Enterohaemorrhagic *Escherichia coli* colonisation of the human colonic epithelium and the innate immune response to infection

by
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Abstract

Enterohaemorrhagic *Escherichia coli* (EHEC) are of great public health importance, causing diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome. A wealth of evidence from cell line and animal studies has linked colonic pathogenesis with EHEC induction of mucosal attaching/effacing (A/E) lesions, which are characterised by intimate bacterial attachment to intestinal epithelial cells (IECs), microvillous effacement and actin polymerisation. However, the clinical relevance of A/E lesions has been called into question by the fact that adherent bacteria have not been located on colonic biopsies taken from EHEC-infected patients. In addition, *in vitro* organ culture (IVOC) of human intestinal biopsies with EHEC has not yielded signs of bacterial colonisation or A/E lesion formation. In this study, we have re-evaluated the colonic IVOC model and demonstrated for the first time that EHEC colonise the colonic epithelium *ex vivo* and induce A/E lesion formation. High levels of oxygen, as used in previous IVOC studies, were found to inhibit EHEC-IEC interactions. Adherence was dependent on expression of the bacterial adhesin intimin and the type III secretion system (T3SS). In contrast, EHEC adherence to the widely-used T84 colonic carcinoma cell line was associated with microvillous effacement but not actin polymerisation, and was not dependent on intimin or the T3SS, highlighting the importance of using physiologically relevant intestinal models when conducting EHEC research.

EHEC pathogenesis has also been linked to induction of an inflammatory response in the colonic mucosa, characterised by recruitment of neutrophils to the epithelium. This process has been associated with expression of the neutrophil chemokine interleukin-8 (IL-8) by cultured IECs following exposure to EHEC flagellin. However, other aspects of the innate immune system, such as expression of antimicrobial peptides (AMPs), have been neglected. We have demonstrated that EHEC induces expression of the AMP human β-defensin-2 (hBD2) and IL-8 in human colonic IECs both *in vitro* and *ex vivo*. Induction was dependent on exposure of IECs to flagellin, and was mediated by mitogen activated protein kinase (MAPK) signalling. Furthermore, we present evidence that IL-8 is secreted apically in polarised T84 cells following apical stimulation, suggesting that this cytokine may be secreted into the lumen during infection. Put together, this PhD project has shed new

light on EHEC-IEC interactions and has re-established the IVOC model as an important tool for future EHEC research.

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Ab – antibody

AE – aerobic

A/E – attaching and effacing

AI – autoinducer

AMP – antimicrobial peptide

AP-1 – activator protein-1

AQP – aquaporin

AR – acid resistance

Bfp – bundle-forming pili

bp – base pairs

BSA – bovine serum albumin

Cah – calcium-binding antigen 43 homologue

CCR – CC-chemokine receptor

CD – Crohn's disease

CDC – Centres for Disease Control and Prevention

CFU – colony-forming units

CL – containment level

Ct – cycle threshold

DC – dendritic cell

ddH_2O – diluted and deionised water

DAEC – Diffusely-adherent *E. coli*

DAPI – 4',6-diamidino-2-phenylindole

DMSO – dimethyl sulphoxide

DMEM – Dulbecco's modified Eagle's medium

DRA – downregulated in adenoma

EA – ethanolamine

EAEC – Enteroaggregative *E. coli*

EAF – EPEC adherence factor

ECDC – European Centre for Disease Prevention and Control

ECM – extracellular matrix

ECP – *E. coli* common pilus

EEA - European Economic Area

Eha – Enterohaemorrhagic *E. coli* autotransporter

EHEC – Enterohaemorrhagic *E. coli*

EIEC – Enteroinvasive *E. coli*

ELF – *E. coli* YcbQ laminin-binding fimbriae

EPEC – Enteropathogenic *E. coli*

ER – endoplasmic reticulum

ERAD – endoplasmic reticulum-associated degradation

Esp – *E. coli* secreted protein

ETEC – Enterotoxigenic *E. coli*

FAE – follicular-associated epithelium

FAS – fluorescent actin stain

FBS – foetal bovine serum

FITC – fluorescein isothiocyanate

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

Gb3 – globotriaosylceramide

GI – gastrointestinal

Grl – global regulator of Ler (LEE-encoded regulator)

hBD – human beta-defensin

HC – haemorrhagic colitis

HCP – haemorrhagic coli pilus

HGT – horizontal gene transfer

HN-S – histone-like nucleoid structuring

HUS – haemolytic uraemic syndrome

H_2O_2 – hydrogen peroxide

IBD – inflammatory bowel disease

IEC – intestinal epithelial cell

IFN – interferon

Iha – *Vibrio cholerae* iron-regulated gene A homologue adhesin

IKK – IκB kinase

IL – interleukin

IVOC – *in vitro* organ culture

LB – Luria Bertani

LEE – locus of enterocyte effacement

Ler – LEE-encoded regulator

Lpf – long polar fimbriae

LPS - lipopolysaccharide

Lrp – leucine-responsive regulatory protein

MA – microaerobic

MAMP – microbe-associated molecular pattern

MAPK – mitogen-activated protein kinase

MIP3α – macrophage inflammatory protein 3α

ND – none detected

NF-κB – nuclear factor κB

NHE – Na^+/H^+ exchanger

NI – non-infected

Nle – non-LEE-encoded effector

NMEC – Neonatal meningitis *E. coli*

NO – nitric oxide

NT – no template

N-WASP – neuronal Wiskott-Aldrich syndrome protein

OD – optical density

Omp – outer membrane protein

PAI – pathogenicity island

PE – phosphatidylethanolamine

PI – post-infection

PCR – polymerase chain reaction

pIVOC – polarised *in vitro* organ culture

POL2A – RNA Polymerase II polypeptide A

PRR – pattern recognition receptor

qPCR – quantitative polymerase chain reaction

Qse – quorum-sensing *E. coli* regulator

RPM – revolutions per minute

RPS3 – ribosomal protein S3

RT-PCR – reverse transcription polymerase chain reaction

SCID – severe-combined immuno-deficient

SCFA – short-chain fatty acids

SE – standard error of the mean

SEM – scanning electron microscopy

SGLT – sodium-dependent glucose transporter

SNP – single nucleotide polymorphism

SPATE – serine protease autotransporters of *Enterobacteriaceae*

sRNA – small RNAs

STEC – Shiga toxin-producing *E. coli*

Stx – Shiga toxin

TBE – Tris-borate-EDTA

TccP – Tir-cytoskeleton coupling protein

TER – transepithelial electrical resistance

TEM – transmission electron microscopy

Tir – translocated intimin receptor

TJ – tight junction

TLR – Toll-like receptor

TNF – tumour necrosis factor

T3S(S) – type III secretion (system)

UC – ulcerative colitis

UK – United Kingdom

UPEC – Uropathogenic *E. coli*

USA – United States of America

USDA – US Department of Agriculture

VDC – vertical diffusion chamber

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CHAPTER ONE

INTRODUCTION

1.1 | Enterohaemorrhagic *E. coli*

The life of humans would be very different without the existence of *Escherichia coli*. *E. coli* bacteria colonise the gastrointestinal (GI) tract within hours of birth and establish themselves as the predominant facultative anaerobe of the microbiota. The majority of *E. coli* strains engage in a mutually beneficial relationship with the host that provides a stable environment for the bacteria and improved digestion and protection from pathogenic infection for the host (Bentley and Meganathan, 1982; Reid *et al.*, 2001). However, a number of strains have acquired genes that confer the ability to cause disease in humans. Since the isolation of the first pathogenic *E. coli* type in 1945 (Bray, 1945), the species has emerged as one of the largest contributors to food-borne illness worldwide. *E. coli* strains are genetically diverse and confer a range of diseases including diarrhoea, meningitis, urinary tract infections and haemolytic uraemic syndrome (HUS). Hundreds of millions of people are now estimated to be affected by pathogenic *E. coli* every year (Croxen and Finlay, 2010). Thus, in the past seventy years, our regard for this species has shifted from harmless commensal to dangerous pathogen that constitutes a huge public health and economic burden worldwide.

Pathogenic *E. coli* strains are broadly classified into nine pathotypes, each colonising a specific niche in the human body (Figure 1.1). Five of these pathotypes – Enterohaemorrhagic *E. coli* (EHEC), Enteropathogenic *E coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), and Diffusely-adherent *E. coli* (DAEC) – colonise the GI tract and induce diarrhoea. Uropathogenic *E. coli* (UPEC) and Neonatal meningitis *E. coli* (NMEC) are extra-intestinal pathotypes that are the leading cause of urinary tract infections and neonatal meningitis, respectively. Finally, adherent-invasive *E. coli* (AIEC) colonise the small intestine and are thought to play a role in Crohn's Disease (CD) pathogenesis (Strober, 2011).

Of the hundreds of pathogenic *E. coli* strains that affect humans, those belonging to the EHEC pathotype pose the largest public health threat in the developed world. EHEC are the fourth most commonly reported bacterial cause of enteric infection in the UK (ECDC, 2014). It is considered a colonic pathogen that causes diarrhoea by

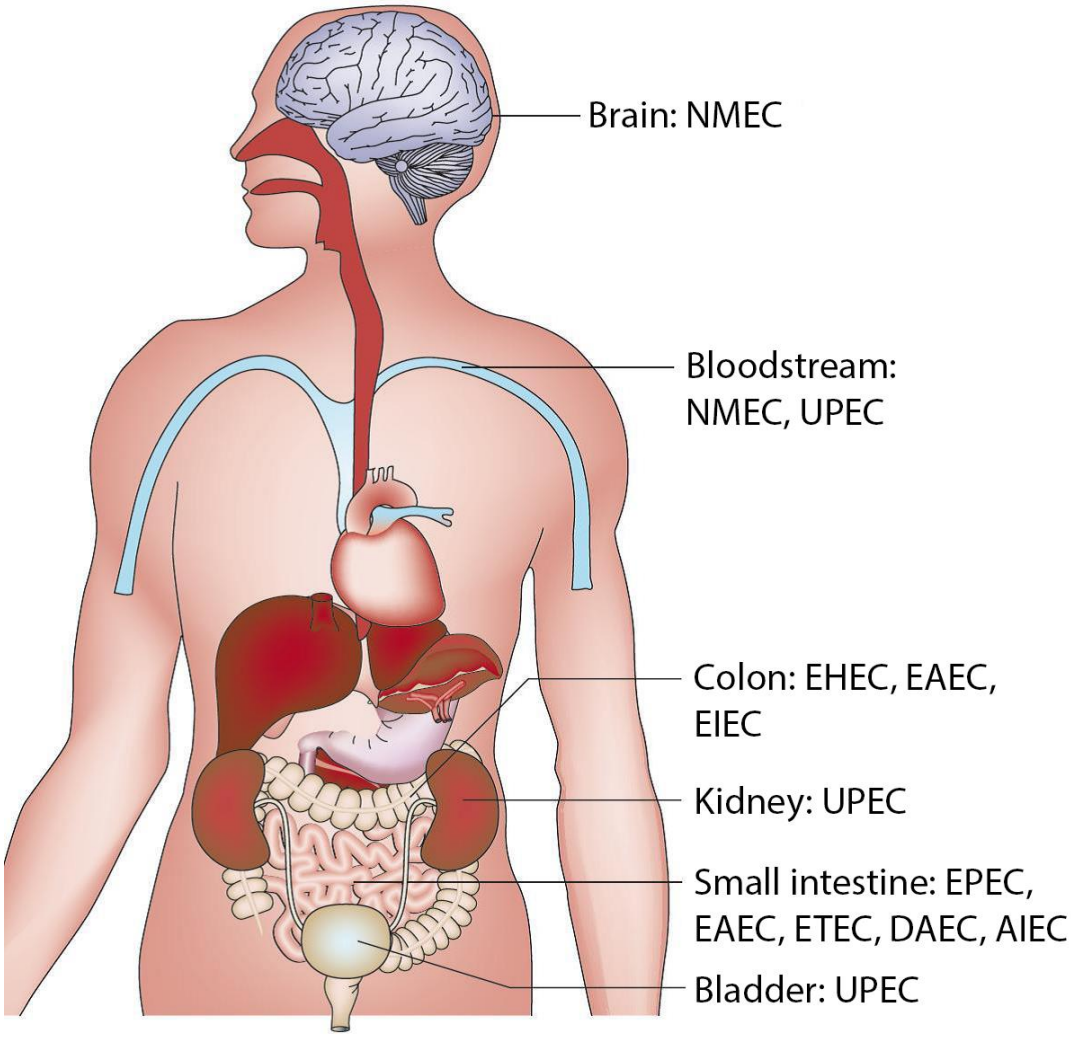

Figure 1.1. Sites of pathogenic *E. coli* colonisation. Modified from Croxen and Finlay, 2010.

forming attaching and effacing (A/E) lesions on intestinal epithelial cells (IECs). EHEC bacteria also produce Shiga toxins (Stxs), which damage cells of the colonic and renal microvasculature to induce haemorrhagic colitis (HC) and HUS, respectively. EHEC-associated HUS is the leading cause of acute kidney failure in children in the United Kingdom (UK), and can be fatal (Lynn *et al.*, 2005). Furthermore, there is no treatment for EHEC infection or HUS, and the use of antibiotics is contraindicated (Goldwater and Bettelheim, 2012). Therefore, EHEC research is crucial in order to develop an effective means to combat infection. This chapter provides an overview of our current understanding of EHEC infection

biology, with emphasis on intestinal pathogenesis and the innate immune response to infection.

1.2 | Emergence and evolutionary origin

In 1983, Riley and colleagues investigated a large food-poisoning outbreak in the United States of America (USA) that was associated with an unusual form of gastrointestinal illness, characterised by severe abdominal pain and HC (Riley *et al.*, 1983). They identified the causative agent as an *E. coli* strain belonging to the serotype O157:H7, which had only once before been isolated from a case of HC in 1975. Retrospective analysis of historical isolates did not implicate *E. coli* O157:H7 in any other past cases (Day *et al.*, 1983; Johnson *et al.*, 1983), indicating that this serotype was an emerging pathogen. Konowalchuk and co-workers reported that EHEC O157:H7 produced a substance that had a cytotoxic effect on Vero African Monkey kidney cells, which they termed verotoxin (Konowalchuk *et al.*, 1977). O'Brien *et al*. also discovered this toxin and demonstrated that it showed sequence homology to Shiga toxin (Stx), which is produced by *Shigella dysenteriae* type 1, and thus named it Shiga-like toxin (O'Brien *et al.*, 1983). Both verotoxin and Shiga toxin are still used to describe this particular type of EHEC toxin, but only the latter term will be used in this thesis. Later that same year, Karmali *et al*., reported an association between HUS and the presence of Stx and Stx-producing *E .coli* (STEC) in the stools of sufferers (Karmali *et al.*, 1983), providing the first indication of a causal role for Stx in development of HUS.

Since the discovery of *E. coli* O157:H7, more than 500 other STEC serotypes have been identified (Blanco *et al.*, 2004). However, not all STEC serotypes are capable of causing disease in humans; only those which harbour additional virulence determinants are considered pathogenic (Nataro and Kaper, 1998). The most important of these is the locus of enterocyte effacement (LEE), a large genetic region that encodes most of the genes necessary for the A/E phenotype (McDaniel *et al.*, 1995). This has led to the use of the term STEC to denote any *E. coli* strain containing *stx* genes and EHEC for those that harbour both *stx* and the LEE (Nataro and Kaper, 1998).

EHEC appears to have evolved from EPEC, since phylogenetic data shows that EHEC O157:H7 and EPEC O55:H7 share a recent common ancestor (Whittam *et al.*, 1993). EPEC also possesses the LEE and triggers A/E lesion formation upon infection of IECs (McDaniel *et al.*, 1995), but does not harbour *stx* genes. Stxs are encoded on lambdoid prophages incorporated into the bacterial chromosome (Makino *et al.*, 1999; Yokoyama *et al.*, 2000), indicating that evolution from EPEC to EHEC likely resulted from horizontal gene transfer (HGT) of *stx* following prophage infection. Interestingly, acquisition of *stx* appears to have occurred several times in evolutionary history, as EHEC strains do not fall into a single phylogenetic group (Reid *et al.*, 2000). This indicates that the EHEC pathotype represents a collection of genetically diverse strains that confer a similar virulence phenotype.

The acquisition of *stx* is not the only example of EHEC gaining virulence through HGT. Indeed, the genomes of pathogenic *E. coli* are striking in that many virulence-associated genes are located in regions of DNA originating from other organisms, termed pathogenicity islands (PAIs). PAIs, such as the LEE in EHEC, are discernible from native genetic material in that they harbour markedly different G+C contents and are found in association with mobile genetic elements (e.g. bacteriophages, plasmids, transposons, insertion sequences). The high level of HGT in *E. coli* drives the continuous emergence of new strains, each harbouring a unique repertoire of virulence genes (Bugarel *et al.*, 2010). This diversity between strains complicates the study of EHEC virulence as it is difficult to infer meaning from analysis of a small number of isolates. Researchers therefore tend to focus their attention on O157:H7 strains (e.g. EDL933, 86-24) as this serotype is most associated with disease in humans. As a result, non-O157:H7 strains are less well-characterised.

The O157:H7 serogroup comprises hundreds of genetically distinct strains, and studies have shown that certain strain types are more associated with disease in humans. In their phylogenetic study, Manning *et al*. developed a system for classifying O157 strains based on detection of single nucleotide polymorphisms (SNPs) in 96 loci in 83 genes (Manning *et al*., 2008). Using this method, they classified over 500 strains isolated from clinical cases in the USA into a total of 9 clades, and found that strains belonging to clade 8 were significantly associated with severe disease presentation (i.e. development of HUS). These clades also differed in

the frequency and distribution of *stx* genes: 100% and 57.6% of clade 8 strains harboured the *stx2* and *stx2c* genes, respectively (Manning *et al*., 2008), which encode the Stx subtypes most associated with severe disease (Karch *et al*., 2006; Orth *et al*., 2007).

