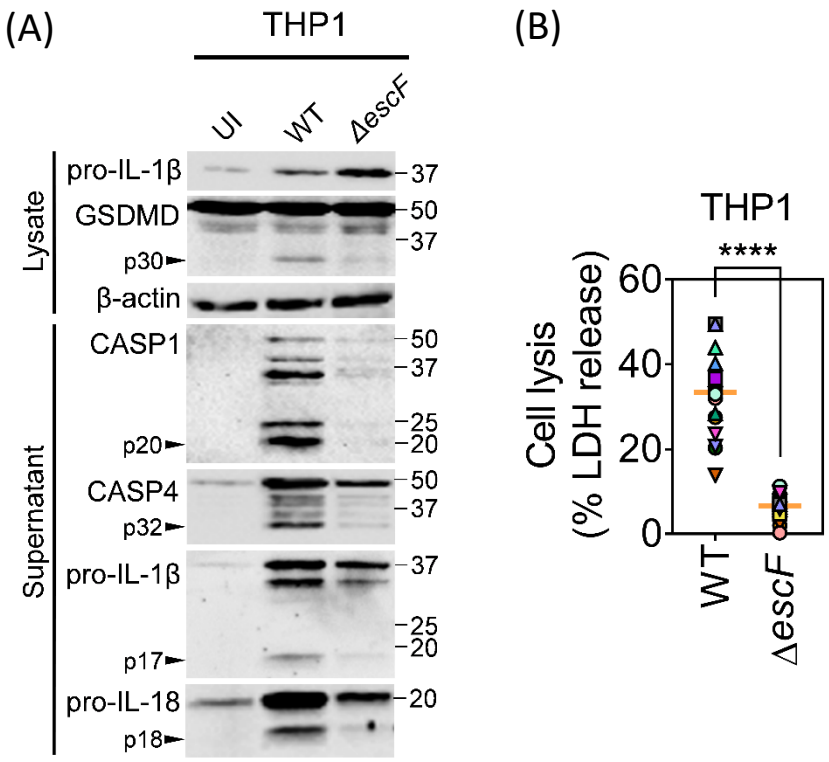

Figure 3.7. EPEC induces rapid inflammasome activation, pyroptosis and cytokine processing in THP1 cells

(A) Representative immunoblots from THP1 cells left uninfected (UI) or infected with EPEC (WT) or T3SS-deficient Δ*escF* EPEC for 4 h at an MOI of 10. Blots show caspase-1, caspase-4, IL-1β, and IL-18 in culture supernatants and IL-1β, GSDMD and β-actin in cell lysates.

(B) Cell death measured by LDH release from THP1 cells infected with WT or Δ*escF* EPEC for 4 h, at an MOI of 10.

Matching shapes and colours of symbols in graphs denote data from independent experiments. Mean indicated by horizontal bars. **** *P<0.0001* by paired Student's *t*-test. Immunoblots are representative of experiments performed 5 times.

3.6 EPEC infection induces T3SS-dependent inflammasome activation and pyroptosis in primary human macrophages

Differential expression of inflammasome sensors and substrates results in distinct outcomes following inflammasome activation in different cell types. Indeed, the expression and regulation of many of the inflammasome pathways varies significantly between different cell lines even within one host species. Therefore, the next aim was to establish whether EPEC also induced rapid T3SS-dependent inflammasome activation in primary human monocyte-derived macrophages (MDMs), as these provide a physiologically relevant model for EPEC infection. Primary human MDMs were infected with WT EPEC or the Δ*escF* mutant. Western blot analysis was used to assess caspase activation and cytokine processing, and LDH-release was quantified as a measure of cell death.

Similarly to THP1 cells, primary human MDMs underwent rapid pyroptosis within 4 h of infection in a T3SS-dependent manner (Figure 3.8A). Additionally, immunoblot analyses confirmed the proteolytic cleavage and activation of caspase-1 and caspase-4 and the cleavage of the proinflammatory cytokine IL-18 into its active p18 form (Figure 3.8B). This confirmed that EPEC infection induced rapid inflammasome activation and pyroptosis in both primary human macrophages and immortalised THP1 macrophages, demonstrating that the inflammasome response to EPEC infection is consistent between these different host cell lines.

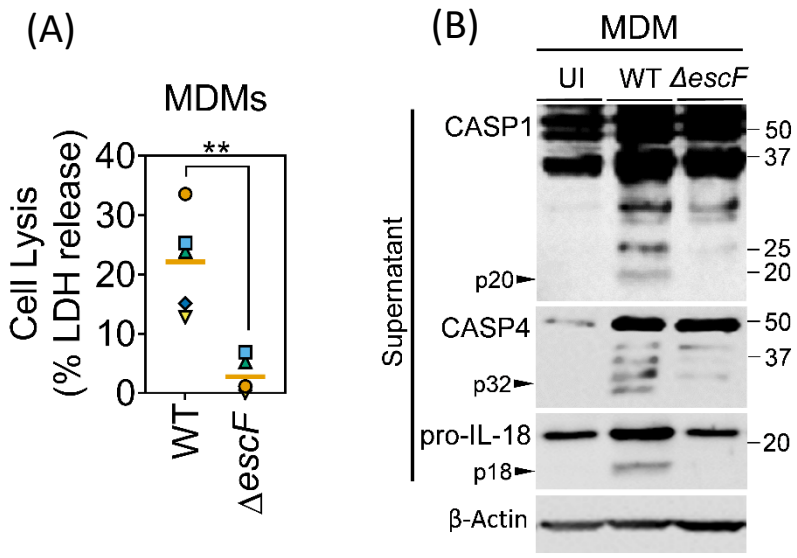

Figure 3.8. EPEC induces rapid inflammasome activation, pyroptosis and cytokine processing in primary MDMs

(A) Pyroptosis measured by LDH release from primary human monocyte derived macrophages (MDMs) infected with WT or Δ*escF* EPEC for 4 h at an MOI of 10.

(B) Representative immunoblots from primary MDMs left uninfected (UI) or infected with WT or Δ*escF* EPEC for 4 h at an MOI of 10. Blots show caspase-1, caspase-4 and IL-18 in culture supernatant and β-actin in the cell lysates.

Matching shapes and colours of symbols in graph (A) denote data from independent donors. Mean indicated by horizontal line (*n= 6* independent repeats from 4 independent donors) ** *P<0.01* by paired Student's *t*-test. Immunoblots are representative of 3 repeats from 3 independent donors.

3.7 EPEC infections induce T3SS-dependent inflammasome assembly in THP1 macrophages

Inflammasomes are multimeric protein complexes that assemble in the host cell cytosol after sensing bacterial PAMPs or DAMPs, where they serve as a scaffold to recruit and activate caspase-1. Inflammasome assembly typically requires activation of an upstream sensor, a downstream effector and, in most cases, the adaptor ASC. Upon activation, inflammasome sensors self-oligomerise and trigger the assembly of ASC via pyrin domain (PYD)–PYD interactions (657). ASC fibrils then assemble into large structures, termed ASC 'specks' or 'foci' (453) and recruit pro-caspase-1, leading to proximity-induced autoproteolytic activation of caspase-1. Once assembled, ASC specks can reach a size of around 1 µm and, therefore, can be utilised as a marker for inflammasome activation and detected by immunofluorescence microscopy (658). ASC-speck formation was used as a readout for inflammasome activation in response to EPEC infection of THP1 cells. Using a THP1 cell line overexpressing a fluorescently tagged version of ASC (THP1$^{ASC\text{-}mRFP}$), the change in ASC distribution was visualised using immunofluorescent microscopy (Figure 3.9A). During infection with the T3SS mutant Δ*escF* EPEC strain, ASC-mRFP remained diffused throughout the cell cytoplasm, however upon WT EPEC infection ASC-mRFP localisation shifted from a diffuse signal to a single inflammasome speck (Figure 3.9A). The accumulation of ASC specks was quantified, and counts revealed that WT EPEC infections induced inflammasome assembly in 12.5% of infected cells, compared to 4% in Δ*escF* infections (Figure 3.9B). However, it has recently been found that after pyroptosis, ASC specks can be released from cells into the extracellular space (447), therefore, the number of ASC positive cells upon WT EPEC infection may be underrepresented. Nevertheless, these results establish that both EPEC-induced cell death and inflammasome activation are dependent on the T3SS or secreted EPEC effector proteins.

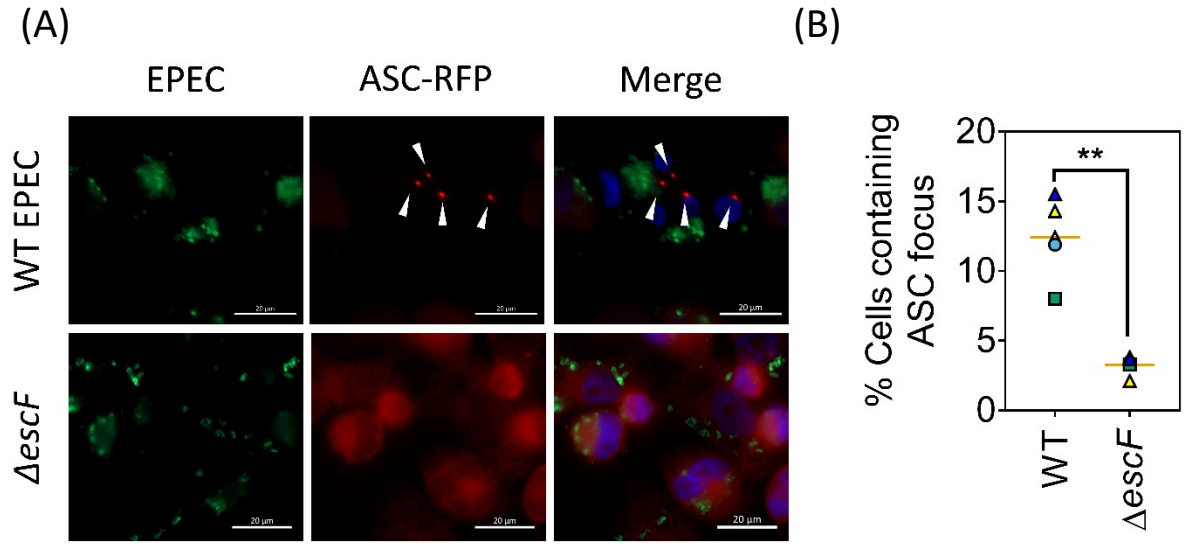

Figure 3.9. EPEC infections induce T3SS-dependent inflammasome assembly

(A) Representative immunofluorescence images from THP1 cells stably expressing mAsc-RFP (Red) (THP1$^{mAsc\text{-}RFP}$) infected with indicated EPEC strains at MOI:10 for 4 h. Bacteria were visualised with anti-0127:H6 antibody (Green) and cell nuclei were stained with DAPI (Blue). Arrowheads indicate re-localisation of ASC into inflammasome foci. Scale bar = 20 µm.

(B) Quantification of inflammasome ASC foci formation in THP1 cells infected with WT and *ΔescF* EPEC at an MOI of 10 for 4 h. >100 host cells were counted from randomly selected fields and % cells showing events were obtained for each experiment. Matching shapes and colours of symbols in graphs denote data from independent experiments. Mean indicated by horizontal line. ** *P<0.01* by paired Student's *t*-test (*n= 5* independent repeats).

3.8 EPEC infection induces rapid NLRP3-dependent inflammasome activation in human macrophages

Bacterial infections have been shown to activate a number of distinct inflammasomes, each of which contain a unique sensor protein that respond to various pathogenic stimuli. Previously cytokine responses to *C. rodentium* infection, the *in vivo* model for A/E pathogens, were demonstrated to be both NLRC4- and NLRP3-dependent in the mice (126). To assess the role of the NLRP3 inflammasome in response to EPEC infection of human cells, the NLRP3 specific inhibitor MCC950 was utilised. MCC950 is a potent, selective inhibitor of the NLRP3 inflammasome, and inhibits both canonical and non-canonical NLRP3, but not the AIM2, NLRC4 or NLRP1 inflammasomes (659).

Interestingly, despite expressing the full-length isoform of human NAIP, inflammasome activation in primary human MDMs infected with WT EPEC was dependent on NLRP3 (Figure 3.10). Inhibition of the NLRP3 inflammasome by MCC950 was sufficient to prevent both EPEC-induced caspase-1

activation (Figure 3.10B) and pyroptosis (Figure 3.10A). These results suggested that the NLRP3 inflammasome was primarily responsible for inflammasome signalling in response to EPEC infections, ruling out a role for NLRC4-, AIM2-, and NLRP1-mediated responses.

The next objective was to assess whether similar NLRP3-dependent responses occurred in THP1 cells, because this would enable a more in depth genetic and mechanistic investigation of this pathway. Treating THP1 cells with MCC950 during infection also prevented EPEC-induced inflammasome activation, GSDMD-cleavage, cytokine processing (Figure 3.11A), and pyroptosis (Figure 3.11B) in PMA-differentiated THP1 macrophages. The requirement for NLRP3 in EPEC-induced GSDMD-cleavage and pyroptosis was particularly interesting as previous work characterising inflammasome responses to A/E pathogens in mouse macrophages identified a non-canonical caspase-4/11-dependent, NLRP3-caspase-1-independent form of pyroptosis (123, 124, 504). In this context, caspase-4/11 acted as a direct sensor for cytosolic LPS, and directly cleaved GSDMD to induce pyroptosis independently of NLRP3.

To investigate whether cytosolic LPS can activate caspase-4 and induce pyroptosis in human macrophages independently of NLRP3, LPS was transfected into THP1 cells using a lipid reagent (Lipofectamine-2000). LPS transfection induced rapid pyroptosis within 5 h of treatment (~60 ± 10 %; Figure 3.11D-E), whereas lipofectamine treatment alone, in the absence of LPS, induced significantly less cell death (~10 ± 10; Figure 3.11E). Notably, unlike EPEC-induced pyroptosis, MCC950 was unable to inhibit pyroptosis induced by LPS transfection (Figure 3.11D). This confirmed that cytosolic LPS induces caspase-4-dependent pyroptosis in THP1 cells independently of NLRP3. These results, therefore, indicate that EPEC infections activate pyroptosis in human macrophages in a manner mechanistically distinct from pyroptosis induced by transfected LPS.

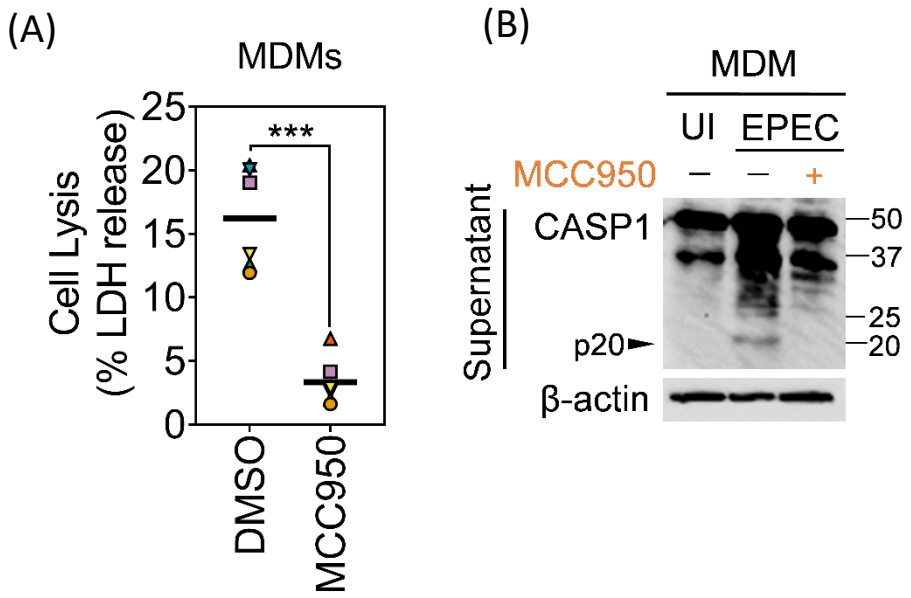

Figure 3.10. EPEC infections induce NLRP3-dependent inflammasome activation and pyroptosis in primary human MDMs

(A) Cell death measured by LDH release from primary MDMs treated with MCC950 (5 μM) or DMSO control, infected with WT EPEC for 4 h at an MOI of 10. (*n= 6* independent repeats from 4 independent donors).

(B) Representative immunoblots from primary MDMs left uninfected (UI) or infected with WT EPEC in the presence or absence of MCC950 (5 μM), for 4 h at an MOI of 10. Blots show caspase-1 in culture supernatants and β-actin in cell lysates.

Matching shapes and colours of symbols in graphs denote data from independent donors/experiments. Horizontal line represents mean. *** *P<0.001* by paired Student's *t*-test Immunoblots are representative of experiments performed 3 times.

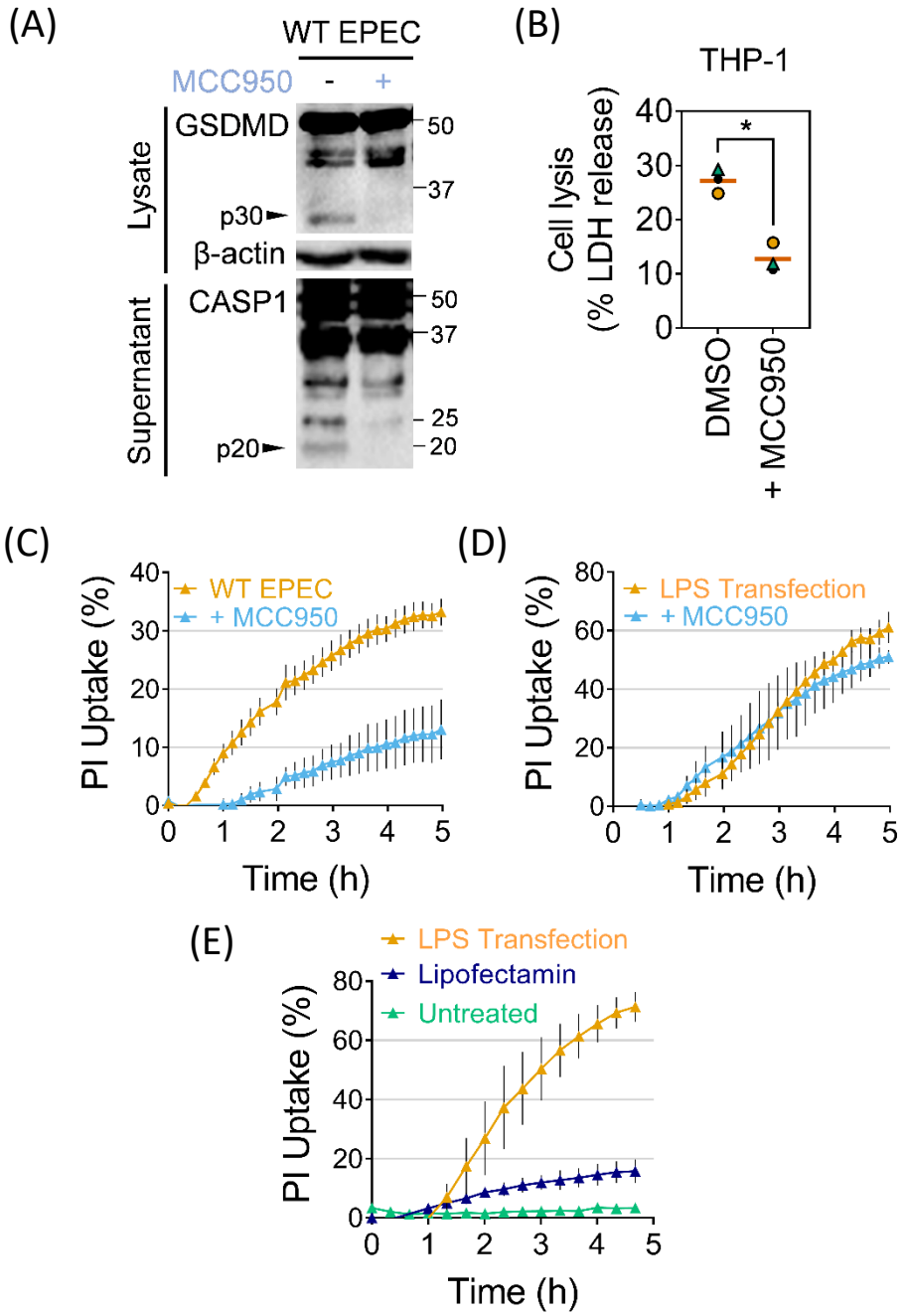

Figure 3.11. EPEC infection induces NLRP3-dependent pyroptosis in THP1 cells

(A) Representative immunoblots from THP1 cells infected with WT EPEC at an MOI of 10 for 4 h, treated with or without MCC950 (5 μM).

(B) Cell lysis measured by LDH release from THP1 cells infected with WT EPEC at an MOI of 10 for 4 h treated with MCC950 (5 μM) or DMSO control. Matching shapes and symbols in graph denote data from independent experiments. Mean indicated by horizontal bar. (*n*= 3 independent repeats)

(C) Real-time PI uptake in THP1 cells infected with EPEC at an MOI of 10 for 5 h, treated with MCC950 (5 μM) or DMSO control. Mean ± SEM plotted from 3 independent experiments.

(D) Real-time PI uptake of THP1 cells treated with MCC950 (5 μM) or DMSO control and transfected with LPS (5 μM) using lipofectamine-2000 for 5 h. Mean ± SEM plotted. (*n= 3* independent experiments).

(E) PI uptake of THP1 cells left untreated, treated with lipofectamine-2000 or transfected with LPS (5 μM) using lipofectamine-2000 for 5 h. Mean ± SEM plotted. (*n= 2* independent experiments).

Immunoblots are representative of experiments performed three times. **P<0.05*, by two-tailed paired Student's *t*-test.

3.9 EPEC induces caspase-4-dependent pyroptosis and caspase-1 activation in primary MDMs

The results presented in this study have established that EPEC infections of human macrophages induce rapid activation of the NLRP3-caspase-1 inflammasome, resulting in pro-inflammatory pyroptosis within 4 h of infection. EPEC-induced pyroptosis was inhibited by either YVAD (Figure 3.3B) or MCC950 (Figure 3.10, 3.11A-C), however, non-canonical caspase-4-dependent pyroptosis induced by cytosolic LPS was not inhibited by MCC950 (Figure 3.11D). These results are consistent with the knowledge that, in response to cytosolic LPS, activated caspase-4 can cleave GSDMD directly to induce pyroptosis, independently of NLRP3 and caspase-1 (445, 498). Taken together, this indicates that in contrast to the caspase-4 non-canonical-inflammasome pathway induced by cytosolic LPS, the NLRP3-ASC-caspase-1 inflammasome is primarily responsible for GSDMD processing and pyroptosis during EPEC infection. However, immunoblots from macrophages infected with WT EPEC suggested proteolytic activation of caspase-4 occurred in both primary MDMs and THP1 cells in a T3SS-dependent manner (Figure 3.7A, 3.8B). This raised the question of whether EPEC infection activated a non-canonical caspase-4-dependent NLRP3 inflammasome, in which caspase-4 induced caspase-1 activation independently of pyroptosis.

Caspase-4 has previously been shown to play an important role in sensing Gram-negative pathogens such as *Legionella* and *Francisella* in primary human MDMs (496, 513). Therefore, the next aim was to establish whether caspase-4 is involved upstream of the NLRP3-ASC-caspase-1 inflammasome during EPEC infection of primary human MDMs. To experimentally verify the role of caspase-4 in primary MDMs, siRNA was utilised to transiently reduce the expression of *CASP4*. Immunoblots of cell lysates confirmed the relative reduction in caspase-4 protein levels after siRNA transduction (Figure 3.12A), and LPS transfection phenotypically verified the knock-down, with caspase-4 silenced cells being resistant to LPS-induced pyroptosis and IL-1β processing (Figure 3.12B). Importantly, silencing of *CASP4* significantly reduced the level of EPEC-induced pyroptosis, as measured by LDH release, compared to CTRL siRNA transfected cells (Figure 3.12C). Caspase-4 was also required for IL-1β processing during EPEC infection of primary MDMs (Figure 3.12C). This suggested that EPEC infection in primary MDMs induced caspase-4-dependent pyroptosis and IL-1β release. These results, in combination with the previously observed requirement for NLRP3-signalling (Figure 3.10), establish that EPEC infection of human macrophages induces a form of rapid pyroptosis that is dependent on both caspase-4 and NLRP3.

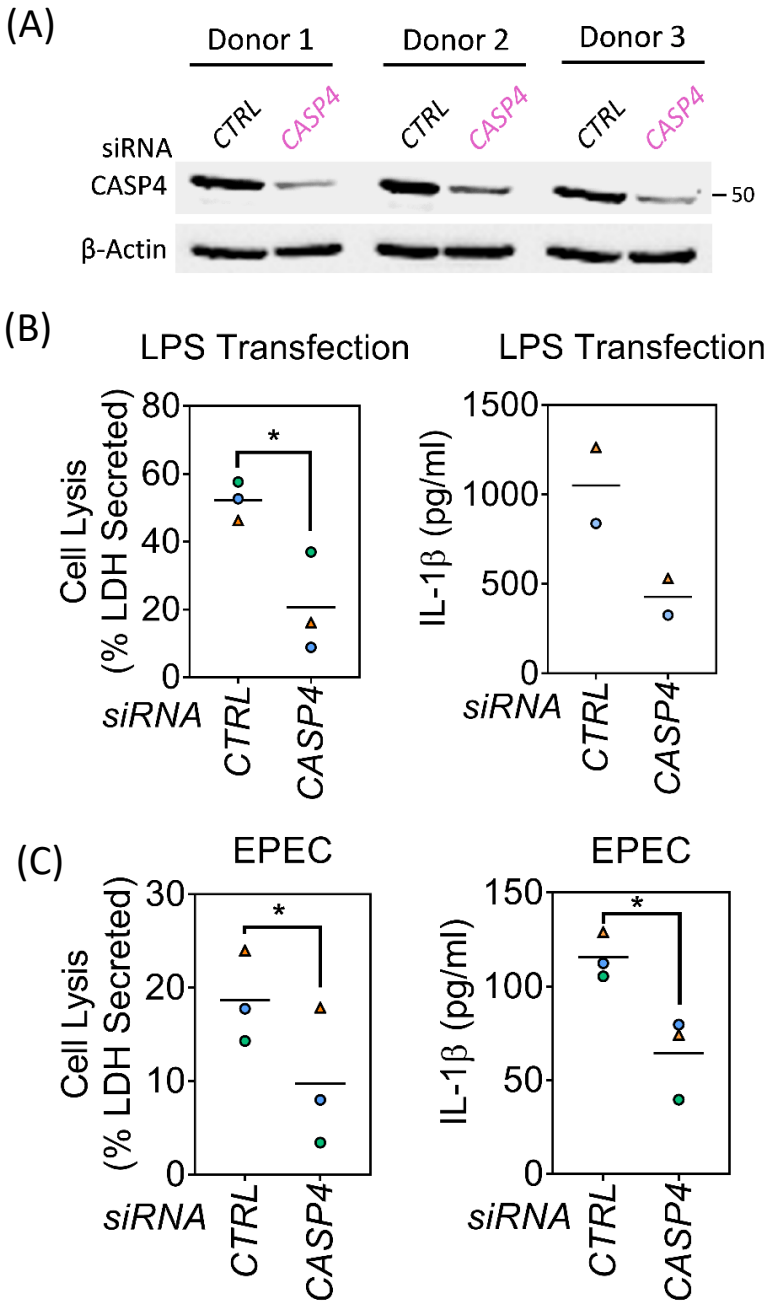

Figure 3.12. EPEC infections induce caspase-4-dependent pyroptosis and caspase-1 activation in primary MDMs

(A) Immunoblots of primary human MDMs from three independent donors transfected with non-targeting (CTRL) or *CASP4* siRNA, showing caspase-4 and β-actin in cell lysates.

(B) Cell lysis as measured by LDH release (Left) and IL-1β as measured by ELISA (Right) from primary MDMs transfected with non-targeting (CTRL) or *CASP4* siRNA, transfected with LPS (5μM) using lipofectamine-2000 for 4 h.

(C) Cell lysis as measured by LDH release (Left) and IL-1β as measured by ELISA (Right) from primary MDMs transfected with non-targeting (CTRL) or *CASP4* siRNA, infected with EPEC at MOI 10 for 4 h.

Matching shapes and colours of symbols in graphs denote data from independent donors. Graphs show mean (horizontal bars), from 2-3 independent repeats. * *P<0.05* by paired Student's *t*-test.

3.10 Stable silencing of inflammasome components in THP1 macrophages

EPEC infection of human macrophages induced a form of rapid pyroptosis that was dependent on both caspase-4 and NLRP3-mediated caspase-1 activation. In order to investigate the host cell signalling cascade responsible for activating pyroptosis in response to EPEC infections, key inflammasome signalling components were selectively silenced in THP1 cells using an optimised miRNA30E short-hairpin RNA (shRNA) strategy, as described previously (607). Expression of caspase-4 (*CASP4*^{miR}), ASC (*ASC*^{miR}) and GSDMD (*GSDMD*^{miR}) was silenced using miRNA30E in THP1 cells, which was demonstrated by the reduced protein expression in cell lysates (Figure 3.13).

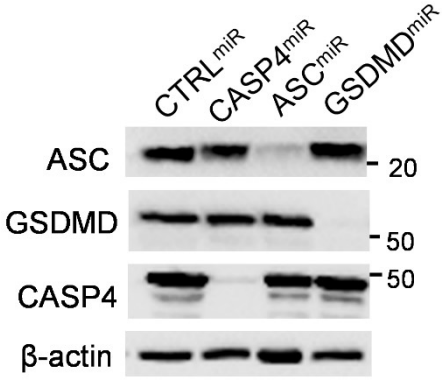

Figure 3.13. Stable silencing of genes using miRNA30E in THP1 cells
(A) Representative immunoblots from THP1 cells stably expressing non-targeting (CTRL) or miRNA30E (miR) against indicated genes showing silencing of protein expression.

3.11 EPEC infection induces GSDMD-dependent pyroptosis in human macrophages

Recently it has been found that cleavage of GSDMD critically determines pyroptosis (445). In fact, cleavage of GSDMD alone is sufficient to drive pyroptosis due to the intrinsic pyroptosis-inducing activity of the cleaved gasdermin N-terminal PDF domain (521). To confirm whether pyroptosis was the predominant form of cell death induced during EPEC infections, CTRL^{miR} and *GSDMD*^{miR} THP1 cells were infected with WT EPEC. As expected, GSDMD knock-down prevented EPEC-induced cell death within the first 5 h of infection (Figure 3.14A-B). Similarly, GSDMD silencing prevented non-canonical inflammasome-dependent pyroptosis in response to LPS transfection (Figure 3.14C-D).

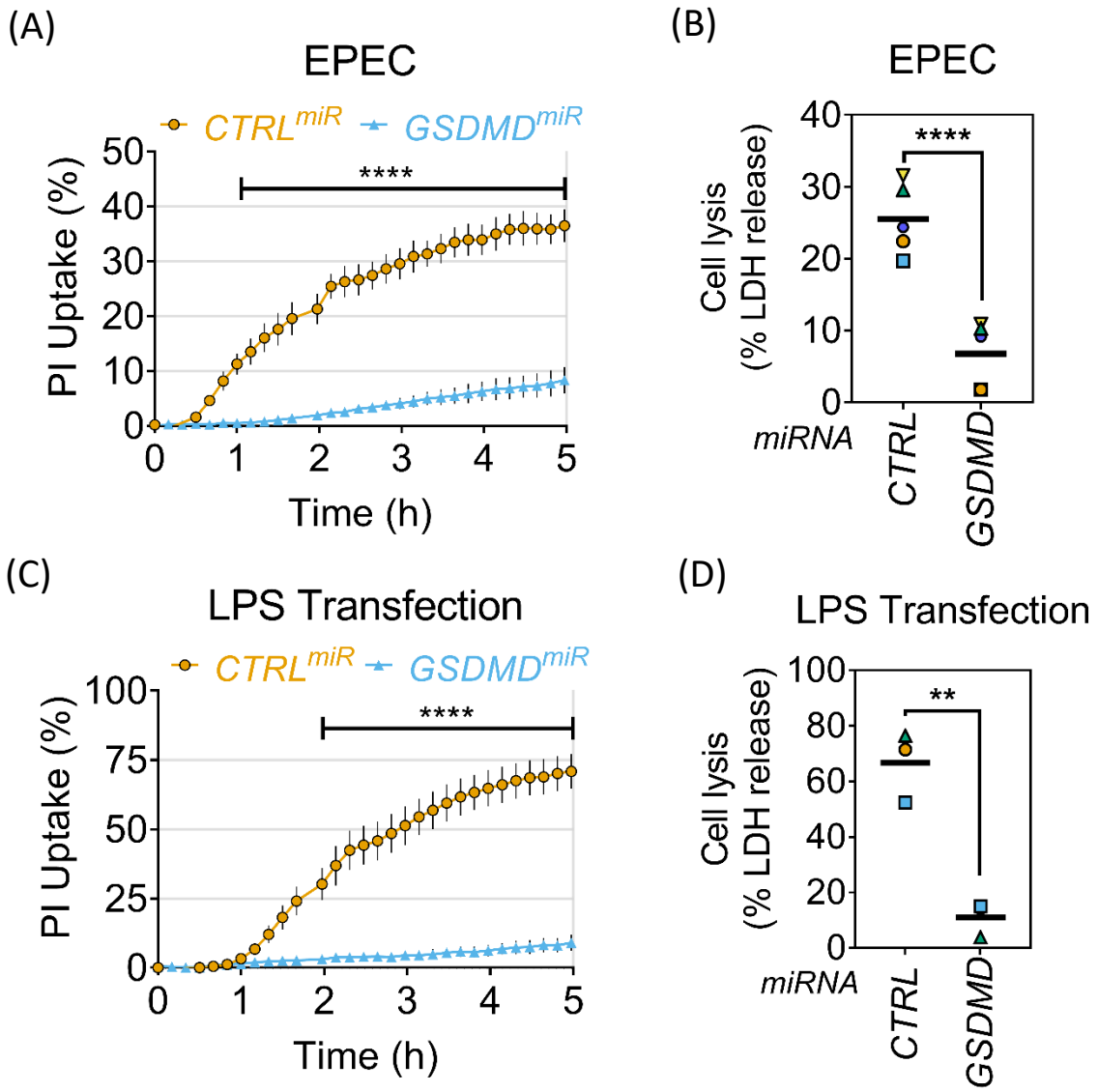

Figure 3.14. EPEC infection induces GSDMD-dependent pyroptosis in THP1 macrophages

(A) Real-time uptake of PI in THP1 cells stably expressing miRNA against GSDMD (*GSDMD*^{miR}) or non-targeting control miRNA (CTRL^{miR}) infected with WT EPEC at an MOI of 10 over 5 h. Graph shows mean ± SEM (*n= 3* independent repeats). **** *P<0.0001* by two-way ANOVA with FDR-based correction for multiple comparisons.

(B) LDH release from THP1 cells stably expressing miRNA against GSDMD (*GSDMD*^{miR}) or non-targeting control miRNA (CTRL^{miR}) infected with WT EPEC at an MOI of 10 for 4 h. (*n = 5* independent repeats)

(C) Real-time uptake of PI in THP1 cells stably expressing miRNA against GSDMD (*GSDMD*^{miR}) or non-targeting control miRNA (CTRL^{miR}), transfected with LPS (5 µg/ml) using lipofectamine-2000. Graph shows mean ± SEM (*n= 3* independent repeats). **** *P<0.0001* by two-way ANOVA with FDR-based correction for multiple comparisons.

(D) LDH release from THP1 cells stably expressing non-targeting (CTRL^{miR}) or miRNA against GSDMD (*GSDMD*^{miR}), transfected with LPS (5 µg/ml) using lipofectamine-2000. Data represents mean ± SEM from 3 independent experiments.

Matching shapes and colours of symbols in graphs in (B and D) denote data from independent experiments, horizontal lines represent mean. ** *P<0.01,* **** *P<0.0001* by paired Students *t*-test.

3.12 EPEC infection induces ASC-dependent pyroptosis in human macrophages

NLRP3-dependent caspase-1 activation by canonical stimuli requires the adaptor protein ASC (634). ASC plays a critical role in the activation and assembly of most inflammasomes, and is essential for signalling by PYD-containing receptors, including NLRP3 (AIM2 and Pyrin) (660). Conversely, although non-canonical caspase-4 activation also induces lysis of the host cell, caspase-4-dependent pyroptosis has previously been shown to occur independently of ASC (123, 496, 497). Therefore, the requirement for ASC in pyroptosis differs depending on the inflammasome activation pathways. To further characterise the mechanism of EPEC-induced pyroptosis, *ASC*^{miR} THP1 cells were infected with EPEC for 4 h and cell death was monitored by PI uptake and LDH release (Figure 3.15). Interestingly, ASC silencing markedly inhibited EPEC-induced pyroptosis (Figure 3.15A-B), whereas pyroptosis induced by LPS-transfection, as a positive control for the non-canonical caspase-4 inflammasome, occurred independently of the ASC adaptor (Figure 3.15C-D). These results establish that EPEC infections induce ASC-dependent pyroptosis in human macrophages. This is consistent with the requirement for NLRP3, as shown previously, confirming that EPEC-induced pyroptosis requires ASC-NLRP3 signalling.