Analysis of EHEC O157:H7 isolates from cases in Europe has revealed an association between virulence and bacterial phage type (PT). Phage typing has been used since the late eighties as a method of distinguishing between individual EHEC strains, and is based on characterising the susceptibility profile of an isolate to a panel of 16 bacteriophages (Ahmed *et al*., 1987; Khakhria *et al*., 1990). In the UK and Ireland, the chance of developing infantile HUS is significantly greater if the patient is infected with a strain belonging to PT21/28 compared with other PTs (Lynn *et al*., 2005). Furthermore, PT21/28 accounts for more cases of human illness in Scotland compared with other PTs (57% of all cases between 1998 and 2008) (Health Protection Scotland, 2009). It has been suggested that the increased virulence of PT21/28 is attributable to the fact that this PT is shed from cattle at higher levels than other PTs (a so-called 'supershedder'), which would lead to higher levels of these strains in the environment and therefore increased chances of transmission to humans (Chase-Topping *et al*., 2007; 2008). In addition, the number of PT21/28 strains harbouring both *stx2* and *stx2c* is relatively high compared with PT32, the second-most prevalent PT (90% and 28%, respectively) (Xu *et al*., 2012), which may explain the increased disease severity in patients infected with PT21/28.

1.3 | Epidemiology

EHEC is one of the most common food-borne pathogens in the developed world. It is estimated that approximately 265,000 EHEC infections occur every year in the USA (CDC, 2015), whilst around 3,500 confirmed cases are reported annually in the European Economic Area (EEA) (ECDC, 2014). The actual number of cases in the EEA is likely to be higher, since not all cases are reported and therefore cannot be included in epidemiological analyses (ECDC, 2014). EHEC is the fourth most reported bacterial cause of food- and water-borne zoonosis in the EEA, after *Campylobacter* spp., *Salmonella enterica* and *Shigella* spp. (ECDC, 2014).

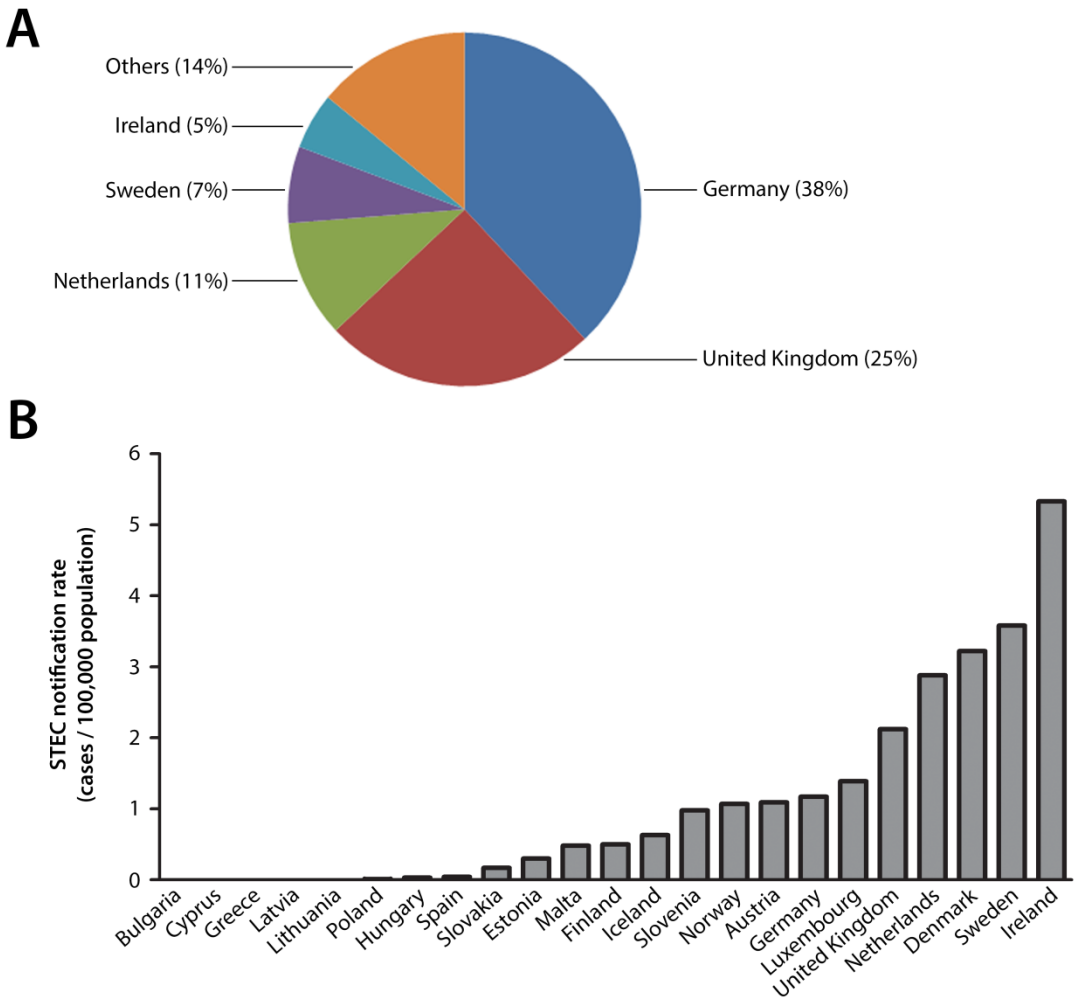

Figure 1.2. Incidence of EHEC infections in the EEA. (A) Proportion of total cases reported by individual countries between 2008 and 2012. **(B)** Median annual notification rates for the years 2008-2012. Some EEA countries are excluded due to insufficient data. Based on data from ECDC, 2014.

Interestingly, the UK accounts for approximately 25% of the total number of EHEC cases reported in the EEA (6,448 of 25,864 cases reported between 2008 and 2012) and has one of the highest notification rates of all EEA countries (Figure 1.2). This suggests that EHEC infection is particularly prevalent in the UK, although it should be noted that differences in diagnostic methods between countries may influence this data (ECDC, 2014). EHEC is common in other developed countries including Canada, Japan and Australia, as well as in developing countries such as Argentina

(Croxen *et al.*, 2013). Sporadic infection accounts for the majority of EHEC cases, although epidemic infections also occur (Byrne *et al.*, 2015).

Public Health England (PHE) recently published an epidemiological report on EHEC infections in England based on data collected since the introduction of the National Enhanced Surveillance System for STEC (NESSS) in 2009 (Byrne *et al.*, 2015). The NESSS is designed to collate microbiological, demographic, clinical and exposure data in order to inform epidemiology and improve outbreak detection. The NESSS report shows that more than 40% of the total confirmed EHEC cases occurred in children <15 years of age, with the highest incidence observed in 1-4 year olds. This age group was not found to be disproportionately affected by *Campylobacter* or *Salmonella* (7.0 and 17.8% of total cases, respectively), indicating that the trend does not apply to all food-borne illnesses. The high rate of infantile EHEC illnesses was attributed to the infectious nature of EHEC, which is thought to facilitate transmission among children *via* person to person contact at schools and childcare facilities. Serogroup O157 was responsible for 98.8% of EHEC cases in England (Byrne *et al*., 2015), which is notably high when compared with figures reported for the EEA as a whole (34%) and the USA (36%) (CDC, 2015; ECDC, 2014). However, standard diagnostic testing for STEC in England often does not include screening for non-O157 strains (Byrne *et al.*, 2015), and so it is possible that non-O157 cases are under-represented. As mentioned in the previous section, the most common O157:H7 phage type is PT21/28 (50% of all cases in Scotland in 2008), followed by PT8 and PT32 (both 11%) (Health Protection Scotland, 2009). According to the ECDC (2014), the most commonly isolated non-O157 serogroups in the EEA are O26, O91, O103 and O145; whilst the US Department of Agriculture (USDA) has named O26, O45, O103, O111, O121 and O145 as the most important non-O157 disease-causing serogroups in the USA (USDA, 2012).

The transmission route of EHEC is faecal-oral, with an estimated infectious dose of <100 organisms (Tuttle *et al.*, 1999). Cattle and other farm animals often serve as a reservoir host for EHEC. Accordingly, the most common route of human exposure to EHEC is through consumption of food products contaminated with infectious livestock faeces. Foods that have been implicated in EHEC transmission include ground beef, sausage, unpasteurised milk and apple juice, and a variety of vegetables

(Chauret, 2011). Exposure by direct contact with reservoir hosts in petting zoos, agricultural shows and farm settings is also an important mode of transmission, accounting for 34.8% of EHEC cases in England between 2009 and 2012 (Byrne *et al.*, 2015). As mentioned above, person to person transmission is also common, particularly during outbreaks.

1.4 | Disease symptoms and treatment

The progression of EHEC-associated disease from ingestion to clinical outcome is summarised in Figure 1.3. Diarrhoea is reported in most cases (92.8% in England between 2009 and 2012), and is often accompanied by other complaints such as abdominal cramps, fever and vomiting (79.2, 32 and 37.3%, respectively) (Byrne *et al.*, 2015). These symptoms typically ensue within 3-4 days of ingesting the infective dose. Bloody diarrhoea, a sign of HC, develops in around 61% of cases around 3 days later. It is the detection of blood in the stool that causes most patients to seek medical attention. In most cases, symptoms will begin to improve 4-10 days after the onset of illness. However, in 5-15 of cases, alleviation of gastrointestinal symptoms is followed by development of HUS (Byrne *et al.*, 2015; Trachtman *et al.*, 2012). HUS is characterised by haemolytic anaemia, thrombocytopaenia, and acute kidney failure. The syndrome is more likely to develop in children, and is the leading cause of acute infantile kidney dysfunction. There is no treatment for HUS other than dialysis, which is performed in 30-40% of cases for approximately 10 days (Trachtman *et al.*, 2012). Approximately 1 in 4 HUS sufferers also develop neurological dysfunction leading to apnoea, coma, seizures, cortical blindness and haemiparesis (Trachtman *et al.*, 2012). Additional sequelae of HUS include chronic renal dysfunction, colonic strictures, hypertension, stroke, gallstones and diabetes mellitus. (Byrne *et al.*, 2015; ECDC, 2014; Lynn *et al.*, 2005; Spinale *et al.*, 2013; Tarr *et al.*, 2005). HUS carries a mortality rate of up to 5%, with death usually resulting from the aforementioned neurological complications (Kavanagh *et al.*, 2014; Trachtman *et al.*, 2012).

There is currently no effective treatment for EHEC infection. The use of antibiotics is not recommended, as some clinical studies have indicated that antibiotic treatment

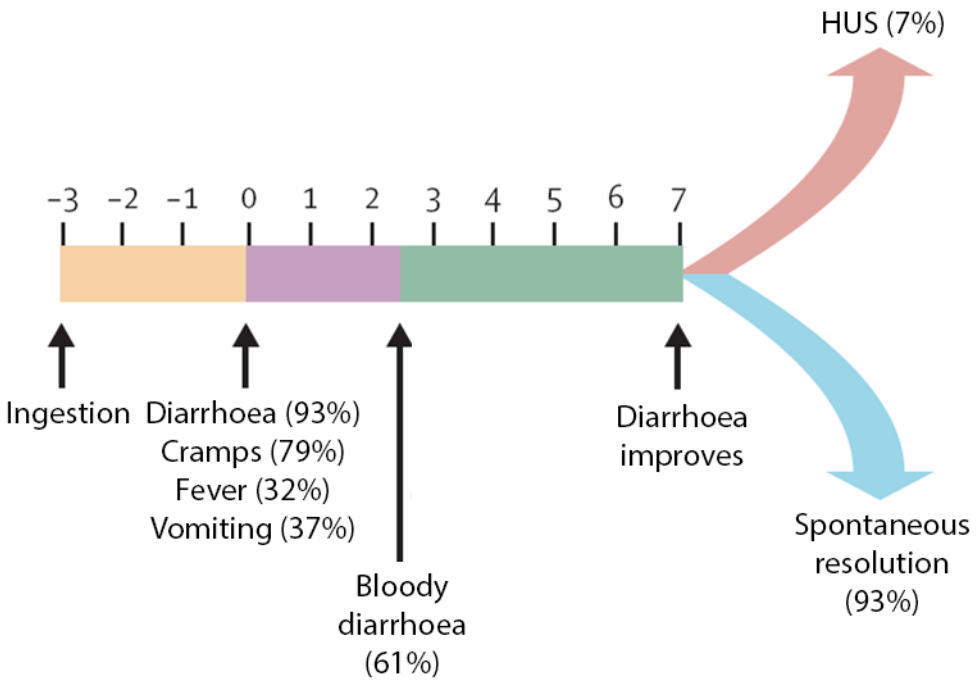

Figure 1.3. Progression of clinical symptoms over time (days) following EHEC infection. Adapted from Tarr *et al*. (2005). Percentages represent proportion of individuals presenting symptom relative to total number of cases (Byrne *et al*., 2015).

is a risk factor for HUS (Smith *et al.*, 2012; Wong *et al.*, 2000). This has been attributed to antibiotics triggering the expression and release of Stx in bacteria *via* activation of the stress-induced Stx phage lytic cycle (Tarr *et al.*, 2005; Zhang *et al.*, 2000). However, other studies show no negative correlation between antibiotic therapy and HUS (Bielaszewska *et al.*, 2012; Safdar *et al.*, 2002), and so the use of antibiotics to treat STEC infection remains controversial. Much effort has been applied to developing a vaccine against EHEC, and several candidates have performed well in animal studies (Bentancor *et al.*, 2009; Gu *et al.*, 2009). Vaccination of cattle has also been explored as a method of preventing transmission to humans (McNeilly *et al.*, 2010; McNeilly *et al.*, 2008; Potter *et al.*, 2004; Tahoun *et al.*, 2015). Many vaccine candidates target Stx and/or bovine IEC adherence factors such as intimin, translocated intimin receptor (Tir), *E. coli* secreted protein A (EspA) and flagellin; or a combination of the above. The economic cost of producing any successful vaccine is likely to influence the extent of its use.

Many alternative therapies are being investigated, which have recently been reviewed by Goldwater and Bettelheim (2012). Several studies have focussed on development of antibodies or synthetic molecules that bind to Stx and prevent it from binding to host cells, thereby inhibiting its pathological effects (Islam and Stimson, 1990; Trachtman *et al.*, 2003; Watanabe-Takahashi *et al.*, 2010). Others have identified molecules, such as manganese and compound 75, which inhibit intracellular transport of Stx and cytotoxicity in host cells (Mukhopadhyay and Linstedt, 2012; Saenz *et al.*, 2007). Another strategy being investigated is the use of probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* spp. to prevent EHEC growth or interaction with the intestinal epithelium (Medellin-Pena and Griffiths, 2009; Mogna *et al.*, 2012; Sherman *et al.*, 2005). Research into these potential therapies is still rudimentary, with few having progressed to clinical trials and none demonstrating a protective effect in humans.

1.5 | Virulence

EHEC possesses two main virulence traits: A/E lesion formation and Stx production. Our current understanding of the mechanisms underlying each of these is reviewed in this section.

1.5.1 | A/E lesion formation

EHEC belongs to a family of pathogens, referred to as attaching and effacing (A/E) bacteria, that forms distinctive histopathological A/E lesions on IECs. Other members of the family include the closely-related human pathogen enteropathogenic *E. coli* (EPEC), rabbit diarrhoeagenic *E. coli* (RDEC-1), and the murine pathogen *Citrobacter rodentium*. A/E lesions were first described by Staley and colleagues following infection of newborn piglets with EPEC (Staley *et al.*, 1969), and are distinguishable on IECs by the presence of intimately adherent bacteria and loss of microvilli around the attachment site. However, our understanding of the mechanisms underlying A/E lesion formation was minimal until the late eighties, when Knutton and colleagues developed a simple *in vitro* test for detecting A/E lesions. Using transmission electron microscopy (TEM) to visualise bacteria adhered

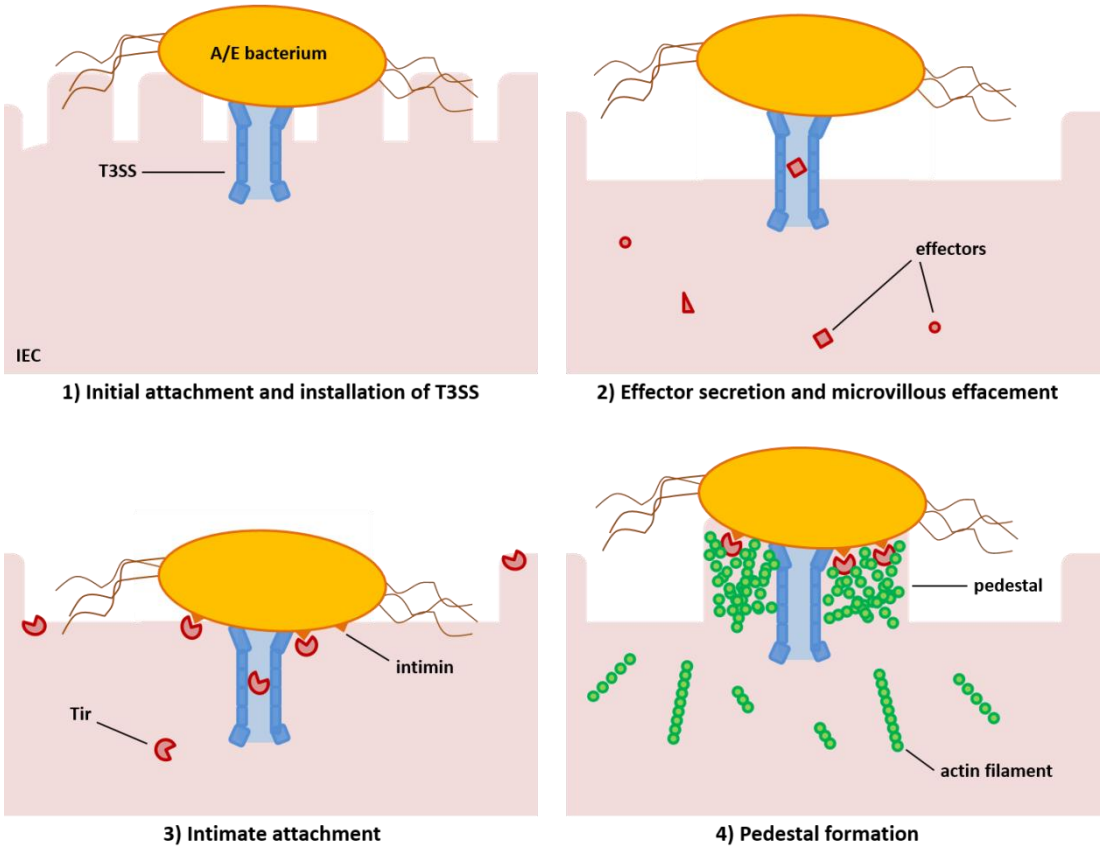

Figure 1.4. A/E lesion formation on IECs.

to cultured cell lines, the authors showed that A/E lesions were associated with polymerised actin beneath the site of bacterial attachment (Knutton *et al.*, 1987). Soon afterwards, the same group introduced the fluorescent actin stain (FAS) as a new method for detecting polymerised actin that was less labour-some than TEM. (Knutton *et al.*, 1989). The FAS test subsequently became the standard diagnostic test for A/E bacteria and provided researchers with a tool with which to identify the virulence factors necessary for A/E pathogenesis *in vitro*.

The events leading to A/E lesion formation have been well-characterised using cultured cell lines (Nataro and Kaper, 1998), and are outlined in Figure 1.4. First, EHEC forms an initial attachment to an IEC *via* one or more adhesins (1). The bacterium then inserts its type III secretion system (T3SS) into the host cell, which is used to inject a barrage of 'effector' proteins into the cytosol. These effectors subvert host cell processes resulting in several morphological changes, which include the

localised effacement of microvilli (2). Microvillous effacement likely facilitates bacterial adhesion to the IEC surface, and also plays a role in induction of diarrhoea by reducing the absorptive surface area of the epithelium (Viswanathan *et al*., 2009). EHEC also secretes its own receptor, the translocated intimin receptor (Tir), into the host cell. Tir inserts into the IEC plasma membrane, where it binds to intimin on the bacterial outer membrane to form an intimate attachment (3). Tir then works with other effector proteins to recruit polymerised actin (4), which elevates the bacterium on an actin-rich pedestal-like structure. Several effector proteins also disrupt epithelial barrier function and subvert the transport of ion and water across the epithelium, which also contributes to the onset of diarrhoea (Viswanathan *et al.*, 2009).

Most of the proteins involved in T3S and A/E lesion formation are encoded on the LEE (Mellies *et al.*, 2007). Several effector proteins are also encoded on this PAI, although an increasing number of non-LEE-encoded effectors (Nle) are being discovered (Wong *et al.*, 2011). The role of these virulence factors in each stage of A/E lesion formation on human model epithelia is reviewed below.

1.5.1.1 | Initial attachment

The mechanisms of EHEC initial attachment to IECs are poorly defined. In EPEC, adherence is thought to be primarily mediated by binding of the bundle-forming pilus (Bfp), a class of type-IV pilus, to an unidentified host receptor (Cleary *et al.*, 2004; Giron *et al.*, 1991). However, EHEC does not possess the plasmid encoding Bfp and must therefore associate with IECs *via* a different mechanism. A whole host of putative adhesins has been described (Table 1.1), many of which were identified by screening the sequenced genome of EHEC O157:H7 for homologues to known adherence factors. Two long polar fimbriae genes (*lpfA1* and *lpfA2*), showing sequence homology to the Lpf operon in *Salmonella enterica* serovar Typhimurium (Perna *et al*., 2001), have been characterised in some detail. Double deletion of *lpfA1* and *lpfA2* leads to partially reduced EHEC adherence to HeLa cervical carcinoma cells, but only when the regulators histone-like nucleoid structuring protein (HN-S) and Ler are also deleted (Torres *et al*., 2008). Significantly reduced EHEC adherence to T84 cells was also observed following deletion of *lpf1* or *lpf2* in a later study

Bacterial factor	Description	Host binding partners	Role in adherence	Reference(s)
Lpf	type IV pili	fibronectin, laminin, collagen IV	adherence to HeLa cells (inhibits adherence to human small intestinal tissue)	Torres *et al.*, 2008; Fitzhenry *et al.*, 2002; Farfan *et al.*, 2011
HCP		fibronectin, laminin	biofilm formation, adherence to T84, Caco-2, HT-29 and HeLa cells	Xicohtencatl-Cortes *et al.*, 2007; 2009
ECP	type I fimbriae	unknown	adherence to HeLa cells	Rendón *et al.*, 2007
ELF	fimbriae	laminin (not fibronectin or collagen IV)	adherence to HeLa cells	Samadder *et al.*, 2009
F9	type I pili	fibronectin (not fibrinogen, elastin or hyaluronic acid)	adherence to bovine IECs	Low *et al.*, 2006
intimin	adhesin mediating intimate attachment	β1-integrins, nucleolin	adherence to HeLa cells	Frankel *et al.*, 1996; Sinclair and O'Brien, 2002; Sinclair *et al.*, 2006
EhaA	autotransporter	unknown	biofilm formation, adherence to bovine rectal IECs	Wells *et al.*, 2008
EhaB	autotransporter	laminin, collagen I (not collagen III, collagen IV, fibronectin)	biofilm formation	Wells *et al.*, 2009
EhaJ	autotransporter	collagen I, II, III and V, fibronectin, fibrinogen, laminin (not collagen IV, elastin, heparin)	biofilm formation	Easton *et al.*, 2011
EspA	T3SS apparatus	unknown	adherence to HeLa cells	Ebel *et al.*, 1998
EspP	Serine protease autotransporter	unknown	biofilm formation, adherence to T84 cells and bovine rectal IECs	Dziva *et al.*, 2007; Puttamreddy *et al.*, 2010
Cah	autotransporter	unknown	biofilm formation (inhibits adherence to HeLa cells)	Torres *et al.*, 2002; 2005a
flagellin	involved in motility	mucin I and II	adherence to bovine IECs	Erdem *et al.*, 2007; Mahajan *et al.*, 2009; Sherman and Soni, 1988
OmpA	outer membrane protein	unknown	adherence to HeLa and Caco-2 cells	Torres *et al.*, 2005a; Torres and Kaper, 2003
Iha		unknown	adherence to HeLa cells	Tarr *et al.*, 2000
ToxB		unknown	adherence to Caco-2 cells	Tatsuno *et al.*, 2001; Stevens *et al.*, 2004

Table 1.1. List of putative adhesion factors expressed by EHEC.

(Farfan *et al.*, 2013). It has been proposed that Lpf may confer bacterial adherence to IECs by binding to extracellular matrix (ECM) proteins, as EHEC has been shown to attach to surfaces coated with fibronectin, laminin and collagen IV in an Lpf-dependent manner (Farfan *et al.*, 2011). However, EHEC adherence to human small intestinal biopsies during *in vitro* organ culture (IVOC) was actually enhanced in *lpf*-deficient mutants compared with wild-type (Fitzhenry *et al.*, 2006), and so the role of these fimbriae in adherence to IECs remains unclear.

Several other fimbrial adhesins have been identified in EHEC. The haemorrhagic coli pilus (HCP) is a type IV pilus that binds to ECM proteins and has been shown to facilitate EHEC biofilm formation and adherence to several human epithelial cell lines (Xicohtencatl-Cortes *et al.*, 2009; Xicohtencatl-Cortes *et al.*, 2007). The *E. coli* common pilus (ECP) is a type I pilus found in both commensal and pathogenic bacteria, and has been shown to play a role in EHEC O157:H7 adherence to HeLa cells (Rendón *et al.*, 2007). *E. coli* YcbQ laminin-binding fimbriae (ELF) bind to laminin (but not fibronectin or collagen IV) and are also involved in EHEC adherence to HeLa cells (Samadder *et al.*, 2009). Finally, signature-tagged mutagenesis screening for bovine colonisation factors has led to discovery of novel 'F9' fimbrial adhesins in EHEC serotypes O157:H7 (Dziva *et al.*, 2004) and O26:H- (van Diemen *et al.*, 2005). F9 was found to bind fibronectin (but not fibrinogen, elastin or hyaluronic acid) and promote adherence to bovine epithelial cells (Low *et al.*, 2006); however, the role of F9 fimbriae in adherence to human IECs has not been reported.

Many non-fimbrial bacterial factors have also been linked to initial attachment of EHEC to human IECs. The outer membrane protein intimin serves as an essential mediator of intimate attachment (see section 1.5.1.2), providing a ligand for the bacterial receptor Tir. However, this adhesin has also been shown to interact with host proteins. The IEC surface molecule β1-integrin anchors the intestinal epithelium to the underlying basement membrane (Humphries *et al.*, 1993). Solid-phase binding analysis of EPEC intimin has revealed an interaction between its cell-binding domain and β1-integrins (Frankel *et al.*, 1996). In addition, it has been demonstrated that EPEC infection of T84 human colonic carcinoma cells triggers a disruption of cell polarity, resulting in redistribution of basolaterally-localised β1-integrin to the apical

membrane, allowing bacterial access (Muza-Moons *et al.*, 2003). *In vitro* binding of EHEC O157:H7 intimin to the host laminin receptor, nucleolin, has also been demonstrated (Sinclair and O'Brien, 2002). Antibodies raised against nucleolin inhibited EHEC adherence to HeLa cells (Sinclair and O'Brien, 2002), and nucleolin was detected beneath EHEC adhered to intestinal epithelium of piglets, neonatal calves and mice (Sinclair *et al.*, 2006). Exposure of HeLa cells to Stx increased surface expression of nucleolin, which resulted in increased EHEC adherence (Robinson *et al.*, 2006). These studies suggest that intimin may mediate the initial attachment of EHEC to IECs *via* binding to β1-integrin and/or nucleolin. Further studies using human intestinal cell lines and/or tissue are necessary to confirm a role for these interactions during intestinal EHEC infection.

E. coli secreted protein A (EspA) is the main constituent of the T3SS translocation filament, which is fundamental for intimate attachment (section 1.5.1.2) and may also play a role in initial attachment. Ebel and colleagues (1998) showed that deletion of *espA* completely abrogated adherence of EHEC O26:H^- to HeLa cells. Furthermore, microscopic analysis of wild type EHEC revealed the presence of vast numbers of EspA-rich filaments connecting the bacteria to the brush border surface. Importantly, these filaments were particularly numerous on bacteria that had not yet induced actin pedestal formation, which suggests that EspA-rich filaments are involved in the early stages of attachment (Ebel *et al.*, 1998). However, a definitive role for the EspA filament in initial attachment has not been demonstrated, although this could be due to the difficulty in distinguishing between events leading to initial and intimate attachment.

Autotransporters are a group of proteins found on the outer membrane of Gram-negative bacteria (Henderson *et al.*, 2004). Enterohaemorrhagic *E. coli* autotransporters (Eha) A, B and J promote biofilm formation and bind to ECM proteins. However, although EhaA may play a role in EHEC adherence to bovine rectal IECs, all three Eha proteins have failed to mediate adherence to human epithelial cells. (Easton *et al.*, 2011; Wells *et al.*, 2009; Wells *et al.*, 2008). *E. coli* secreted protein P (EspP) is a serine protease autotransporter that promotes biofilm formation by EHEC and mediates adherence to T84 cells and bovine IECs (Dziva *et al.*, 2007; Puttamreddy *et al.*, 2010). Finally, the calcium-binding antigen 43

homologue (Cah) autotransporter expressed by EHEC O157:H7 promotes cell-to-cell aggregation and biofilm formation but confers a decrease in adherence to HeLa cells (Torres *et al.*, 2002). Cah also failed to influence EHEC adherence to Caco-2 cells (Torres *et al.*, 2005a), suggesting that this autotransporter does not play a role in initial attachment.

Other putative adhesins include flagellin, outer membrane protein A (OmpA), *Vibrio cholerae* iron-regulated gene A homologue adhesin (Iha), and ToxB. Whilst EPEC (H6) flagellin mediates adherence of EPEC to HeLa cells (Girón *et al.*, 2002), EHEC adherence was not influenced by antibody-mediated blocking of H7 flagellin (Sherman and Soni, 1988). Conversely, a role for H7 flagellin in EHEC adherence to bovine IECs and mucus has been demonstrated in several studies (Erdem *et al.*, 2007; Mahajan *et al.*, 2009). Both OmpA and Iha enhance EHEC adherence to HeLa cells (Tarr *et al.*, 2000; Torres *et al.*, 2005a; Torres and Kaper, 2003). ToxB contributes to EHEC O157:H7 adherence to Caco-2 cells, although this appears to occur indirectly through enhancement of T3S (Tatsuno *et al.*, 2001), which is essential for the intimate adherence phenotype (see section 1.5.1.2). ToxB is not required for EHEC intestinal colonisation of cattle or sheep (Stevens *et al.*, 2004).

Interestingly, the host cell membrane phospholipid, phosphatidylethanolamine (PE), has been shown to play a role in EHEC adherence to human epithelial cells. Using solid phase and liposome aggregation assays, Barnett-Foster and colleagues (1999) demonstrated that EHEC and EPEC preferentially bind to PE on the surface of HeLa cells. The same research group later showed that EHEC adherence triggered an increase in surface expression of PE *via* induction of apoptosis (Barnett Foster *et al.*, 2000). This data suggests that EHEC may use PE as a receptor for attaching to human epithelial cells. It remains to be determined whether EHEC interacts with PE on intestinal epithelial cells. Whilst EPEC binding to PE appears to involve Bfp (Barnett Foster *et al.*, 1999), no PE binding partners have thus far been described in EHEC.

Despite the large number of bacterial factors implicated in EHEC initial attachment to host cells, none except intimin and EspA (which are also involved in intimate attachment) have proven to be essential for adherence. One possible explanation for this could be that EHEC uses several adhesins that together confer a cumulative

effect on adherence. In addition, the majority of functional EHEC adhesin studies have been performed using non-intestinal cell lines (i.e. HeLa cells), so the potential influence of host intestinal factors has not been taken into account. More research using physiological IEC models is needed in order to elucidate the role of these adhesins during infection, as well as the factors influencing their expression and function.

1.5.1.2 | Intimate attachment

Intimin was the first bacterial protein found to be involved in EPEC-mediated A/E lesion formation (Jerse *et al.*, 1990), and an EHEC homologue was identified soon after (Yu and Kaper, 1992). This 102 kDa protein consists of an N-terminus that is conserved across EHEC and EPEC strains, and a variable C-terminus that confers receptor binding activity (Batchelor *et al.*, 2000). Five intimin types have been described (α, β, γ, δ and ε), based on antigenic differences in the C-terminus region. The most common subtypes found in EHEC are γ, expressed by EHEC O157; and β, expressed by some non-O157 serogroups including O26 (McGraw *et al.*, 1999). Intimin is essential for EHEC A/E lesion formation on HeLa cells (McKee *et al.*, 1995) and for *in vivo* colonisation of infant rabbits, gnotobiotic piglets and neonatal calves (Dean-Nystrom *et al.*, 1998b; Donnenberg *et al.*, 1993; Ritchie *et al.*, 2003). Furthermore, IVOC experiments using human intestinal tissue have shown that intimin is required for EHEC A/E lesion formation on small intestinal epithelium (Fitzhenry *et al.*, 2002), although no adherence to colonic epithelium (the site of EHEC-associated pathology) was observed using this model (Phillips *et al.*, 2000).

Initially, it was assumed that intimin mediated intimate attachment of A/E bacteria by interacting with a host cell receptor. Rosenshine and colleagues were the first to identify a 90 kDa protein on the host epithelial cell surface, which they termed Hp90, interacting with intimin during EPEC infection of HeLa cells (Rosenshine *et al.*, 1992; Rosenshine *et al.*, 1996). However, in 1997, Kenny *et al*. discovered that Hp90 was not a host derived-receptor but a bacterial protein translocated into the host cell *via* the T3SS, and thus renamed the protein translocated intimin receptor (Tir) (Kenny *et al.*, 1997). This was the first recorded example of a bacterium injecting its own receptor into a host cell to mediate adherence. Tir has since been described in

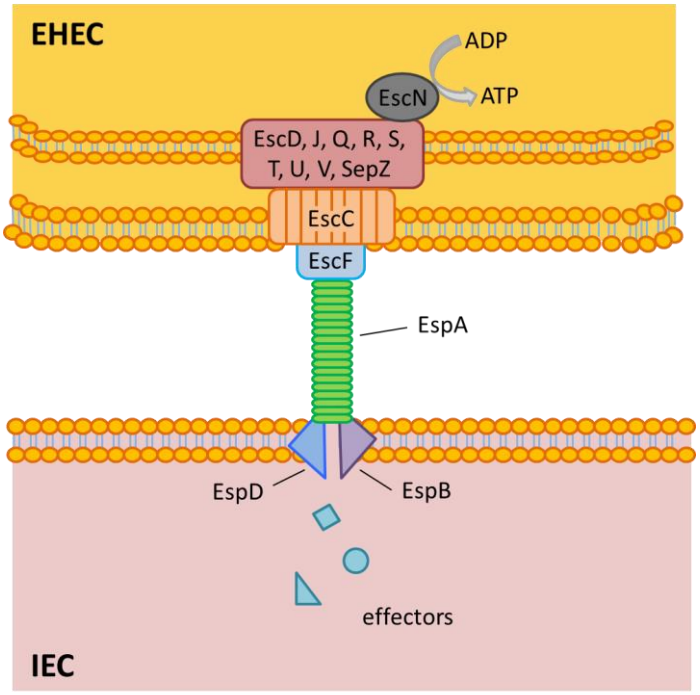

Figure 1.5. Structure of T3SS in A/E pathogens. Based on Pallen *et al*. (2005) and Garmendia *et al*. (2005)

EHEC (Deibel *et al.*, 1998), and the protein is essential for A/E lesion formation during infection of HeLa cells (DeVinney *et al.*, 1999) and IVOC of human small intestinal tissue (Schüller *et al.*, 2007). Following translocation into the host cytosol, Tir inserts into the host plasma membrane in a hairpin loop topology, such that the N- and C-terminal portions are cytosolic whilst the central region traverses the membrane for intimin binding (Luo *et al.*, 2000). Binding of Tir to intimin mediates intimate attachment of the bacterium to within 10 nm of the host cell membrane (Frankel *et al.*, 2001).