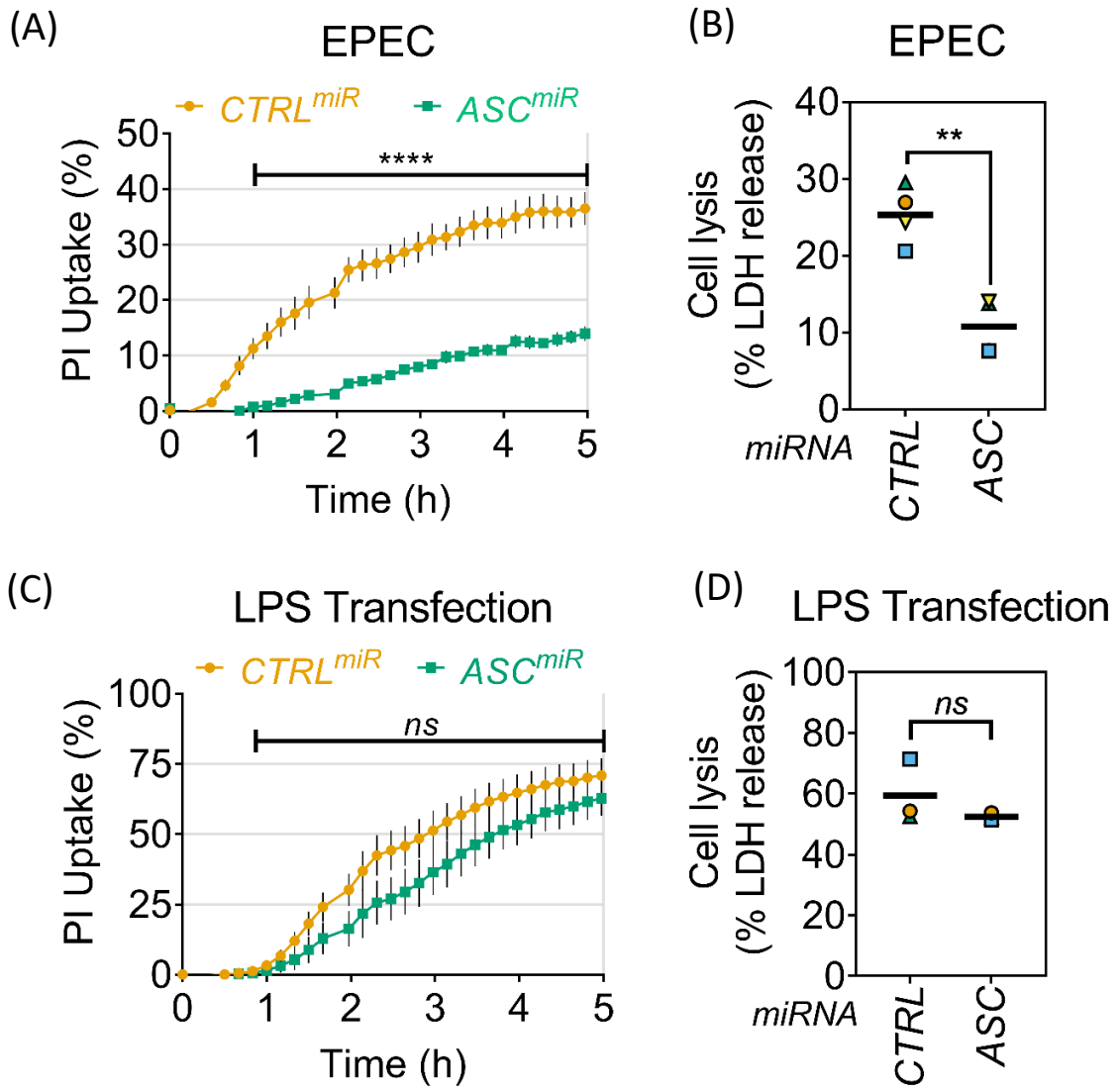

Figure 3.15. EPEC infections induce ASC-dependent pyroptosis in THP1 macrophages

(A) Real-time uptake of PI in THP1 cells stably expressing miRNA against ASC (ASC^{miR}) or non-targeting control miRNA ($CTRL^{miR}$) infected with WT EPEC at an MOI of 10 over a 5 h infection. Graph shows mean ± SEM (*n= 3* independent repeats). **** *P<0.0001* by two-way ANOVA with FDR-based correction for multiple comparisons.

(B) LDH release from THP1 cells stably expressing miRNA against ASC (ASC^{miR}) or non-targeting ($CTRL^{miR}$) miRNA, infected with WT EPEC at an MOI of 10 for 4 h.

(C) Real-time PI uptake in THP1 cells stably expressing miRNA against ASC (ASC^{miR}) or non-targeting ($CTRL^{miR}$) miRNA, transfected with LPS (5 µg/ml) using lipofectamine-2000. Graph shows mean ± SEM (*n= 3* independent repeats).

(D) LDH release from THP1 cells stably expressing non-targeting ($CTRL^{miR}$) or miRNA against ASC (ASC^{miR}), transfected with LPS (5 µg/ml) using lipofectamine-2000. Data represents mean ± SEM from 3 independent experiments.

Matching shapes and colours of symbols in graphs in (B and D) denote data from independent experiments, horizontal lines represent mean. ** *P<0.01* by paired Students *t*-test. *ns*= non-significant.

3.13 EPEC infection induces caspase-4-dependent pyroptosis in human macrophages

The observation that EPEC-induced pyroptosis was inhibited by either the NLRP3 inhibitor MCC950 or genetic silencing of ASC, suggested that EPEC infection activated an ASC-NLRP3-CASP1 inflammasome. However, immunoblot analysis suggested proteolytic activation of caspase-4 occurred in response to EPEC infection (Figure 3.7A, 3.8B), and experiments in MDMs identified a role for caspase-4 in EPEC-induced pyroptosis (Figure 3.12). Therefore, to determine the contribution of caspase-4 to THP1 cell death, $CTRL^{miR}$ and $CASP4^{miR}$ THP1 cells were infected with EPEC, or transfected with LPS as a control for the caspase-4 non-canonical inflammasome. Caspase-4 silencing was phenotypically validated as $CASP4^{miR}$ THP1 cells were resistant to pyroptosis induced by cytosolic LPS (Figure 3.16 C-D). Similarly to MDMs, stable silencing of caspase-4 prevented EPEC-induced pyroptosis in THP1 cells (Figure 3.16 A-B).

Collectively, these results demonstrated that EPEC infection of human macrophages induced a form of rapid pyroptosis that was dependent on caspase-4, ASC, GSDMD and NLRP3. The requirement for both caspase-4 and ASC in EPEC-mediated pyroptosis was surprising, as caspase-4-dependent pyroptosis typically occur independently on ASC. Therefore, to further investigate the signalling cascade that occurred upstream of EPEC-induced pyroptosis western blot analysis was used to establish the of hierarchy of protein activation. Utilising miRNA30E knock-down THP1 cells, the mechanism of inflammasome activation in response to EPEC infection could be systematically studied. However, initially, it was important to verify that these cell lines responded normally to canonical and non-canonical NLRP3-inflammasome agonists.

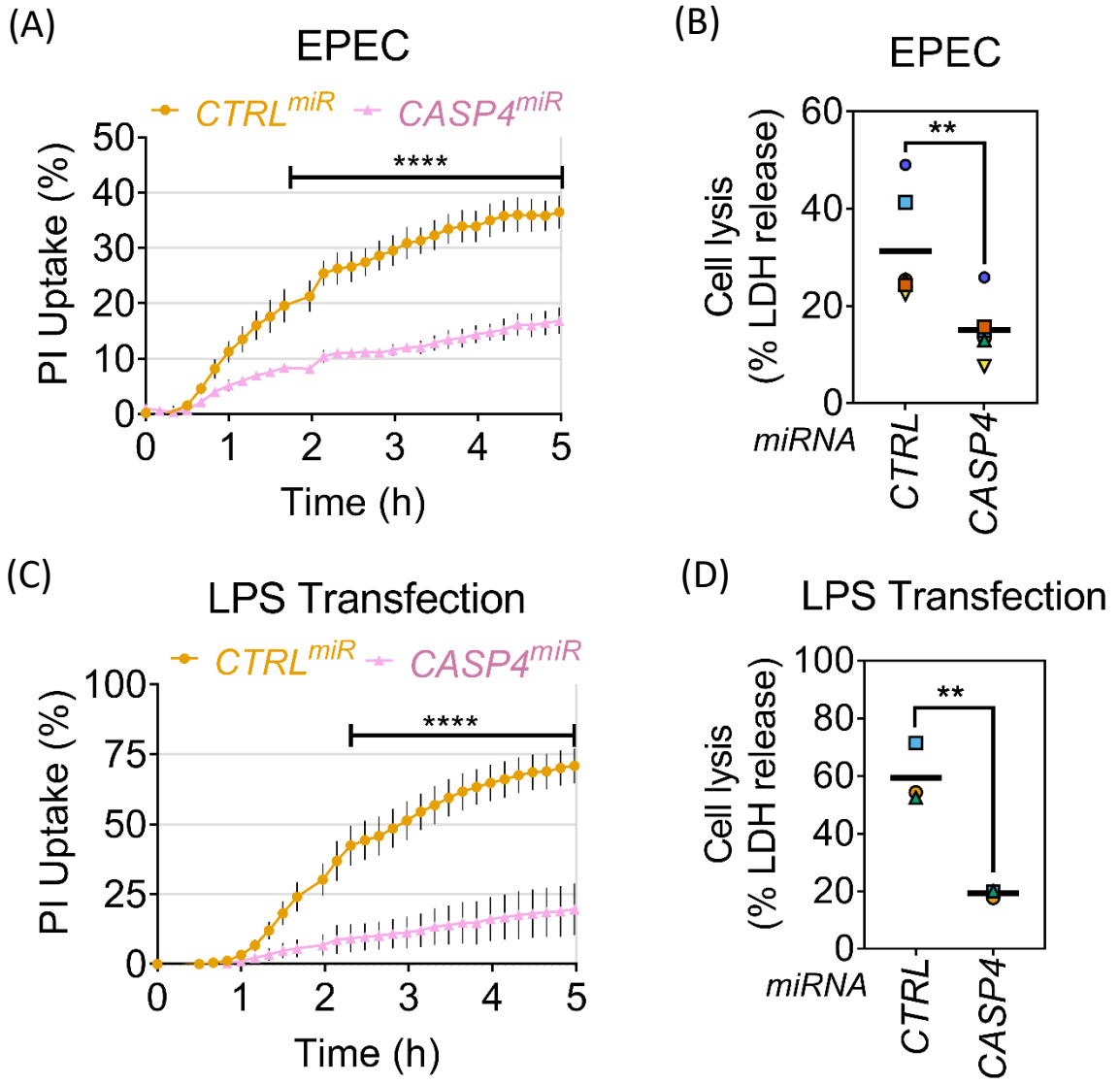

Figure 3.16. EPEC infection induces caspase-4-dependent pyroptosis in THP1 cells

(A) Real-time uptake of PI in THP1 cells stably expressing miRNA against caspase-4 (*CASP4*^{miR}) or non-targeting (CTRL^{miR}) miRNA, infected with WT EPEC at an MOI of 10. Graph shows mean ± SEM (*n= 3* independent repeats). **** *P<0.0001* by two-way ANOVA with FDR-based correction for multiple comparisons.

(B) LDH release from THP1 cells stably expressing miRNA against caspase-4 (*CASP4*^{miR}) or non-targeting (CTRL^{miR}) miRNA, infected with WT EPEC at an MOI of 10 for 4 h.

(C) Real-time uptake of PI in THP1 cells stably expressing miRNA30E against caspase-4 (*CASP4*^{miR}) or non-targeting (CTRL^{miR}) miRNA, transfected with LPS (5 µg/ml) using lipofectamine-2000. Graph shows mean ± SEM (*n= 3* independent repeats). **** *P<0.0001* by two-way ANOVA with FDR-based correction for multiple comparisons.

(D) LDH release from THP1 cells stably expressing non-targeting (CTRL^{miR}) or miRNA against caspase-4 (*CASP4*^{miR}), transfected with LPS (5 µg/ml) using lipofectamine-2000. Data represents mean ± SEM from 3 independent experiments.

Matching shapes and colours of symbols in graphs in (B and D) denote data from independent experiments, horizontal lines represent mean. ** *P<0.01* paired by Students *t*-test.

3.14 GSDMD is required for secretion of IL-1β and the caspase-1 (p20) domain upon inflammasome activation

Potassium ions (K^+) efflux from cells has previously been identified as the universal activating signal for canonical-NLRP3 inflammasome activation (490, 661). Nigericin is a lipophilic ionophore that functions as a K^+/H^+ exchanger and induces rapid K^+ efflux from cells. K^+ efflux causes NLRP3 monomers to oligomerise and interact with the pyrin domain (PYD) of ASC. The adaptor ASC then recruits the pro-caspase-1 via its CARD domain, enabling autocatalysis and activation of caspase-1 (Figure 3.17A).

To characterise the effect of *GSDMD*-silencing on canonical NLRP3 inflammasome activation, PMA differentiated $CTRL^{miR}$ and $GSDMD^{miR}$ THP1 cells were stimulated with LPS for 3 h to induce transcription of NLRP3 and related pro-inflammatory cytokines, then treated with nigericin (10 μM) for 45 min. Immunoblot analysis of cell lysates and supernatants demonstrated that canonical NLRP3 activation induced caspase-1 cleavage and IL-1β processing in both $CTRL^{miR}$ and $GSDMD^{miR}$ THP1 cells. However, although $GSDMD^{miR}$ cells were able to activate caspase-1 in response to canonical stimuli, unlike $CTRL^{miR}$ cells in which processed caspase-1 (p20) and IL-1β were secreted from cells into the extracellular supernatants (Figure 3.17B), in $GSDMD^{miR}$ THP1 these active fragments were retained within the cells, and could be seen within the cell lysate fraction of immunoblots (Figure 3.17B). Importantly, the level of caspase-1 activation was similar between $CTRL^{miR}$ and $GSDMD^{miR}$ THP1 cells, as demonstrated by immunoblots of pooled cell fractions where cell supernatants and lysates were combined (Figure 3.17C). These results demonstrated that caspase-1 activation downstream of the canonical NLRP3 inflammasome occurred independently of GSDMD. However, GSDMD was required for secretion of the caspase-1 p20 domain and pro-inflammatory cytokines into the cell supernatant.

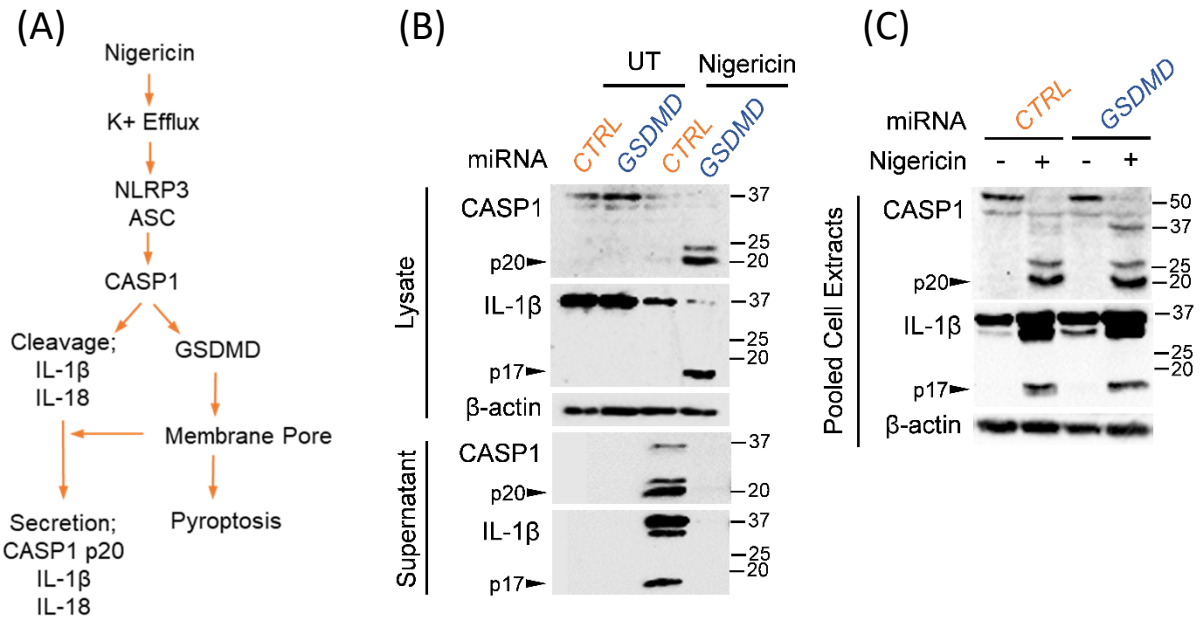

Figure 3.17. GSDMD is required for the secretion of proinflammatory cytokines and caspase-1 p20 domain

(A) Schematic representation of the canonical NLRP3-inflammasome pathway induced by nigericin treatment.

(B) Representative western blots from THP1 cells stably expressing miRNA against GSDMD (*GSDMD*^{miR}) or non-targeting miRNA (CTRL^{miR}), primed with LPS (250 ng/ml) for 3 h, then either left untreated (UT) or treated with Nigericin (10 μM) for 45 min. Immunoblots show caspase-1, and IL-1β in culture supernatant and caspase-1, IL-1β and β-actin in cell lysates.

(C) Representative immunoblots of pooled cells extracts from THP1 cells stably expressing non-targeting (CTRL^{miR}) or miRNA against GSDMD (*GSDMD*^{miR}), primed with LPS (250 ng/ml) for 3 h, then either left untreated (-) or treated with Nigericin (+) for 45 min. Blots show caspase-1, IL-1β and β-actin in pooled cell extracts. Blots are representative of two independent repeats.

3.15 Canonical NLRP3 inflammasome activation requires ASC but not caspase-4 or GSDMD in THP1 cells

Nigericin-induced activation of the canonical NLRP3 inflammasome has been extensively characterised, and previous research has consistently demonstrated that activation is dependent on the ASC adaptor protein, but occurs independently of caspase-4 or GSDMD (662-665). Therefore, to validate the inflammasome responses of the newly synthesised miRNA30E silenced *ASC*^{miR} and *CASP4*^{miR} THP1 cells, canonical inflammasome activation was induced using nigericin. As expected, stable silencing of ASC in THP1 cells (*ASC*^{miR}) prevented nigericin-induced caspase-1 activation and downstream GSDMD cleavage and cytokine processing (Figure 3.18A). Similarly, NLRP3-dependent activation of caspase-1 was inhibited by extracellular potassium chloride (KCl), as this prevents the efflux of K^+ from cells (Figure 3.18B). However, silencing of caspase-4 (*CASP4*^{miR}) had no impact on nigericin-induced caspase-1 activation (Figure 3.18B). Collectively, these results

not only confirmed the previously described canonical NLRP3 pathway, but more importantly phenotypically characterised the miRNA30E expressing THP1 knock-down lines.

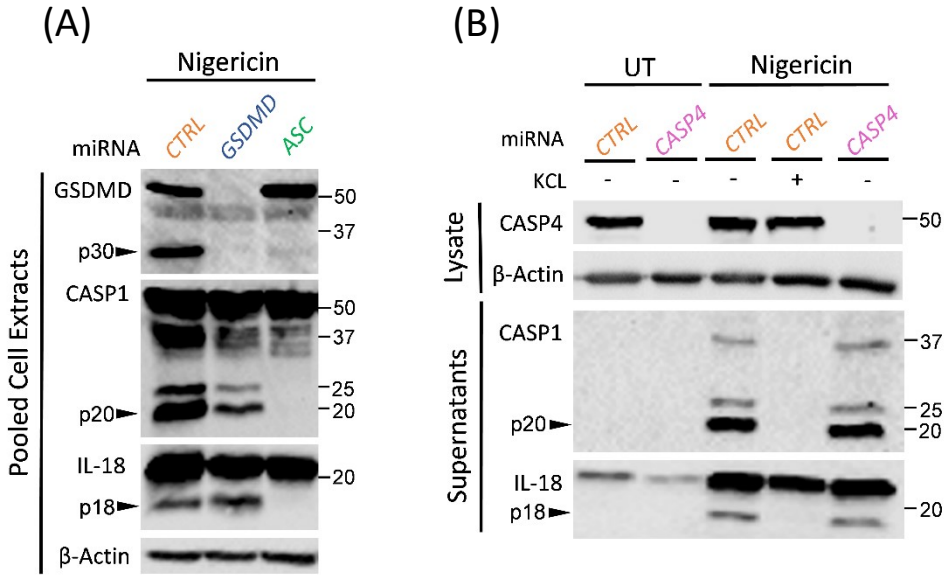

Figure 3.18. Canonical NLRP3 inflammasome activation requires ASC, but not caspase-4 or GSDMD

(A) Representative western blots from THP1 cells stably expressing non-targeting (CTRL^{miR}) or miRNA against indicated genes, primed with LPS (250 ng/ml) for 3 h, then treated with Nigericin (10 μM) for 45 min. Immunoblots for GSDMD, caspase-1, and IL-18 in pooled cell extracts. Blots are representative of two independent repeats.

(B) Representative western blots from THP1 cells stably expressing non-targeting (CTRL^{miR}) or miRNA against indicated genes primed with LPS (250 ng/ml) for 3 h, then either left untreated (UT) or treated with nigericin (10 μM) for 45 min. Where indicated cells were incubated with KCl (20 mM) 30 min prior to treatment. Immunoblots for caspase-1 and IL-18 in the cell supernatant and caspase-4 and IL-1β in cell lysates. Data is representative of three independent repeats.

3.16 Non-canonical NLRP3-driven caspase-1 activation requires both caspase-4 and GSDMD

The work in this study has confirmed the requirement for both caspase-4 and GSDMD in membrane damage induced by LPS transfection (Figure 3.12, 3.114C-D, 3.16C-D). It has previously been reported that in the non-canonical inflammasome pathway, GSDMD functions downstream of caspase-4 to induce caspase-1 activation (123). Cytosolic LPS induces non-canonical NLRP3-inflammasome activation in THP1 macrophages (501), therefore to test this pathway, and experimentally validate the CTRL^{miR}, *CASP4*^{miR}, *ASC*^{miR} and *GSDMD*^{miR} THP1 cells, LPS was transfected into miRNA30E expressing THP1 cell lines, and cell extracts were analysed by immunoblotting (Figure 3.19).

LPS transfection in CTRL^{miR} THP1 cells induced activation of caspase-4 and the subsequent cleavage and activation of caspase-1 and GSDMD (Figure 3.19B). As caspase-4 functions as the receptor for cytosolic LPS, silencing of caspase-4 (*CASP4*^{miR}) prevented both caspase-1 activation and GSDMD cleavage upon LPS transfection. ASC silencing however, did not prevent GSDMD cleavage in response to cytosolic LPS, but did inhibit caspase-1 activation (Figure 3.19B). GSDMD silencing prevented caspase-1 activation in response to cytosolic LPS (Figure 3.19A), confirming that GSDMD functioned downstream of caspase-4, and played a fundamental role in both the induction of pyroptosis and the activation of caspase-1 during the caspase-4 non-canonical inflammasome response in THP1 macrophages.

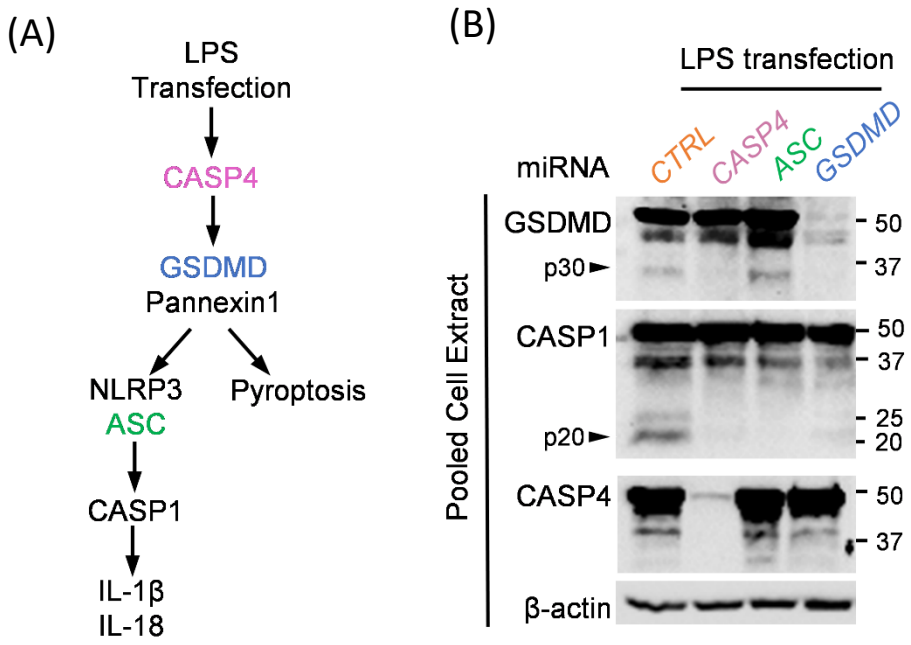

Figure 3.19. Non-canonical NLRP3-caspase-1 inflammasome activation requires caspase-4 and GSDMD

(A) Schematic representation of the non-canonical NLRP3 pathway induced by LPS transfection.
(B) Immunoblots from THP1 cells stably expressing non-targeting (CTRL^{miR}) or miRNA against indicated genes transfected with LPS (5 µg/mL) using Lipofectamine-2000 for 4 h. Blots show GSDMD, caspase-1, and caspase-4 in pooled cell extracts. Data is representative of three independent repeats.

3.17 EPEC infection activates caspase-4-dependent non-canonical NLRP3-inflammasome signalling in human macrophages

During EPEC infections of human macrophages expression of both caspase-4 and ASC are required to mediated pyroptosis (Figure 3.12, 3.15, 3.16). This distinguished EPEC-induced pyroptosis from the typical non-canonical or canonical NLRP3 activation pathways. The next aim was to establish the role of GSDMD, ASC and caspase-4 in inflammasome signalling during EPEC infections. Cell death analysis of EPEC infections revealed that stable silencing of GSDMD (*GSDMD*^{miR}) attenuated pyroptosis induced by EPEC (Figure 3.14), this formally validated that EPEC induced GSDMD-dependent pyroptosis in human macrophages. However, whether GSDMD also played a role upstream of EPEC-induced NLRP3 inflammasome activation remained to be established. Immunoblot analysis of EPEC infection of *GSDMD*^{miR} THP1 cells demonstrated that GSDMD was also required for caspase-1 activation (Figure 3.20B). This pointed to a GSDMD-dependent non-canonical NLRP3 activation pathways during EPEC infection, as GSDMD was not required for caspase-1 activation induced by canonical NLRP3 stimuli in these cells (Figure 3.18A). Interestingly,

EPEC infection in *ASC*^{miR} THP1 cells demonstrated that ASC was also required for both EPEC-induced caspase-1 activation and GSDMD cleavage (Figure 3.20B), verifying the previously observed requirement for ASC in EPEC-induced pyroptosis (Figure 3.15). Immunoblot analysis from pooled cell extracts of caspase-4 silenced THP1 cells infected with EPEC confirmed that caspase-4 was required for both GSDMD cleavage (p30) and capase-1 activation (p20) during EPEC infection (Figure 3.20C). This suggested that caspase-4 orchestrated GSDMD- and ASC-dependent caspase-1 activation (Figure 3.20A). Together, these results suggest that EPEC infections activate a rapid atypical inflammasome signalling pathway in human macrophages, in which caspase-4 is required for caspase-1 activation but is not sufficient for pyroptosis. This non-canonical caspase-4-mediated activation of caspase-1 was dependent on GSDMD, however, in this context caspase-4 activation was not sufficient to induce GSDMD cleavage or pyroptosis, as ASC silencing prevented the cleavage of GSDMD and the release of the PFD (p30) domain (Figure 3.20B), thus ASC-caspase-1 signalling was responsible for GSDMD cleavage during EPEC infection.

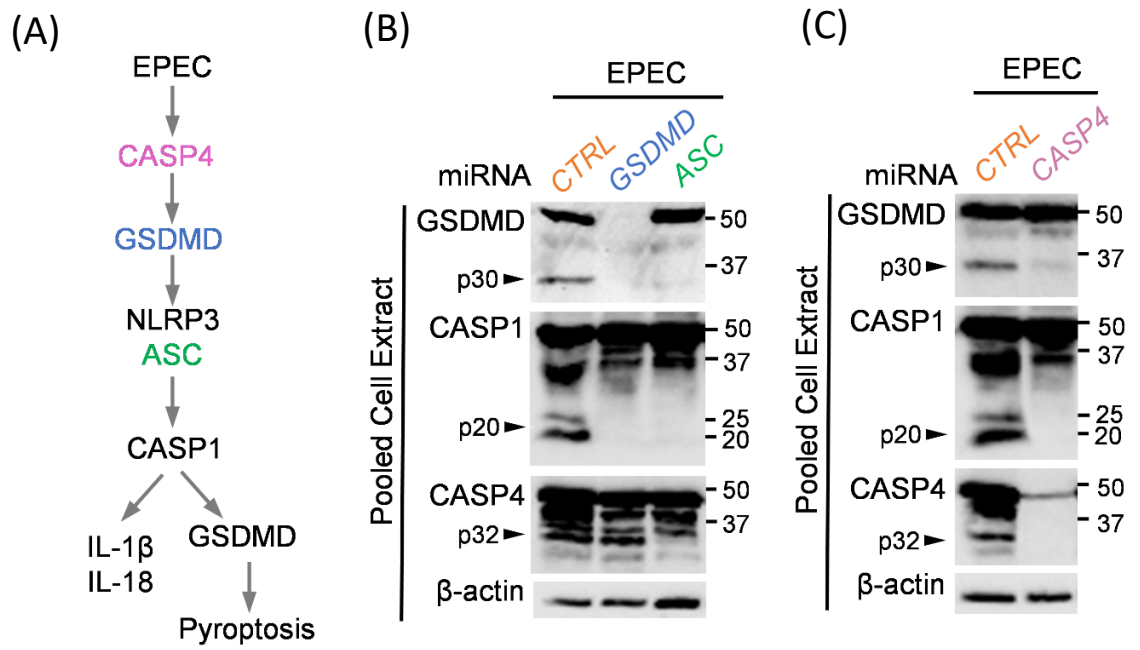

Figure 3.20. EPEC infection induces a caspase-4-dependent non-canonical inflammasome in human macrophages

(A) Schematic representation of EPEC-induced inflammasome activation of the non-canonical NLRP3 inflammasome.

(B) Representative immuneblots from THP1 cells stably expressing non-targeting (CTRL^{miR}) or miRNA against GSDMD (*GSDMD*^{miR}) or ASC (*ASC*^{miR}), infected with EPEC at an MOI of 10 for 4 h. Immunoblots for GSDMD, caspase-1, caspase-4 and β-actin in pooled cell extracts are shown. Data is representative of three independent repeats.

(C) Representative western blots from THP1 cells stably expressing non-targeting (CTRL^{miR}) or miRNA against CASP4 (*CASP4*^{miR}) infected with EPEC at an MOI of 10 for 4 h. Immunoblots for GSDMD, caspase-1, caspase-4 and β-actin in pooled cell extracts are shown. Data is representative of three independent repeats.

3.18 Caspase-4 silencing does not inhibit NF-κB signalling in THP1 macrophages

During infection, expression of some NLRs/ALRs, and pro-IL-1β are transcriptionally induced by upstream sensory pathways, meaning ineffective priming of cells can result in low expression of these inflammasome signalling components dampening inflammatory responses. Pro-IL-1β and NLRP3 are robustly induced by proinflammatory ligands such as LPS or TNF that activate NF-κB signalling and promoter activation (494, 666). Caspase-4 has previously been reported to function in NF-κB-dependent signalling pathways (667). This study suggested LPS-sensing led to caspase-4-dependent NF-kB transcriptional up-regulation and secretion of cytokines including IL-1β. Therefore, *CASP4*^{miR} THP1 cells were tested for signalling defects using a range of chemical agonists to characterise alterations in receptor-mediated signalling compared to that of the non-targeting control (CTRL^{miR}) THP1 cells (Figure 3.21A). Interestingly, immunoblots for pro-IL-1β in cell lysates demonstrated that caspase-4 functioned downstream of NOD1 and NOD2 signalling pathways, as deficiencies in γ-D-glutamylmesodiaminopimelic acid (IE-DAP) and muramyl dipeptide (MDP) induced IL-1β transcription were evident in the *CASP4*^{miR} cells. However, TLR-induced NF-κB nuclear translocation and activation were not inhibited in PAM (TLR-1/2) or LPS (TLR4) primed cells, as the levels of pro-IL-1β in the cell lysates upon TLR induction were unaffected by caspase-4 silencing (Figure 3.21A). NOD1 and NOD2 are intracellular surveillance proteins that detect bacterial peptidoglycan; NOD1 and NOD2 respond to iE-DAP and MDP respectively. Upon activation, assembly of NOD1 and NOD2 signalosomes ultimately culminates in the activation of the NF-κB transcription factor, which drives proinflammatory gene regulation (668). However, there is no current evidence for NOD1 or NOD2 signalling in caspase-4 activation. These pathways are likely to be dispensable in EPEC-induced NF-κB signalling and caspase-4 activation, because EPEC is primarily an extracellular pathogen that stimulates cell-surface receptors such as TLRs to induce NF-κB transcriptional induction. Upon infection, bacterial PAMPs including LPS, flagellin, lipoproteins, and CpG DNA stimulate host cell TLRs, leading to a broad immune response via the activation of NF-κB (669-671). Indeed, when very low concentrations of LPS were used in conjunction with NOD1 and 2 agonists, there was no evident defect in NF-κB signalling in caspase-4 silenced cells (Figure 3.21A). Furthermore, upon treatment with extracellular LPS from *E. coli* O111:B4, CTRL^{miR} and *CASP4*^{miR} THP1 cells triggered equivalent transcriptional induction of the core inflammasome components NLRP3 and ASC (Figure 3.21B). These results suggest that the inhibition of inflammasome responses

in caspase-4 silenced cells can not be attributed to inefficient priming, but instead that caspase-4 plays a fundamental role within the inflammasome activation pathway.

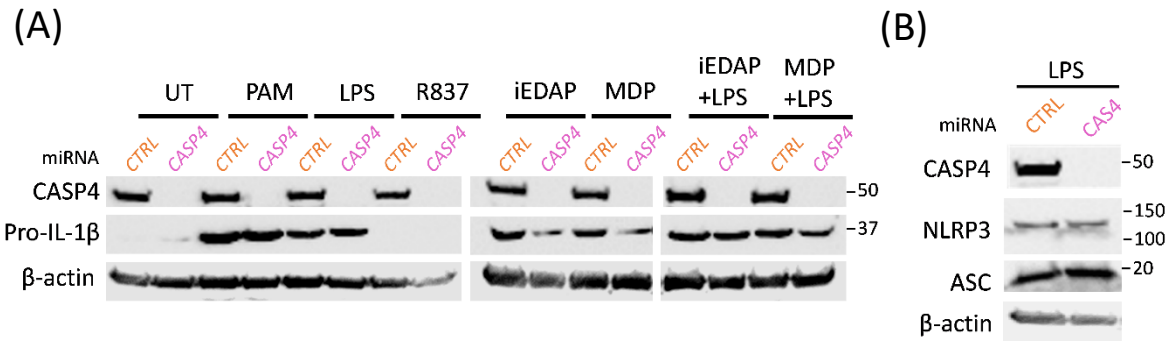

Figure 3.21. Caspase-4 silencing does not inhibit Toll-like receptor induced IL-1β transcription

(A) Representative immunoblots from THP1 cells expressing non-targeting miRNA (CTRL^{miR}) or caspase-4 miRNA (*CASP4*^{miR}), primed with chemical agonists; Pam3CSK4 (PAM 100 ng/ml), LPS (1 µg/ml), R837 (5 µg/ml), IE-DAP (1 µg/ml), MDP (1 µg/ml), IE-DAP+LPS (1 µg/ml + 5 ng/ml respectively) or MDP+LPS (1 µg/ml + 5 ng/ml respectively) for 4 h. Cell lysates were collected and analysed for indicated proteins by immunoblotting. Blots are representative of two independent repeats,

(B) Western blots from THP1 cells stably expressing non-targeting (CTRL^{miR}) or miRNA against CASP4 (*CASP4*^{miR}) were treated with LPS (250 ng/ml) 3 h. Blots for caspase-4, NLRP3, ASC and β-actin in the cell lysates. Blots are representative of three independent repeats.

3.19 Transient knock-down of ASC and caspase-4 prevents EPEC-induced pyroptosis and inflammasome activation in THP1 macrophages

Collectively the results presented so far have suggested that EPEC infection provokes rapid ASC- and caspase-4-dependent inflammasome activation and pyroptosis in human macrophages. Thus far, stably transduced THP1 cell lines expressing optimised shRNA against *ASC* and *CASP4* have been utilised to characterise this pathway. However, shRNA sequences have previously been reported to occasionally mediate off-target effects. Although these effects are typically milder than those on the target gene, some adverse off-targeting events can significantly alter the results of experiments and even contribute to cellular toxicity (672). Therefore, in order to validate the result here, which identified a role for both caspase-4 and ASC in EPEC-induced pyroptosis, transient knock-down of caspase-4 and ASC were induced utilising independent siRNA oligonucleotides to supress expression of the target genes and independently validate these findings.

Silencing of target genes was visualised by immunoblots of cell lysates (Figure 3.22A). siRNA treated THP1 cells were infected with EPEC at an MOI of 10 for 4 h and pyroptosis was measured by LDH

release. In agreement with the results for miRNA-expressing cells, transient silencing of *CASP4* and *ASC* with siRNA also attenuated pyroptosis induced by EPEC (Figure 3.22A). As expected, pyroptosis triggered by transfected LPS was caspase-4-dependent but ASC-independent (Figure 3.22B), whereas pyroptosis induced by nigericin treatment was ASC-dependent but caspase-4-independent (Figure 3.22C). Independent validation for inflammasome activation was obtained by quantifying IL-1β release as measured by ELISA from EPEC-infected *CASP4* and *ASC* siRNA silenced macrophages (Figure 3.23). These results confirmed that both caspase-4 and ASC were required for IL-1β processing in response to EPEC infection (Figure 3.23A). However, nigericin induced canonical ASC-NLRP3-CASP1 processing of IL-1β occurred independently of caspase-4 (Figure 3.23B).

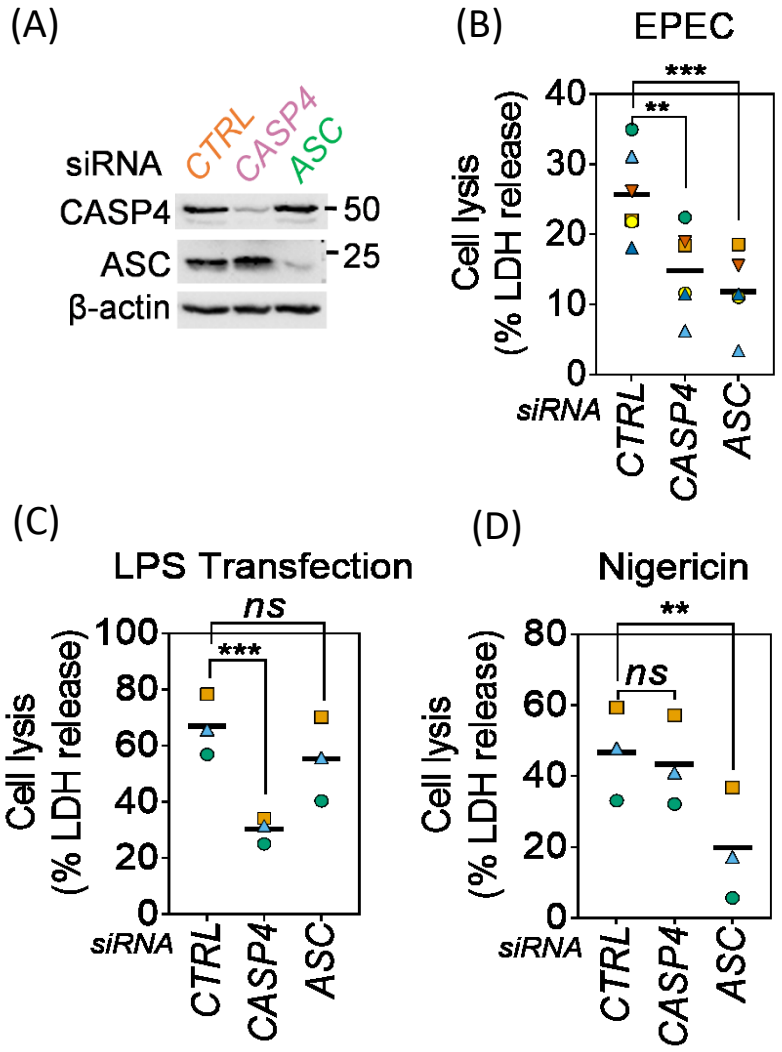

Figure 3.22. siRNA knock-down of caspase-4 and ASC prevents EPEC-induced pyroptosis

(A) Representative immunoblot of cell lysates from THP1 cells transfected with non-targeting (CTRL) or indicated siRNAs showing silencing of protein expression. Blots are representative of 4 independent repeats.