The T3SS is a 3.5 MDa complex found in many Gram-negative bacterial species. It is comprised of approximately 25 proteins that span the inner and outer membrane and mediate secretion of proteins out of the organism (Puhar and Sansonetti, 2014). The structure comprises a cytoplasmic domain containing an ATPase known as EscN; a trans-membrane ‘needle’ complex made from many proteins such as EscF, EscC, EscJ and EscD; and an extracellular translocon consisting of a hollow EspA-

Effector	Function	Reference(s)
LEE-encoded		
EspB	translocation pore component, disrupts phagocytosis	Hamaguchi *et al.*, 2008; Iizumi *et al.*, 2007
EspF	disrupts TJ activity, disrupts SGLT1 and NHE3 channelling, disrupts AQP channelling, disrupts mitochondria function, promotes apoptosis, disrupts phagocytosis	Viswanathan *et al.*, 2004; Hodges *et al.*, 2008; Guttman *et al.*, 2007; Nougayrede and Donnenberg, 2004; Quitard *et al.*, 2006
EspG	Disrupts DRA channelling, disrupts AQP channelling	Gill *et al.*, 2007; Guttman *et al.*, 2007
EspH	involved in pedestal formation, disrupts phagocytosis, promotes apoptosis	Tu *et al.*, 2003; Dong *et al.*, 2010; Wong *et al.*, 2012
EspZ	Inhibits T3SS effector translocation and apoptosis signalling	Berger *et al.*, 2012; Shames *et al.*, 2010
Map	suppresses pedestal formation, disrupts TJ activity, disrupts SGLT1 channelling, disrupts mitochondria function, promotes apoptosis	Kenny *et al.*, 2002; Thanabalasuriar *et al.*, 2010; Dean *et al.*, 2006
Tir	intimate attachment, involved in pedestal formation, disrupts SGLT1 channelling	DeVinney *et al.*. 1999; Kenny *et al.*, 1999; Dean *et al.*, 2006
Non-LEE-encoded		
EspJ	disrupts phagocytosis	Marches *et al.*, 2008; Kurushima *et al.*, 2010
EspM	involved in stress fibre formation, suppresses pedestal formation, disrupts TJ activity, suppresses apoptosis signalling	Simovitch *et al.*, 2010; Arbeloa *et al.*, 2008; 2010; Wong *et al.*, 2012
EspV	disrupts cytoskeleton	Arbeloa *et al.*, 2011
NleA/EspI	disrupts TJ activity and COPII-dependent protein trafficking	Thanabalasuriar *et al.*, 2010
NleB	disrupts NF-κB activity, suppresses apoptosis signalling	Li *et al.*, 2013; Newton *et al.*, 2010; Pearson *et al.*, 2013
NleC	disrupts NF-κB activity, disrupts MAPK signalling	Baruch *et al.*, 2011; Pearson *et al.*, 2011; Yen *et al.*, 2010; Mühlen *et al.*, 2011; Sham *et al.*, 2011
NleD	suppresses MAPK signalling, suppresses apoptosis signalling	Baruch *et al.*, 2011; Marchés *et al.*, 2005
NleE	disrupts NF-κB activity	Nadler *et al.*, 2010; Newton *et al.*, 2010
NleF	suppresses apoptosis signalling	Blasche *et al.*, 2013
NleH1	disrupts NF-κB activity, suppresses apoptosis signalling	Gao *et al.*, 2009; Pham *et al.*, 2012; Hemrajani *et al.*, 2010
NleH2	promotes NF-κB activity, promotes MAPK signalling, suppresses apoptosis signalling	Gao *et al.*, 2009; Pham *et al.*, 2012; Hemrajani *et al.*, 2010
TccP	involved in pedestal formation, disrupts TJ activity	Campellone *et al.*, 2004; Garmendia *et al.*, 2004; Viswanathan *et al.*, 2004

Table 1.2. Function of EHEC-secreted effectors. Only effectors with known functions in EPEC or EHEC are included.

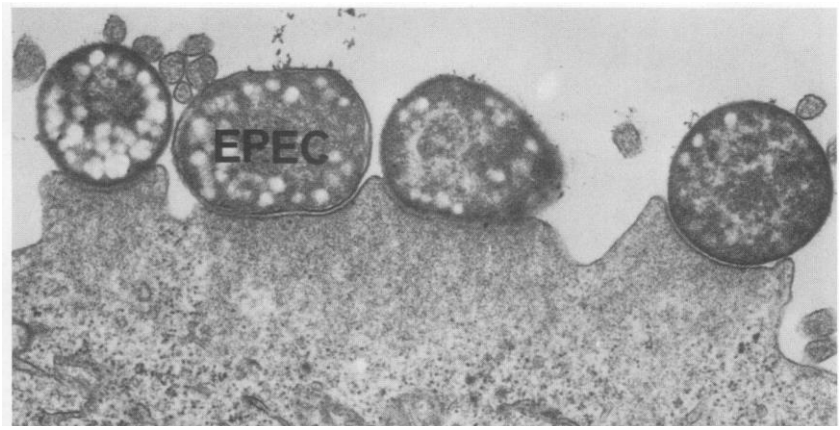

Figure 1.6. Actin pedestals in EPEC-infected Caco-2 cells. Taken from Knutton *et al*. (1989).

rich filament and a terminal EspB-EspD pore (Figure 1.5). The translocation pore inserts into the host plasma membrane and bacterial proteins are passed through the translocon into the cytosol. In addition to Tir, the T3SS secretes a barrage of effector proteins, which have a wide range of effects on the host cell (Table 1.2).

1.5.1.3 | Pedestal formation

TEM analysis of EPEC- and EHEC-infected IECs reveals a dense actin-rich region beneath the site of bacterial attachment (Knutton *et al.*, 1989; Knutton *et al.*, 1987). This actin-rich region often extends out of the cell by up to 10 µm, elevating the bacterium on a pedestal-like structure (Figure 1.6). Using HeLa cells as an *in vitro* model, scientists discovered that actin recruitment was mediated by the action of Tir (Kenny, 1999). Tir indirectly recruits host-derived neuronal Wiskott-Aldrich syndrome protein (N-WASP) to the site of adherence, which in turn recruits actin-related protein-2/3 (Arp2/3) complex to nucleate actin polymerisation (Campellone *et al.*, 2004; Garmendia *et al.*, 2004). EPEC and EHEC Tir recruit N-WASP *via* different signalling pathways. EPEC O127:H6 Tir is phosphorylated and binds to the host adaptor protein, Nck, to mediate activation of N-WASP (Kenny, 1999). In contrast, EHEC O157:H7 Tir stimulates a second secreted bacterial effector protein, Tir-cytoskeleton coupling protein (TccP), also known as $EspF_U$, to recruit N-WASP (Campellone *et al.*, 2004; Garmendia *et al.*, 2004). Activation of TccP by EHEC Tir

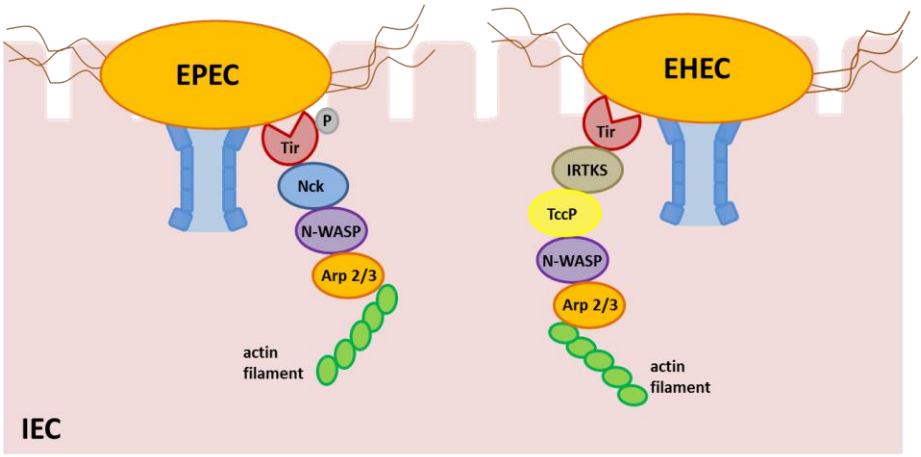

Figure 1.7. Actin recruitment pathways employed by EPEC and EHEC

also occurs indirectly *via* a host linker protein known as insulin receptor tyrosine kinase substrate (IRTKS) (Vingadassalom *et al.*, 2009; Weiss *et al.*, 2009) (Figure 1.7). TccP is the only EHEC effector protein other than Tir that is essential for actin recruitment. Since these initial studies, it has emerged that most non-O157 EHEC strains are able to utilise both the Nck and TccP pathways to recruit N-WASP and induce actin pedestal formation (Frankel and Phillips, 2008; Ogura *et al.*, 2007). The existence of several mechanisms for actin recruitment in A/E pathogens suggests that strong selective pressure is exerted on expression of this phenotype.

Several effector proteins have been shown to influence pedestal formation. Overexpression of *espH* in EHEC results in dramatic elongation of pedestals during infection of HeLa cells, and deletion of *espH* in EPEC inhibits pedestal formation (Tu *et al.*, 2003). Conversely, Δ*espM* EHEC and Δ*map* EPEC form elongated actin pedestals on HeLa cells (Kenny *et al.*, 2002; Simovitch *et al.*, 2010), suggesting that these two effectors may attenuate pedestal formation. The relevance of effector-mediated control of pedestal formation is yet to be elucidated.

Since the discovery of EHEC/EPEC-associated actin pedestals (Knutton *et al.*, 1989), this phenotype has served as the primary marker for A/E lesions. However, data from studies using human small intestinal IVOC has put into question the importance of actin pedestals in A/E lesion formation. In 2004, Garmendia *et al*. reported that, although infection of small intestinal biopsies with EHEC O157:H7 Δ*tccP* resulted in

impaired recruitment of N-WASP and α-actinin (another constituent of actin pedestals), mutants were still capable of forming intimate attachments and inducing microvillous effacement (Garmendia *et al.*, 2004). Furthermore, it has been shown that atypical EPEC serotype O125:H6, which is unable to recruit actin, is able to form A/E lesions that are indistinguishable from those induced by EHEC O157:H7 during small intestinal IVOC (Bai *et al.*, 2008). Moreover, T84 human colonic carcinoma cells infected with EHEC show signs of intimate attachment but not pedestal formation (Ismaili *et al.*, 1995). These studies indicate that actin recruitment is not required for formation of A/E lesions. However, inhibition of Tir phosphorylation (i.e. actin recruitment) in *C. rodentium* resulted in impaired colonisation and mucosal attachment during infection of mice (Mallick *et al.*, 2014). In addition, TccP/EspF$_U$ EHEC mutants demonstrated attenuated colonisation and expansion at later stages of infection of rabbits and gnotobiotic piglets (Ritchie *et al.*, 2008). These data suggest that actin pedestal formation, whilst not essential for A/E lesion formation, may stabilise adherence to the intestinal epithelium and promote expansion of bacterial colonisation. Another hypothesis is that actin pedestals serve as a localised signalling hub that coordinates spatial and temporal control of effector protein activity (Lai *et al.*, 2013), since many effectors are known to congregate at this site following translocation (Munera *et al.*, 2012). More studies are warranted in order to fully elucidate the role of actin pedestal formation during EHEC infection.

1.5.1.4 | Microvillous effacement

Micovillous effacement on IECs is thought to contribute to diarrhoea by reducing the surface area available for nutrient absorption and thereby increasing the osmolarity of the intestinal lumen (Hodges and Gill, 2010). Despite its important role in A/E pathogenesis, the cause of microvillous effacement has not been elucidated. EPEC-induced microvillous effacement on human small intestinal mucosa appears to be dependent on expression of the T3SS (Shaw *et al.*, 2005), suggesting that one or more effectors are involved. EPEC Tir has been implicated, although conflicting data exists depending on model system used: Tir was found to be essential for microvillous effacement in Caco-2 cells (Dean *et al.*, 2006; Dean *et al.*, 2013) but not during human or porcine IVOC (Girard *et al.*, 2005; Shaw *et al.*, 2005). Furthermore,

whilst EPEC-associated microvillous effacement on Caco-2 cells has been attributed to concerted action of Tir, Map and EspF (Dean *et al.*, 2006), induction of the same phenotype on human small intestinal mucosa was attenuated by deletion of *espF* and *espH* but not not *tir* or *map* (Shaw et al., 2005). To the best of our knowledge, the cause of microvillous effacement by EHEC has yet to be investigated.

1.5.1.5 | Disruption of IEC function

In addition to microvillous effacement, EHEC and EPEC employ other strategies to induce diarrhoea such as disruption of epithelial barrier function, ion transport and water transport (Figure 1.8). Induction of diarrhoea is likely to benefit EHEC colonisation by displacement of resident microflora and also facilitates host transmission (Lupp *et al.*, 2007). These interferences with host cell processes are mediated by the action of effector proteins and require a functional T3SS (Gill *et al.*, 2007), and so the ability to induce diarrhoea is inexorably linked to A/E lesion formation. Most of the data on effector function are based on EPEC studies, with relatively little experimental work conducted using EHEC strains. Although all A/E pathogens share a core repertoire of 21 effectors, the number expressed by EHEC is thought to be almost three times that of EPEC (Tobe *et al.*, 2006). Therefore, it is difficult to ascertain how much of the data on EPEC can be applied to EHEC. Moreover, few studies have been verified *ex vivo* or *in vivo* using human intestinal tissue or animal models, respectively.

1.5.1.5.1 | Epithelial barrier function

The intestinal epithelium acts as a physical barrier between the external environment and the internal tissues. The selective permeability of this cell-thick layer is mediated by tight junctions (TJ), intercellular complexes formed by interaction of transmembrane proteins (i.e. occludins, claudins and zonula occludens proteins) with the actin cytoskeleton. TJs serve a dual purpose; they seal the intercellular space and selectively permit migration of lymphocytes and solutes across the epithelium, and they also maintain the spatial segregation of apical and basolateral membrane-bound

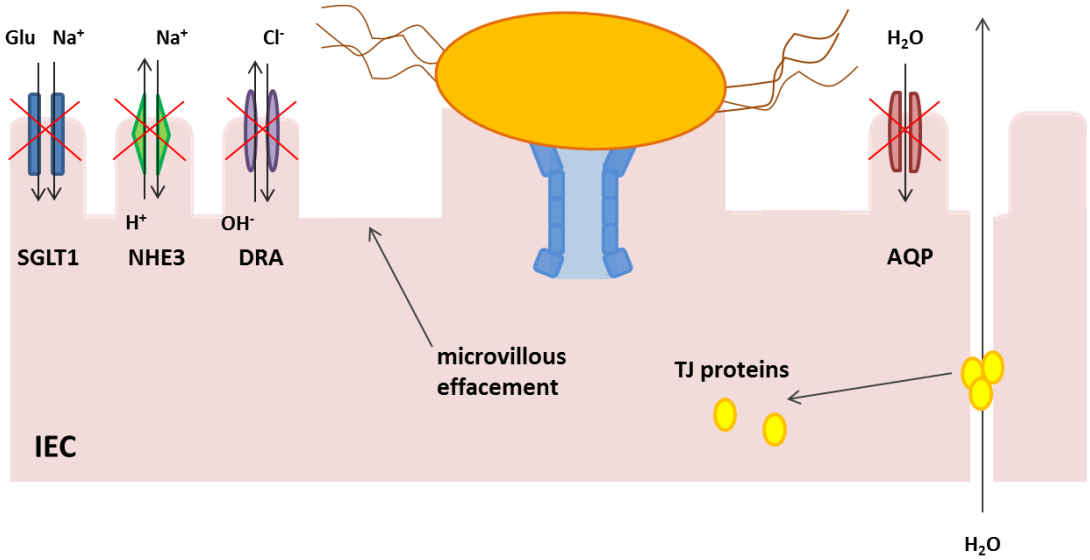

Figure 1.8. Mechanisms contributing to diarrhoea during EPEC/EHEC infection.

molecules (Turner, 2009). Thus, TJs play an essential role in host defence against enteric infection. Several studies have reported that EHEC impairs epithelial barrier function, determined by a reduction in transepithelial electrical resistance (TER), during infection of polarised T84 and Caco-2 cells (Li *et al.*, 1999; Philpott *et al.*, 1997; Viswanathan *et al.*, 2004). Interestingly, whilst EPEC-mediated disruption of barrier function has been attributed solely to the action of the effector protein EspF, EHEC employs TccP/EspF$_U$ as well as EspF to induce this effect in both cell lines (Viswanathan *et al.*, 2004). Barrier function is impaired by EspF- and TccP-mediated redistribution of occludin away from TJs (Viswanathan *et al.*, 2004). EspM, found in EHEC but not EPEC, induced loss of zonula occludens protein 1 (ZO-1) from TJs in HeLa cells but did not influence TER in MDCK canine kidney cells (Simovitch *et al.*, 2010). In EPEC, NleA and Map contribute to TJ disfunction in T84 and Caco-2 cells by interfering with COPII-dependent cell protein trafficking pathways (Thanabalasuriar *et al.*, 2010). Furthermore, EPEC interferes with the actomyosin ring that controls TJ activity (Yuhan *et al.*, 1997). These additional mechanisms for altering barrier function have yet to be investigated in EHEC.

1.5.1.5.2 | Ion transport

Secreted EPEC effectors impair the activity of both chloride and sodium exchangers. This results in a rise in luminal ion concentrations, which hinders water uptake by the epithelium. Sodium ions typically enter IECs *via* one of two membrane-bound exchangers: Na^+/H^+ exchangers (NHEs) or sodium-dependent glucose transporter 1 (SGLT1). Hecht and colleagues (2004) have demonstrated that infection of Caco-2, T84 and HT-29 cells with EPEC results in varying effects on NHE channelling; reporting enhanced activity for NHEs 1 and 2 and reduced activity for NHE3. This reduction in NHE3 activity is mediated by EspF (Hodges *et al.*, 2008). SGLT1 channelling in Caco-2 cells is reduced during EPEC infection *via* an unknown mechanism that requires expression of Tir, Map, EspF and intimin (Dean *et al.*, 2006). Choride uptake in Caco-2 and T84 cells is also inhibited by EPEC infection *via* EspG-mediated internalisation of the Cl^-/OH^- exchanger, downregulated in adenoma (DRA), into the cytoplasm (Gill *et al.*, 2007). Disruption of anion secretion has been reported in T84 cells during infection with EHEC O157:H7, as assessed by voltage clamp analysis (Li *et al.*, 1999). Aside from this study, EHEC-mediated subversion of ion channel activity has not been investigated.