(B) LDH release from THP1 cells transfected with non-targeting (CTRL) or indicated siRNA and infected with EPEC at an MOI of 10 for 4 h. (*n = 6* independent repeats)

(C) Cell lysis measured by LDH release from THP1 cells transfected with non-targeting (CTRL) siRNA or siRNA targeting caspase-4 (*CASP4)* or ASC *(ASC)*, transfected with LPS (5 µg/ml) using Lipofectamine-2000 for 4 h. (*n = 3* independent repeats)

(D) Cell lysis measured by LDH release from THP1 cells transfected with indicated siRNAs, primed for with LPS for 3 h (250 ng/ml) and then treated with nigericin (10 µM) for 45 min. (*n = 3* independent repeats)

Matching shapes and colours of symbols in graphs denote data from independent experiments, horizontal lines represent mean. *** *P<0.001,* ** *P<0.01* by one-way ANOVA. *ns*= non-significant.

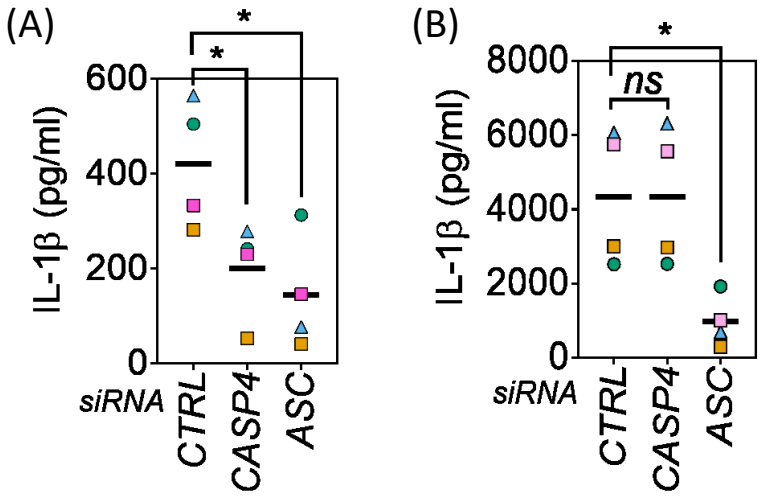

Figure 3.23. siRNA knock-down of caspase-4 and ASC prevents EPEC-induced IL-1β processing
Quantification of IL-1β secretion by ELISA from THP1 cells transfected with non-targeting (CTRL) or indicated siRNAs, infected with EPEC for 4 h (A), or primed for with LPS for 3 h (250 ng) and then treated with nigericin (10 μM) for 45 min (B). Matching shapes and colours of symbols in graphs denote data from independent experiments (*n = 3-4* independent repeats). * *P<0.5* by one-way ANOVA. *ns*= non-significant.

3.20 EPEC-induced pyroptosis occurs independently of caspase-8

Recently a number of independent studies have identified a critical role for caspase-8 within the non-canonical and canonical NLRP inflammasome activation pathway. Caspase-8 has been shown to play a role in both the transcriptional priming and post-translational activation of the NLRP3 inflammasome (543). Caspase-8 can also be recruited to inflammasome complexes in response to *Salmonella* infections, where it is activated by NLRC4 and ASC (544). Similarly, in epithelial cells NLRP3 and ASC have been shown to form a conserved non-canonical platform for caspase-8 activation, independently of caspase-1, that regulates apoptosis (673).

In resting cells, caspase-8 suppresses RIPK3-RIPK1-dependent necroptosis, by restricting RIPK1 and RIPK3 activities. For this reason, disruption of caspase-8 expression has been shown to cause embryonic death in mice (674). Therefore, stable silencing of caspase-8 in cells was not possible, however, transient silencing of caspase-8 in THP1 cells was achieved using siRNA transfection (Figure 3.24A). To establish whether caspase-8 also functioned within the unconventional inflammasome pathway stimulated by EPEC, THP1 cells were transfected with *CASP8* siRNA and infected with EPEC. Silencing of caspase-8 did not affect EPEC-induced pyroptosis (Figure 3.24B).

Similarly, caspase-8 silencing did not inhibit cell death induced by LPS-transfection (Figure 3.24C). Together these results indicate that caspase-8 does not play a significant role in either LPS- or EPEC-induced pyroptosis in THP1 macrophages.

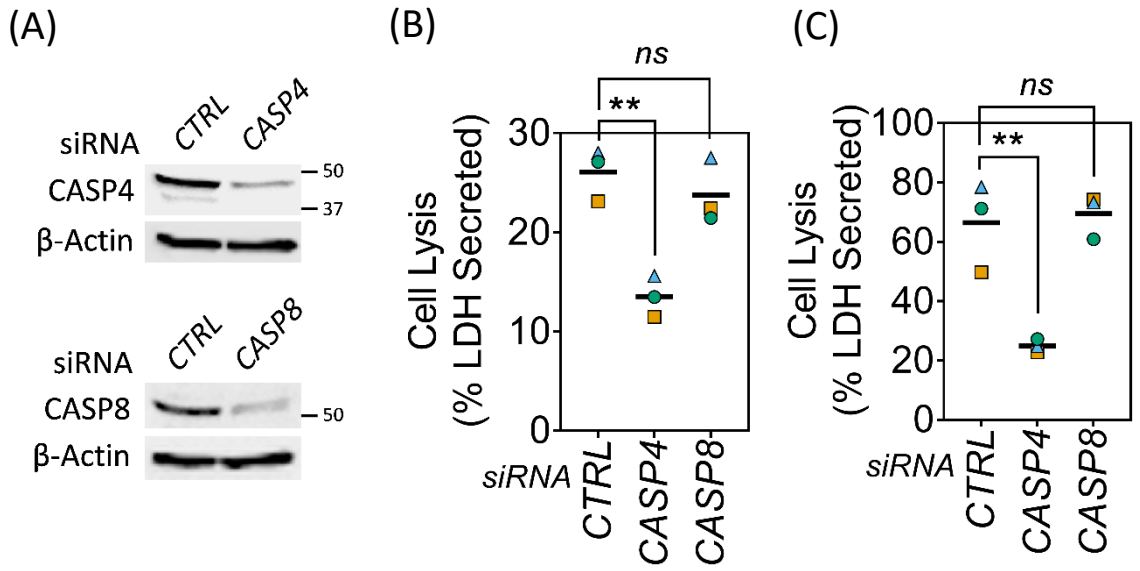

Figure 3.24. Caspase-8 is not required for EPEC-induced cell death

(A) Immunoblots of cell lysates from THP1 cells transfected with non-targeting (CTRL), *CASP4* or *CASP8* siRNA. Blots are representative of 3 independent repeats.

(B) Quantification of pyroptosis by LDH release assay from THP1 cells transfected with non-targeting (CTRL) or indicated siRNAs and infected with EPEC for 4 h

(C) LDH release from THP1 cells transfected with non-targeting (CTRL), *CASP4* or *CASP8* siRNA, transfected with LPS (5 µg/ml) using Lipofectamine-2000 for 4 h.

Matching shapes and colours of symbols in graphs denote data from independent experiments. Horizontal lines represent mean. n = 3 independent repeats. ** *P<0.01* by one-way ANOVA. *ns*= non-significant.

3.21 EPEC-induced pyroptosis occurs independently of RIPK1 and MLKL

Within host cells RIPK1 functions as a key regulator of both survival and death in response to various cellular stresses. However, recent studies have identified a role for RIPK1 in NLRP3 inflammasome activation induced by endoplasmic reticulum stress (675-677). Similarly, RIPK3- and MLKL-dependent necroptotic signalling has been shown to occasionally function in the NLRP3-inflammasome pathways to drive IL-1β inflammatory responses (678-680). To determine whether RIPK1 or MLKL signalling plays a role in EPEC-induced NLRP3 activation, RIPK1 and MLKL were knocked down in THP1 cells using miRNA30E. Immunoblots from CTRL^{miR}, *RIPK1*^{miR} and *MLKL*^{miR} THP1 cell lysates confirmed the gene repression (Figure 25A). CTRL^{miR} *MLKL*^{miR} and *RIPK1*^{miR}

THP1 cells were infected with WT EPEC for 5 h. Silencing of RIPK1 or MLKL did not inhibit EPEC-induced pyroptosis as measured by LDH release, (Figure 3.25B), and immunoblot analysis confirmed that in response to EPEC infection, RIPK1 and MLKL were not required for NLRP3-driven caspase-1 activation, or the processing of pro-IL-1β and pro-IL-18 into their mature bioactive fragments (Figure 3.25C).

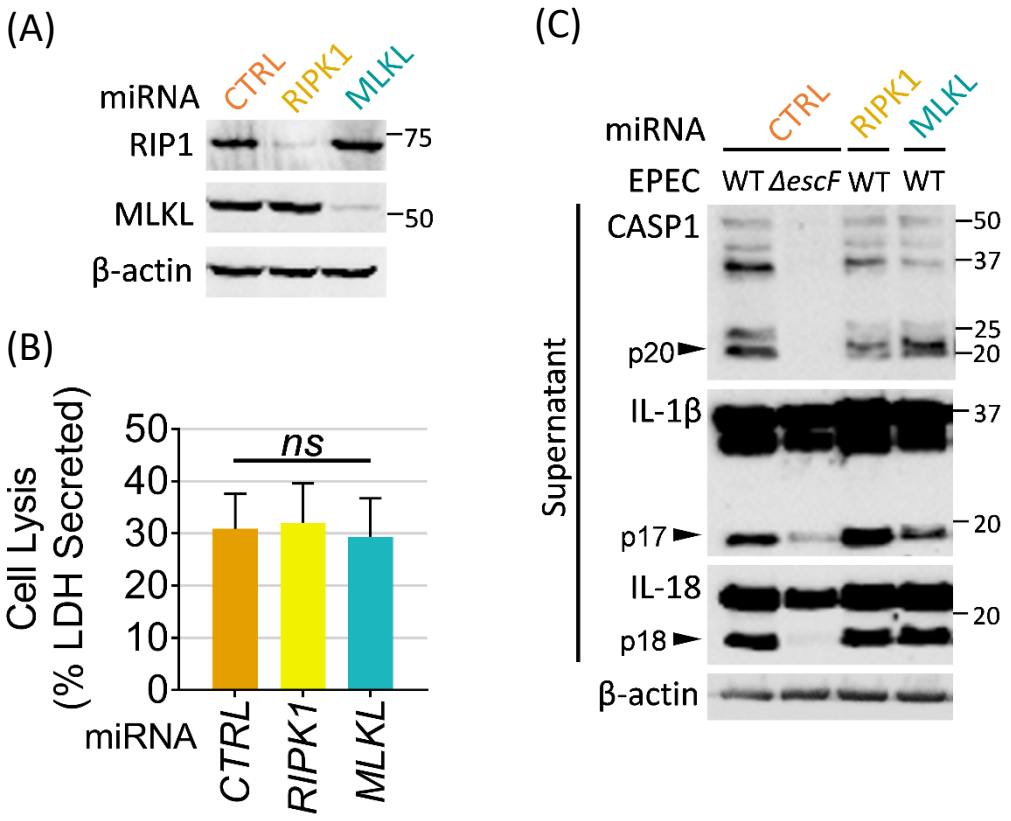

Figure 3.25. RIPK1 and MLKL are not required for EPEC-induced NLRP3 activation and pyroptosis

(A) Representative immunoblots from THP1 cells stably expressing non-targeting (CTRL^{miR}) or miRNA (miR) against indicated genes, showing silencing of protein expression. Blots are representative of 3 independent repeats.

(B) Quantification of pyroptosis by LDH release from indicated THP1 cells infected with EPEC for 5 h. Graph denotes data from three independent experiments. *ns* = non-significant by one-way ANOVA.

(C) Immunoblots from THP1 cells stably expressing non-targeting (CTRL^{miR}) or miRNA against RIPK1 (*RIPK1*^{miR}) or MLKL (*MLKL*^{miR}) infected with EPEC at an MOI of 10 for 5 h. Immunoblots for caspase-1, IL-1β and IL-18 in cell supernatants and β-actin in cell lysates are shown. Data is representative of two independent repeats.

3.22 EPEC-induced caspase-11 activation requires LPS-sensing

Recent studies on the activation of the non-canonical inflammasome indicate that mouse caspase-11 and its human counterparts, caspase-4 and caspase-5, are oligomerised and activated by direct interaction with intracellular LPS. It has previously been shown that caspase-4 is highly expressed in human monocytes and is responsible for LPS-induced pyroptosis (446), suggesting that endogenous caspase-4, rather than caspase-5, mediated intracellular LPS inflammatory responses and cell death. As human caspase-4 and murine caspase-11 have previously been shown to be functional homologues, the next aim was to determine whether murine caspase-11 could restore EPEC-induced toxicity in caspase-4 silenced cells. *CASP4*^{miR} THP1 cells were reconstituted with mouse caspase-11, or a catalytically inactive caspase-11 mutant (Cas11^{CM}) (Figure 3.26A), and infecting with EPEC, or transfected with LPS. Interestingly, caspase-11 expression restored pyroptosis induced by both cytosolic LPS (Figure 3.26B), and EPEC (Figure 3.26C), which suggested that mouse caspase-11 could substitute for human caspase-4 in the context of EPEC-induced pyroptosis. Importantly, the Cas11^{CM} failed to complement caspase-4 silencing, meaning the catalytic activity of capsase-11 was required to induce pyroptosis (Figure 3.26B-C).

Recently, it was discovered that cytosolic LPS could directly bind to and thereby activate caspase-4/5/11. Through extensive mutagenesis analyses Shi *et al* identified three caspase-11 mutations, K19E, K52E/R53E/W54A (KRW) and K62E/K63E/K64E (KKK), which abrogated LPS binding by caspase-11. Although human caspase-4 and murine caspase-11 have been shown to be functional homologues, they share less sequence homology than, for example, functional orthologues of apoptotic caspases, and these differences may indicate specialisation of their functions. Indeed, the LPS-binding residues identified in caspase-11 are not well conserved within human caspase-4 (Figure 3.27). Therefore, to establish whether direct sensing of EPEC LPS during infection was responsible for the inflammasome responses in human macrophages, *CASP4*^{miR} THP1 cells were reconstituted with a mouse caspase-11 LPS-binding mutant (Cas11^{KE}), with mutated residues K62E/K63E/K64E; which have previously been shown to be necessary for LPS binding and caspase-11 activation in response to cytosolic LPS (446). Interestingly, Cas11^{KE} expression in *CASP4*^{miR} THP1 cells failed to restore pyroptosis upon LPS transfection (Figure 3.26B), or EPEC infection (Figure 3.26C). Altogether, this suggests that EPEC infections induce LPS-dependent, unconventional, caspase-4-dependent inflammasome activation in human macrophages, which requires the NLRP3-ASC-Caspase-1 inflammasome for pyroptosis.

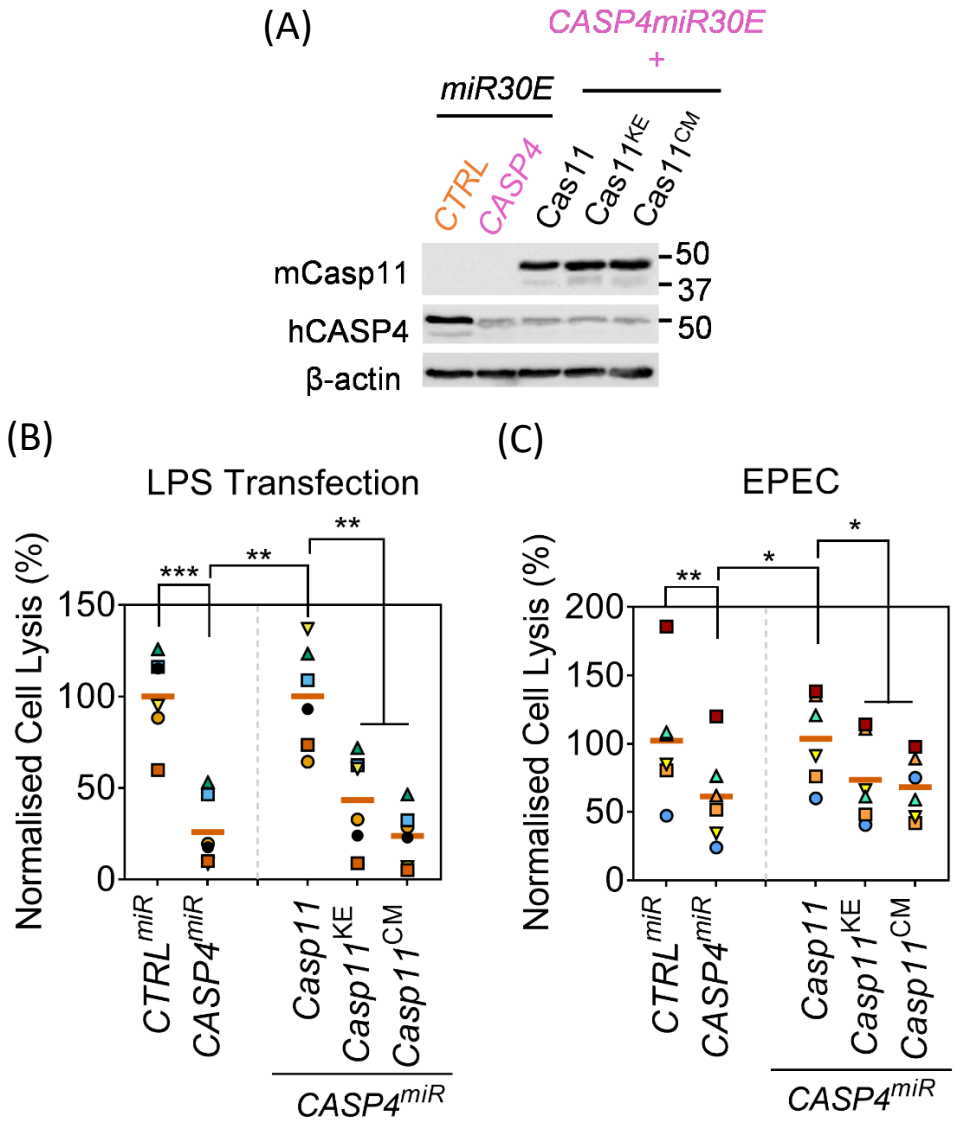

Figure 3.26. EPEC-induced caspase-11 activation requires LPS-sensing

THP1 cells stably transduced with non-targeting (CTRL^{miR}) or miRNA targeting CASP4 (*CASP4*^{miR}), expressing mouse caspase-11 (Cas11), caspase-11 LPS binding mutants (Cas11^{KE}) or a caspase-11 catalytic mutant (Cas11^{CM}).

(A) Immunoblots of cell lysates showing *CASP4* miRNA knock-down and caspase-11 expression in indicated stably transduced THP1 cells.

(B) Cell lysis as measured by LDH release from indicated THP1 cells, transfected with LPS (5 µg/mL) using lipofectamine-2000 for 4 h.

(C) Cell lysis as measured by LDH release from indicated THP1 cells, infected with EPEC at a MOI of 10 for 4 h.

Matching shapes and colours of symbols in graphs denote data from independent experiments, Horizontal lines represent means (*n*= 6 independent repeats). * *P<0.05, ** P<0.01, *** P<0.001* by one-way ANOVA.

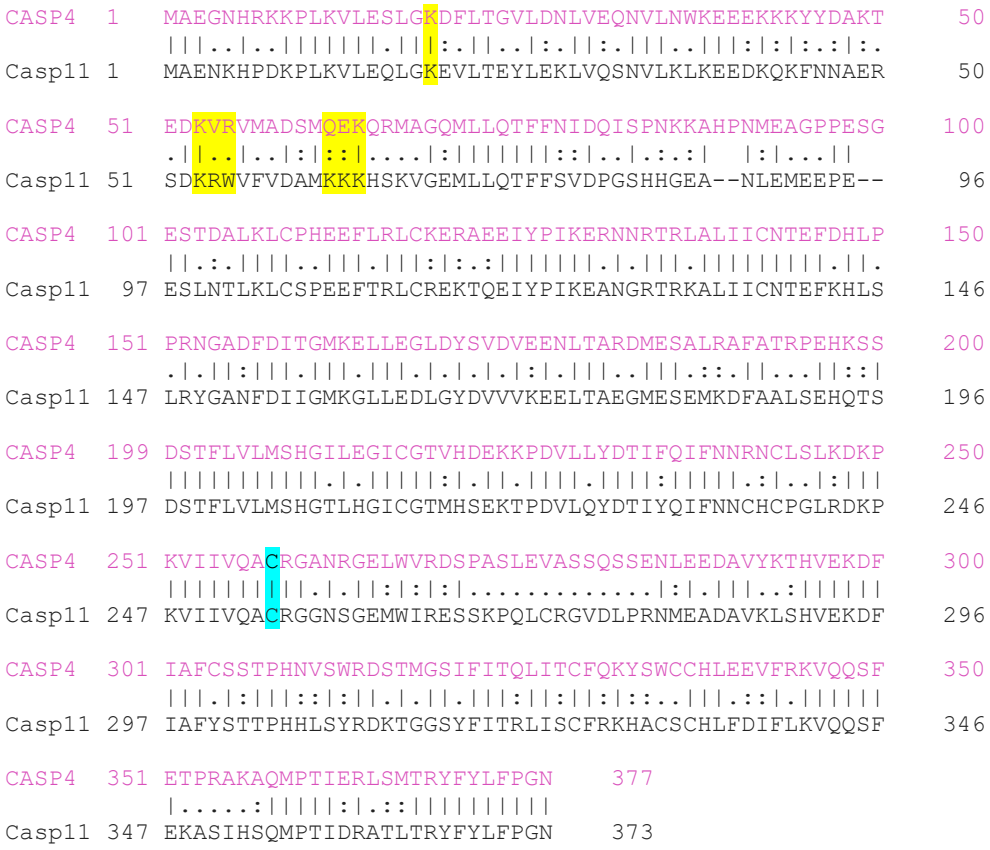

Figure 3.27. Alignment of human caspase-4 and mouse caspase-11

Human caspase-4 (ID: P49662) and mouse caspase-11 (ID: P70343) sequence alignment using the ClustalW2 algorithm. Residues important for LPS binding to caspase-11 are highlighted in yellow, catalytic cysteine residue is highlighted in blue.

3.23 EPEC-induced pyroptosis occurred independently of pannexin-1 and the P2X7 receptor

Intracellular LPS has been shown to activate the non-canonical inflammasome and induce caspase-4/11-dependent pyroptosis. In murine macrophages the signalling pathway downstream of caspase-11 is dependent the pannexin-1 channel and subsequent ATP release, which in turn activates the purinergic P2X7 receptor to mediate cytotoxicity (500). It has previously been shown that in the absence of pannexin-1 pyroptosis induced by cytosolic LPS is abrogated. EPEC infections, however, induced a novel caspase-4-dependent non-canonical inflammasome, which was dependent on LPS-sensing by caspase-4 and also required the ASC-NLRP3 axis to mediate pyroptosis. To determine whether the induction of pyroptosis in response to EPEC infection was also dependent on pannexin-1, probenecid a specific inhibitor of the pannexin-1 channel, was used (681). Real-time PI uptake analysis of THP1 cells incubated in probenecid and transfected with LPS

revealed that, in agreement with studies in murine macrophages, pyroptosis induced by LPS transfection in human macrophages was dependent on pannexin-1 signalling (Figure 3.28B). Importantly, probenecid failed to inhibit EPEC-induced pyroptosis (Figure 3.28A). The different requirement for pannexin-1 signalling in LPS-induced pyroptosis and EPEC-induced pyroptosis further differentiates these inflammasome signalling pathways. This again demonstrated that EPEC-induced pyroptosis was mechanistically distinct from that induced by cytosolic LPS (Figure 3.28C).

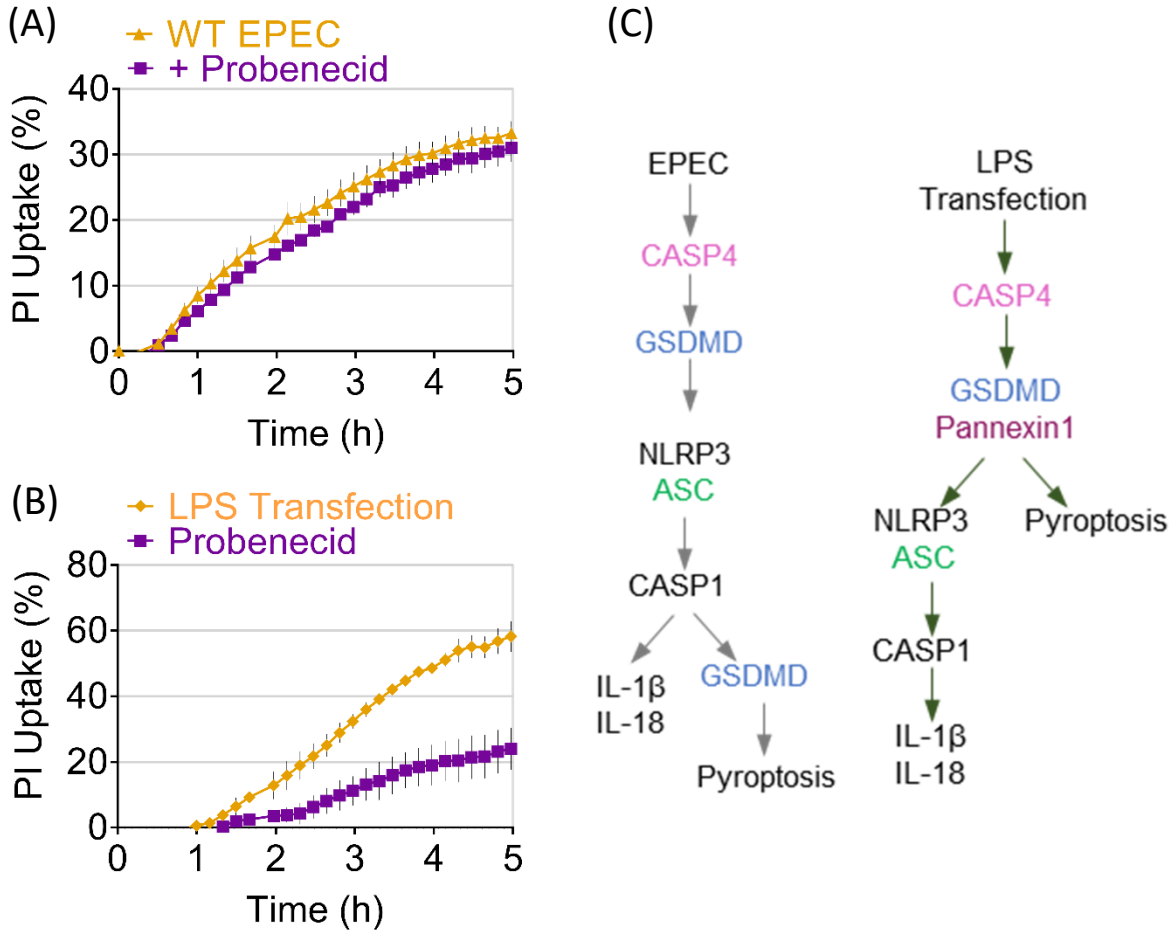

Figure 3.28. EPEC-induced pyroptosis occurs independently of the pannexin-1 channel

(A) Real-time PI-uptake assay from THP1 cells infected with EPEC at an of MOI of 10 in the absence or presence of probenecid (100 μM) for 5 h. Graph represent mean ± SEM (*n*= 3 independent experiments)

(B) Real-time PI-uptake assay from THP1 cells transfected with LPS (5 μg/mL), in the absence or presence of probenecid (100 μM) for 5 h. Mean ± SEM from *n*= 3 independent experiments are plotted.

(C) Comparison of EPEC- and LPS-induced activation of the non-canonical NLRP3-inflammasome, and pyroptosis.

Discussion

Activation of programmed cell death pathways is an important part of the innate immune response to bacterial infections. However, the mechanisms by which host cells, specifically human macrophages, activate these pathways in response to A/E pathogens is largely uncharacterised. The results in this study has shown infection of human macrophages with EPEC triggers a rapid atypical caspase-4-dependent NLRP3 signalling pathway which is distinct from that stimulated by LPS transfection. Although previous studies have characterised non-canonical inflammasome signalling pathways in response to infection of mouse macrophages with extracellular bacteria such as EPEC, EHEC or non-pathogenic *E. coli,* these were shown to induce caspase-11 signalling and inflammasome activation 15–20 h post-infection (123, 504, 571). However, these previous studies used bacteria grown in conditions that failed to induce transcription of the key EPEC virulence genes. In contrast, the work presented here used DMEM-primed EPEC, to demonstrate that the presence of transcriptionally regulated virulence factors during EPEC infection drives the activation of a rapid, mechanistically distinct, inflammasome pathway in human macrophages, that occurs within the first 2 – 4 h of infection, in a manner that is entirely dependent on the T3SS (Figure 3.1-3.9).

Infections of immortalised mouse bone-marrow derived macrophages (iBMDMs) with A/E pathogens, such as EPEC, expressing the T3SS have previously been shown to rapidly activate caspase-1 via NLRC4 leading to robust pyroptosis and cytokine processing (481, 483, 572). However, different expression of inflammasome sensors and caspase substrates within cell types results in distinct outcomes following inflammasome activation, and the regulation of many inflammasome pathways varies significantly between different hosts, as well as between immortalised cell lines and their primary cell counterparts. For example, expression of human NAIP (hNAIP) is higher in primary macrophages from human donors compared with hNAIP expression in monocytic tumor cell lines such as THP1 and U937, which are insensitive to the hNAIP agonist cytosolic flagellin (682). This could potentially account for the concomitant activation of NLRP3 seen in these immortalised cell lines, compared to the strong activation of NLRC4 in primary macrophages. However, importantly, based on my studies, EPEC-induced inflammasome activation and cell death in primary human $CD14^+$ monocyte-derived macrophages (MDMs), which express hNAIP, was found to be dependent on the NLRP3 inflammasome, as pyroptosis could be inhibited by the NLRP3 specific inhibitor MCC950 or silencing of ASC (Figure 3.10, 3.15). The NAIP–NLRC4

innate immune axis requires specific NAIP receptors that function as a ligand-specific receptor and undergoes oligomerisation to activate the NLRC4 adapter and induce caspase-1 activation. Unlike mice which encode four functional NAIPs—NAIP1, NAIP2, NAIP5, and NAIP6; humans have a single NAIP gene that is involved in the detection of flagellins, rods and needle proteins from various Gram-negative pathogens (575). My results demonstrate that EPEC is not detected by the human NAIP-NLRC4 system during infection of human macrophages, but instead, the NLRP3 inflammasome is required to induce inflammatory responses and pyroptosis. This, therefore, highlights a key divergence in the inflammasome signalling responses to infection between host species and underscores the importance of using relevant infection models to characterise immune responses to host specific pathogens. Indeed, recent studies characterising inflammasome signalling in human myeloid cells have uncovered surprising differences in human MDMs compared to mouse macrophages during bacterial infections. For example, the role of NLRP3 in the detection of cytosolic DNA, which contrasts with its roles in mouse macrophages (683). Similar differences have also been reported between human and mouse NLRC4 signalling pathways (684). The results here expand on this concept by identifying a new role for the human caspase-4 non-canonical-NLRP3 inflammasome in the context of EPEC infections.

The indispensable role for caspase-4 and NLRP3 in detecting EPEC in MDMs adds to the diverse range Gram-negative bacteria are sensed by human caspase-4, including *Francisella, Legionella* and *Yersinia* (496, 513). Interestingly, these studies found differential roles for caspase-4 in detecting natural infection by bacteria versus transfected LPS (496, 513). Similarly, my work demonstrates that EPEC infections induce atypical NLRP3 activation during natural infection of human macrophages, that differed from caspase-4-signalling upon LPS-transfection, since EPEC infection-induced pyroptosis had a dual requirement of both caspase-4 and NLRP3-caspase-1. EPEC-induced pyroptosis is further differentiated from that induced by cytosolic LPS by the requirement for pannexin-1. In agreement with the study by Yang *et al,* who demonstrated that cytosolic LPS induced caspase-11-dependent cleavage of the pannexin-1 channel to mediate cytotoxicity (500), here I confirmed that LPS transfection-induced pyroptosis was effectively prevented by the pannexin-1 inhibitor probenecid (Figure 2.28B). In contrast, EPEC-induced pyroptosis was unaffected by inhibition of the pannexin-1 channel (Figure 2.28B), demonstrating that EPEC infection induced a mechanistically distinct mechanism of pyroptosis, that is dependent on GSDMD but not pannexin-1. My results, therefore, support the hypothesis that diverse pathways occur downstream of caspase-4 activation to induce GSDMD-dependent pyroptosis, and may explain the

different models previously proposed for non-canonical mediated pyroptosis (445, 500) (Figure 1.9).

Emerging evidence suggests human caspase-4 is the functional orthologue to mouse caspase-11, due to their functional similarities. The data presented in my study supports this notion, as caspase-11 was shown to functionally substitute for caspase-4 in inducing rapid pyroptosis in THP1 cells upon either LPS transfection or EPEC infection. Both human caspase-4 and its mouse counterpart caspase-11 have previously been shown to be direct sensors for cytosolic LPS (446). This indicated that LPS sensing was the initial stimulus driving the rapid cell death response to EPEC infection in human macrophages. Interestingly, although LPS sensing by caspase-4/11 was required for pyroptosis induced by either EPEC infection, or LPS transfection, the signalling pathways downstream of LPS sensing diverged depending on the activating stimulus. This indicates that caspase-4 may differentially recognise LPS, depending on its physical form or route of delivery into host cells. Considering that EPEC is typically non-cytosolic, a major question that remains is the manner in which LPS gains access to the cytosol, particularly within the first few hours of infection to induce this rapid inflammasome response. The requirement for a functional T3SS in inducing this rapid inflammasome signalling pathway implicates EPEC effector driven signalling mechanism in this process. The mechanism and effectors responsible for this will be further investigated in Chapter 4.

My study, along with numerous others (446, 496, 501, 503), has demonstrated that caspase-4 is responsible for mediating inflammasome response to Gram-negative bacterial infections in human cells, however, the contribution of caspase-5 in LPS recognition during bacterial remains unclear. A number of studies have identified caspase-4 as the principal activator of the non-canonical NLRP3 inflammasome in response to Gram-negative bacteria. However, a more recent study has suggested that in human monocytes caspase-5 rather than caspase-4 is required for inflammasome activation in response to *P. aeruginosa* OMVs, whereas transfected *P. aeruginosa* LPS induces inflammasome responses via caspase-4 (685). This provides further evidence supporting the hypothesis that LPS is differentially recognised by host cells depending on its physical form or delivery mechanism. Although caspase-5 is not well expressed in THP1 cells (501), its presence and newly identified role in MDMs highlights the possibility that caspase-5 may also play a role in EPEC-mediated inflammasome activation, and this requires further analysis. If there is a level of functional redundancy between caspase-4 and caspase-5 signalling, simultaneous silencing of both caspase-4 and caspase-5 may further inhibit inflammatory responses during EPEC infections. Additional work

is needed to establish the potential role of caspase-5 during EPEC infection of MDMs. Unlike caspase-4, which is constitutively expressed in human macrophages, expression of caspase-5 is inducible (686). It will therefore be important to establish whether caspase-5 expression is upregulated in response to EPEC infections, and whether this plays a role in EPEC-induced pyroptosis.

Another interesting observation is the dual involvement of GSDMD in EPEC-induced macrophage pyroptosis. GSDMD expression was required for caspase-1 activation by EPEC (Figure 3.20), and the cleavage of GSDMD, via the NLRP3-caspase-1 inflammasome, led to pyroptosis (Figure 3.14). The mechanism by which caspase-4 and GSDMD mediate NLRP3 activation independently of pyroptosis during EPEC infections is still unclear, however, there are a number of intriguing possibilities. Recently, it has been demonstrated that pyroptosis is not the only outcome of GSDMD activity. In addition to causing membrane rupture, GSDMD pores can facilitate inflammatory cytokine secretion from cells prior to cell lysis. Furthermore, oxidized lipids (oxPAPC) derived from dead mammalian cells, the N-acetyl glucosamine (NAG) fragment of bacterial peptidoglycan (PGN), and bacterial LPS, have all been reported to induce the inflammasome-mediated release of IL-1 from living monocytes and macrophages in a GSDMD-dependent manner (515, 559, 687, 688). Additionally, single-cell analysis has revealed a new model of pyroptotic cell death that comprises two phases. The first phase involves the opening of GSDMD-dependent ion channels or restricted pores that are insufficient to induce pyroptosis (689). This is then followed by osmotic cell swelling, rupture of the plasma membrane and full permeability. Whether there is a certain threshold in which the amount of GSDMD pores either promotes active secretion of cytokines and ion exchange from living cells, or membrane rupture and pyroptosis, is still unclear. Nevertheless, this supports a model in which EPEC infections induce caspase-4-dependent GSDMD processing in human macrophages at a level that is undetectable via western blot, which enables cells to remain viable rather than undergoing instantaneous pyroptosis. However, this low-level GSDMD pore formation facilitates K^+ efflux from cells and downstream NLRP3-mediated caspase-1 activation. Active caspase-1 enhances GSDMD cleavage, increasing the number of GSDMD pores, ultimately leading to membrane rupture and pyroptosis. Here I confirm that ASC- and NLRP3-dependent caspase-1 activity downstream of caspase-4 and GSDMD is required to promote detectable cleavage of GSDMD into its ~ 30-kDa N-terminal pore-forming effector domain (Figure 3.11A, 3.20B), and this increase in GSDMD processing was required for membrane rupture and LDH release from cells (Figure 3.11B, 3.15B). Another possibility is that differentially activated un-

cleaved GSDMD might function to activate the NLRP3 inflammasome via a currently uncharacterised mechanism. Previous work aiming to determine which GSDMD variants were responsible for pore formation, identified two functionally distinct groups of GSDMD variants. One group which consisted of full-length GSDMD or its C-terminal fragment exhibited low levels of membrane permeability when expressed in cells, as detected by susceptibility to membrane impermeant dyes (559). It is, therefore, possible that activated, but un-cleaved, GSDMD is responsible for somehow mediating low-level membrane permeability and subsequent NLRP3-caspase-1 activation during EPEC infections. Finally, the N-terminal pore-forming domain of GSDMD has also recently been shown to bind to cardiolipin, a lipid moiety found on bacterial plasma membranes as well as on the mitochondrial inner membrane (519, 520). Indeed, the recombinant protein of the GSDMD N-terminal domain induces robust killing of *E. coli*, whereas the full-length protein does not (520). Whether GSDMD functions to activate the NLRP3 inflammasome via EPEC lysis and the subsequent release of bacterial PAMPs into the host cell cytosol is another possibility that requires further investigation. Further work utilising uncleavable, or oligomerisation deficient forms of GSDMD, may help to clarify the mechanisms responsible for this unconventional inflammasome activation pathway induced by EPEC infections.

In addition to characterising an atypical caspase-4-dependent NLRP3 signalling pathway, my results have underscored the importance of ASC within the non-canonical NLRP3 inflammasome. Within the inflammasome field, there are some inconsistencies centred around the role of ASC during non-canonical inflammasome activation. Immunofluorescent microscopy studies in mouse macrophages have previously shown that upon infection with Gram-negative bacteria, caspase-11 was required for ASC speck formation and thus NLRP3 inflammasome-mediated activation of caspase-1 (690). Here using THP1 cells expressing fluorescently tagged ASC (THP1$^{mAsc\text{-}RFP}$), I confirm that ASC speck accumulation could be seen during infection with WT EPEC, but not the T3SS mutant strain (*ΔescF*) (Figure 3.9). In contrast, using a biochemical assay to measure ASC dimer and oligomer formation, Rathinam *et al*. concluded that during infection of mouse BMDMs with un-primed EHEC caspase-11 did not induce ASC oligomerisation, instead an alternative model was proposed in which caspase-1 activation was enhanced through direct heterodimerisation with caspase-11 (504). Although both models are consistent with the reported functions of caspase-11 in promoting caspase-1-mediated cell death and pro-IL-1β maturation, they deviate in the mechanisms of NLRP3 inflammasome assembly and activation downstream of non-canonical stimuli. Using genetic silencing of ASC, in this study I have demonstrated that downstream of

caspase-4 and GSDMD, ASC played an essential role in caspase-1 activation. This confirmed that during EPEC infection caspase-4 activation alone is not sufficient to induced caspase-1 processing in the absence of ASC, despite previous work demonstrating that caspase-4/11 co-immunoprecipitated with endogenous caspase-1 from macrophages treated with LPS+CTB (Cholera toxin B) or *E. coli* (123), and the fact that caspase-11 has been shown to form complexes with caspase-1 when co-transfected into cells (691).

Caspase-8 has previously been shown to play an integral role in inflammasome activation in response to Gram-negative pathogens such as *Salmonella* (544) and *Yersinia* (546, 583). However, caspase-8 was dispensable for EPEC-induced macrophage cell-death (Figure 3.24). In human monocytes, LPS has also been shown to trigger an "alternative inflammasome". Within these cells, inflammatory responses are propagated by RIPK1, FADD and caspase-8 signalling upstream of NLRP3 (688). The inflammasome pathway activated upon EPEC infection in human macrophage cells is distant from this LPS induced mechanism in human monocytes because it occurs independently of both RIPK1 and caspase-8 (Figure 3.24, 3.25). The reason for these different response mechanisms to LPS remains to be fully characterised. This, however, provides further evidence for cell-type-specific responses to inflammasome stimuli. Although not required for EPEC-induced pyroptosis, caspase-8 has also been implicated in NLRP3-mediated IL-1β processing (544). Whether caspase-8 has an important role in regulating cytokine responses during EPEC infection still remains to be established.

During *in vivo* infections host cells are typically exposed to numerous bacterial PAMPs simultaneously, meaning both TLRs and NLRs are activated quick succession. In this work I used naïve macrophages prior to infection to recapitulate an infection model in which simultaneous sensing of microbial ligands and pathogen virulence factors occurs. Previous work with mouse BMDMs has shown activation of caspase-11 requires transcriptional priming prior to activation (692). However, unlike caspase-11 in mouse macrophages, in human macrophages caspase-4 is constitutively expressed (446). This may, therefore, partially account for the non-canonical pyroptosis observed upon EPEC infection within the first hour of infection (Figure 3.2A), even in the absence of an initial priming stimuli. The level of NLRP3 protein expression in resting macrophages was initially thought to be insufficient for NLRP3 activation, and transcription priming has been shown to play an important role in NLRP3 inflammasome activation (486). However, emerging evidence has demonstrated that priming macrophages for a short extent of time (~10 min), is sufficient for to enhances NLRP3 inflammasome activation (693, 694), and this was attributed to

post-transcriptional regulation of NLRP3 rather than transcriptional upregulation. In agreement with this concept, simultaneous stimulation of TLRs and NLRP3 with chemical activists such as ATP has previously been shown to lead to a rapid assembly of the NLRP3 inflammasome complex within 30 min, that occurs independently of new protein synthesis (695). Collectively these results indicate that human macrophages have the capacity to activate a more immediate non-canonical inflammatory response upon challenge with a caspase-4 agonist such as LPS. The implications of this *in vivo* remain to be determined, but this may facilitate a more acute inflammatory response to Gram-negative bacterial infections. However, this could result in humans being more susceptible to inflammasome hyperactivation leading to conditions such LPS-induced septic shock and may explain why the LPS dose required to induce severe disease in humans is several orders of magnitude lower than the dose required to induce shock in mice (642, 643).

Interestingly, macrophages that evade EPEC-induced pyroptosis were shown to be susceptible to late-stage cell death via a mechanistically distinct, programmed necroptotic cell death pathway. In the absence of caspase-1/4-induced pyroptosis, macrophages infected with EPEC underwent a late phase of cell-autonomous necroptosis. Importantly, this work establishes that EPEC induced necroptosis is an effector driven process that required RIPK1 activity to induce cell death. However, it is likely that the caspase-4-dependent inflammasome pathway is the principal mechanism induced during EPEC infection of human macrophages. Whether this subsequent necroptotic cell death pathway would be sufficient to compensate for EPEC infections that are able to bypass or suppress the initial stage of pyroptotic cell death remains to be experimentally verified. Nevertheless, the capacity to activate diverse forms of programmed cell death has previously been reported in the clearance of pathogens by necroptosis when apoptosis is actively inhibited (696)

Previous studies of EPEC effector modulation of programmed cell death pathways have focused on epithelial cells, where the synergistic activity of effectors typically promotes epithelial cell survival (240, 598, 697). However, my results illustrate, for the first time, novel mechanistic strategies in which human macrophages can potentially suppress EPEC infection by the induction of programmed cell death pathways and the generation of pro-inflammatory cytokines. The identification that EPEC infection induced caspase-1 activation is of special significance in light of earlier work showing the important role caspase-1 has in response to enteropathogenic bacterial infections *in vivo* (126). Both NLRP3 and NLRP4 inflammasomes have been shown to have important roles in the immune response to *C. rodentium* infections *in vivo* (126). However, here I have demonstrated that during EPEC infections of human cells NLRP3 is the principle inflammasome

responsible for mediating inflammatory responses. This concept is further supported by the fact that unlike many Gram-negative bacteria, both flagellins and the T3SS needle protein EscF from EPEC have previously been reported to be intrinsically inactive in binding to NAIP5/6 (483) and NAIP1/hNAIP (573). The protective role of caspase-11-induced non-canonical NLRP3 inflammasome activation has also been shown during *C. rodentium* infection-induced colitis. Notably, in mice that have increased caspase-11 expression, non-canonical NLRP3 inflammasome activation has been shown to mediate increased protection from infection (124). This suggests that the activation of caspase-4/11 may function to protect the host from infections with A/E pathogens, and therefore the activation of these pathways *in vivo* may be detrimental to the pathogen.

The results presented in this study provide compelling evidence that EPEC infections activate a novel caspase-4-dependent inflammasome pathway in human macrophages to induce inflammatory responses and pyroptotic cell death. However, there are some limitations within this work that should be addressed in the future to help characterise this pathway further. Firstly, the work here describes a model in which caspase-4 acts upstream of GSDMD to activate the NLRP3-caspase-1 inflammasome. Using targeted gene silencing, the hierarchy of this activation pathway has been established (Figure 3.20). However, in this study western blot analysis was completed 4 h post infection, after pyroptosis had been induced. The use of western blot analysis of EPEC infected cells at earlier time points during infection, or time-course analysis of protein cleavage and activation would provide further evidence of this activation cascade. Theoretically, caspase-4 cleavage and activation should be observed prior to either GSDMD cleavage or caspase-1 activation. Furthermore, if caspase-4 activation, in this context, is insufficient to induce membrane rupture and pyroptosis the active fragment of caspase-4 should be retained within cell lysates prior to caspase-1 activation.

Completing immunofluorescence analysis at earlier infection timepoints would also provide more comprehensive assessment of inflammasome speck formation during EPEC infections. The accuracy of ASC specks quantifications completed in this study was limited by the fact that after inflammasome activation occurs, ASC specks can be released from cells into the extracellular space (447). Therefore, the number of ASC-speck positive cells counted upon EPEC infection may be underrepresented in this work. In future experiments, utilising live cell immunofluorescence imaging, rather than fixed time-point analysis, would enable inflammasome complex formation to be visualised in real time, and would allow accurate temporal analysis of inflammasome speck

formation, membrane rupture (as measured by PI uptake), and ASC-speck release into the extracellular matrix to be established.

Throughout this study stably transduced THP1 cell lines expressing optimised shRNA, or transient knock-down of proteins in MDMs using siRNA, have been utilised to characterise the role of inflammasome associated proteins, including; caspase-4, caspase-8, ASC and GSDMD, during EPEC infections. It has previously been established that RNAi can have off-target effects (698, 699), and can induce silencing of non-target mRNAs, often via interactions with the 3'UTR (700, 701). Furthermore, the use of RNAi to reduced transcript levels may not necessarily result in a loss of phenotype. This is particularly relevant in situations where a reduction in protein expression is not necessarily rate limiting, which may occur in the case of enzymes such as caspases. Therefore, additional characterisation using techniques that enable stable genetic protein knock-outs (KO) cell lines should be utilised, such as the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) systems (702). The application of CRISPR/Cas9 for mammalian gene editing has recently revolutionised the gene editing field (702-704). Unlike miRNA silencing, CRISPR knockouts entirely prevent protein expression, this therefore removes the possibility that confounding effects from residual protein expression after knock-down may occur. In this study attempts to synthesis miRNA caspase-1 knock-down THP1 lines were unsuccessful (data not shown), and therefore synthesising a caspase-1 knock-out cell line using CRISPR/Cas9 would help to confirm the proposed role of caspase-1 during EPEC-induced pyroptosis.

Another important constraint of RNAi to knock-down gene expression, is that this technique does not enable effective complementation of the knock-down cells, thus limiting the comprehensive analysis of overall protein function. Although strategies have been developed to facilitate gene replacement in miRNA expressing cells, these are primarily based on silent modification of the shRNA-targeted region to efficiently re-express recombinant genes (705). However, this may affect the expression or function of the recombinant genes (706). Therefore, another significant advantage of utilising CRISPR-Cas9 mediated genome editing for further analysis is that it would provide a mechanism to investigate modified proteins by introducing targeted mutations within the genome of human macrophages. This would enable more comprehensive assessment of protein functions to be investigated, such as catalytic inactive caspases, caspase-4 LPS-binding mutants, or uncleavable GSDMD mutants.

Despite the fundamental role the non-canonical NLRP3 inflammasomes play in the immune response to enteropathogenic bacterial infections, the mechanisms by which these extracellular pathogens are recognised by caspase-4 remains to be fully established. The results thus far have underscored the importance of considering the role of secreted virulence factors in this host-pathogen interaction, as the presence of the T3SS during EPEC infection was shown to be paramount in initiating this rapid inflammasome response. It is therefore likely that the T3SS itself or a T3SS secreted effector protein is responsible for activating this rapid, atypical non-canonical inflammasome pathway, and this will be investigated further in the next chapter.

Chapter 4 –

EPEC intimin:Tir interaction is essential for inflammasome activation in human macrophages

Introduction

Macrophages can promote host-defence by sensing and responding to infection via inflammasomes, which are signalling platforms that activate caspase-1 (443, 449). Here, systematic investigation of the mechanisms of EPEC-mediated inflammasome activation in primary human monocyte-derived macrophages found an essential role for caspase-4 in rapid inflammasome activation by EPEC expressing the T3SS and other LEE- and non-LEE encoded virulence factors. Furthermore, EPEC-induced pyroptosis required LPS sensing by caspase-4, but was mechanistically distinct from pyroptosis induced by cytosolic LPS.

Previous work characterising inflammasome-mediated responses to A/E pathogens has established the activation of non-canonical caspase-4-dependent inflammasomes over a 15-18 hour period, via a mechanism that was independent of the T3SS (504, 532, 571). These studies, however, did not induce expression of the LEE and non-LEE virulence locus by the pathogens prior to infections. In fact, infection with uninduced EPEC, EHEC or *C. rodentium* activated an inflammasome response similar to that of non-pathogenic *E. coli* strains (123, 497, 571, 639). The LEE is highly conserved between A/E pathogens and *in vivo* infection studies have demonstrated the essential nature of both the T3SS and several LEE-encoded effector proteins during infection (122). LEE expression is a highly regulated process that can be activated by a number of stimuli (163), including the presence of bicarbonate ions within the growth medium (177). Elevated expression of the T3SS and effectors encoded by the LEE and the non-LEE virulence regulons can be induced by 'priming' EPEC in low glucose DMEM, this mimics the transcriptional regulation induced during *in vivo* infections (173). Throughout this project, in data shown so far, DMEM-primed EPEC were used for infections, enabling any effector-mediated responses to be characterised. The expression of the LEE and non-LEE genes is fundamental to EPEC virulence and is required for assembly of the T3SS and secretion of effectors including Tir and expression of intimin on the bacterial surface. The next aim of this project was therefore to validate the role of EPEC LEE- and non-LEE encoded virulence factors in activating the rapid non-canonical NLRP3 inflammasome in human macrophages, and to identify the T3SS-dependent stimulus responsible for inducing this unconventional inflammasome pathway.

Results

4.1 Expression of EPEC LEE virulence factors drives rapid pyroptosis in human macrophages

The work in the previous chapter identified a fundamental role for the T3SS in inducing rapid pyroptosis in human macrophages infected with DMEM-primed bacteria (Figure 3.1-3.2), this contrasted previous studies that characterised a T3SS-independent non-canonical inflammasome response to A/E pathogens grown in LB prior to infection of mouse macrophages (28, 31, 45, 81). To address these differences, the inflammasome responses induced by DMEM-primed (D) LEE-expressing, and LB grown (L) LEE non-expressing EPEC were compared. I reasoned that roles for the T3SS would be evident only under LEE-inducing conditions, potentially explaining the T3SS-independent inflammasome activation pathway previously characterised in response to LB grown bacteria. In agreement with previous work (177), EPEC LEE expression was markedly upregulated upon bacterial-priming in DMEM (Figure 4.1A). By comparing the expression of the LEE-encoded effector protein Tir as a readout for LEE-expression, DMEM-primed EPEC was shown to express notably more Tir than LB-grown cultures (Figure 4.1A). This confirmed that EPEC virulence gene expression was highly upregulated in DMEM-primed cultures.

Directly comparing the levels of pyroptosis induced by WT and *ΔescF* EPEC grown in DMEM or LB, confirmed that the role of the T3SS in pyroptosis is only evident upon infection with DMEM-primed bacteria. EPEC cultured in LB prior to infection induced very little (~5 ± 3 %) cell death within the first 5 h of infection, and no significant difference in cell death between WT and *ΔescF* infections was evident (Figure 4.1B). Notably, LB-grown EPEC did induce measurable cell death in THP1 cells 18 h post-infection that was independent of the T3SS (Figure 4.1B). However, DMEM-priming of WT EPEC induce rapid cell death at 5 h post infection, that was not evident in infections with LB-grown WT EPEC (Figure 4.1B); this difference was therefore a result of expression of the LEE regulon. In agreement with previous results in this study, the *ΔescF* mutant failed to induce pyroptosis at 5 h, even when cultured in DMEM, confirming that the rapid pyroptosis induced during EPEC infections was dependent on the T3SS and transcription of EPEC virulence genes. Furthermore, increased expression of the LEE regulon and the presence of a functional T3SS during infection induced significantly higher cell death up to 18 h post infection (Figure 4.1B). Together, these results demonstrate that EPEC infections induce a rapid form of T3SS-dependent pyroptosis that requires expression of the LEE virulence locus. However, in the absence of LEE expression,

EPEC induces a delayed form of cell death which occurs independently of the T3SS (Figure 4.1B), and is consistent with previous findings for LB-grown A/E pathogens in mouse macrophages (123, 571).

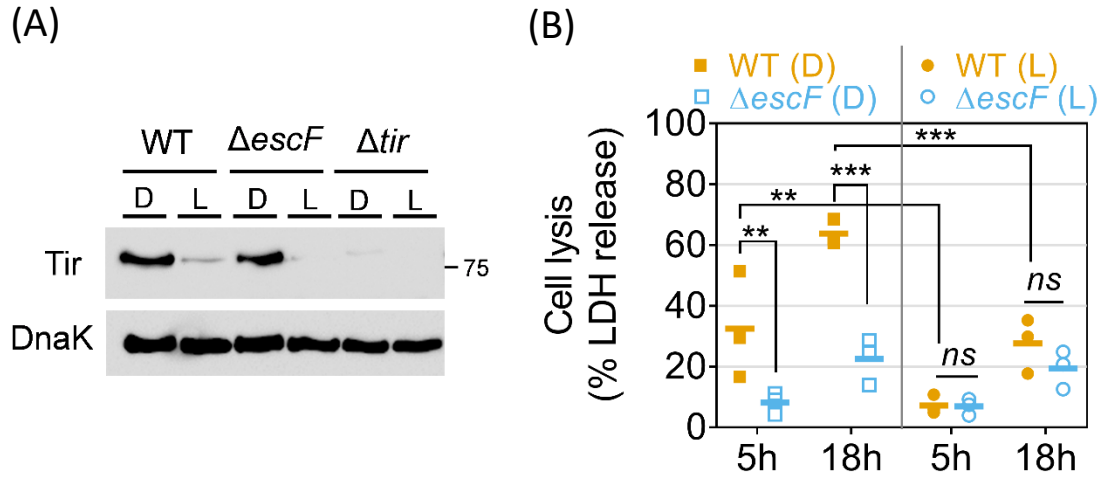

Figure 4.1. Expression of EPEC virulence factors drives rapid pyroptosis in human macrophages

(A) Expression of Tir and DnaK (as a house-keeping gene) in indicated strains of EPEC grown overnight in LB broth, then sub-cultured 1:50 into DMEM and grown statically for 3h (D), or grown in LB (L). Immunoblots of bacterial cell lysates. Blots are representative of two experimental repeats.

(B) LDH release measurements from THP1 cells infected at an MOI of 10 with DMEM-primed (D) or LB-grown (L) WT or *ΔescF* EPEC. LDH measured 5 h or 18 h post infection (*n*= 3 independent experiments). Mean indicated by horizontal bars. ** *P<0.01* *** *P<0.001* by two-way ANOVA.

4.2 Expression of EPEC virulence factors drives rapid inflammasome activation in human macrophages

EPEC-induced cell death, within the first 5 h of infection, was dependent on the expression of EPEC virulence factors. To establish whether DMEM-induced gene expression was also required to mediate rapid inflammasome activation in human macrophages, DMEM-primed and LB-grown EPEC were used to infect THP1 cells (Figure 4.2A) and primary MDMs (Figure 4.2B). Immunoblot analysis of infected cell lysates and supernatants revealed that at 5 h post infection only DMEM-primed EPEC induced inflammasome activation in THP1 cells, which was verified by the proteolytic cleavage of both caspase-4 and caspase-1, and the associated cleavage and activation of the caspase substrates GSDMD and IL-18 (Figure 4.2A). Interestingly, immunoblots of cell extracts from 18 h-long infections revealed that a delayed inflammasome activation pathway occurred later during infection that was independent of both virulence gene expression and the T3SS (Figure

4.2A). Concordant results were obtained in primary MDMs in which DMEM-induced gene expression was required for rapid inflammasome activation at 5 h (Figure 4.2B). The delayed inflammasome activation by LB-grown EPEC was also evident in primary MDMs 18 h post infection, and importantly, this inflammasome driven caspase-1 activation and cytokine processing occurred independently of the T3SS (Figure 4.2B). Collectively, these results distinguish between two pathways of inflammasome activation. Interestingly, DMEM-primed EPEC induces T3SS-dependent inflammasome activation and pyroptosis in human macrophages, which is markedly accelerated, and distinct from T3SS-independent late activation at 18 h.

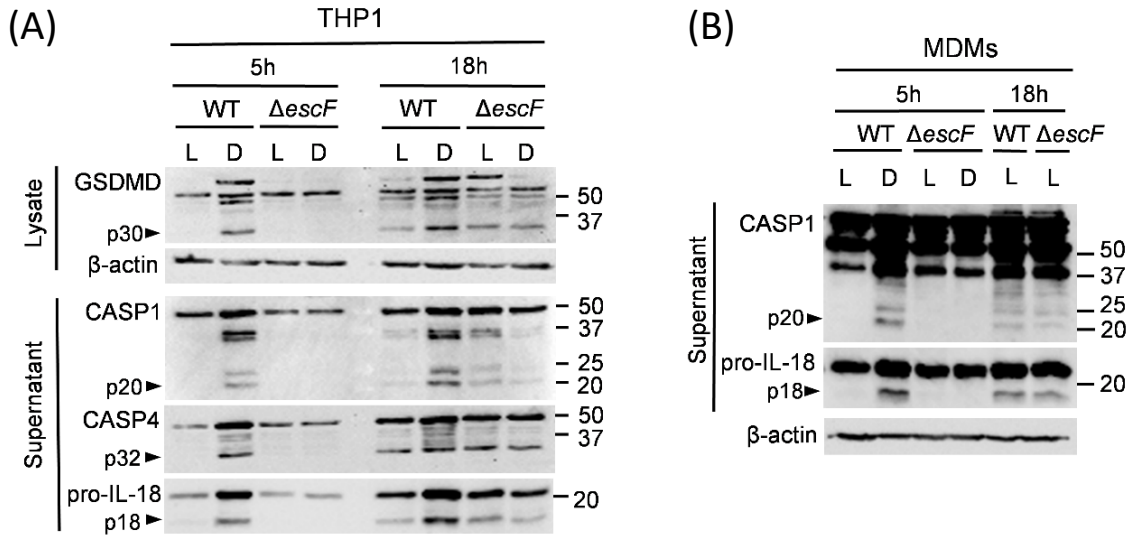

Figure 4.2. Expression of EPEC virulence factors drives rapid inflammasome activation in human macrophages

(A) Representative immunoblots from THP1 cells infected with DMEM-primed (D) and LB-grown (L) WT or *ΔescF* EPEC at an MOI of 10 for 5 h or 18 h. Immunoblots for caspase-1, caspase-4 and IL-18 in culture supernatant and immunoblots for GSDMD and β-actin in cell lysates are shown. Data is representative of three independent repeats.

(B) Representative immunoblots from primary MDMs infected for 5 h or 18 h with DMEM-primed (D) and LB-grown (L) WT or *ΔescF* EPEC at MOI: 10. Immunoblots for caspase-1 and IL-18 in culture supernatant and immunoblots for β-actin in cell lysates are shown. Data is representative of three independent repeats.

4.3 Expression of EPEC virulence factors drives a rapid mechanistically distinct form of pyroptosis in human macrophages

In addition to rapid T3SS-dependent pyropotosis, EPEC infection induced a delayed T3SS-independent form of inflammasome activation and pyroptosis. Previous work has described LPS- or OMV-dependent non-canonical inflammasome driven pyroptosis in macrophages, that occurred

independently of virulence factors (497, 526, 532). The results in this study have characterised a rapid T3SS-dependent form of inflammasome activation that required caspase-4, ASC and GSDMD to mediate pyroptosis. To investigate whether delayed T3SS-independent pyroptosis also required both caspase-4 and ASC, CTRL^{miR} *CASP4*^{miR}, *ASC*^{miR}, and *GSDMD*^{miR} THP1 macrophages were infected with LB-grown EPEC cultures for 18 h and LDH secretion was used as a measure of cell lysis. Unlike pyroptosis induced by DMEM-primed LEE expressing EPEC (Figure 4.3A), pyroptosis induced by LB grown EPEC was caspase-4-dependent but ASC-independent (Figure 4.3B). This pathway was consistent with the non-canonical pathway induced by LPS-transfection, and suggested that in this context, caspase-4 was directly responsible for inducing pyroptosis (Figure 4.3C). These results confirmed that bacterial expression of virulence-associated genes not only alters the kinetics, but also the mechanism of pyroptosis in human macrophages, and that non-pathogenic *E. coli* (*ΔescF*) are markedly defective in inflammasome activation.

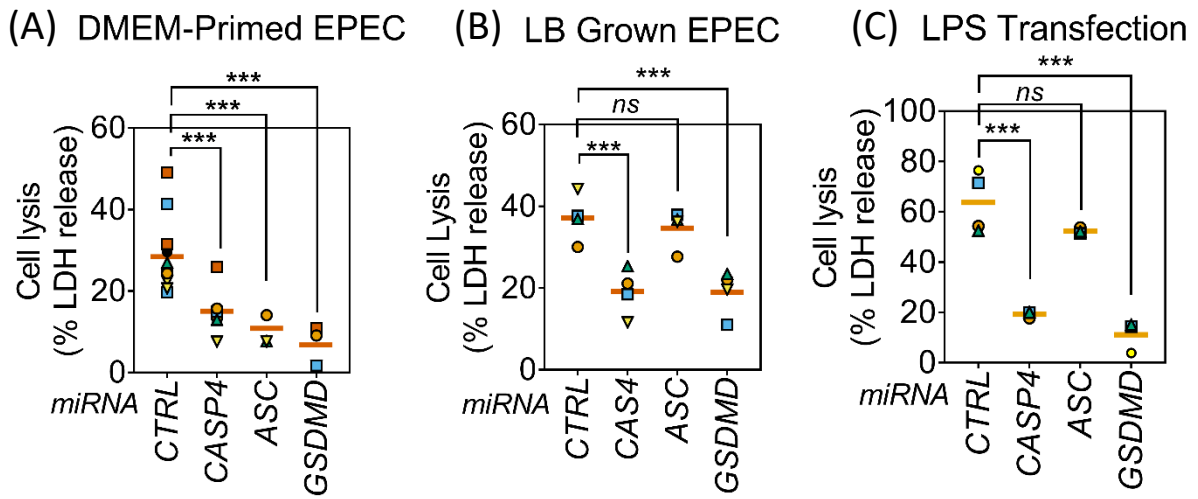

Figure 4.3. Expression of EPEC virulence factors drives rapid ASC-dependent inflammasome activation in human macrophages

THP1 cells stably expressing non-targeting (CTRL^{miR}) or miRNA against indicated genes.

(A) LDH release and a measure of cell lysis from indicated THP1 miRNA-expressing cells infected with DMEM-primed EPEC for 4 h. (*n = 4-6* independent repeats)

(B) LDH release assay from indicated THP1 miRNA-expressing cells infected with LB-grown EPEC for 18 h. (*n = 4* independent repeats)

(C) LDH release and a measure of cell lysis from indicated THP1 miRNA-expressing cells transfected with LPS for 4 h. (*n = 3-4* independent repeats)

Matching shapes and colours of symbols in graphs denote data from independent experiments. **** P<0.001* by one-way ANOVA and Dunnett's test for indicated comparison. *ns* = non-significant.

4.4 EPEC non-LEE virulence factors are not required to induce pyroptosis in THP1 macrophages

A number of recent studies have identified non-LEE encoded EPEC effector proteins that function to subvert host immune responses, including inflammasome activation (Figure 1.8). However, the inflammasome activating stimulus still remains unclear. Therefore, to investigate whether these non-LEE effectors were required for inflammasome activation, THP1 macrophages were infected with WT EPEC, or EPEC mutant strains lacking non-LEE pathogenicity islands encoding one or more known effector protein (Figure 4.4A). Consistent with previous work on NleA/EspI and NleF in suppressing inflammasome activation (226, 228), pyroptosis by the *ΔPP6* strain (which lacks NleA, NleF and NleH) was higher as compared to WT EPEC (Figure 4.4B). Interestingly, the *ΔPP4* EPEC strain, lacking NleD, NleC, NleB2, NleG and NleI, induced less cell death than the WT infection. However, the *ΔPP4* mutant induced three-fold more death that the T3SS mutant *ΔescF* strain, indicating that although these effector proteins may contribute to EPEC-mediated cytotoxicity, they are not the principle activating stimuli.

(A)

Pathogenicity Island	Effectors
PP2	EspJ, NleH1, Cif*
PP4	NleD, NleC, NleB2, NleG, NleI NleH3*
PP6	NleF, NleH2, NleA (EspI), EspO*
IE2	NleE2, EspL1*, NleB3*,
IE6	NleE1, NleB1, EspL2

** Indicates Pseudogene*

(B)

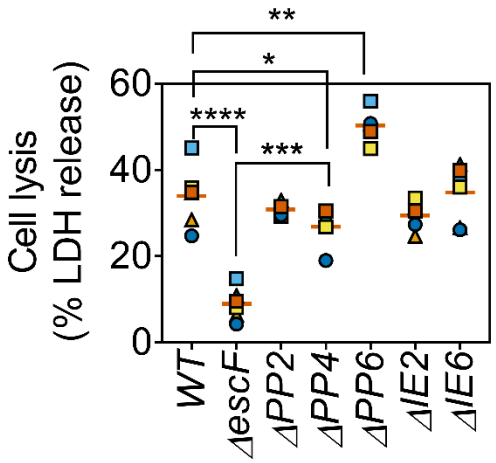

Figure 4.4. EPEC non-LEE virulence factors are dispensable for EPEC-induced pyroptosis
(A) Table of EPEC pathogenicity islands and associated effector proteins.
(B) LDH release assay from THP1 cells infected with indicated EPEC strains, at an MOI of 10 for 5 h. Mean is plotted from *n*= 5 independent experiments.
Matching shapes and colours of symbols in graphs denote data from independent experiments. ***** P< 0.0001*, **** P<0.001*, ***P<0.01*, **P<0.05* by one-way ANOVA.

4.5 Translocated intimin receptor (Tir) is essential for EPEC-induced cytotoxicity in human macrophages

Deletion of non-LEE pathogenicity islands did not markedly affect EPEC-induced pyroptosis in THP1 cells (Figure 4.4B), indicating that either the T3SS itself or LEE encoded effectors were responsible for inducing the rapid inflammasome activation and pyroptosis. Previous work on effector translocation dynamics has demonstrated that EPEC effectors exhibited distinct kinetics of effector translocation, with Tir being the first effector protein translocated into host cells within the first hour of infection (197). As EPEC infections induced rapid cell death in macrophages, effectors secreted early within the course of infection are likely to play the most significant roles (Figure 3.1, 3.2), therefore, the role of Tir in EPEC-mediated cytotoxicity was examined.

Deletion of Tir severely attenuated EPEC-induced pyroptosis both in primary MDMs (Figure 4.5A) and THP1 cells (Figure 4.5B). In fact, the *Δtir* strain induced cell death as poorly as the *ΔescF* T3SS mutant. This indicated that the secretion of Tir into host cells via the T3SS was required to induce pyroptosis. Importantly, complementation of the *Δtir* EPEC with a plasmid that allows for IPTG inducible *tir* expression (pTir) was able to restore EPEC-induced pyroptosis (Figure 4.5).

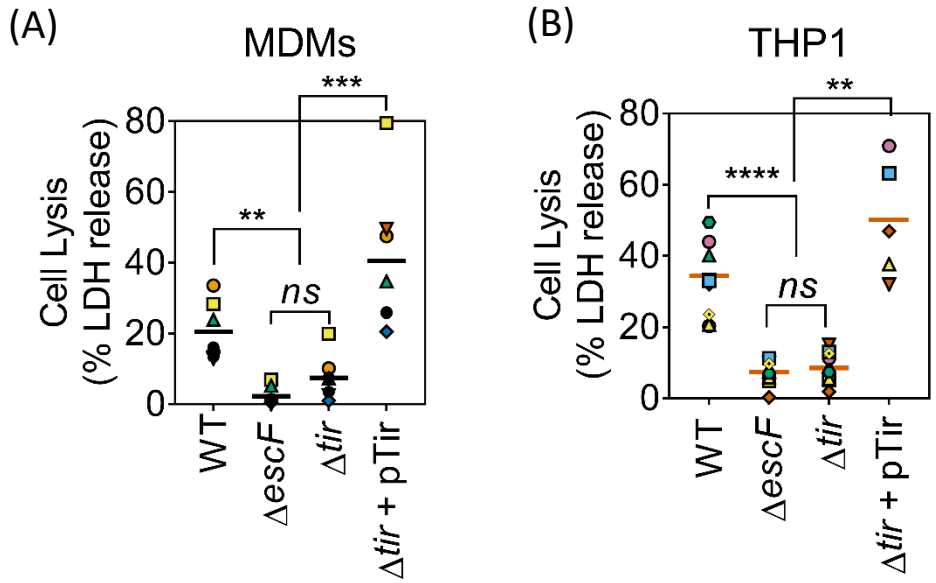

Figure 4.5. Tir is essential for EPEC-induced cell death in human macrophages
Graph shows LDH release assay from primary MDMs (A) and THP1 macrophages (B) infected with WT EPEC, *ΔescF*, *Δtir*, and *Δtir* + $pTir_{EPEC}$, at an MOI of 10 for 4 h. IPTG (0.1 mM) was added 30 min prior to infection to induce Tir expression from pSA10 plasmid (pTir). Mean ± SEM plotted from *n = 7* (A) and *n = 5-8* (B) independent experiments.
Matching shapes and colours of symbols in graphs denote data from independent donors/experiments. Means are indicated by horizontal lines. **** *P< 0.0001*, *** *P<0.001*, **P<0.01*, by one-way ANOVA. *ns* = non-significant.

In agreement with these results, Tir was also required to induced membrane disruption and PI uptake in EPEC infected THP1 macrophages (Figure 4.6), Notably, Tir complementation induced more PI uptake than even WT EPEC (Figure 4.6A), indicating that amplified secretion of Tir via an inducible promotor further increased pyroptotic cell death.

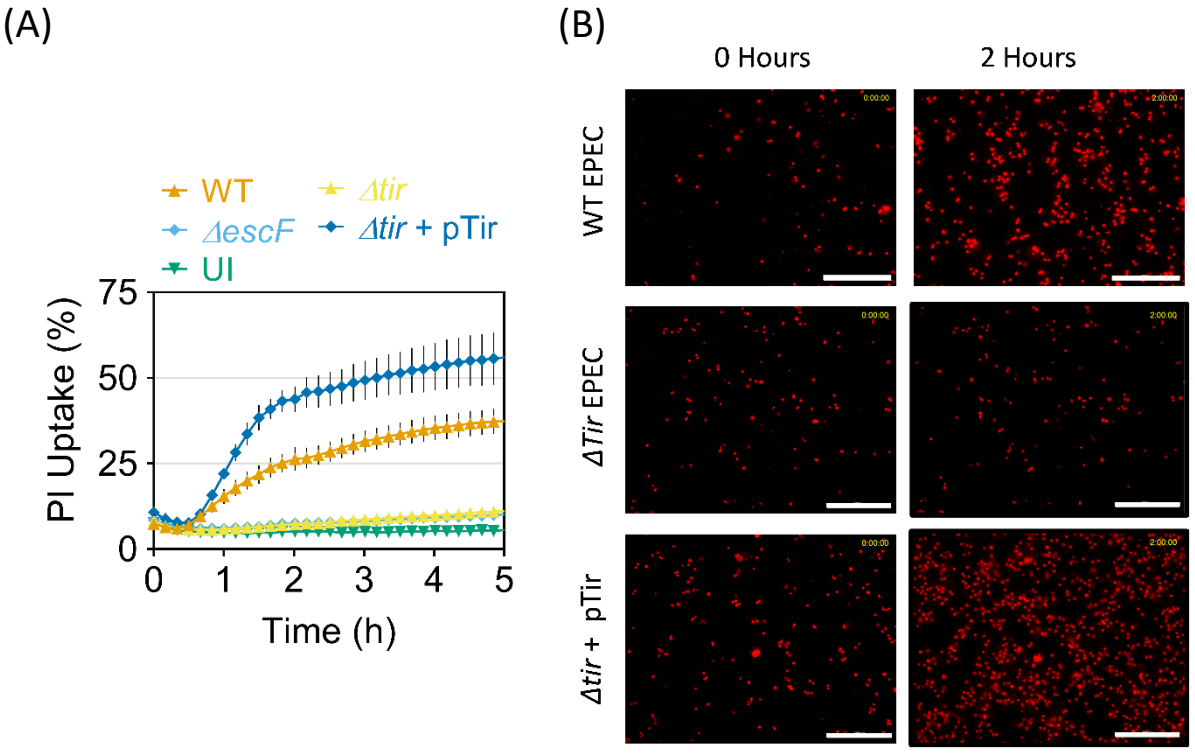

Figure 4.6. Tir is essential for EPEC-induced membrane rupture in THP1 macrophages

(A-B) Cytotoxicity as measured by real-time Propidium Iodide Uptake (PI).

(A) Real-time PI uptake measured over 5 h from THP1 cells infected with indicated EPEC strains at MOI:10, IPTG (0.1 mM) was added 30 min prior to infection to induce Tir expression from pSA10 plasmid (pTir). Values are calculated as a percentage of a Triton-X treated positive control. (Mean ± SEM; *n*= *6* independent experiments).

(B) Representative images of EPEC-induced PI uptake. Real-time Immunofluorescence imaging of THP1 cells infected with WT, Δ*tir* and Δ*tir* + pSA10:Tir EPEC strains. PI uptake (Red). Scale bar = 200 µm.

4.6 Tir is essential for EPEC-induced inflammasome activation and assembly

To verify the role of Tir in inflammasome activation, western blot analysis for caspase-1/4 activation and caspase substrate cleavage was completed. Infection with an EPEC strain lacking Tir (Δ*tir*) failed to induce caspase-1 and caspase-4 activation in primary MDMs (Figure 4.7A) or THP1 macrophages (Figure 4.7B), as shown from the lack of cleaved (p20) band of caspase-1, and the cleaved (p32) band of caspase-4 by immunoblot. However, expression of Tir, via a plasmid that allows for IPTG inducible gene expression (pTir), restored inflammasome activation (Figure 4.7A-B). Similarly, expression of *tir* was required to instigate the proteolytic cleavage and activation of inflammatory cytokines IL-1β and IL-18 (Figure 4.7A-B). EPEC *Δtir* also failed to induce inflammasome assembly as measured by ASC speck formation in THP1 cells expressing a fluorescently tagged ASC (THP1$^{ASC\text{-}mRFP}$). The accumulation of ASC foci was quantified, and counts revealed that WT EPEC infections induced significantly more ASC foci than either *ΔescF* or *Δtir* strains (Figure 4.7C-D). Collectively, these results establish that EPEC infections trigger a Tir-dependent atypical caspase-4-NLRP3 inflammasome signalling pathway to induce rapid pyroptosis in human macrophages.