1.5.1.5.3 | Water transport

Aquaporins (AQPs) are membrane-bound protein channels that transport water across epithelia. At least thirteen AQPs are present in mammals, with AQP1, 3, 4 and 7 known to be expressed in the human colon (Abascal *et al.*, 2014). At present, little is known about regulation of aquaporins during enteric infection. However, infection of mice with *C. rodentium* triggered redistribution of AQP2 and 3 from the plasma membrane to the cytoplasm of colonocytes (Guttman *et al.*, 2007). Surface expression of AQPs was re-established in mice that recovered from infection. This redistribution of AQPs was partially dependent on expression of EspF and EspG, since AQP2 and 3 remained on the surface of colonocytes following infection with Δ*espF and* Δ*espG* mutant strains. It is yet to be established whether EHEC infection affects water transport by aquaporins in humans.

1.5.1.6 | Regulation of A/E gene expression

Most of the genes involved in A/E lesion formation are located in the locus of enterocyte effacement (LEE). The LEE consists of 41 genes arranged across five transcriptional units (LEE1-5); with LEE1, LEE2 and LEE3 encoding structural components of the T3SS, LEE4 encoding the translocation pore, and LEE5 encoding intimin and Tir (Elliott *et al.*, 1998). Transcriptional regulation of the LEE is orchestrated by three self-encoded core regulators and an array of non-LEE-encoded proteins (Figure 1.9) (reviewed in Connolly *et al.*, 2015; Mellies *et al.*, 2007). The LEE-encoded regulator (Ler) is found on LEE1 and is the master regulator of *LEE2-5* transcription. Deletion of *ler* compromises EHEC virulence, demonstrated by reduced effector secretion and actin pedestal formation during infection of HeLa cells (Elliott *et al.*, 2000). Ler activates *LEE* transcription by antagonising the activities of the global gene repressor, HN-S (Bustamante *et al.*, 2001). Also encoded on the LEE are the global regulators of Ler, GrlA and GrlR, which activate and repress *ler* activity, respectively (Iyoda *et al.*, 2006). Non-LEE encoded regulators of LEE transcription include the Pch proteins (Iyoda and Watanabe, 2004), quorum-sensing in *E. coli* (Qse) signalling proteins (Reading *et al.*, 2007; Sperandio *et al.*, 2002), and GadE, a regulator involved in resistance to acid stress (Kailasan Vanaja *et al.*, 2009). Interestingly, bacteriophage-derived proteins have also been shown to influence LEE expression. Xu and colleagues (2012) have reported that the Stx2 prophage regulator *c*II, found in most virulent EHEC strains, is capable of inhibiting *ler* activity and expression of *LEE*-encoded effectors, suggesting that Stx2 lysogeny may suppress A/E lesion formation in EHEC. Thus, the mechanisms controlling the A/E phenotype are manifold.

In recent years, it has become increasingly clear that a vast array of external cues influences A/E gene expression in EHEC. Many of these cues have been shown to interact with the regulatory mechanisms described above to promote or repress transcription of A/E genes. These interactions are summarised in Figure 1.9B and discussed in more detail below.

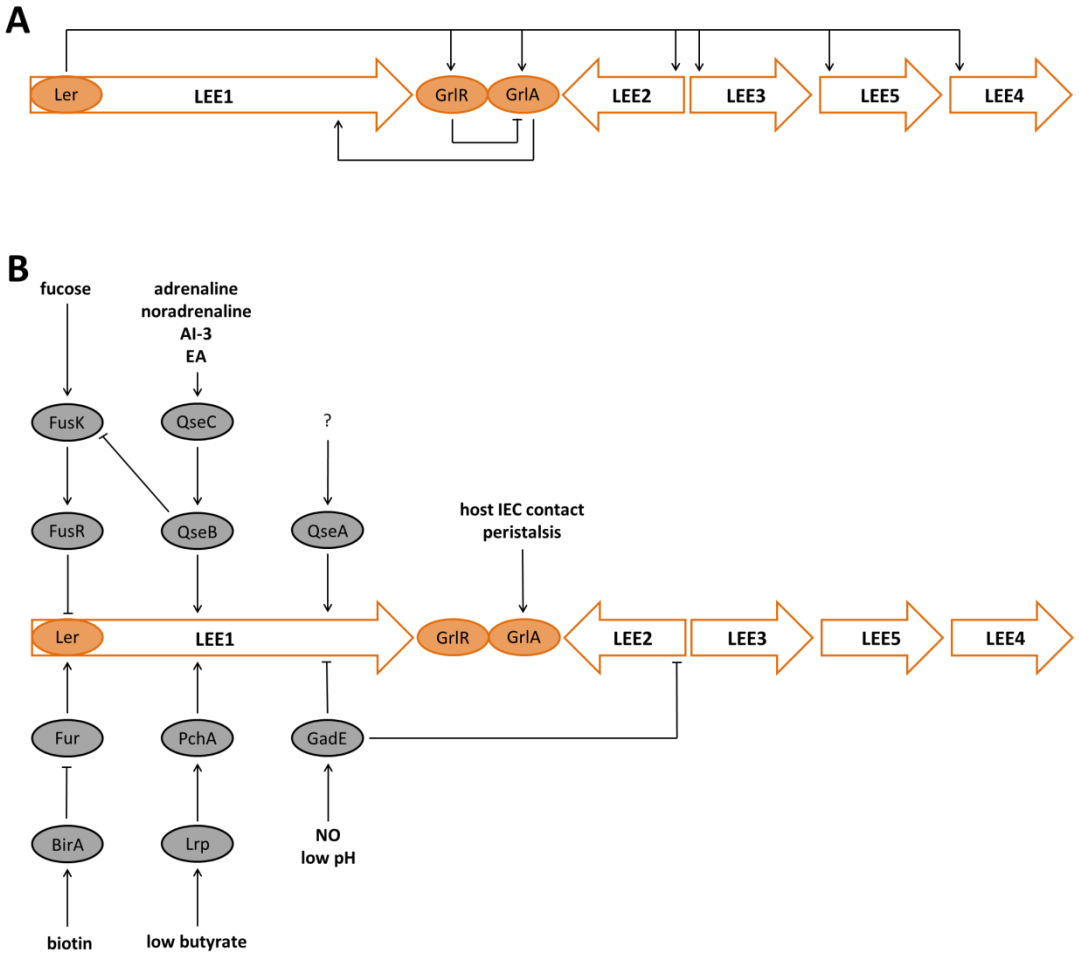

Figure 1.9. Regulation of the LEE in EHEC. (**A**) Core regulation by LEE-encoded regulators. (**B**) Influence of external cues on LEE transcription and the regulatory pathways involved. Pointed and blunt arrows denote positive and negative regulation, respectively.

1.5.1.6.1 | Acid stress

EHEC are exposed to rapidly increasing and decreasing acidity as they enter and leave the stomach. House and colleagues (2009) have postulated that acid stress triggers virulence gene expression in EHEC O157:H7. The authors reported that exposure to buffered-adaptive acid stress (incubation for a brief period at pH 5.0 followed by pH 3.0 for 30 minutes) significantly enhanced the ability of EHEC to adhere to Caco-2 and HeLa cells. However, this adherence was not intimin-dependent, and microarray analysis revealed variable effects on *LEE*-encoded T3SS genes, suggesting that acid stress did not induce A/E-associated virulence.

Conversely, several other research groups have demonstrated that acid stress negatively regulates A/E lesion gene expression. It has been shown that inactivation of GadE, one of the two main proteins known to mediate acid resistance (AR) in response to acidic conditions, results in up-regulation of many LEE-encoded genes and enhanced adherence to Caco-2 cells (Kailasan Vanaja *et al.*, 2009; Tatsuno *et al.*, 2003). GadE-mediated inhibition of LEE gene expression was dependent on Ler (Kailasan Vanaja *et al.*, 2009), and a later study confirmed that GadE binds to promoter regions on both *LEE1* and *LEE2* (Tree *et al*., 2011). This data suggests that GadE inversely coordinates AR and LEE gene expression depending on environmental pH levels; acting to inhibit LEE expression in favour of AR during acid stress, and vice versa in times of low acid stress (Kailasan Vanaja *et al.*, 2009). Interestingly, a recent study has demonstrated that EHEC activates GadE-mediated repression of *LEE* expression in response to nitric oxide (NO) (Branchu *et al.*, 2014), which is found in gastric juices, further highlighting the important role of GadE in regulation of A/E pathogenesis.

1.5.1.6.2 | Butyrate

During passage through the intestine, EHEC encounters a vast array of molecules produced by the resident microflora and the host. Bacterial metabolites include the short chain fatty acids (SCFA); of which acetate, propionate and butyrate are most abundant in the GI tract (Cummings *et al.*, 1987). SCFAs are utilised by the host to promote normal bowel function; in particular, butyrate is the preferred energy source for colonocytes and is thought to be protective against colitis and colorectal cancer (Hamer *et al.*, 2008). Interestingly, Nakanishi and colleagues (2009) have shown that EHEC uses butyrate to stimulate virulence gene expression. EHEC exposure to butyrate at levels comparable to those found in the terminal ileum (1.25 to 40 mM) resulted in enhanced adherence to Caco-2 cells and increased expression of LEE-encoded genes *tir* and *espB*. Induction of these genes by butyrate was dependent on expression of *ler*, as well as the gene encoding PchA. PchA is a known positive regulator of *ler* expression (Iyoda and Watanabe, 2004) and shares sequence homology to the Per regulators of LEE transcription found in EPEC (Porter *et al.*, 2004). In addition, the protein responsible for sensing butyrate in EHEC has been

identified as the global regulator, leucine-responsive regulatory protein (Lrp) (Nakanishi *et al.*, 2009). As no recognition site for Lrp could be identified in or around the *pchA* gene sequence, the authors concluded that Lrp must induce *LEE* expression *via* indirect regulation of *pchA*. Butyrate has also been shown to induce EHEC production of flagella (Tobe *et al.*, 2011).

1.5.1.6.3 | Quorum-sensing signalling molecules

The idea that quorum-sensing (QS) plays a role in A/E virulence gene regulation was first proposed by Sperandio and colleagues, who found that exposure of EHEC to preconditioned medium containing EHEC secreted products resulted in induction of *ler* and other LEE-encoded genes. (Sperandio *et al.*, 2002; Sperandio *et al.*, 1999). This effect was mediated by the action of a novel regulatory protein, which the authors named quorum-sensing *E. coli* regulator A (QseA). This discovery stimulated a wave of research into QS-mediated control of EHEC virulence, and several additional regulatory cascades have been discovered. A two-component system, involving the sensory kinase QseE and the response regulator QseF, induces expression of the non-LEE-encoded gene *tccP* (Reading *et al.*, 2007); whilst a second two component system, involving QseC and QseB, triggers expression of genes encoding Ler, flagellin, and other motility factors (Clarke and Sperandio, 2005; Hughes *et al.*, 2009). These two regulatory systems appear to be linked, since stimulation of QseC results in activation of QseE (Reading *et al.*, 2007). Furthermore, both systems are triggered upon detection of the host hormones adrenaline and noradrenaline, and the QseCB pathway is also responsive to the bacterial signalling molecule, autoinducer-3 (AI-3) (Clarke *et al.*, 2006; Reading *et al.*, 2007). AI-3 is abundant in the intestines, as it is secreted by the resident microflora (Sperandio *et al.*, 2003; Walters *et al.*, 2006). Both adrenaline and noradrenaline are also found in the GI tract, where they modulate smooth muscle contraction, submucosal blood flow, and ion secretion (Furness, 2000; Horger *et al.*, 1998). Based on these data, it appears that EHEC uses host hormones and/or secreted bacterial signalling molecules as a spatial cue to stimulate expression of the *LEE*, *tccP* and flagella genes upon entry into the intestine. At present, the external cue(s) responsible for QseA-mediated regulation of the *LEE* have not been resolved.

Recently, ethanolamine (EA) has been highlighted as another stimulus for A/E gene regulation *via* the QS pathways. EA, the breakdown product of phosphatidylethanolamine (PE), is an essential constituent of all cell membranes and is abundant in the intestinal lumen due to the constant shedding of IECs (Cotton, 1972). EHEC possesses an operon (the *eut* operon) that allows it to utilise EA as a nitrogen source, which gives it a competitive advantage over other bacterial species (Bertin *et al.*, 2011). In a study by Kendall *et al*. (2012), EHEC exposure to EA triggered expression of *ler*, *eae*, and *espA*; as well as enhanced actin pedestal formation on HeLa cells. EA exposure was also associated with higher transcript levels of *qseA*, *qseE* and *qseC*, suggesting that EA induces the A/E phenotype *via* QS. Deletion of the *eutB* gene did not affect EA-mediated induction of *ler* expression, indicating that EA-mediated induction of A/E gene expression is not the indirect result of EA metabolism.

1.5.1.6.4 | Fucose

Fucose is one of the preferred carbon sources for *E. coli* in the human gut (Fabich *et al.*, 2008). It is made abundantly available in the intestinal mucus by the commensal bacterium *Bacteroides thetaiotaomicron*, due to its ability to cleave fucose from mucin glycoproteins (Fischbach and Sonnenburg, 2011). In a recent study, Pacheco *et al*. (2012) reported that EHEC exposure to fucose at levels found in the mucus layer resulted in direct suppression of *ler* transcription *via* the action of a novel two-component signalling system, termed FusKR. In the same study, FusKR activity was inhibited by the aforementioned QseBC and QseEF regulatory pathways, thereby demonstrating another mechanism for QS-mediated induction of A/E genes. Based on this data, the authors proposed a model whereby EHEC uses fucose as a cue to suppress A/E gene expression whilst in the mucus *via* FusKR; once at the intestinal epithelium, exposure to reduced fucose levels and/or induction of Qse signalling results in the lifting of FusKR-mediated repression of *LEE* transcription, allowing expression of A/E genes to ensue.

1.5.1.6.5 | Biotin

Biotin is an essential nutrient for maintaining cellular function in all living organisms. Bacteria are able to obtain biotin either from the environment or by synthesising it themselves, whereas humans lack the biotin synthesis machinery and must therefore rely on intestinal absorption (Said, 2009). The extent of biotin absorption in the small intestine leads to relatively low levels of biotin present in the colon (Yang *et al.*, 2015). In a recent study by Yang and colleagues (2015), the role of biotin in EHEC virulence gene expression was examined. The study showed that exposure of EHEC to high levels of biotin abrogated adherence to HeLa and Caco-2 cells, colonisation of mice, and expression of *ler*, *eae*, *tir*, and genes encoding T3S apparatus proteins. This effect was dependent on bacterial expression of *birA*, a regulatory protein that represses biotin synthesis when the nutrient is abundant in the environment. BirA was found to negatively regulate the global regulator protein, Fur, which in turn was shown to stimulate LEE gene expression. The authors proposed a model whereby EHEC exposure to reduced biotin levels in the colon leads to a reduction in BirA-mediated repression of Fur, enabling Fur to stimulate expression of LEE genes encoding A/E lesion formation. Interestingly, A/E gene expression in EPEC was unaffected by biotin, and it was suggested by the authors that this may account for the difference in tissue tropism exhibited between EPEC and EHEC (Yang *et al.*, 2015). No evidence for Fur binding to promoter regions on the LEE, nor to those on regulatory genes such as GrlA/R or QseA, was observed; indicating that Fur likely induces *LEE* expression indirectly *via* an unidentified regulator.

1.5.1.6.6 | Oxygen

Upon entry into the GI tract, EHEC is exposed to rapidly decreasing oxygen concentrations. Non-invasive imaging of oxygen in mice suggests that colonic oxygen concentrations are around 1.4% of atmospheric pressure, which is considered microaerobic (MA) (He *et al.*, 1999). To investigate the effect of microaerobiosis on EHEC expression of A/E lesion genes, Schüller and Philips (2010) developed a vertical diffusion chamber (VDC) system to perform infections under MA conditions. The authors found that EHEC displayed enhanced adherence to polarised T84 cells and production of Tir, EspA and EspB in MA conditions compared with

aerobic (AE) conditions. Increased Tir translocation into the host membrane, a marker for intimate attachment and A/E lesion formation, was also observed during MA infection of Caco-2 cells. Adherence of an *espA* mutant was not affected by microaerobiosis, suggesting that oxygen regulates expression of *LEE*-encoded genes. Girard and colleagues (2007) also reported a positive effect of reduced oxygen levels on EHEC adherence during bovine intestinal IVOC. Put together, it appears that the reduction in environmental oxygen concentration experienced upon entry into the intestine triggers expression of the A/E phenotype by EHEC. To date, the mechanisms underlying this effect are yet to be explored.

1.5.1.6.7 | Contact with IECs

During their study of oxygen-mediated effects on the A/E lesion phenotype, Schüller and Phillips (2010) noted that EHEC expression of the T3S proteins Tir, EspA and EspB was induced only in the presence of an IEC monolayer. Moreover, incubation with preconditioned T84 cell medium was not sufficient to induce T3SS activity, suggesting that physical contact with host IECs was required. In agreement with these findings, Roe *et al*. (2004) have shown that centrifugation of EHEC bacteria onto embryonic bovine lung (EBL) epithelial cells is sufficient to induce bacterial expression of Tir in an environment where T3SS expression is usually repressed (i.e. during incubation in M9 medium). The impact of host cell contact on A/E virulence gene expression has recently been studied in detail by Alsharif and colleagues (2015), who reported that EHEC exhibited higher levels of *LEE1* promoter activity when adhered to HeLa cells compared with glass slides. Induction of *LEE1* expression and actin pedestal formation was dependent on GrlA, and was not affected by inhibition of host cell signalling. In addition, the authors demonstrated that exposure of EHEC to fluid shear, at levels comparable to those found at the exposed brush border (up to 5 dynes/cm^3), further induced *LEE1* activation. These data suggest that EHEC are able to sense physical contact with IECs and mechanical fluctuations associated with peristalsis as a cue for enhancing expression of genes responsible for A/E lesion formation. At present, it is not known how GrlA is activated in response to these cues, although it has been established that GrlR is not involved.

It is clear from the available data that regulation of A/E lesion formation in EHEC is a complex process that involves sensing of a variety of environmental cues using several regulatory pathways. The challenge remains to unravel how these pathways interact to coordinate spatial and temporal expression of the A/E lesion phenotype as EHEC travels through the GI tract.

1.5.1.7 | Clinical relevance of A/E lesion formation

Although A/E lesion formation has been well-characterised using human carcinoma cell lines such as HeLa, very few studies have examined EHEC pathogenesis on human intestinal tissue. In 2000, Phillips *et al*. employed the IVOC model to investigate A/E lesion formation by EHEC strain 85-170 and EPEC strain E2348/69 at various human intestinal sites (Phillips *et al.*, 2000). The authors reported that, whilst EPEC triggered A/E lesion formation on biopsies from the proximal and distal small intestine, EHEC-induced A/E lesions were restricted to the follicular-associated epithelium (FAE) of Peyer's patches in small intestinal biopsies. No EHEC adherence to colonic biopsies was observed, which is striking as EHEC-induced intestinal damage is localised to the transverse colon (Griffin *et al.*, 1990; Kelly *et al.*, 1987; Malyukova *et al.*, 2009; Shigeno *et al.*, 2002). To address this discrepancy, the same research group extended the scope of their initial study by performing IVOC on biopsies from a wider range of colonic sites (caecum to rectum) and using several different EHEC strains. However, A/E lesions were observed in only 4 out of 113 colonic IVOCs (Chong *et al.*, 2007). To determine whether adherence to FAE was required to prime EHEC for colonic colonisation, a two-step IVOC experiment was performed in which FAE-containing terminal ileal biopsies were infected with EHEC and then co-cultured with colonic biopsies. Results showed that priming of EHEC on FAE enhanced colonic colonisation. However, bacteria bound to colonic biopsies exhibited a non-intimate adherence phenotype inconsistent with A/E lesions (Chong *et al.*, 2007). Put together, these data indicate that EHEC do not form A/E lesions on human colonic epithelium *ex vivo*, and therefore cast doubt on their role in colonic pathogenesis *in vivo*.

Several limitations to the aforementioned studies exist, which may have affected the ability of EHEC to form A/E lesions on colonic biopsies. Firstly, all of the EHEC

strains tested were Stx-negative, which may be relevant as Stx has been shown to enhance EHEC adherence to HeLa cells and colonisation of mice (Robinson *et al.*, 2006; section 1.5.2.3). Secondly, these IVOC experiments were performed under 95% oxygen in order to maintain oxygenation of the explanted intestinal tissue, which may have inhibited expression of A/E genes (section 1.5.1.6.6). Thirdly, only biopsies derived from paediatric donors have been used for IVOC, and so it is currently undetermined whether EHEC colonise intestinal biopsies isolated from adults. To date, the effect of age on EHEC adherence has not been studied.

In vivo animal studies have shown that EHEC forms colonic A/E lesions following infection of gnotobiotic piglets, infant rabbits and neonatal calves (Dean-Nystrom *et al.*, 1997; Francis *et al.*, 1986; Pai *et al.*, 1986; Tzipori *et al.*, 1986). These studies were performed using young animals, which lack an intestinal microbiota, in order to avoid the effects of colonisation resistance. In contrast, clinical examination of human intestinal samples from EHEC-infected patients has not yielded evidence of adherent bacteria (Kelly *et al.*, 1987; Malyukova *et al.*, 2009), although it should be noted that samples are generally taken at a progressed stage of disease when colonisation may have diminished or be obscured. More *ex vivo* human tissue studies are needed in order to clarify whether A/E lesions have clinical relevance to human disease during EHEC infection.

1.5.2 | Shiga toxin production

Shiga toxin (Stx) production distinguishes EHEC from all other A/E pathogens. Stxs are a family of exotoxins whose name derives from the first bacterial species found to produce the toxin, *Shigella dysenteriae* type I (Olitsky and Kligler, 1920). Whilst Stx from *Shigella* is considered the prototype, EHEC produce two antigenically and structurally distinct toxins termed Stx1 and Stx2 (Bergan *et al.*, 2012). Stx1 exhibits 99% sequence homology to Stx produced by *S. dysenteriae*, whereas Stx2 shares approximately 56% homology (Jackson *et al.*, 1987a; Jackson *et al.*, 1987b; Strockbine *et al.*, 1986). Several Stx1 and Stx2 subtypes exist (see Table 1.3), and EHEC are capable of harbouring one or several of these subtypes. Stx2 is more closely associated with disease in humans compared with Stx1 (Boerlin *et al.*, 1999; Werber *et al.*, 2003). Whilst the characteristics of each individual subtype are yet to

Subtype	Source	A subunit homology	B subunit homology	Reference(s)
Stx	*S. dysenteriae* Type 1	prototype	prototype	Strockbine *et al*., 1988
Stx1(a)	*E. coli* O157:H7	99%	100%	Calderwood *et al*., 1987; Jackson *et al*., 1987b
Stx1c	*E. coli* O128:H2	97%	97%	Zhang *et al*., 2002
Stx1d	*E. coli* ONT:H19	94%	92%	Bürk *et al*., 2003
Stx2(a)	*E. coli* O157:H7	prototype	prototype	Jackson *et al*., 1987a
Stx2b	*E. coli* O118:H12	94%	89%	Piérard *et al*., 1998
Stx2c	*E. coli* O157:H7	100%	97%	Schmitt *et al*., 1991
Stx2d	*E. coli* O91:H21	99%	97%	Teel *et al*., 2002
Stx2e	*E. coli* O139	94%	87%	Gyles *et al*., 1988; Weinstein *et al*., 1988
Stx2f	*E. coli* O128:H2	71%	83%	Schmidt *et al*., 2000
Stx2g	*E. coli* O2:H25	96%	94%	Leung *et al*., 2003

Table 1.3. List of Stx subtypes. Adapted from Bergan *et al*. (2012)

be elucidated in detail, all Stx2 subtypes have been found in human EHEC isolates except for Stx2g, which was derived from a cattle isolate (Bergan *et al.*, 2012; Leung *et al.*, 2003). Stx2a- and Stx2c-producing strains are most often associated with HUS compared with strains producing other Stx subtypes (Karch *et al.*, 2006; Orth *et al.*, 2007). Stx2e has been linked to oedema disease in pigs (Weinstein *et al.*, 1988). Interestingly, Stx2d cytotoxicity is enhanced by exposure to human and mouse intestinal mucus (Melton-Celsa *et al.*, 1996) suggesting that Stx activity may be modulated by external cues.

Stx is a holotoxin with an AB_5 molecular structure. The 32-kDa A subunit is located in the centre of a doughnut-shaped ring of five identical 7.7-kDa B subunits (Fraser *et al.*, 1994). The A subunit is responsible for the toxin's enzymatic activity, whilst the B subunits mediate binding to the host cell surface glycolipid, globotriaosylceramide (Gb3; also known as CD77) (Lindberg *et al.*, 1987). Stx binding to Gb3 triggers internalisation of the toxin, retrograde transport through the cell, and inhibition of protein synthesis and apoptosis (Figure 1.10). Gb3 is highly expressed in the microvasculature of the colon and the kidney, and it is thought that

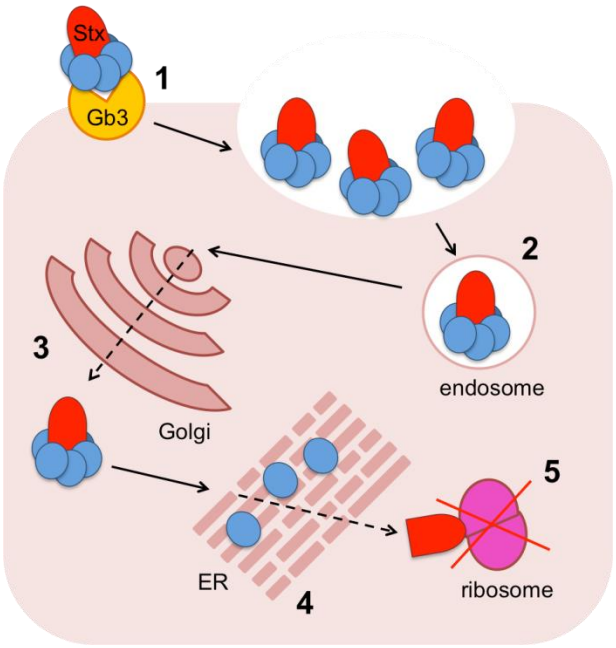

Figure 1.10. Intracellular Stx transport within Gb3-positive cells. (1) The B subunits (blue) of Stx bind to host cell receptor Gb3; **(2)** the B subunits trigger membrane invagination leading to internalisation of the toxin into an early endosome; **(3**) Stx is transferred from the endosome to the Golgi apparatus, where it begins its migration to the endoplasmic reticulum (ER) *via* the retrograde transport pathway; **(4)** once in the ER, the A subunit (red) dissociates from the B subunits and is targeted for ER-associated degradation (ERAD); **(5)** the ERAD pathway releases the A subunit into the cytosol, at which point the subunit refolds, thus preventing proteasomal degradation. This is followed by ribosomal binding and cleavage of a single adenine residue of the 28S rRNA by N-glycosidase activity. Disruption of protein synthesis leads to activation of a ribotoxic response and subsequent apoptosis (Johannes and Romer, 2010; Pacheco and Sperandio, 2012).

Stx-mediated cytotoxic action at these sites is responsible for the onset of HC and HUS, respectively (Tarr *et al.*, 2005). Stx2 exhibits 400 times greater renal toxicity in mice compared with Stx1 (Tesh *et al.*, 1993), which may explain the closer association of Stx2-producing strains with HUS.

1.5.2.1 | Regulation of Stx expression and release

Stx is encoded within the late genes of lambdoid prophages integrated into the EHEC chromosome (Johansen *et al.*, 2001). Expression of *stx* is controlled by phage-encoded promoters: the p_{stx} promoter induces expression of *stx* genes only, whereas P_R and P_R' control expression of all phage genes including *stx* and the genes involved in bacterial cell lysis (Figure 1.11). Induction of *stx1* expression is mainly mediated by p_{stx}, which is subject to negative regulation by the global regulator protein Fur (Calderwood and Mekalanos, 1987). In contrast, *stx2* expression is regulated by P_R and P_R' without the involvement of p_{stx} (Wagner *et al.*, 2001b). P_R and P_R' expression are repressed in homeostatic conditions by the regulatory protein *c*I, which binds to an 'operator site' (O_R) upstream of the promoters (Waldor and Friedman, 2005). Removal of *c*I-mediated repression results in activation of P_R and P_R', which in turn induce expression of *stx2* (Tyler *et al.*, 2004). This process also leads to expression of genes involved in the phage lytic cycle, meaning that induction of *stx2* is inexorably linked to bacterial cell lysis (Neely and Friedman, 1998; Wagner *et al.*, 2001b). This differential regulation of *stx1* and *stx2* expression results in different cellular localisation: whereas Stx1 remains predominantly intracellular within the periplasmic space, Stx2 is mainly found extracellularly in bacterial supernatants (Shimizu *et al.*, 2009; Strockbine *et al.*, 1986). However, both Stx1 and Stx2 have been identified within outer membrane vesicles (OMVs) secreted by EHEC (Kolling and Matthews, 1999), indicating an alternative pathway of toxin release to cell lysis. Once released into the intestinal lumen, Stx traverses the epithelium by one or more putative mechanisms (reviewed in Schüller, 2011) and interacts with Gb3-positive cells in the colon and kidney, leading to systemic disease.

Bacterial expression of *stx1* and *stx2* is induced a by a number of external cues, which are discussed below.

1.5.2.1.1 | Iron

Iron is an essential nutrient for all living organisms, and enteric pathogens must acquire iron from the external GI environment in order to survive (Skaar, 2010). In 1982, O'Brien and colleagues reported that incubation of certain *E. coli* strains in iron-depleted broth resulted in production of a toxin that could be neutralised with

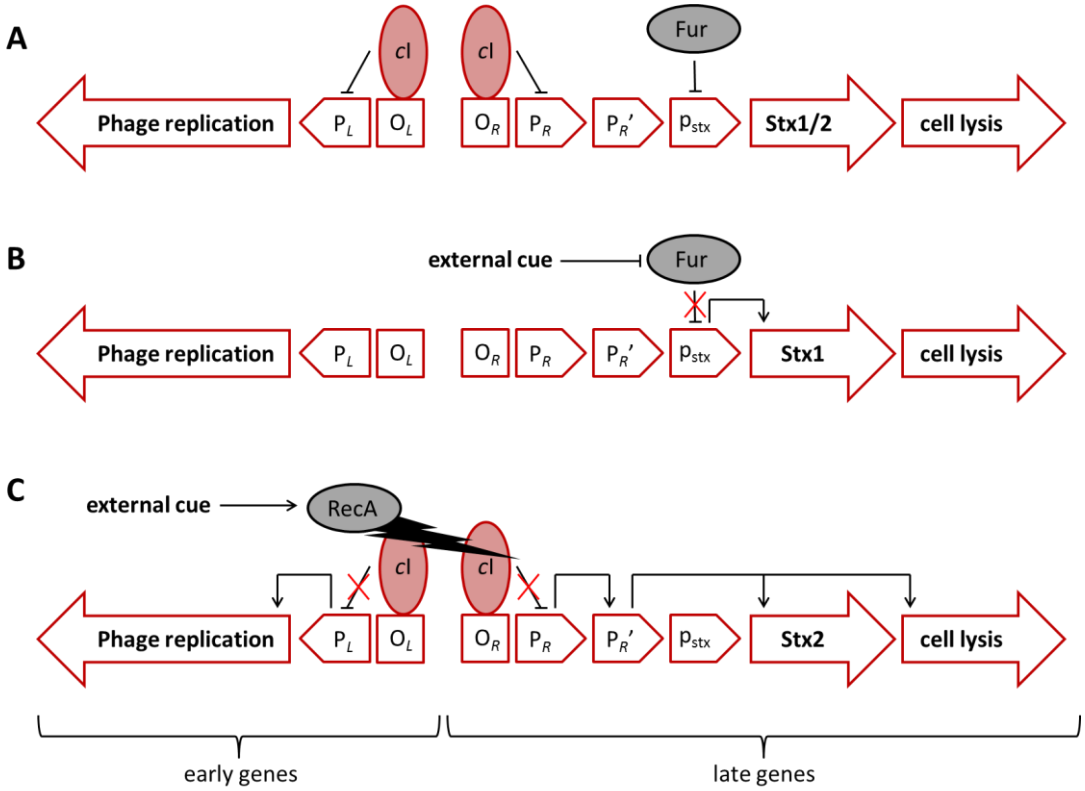

Figure 1.11. Regulation of Stx expression. (A) Repression of Stx phage during lysogeny **(B)** Induction of *stx1* expression (based on Wagner *et al*., 2002) **(C)** Induction of *stx2* expression (based on Pacheco and Sperandio *et al*., 2012). Phage encoded and native regulators are shown in red and grey, respectively. Pointed and blunt arrows denote positive and negative regulation, respectively.

antibodies raised against the *Shigella dysenteriae* type I toxin (O'Brien *et al*., 1982). Following the subsequent discovery of the EHEC pathotype, it was demonstrated that low iron concentrations induces expression of *stx1* in EHEC by releasing Fur-mediated repression of p_{stx} (Calderwood and Mekalanos, 1987). As Stx-mediated damage to the colonic microvasculature is thought to cause HC, it is possible that EHEC produces Stx1 under low iron conditions as a means of triggering the migration of iron-rich blood into the lumen. However, this strategy would require a mechanism for Stx1 release from the bacteria, which is yet to be presented. Iron does not appear to play a role in regulation of Stx2 expression (Sung *et al*., 1990).

1.5.2.1.2 | DNA damage

In the years following the discovery of EHEC, several research groups noted that EHEC production of Stx2 was enhanced by DNA-damaging agents such as UV radiation, mitomycin C and antibiotics (Acheson and Donohue-Rolfe, 1989; Al-Jumaili *et al.*, 1992; Muhldorfer *et al.*, 1996). Mühldorfer and colleagues (1996) demonstrated that mitomycin C-mediated induction of *stx2* expression was dependent on RecA, a protein involved in the SOS response. The SOS response is a conserved bacterial mechanism for coping with conditions that cause DNA damage, and mediates DNA repair, inhibition of cell division and induction of prophages (Little and Mount, 1982). Upon induction of the SOS response, RecA cleaves *c*I to de-repress P_R and P_R', resulting in expression of *stx2* and the rest of the phage genome (Koudelka *et al.*, 2004). This association between the SOS response and *stx2* expression is thought to explain why treatment of EHEC infections with certain antibiotics increases the risk of developing HUS (Pacheco and Sperandio, 2012).

1.5.2.1.3 | QS signalling ligands

In addition to its effect on LEE and flagellar gene expression (section 1.5.1.6.3), adrenergic QS signalling has been shown to regulate *stx2* expression. In 2001, Sperandio and colleagues reported that inhibition of QS pathways in EHEC results in reduced expression of *stx2*, as well as *recA* and other SOS response regulators (Sperandio *et al.*, 2001). Hughes *et al*. (2009) later confirmed the role for QS in *stx2* regulation by showing that exposure to adrenaline or AI-3 results in expression of *stx2* in wild-type but not Δ*qseC* EHEC. The authors also found that QseC-mediated induction of *stx2* expression occurs *via* QseF. In addition, EA triggers expression of *stx2*, together with *qseC*, *qseF* and *qseA* (Kendall *et al.*, 2012). Put together, these studies indicate that EHEC detection of adrenaline, AI-3 and EA by QS results in induction of SOS response proteins, which in turn trigger production of Stx2. It is currently unclear whether detection of these external stimuli leads to induction of other SOS response-associated effects.

1.5.2.1.4 | Hydrogen peroxide and NO

Neutrophils produce DNA damaging agents such as hydrogen peroxide (H_2O_2) and NO (Carreras *et al.*, 1996), and so investigators have examined the role of these molecules in Stx regulation. Wagner and colleagues (2001a) showed that incubation of EHEC with human neutrophils or H_2O_2 resulted in enhanced *stx2* expression. This effect has since been corroborated by Los *et al*. (2010), who suggested that oxidative stress conditions in the GI tract may contribute to Stx production by EHEC. In contrast, NO has been shown to inhibit *stx2* expression in EHEC exposed to mitomycin C. This effect was associated with a reduction in *recA* expression, indicating that NO negatively affects *stx2* expression by repressing the SOS response (Vareille *et al.*, 2007). Put together, this data suggests that neutrophils can modulate *stx2* expression during EHEC infection by secretion of H_2O_2 or NO, respectively. However, a role for neutrophils or secreted factors on Stx production during *in vivo* infection has not been demonstrated.