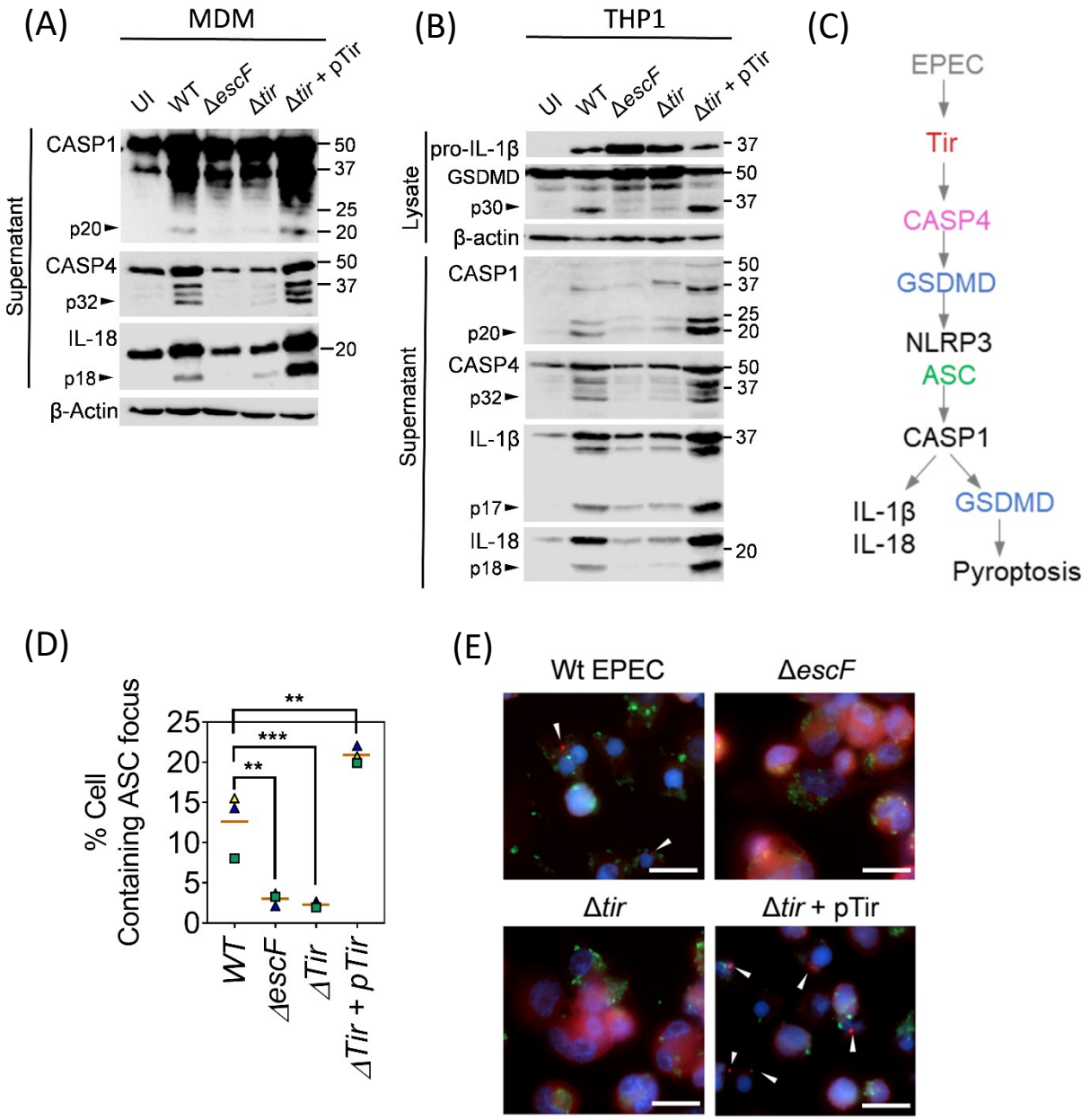

Figure 4.7. Tir is essential for EPEC-induced inflammasome activation in human macrophages

(A) Representative western blots for indicated proteins from primary MDMs infected with indicated EPEC strains at an MOI:10 for 4 h. IPTG (0.1 mM) was added 30 min prior to infection to induce Tir expression from pSA10 plasmid (pTir). Blots are representative of 3 independent repeats.

(B) Representative western blots for indicated proteins from THP1 cells infected with indicated EPEC strains at an MOI:10 for 4 h. IPTG (0.1 mM) was added 30 min prior to infection to induce Tir expression from pSA10 plasmid (pTir). Blots are representative of 6 independent repeats.

(C) Schematic representation of Tir-induced inflammasome activation of the non-canonical NLRP3 inflammasome.

(D) Quantification of inflammasome ASC foci formation in THP1$^{ASC\text{-}mRFP}$ cells infected with indicated EPEC strains at an MOI of 10 for 4 h. Matching shapes and colours of symbols in graphs denote data from independent experiments . >100 host cells were counted from at least 4 randomly selected fields and % cells showing events were obtained for each experiment. *n*= 3 independent repeats *** *P<0.001,* **P<0.01,* by one-way ANOVA.

(E) Representative immunofluorescence images of THP1 cells stably expressing mAsc-RFP (Red) infected with indicated EPEC strains. Bacteria were visualised with anti-O127:H6 antibody (Green) and cell nuclei were stained with DAPI (Blue). Arrows indicate inflammasome foci. Scale bar= 20 µm.

4.7 Tir expression correlates with inflammasome activation and pyroptosis

The expression and translocation of Tir during EPEC infection was required for inflammasome activation and pyroptosis during infection of human macrophages. The next aim was therefore to assess whether Tir was directly responsible for inducing these responses. THP1 cells were infected with Δ*tir* EPEC strains complemented with a pSA10:Tir plasmid that allows for IPTG inducible *tir* expression (pTir). 30 min prior to infection EPEC cultures were incubated with a range of IPTG concentrations to induced increasing levels of *tir* expression (Figure 4.8A). Interestingly, the level of *tir* expression directly correlated with the induction of pyroptosis (Figure 4.8A), and the activation of the non-canonical inflammasome (Figure 4.8B) during infection of THP1 cells. These results indicate that Tir itself may function as the activating stimulus to induce inflammasome activation during EPEC infection.

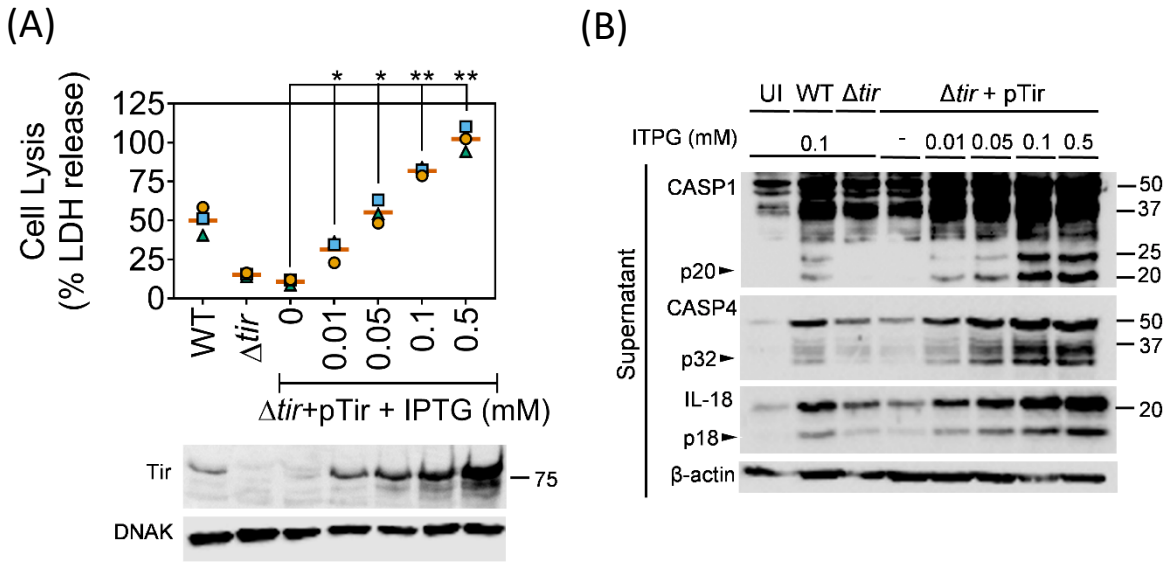

Figure 4.8. Tir expression correlates with inflammasome activation and pyroptosis

(A) Quantification of cell lysis (LDH release) from THP1 cells infected for 4 h with Δ*tir* EPEC expressing Tir from a plasmid that allows for IPTG-inducible *tir* expression (pTir). Bacteria treated with indicated concentrations of IPTG for 30 min before infection. LDH release (*n= 3* experiments) and representative immunoblots (bottom) for Tir and DnaK from bacteria lysates are shown.

(B) Representative western blots for indicated proteins from THP1 cells infected for 4 h with Δ*tir* EPEC expressing Tir from a plasmid that allows for IPTG-inducible *tir* expression (pTir). Bacteria were treated with indicated concentrations of IPTG for 30 min before infection. Blots show caspase-1, caspase-4 and IL-18 in culture supernatants and β-actin in cell lysates.

Matching shapes and colours of symbols in graph in (A) denote data from independent experiments, * *P<0.05,* ** *P<0.01,* *** *P<0.001* by one-way ANOVA.

4.8 Deletion of *tir* does not inhibit bacteria attachment or effector translocation

Tir is an essential virulence factor, that is required to mediate attachment to intestinal epithelial cells and induce characteristic A/E legions during infection, with deletion of the *tir* gene resulting in the loss of the A/E phenotype (263) and virulence during *in vivo* models (266, 707). As Tir has previously been shown to be fundamental to bacterial attachment to the host epithelium during infection, it was important to establish whether deletion of *tir* prevented efficient attachment of bacteria to macrophages, because this might account for the observed difference in inflammasome activation and pyroptosis induced during infection. CFU counts of bacteria recovered from THP1 cells infected with WT, *ΔescF* and *Δtir* EPEC strains the 2 h post infection showed no difference between levels of initial attachment to the host cells (Figure 4.9A), indicating that interaction with phagocytic macrophages does not require Tir-mediated intimate attachment. This was consistent with previous studies, which demonstrated *Δtir* EPEC interacted with macrophages in a manner similar to both WT and secretion-deficient EPEC strains, and that attachment to macrophages does not require Tir-mediated signalling (708). Therefore, the observed differences in inflammasome activation and pyroptosis could not be attributed to differences in bacterial numbers or attachment to host cells.

Upon EPEC infection, Tir is the first effector protein injected into host cells via the T3SS and upon translocation Tir inserts itself into the host cell plasma membrane. Within the host cell membrane Tir functions as a receptor for the EPEC outer membrane protein intimin. It has previously been suggested that the subsequent translocation of additional T3SS-effector proteins into host cells is reliant on this initial phase of Tir translocation (196, 709). However, more recently, it has been established that EPEC effectors were efficiently translocated into host cells in the absence of Tir, indicating that Tir is not required for effector translocation during EPEC infection (197). This suggests that the phenotypes observed with the Tir mutant strain were not a consequence of ineffective translocation of other effector proteins. In support of this qRT-PCR was used to demonstrate that EPEC *Δtir* retained effector driven NF-κB inhibition similar to that seen in WT EPEC infections (figure 4.9A). A number of secreted EPEC effector proteins inhibit the critical NF-κB and MAPK signalling cascades to regulate host inflammatory signals downstream of cytokine receptors or pathogen sensing (Figure 1.5). The T3SS mutant *ΔescF* EPEC strain is unable to secrete these effectors into host cells so fails to suppress NF-κB signalling. Accordingly, *ΔescF* EPEC infection of THP1 cells induces markedly higher transcription of the NF- κB-dependent cytokines IL-1β and IL-6 than WT or *Δtir* EPEC infections (Figure 4.9B). This was also made evident by reduced levels of pro-

IL-1B in cell lysates from WT and *Δtir* infections compared to that seen in *ΔescF* infections (Figure 4.7B). These results confirmed that the *Δtir* EPEC mutant strain encoded a functional T3SS and retained the ability to effectively translocate additional effector proteins into host macrophages.

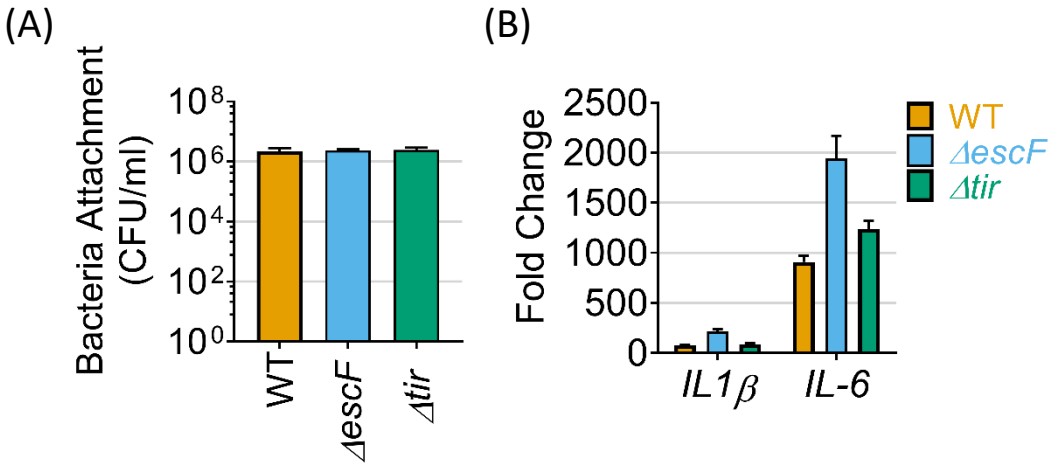

Figure 4.9. Tir is not required for bacterial attachment or effector translocation during infection of THP1 cells

(A) Quantification of bacterial adherence to THP1 cells infected with indicated EPEC strains at an MOI of 10 for 2 h. The percentage of adherent or internalised bacteria was determined by CFU counts from infected cells. Results are presented as mean±SD from two independent experiments.

(B) Graph shows mRNA fold change relative to GAPDH for indicated cytokines from THP1 cells infected with indicated EPEC strains for 5 h. (*n*= 2 independent experiments; Experiments performed with the assistance of Dr Avinash Shenoy).

4.9 Tir expression induces actin polymerisation at the site of infection in THP1 macrophages

In addition to its function as a bacterial receptor Tir serves as a signalling effector, where upon infection intimin-mediated Tir clustering induces recruitment of host adaptors and actin nucleators to the site of infection, activating actin polymerisation. During infection Tir signalling plays an integral role in attachment to intestinal epithelial cells (707, 710). However, the physiological role of Tir signalling in myeloid cells is not well defined. To establish whether Tir signalling induced actin polymerisation in phagocytic macrophages, THP1 cells were infected with WT, *ΔescF* and *Δtir* EPEC strains and actin polymerisation was visualised by immunofluorescent microscopy (Figure 4.10). In agreement with previous research, EPEC-induced actin polymerisation was entirely *tir*-dependent, as both the T3SS (*ΔescF)* and Tir (*Δtir)* mutant EPEC strains failed to induced actin polymerisation at

the site of bacterial attachment; however, both the WT an *Δtir+*pTir strains displayed robust actin polymerisation (Figure 4.10). Importantly, these results demonstrated that Tir induced significant F-actin accumulation beneath attached EPEC during infection of phagocytic macrophage cells. The observed actin polymerisation was not a result of phagocytic cup formation, as the Tir mutant strain, which cannot form actin pedestals (263), showed no significant F-actin rearrangement at the site of bacteria contact.

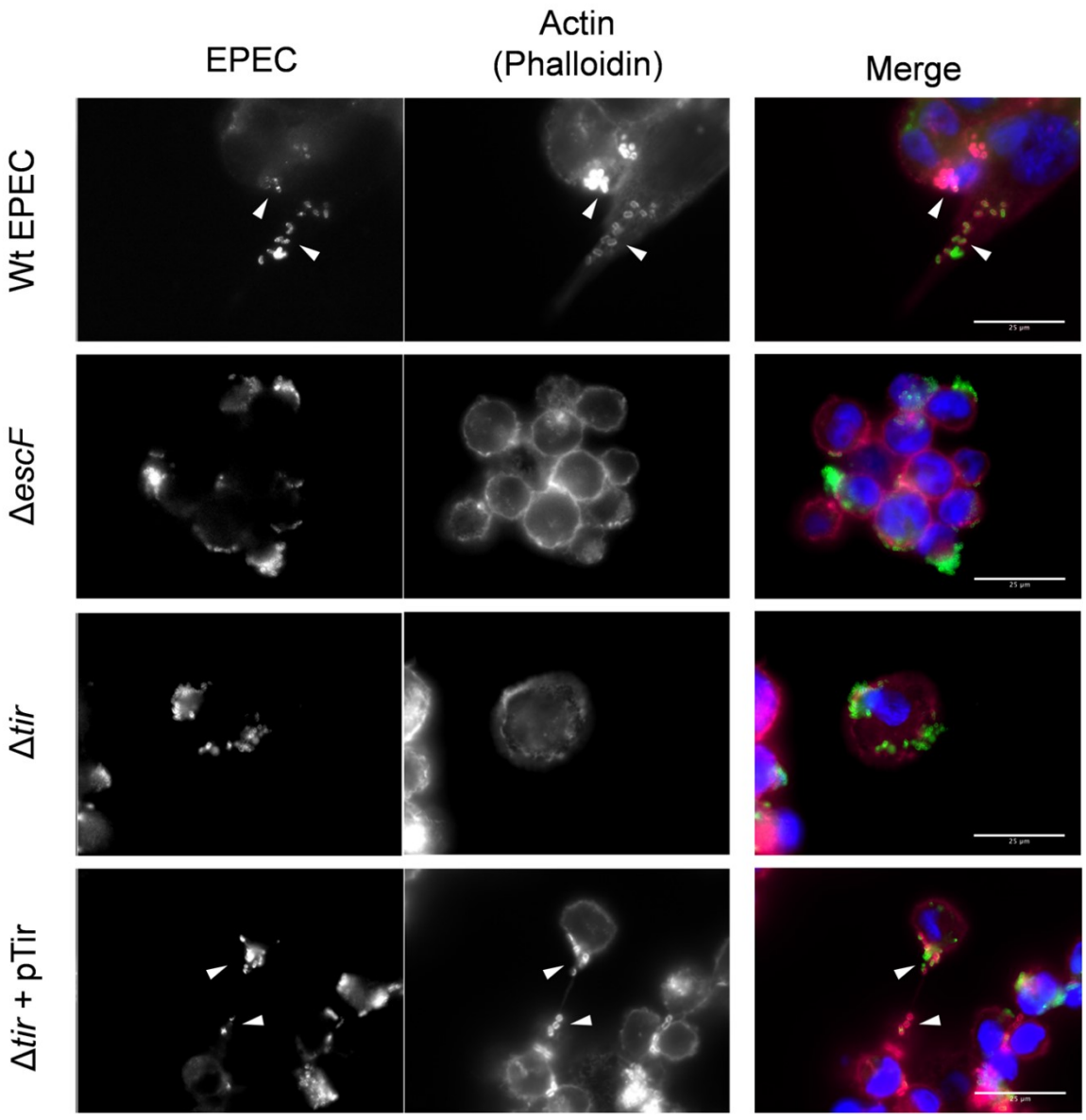

Figure 4.10. Tir induces actin polymerisation during infection of THP1 macrophages
Representative immunofluorescence images of THP1 cells infected with WT EPEC, *ΔescF*, *Δtir* and *Δtir*+pTir EPEC for 2 h. IPTG (0.1 mM) was added 30 min prior to infection to induce Tir expression from pSA10 plasmid (pTir). Bacteria were visualised with anti-O127:H6 antibody (Green), actin was stained with phalloidin (Magenta) and cell nuclei were stained with DAPI (Blue). Arrow heads indicate polymerised F-actin beneath bacteria. Images are representative of 3 experimental repeats. Typically, ~20-50 host cells were imaged from at least 4 randomly selected fields per experiment. Scale bar = 25 µm.

4.10 Secreted effector proteins inhibit phagocytosis of EPEC by THP1 macrophages

It is well established that several EPEC effector proteins including EspF, EspB, EspH, EspG, and EspJ are able to inhibit phagocytosis (216, 221, 624, 711). Previously WT EPEC or EPEC strains lacking only Tir were shown to be phagocytosed less effectively than a T3SS mutant due to the secretion of these effectors (708), supporting a pedestal-independent mechanism of phagocytosis evasion. However, in contrast, more recent research indicated that the capacity of EPEC to polymerise actin into pedestals correlated with resistance to phagocytosis (712), Therefore, to determine if there was a discernible difference in the rate of phagocytosis of WT, *ΔescF* and *Δtir* EPEC strains, differential inside-outside staining of infected THP1 cells was used to quantify the proportions of extracellular versus intracellular bacteria 2 h and 4 h post infection (Figure 4.11A). Extracellular WT and *Δtir* EPEC were both found in greater quantities than the T3SS deficient *ΔescF* strain 2 h and 4 h post infection (Figure 4.11B). Interestingly, there was no significant difference in the ability of WT or the *Δtir* mutant to inhibit internalisation, as approximately 85% remained extracellular 2 h post infection. In contrast, the T3SS mutant strain was internalised at a significantly higher rate. These results indicated that the ability of WT and *Δtir* EPEC to secrete anti-phagocytic effector proteins, rather than Tir-mediated pedestal formation, provided an increased resistance to phagocytosis in THP1 cells. Furthermore, this confirmed that the difference observed in caspase-4 inflammasome activation between WT and *Δtir* EPEC were not a consequence of different quantities of intercellular bacteria.

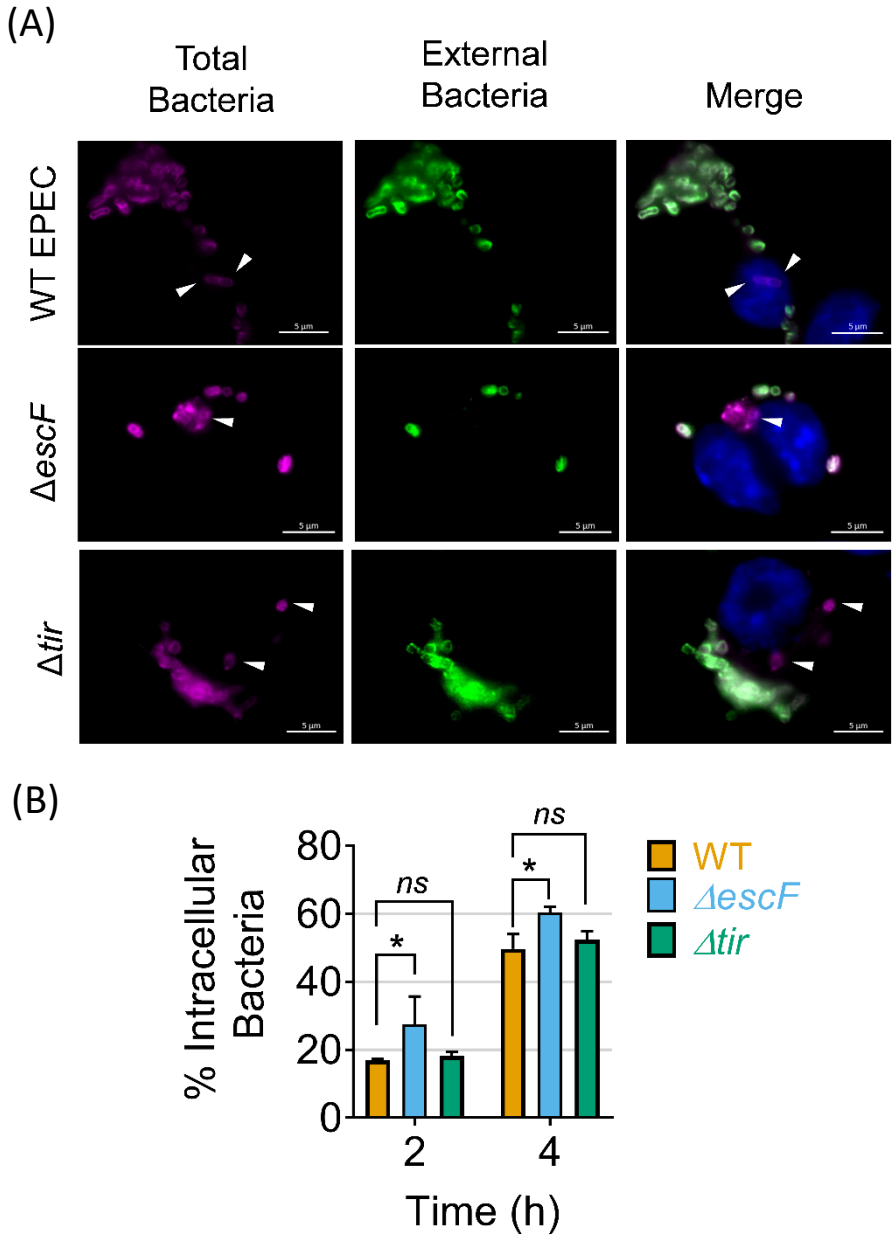

Figure 4.11. Secreted effector proteins inhibit phagocytosis

(A) Representative images from THP1 macrophages infected with indicated EPEC strains for 2 h, fixed, and differentially stained to determine the total number of cell-associated bacteria (purple), and the number of external bacteria (green). Cell nuclei were stained with DAPI (Blue). Scale bar = 5 µm.

(B) The percentage (%) of internalised bacteria from THP1 cells infected with indicated EPEC strains at an MOI of 10. Intracellular bacteria were quantified at the depicted times. Each data point represents the mean ± SEM calculated from >100 cells from 2-3 coverslips, from 3 independent experiments. *$p<0.05$, by two-way ANOVA.

4.11. Intimin-mediated Tir clustering is essential for inflammasome activation in human macrophages

Collectively these results indicate that EPEC Tir is essential for inducing inflammasome activation and pyroptotic cell death in human macrophages. Following delivery of Tir into the host cell, its central domain binds intimin (*eae*), which is also encoded on the LEE. Intimin-mediated Tir clustering is crucial for activating Tir and inducing the signalling events that lead to pedestal formation (263, 624, 713, 714). In fact, translocation or expression of Tir in mammalian cells by itself has no discernible effect on actin architecture (715, 716). Therefore, to establish whether the translocation of Tir alone, or rather downstream intimin-mediated Tir signalling events were responsible for inducing inflammasome activation, an EPEC mutant strain lacking intimin (*Δeae*) was utilised. Intimin deficient EPEC can deliver Tir and other effectors into cultured cells without activating Tir signalling or inducing actin polymerisation (270, 273). Interestingly, deletion of intimin markedly reduced the level of EPEC-induced cell death, as measured by LDH release during EPEC infection of either primary MDMs (Figure 4.12A), or THP1 cells (Figure 4.12B). EPEC-induced non-canonical inflammasome activation was also dependent on intimin, as demonstrated by the absence of cleaved caspase-1 and GSDMD fragments in western blots from THP1 cells infected with either *Δtir* or *Δeae* EPEC strains (Figure 4.12C). The intimin mutant strain was also deficient in inducing ASC-dependent inflammasome assembly (Figure 4.12D). These results suggested that Tir alone was not sufficient to mediate inflammasome activation and cell death, but rather both intimin and Tir had fundamental roles in activating a rapid non-canonical inflammasome in human macrophages.

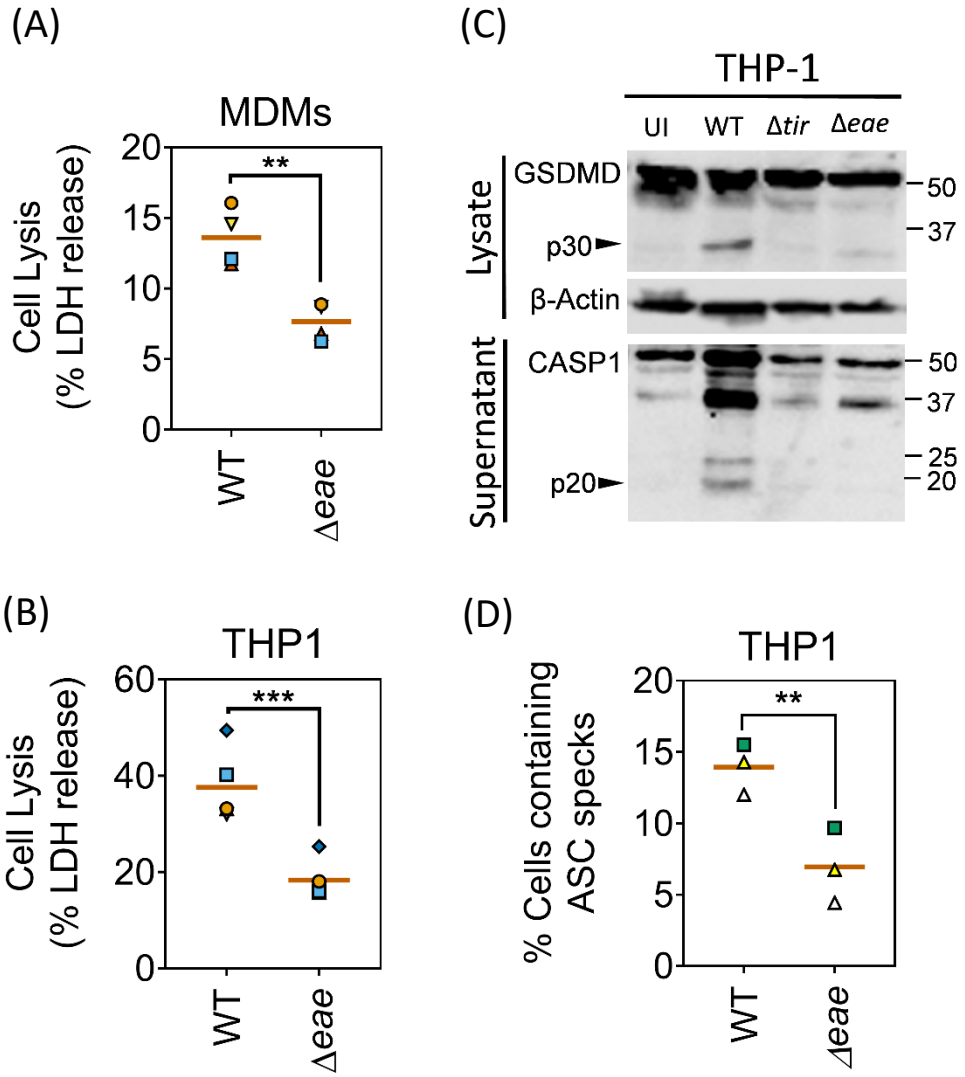

Figure 4.12. Tir-intimin binding is essential for EPEC-induced inflammasome activation in human macrophages

(A) Graph shows cell death measured by LDH release from primary MDMs infected with EPEC (WT) or intimin-deficient EPEC (*Δeae*) at an MOI of 10 for 4 h. (Mean ± SEM for *n*= 4 independent experiments)

(B) Graph shows LDH release from PMA differentiated THP1 cells infected with WT or *Δeae* EPEC at an MOI of 10 for 4 h. (Mean ± SEM for *n*= 4 independent experiments)

(C) Representative immunoblots from THP1 cells infected with WT, *Δtir* or *Δeae* EPEC at an MOI of 10 for 4 h. Blots show caspase-1 in culture supernatant, and GSDMD and β-actin in the cell lysates.

(D) Quantification of inflammasome ASC foci formation in THP1$^{mAsc\text{-}RFP}$ cells infected with indicated EPEC strains at an MOI of 10 for 4 h. ~100-200 host cells were counted from at least 4 randomly selected fields and % cells showing events were obtained for each experiment. (Mean ± SEM for *n*= 3 independent experiments)

Matching shapes and colours of symbols in graphs denote data from independent experiments. Mean indicated by horizonal bars. ** *P<0.01* *** *P<0.001* by two-tailed paired Student's *t*-test. Immunoblots are representative of experiments performed 3 times.

4.12. Tir induces non-canonical inflammasome activation independently of other EPEC effector proteins

In this study EPEC infections were shown to induce a novel inflammasome activation pathway in human macrophages, in which inflammasome activation is dependent on the EPEC effector proteins Tir and intimin. The next question was to establish whether the interaction between Tir and intimin alone was sufficient to induce inflammasome activation, or whether other EPEC virulence factors play an essential role in this pathway. Ectopic expression of Tir has previously been shown to be inefficient, as mammalian-expressed Tir fails to effectively localise to the plasma membrane and has severely reduced signalling efficiency (283). Furthermore, Tir expressed in mammalian cells does not undergo full modification and is not efficiently phosphorylated (301). As the results here have demonstrated that Tir interaction with intimin is essential in generating inflammasome activation, Tir localisation to the plasma membrane is required, meaning ectopic expression of Tir in human cells could not be utilised to investigate this pathway.

Therefore, to assess the role of the Tir-intimin axis independently of additional EPEC virulence genes a number of synthetic *E. coli* K12 injector (SIEC) strains were used (Figure 1.3, (302)). Inflammasome activation was assessed in THP1 cells infected with various strains: SIEC, which expresses the EPEC T3SS in the absence of any additional EPEC virulence factors; SIECeLEE5, which expresses a functional T3SS and eLEE5 (encoding: Tir, CesT and intimin), and SIECΔp1eLEE5, which expresses a promoter-less eLEE1 and therefore cannot assemble a functional T3SS (Figure 4.13). Similarly to WT EPEC, infection of THP1 cells with SIECeLEE5 induced rapid pyroptosis, which was markedly diminished in the absence of eLEE5 that encodes Tir, its chaperon CesT and intimin (eae) (Figure 4.13A). Furthermore, western blot analysis of THP1 cells infected with EPEC and SIEC strains demonstrated that the SIECeLEE5 strain induced caspase-1 and caspase-4 activation, GSDMD cleavage and cytokine processing to a similar degree as WT EPEC infection (Figure 4.13B). Inflammasome activation was dependent on the presence of a functional T3SS as SIECΔp1eLEE5 failed to stimulate caspase cleavage (Figure 4.13B). This confirmed that Tir signalling was both necessary and sufficient for inflammasome activation in human macrophages, even in the absence of other EPEC virulence factors.

Interestingly, western blot analysis of SIEC infected THP1 cells, but not SIECΔp1eLEE5, revealed the presence of active caspase-1 (Figure 4.14B), this suggested that in the absence of other EPEC virulence proteins the T3SS alone is able to induce moderate caspase-1 activation and pyroptosis,

although pyroptosis and GSDMD cleavage induced by the SIEC strain was significantly lower than the Tir and intimin expressing SIECeLEE5 bacteria (Figure 4.14A).

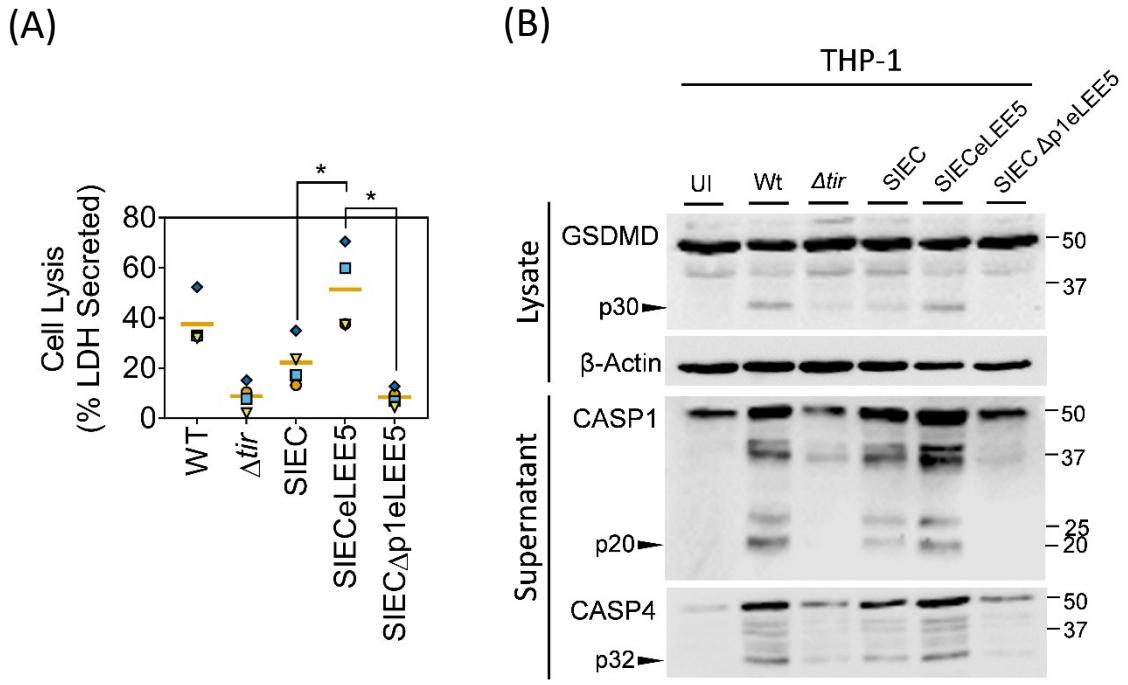

Figure 4.13. Expression of Tir and intimin is sufficient to induce pyroptosis and inflammasome activation

(A) LDH release assay from THP1 cells infected with synthetic injector *E. coli* (SIEC) strains or WT or Δ*tir* EPEC at an MOI of 10 for 4 h. SIEC strains were treated with (0.1 mM) IPTG for 30 min prior to infection to induce the expression of eLEE operons in SIEC strains. (*n= 5* independent experiments)

(B) Western blots of indicated proteins from THP1 cells left uninfected (UI), or infected with WT or *Δtir* EPEC, or indicated SIEC strains at an MOI of 10 for 4 h. SIEC strains were treated with (0.1 mM) IPTG for 30 min prior to infection to induce the expression of eLEE operons.

Matching shapes and colours of symbols in graphs denote data from independent experiments. Immunoblots represent 2 independent experiments. * *P<0.05*, by one-way ANOVA.

The observation that SIEC infections induced caspase-1 activation in the absence of Tir and intimin was unexpected, because previous results demonstrated that during EPEC infections the presence of a functional T3SS in the absence of either Tir or intimin was not sufficient to induce inflammasome activation, GSDMD cleavage or pyroptosis (Figure 4.7A-B 4.12C). This implies that another EPEC effector may block this T3SS-pathway. To further characterise the inflammasome pathways activated by SIEC bacteria CTRL^{miR}, *CASP4*^{miR} and *ASC*^{miR} THP1 cells were infected with SIEC, SIECeLEE5 and SIECΔp1eLEE5, and pyroptosis was measured by LDH release. Unsurprisingly,

the SIECΔp1eLEE5 failed to induce cell death (Figure 4.14). However, infections with SIECeLEE5 strain, induced rapid pyroptosis in THP1 cells that was dependent on both caspase-4 and ASC (Figure 4.14). This was consistent with the type of cell death induced by WT EPEC infections and verified a mechanism by which Tir and intimin induce an atypical non-canonical inflammasome, that requires both caspase-4 and ASC to induce pyroptosis. Interestingly, although infections with the SIEC strain induced significantly less cell death than the SIECeLEE5 strain (approx.- 12% vs 37%), the cell death induced by SIEC was mechanistically distinct from SIECeLEE5-induced pyroptosis. SIEC infections induced caspase-4-independent, but ASC-dependent cell death in THP1 macrophages (Figure 4.14). These results indicate that SIEC activates a canonical ASC-dependent inflammasome pathway in THP1 macrophages that is driven by the T3SS. Importantly, this T3SS inflammasome activation was much less prominent than Tir induced pyroptosis, and was only evident in SIEC infections, not during infections with *Δtir* or *Δeae* EPEC that expressed functional T3SS (Figure 4.7A-B 4.12C). This suggests that additional EPEC encoded proteins may function to suppress this secondary canonical inflammasome pathway downstream of the T3SS during WT EPEC infections.

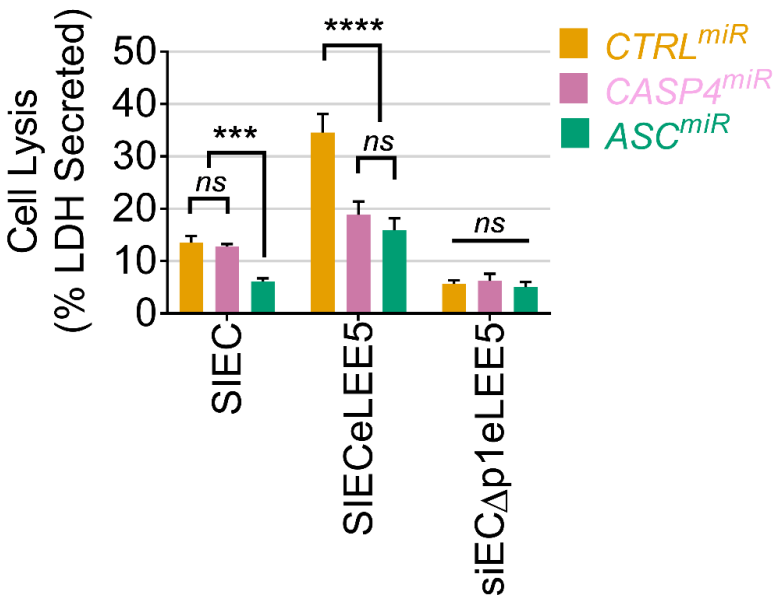

Figure 4.14. Tir and intimin expression by non-pathogenic K12 *E. coli* induces caspase-4- and ASC-dependent pyroptosis in THP1 macrophages

Cell lysis as measured by LDH release from THP1 cells stably transduced with non-targeting miRNA (CTRL^{miR}) or miRNA targeting CASP4 (*CASP4*^{miR}) or ASC (*ASC*^{miR}), infected with indicated synthetic injector *E. coli* (SIEC) strains, at an MOI of 10 for 4 h. Graph shows mean ± SEM (*n= 3* independent experiments). ****p<0.001, ****p<0.0001* by two-way ANOVA.

Discussion

Collectively the data presented in this study has demonstrated that early macrophage pyroptosis relies entirely on the expression of the EPEC virulence regulon, which not only accelerated caspase-4-dependent inflammasome activation, but also led to atypical pyroptosis. By culturing EPEC in conditions that stimulate physiological effector secretions prior to infection, a novel inflammasome pathway was identified. However, in agreement with previously published work (123, 497, 504, 571, 639), culturing EPEC in LB prior to infection induced delayed inflammasome activation late in the course of infection (~18 h) (Figure 4.1, 4.2). This was most likely a consequence of endocytosis of LPS-containing OMVs, that activate caspase-4 to induce pyroptosis, as reported previously (532, 533). Notably, there was no dependence on the T3SS in these conditions. Consistent with this, the results here demonstrate that WT and *ΔescF* EPEC strains induce comparable levels of pyroptosis in THP1 cells when cultured in LB prior to infection. In this context, both WT and *ΔescF* EPEC effectively function as non-pathogenic *E. coli* strains, due to insufficient transcription of the T3SS and other Ler encoded virulence genes. This may explain why pathogenic enterobacterial *E. coli* strains cultured in LB have previously been reported to activate the non-canonical inflammasome in a similar manner to non-pathogenic *E. coli* during infection of mouse macrophages (497, 532, 543).

Previously, the T3SS has been shown to have a direct physiological role in mediating phagosomal escape of intracellular Gram-negative bacterial pathogens (717-719). After internalisation pathogens can shed PAMPs including LPS from their cell surface into the cytosol where it directly binds to the CARD domain of caspase-4, initiating oligomerisation and activation (496, 498, 720). However, the role the T3SS has in facilitating LPS translocation into the host cell cytosol during infection with extracellular or vacuolar bacterial pathogens such as EPEC and EHEC has yet to be established. It has previously been suggested that pore formation by bacterial toxins, such as the needle component of the T3SS, could induce K^+ efflux that leads to NLRP3 inflammasome activation and caspase-1-mediated inflammatory responses (721). Another possibility was that LPS could be actively secreted into host cells via the T3SS to activate caspase-4. However, the results obtained here establish that during EPEC infections the T3SS alone is not responsible for inducing inflammasome activation, rather the T3SS effector protein Tir was shown to be essential for EPEC-mediated activation of the caspase-4 non-canonical-NLRP3 inflammasome in human macrophages (Figure 4.5-4.8). Interestingly, the bacterial outer membrane protein intimin, which mediates Tir

clustering and tyrosine phosphorylation, was also necessary (Figure 4.12). Translocation of Tir during infection is not affected by the absence of intimin (197), thus, both Tir and intimin are required to propagate inflammasome responses. As Tir- and intimin- deficient EPEC strains retain the ability to express a functional T3SS, inflammasome activation during WT EPEC infection could not be directly attributed to the T3SS pore or secretion of LPS moieties.

Tir and intimin have an essential role in mediating the intimate attachment of bacteria to host epithelial cells during infection. However, in addition to interactions with enterocytes, EPEC also interacts with infiltrating phagocytic immune cells such as macrophages, neutrophils, and dendritic cells (131, 342, 461, 722). The role of both Tir and intimin have been extensively characterised in the context of EPEC infection in epithelial cells, but their function during infection of macrophages has not been well established. As Tir is highly conserved among A/E pathogens and is an essential virulence determinant, it is an attractive target for the host immune system. However, the mechanism by which Tir induces caspase-4-dependent inflammasome activation in macrophages remains to be established. The LPS binding capacity of caspase-11 was shown to be required for inflammasome activation in response to EPEC, suggesting that the activity of Tir and intimin may function to promote the cytosolic access of LPS during infection. The mechanism by which Tir and intimin may mediate this cytosolic access to LPS, however remains unclear. The results in this study demonstrated that bacterial internalisation did not correlate with macrophage survival as the *Δtir* strain that was markedly defective in inducing pyroptosis, was phagocytosed similarly to WT EPEC (Figure 4.11). However, the methods used to establish the level of bacterial internalisation in the project are limited by the ability to efficiently distinguish individual EPEC bacterium. EPEC expresses BFP which causes characteristic microcolony formation, and this makes visually identifying individual bacterium challenging. Furthermore, inside-outside staining does not differentiate between live and dead bacterium, therefore, to more effectively address the possibility that live EPEC can escape the vacuole in a T3SS- or Tir-dependent manner antibiotic protection assays are required.

EPEC is typically an extracellular pathogen and secretes a number of effector proteins to inhibit phagocytosis during infection, meaning the percentage of internalised bacteria is typically lower compared with non-pathogenic *E. coli* strains (624). However, whether the small percentage of internalised bacteria reside within the vacuole or escape into the cytosol during infection has not been well established and requires further investigation. Importantly, more recent work by our lab has demonstrated that the vast majority (~95%) of intracellular EPEC remain vacuolar during

infection of human macrophages, and the expression of Tir does not alter the rate of vacuole escape during EPEC infection (Slater *et al.,* Unpublished data). These findings suggest that the differential internalisation or vacuolar escape are not responsible for the difference in pyroptosis induced by WT and *Δtir* strains. Tir-induced caspase-4 activation must, therefore, be mediated through a novel mechanism. As such it is currently unclear how EPEC LPS is available to caspase-4 in the cytoplasm. Further research is required to characterise this mechanism. One possibility is that guanylate binding proteins (GBPs) accelerate the recognition of LPS without releasing bacteria from the vacuole, as has previously been demonstrated with *Chlamydia muridis* (527, 531). In the context of *C. muridis* infections, GBPs enhance the detection of LPS by caspase-4, and therefore the role of GBPs in EPEC recognition should be investigated further through use of specific inhibitors and gene silencing. Additionally, although the expression of Tir and intimin does not alter the rate of vacuole escape during EPEC infection (Slater *et al.,* Unpublished data), whether Tir:intimin mediated intimate attachment of bacteria to the host cell membrane facilitates the translocation of LPS aggregates or OMVs into the host cytosol during infection has not been experimentally tested. Further work using high-resolution confocal microscopy and transmission electron microscopy (TEM) to visualise the sub-cellular location of LPS-complex during EPEC infections, as described previously (723), would provide valuable insights into this activation pathway.

A/E pathogens typically secrete 25-30 effectors which subvert various host processes, including phagocytosis, activation of NF-κB, MAPKs, type I interferons and cell death (Table 1.3). Among these, Tir is the first effector translocated into host cells during infection and has been reported to impact T3SS effector translocation into HeLa cells (197, 724). Although Tir-deficient strains retain the ability to translocate all other known effector proteins, there is some evidence that effectors are translocated at non-physiological levels in Tir-deficient strains, resulting in differences in over-all receptor abundance in host cells during infection (197, 724). Therefore, it becomes difficult to directly attribute the loss of inflammasome activation to the absence of Tir, as the indirect effects associated with the altered translocation of other effector proteins could potentially confound results. To mitigate these, the ability of Tir-deficient strains to target other effector regulated host responses in macrophage cells was established, including inhibition of NF-κB transcriptional pathways and phagocytosis, and was shown to be consistent with that of WT EPEC (Figure 4.9, 4.11). This indicated that the secretion of other effector proteins was not significantly altered in the absence of Tir, suggesting that Tir itself, or Tir-dependent signalling pathways were responsible for inducing inflammasome responses in macrophages. This was confirmed using SIEC strains, in which

the presence of the T3SS, Tir, and intimin in the absence of any other EPEC associated factors was sufficient to induce rapid caspase-4-dependent non-canonical inflammasome activation and, caspase-4- and ASC-dependent pyroptosis (Figure 4.13, 4.14).

Interestingly, infections of macrophages with SIEC strains expressing only a T3SS were shown to induce a detectable level of pyroptosis, that was not evident in EPEC strains expressing the T3SS in the absence of Tir and intimin. This indicated that EPEC encodes additional factors that function to subvert T3SS-induced toxicity. The mechanism of cell death induced by the T3SS was distinct from that induced by Tir and intimin during EPEC infections, as there was no dependency on caspase-4 (Figure 4.14). T3SS-induced cell death was dependent on ASC, and may, therefore, be mediated by the canonical NLRP3 inflammasome. However, other ASC-dependent inflammasome pathways cannot yet be ruled out. There are a number of EPEC encoded proteins that potentially function to inhibit this T3SS toxicity. One candidate is the EPEC SPATE family effector, EspC, which is expressed by EPEC, but not the SIEC strains. Previous research has demonstrated that the EPEC EspC targets EspA-EspD subunits of the T3SS translocon upon host cell-contact. This effectively down-regulates pore formation and reduces EPEC-induced cytotoxicity in epithelial cells (75). Although the authors of this study did not identify the mechanisms of cytotoxicity and indeed whether this is inflammasome-dependent pyroptosis, these results in combination with my work support a model in which the T3SS pore has the potential to activate NLRP3 by promoting K^+ efflux, while EspC functions to regulate this pore-formation and downstream pyroptosis during WT EPEC infections.

EPEC secretes a number of non-LEE encoded effector proteins that have been reported to manipulate immune responses. In particular, NleF has previously been shown to inhibit caspase-4 in human and mouse IECs (228). NleF may, therefore, function to limit caspase-4 activity during EPEC infection of macrophages, and this could potentially explain why caspase-4 activity alone was not sufficient to mediate efficient GSDMD cleavage and pyroptosis. However, as Tir-induced inflammasome activation during SIEC infections, which do not express NleF, was also dependent on both caspase-4 and ASC, it is likely that other mechanisms are responsible for this atypical pathway. Additionally, the EPEC effector protein NleA can inhibit NLRP3 inflammasomes in human THP1 cells (226). In naïve cells, ubiquitylation restrains NLRP3 activity, which is relieved by its deubiquitylation by BRCC3 (495). NleA was shown to interact with NLRP3 impairing its deubiquitylation, and consequently reducing oligomeric inflammasome formation and downstream IL-1β secretion (226). However, this study utilised a TOE-A5 E2348/69-derivative strain that was deleted for all effector proteins encoded on the PP6 PAI (*nleH*/ *nleA*(*espI*)/ *nleF*/ *espO*), and complemented this strain with

plasmid-encoded NleA to characterise the effector function (226). Plasmid-mediated expression of effector proteins during EPEC infections is often associated with over-expression and increased effector translocation into host cells, which may amplify effector phenotypes and create confounding artefacts. Indeed, in macrophages, only the combined deletion of NleF and NleA (and NleH & EspO as with the ΔPP6 mutant) increased EPEC-induced pyroptosis (Figure 4.4). Deletion of NleA or NleF in isolation did not induce a significant increase in toxicity (data not shown). As both caspase-4 and NLRP3 function in a single activation cascade to induce pyroptosis upon EPEC infection, it follows that the inhibitory function of these effectors is only relieved when both are removed.

Although the inhibitory function of these effector proteins has been established, EPEC infections still induced robust inflammasome activation in human macrophages. It is likely that a higher abundance of caspase-4 and NLRP3 in macrophages reduces the inhibitory effects of individual EPEC effectors, compared to that reported for infection of IEC or when effectors are over-expressed on plasmids. Furthermore, EPEC effectors have distinct kinetics of effector translocation and reach different maximal levels in the host cells (197). During EPEC infections Tir is the principal effector proteins injected into host cells, and it is translocated at a higher rate than any other effector (197). Whereas the translocation efficiency of chromosomally encoded NleA and NleF are significantly lower. Effector translocation efficiencies directly correlate with the temporal order of translocation (197). Therefore, Tir, as the primary effector protein secreted into host cells, can mediate rapid inflammasome activation early during infection, whereas the physiological effects of NleA and NleF may only be apparent later in infection when the concentration of these inhibitors within host cells reaches a certain threshold. Interestingly, cells that do not undergo Tir-mediated pyroptosis were found to undergo a late phase of cell-autonomous necroptosis. The secretion of these inhibitory effectors may contribute the switch in cell death mechanism in a time-dependent manner. Plasmid-mediated over-expression of NleA and NleF during EPEC infection could potentially alter the dynamics of this pathway and inhibit Tir-dependent non-canonical inflammasome activation, however, this remains to be experimentally verified.

During EPEC infection, the interaction of Tir and intimin have been shown to regulate a range of processes, including intimate attachment to host cells, the formation of attaching and effacing lesions, and localised actin assembly. However, the mechanism by which Tir and intimin function to induce caspase-4 activation remains to be elucidated. This will be investigated further in Chapter 5.

Chapter 5 –

Tir-induced actin polymerisation triggers rapid inflammasome activation in human macrophages.

Introduction

Collectively the results in this study have established that LEE-encoded Tir and intimin are both required and sufficient to activate a rapid caspase-4-dependent non-canonical inflammasome pathway in primary human monocyte-derived macrophages. Within host cells, Tir serves two principle functions: tethering the bacteria to the host cell and providing a direct connection to the host's cytoskeleton. Tir is an essential virulence determinant during EPEC infection. During EPEC infection Tir is integrated into the host cell plasma membrane in a hairpin loop conformation, and functions as a receptor for intimin (261, 271). This interaction between Tir and intimin forms characteristic A/E lesions which firmly anchor the bacterium to the host cell facilitating infection (272, 287). Within the host cell plasma membrane Tir also functions as a signalling effector and coordinates the recruitment of numerous host adaptor proteins and actin nucleators to trigger localised actin assembly at the host cell membrane (269, 274).

It was initially believed that the Tir-induced actin signalling pathways were also responsible for A/E lesion formation. This has subsequently been challenged because the tyrosine residues required for actin polymerisation are dispensable for bacterial attachment and A/E lesion formation on mucosal surfaces, both in EPEC infections of human tissue (265), and *in vivo C. rodentium* infections (312). Additionally, an EPEC mutant strain expressing intimin and Tir, but lacking all other LEE effectors, has been shown to trigger robust actin polymerisation in cultured cells but was unable to form A/E lesions on human intestinal *in vitro* organ cultures (68), suggesting that the physiological role of Tir-mediated actin polymerisation is currently not well defined.

During *in vivo* infections however, Tir-induced tyrosine-dependent actin polymerisation has been shown to have a direct role in the modulation of immune responses. Despite being dispensable for colonisation, Tir tyrosine residues Y474 and Y54 (Y471 and Y451 in *C. rodentium*) were required to propagate normal inflammation and neutrophil recruitment in response to *C. rodentium* infection (313). Therefore, the next aim of this study was to establish whether Tir-induced actin polymerisation pathways were required for inflammasome activation in human macrophages. Additionally, although Tir is highly conserved within A/E pathogens, EPEC and EHEC Tir use divergent mechanisms of signalling to induce actin polymerisation during infection (725, 726). Therefore, whether EHEC Tir is also able to activate non-canonical inflammasome signalling in human macrophages was investigated.

Results

5.1 EPEC Tir-induced actin polymerisation is required for inflammasome activation

Previous work has established that binding of Tir by intimin induces Tir clustering, leading to downstream signalling events that result in the formation of actin-rich structures underneath adherent bacteria (283). However, the work in this study has identified a novel outcome of Tir-intimin interactions in inducing rapid non-canonical inflammasome activation in human macrophages. In order to test the hypothesis that Tir-induced actin polymerisation is required for inflammasome activation, THP1 cells were infected with EPEC expressing chromosomally mutated $Tir^{Y454A/Y474A}$ (TirAA) (604), which can interact with intimin, but cannot recruit N-WASP to the sight of infection, or activate the actin polymerisation cascade in THP1 cells (Figure 5.1).

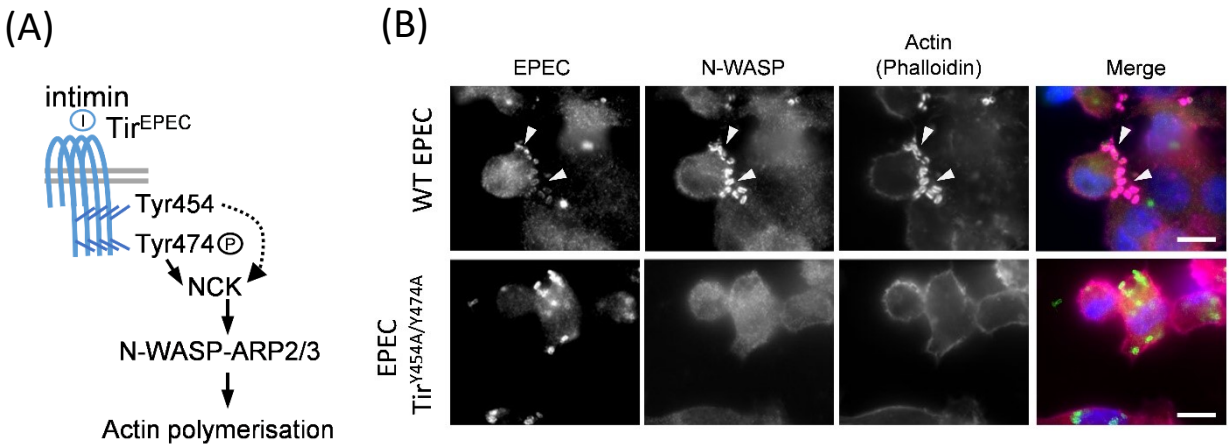

Figure 5.1. Tir tyrosine residues Y474 and Y454 mediate actin polymerisation in THP1 macrophages

(A) Schematic showing the mechanisms of N-WASP and ARP2/3-dependent actin polymerisation induced by Tir tyrosine residues Y474 and Y454 upon clustering by intimin.

(B) Representative images from immunofluorescence microscopy of THP1 cells infected with WT EPEC and EPEC expressing Tir tyrosine mutant $Tir^{Y454A/Y474A}$ at an MOI of 10 for 2 h. Bacteria were visualised with anti-O127:H6 antibody (green), N-WASP was stain with anti-WASL antibody (red) and actin was stained with phalloidin (magenta). Arrowheads indicate N-WASP recruitment to the site of infection and bacteria with actin-rich pedestals. Images are representative of three independent repeats. Scale bar = 20μm.

Mutation of these tyrosine residues, therefore, provided a method to genetically uncouple Tir delivery and intimin binding from Tir-induced actin polymerisation during infection. To establish the role of the Tir-mediated actin polymerisation cascade in inflammasome activation, primary human MDMs were infected with WT EPEC expressing Tir or the TirAA mutant. Remarkably, EPEC expressing Tir tyrosine mutants Y454A/Y474A induced significantly less pyroptosis in primary MDMs than WT EPEC (~77% reduction; Figure 5.2A), and similarly to the *Δtir* mutant strain, was unable to activate caspase-1 upon infection (Figure 5.2B). Consistent with these results, pyroptosis was also attenuated in THP1 cells (~75% reduction; Figure 5.2C-D), and both inflammasome activation and downstream cytokine processing was entirely dependent on the Tir tyrosine residues Y454 and Y474 (Figure 5.2E). These findings pointed to an essential role for Tir-driven actin polymerisation in inflammasome activation.

To further investigate the role of actin polymerisation, EPEC strains expressing individual tyrosine mutants Tir^{Y454A} (Tir AY) and Tir^{Y474A} (Tir YA) were utilised. Mutation of tyrosine residue Y474, the principle residue responsible for actin polymerisation, prevented EPEC-induced pyroptosis and inflammasome activation to a similar extent as the *Δtir* and TirAA EPEC strains, in both primary MDMs (Figure 5.3A-B) and THP1 cells (Figure 5.3C-D). Mutation of tyrosine Y454 (Tir AY), also reduced EPEC-mediated pyroptosis and inflammasome activation, although to a lesser extent than the Y474 mutant (Figure 5.3), suggesting that the tyrosine residue Y454 has an important but not essential role in inflammasome activation.

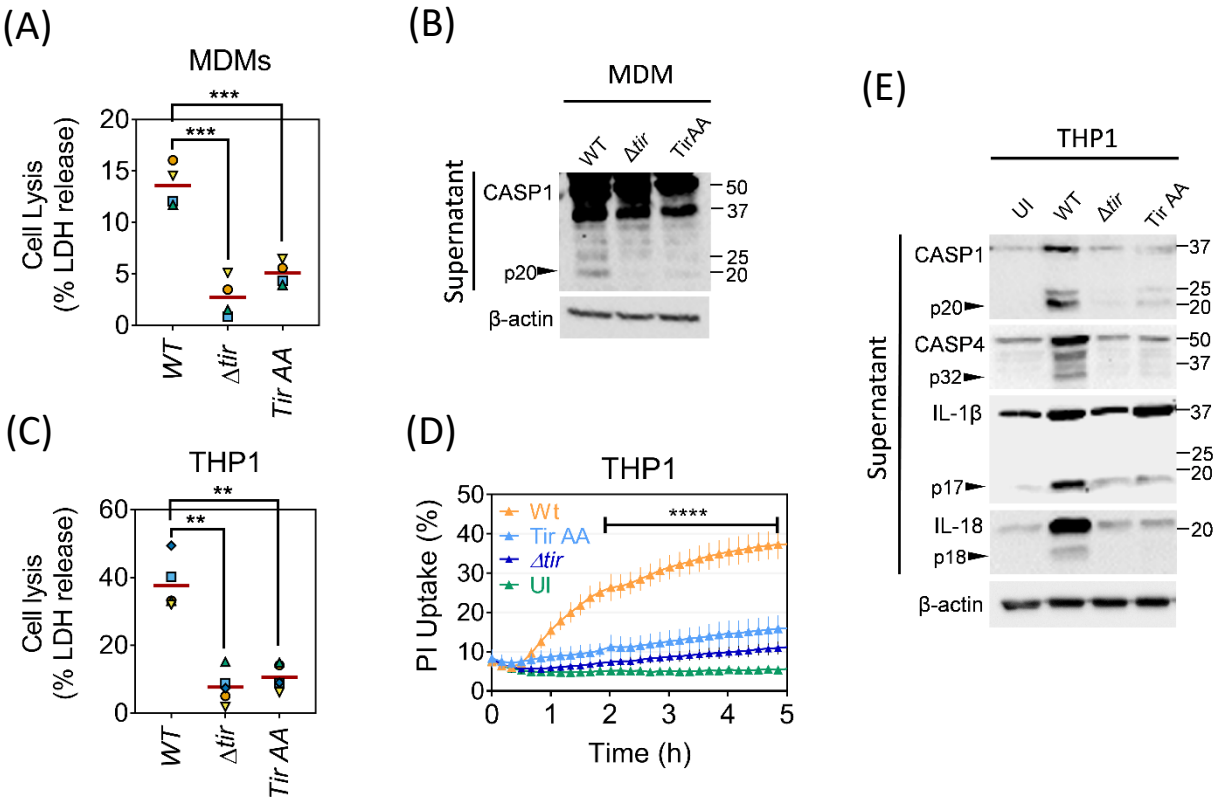

Figure 5.2. Tir tyrosine residues Y454 and Y474 are required for EPEC-induced inflammasome activation in human macrophages

(A) Cell lysis measured by LDH release of primary human MDMs infected with WT EPEC, *Δtir* EPEC, and EPEC expressing Tir$^{Y454A/Y474A}$ (TirAA), at an MOI of 10 for 4 h. (*n* = 4 independent donors). ** *P<0.01* *** *P<0.001* by one-way ANOVA.

(B) Representative western blots from primary human MDMs infected with WT EPEC, *Δtir* EPEC, and EPEC expressing Tir$^{Y454A/Y474A}$ (TirAA), at an MOI of 10 for 4 h. Blots show caspase-1 in cell supernatants, and β-actin in cell lysates. Blots are representative of 3 independent repeats from independent donors.

(C) Cell lysis measured by LDH release from THP1 cells infected with indicated EPEC strains at an MOI of 10 for 4 h. (*n*= 5 independent repeats). ** *P<0.01* by one-way ANOVA.

(D) Real-time PI uptake measured over 5 h from THP1 cells infected with indicated EPEC strains at MOI:10. Graphs show mean ± SEM; *n= 6* independent experiments. **** *P<0.0001* by two-way ANOVA with FDR-based correction for multiple comparisons for indicated comparisons between WT and other strains.

(E) Representative western blots from THP1 cells, either left uninfected (UI) or infected with WT EPEC, *Δtir* EPEC, and EPEC expressing Tir $^{Y454A/Y474A}$ (TirAA), at an MOI of 10 for 4 h. Blots show caspase-1, caspase-4, IL-1β and IL-18 in cell supernatants, and β-actin in cell lysates. Blots are representative of 4 independent repeats.

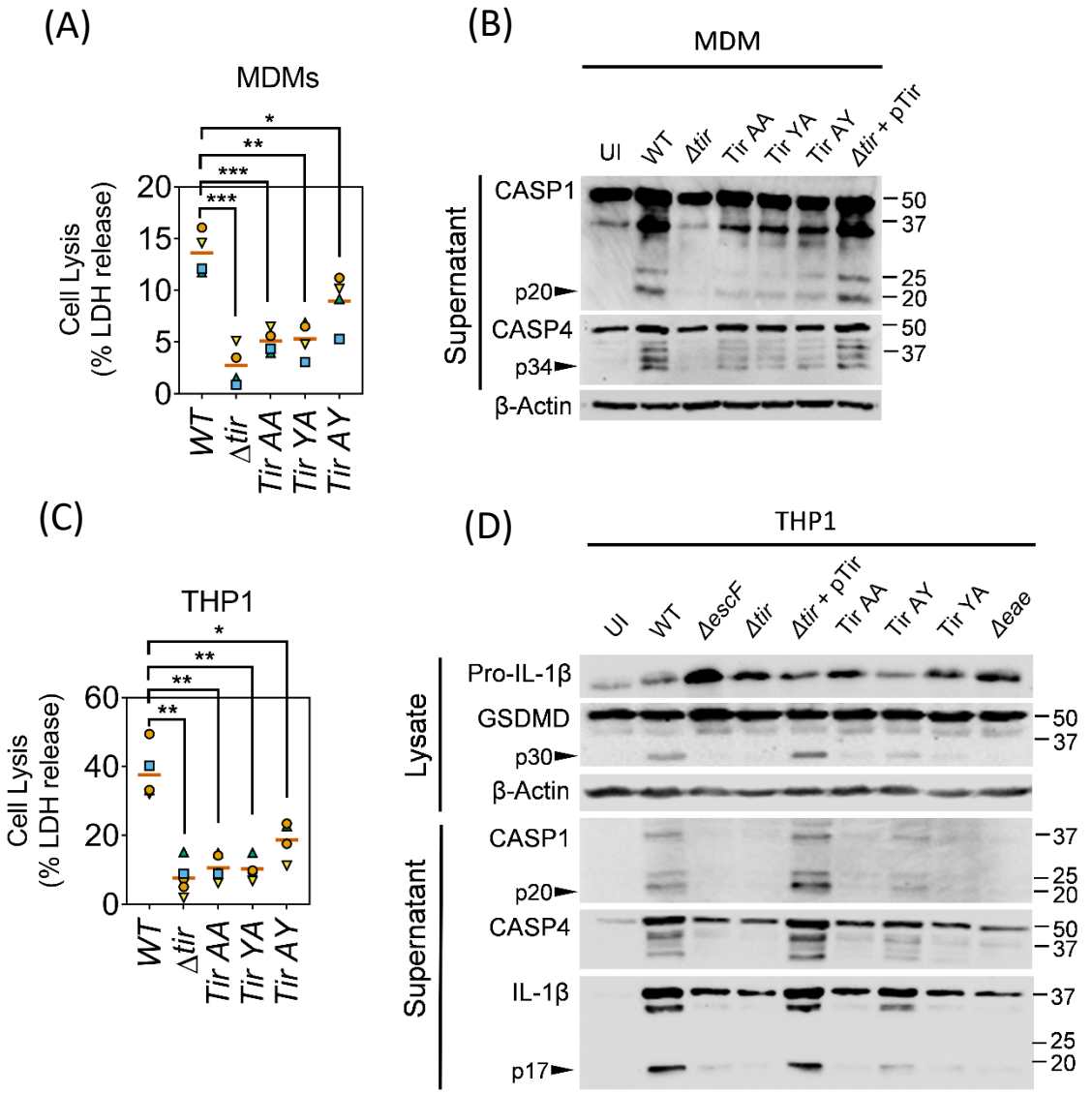

Figure 5.3. Tir residues Y474 and Y454 have important roles in inflammasome activation in human macrophages

(A) Cell lysis measured by LDH release of primary human MDMs infected with WT EPEC, *Δtir* EPEC, and EPEC expressing Tir$^{Y454A/Y474A}$ (TirAA), Tir^{Y474A} (Tir YA) or Tir^{Y454A} (Tir AY), at an MOI of 10 for 4 h. (*n* = 4 independent repeats from independent donors). * *P<0.05* ** *P<0.01* *** *P<0.001* by one-way ANOVA.

(B) Representative western blots from primary human MDMs infected with indicated EPEC strains at MOI: 10 for 4 h. IPTG (0.1 mM) was added 30 min prior to infection to induce Tir expression from pSA10 plasmid (pTir). Blots show caspase-1 and caspase-4 in cell supernatants, and β-actin in cell lysates. Blots are representative of 2 independent repeats.

(C) Cell lysis measured by LDH release from THP1 cells infected with indicated EPEC strains at an MOI of 10 for 4 h. (*n*= 4-6 independent repeat). . * *P<0.05* ** *P<0.01* by one-way ANOVA.

(D) Representative western blot from THP1 cells, either left uninfected (UI) or infected with indicated EPEC strains at an MOI of 10 for 4 h. Blots show caspase-1, caspase-4, IL-1β and IL-18 in cell supernatants, GSDMD and β-actin in cell lysates. Blots are representative of 3 independent repeats.

5.2 EPEC and EHEC Tir-dependent actin polymerisation drives inflammasome activation, pyroptosis and cytokine processing

The results presented earlier have demonstrated that the Tir C-terminal tyrosine residues responsible for mediating actin polymerisation (Y454 and Y474) were also required to induce rapid non-canonical inflammasome activation during EPEC infection of human macrophages. This suggested that actin polymerisation downstream of Tir signalling was required to induce inflammasome activation and pyroptosis. However, this strategy utilises mutated Tir constructs, therefore inhibition of alternative Tir tyrosine-dependent signalling pathways could not be discounted. The next aim was to functionally separate Tir from the downstream actin polymerisation cascade. To do this I utilised Tir from EHEC O157:H7. During EPEC infections the clustering of EPEC Tir in the plasma membrane of host cells is sufficient to induce actin pedestal formation (283), however, EHEC Tir translocation alone is not sufficient to induce actin polymerisation. EHEC Tir signalling required an additional effector protein; TccP (Figure 3.4A). This provides a model in which the presence and absence of TccP can functionally separate Tir from the downstream actin polymerisation pathway.

The effector protein TccP is located within prophage CP-933U, which is present in EHEC but not EPEC E2348/69. Therefore, complementing the EPEC *Δtir* mutant with a plasmid encoding Tir^{EHEC} could not restore N-WASP recruitment or the subsequent actin polymerisation during infection of THP1 macrophages (Figure 5.4B). However, co-expression of Tir^{EHEC} and TccP in EPEC *Δtir* restored both N-WASP recruitment and actin polymerisation at the site of infection. Expression of TccP in the absence of Tir was not sufficient to induce actin polymerisation (Figure 5.4B).

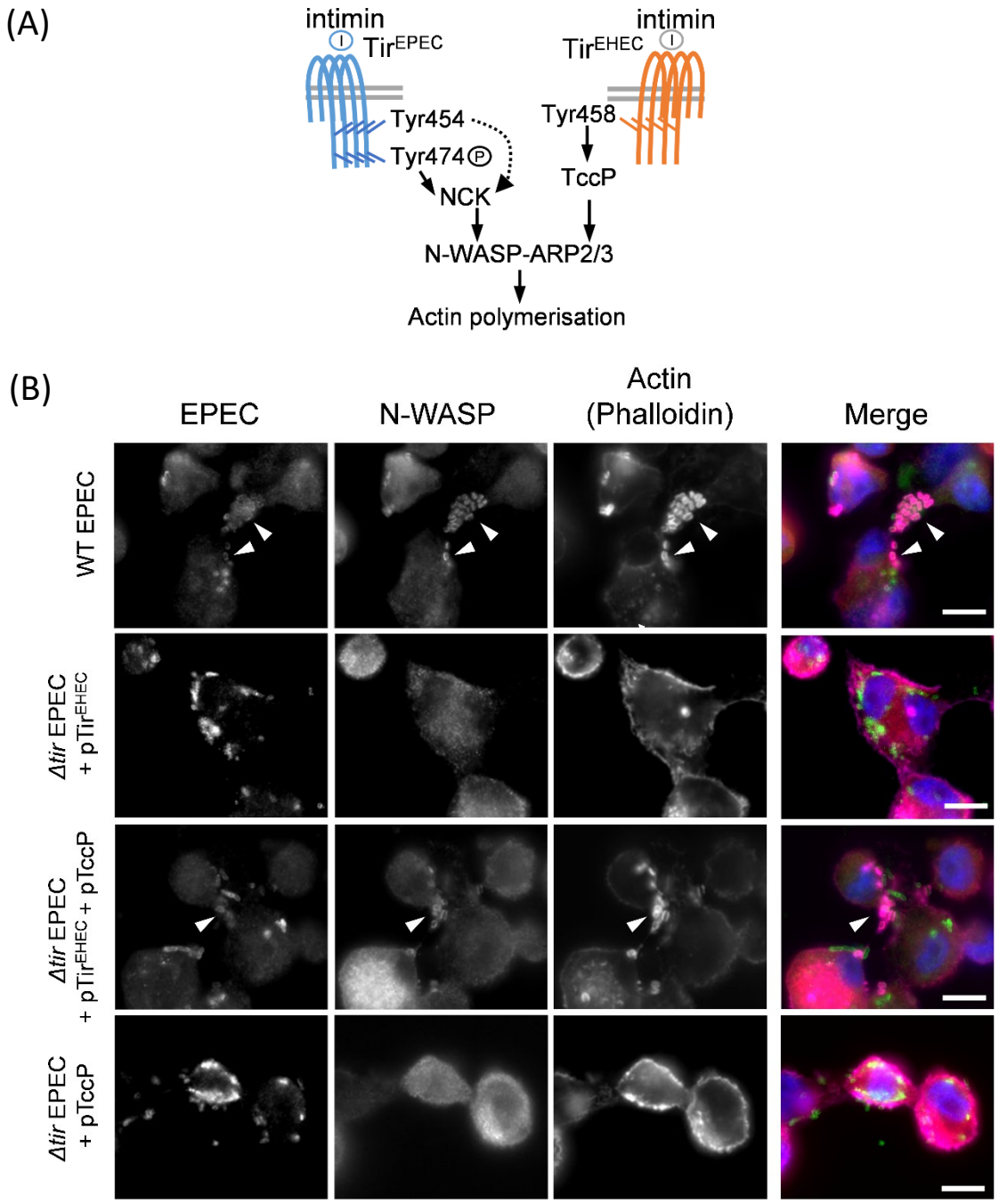

Figure 5.4. EHEC Tir-dependent actin polymerisation requires the effector protein TccP

(A) Schematic representation of the distinct mechanisms of EPEC and EHEC Tir-induced actin polymerisation. EPEC Tir relies on the phosphorylation of a tyrosine residue, Y474, to recruit the host adaptor protein Nck, N-WASP and the Arp2/3 complex, triggering actin polymerisation. EHEC Tir promotes Nck-independent actin polymerisation via a conserved NPY^{458} motif that employs the non-LEE encoded effector TccP to recruit N-WASP and Arp2/3 and induce actin polymerisation

(B) Representative images from immunofluorescence microscopy of THP1 cells infected with WT EPEC, *Δtir* EPEC, *Δtir* + pACYC:Tir^{EHEC}, *Δtir* + pACYC:Tir^{EHEC} and pSA10:TccP, and *Δtir* + pSA10:TccP at an MOI of 10 for 2 h. IPTG (0.1 mM) was added 30 min prior to infection to induce TccP expression from the pSA10 plasmid. Bacteria were visualised with anti-O127:H6 antibody (Green), N-WASP was visualised with Anti-WASL antibody (Red) and actin was stained with phalloidin (Pink) Arrowheads indicate N-WASP recruitment to the site of infection and bacteria with actin-rich pedestals. Scale bar = 20μm. Images are representative of three independent repeats.

Exploiting these strains, the role of Tir-induced actin polymerisation in inflammasome activation was assessed. THP1 cells were infected with EPEC *Δtir* strains expressing Tir^{EHEC} alone, TccP alone, or co-expressing Tir^{EHEC} and TccP, and inflammasome activation was quantified by cell-death assays (Figure 5.5A) and immunoblot analyses of GSDMD, caspase-1, caspase-4, IL-1β and IL-18 (Figure 5.5B). The expression of EHEC Tir alone in the absence of TccP failed to induce inflammasome activation or pyroptosis, indicating that Tir translocation was not sufficient to induce inflammasome activation. The expression of TccP in the absence of Tir also failed to activate inflammasomes (Figure 5.5). However, infection of THP1 cells with EPEC *Δtir* co-expressing Tir^{EHEC} and TccP induced robust caspase-1 and caspase-4 activation (Figure 5.5B), and the associated GSDMD cleavage induced pyroptosis (Figure 5.5A), as well as IL-1β and IL-18 cytokine processing (Figure 5.5B). Taken together, this verified that Tir-driven actin polymerisation was responsible for rapid non-canonical inflammasome activation in human macrophages.