1.5.2.1.5 | Regulation of Stx expression levels

A seminal paper by Wagner *et al*. has shed light on possible mechanisms by which the Stx-encoding bacteriophage itself may influence the level of Stx expressed in the bacterial host (Wagner *et al*., 2002). By constructing lysogens containing mutant H-19B prophages (a Stx1-encoding prophage isolated from O26:H11 strain H19), the authors demonstrated several key findings. One of these was that mutation of the *P* gene, which is essential for prophage replication, resulted in a significant reduction in Stx1 production following mitomycin C-mediated induction. This indicated that prophage replication is a major contributor to phage-regulated Stx1 production, likely by increasing the copy number of *stx1* and therefore the amount expressed following induction. This was corroborated by the observation that prophage induction by mitomycin C results in increased detection of *stx1A* by Southern hybridisation in wt but not Δ*P* H19. More recently, a study by Fogg *et al*. confirmed a cumulative effect of prophage burden on Stx production in EHEC by comparing *stx* transcription and translation in *E. coli* K12 single and double isogenic lysogens of Stx phage $\varphi 24_B$ (Fogg *et al*., 2012). Not only did the double lysogen express higher levels of toxin compared with the single lysogen, but they were also more sensitive

to induction by the antibiotic norfloxacin. These data indicate that prophage/*stx* copy number is likely to influence both the degree of induction and the level of Stx produced by induced bacteria.

A second key finding from the study by Wagner and colleagues was that deletion of the *S* gene involved in phage-mediated lysis resulted in prolonged accumulation of Stx1 relative to wt cells, indicating that the timing of cell lysis (and therefore Stx release) is also likely to influence the quantity of Stx produced (Wagner *et al*., 2002). In many lambdoid phages, the *S* gene can be translated in one of two methionine start sites, yielding either a short product that induces lysis or a longer product that inhibits lysis (Neely and Friedman, 1998). It is thought that the relative concentration of these two products dictates the time of bacterial lysis, which would in turn affect the amount of Stx produced before lysis. However, genetic analysis of the *S* gene located on the H19B prophage did not reveal the same conserved dual translational start site found in the *S* gene of other start sites (Neely and Friedman, 1998), and it remains to be determined whether this prophage possesses another mechanism for *S*-mediated control of cell lysis.

Further investigation into how prophage-mediated control of *stx* expression and cell lysis may influence Stx expression levels is of great interest in that it may yield knowledge of prophage-specific molecular interactions that can be exploited for therapeutic inhibition of Stx expression. In this regard, it would prove particularly useful to shed light on how this phenomenon is influenced by external stimulatory or inhibitory factors.

1.5.2.2 | Stx interaction with the human intestinal epithelium

Most functional Stx research has focussed on its effect on Gb3-expressing cells, as Stx-mediated damage to these cells is linked to development of HC and HUS (Johannes and Römer, 2010). However, Gb3-negative IECs represent the first point of contact between Stx and the host, and so may play an equally important role in EHEC pathogenesis.

1.5.2.2.1 | Internalisation of Stx into IECs

Analysis of human intestinal tissue has revealed that IECs do not express Gb3 on their surface (Björk *et al.*, 1987; Holgersson *et al.*, 1991; Miyamoto *et al.*, 2006; Schüller *et al.*, 2004). This lies in contrast to most commonly-used colonic carcinoma cell lines (e.g. Caco-2, HT-29), which express Gb3 and are susceptible to Stx cytotoxicity (Jacewicz *et al.*, 1995; Schüller *et al.*, 2004). On the other hand, T84 colonic carcinoma cells do not express Gb3 (Jacewicz *et al.*, 1995; Miyamoto *et al.*, 2006; Schüller *et al.*, 2004), which makes them the most physiologically accurate *in vitro* model with which to study Stx interactions with the human intestinal epithelium.

Interestingly, it has been reported that incubation of T84 cells with Stx results in internalisation of the toxin, despite the absence of the Gb3 receptor (Philpott *et al.*, 1997; Schüller *et al.*, 2004). Intracellular Stx was visualised in association with the Golgi apparatus and the ER, suggesting intracellular toxin trafficking *via* retrograde transport (Philpott *et al.*, 1997) similar to that described in Gb3-positive cells (Figure 1.10; Johannes and Römer, 2010). However, T84 cells do not show reduced protein synthesis or apoptosis following Stx internalisation, indicating that ERAD retrotranslocation of the toxin from the ER to the cytosol is likely disrupted (Schüller *et al.*, 2004). Importantly, addition of Stx to the apical side of polarised T84 cell monolayers results in translocation to the basolateral compartment without affecting epithelial barrier function (Maluykova *et al.*, 2008; Philpott *et al.*, 1997; Tran *et al.*, 2014). Further characterisation of Stx internalisation in these cells has implicated a role for a clathrin-independent endocytosis pathway resembling macropinocytosis (MPC), a non-receptor-mediated method of taking up extracellular solute macromolecules within large actin-coated vesicles (Malyukova *et al*., 2009). Toxin uptake was significantly reduced by known inhibitors of MPC during incubation of T84 cells with purified Stx and Stx-negative EHEC, and the bacterial serine protease EspP was found to be sufficient to stimulate toxin internalisation *via* this route (Malyukova *et al*., 2009; In *et al*., 2013). However, a recent study of Stx translocation using a wild-type EHEC infection model reported no effect of MPC inhibition on toxin transport through T84 cells (Tran *et al*., 2014), thereby casting uncertainty about the physiological relevance of this transport route. Nevertheless, it is clear that Gb3-independent Stx translocation through IECs is a reproducible

phenomenon *in vitro*, which may explain how Stx traverses the epithelium and causes systemic pathology during infection (Schüller, 2011).

1.5.2.2.2 | Stx and EHEC adherence

A role for Stx in EHEC adherence to epithelial cells has been postulated by Robinson and colleagues (2006), who showed that infection of HeLa cells with Stx-negative EHEC strain TUV 89-2 resulted in reduced adherence compared with the Stx-producing 86-24 wild-type strain. In the same study, TUV 89-2 colonisation of mice was also significantly lower than that of 86-24. The authors hypothesised that host cell exposure to Stx induced surface expression of the putative intimin receptor nucleolin, which in turn promoted bacterial adherence. This theory was supported by data showing that incubation of HeLa cells with Stx resulted in increased surface expression of nucleolin (Robinson *et al.*, 2006). Stx-enhanced EHEC adherence has also been demonstrated in HT-29 cells (Xicohtencatl-Cortes *et al.*, 2007). However, infection of infant rabbits with an Δ*stx2* mutant of EHEC 905 resulted in similar levels of intestinal colonisation to the wild type, suggesting that Stx2 does not influence EHEC adherence to the intestinal epithelium in this model (Ritchie *et al.*, 2003). Therefore, the role of Stx in EHEC adherence remains controversial.

1.5.2.2.3 | Stx and the innate immune response

Many studies have examined the role of Stx in regulation of the innate immune response by IECs, with data supporting both stimulatory and inhibitory effects. This data is discussed in more detail in section 1.6.1.2.

1.5.2.3 | Role of Stx outside the human host

Despite a wealth of research into the role of Stx during EHEC infection of humans, there is very little evidence from these studies to suggest where the evolutionary advantage for Stx prophage carriage actually lies. EHEC infection of humans is a relatively rare occurrence compared to infection of cattle. Indeed, it is generally accepted that ruminants represent the primary hosts for EHEC and that infection of humans is an incidental occurrence (Dean-Nystrom *et al.*, 1998a; Hancock *et al.*,

1998). Therefore, the role of Stx prophage carriage in EHEC survival is most likely related to life within the ruminant host or in the external environment. Interestingly, EHEC infection of cattle is generally asymptomatic and does not result in the Stx-associated pathology (i.e. HC, HUS) that arises during human infection (reviewed in Smith *et al*., 2002), despite the fact that Stx is present in cow pats at detectable levels (Ball *et al*., 1994; Hyatt *et al*., 2001) and that Stx expression in EHEC O157:H7 has been linked with increased faecal shedding in cattle (Hoffman *et al*., 2006). This suggests that Stx interacts differently with cattle compared with humans, perhaps in a way that confers a selective advantage to the host bacterium.

One hypothesis for the selective advantage of the Stx prophage is that it facilitates EHEC transmission, colonisation and/or survival within the ruminant host. As previously mentioned in section 1.5.2.2.2, Stx has been shown to promote colonisation of mice and adherence to immortalised epithelial cells (Robinson *et al*., 2006), and so it is possible that Stx may also facilitate colonisation of the lower intestinal epithelium in ruminants. In addition, Stx has been shown to modulate various aspects of the bovine immune system. Menge *et al*. reported that purified Stx1 induced apoptosis in stimulated immortalised bovine B cells and hindered activation of peripheral blood mononuclear cells isolated from bovine blood (Menge *et al*., 1999). In a later study, the same group demonstrated that ileal intraepithelial lymphocytes (IELs) were also affected by Stx: exposure to purified Stx1 resulted in significant loss of the $Gb3^+$ subset of IELs and reduced mitogen-mediated activation in the remaining cells (Menge *et al*., 2004). Ileal IELs have also been shown to exhibit elevated expression of interleukin 4 (IL-4) in the presence of Stx1 (Moussay *et al*., 2006), and primary bovine mucosal macrophages show reduced surface expression of CD14 and CD172a and elevated expression of a range of cytokines (Menge *et al*., 2015). All of these cell types have been shown to express Gb3 on their surface and bind Stx1 (Stamm *et al*., 2002; Menge *et al*., 2004; 2015). Put together, these findings suggest that Stx1 impairs the initiation of the adaptive and/or inflammatory response in cattle by targeting a range of immune cells including lymphocytes and macrophages. This likely benefits EHEC by preventing immune-mediate killing, and therefore may represent the selective force behind EHEC retention of the Stx prophage.

Aside from the advantages conferred by direct interactions with the ruminant host, it has been suggested that the Stx prophage may confer protection against predation by other inhabitants of the GI tract. For example, the rumen is populated by a range of protozoan species that graze on bacteria, including those from the genera *Eudiplodinium*, *Metadinium*, *Polyplastron*, *Isotrichia*, *Entodinium* and *Diplodinium* (Rasmussen *et al*., 2005). Interestingly, Steinberg and Levin reported that lysogeny of *E. coli* K12 with a Stx2-producing prophage resulted in augmented survival within the food vacuoles of the ciliated protozoan, *Tetrahymena pyriformis* (Steinberg and Levin, 2007). In addition, intracellular survival of EHEC O157:H7 harbouring *stx1* and *stx2* was higher compared with K12, but O157:H7 strains containing *stx1* alone or no *stx* genes showed no observable fitness advantage over K12 following ingestion by the ciliate. Lysogeny of K12 with a Stx2 prophage encoding a non-functional *stx2* gene resulted in enhanced survival compared with K12 but to a lesser extent than that observed with the lysogen containing functional *stx2*. Further to this study, Stoffa and Koudelka showed that Stx2 produced by EHEC EDL933 whilst encapsulated within *Tetrahymena thermophila* phagocytic vesicles resulted in killing of the protozoan cell by inhibition of protein synthesis (similar to its effect on mammalian cells), but only when the toxin was released *via* prophage-mediated lysis of the bacterium and not as a result of bacterial digestion by the host (2012). This suggests a possible explanation as to why *stx* is almost exclusively retained within a lytic prophage in EHEC, since phage-mediated lysis appears to be essential for toxin-mediated attack on bacteriophagic protozoa (Stoffa and Kouldelka, 2012).

This data suggests that possession of Stx-producing prophages confers protection against bacteriophagic protozoa, which could lead to increased success within the ruminant host (Steinberg and Levin, 2007). Furthermore, residing within protozoa could also result in sheltering from other hazards associated with the GI tract (e.g. predation, microbial toxins, host immune response) or the external environment (e.g. desiccation, predation, biocides) (Brandl, 2006). Thus, it seems that the selective advantage of harbouring the Stx prophage may be driven by its ability to enhance survival in environments other than the human host.

1.6 | Interaction with the innate immune system

Enteric innate immune defences play a pivotal role in protecting humans from microbial infection, and many of these defences are orchestrated by the intestinal epithelium (Figure 1.12A). Specialised goblet cells secrete heavily-glycosylated proteins, called mucins, into the lumen, which form a dense layer of gel-like mucus that lines the epithelium (Linden *et al.*, 2008). The mucus layer serves the dual purpose of providing a niche for the commensal microflora and forming a physical barrier between luminal microbes and the intestinal epithelium (Birchenough *et al.*, 2015; Juge, 2012). Intestinal epithelial enterocytes secrete a range of microbicidal molecules, called antimicrobial peptides (AMPs), into the lumen. Some of these AMPs (e.g. human β-defensin (hBD)-1) are constitutively produced and retained in the mucus layer, forming a biochemical barrier (Dommett *et al.*, 2005; Mukherjee and Hooper, 2015). These preventative measures represent the front line of defence against infection by pathogenic microbes.

The intestinal epithelium is also capable of mounting further innate immune defence mechanisms in the event of microbial attack (Figure 1.12B). IECs express pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), which recognise microbe-associated molecular patterns (MAMPs) associated with bacterial cell walls or membranes (e.g. lipopolysaccharide (LPS), peptidoglycan, flagella or nucleic acids). Stimulation of PRRs by MAMPs triggers a cascade of pro-inflammatory signal transduction pathways. These include the nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, which lead to activation of the transcription factors activator protein-1 (AP-1) and NF-κB, respectively (Li and Verma, 2002; Morrison, 2012). These transcription factors stimulate expression of a range of genes such as those encoding cytokines (e.g. interleukin (IL)-8, interferon (IFN)-γ, tumour necrosis factor (TNF)-α) and inducible AMPs (e.g. hBD2, LL-37). AMP production results in direct microbial killing *via* cell lysis, whilst secretion of cytokines leads to recruitment of specialised innate immune cells (e.g. dendritic cells (DCs), neutrophils, macrophages) that phagocytose microbes and secrete DNA-damaging agents (e.g. NO, H_2O_2). These immune cells migrate across the epithelium through TJs and attack invading microbes in the

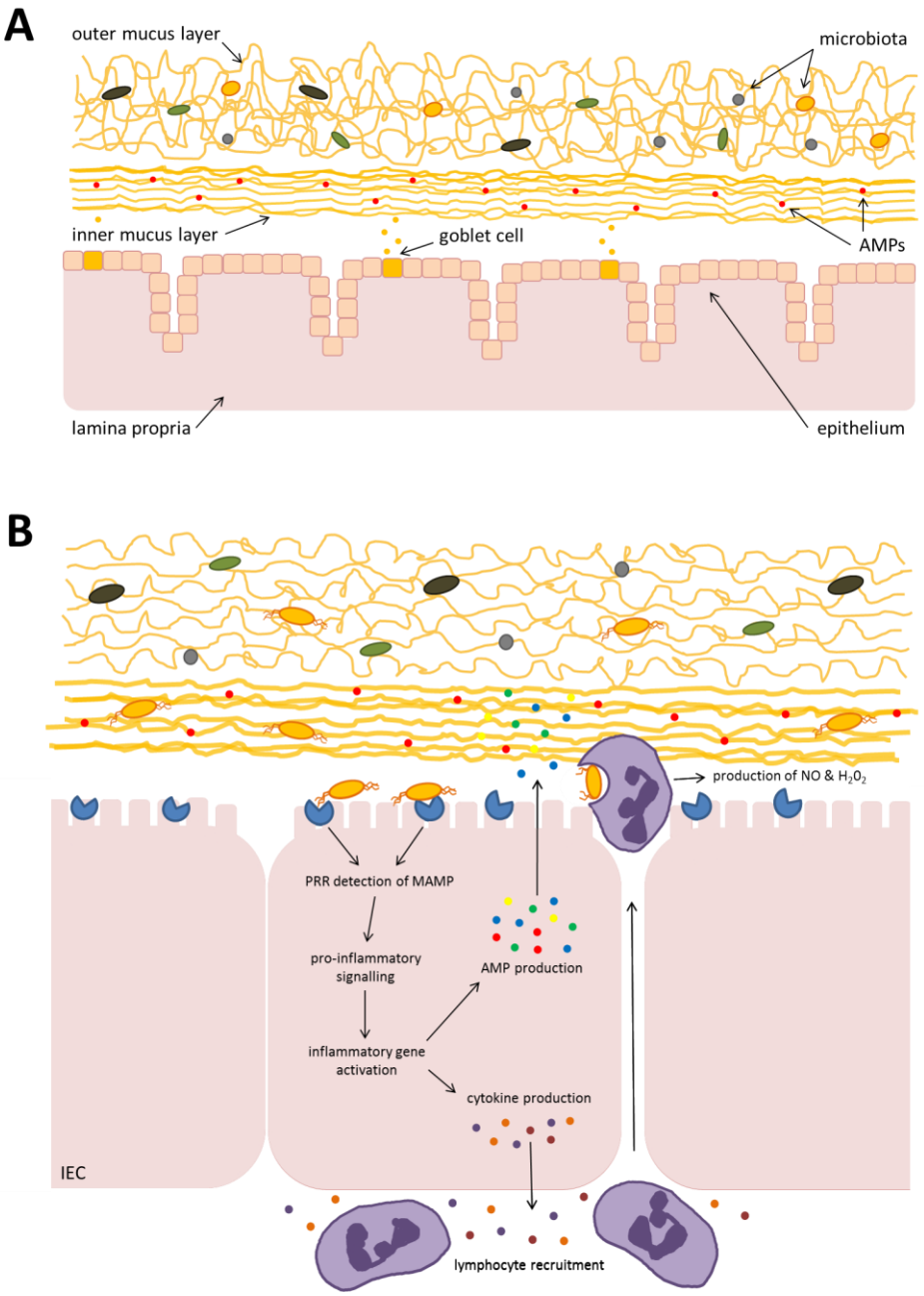

Figure 1.12. Innate immune defence in the colon. (A) Mechanisms employed during homeostasis. IECs secrete mucins and AMPs to form a dense sterile inner mucus layer and a loose microbiota-rich outer layer. **(B)** Mechanisms employed during microbial attack. Detection of MAMPs by PRRs induces an inflammatory response characterised by production of inducible AMPs and recruitment of lymphocytes.

lumen. These defence measures are collectively termed the inflammatory response (Bergstrom *et al.*, 2012; Brown *et al.*, 2013).

In addition to inducing inflammation, infected IECs are capable of undergoing cell death *via* apoptosis as a mechanism of killing and/or displacing invading bacteria. Apoptosis is induced following microbial stimulation of 'death receptors' on the IEC membrane (Wong Fok Lung *et al.*, 2014).