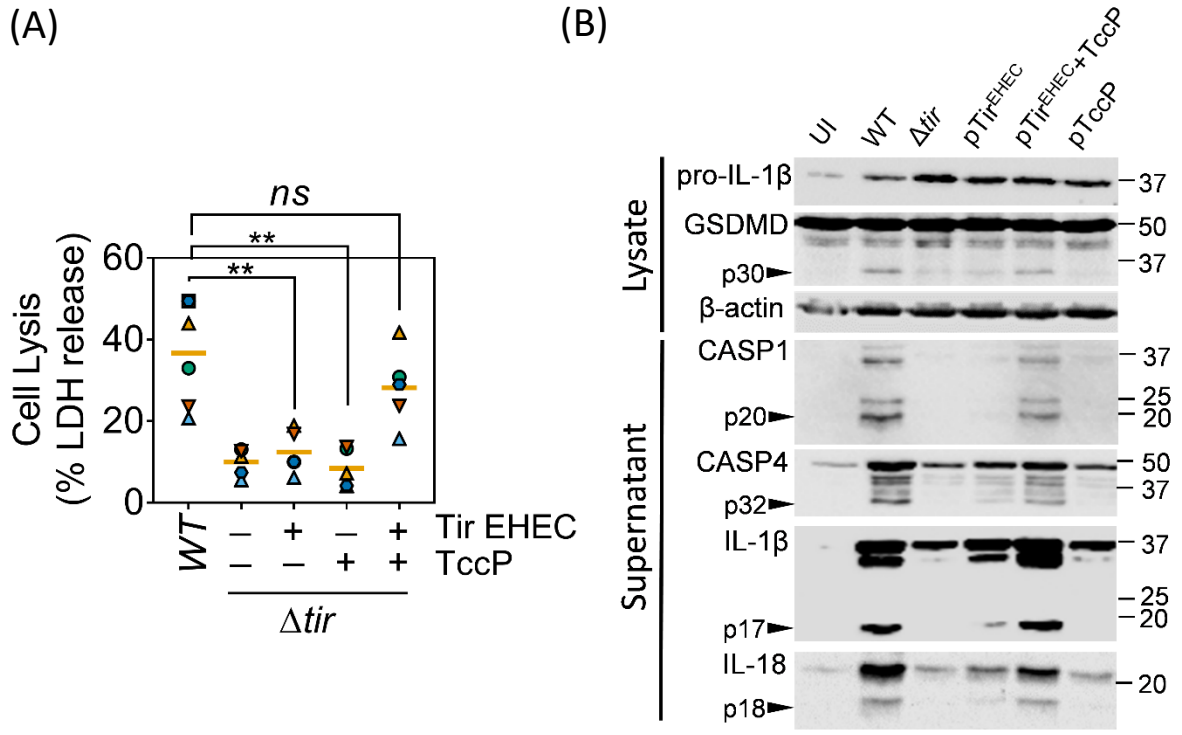

Figure 5.5. EHEC Tir-dependent actin polymerisation drives pyroptosis and inflammasome activation

(A) Graph represents cell lysis measured by LDH release from THP1 cells infected with WT EPEC, and *Δtir* EPEC expressing pACYC:Tir^{EHEC} and/or pSA10:TccP at an MOI of 10 for 4 h. IPTG (0.1 mM) was added 30 min prior to infection to induce TccP expression from pSA10 plasmid. (*n*= 5 independent experiments).

(B) Representative immunoblots from THP1 macrophages left uninfected (UI) or infected with indicated strains of EPEC expressing Tir from EHEC (pTir^{EHEC}), and/or expressing EHEC TccP (pTccP). Blots show caspase-1, caspase-4, IL-1β and IL-18 in culture supernatants and IL-1β, GSDMD and β-actin in cell lysates.

Matching shapes and colours of symbols in graphs denote data from independent donors/experiments. Immunoblots are representative of experiments performed three times. ** *P<0.01* by one-way ANOVA *ns*= non-significant.

5.3 EPEC-induced inflammasome activation is dependent on actin polymerisation.

The identification that Tir-driven actin polymerisation induced rapid atypical non-canonical inflammasome activation in human macrophages was particularly interesting, as it provided evidence of a novel mechanism of pathogen induced inflammasome responses. To further validate this pathway I utilised cytochalasin-D (Cyto-D), a widely used inhibitor of actin dynamics (727). Cyto-D binds to the barbed ends of actin filaments with high affinity and inhibits both the polymerisation and depolymerisation of actin subunits (728-730), and inhibits G- and F-actin binding to cofilin (731). My earlier findings pointed to an essential role for Tir-driven actin polymerisation in inflammasome activation. To test whether the direct manipulation of the actin

nucleation machinery by EPEC was responsible for inducing inflammasome activation, THP1 cells were pre-incubated with Cyto-D prior to infection. Treatment with Cyto-D impaired pyroptosis induced by EPEC infection, as measured by real-time PI uptake (Figure 5.6A), and LDH release (Figure 5.6C). This was consistent with the theory that EPEC-induced pyroptosis required actin polymerisation to stimulate non-canonical inflammasome activation. Importantly, Cyto-D did not inhibit pyroptosis induced by LPS transfection (Figure 5.6B), demonstrating that actin polymerisation played a role in the inflammasome activation pathway activated in response to EPEC infection, but was dispensable for caspase-4 activation induced by cytosolic LPS. Furthermore, this confirmed that Cyto-D treatment specifically inhibited EPEC-induced pyroptosis and did not mediate a universal inhibitory response to inflammasome activation.

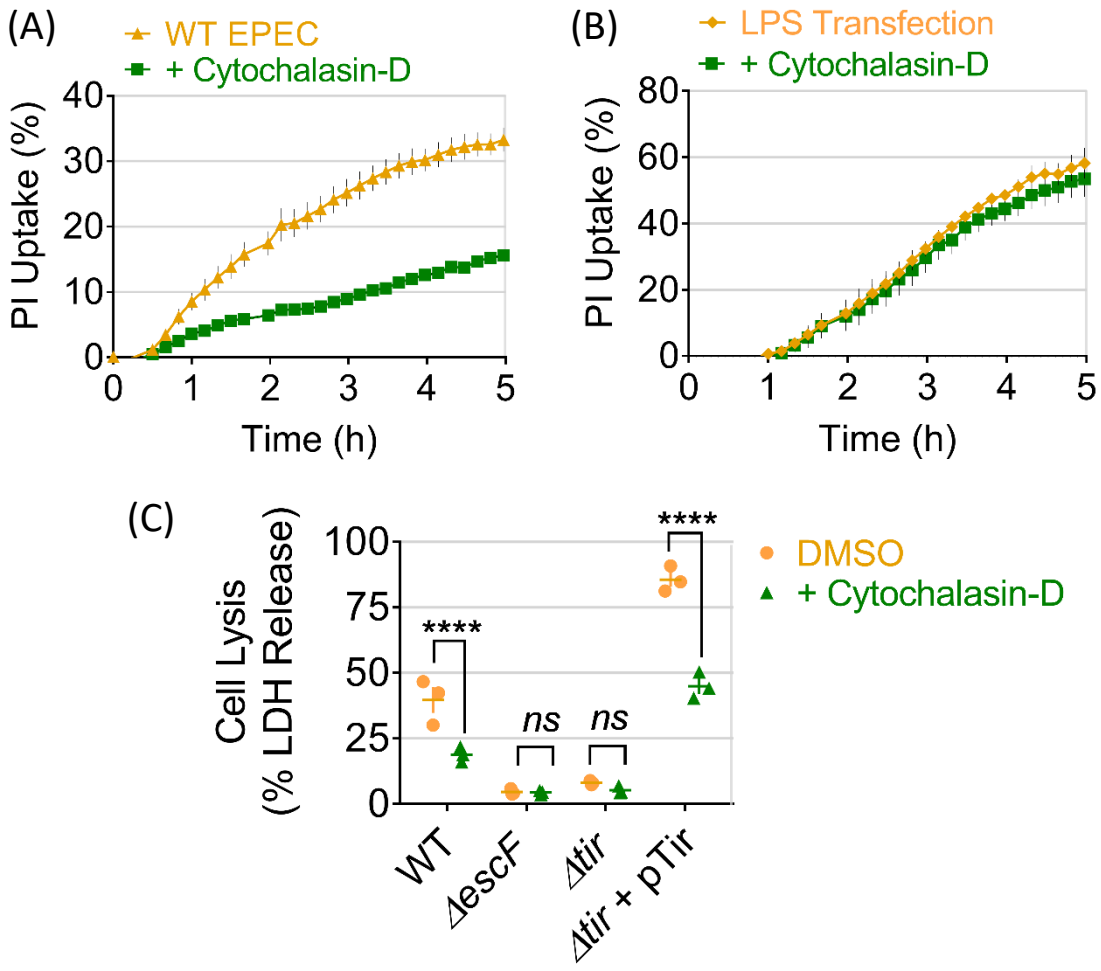

Figure 5.6. Inhibition of actin polymerisation prevents EPEC-induced pyroptosis in THP1 macrophages

(A) Real-time PI uptake assay from THP1 cells infected with WT EPEC at an MOI of 10 over a 5 h infection, in the absence or presence of cytochalasin-D (200 nM). Graphs shown mean ± SEM from *n*= 3 independent experiments.

(B) Real-time PI uptake assay from THP1 transfected with LPS (5 µg/mL), in the absence or presence of Cytochalasin-D (200 nM) for 5 h. Mean ± SEM from *n*= 3 independent experiments are plotted.

(C) Cell lysis measured as LDH release from THP1 cells infected with indicated EPEC strains in the absence or presence of cytochalasin-D (200 nM). IPTG (0.1 mM) was added 30 min prior to infection to induce Tir expression from pSA10 plasmid (pTir). Graph shows mean ± SEM *n= 3* independent experiments **** *P<0.0001*, *ns* – non-significant by two-way ANOVA.

5.4 EspJ can inhibit EPEC-induced pyroptosis in human macrophages

Upon interaction with the bacterial surface protein intimin, EPEC Tir is phosphorylated on residue Y474 (269). Phosphorylation is essential for Tir driven actin polymerisation, as only phosphorylated Tir is able to bind to the cellular adaptor Nck (260, 280, 282). Tir tyrosine phosphorylation and Nck-dependent actin pedestal formation, is mediated by a range of redundant host tyrosine kinases. Recently, the T3SS effector protein EspJ has been shown to block non-receptor tyrosine kinases and inhibit actin polymerisation induced by EPEC (237). Therefore, to establish whether EspJ could also inhibit EPEC-induced pyroptosis, EspJ was over-expressed in WT EPEC using a pSA10:EspJ plasmid that allows for IPTG inducible *espJ* gene expression (pEspJ). Consistent with its role in inhibiting actin polymerisation, overexpression of EspJ inhibited pyroptotic cell death during EPEC infection of THP1 macrophages as measured by LDH release (Figure 5.7).

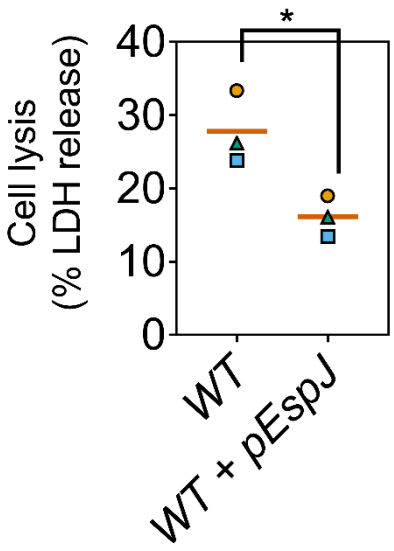

Figure 5.7. EspJ inhibits EPEC-induced pyroptosis in human macrophages
Cell Lysis as measured by LDH release from THP1 cells infected with WT EPEC and WT EPEC expressing pSA10:EspJ at an MOI of 10 for 4 h. IPTG (0.5 mM) was added 30 min prior to infection to induce EspJ expression from pSA10 plasmids (pEspJ). Mean ± SEM (*n= 3* independent experiments). * *P<0.05* by two-tailed paired Student's *t*-test.

5.5 Inflammasome components are not required for Tir-induced actin polymerisation

Earlier work presented in this study utilised targeted gene-silencing of key inflammasome components to characterise the inflammasome signalling cascade induced by EPEC infection. EPEC-induced inflammasome activation was shown to require caspase-4, ASC and GSDMD to propagate inflammasome activation and pyroptosis. Here, inflammasome activation was also shown to be dependent on Tir-mediated actin polymerisation. Therefore, it was important to establish whether silencing of *caspase-4, ASC* or *GSDMD* prevented host cell induced actin polymerisation. This was particularly important for caspase-4 as is the mouse model, caspase-11, the functional homologue of human caspase-4, has been shown to physically interact with AIP1 (also known as WDR1), and this interaction potentiates cofilin-mediated actin depolymerisation (561) to augment the remodelling of the actin cytoskeleton (560). Caspase-4 has also been shown to mediate cofilin phosphorylation. If caspase-4 silencing inhibited EPEC-induced actin polymerisation, this would account for the previously identified requirement of caspase-4 in EPEC-induced inflammasome signalling. Immunofluorescent analysis of EPEC infected CTRL^{miR}, *CASP4*^{miR}, *ASC*^{miR} and *GSDMD*^{miR} THP1 macrophages was used to assess that capacity of these cell lines to undergo actin polymerisation in response to EPEC infection. Importantly, stable silencing of caspase-4, ASC and GSDMD did not inhibit Tir-induced actin polymerisation in THP1, as F-actin accumulated at the site of infection at the rate consistent with that observed in CTRL^{miR} THP1 cells (Figure 5.8). This verified that these proteins are not required for EPEC-mediated actin polymerisation, and therefore function downstream of actin polymerisation during EPEC-induced inflammasome activation.

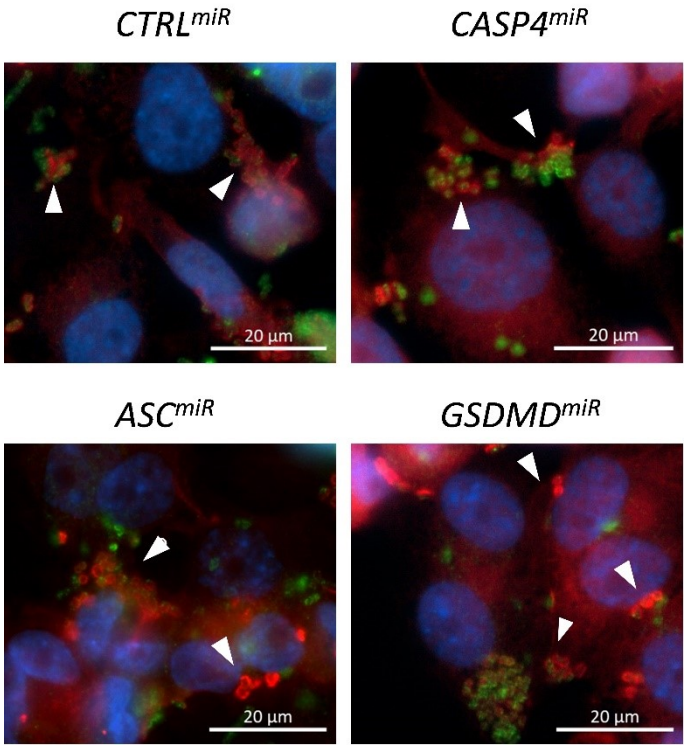

Figure 5.8. Inflammasome components are not required for Tir-induced actin polymerisation
Representative images from immunofluorescence microscopy of THP1 cells stably transduced with non-targeting (CTRL^{miR}) or miRNA targeting CASP4 (*CASP4*^{miR}), ASC (*ASC*^{miR}) or GSDMD (*GSDMD*^{miR}), infected with WT EPEC for 2 h. Bacteria were visualised with anti-O127:H6 antibody (Green), cell nuclei were stained with DAPI (Blue), and actin was stained with phalloidin (red) Arrowheads indicate bacteria-induced actin polymerisation. Images are representative of two independent repeats. Scale bar = 20 µm.

5.6 Cofilin-1 is not required for EPEC-induced inflammasome activation

Previous examination of EPEC-induced actin pedestals identified numerous host cell cytoskeletal, signalling, and adapter proteins that are recruited to the site of bacterial attachment in a Tir-dependent manner (207, 304, 305, 732). Among these proteins is the actin depolymerising factor cofilin (ADF). In host cells cofilin attaches to F-actin and promotes depolymerisation, whereas phosphorylation of cofilin prevents its association with actin, thereby stabilising actin filaments (733). Previous research has also identified that the CARD domain of mouse caspase-11 associates with AIP-1 and cofilin complexes to facilitate actin depolymerisation (560). Furthermore, in the context of lysosome acidification, cofilin associated with the actin that surrounds phagocytic vesicles was shown to recruit caspase-11 (568).

The work in this study identified a mechanism of caspase-4 driven NLRP3 inflammasome activation downstream of Tir-induced actin polymerisation. This raised the possibility that the association of human caspase-4 with cofilin localised to the EPEC pedestal might be necessary for EPEC induced inflammasome activation and pyroptosis. To test this theory, expression of Cofilin-1 (CFL1) was transiently knocked down in THP1 cells using siRNA. Non-targeting CTRL and *CASP4* siRNA were used as controls. siRNA silencing of cofilin-1 was confirmed by western blot analysis of cell lysates (Figure 5.9A). Silencing of cofilin-1 in THP1 cells infected with WT EPEC did not inhibit EPEC-induced inflammasome activation, as caspase-1 and caspase-4 cleavage was evident in immunoblots of infected cell supernatants (Figure 5.9B). Although western blot analysis indicated that Cofilin-1 silencing inhibited EPEC-induced GSDMD cleavage, there was no significant difference in EPEC-induced pyroptosis (Figure 5.9C). Similarly, cofilin-1 did not play a role in the non-canonical inflammasome pathway activated by LPS transfection (Figure 5.9C). These results suggest that cofilin-1 does not play a significant role in mediating EPEC-induced inflammasome activation.

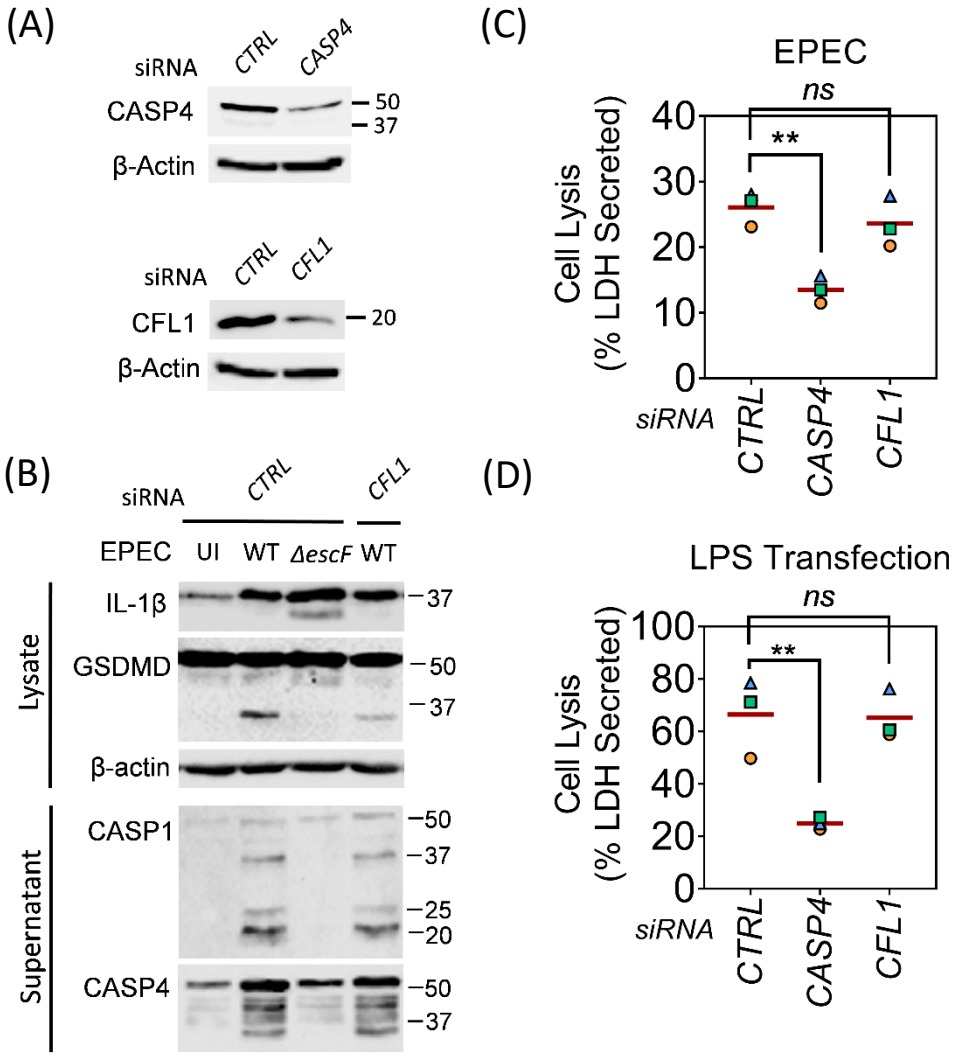

Figure 5.9. Cofilin-1 knock-down does not prevent EPEC-induced inflammasome activation or pyroptosis

(A) Immunoblot of cell lysates from THP1 cells transfected with non-targeting (CTRL), cofilin-1 (*CF1*) or caspase-4 (*CASP4*) siRNAs.

(B) Western blots of indicated proteins from THP1 cells transfected with non-targeting (CTRL), cofilin-1 (*CF1*) or caspase-4 (*CASP4*) siRNAs, left uninfected (UI) or infected with indicated EPEC strains at an MOI of 10 for 5 h.

(C) Quantification of LDH release from THP1 macrophages transfected with non-targeting (CTRL) or indicated siRNA infected with EPEC at an MOI of 10 for 4 h. (*n*= 3 independent experiment)

(D) Quantification of LDH release from THP1 cells transfected with non-targeting (CTRL) or indicated siRNA and transfected with LPS (5 µg/ml) using Lipofectamine-2000 for 4 h, (*n*= 3 independent experiments)

Matching shapes and colours of symbols in graphs denote data from independent experiments. ** *P<0.01* by one-way ANOVA. *ns*- non-significant. Immunoblots are representative of 2 independent repeats.

5.7 Effector-induced modification of actin dynamics is not a universal inflammasome activator

Tir-driven actin polymerisation was shown to be the activating signal required to induce inflammasome activation and pyroptosis during EPEC infection of human macrophages. Tir, however, is one of many bacterial effectors that modulate the state of the actin cytoskeleton to propagate infection. A number of EPEC and EHEC bacterial effectors activate cytoskeletal changes by activating Rho GTPase signalling, these including EspM, EspT and Map. Therefore, if pathogen-mediated actin polymerisation was a universal activating signal for non-canonical inflammasome activation, expression of these actin modifying WxxxE-effector proteins during infection of THP1 cells may induce similar inflammasome pathways. The EPEC E2348/69 strain used in this study expresses Map, but does not express either EspM or EspT (323). However, overexpression of Map during infection was shown to significantly increase its ability to induce actin rich filopodia (209). Therefore, to establish whether WxxxE effector driven actin modifications could activate inflammasome pathways in a similar way to Tir, THP1 macrophages were infected with EPEC $Tir^{Y454A/Y474A}$ (TirAA, which was unable to induce inflammasome activation; Figure 5.2) expressing *map, espM2* and *espT* on pSA10 plasmids that enable IPTG inducible gene expression.

Immunofluorescence analysis of THP1 cells infected with TirAA overexpressing Map, EspM2 and EspT exhibited alterations to actin structures consistent with previous studies. Overexpression of Map induced actin-rich filopodia, EspT triggered membrane ruffles and lamellipodia, and EspM induced the formation of parallel stress fibres (Figure 5.10A). The capacity of WxxxE-effectors to induce distinct actin rich structures in host cells, however, did not correlate with an increased level of toxicity. THP1 cells infected with EPEC $Tir^{Y454A/Y474A}$ expressing Map or EspT did not induce more cell death than $Tir^{Y454A/Y474A}$ expressing a pSA10 empty control plasmid (Figure 5.10B), confirming that regulation of actin polymerisation pathways by these effectors did not induce cell death in THP1 cells. The expression of EspM during infection mediated a marginal increase in cytotoxicity, however the level of cell death was still markedly reduced compared to WT EPEC infections (Figure 5.10B). Collectively, these results established that disruption of the actin cytoskeleton by bacteria effectors is not always sufficient for non-canonical inflammasome activation. Tir-induced actin polymerisation, therefore, provides a unique mechanism of rapid inflammasome activation in human macrophages.

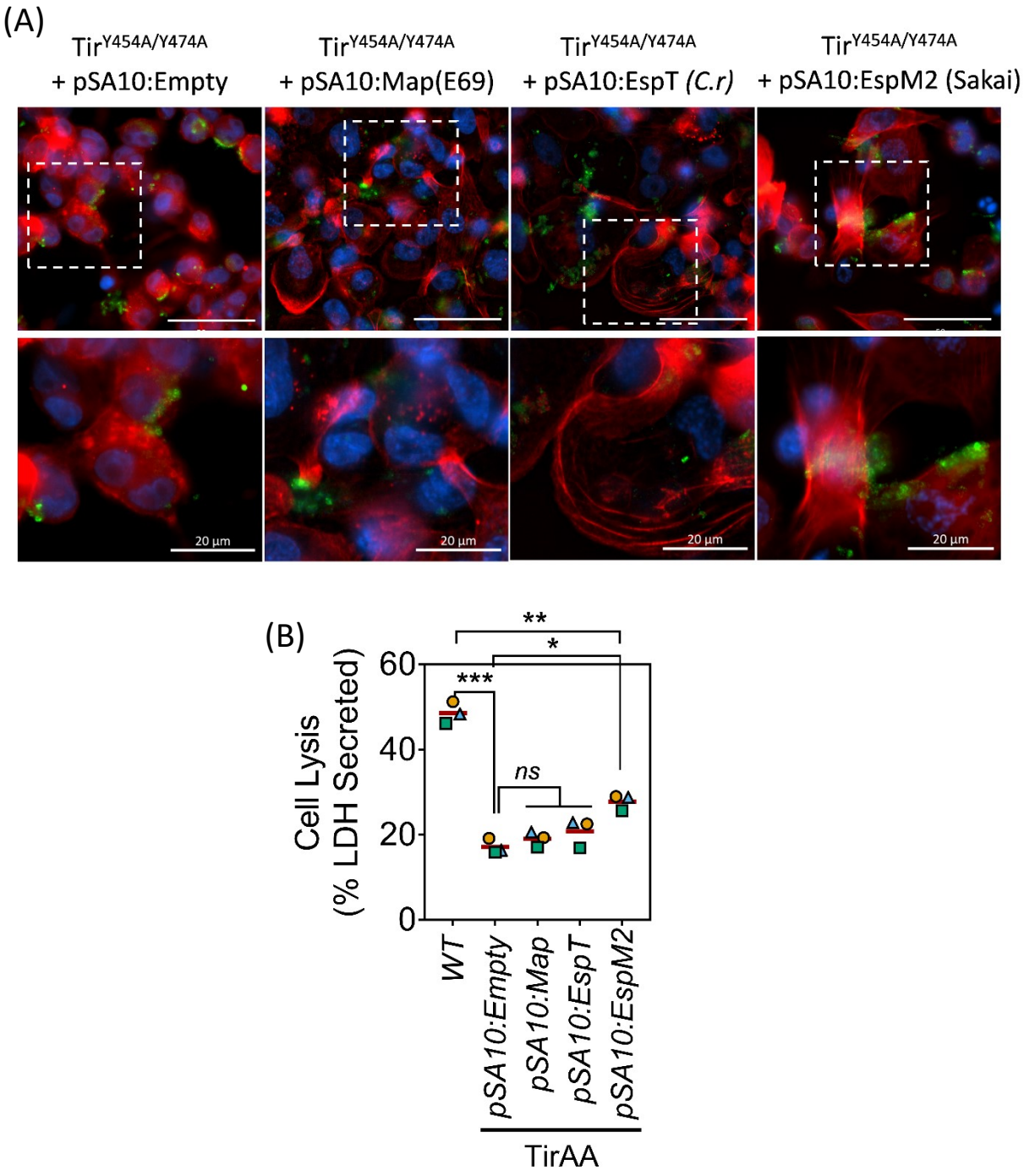

Figure 5.10. WxxxE-Effector-induced actin remodelling does not induce pyroptosis

(A) Representative images from immunofluorescence microscopy of THP1 cells Infected with EPEC Tir$^{Y454A/Y474A}$ expressing indicated pSA10 plasmids for 2 h. IPTG (0.1 mM) was added to bacteria cultures 30 min prior to infection to induce expression from pSA10 plasmids. Bacteria were visualised with anti-O127:H6 antibody (green), cell nuclei were stained with DAPI (Blue), and actin was stained with phalloidin (red). Scale bars = 50 µm Top Row, or 20 µM Bottom Row.

(B) Graph shows cells death as measured by LDH release from THP1 macrophages infected with WT EPEC or EPEC Tir $^{Y454A/Y474A}$ (TirAA) expressing pSA10:Empty. pSA10:Map from EPEC E2348/69, pSA10:EspT from *C. rodentium,* and pSA10:EspM2 from EHEC Sakai, at an MOI of 10. Cell lysis was measured 4 h post infection. Matching shapes and colours of symbols in graphs denote data from independent experiments. (*n= 3* independent experiments) **** P<0.001 ** P<0.01* by one-way ANOVA. *ns*= non-significant.

5.8 Ectopic expression of membrane-targeted FcγR-Tir constructs is toxic to THP1 macrophages

Previous research in the Frankel lab has shown that transfection of a FcγR-Tir^{EPEC} chimeras, in which the C-terminus of Tir is fused to the membrane targeted human FcγIIa receptor, localises to the host cell membrane and induces actin pedestal formation in HeLa cells when clustered by IgG-opsonised beads (Guenot, *et al.,* unpublished data). Therefore, expression of these constructs in THP1 cells would enable Tir-induced actin polymerisation to be investigated independently of all other bacterial derived PAMPs. FcγRTir chimeras were cloned into the pMX-cmv mammalian expression plasmids tagged with mCherry and transduced into THP1 macrophages. Interestingly, expression of FcγRTir-EPEC-mCherry constructs that expressed the Wild-Type Tir C-terminal, containing residues Y454 and Y474, was toxic to THP1 cells. However, expression of either FcγTir$^{Y454F/Y474F}$ or FcγRTir-EHEC proteins, which are unable to induced Tir-mediated actin polymerisation, did not affect THP1 viability. This suggested that the capacity of these constructs to induce actin polymerisation correlated with their toxicity.

To confirm the previous observations that FcγRTir constructs induced actin pedestal formation in HeLa cells, HeLa cells were transfected with pMX-cmv-FcγTir-mCherry and FcγTir$^{Y454F/Y474F}$-mCherry plasmids. Preliminary work was done to characterise these cells. Hela cells expressing FcγTir constructs were incubated for 90 min with 3 µm beads opsonised with IgG, and phenotypes were analysed by epifluorescence microscopy. Actin pedestal-like structures were observed under opsonised beads in cells expressing FcγTir (Figure 5.11). In contrast, HeLa cells expressing FcγRTir$^{Y454F/Y474F}$ recruited the FcγRTir$^{Y454F/Y474F}$-mCherry tagged constructs to the site of bead attachment, but failed induce actin pedestal formation. Collectively, these results indicated that expression of Tir complexes capable of inducing actin pedestal formation within human macrophages is detrimental to cell viability and, therefore, alternative strategies are required to investigate Tir-induced signalling pathways in the absence of bacterial infections.

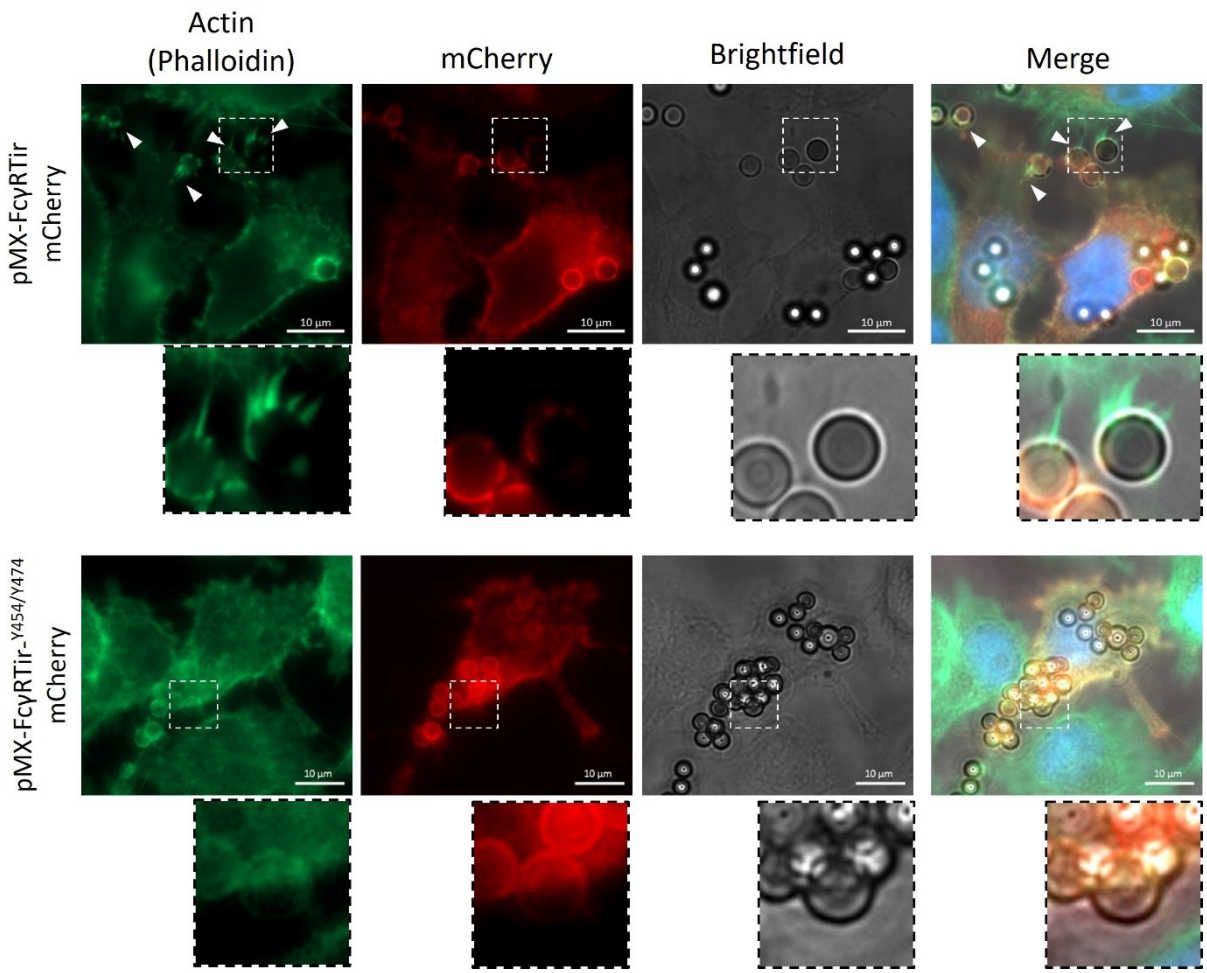

Figure 5.11. HeLa cells expressing FcγRTir constructs induce actin pedestals when challenged with IgG-opsonised beads

Representative immunofluorescence images, from HeLa cells expressing FcγRTir-mCherry and FcγRTir$^{Y454F/Y474F}$-mCherry receptor constructs incubated with IgG coated beads for 90 min. F-actin structures were stained with Phalloidin (Green) and cell nuclei were stained with DAPI (Blue), mCherry-tagged FcγRTir receptors (Red) and beads are shown in brightfield Scale bars = 10 µm. White arrowheads indicate pedestal-like structures. Data is representative of two experimental repeats.

Discussion

The results in this chapter identify Tir-mediated actin polymerisation as the activating signal responsible for non-canonical inflammasome activation during EPEC infection of human macrophages. Caspase-4 is the principal regulator responsible for both EPEC-induced inflammasome activation and pyroptosis. Therefore, collectively these results provide evidence for a link between Tir-induced cytoskeleton remodeling and caspase-4 activation.

Tir translocation during EPEC infection has been reported to regulate a multitude of cellular processes during infections, including; mediating the intimate attachment of bacteria to host cells (261), stimulating actin pedestal formation (274), and inhibiting the production of proinflammatory cytokines through the recruitment of phosphatases SHP-1 and -2 (734). This study aimed to identify the mechanism by which Tir induced rapid non-canonical inflammasome activation in human macrophages. Utilising EPEC strains that expressed genetically mutated Tir proteins, lacking the tyrosine residues required to induce actin polymerisation, Tir-mediated actin polymerisation was shown to be essential for inflammasome activation (Figure 5.1, 5.2). These Tir constructs have previously been shown to intimately attach to host cells in both *in vitro* (604) and *in vivo* (312, 313) infections, and retain the residues required for SHP-1/2 recruitment (735). These findings demonstrate that Tir-mediated actin pedestal formation, rather than other Tir-dependent processes, is principally responsible for inducing inflammasome activation.

Tir-dependent actin polymerisation is genetically distinct in EPEC and EHEC, with EHEC circumventing upstream kinase requirements by translocating TccP (283, 289, 290, 725). Despite these different activation pathways, both EPEC and EHEC Tir induced inflammasome activation and pyroptosis in human macrophages. Interestingly, the inhibition and restoration of actin pedestal formation was shown to correlate directly with inflammasome activation and pyroptosis (Figure 5.4, 5.5). As EPEC- and EHEC-induced actin polymerisation pathways converge on N-WASP, to direct Arp2/3-mediated actin nucleation, these results indicate that inflammasome activation occurred downstream of N-WASP and the Arp2/3 complex. Indeed, disruption of actin polymerisation downstream of Arp2/3 using the chemical inhibitor cytochalasin-D prevented EPEC-induced pyroptosis (Figure 5.6). This demonstrated that Tir-proximal events were relatively less important compared to changes in actin dynamics. However, it is important to consider that cytochalasin-D, in addition to preventing Tir-induced actin polymerisation (736), is a universal inhibitor of actin polymerisation, and therefore inhibits numerous host cell responses, including

phagocytosis (737). The absence of pyroptosis in cytochalasin-D treated macrophages therefore cannot be directly attributed to the absence of Tir-mediated actin polymerisation, as it may be a consequence of reduced bacterial internalisation. Although, results with Tir mutant EPEC strains indicate that the rate of phagocytosis does not correlate with pyroptosis (Figure 4.11). Additional work is required to experimentally establish the mechanism in which cytochalasin-D inhibits EPEC induced pyroptosis, this includes immunofluorescence analysis to visulise the absence of actin polymerisation at the site of bacterial attachement in cytochalasin treated cells, and antibiotic protection assays to establish if cytochalasin-D treatment alters the rate of bacterial internalisation.

The results here suggest that Tir-mediated actin disturbance itself might serve as a novel pathogen associated stimulus that is sensed by the non-canonical inflammasome sensor caspase-4. Interestingly, mouse caspase-11, which can localise to sites of bacterial infection (465), has previously been reported to have a role in mediating actin dynamics. During inflammatory responses to infection, caspase-11 alters actin dynamics both through physically interacting with AIP1 (560) and the actin-remodeling protein Flightless-I (738), or via the regulation of cofilin phosphorylation (562, 563). Cofilin is required to regulate the actin-filament dynamics of pedestals during EPEC infections (304). Given these reports, it is plausible that actin polymerisation may be responsible for recruiting caspase-4 to the site of infection, where it can initiate inflammasome responses. Attempts in this study to visualize the cellular location of endogenous caspase-4 during EPEC infections were unsuccessful, due to the limited availability of appropriate caspase-4 antibodies for immunofluorescent. Therefore, to establish whether caspase-4 is recruited to the site of Tir-induced actin polymerisation during EPEC infections, further work utilising stable caspase-4 knock-out THP1 lines expressing fluorescently tagged caspase-4 constructs should be completed. This would provide further insight into the mechanisms in which caspase-4 is activated in response to Tir-mediated actin polymerisation to induce NLRP3-inflammasome activation in the absence of pyroptosis.

One key question that still remains is why activation of caspase-4 during EPEC infection failed to induce cell death. Following phagocytosis of *Staphylococcus aureus* and *L. pneumophila,* caspase-1 and caspase-11 have been shown to play a critical role in lysosome acidification (562, 739). Cofilin associated with the actin surrounding phagocytic vesicles was shown to recruit caspase-11, which in turn, activated caspase-1 in proximity to the phagosome (568). However, caspase-11-mediated recruitment of caspase-1 led to ASC-independent low-level activation of caspase-1, in a manner that did not induce host cell death (568). Although the mechanism by which caspase activation

induced lysosome acidification in the absence of cell death was not fully established, the authors attributed these different outcomes to varying levels of caspase-1 activity and its restricted localisation. Applying these principles to EPEC infections, one possibility is that, similarly to caspase-1 activation in lysosome acidification, varying levels of caspase-4 activity may promote different outcomes. Perhaps, the mechanism of caspase-4 activation during EPEC infections restricts its proteolytic activity, whereas highly active caspase-4, in response to transfected LPS, drives GSDMD-cleavage and pyroptosis. In support of this graded response, other studies have shown that low-level caspase-3 or -8 activation contributes to specific aspects of immune-cell development, activation, and differentiation (740), whereas high-level activation promotes apoptosis (741). If activated caspase-4 is localised to pedestals during infection this might restrict its activity. The importance of caspase localisation to its function has already been demonstrated in the context of pyroptosis, as localising GSDMD near the developing inflammasome directs its cleavage by caspase-1 (498). EPEC-induced actin polymerisation may recruit caspase-4 to the site of infection, thus limiting its access to GSDMD and restricting is pyroptotic potential. This would explain why EPEC-induced pyroptosis required co-operative activities of caspase-4 and caspase-1 for optimal GSDMD cleavage.

Although in this study I demonstrate that siRNA based silencing of cofilin-1 did not inhibit EPEC-induced inflammasome activation and pyroptosis (Figure 5.9), the actin-binding protein AIP1 has been reported to interact with caspase-11 directly and is responsible for the recruitment of caspase-11 to the site of actin polymerisation (560). AIP1 co-localises closely with cofilin *in vivo* in different organisms, suggesting the two proteins co-operate in the reorganisation of the actin cytoskeleton. siRNA targeted knock-down of AIP1 failed to reduce AIP1 protein expression in THP1 cells (data not shown). Therefore, the role of these actin regulators in the context of Tir-mediated actin polymerisation and caspase-4 activation requires further analysis. Caspase-11 also interacts with the actin-remodeling protein Flightless-I, which has been shown to control the localisation and activation status of this proinflammatory caspase, and caspase-11 itself has also been reported to have an intrinsic affinity for F-actin (2). Therefore, caspase-4/11 may have the ability to localise to EPEC-induced F-actin pedestals through a number of redundant mechanisms, suggesting that targeted suppression of individual pathways may not be sufficient to inhibit caspase-4 recruitment and/or activation.

During EPEC infection these actin-binding proteins may play an important role in linking Tir-induced actin polymerisation to caspase-4, but they may also play a role in regulating the activity

of caspase-4. Co-immunoprecipitation results have previously revealed that the C-terminal WD40 propeller of AIP1 and full-length Flightless-1 both interact directly with mouse caspase-11 (560, 738). Interestingly, AIP1, similarly to LPS, binds to the CARD-domain of caspase-11. Although the CARD-domains of caspase-11 and caspase-4 share only 51% identity (513), they have similar interacting partners, and both caspase-4 and caspase-11 have previously been shown to bind to LPS and oxPARC (446, 742). It is, therefore, possible that when activated in human macrophages these actin-associated proteins bind to caspase-4. These interactions may impede LPS-binding, which would effectively raise the threshold required for LPS-induced non-canonical inflammasome activation. This may explain why caspase-4 activation alone is not sufficient to induce pyroptosis during EPEC infections that induce actin polymerisation, yet can readily induce pyroptosis in response to transfected LPS. This concept is not unprecedented, as recently in macrophages, oxPARC was shown to inhibit LPS-induced non-canonical inflammasome activation by binding directly to caspase-4 and caspase-11, which inhibited LPS binding and activation (742). The recent identification of host-derived proteins that are able to interact with caspase-4/11 via its CARD domain is notable. Although mutation of the residues required to mediate LPS binding within the caspase-11 CARD abrogated both LPS and EPEC induced pyroptosis, whether these residues are also required to mediate interactions with other caspase-11-interacting proteins cannot be discounted. Therefore, differential analysis of the interactome of both WT caspase-11 and the LPS-binding mutant caspase-11 during EPEC infection may help to clarify the mechanism responsible for caspase-4/11 activation.

Given these results, it is tempting to speculate that specific forms of actin disturbance might represent a pathogen-associated danger signal that can be sensed by the innate immune system. If this holds true, a link between actin and inflammasome signalling might serve as a general function in mounting inflammatory immune responses to the numerous bacterial pathogens that target the cytoskeleton as an infection strategy (257-259, 743). Interestingly, manipulation of host-actin polymerisation pathways by pathogens have previously been shown to play a role within inflammasome pathways (452, 567). Toxin-induced inhibition of Rho GTPases results in an ineffective regulation of the actin cytoskeleton, which is sensed by Pyrin (452). These toxins specifically target the Rho subfamily and covalently modify a conserved switch-I residue within Rho GTPases. Interestingly, direct Rho-pyrin interactions are not required for inflammasome activation (452). This provides evidence for an indirect mechanism in which disruption of actin signalling through modification of Rho activity induce inflammasome responses. However, as EPEC

does not modify Rho covalently, the pyrin inflammasome is unlikely to be responsible for EPEC-induced caspase-1 activation. This was confirmed by the fact that inhibition of caspase-4 or NLRP3 rescued cells from EPEC-induced pyroptosis (Figure 3.11, 3.16). Research has also demonstrated a role for NLRC4 in actin dynamics during *S.* Typhimurium infection of macrophages (567). NLRC4, in concert with NAIPs, forms inflammasome complexes upon sensing flagellin or the inner rod or needle proteins of the T33 from infecting *Salmonella* (575). Notably, during *Salmonella* infection, inhibition of actin polymerisation with cytochalasin-D was shown to prevent NLRC4-dependent assembly of ASC-containing inflammasome foci, pyroptosis, and IL-1β processing, which correlated with reduced bacterial invasion (567). This work identified actin cytoskeletal remodeling as an effector mechanism of the NLRC4 inflammasome that functioned to limit additional bacterial uptake by inducing mechanical stiffening of the cell via actin polymerisation. Together, this evidence highlights links between actin dynamics and inflammasome-associated caspases during bacterial infection. Similarly, in this study I demonstrate that actin polymerisation is required to mediate inflammasome activation in response to EPEC. However, during EPEC infection caspase-4 is required to mediate these inflammasome responses. Although there is limited evidence directly linking the actin cytoskeleton to caspase-4 during bacterial infections of macrophages, the caspase-4 homologue caspase-11, was shown to restrict the intracellular bacterial growth of *L. pneumophila* by moderating actin dynamics (562). This study demonstrated that the reduced actin dynamics in *Casp11*$^{-/-}$ macrophages was a consequence of reduced flotillin-1 expression, as well as increased phosphorylation of cofilin (562). During EPEC infection, suppression of caspase-4 did not inhibit actin polymerisation (Figure 5.8), however, caspase-4 was required to induce inflammasome activation in response to Tir-driven alterations to the actin cytoskeleton. Collectively this research provides compelling evidence for a direct link between the host cell actin cytoskeleton and inflammasomes.

In addition to inflammasome activation, subversion of the actin cytoskeleton by bacterial virulence factors has been shown to be an important mediator of immune signalling. For example, the *Salmonella* Rho GTPase GEF SopE has been shown to activate the PRRs NOD1 (317), while NOD2 is regulated by Rac1 (316), and localises at the plasma membrane at cortical F-actin structures (316). Consequently, perturbation of F-actin dynamic remodeling and Rho GTPase activity alter NOD1 and NOD2 activation and innate immune responses. A number of EPEC and EHEC effector proteins have been shown to interact with Rho GTPases, and serve as key regulators in actin dynamics, for example the T3SS effectors Map, EspM, EspM2 and EspT (209, 323, 324, 744), which,

along with SopE, belong to the extended WxxxE family of T3SS effector proteins (208). These WxxxE effectors promote a diverse array of actin structures including actin-rich filopodia, membrane ruffles, and lamellipodia (209, 323, 324, 744). Notably, during EPEC infection inflammasome responses were only induced in response to Tir-dependent actin polymerisation, as F-actin remodeling induced by Rho GTPase targeting effectors had no significant impact on EPEC-induced pyroptosis (Figure 5.10).

Actin remodeling in eukaryotic cells is an very dynamic yet highly-regulated process, with numerous host cell signalling molecules all working in co-ordination to control actin polymerisation pathways. Different signalling cascades typically give rise distinct actin-rich cell surface structures, and the actin-nucleating components also influence the type of structure (745, 746). Collectively, my results suggest that the distinct mechanism of Tir-induced actin pedestal assembly specifically activates a caspase-4-dependent non-canonical inflammasome in human macrophages. Tir-driven protein recruitment may be behind the ability of Tir-mediated actin remodeling to induce macrophage cell death. The actin pedestal has long been considered as a central focus that recruits an array of EPEC effectors and host proteins during infection (304, 747). If this localised actin assembly within EPEC pedestals facilitates the recruitment of inflammasome related proteins to the site of bacterial attachment, this could initiate proximity-dependent activation cascades.

Here I have shown that the expression of membrane-targeted Tir constructs is toxic to THP1 cells. Although Tir clustering is required to induce actin polymerisation, by expressing Tir constructs fused to FcγIIa receptors, it is possible that IgGs within the culture medium induced clustering of the FcγRTir constructs and downstream actin polymerisation even in the absence of IgG-opsonized particles. Interestingly, the actin-polymerising ability of these constructs was directly responsible for mediating their cytotoxicity, as FcγRTir constructs that were genetically unable to induce actin polymerisation were not toxic when expressed in THP1 cells. An alternative future approach would be the expression of FcγRTir_EHEC construct in THP1 cells that would unable to induce actin polymerisation in the absence of TccP. Co-transection of these cells with a doxycycline inducible plasmid that enables inducible expression of TccP (pLTet-2xFlag_TccP) would then enable this actin polymerisation pathway to be chemically induced in the absence of infection. This would enable a more comprehensive analysis of Tir-induced actin polymerisation and the downstream signalling pathways in human macrophages in the absence of additional bacteria associated factors. An alternative approach to investigate the role of pathogen-mediated actin disturbances in inflammasome activation is through the use of Vaccinia virus. In many ways, the mechanisms by

which canonical EPEC induces actin pedestal formation, and Vaccinia virus induces actin tail formation are very similar. Consistent with EPEC-Tir signalling, Vaccinia-mediated actin polymerisation is dependent on a pathogen-derived membrane protein, termed A36R, that requires tyrosine phosphorylation to induce downstream N-WASP activation and actin nucleation. A36R signalling requires phosphorylation of tyrosine residues 112 (282, 748). Notably, the sequences flanking Y474 of Tir (EHIYDEVA) and Y112 in A36R (EHIYDSVA) are highly conserved, suggesting that these pathogens utilise homologous mechanisms to induce actin polymerisation during infection. Investigating whether A34R from Vaccinia also has the capacity to induce a similar caspase-4-dependent non-canonical inflammasome pathway in human macrophages would help clarify the molecular mechanism involved in this signalling cascade.

Manipulation of tyrosine kinase signalling is common during bacterial infections. Tyrosine phosphorylation of EPEC Tir is mediated by Abl and Scr family Kinases. Depending on the phosphorylation status of Tir, Nck is recruited and stimulates downstream pedestal formation (275, 276). The effector protein EspJ has previously been shown to disrupt Tir-phosphorylation and downstream actin polymerisation (237). *In vivo* proteomic analysis has also demonstrated that EspJ has immunoregulatory roles during infection, including; modulating proteins linked to the defense response, the regulation of immune cell migration, cytokine responses, and manipulation of the actin cytoskeleton (279). The results obtained in my study suggest that these pathways may be regulated, in part, by the suppression of Tir-mediated actin polymerisation and downstream inflammasome activation (Figure 5.7). Interestingly, it has previously been demonstrated that upon EPEC infection of HeLa cells, Tir retains its ability to induce actin pedestals when EspJ is translocated through the T3SS during infection (237). This was attributed to the spatiotemporal control of effector proteins in concert with the reported hierarchy of EPEC effector protein translocation (197). Indeed, Tir has been shown to be translocated prior to EspJ (199). These results suggest that when chromosomally expressed, EspJ may function to prevent excessive actin polymerisation after the initial stage of EPEC attachment to limit downstream inflammatory responses.

The intestine is a unique immunological environment, in which host cells are continuously exposed to the microorganisms constituting the normal microflora. However, the intestine also provides a potential gateway for pathogenic microorganisms that have the ability to cause disease. Intestinal immune cells have, therefore, evolved mechanisms to distinguish between innocuous microflora and disease-causing pathogens. The mechanisms underlying immune surveillance and inflammasome activation in response to infection of enteric pathogens are not fully understood.

However, here I demonstrate that the EPEC effector protein Tir, which is expressed exclusively by A/E pathogens, activates caspase-4 and the non-canonical inflammasomes during infection. Specifically, Tir-induced actin polymerisation was shown to activate an atypical non-canonical inflammasome pathway in human macrophages. This work, therefore, provides a new mechanistic insight into how the human innate immune system might specifically recognise these pathogenic strains of bacteria during infection of the host. Despite Tir-dependent actin polymerisation being genetically distinct in the prototypical strains EPEC 2348/69 and EHEC EDL933 used in this study (283, 289, 290, 725), Tir derived from both these strains induced inflammasome activation and pyroptosis in human macrophages. Interestingly, strains from EPEC lineage 2 and some EHEC strains utilise both Nck-dependent and TccP/TccP2-dependent pathways for actin polymerisation (300). In fact, a recent screen of clinical and environmental strains has revealed the presence of both pathways seems to be the norm, contrasting the segregation of signalling between EPEC E2348/69 and EHEC EDL933 (298). This raises the possibility that the ability to utilise both the Nck and TccP pathways confers an advantage to these strains. A key question that remains is whether these strains, which utilise both actin polymerization pathways, trigger heightened inflammatory responses via inflammasomes during infection.

As Tir-dependent actin polymerisation pathways are retained across A/E pathogens, they appear to be under intense selective pressure, however, the functional relevance of this actin polymerisation pathway *in vivo* is not clear. Testing the implications of this Tir-dependent inflammasome pathway *in vivo* is limited by the fact mice are resistant to EPEC infection. *In vivo* studies are frequently done using the murine model pathogen *C. rodentium*. Early during *C. rodentium* infection, protective IL-18 secretion is caspase-11-dependent (125, 572). However, at later time points, due to immune cell infiltration, both NLRC4 and NLRP3-dependent inflammasomes take precedence, and play critical roles in host defense, with Nlrp3 and Nlrc4 inflammasome-mediated IL-1β and IL-18 production being required for efficient clearing of *C. rodentium* infections from the gastrointestinal tract (126). As the NLRC4 inflammasome is not activated in human macrophages during EPEC infection, the NLRP3 inflammasome alone is likely to be principally responsible for mediating these immune responses in human hosts. The role for this Tir-dependent Nlrp3 inflammasome activation pathway in the context of *C. rodentium* infection is, therefore, likely to be masked by the activation of Nlrc4. Nevertheless, *C. rodentium* Tir shares 91% amino acid sequence identity with Tir from EPEC E2348/69 (122), and has the ability to trigger actin polymerisation via both the Nck-dependent and Nck-independent pathways via tyrosine residues Y471 (Equivalent to Tir^{EPEC} Y474) and Y541

(Equivalent to Tir^{EPEC} Y454). Previous work using *C. rodentium* to investigate these different Tir signalling pathways *in vivo* has identified a role for Tir-driven actin polymerisation in activating host innate immune responses (313). Host cells infected with *C. rodentium* strains expressing $Tir^{Y451A/Y471A}$ mutants produced significantly less of the chemokines CXCL1 (KC) and CXCL2 (MIP2-alpha) compared cells infected with WT *C. rodentium* (313), and these attenuated inflammatory responses were mirrored by significantly reduced colonic hyperplasia (313). CXCL1 and CXCL2 signal via CXCR2 to promote an influx of neutrophils to the site of infection. Interestingly, in mouse models of inflammation, a lack of IL-1β has been associated with an impairment in the production of these neutrophil chemokines and subsequent neutrophil recruitment (749, 750). As I have demonstrated that Tir-induced actin remodeling triggers inflammasome activation, which drives activation of caspase-1 and the subsequent release of inflammatory cytokines such as IL-1β, this may play an important role in neutrophil recruitment during infection. Consistent with this, *in vivo* infections of mice with *C. rodentium* $Tir^{Y451A/Y471A}$ mutants, that are unable to induce actin polymerisation, were reported to recruit significantly fewer neutrophils 14 days post-infection (313). Collectively, these results support a model in which Tir-induced actin polymerisation promotes inflammasome activation during infection, which is required to initiate efficient neutrophil infiltration and subsequent bacterial clearance (751).

Although neutrophils contribute to the host defense against infection, *in vivo* infections with WT *C. rodentium* and *C. rodentium* expressing mutant $Tir^{Y451A/Y471A}$ resulted in the same colonisation and bacterial clearance dynamics (312), suggesting that the host immune response can compensate for the absence of Tir-mediated neutrophil recruitment late during infection and clear the pathogen. In the context of *C. rodentium*, it is possible that activation of the Nlrc4 inflammasome plays a role in this response and subsequent bacterial clearance. Notably, *Casp1*$^{-/-}$ mice were highly susceptible to *C. rodentium* infection, whereas *Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$ mice initially presented with a milder phenotype that gradually worsened over the course of the infection (126), suggesting that these pathways may have complementary roles. Therefore, characterising the role of Tir-mediated actin polymerisation during infections of *Nlrc4*$^{-/-}$ mice could help clarify the implications of this pathway *in vivo* and may provide a more accurate model for the inflammasome responses induced during EPEC infection of human hosts.

Chapter 6-

Conclusions and Future Perspectives

This project set out to characterise the programmed cell death pathways induced by EPEC in human macrophages and aimed to understand the contribution of secreted EPEC effector proteins to these programmed cell death pathways. Here I have shown that EPEC induces Tir-dependent, rapid non-canonical NLRP3-inflammasome activation in human macrophages. Notably, in contrast to non-pathogenic *E. coli*, EPEC infection triggered both pyroptosis and cytokine processing through the NLRP3-caspase-1-inflammasome. Mechanistically, caspase-4 activation relied on LPS-detection and EPEC-induced actin polymerisation, either via Tir tyrosine phosphorylation and Nck recruitment, or Tir and the Nck-mimicking effector TccP. Collectively this study demonstrated that during physiological infection conditions EPEC induced an atypical caspase-4-NLRP3 signalling pathway stimulated by the co-ordinated activity of LPS and effector-driven actin polymerisation (Figure 6.1).

The importance of inflammasome activation during bacterial infections has been well established, and different inflammasomes have been shown to be paramount in the immune response to diverse bacterial pathogens, for example: the Nlrc4 inflammasome in response to *S.* Typhimurium (462, 463), *L. pneumophila* (467), and *Pseudomonas aeruginosa* (752); Nlrp1b in response to *B. anthracis* (478); Aim2 in response to *F. tularensis* (474), and the Nlrp3 inflammasomes in response to *S.* Typhimurium (462), *S. aureus* (753), *Klebsiella pneumoniae* (754) and *Burkholderia pseudomallei* (755), among others (Reviewed: (570)). This is also true in the context of A/E pathogens such as *C. rodentium*, in which caspase-11, Nlrp3 and Nlrc4 have all been implicated in the immune responses to infection (126, 504). The contribution of inflammasomes to host defence and disease pathogenesis is highlighted by the fact that disruption of the relevant inflammasome typically leads to an increase in bacterial replication and/or a decrease in host survival following infection. Conversely, forced expression of inflammasome ligands or deletion of inflammasome inhibitors in bacterial pathogens that can evade inflammasome detection can limit bacterial infection and promote bacterial clearance (444, 576, 752, 756). Therefore, understanding these complex innate immune pathways, and how they are activated and inhibited in the context of infection is key to developing future treatment strategies.

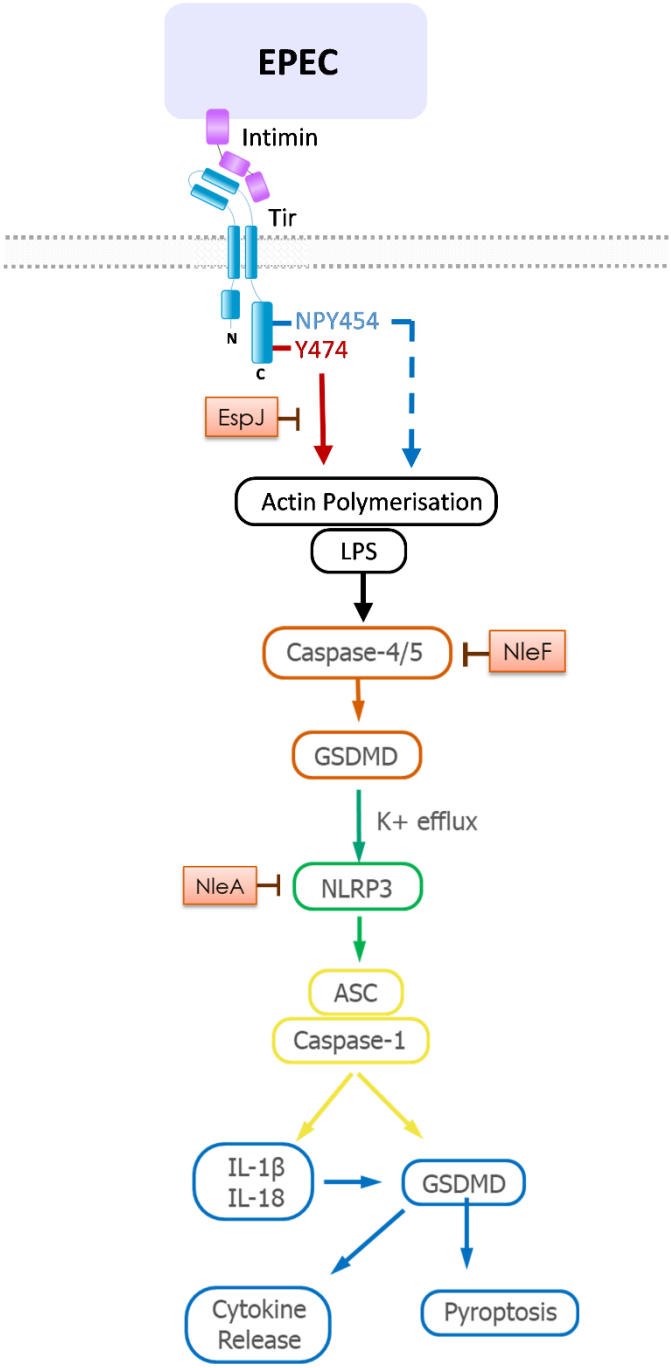

Figure 6.1. Proposed model for EPEC-induced inflammasome activation and pyroptosis

During EPEC infection of human macrophages, Tir- and intimin-mediated actin polymerisation induces rapid caspase-4- and GSDMD-dependent activation of the NLRP3 inflammasome. NLRP3 undergoes oligomerisation with the adaptor protein ASC and induces activation of caspase-1. NLRP3 activation of caspase-1 is required to mediate both cytokine processing and pyroptotic cell death. Effectors that suppress this inflammasome pathway are shown in red (EspJ, NleA and NleF).

A unifying property of inflammasome sensors is that they are all localised in the host cell cytosol. This localisation implies that inflammasomes will respond to pathogens, which access the cytosol as part of their virulence strategy, but will ignore harmless microbes which reside within the extracellular space (757). However, EPEC and other A/E pathogens are predominantly extracellular and therefore the ability of host cells to discriminate between these pathogens and non-pathogens is less well established, despite being crucial to the host innate immune response (126). The results here indicate that inflammasome responses occur in a Tir specific manner during infection of these extracellular bacteria, and therefore this study provides evidence of a novel strategy in which host cells can differentiate between disease-causing pathogens and the microbiota.

The non-canonical inflammasome has now been established as an integral part of innate immune mechanisms during bacterial infections, and the activation of caspase-11 has been shown to provide protection when activated correctly. However, excessive activation has the potential to cause extensive cell death and tissue damage. In addition to their fundamental roles in the immune response to infection, inflammasomes play a crucial role in immune homeostasis, with mutations in key inflammasome related proteins emerging as the underlying cause of various inflammatory, neurologic and metabolic disorders (758, 759). Within the intestine, inflammatory bowel diseases (IBD) such as Crohn's disease (CD), ulcerative colitis (UC), and indeterminate colitis are linked to inflammasome perturbations (760, 761). In this context, the NLRP3 inflammasome has been identified as a key regulator of intestinal immune tolerance, with dysregulation of NLRP3 signalling being linked to disease pathology (761, 762). Additionally, over the last few years, a pivotal role for the non-canonical caspase-4/11-dependent NLRP3 inflammasome in inflammatory bowel diseases has been identified. Intriguingly, in the mouse intestine, caspase-11 possesses two contradicting roles. It is thought to confer protection during chemically induced colitis by mediating intestinal barrier-protective effects (763-765), whereas, conversely, excessive cytosolic localisation of LPS results in hyperactivation of caspase-11 and septic shock (497, 507, 692). Given the evidence linking the caspase-4/11-dependent non-canonical pathway to both pathogen-induce sepsis and dysregulated intestinal inflammation, investigating the role of human caspase-4 within these pathways may lead to the identification of novel molecular targets for the treatment for these inflammatory bowel diseases.

This study revealed a novel role of caspase-4 during EPEC infection. As a receptor for LPS, caspase-4 was required to induce the inflammasome activation cascade during EPEC infection, but was not sufficient to induce pyroptosis. This demonstrated that caspase-4 activation during EPEC infection

was mechanistically distinct from that induced by cytosolic LPS. These results imply that other factors may limit caspase-4 activity during infection preventing pyroptosis. However, the host is still able to instigate a rapid inflammasome-mediated immune response through the activation of the NLRP3-caspase-1 inflammasome, demonstrating a level of redundancy in these host immune pathways. Inflammasome-evasion mechanisms are common pathogenicity strategies utilised by a range of bacterial pathogens, and inflammasomes must be able to evolve rapidly to respond to this.

Typically, inflammasome sensors are capable of transitioning from a fully off state to a fully on state across a narrow range of agonist concentrations, mediated by their highly co-ordinated oligomerisation into a single cytosolic complex. Interestingly, my results support a model in which caspase-4 functions differently from other inflammasome sensors in the fact that it can facilitate different outcomes upon LPS sensing. This is exemplified during EPEC infections, in which caspase-4 activation in response to EPEC Tir- and LPS-driven activation functions differently to caspase-4 activation in response transfected LPS moieties. A more comprehensive understanding of these mechanisms may be achieved utilising single cell analysis assays to establish whether caspase-4 reaches graded levels of activation in response to factors such as different forms of LPS and subsequent access to the lipid-A fraction, varying intracellular LPS concentrations, different mechanisms of delivery, or whether other pathogen- and host-derived factors function to limit caspase-4 activity during infection. Furthermore, identifying the interacting partners associated with caspase-4 during EPEC infection may help elucidate the factors responsible for its altered activity, and provide important mechanistic insights into the regulation and activation of caspase-4 in response to diverse stimuli.

Another important aspect of inflammasomes is their intrinsic autoinhibition and complex regulation. This prevents the unwanted initiation of inappropriate and potentially harmful inflammatory signals when no pathogen is present. For example, NLR autoinhibition is mediated thought their LRR domain (766), which blocks oligomerisation. Similarly, transcriptional regulation of inflammasome sensors, such as caspase-11 in the mouse (123), limits unwanted inflammation in the absence of infection. However, caspase-4 is constantly expressed in human cells, so does not have the same level of intrinsic regulation. Recently, new evidence has highlighted the fact that even non-pathogenic Gram-negative bacteria have the capacity to activate caspase-4/11 due to the release of LPS-laden OMVs (531, 532). Within the intestine, host cells are constantly in contact with bacteria that make up the microbiome, including a number of Gram-negative bacterial species, which are theoretically able to induce caspase-4 activation. However, despite its constant

expression, caspase-4-mediated inflammasome responses are down-regulated in the absence of pathogenic virulence factors. This indicates that additional mechanisms of regulation, such as IFNγ exposure, must exist within human cells to prevent unwanted caspase-4 activation in the absence of infection. This raises the question of how exactly LPS or OMVs gain access to the cytosol, and whether additional proteins are involved in liberating LPS from bacteria or OMVs in order to present it to caspase-4. Binding of pro-caspase-4 to purified meningococcal OMVs has been demonstrated (767). However, given the heterogeneity of OMVs, whether this is consistent among diverse OMVs derived from different Gram-negative pathogens requires further investigation (768, 769).

Both interferon-induced GBPs and IRGB10 have recently been shown to play a critical role in caspase-4/-11-dependent pyroptosis and NLRP3-inflammasome activation in response to intracellular Gram-negative bacterial pathogens (524, 527, 528). Other proteins may also assist in this process and promote LPS recognition by the non-canonical inflammasome. Indeed, it has been suggested that presentation of LPS from intact bacteria, or OMVs, requires modification of the bacterial outer-membrane for optimal activation of the non-canonical inflammasome (284, 524). The work here provides evidence that bacterial-associated effector proteins, such as Tir, drive the rapid caspase-4 activation during infection. This may be a result of Tir induced alterations to actin at the host cell membrane promoting access of LPS to the host cytosol. It is possible that other bacterial-associated or membrane-associated components also facilitate similar mechanisms. However, further work is required to understand the regulation of caspase-4 within human cells, and the potential role of OMVs and GBPs/IRGB10 during EPEC infections in the presence of secreted virulence factors. It would be interesting to establish whether the presence of Tir and intimin not only accelerate inflammasome-dependent pyroptosis, but also bypass the requirement for GPBs and OMVs in caspase-4 activation upon EPEC infection. EPEC may provide a useful tool to help clarify the different mechanisms of caspase-4 regulation and activation when challenged with either pathogenic or non-pathogenic Gram-negative bacteria.

The work here also presented evidence supporting diverse roles for GSDMD in the host immune response. The current model in which pyroptosis is initiated by the insertion of large, non-selective, GSDMD-pores into the host cell plasma membrane is challenged by the dual role GSDMD plays during EPEC infections. GSDMD is first required to activate the NLRP3 inflammasome in response to EPEC infection, then subsequently functions to mediate NLRP3-driven pyroptosis. An intriguing hypothesis is that low-level GSDMD activity mediates ionic flux

through pores of lower stoichiometry, while amplified GSDMD-cleavage increases the availability of the N-terminal pore-forming domain, which may facilitate the formation of larger GSDMD-pore structures to induce cell lysis. Notably, in support of this hypothesis, GSDMD deficiency not only inhibits pyroptotic plasma membrane rupture but has also been shown to abolish early ion exchange in LPS-transfected macrophages, that can occur up to 20 min before plasma membrane rupture (689). It is tempting to speculate that during EPEC infection, initially, GSDMD monomers insert into the plasma-membrane as small oligomers that function as selective pores to allow cytokine secretion and ion exchange, but then upon NLRP3 activation when a certain threshold of GSDMD cleavage is reached, GSDMD further assembles into higher order oligomers that form non-selective GSDMD pores and induce pyroptosis. Indeed, GSDMD N-terminal oligomers formed in GSDMD-overexpressing HEK293 cells were reported to be heterogeneous in size (520). Although this model of GSDMD activity has been suggested before (559, 689), in previous research the initial stage of pore formation is inseparable from the later onset of cell membrane rupture and pyroptosis. Therefore, Tir-mediated atypical inflammasome activation may provide a useful experimental model to dissect the intricacies of GSDMD-mediated inflammatory responses in the future. Furthermore, recent work looking at *S.* Typhimurium infections has revealed that Ca^{+} influx through GSDMD pores functions as a regulatory signal in host cells, and initiates membrane repair mechanisms. This membrane repair is mediated through the recruitment of the membrane-remodeling endosomal sorting complexes required for transport (ESCRT) machinery to the damaged host cell plasma membrane (770). This provides additional evidence that caspase cleavage of GSDMD is not a binary process that leads directly to pyroptosis, but is regulated by additional host cell factors. During EPEC infections this membrane repair machinery could allow cells to restrict caspase-4 induced pyroptosis, by removing GSDMD pores from the plasma membrane, while permitting limited GSDMD-dependent K+ efflux to induced NLRP3 activation. Further work is also needed to asses the impact of EPEC virulence factors such as Tir and intimin on the host cell ESCRTs machinery during infection.

Notably, this study highlighted fundamental differences between inflammasome signalling pathways in human and mouse macrophages. The observation that inflammasome responses during EPEC infection were dependent on the NLRP3 inflammasome was surprising, as T3SS encoding EPEC has previously been shown to activate the Naip2-NLRC4 inflammasome in mouse macrophages, through detection of the T3SS protein EscI (483, 684). This suggests that the human *NAIP* gene (hNAIP) may respond differently to ligands than its mouse counterparts. In support of

this, human macrophages and monocytes cultured *ex vivo* generally support robust *L. pneumophila* replication, which contrasts with the restricted replication seen in most mouse macrophages (771), and this was attributed to the inability of hNAIP to respond to *L. pneumophila* infections to propagate inflammasome activation. Differences between caspase-11 and caspase-4 have also been demonstrated, contrary to mouse caspase-11, the human homologue caspase-4 is able to detect cytosolic tetra-acylated LPS that has been released from *F. novicida* (513). Moreover, it was recently shown that the human pathogen *S. flexneri* antagonizes cell death via the actions of the T3SS effector OspC3, which binds to cleaved caspase-4 preventing its activation, but does not bind to mouse caspase-11 (514). Collectively, this provides compelling evidence that the mouse model, although one of the most important animal systems for the study of the mechanisms underlying human diseases, may not model inflammasome pathways accurately during infection by human pathogens. The observed differences between human and murine inflammasome pathways thus limits the accurate translation of results from mouse infection models to human pathogens and therefore, such results need to be interpreted carefully.

Another significant challenge will be to define the precise role of this Tir-induced macrophage inflammasome pathway *in viv*o. Within host organism specific cell types exhibit unique inflammasome functions, therefore it is important to evaluate how individual cell types contributes to the overall immune response to pathogens. Inflammasomes have been studied primarily in hematopoietic cells, and the principal focus of this study was to characterise EPEC-induced inflammasome pathways in human macrophages. However, recent work has demonstrated that inflammasomes can also have important roles in non-hematopoietic cells *in vivo* (572, 772-775). Recently, EPEC and *C. rodentium* infection in human and mouse intestinal epithelial cells (IECs) have been shown to activate a non-canonical inflammasome pathway (195, 410, 515). Unlike macrophages, IECs poorly express NLRP3 (662-664), and therefore caspase-4/11 has been shown to directly process pro-IL-18 into its mature form (410, 457). Further work is required to determine the molecular underpinnings of this mechanism and to establish whether similar Tir-dependent signalling pathways are responsible for activating inflammasomes in human IECs.

Despite continuous efforts centred on reducing the global burden of disease, infectious diseases continue to have a significant impact on human health worldwide (776, 777), with enteric pathogens, such as A/E pathogens being a significant cause of global morbidity and mortality (777, 778). Manipulation of host cells by T3SS is a common theme amongst enteric bacterial pathogens and plays a fundamental role in virulence (779). Therefore, research into T3SS

effectors and how specific effector proteins can alter host immune responses may help uncover new therapeutic targets for the treatment of these pathogens. The development of new therapies is particularly important given the inexorable rise of antibiotic resistance among enteric pathogens (780-783), combined with a steady decline in the rate of discovery of new antibiotics.

This project has identified a novel role for the effector protein Tir, and Tir-mediated actin polymerisation in inflammasome activation and pyroptosis during EPEC infection. Interestingly, in contrast to this, EPEC effector proteins have previously been shown to target and inhibit inflammatory signalling pathways and programmed cell death to subvert these host immune responses. An interesting hypothesis is that Tir, as the first virulence factor secreted into host cells, induces actin polymerisation and LPS-mediated activation of caspase-4, which stimulates the host immune system, instigating inflammatory responses and eventual bacterial clearance. However, subsequent to this initial rapid innate immune response, additional EPEC effectors are secreted to disrupt these pathways and downregulate inflammation during infection. In this manner, EPEC may orchestrate a complex effector-mediated pattern of inflammation that facilitates infection within the host cells. This could be mediated through a number of mechanisms, such as the inhibition of Tir-mediated actin polymerisation by EspJ (237), the inhibition of caspase-4 by NleF (230), or the inhibition of NLRP3 activation by NleA (226). The co-ordinated action of these effectors may function to protect cells from excessive actin polymerisation and subsequent inflammasome activation. It is not unusual for EPEC to translocate several effectors with complementary functions, as observed for the inhibition of NFκB signalling (81), and so multiple effectors could potentially contribute to this phenotype by disrupting Tir^{EPEC}-dependent signalling and limiting inflammasome responses late in infection. As both caspase-4 and NLRP3 play an essential role in the immune response to EPEC infection, strains that can prevent these inflammatory responses may be more virulent and persist within the host for extended periods. Targeting these strategies may, therefore, provide possible treatment options to assist in the clearance of infection.

Designing strategies to target the virulence of enteropathogens such as EPEC is challenging due to the inherent genomic plasticity of these strains, and also the redundant nature of many of the effector proteins (20). *E. coli* as a species is constantly evolving, and the rapid growth and easy acquisition of new traits by these pathotypes results in bacterial targeted therapies becoming obsolete. Therefore, treatments and preventative methods must constantly be evolved. An attractive alternative approach to the treatment of bacterial infection is the selective amplification

of the innate immune response. The innate immune system functions rapidly during infection and targets a broad range of infectious pathogens. Therefore, the development of new therapeutics that augment the function of the innate immune system could provide a range of effective broad-spectrum antimicrobials. Therapeutics that modify innate immunity are already used for a number of autoimmune diseases, however, the effectiveness of these treatment in counteracting bacterial infections has yet to be experimentally verified (784). To realise the potential advantages of targeting innate immunity during bacterial infection, it is imperative to gain a more comprehensive understanding of the innate immune system. Currently, the precise mechanisms by which immune system targeting therapeutics function *in vivo* is not well defined, and therefore the potential adverse effects that may arise as a result of treatment induced amplification of the innate immune responses during microbial infections requires further investigation. This is particularly important as dysregulation of the innate immune system and the associated inflammatory response can result in adverse events, such as sepsis, that can be detrimental to a host. However, a more comprehensive understanding of the mechanisms behind inflammasomes and sepsis will help to mitigate these obstacles and may create opportunities for the development of novel therapies through the identification of targets for rational drug design.

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