The multitude of antimicrobial defence mechanisms at the disposal of IECs highlights the importance of the intestinal epithelium in preventing and fighting enteric infection. As the intestinal epithelium also serves as the target site for EHEC colonisation, innate immune interactions between IECs and EHEC are likely to play a key role in the outcome of infection. The rest of this section summarises our current knowledge of these interactions.

1.6.1 | Innate immune response to EHEC infection

Intestinal biopsies from EHEC-infected patients display signs of moderate inflammation, characterised by neutrophil infiltration into the lamina propria (Griffin *et al.*, 1990; Kelly *et al.*, 1990). Using an *in vitro* model system involving polarised T84 cells and isolated human neutrophils, Savkovic and colleagues (1996) showed that EPEC-associated neutrophil transmigration across the epithelium is mediated by the chemokine, IL-8. Since then, many studies have reported elevated IL-8 expression levels in human IEC lines (i.e. T84, Caco-2, HT-29) following infection with both EPEC (Khan *et al.*, 2008; Ruchaud-Sparagano *et al.*, 2007; Sharma *et al.*, 2006; Zhou *et al.*, 2003) and EHEC (Bellmeyer *et al.*, 2009; Berin *et al.*, 2002; Dahan *et al.*, 2002; Farfan *et al.*, 2013; Gobert *et al.*, 2008; Ledesma *et al.*, 2010; Miyamoto *et al.*, 2006). EHEC-induced IL-8 expression in T84 cells is dependent on both MAPK and NF-κB signalling pathways (Dahan *et al.*, 2002). In addition, IL-8 has been detected in the blood of EHEC-infected patients suffering from HUS (Fitzpatrick *et al.*, 1992; Murata *et al.*, 1998). These data highlight IL-8 as an important mediator of the innate immune response to EHEC. As such, many researchers use this cytokine as a marker for inflammation when examining EHEC-IEC interactions. To date, EHEC-IEC innate immune interactions have only been

investigated using cultured epithelial cell lines, although EPEC induction of IL-8 in human small intestinal biopsies has been demonstrated (Schüller *et al*., 2009).

Several bacterial factors have been implicated in stimulating the innate immune response during EHEC infection, as discussed below.

1.6.1.1 | Flagellin

Flagellin, a protein that forms part of the flagellum, is considered the primary inducer of the innate immune response during EHEC infection. The flagellum itself is comprised of a long extracellular flagellin-rich filament attached to a molecular motor that is embedded in the bacterial outer membrane(s) (Jarrel and McBride, 2008). This filament is composed of 11 protofilaments, which wrap together to form the final structure. Each protofilament is almost entirely composed of stacked flagellin monomers. Flagellin consists of four linearly connected domains (termed D0-D4) which together form a structure that resembles an upper case greek gamma (Γ) (Yonekura *et al*., 2003) (Figure 1.13). The first two of these domains (D0 and D1) are highly conserved and are embedded within the filament structure, whilst D3 and D4 are more variable and form the external part of the filament (Bardoel and Strijp, 2011). Flagellin is detected exclusively by TLR5, which recognises a discrete conserved site on the D1 domain (Smith *et al*., 2003). As the D1 domain of flagellin is buried within the filament core and therefore inaccessible to TLR5 when in its polymeric state, it is assumed that TLR5 is only capable of recognising monomeric flagellin (Smith *et al*., 2003).

Purified H7 flagellin is capable of inducing IL-8 expression in T84 and Caco-2 cells, as well as in human colonic xenografts (Berin *et al.*, 2002; Miyamoto *et al.*, 2006; Zhou *et al.*, 2003). Moreover, deletion of *fliC*, the gene encoding flagellin, significantly attenuated the IL-8 response to EHEC in T84 and Caco-2 cells (Bellmeyer *et al.*, 2009; Miyamoto *et al.*, 2006). However, it should be noted that deletion of *fliC* did not completely abolish the IL-8 response in T84 cells (Bellmeyer *et al.*, 2009), suggesting that other factors may also play a role. Partial abrogation of the IL-8 response by deletion of *fliC* has also been reported for EPEC (Khan *et al.*, 2008; Schüller *et al.*, 2009). Bocking TLR5 activity in intestinal cell lines inhibited

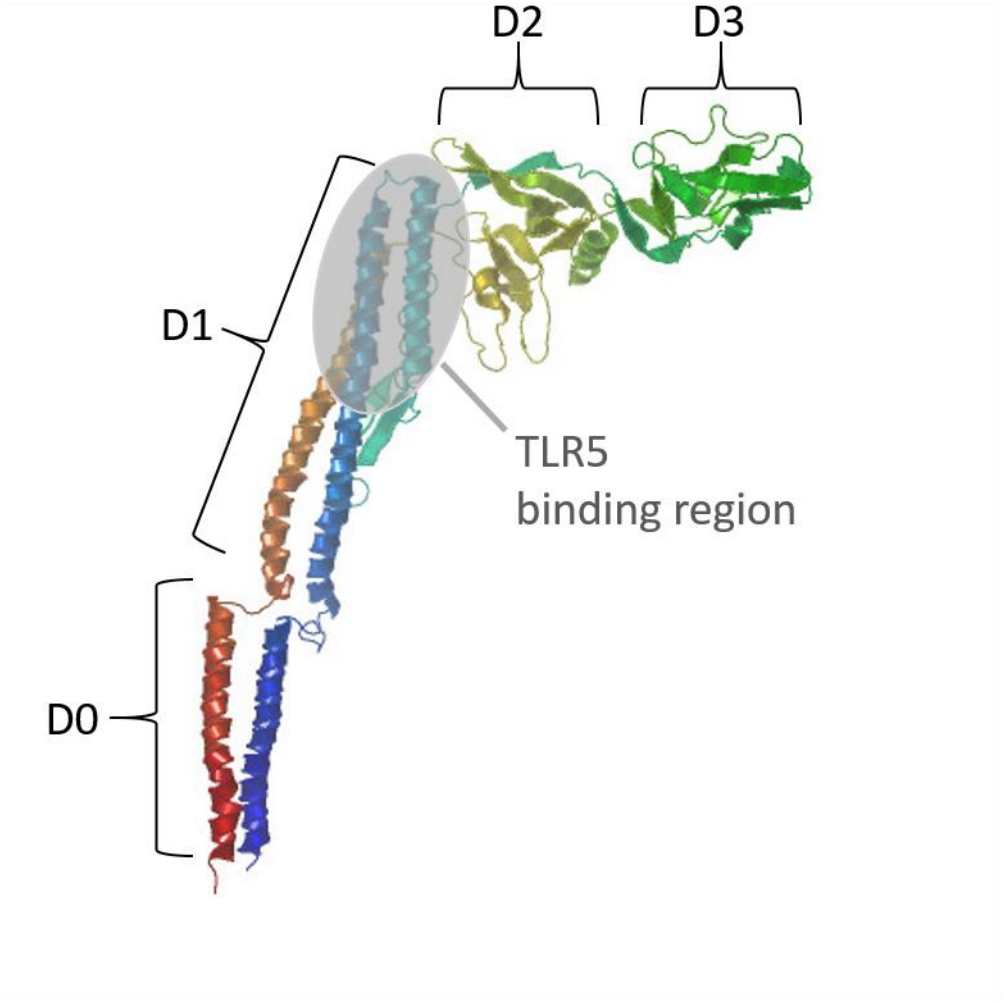

Figure 1.13. Flagellin domain organisation and TLR5 binding region. Adapted from RCSB Protein Data Bank (URL: http://www.rcsb.org/pdb/results/results.do?outformat=&qrid=8FB40A8F&tabtoshow=Current).

the inflammatory response to flagellin from *Salmonella dublin* and *E. coli* K12 (Bambou *et al.*, 2004; Eaves-Pyles *et al.*, 2011). It is therefore assumed that stimulation of IL-8 by EHEC flagellin is also mediated by TLR5, although this has not been confirmed experimentally.

1.6.1.2 | Stx

Yamasaki and colleagues (1999) were the first to implicate Stx in innate immune gene regulation, showing that recombinant Stx1 induced IL-8 expression in Caco-2 cells. Although a stimulatory effect of purified Stx1 and Stx2 has since been demonstrated on HCT-8 and T84 cells (Bellmeyer *et al.*, 2009; Thorpe *et al.*, 2001), this was not confirmed by other studies on Caco-2 cells (Berin *et al.*, 2002; Miyamoto *et al.*, 2006). Similarly, whilst deletion of *stx* in EHEC resulted in reduced IL-8 and NF-κB expression in T84 cells (Bellmeyer *et al.*, 2009), other studies on Caco-2 cells reported no effect of *stx* deletion on the IL-8 response (Berin *et al.*, 2002; Miyamoto *et al.*, 2006). Moreover, Δ*stx* mutants have even been shown to enhance IL-8 and MIP3α expression in T84 cells relative to wild-type bacteria (Gobert *et al.*, 2007). It is difficult to determine the cause of the varying effects of Stx, since contrasting results have been observed from studies using the same cell line and similar Stx concentrations. It is possible that different cell culture methods may lead to differences in internalisation of Stx and/or expression of any Stx-responsive innate immune receptors, which would likely affect the IL-8 response to the toxin. However, the influence of cell culture methods on Stx-IEC interactions has not been examined. Thus, the role of Stx in innate immune gene regulation remains unclear.

1.6.1.3 | Fimbriae

Two EHEC fimbriae, HCP and Lpf, have been associated with the innate immune response to EHEC. Ledesma and colleagues (2010) have shown that purified HCP and recombinant HcpA (the main constituent of HCP) induce expression of IL-8 and TNF-α in T84 and HT-29 cells *via* activation of MAPK signalling proteins p38, ERK1/2 and JNK. However, deletion of *hcpA* did not significantly affect IL-8 expression in HT-29 cells compared with the wild-type strain. In a recent study by Farfan *et al*. (2013), double deletion of the genes encoding Lpf1 and Lpf2 resulted in significantly lower T84 cell expression of IL-8, as well as several other cytokines (IL-6, IL-15. FGF, GM-CSF and IP-10).

1.6.2 | Immune evasion mechanisms

The innate immune response creates a hostile environment in which EHEC must survive and proliferate. The concept that EHEC might secrete effector proteins that subvert the innate immune response first arose from reports stating that inhibition of T3S in EHEC and EPEC resulted in an enhanced IL-8 response to infection (Hauf and Chakraborty, 2003; Khan *et al.*, 2008; Ruchaud-Sparagano *et al.*, 2007). Since then, many effectors have been discovered that interfere with host cell control of inflammation, apoptosis and phagocytosis (Table 1.2) (Santos and Finlay, 2015). Those effectors targeting inflammatory signalling are discussed in more detail below.

EPEC and EHEC effectors repress the inflammatory response by targeting both the NF-κB and MAPK signalling pathways. NF-κB is a transcription factor that comprises a dimer of two Rel homology protein subunits, p50 and p65, together with one or more non-Rel homology proteins. In its inactivated state, NF-κB resides in the cytoplasm bound to the inhibitory protein, IκB. Upon stimulation of the NF-κB pro-inflammatory signalling cascade, the enzyme IκB kinase (IKK) is activated, which in turn acts to dissociate IκB from NF-κB. The de-repressed NF-κB then translocates to the nucleus, where it binds DNA sequences called response elements to induce transcription of inflammatory genes such as IL-8 (Figure 1.14) (Lawrence, 2009). NleE has been shown to suppress the IL-8 response to EPEC and EHEC in several cell lines by preventing degradation of IκB, thereby inhibiting NF-κB activation (Nadler *et al.*, 2010; Newton *et al.*, 2010; Yen *et al.*, 2010). NleB has the same effect during EPEC infection of HeLa and Caco-2 cells, although this effector is thought to play a lesser role compared with NleE (Li *et al.*, 2013; Newton *et al.*, 2010; Pearson *et al.*, 2013). NleH1 and NleH2 both interact with ribosomal protein S3 (RPS3), a non-Rel homology subunit of NF-κB that confers gene regulation specificity. Whilst NleH1 inhibits translocation of RPS3 into the nucleus, NleH2 increases translocation of the subunit (Gao *et al.*, 2009; Pham *et al.*, 2012). Furthermore, these two effectors interact with each other, with relative levels of each determining the overall net effect on RPS3 translocation (Pham *et al.*, 2012). Infection of gnotobiotic piglets with a Δ*nleH1* EHEC mutant resulted in greater disease severity, whilst no effect was observed with the Δ*nleH2* mutant (Gao *et al.*, 2009). Finally, EPEC and EHEC NleC have been shown to cleave the p65 subunit of NF-κB, leading to degradation of NF-

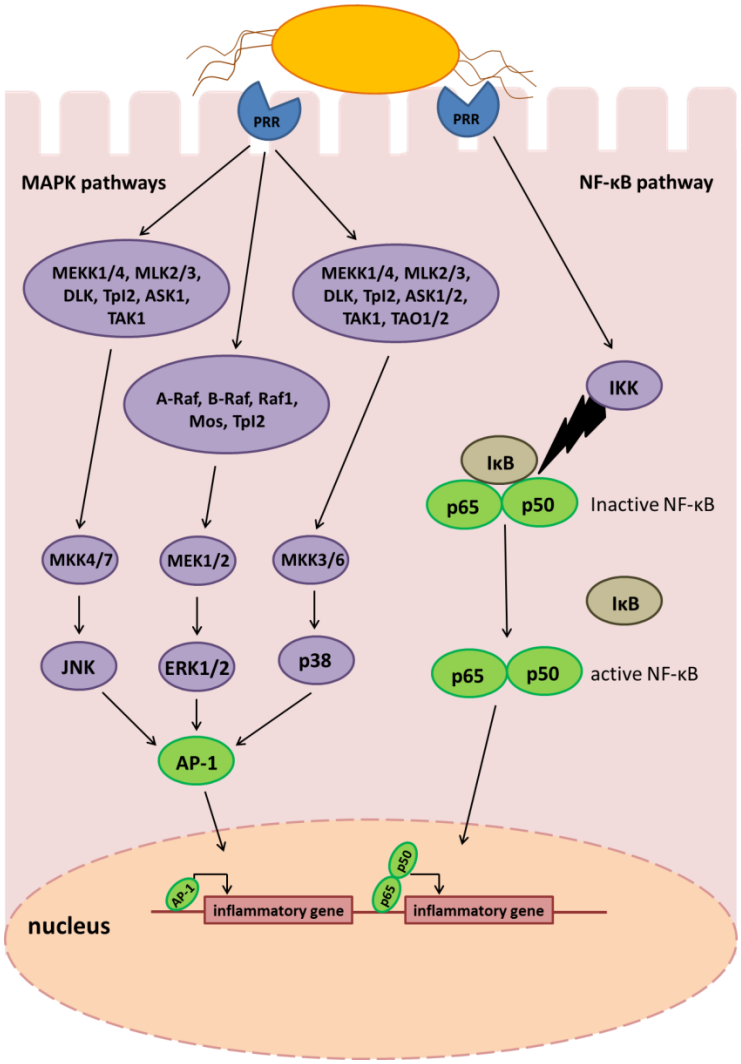

Figure 1.14. NF-κB and MAPK signalling pathways. Transcription factors are shown in green ovals. Positive and negative regulatory proteins are shown in purple and grey ovals, respectively. MAPK pathway based on Morrison *et al*. (2012).

2011; Yen *et al.*, 2010). Ectopically-expressed NleC also targets the p50 and c-Rel subunits in HeLa cells, conferring the same effect on IL-8 expression (Mühlen *et al.*, 2011).

Several different MAPK pathways regulate gene transcription in humans, and two of these – the p38 and JNK pathways – are pro-inflammatory. PRR stimulation initiates the activation of a phosphorylation cascade involving various kinases, beginning with a MAPK kinase kinase (MAPKKK). The activated MAPKKK phosphorylates a MAPK kinase (MAPKK), which in turn phosphorylates either the p38 or JNK

MAPKs (Morrison, 2012). Both of these MAPKs stimulate transcription factors such as AP-1 to induce transcription of pro-inflammatory genes including IL-8 (Figure 1.14) (Pramanik *et al.*, 2003). Baruch and colleagues (2011) have demonstrated that EPEC NleD cleaves both p38 and JNK, which prevents activation of AP-1 during infection of HeLa cells. However, deletion of *nleD* did not reduce the ability of EPEC to repress IL-8 expression. EPEC NleC is capable of inhibiting phosphorylation of p38 in Caco-2 and HT-29 cells, leading to reduced IL-8 production (Sham *et al.*, 2011). Conversely, ectopically-expressed NleH2 has been shown to enhance AP-1-dependent transcription in HeLa cells (Gao *et al.*, 2009), although the mechanism underlying this effect has not been investigated.

1.6.3 | Antimicrobial peptides

Antimicrobial peptides (AMPs) are important components of the enteric innate immune system. These small cationic peptides are capable of directly killing microorganisms by attacking their cell membrane and/or wall (Mukherjee and Hooper, 2015). AMPs are also becoming increasingly recognised as important inflammatory signalling molecules (Yang *et al.*, 2004), and inflammatory bowel disease (IBD) has been associated with enhanced AMP production (Fahlgren *et al.*, 2003; Zilbauer *et al.*, 2010). Whilst some AMPs are constitutively expressed by IECs, others are induced by the presence of microbes or inflammatory stimuli (Dommett *et al.*, 2005). Infection of IECs with a range of pathogens leads to production of inducible AMPs, suggesting that they play an important role in resisting pathogen attack. Despite this, the effect of EHEC infection on AMP production in IECs has not been investigated.

Several AMPs are expressed by IECs in the human colon: lysozyme, human β-defensins (hBDs) 1-4, and LL-37. The mode of action and expression pattern of each of these is summarised below.

1.6.3.1 | Lysozyme

Lysozyme is a glycosidase that damages bacterial cell walls by hydrolysis of peptidoglycans (Callewaert and Michiels, 2010). It is more effective at killing Gram-

positive than Gram-negative bacteria, as peptidoglycans are shielded by the outer cell membrane of the latter (Mukherjee *et al.*, 2008). However, *E. coli* susceptibility to lysozyme-mediated lysis has been demonstrated (Sedov *et al.*, 2011). Lysozyme is expressed in large amounts by specialised Paneth cells found at the base of small intestinal crypts (Peeters and Vantrappen, 1975). Colonic expression of lysozyme is thought to be generally low but elevated levels are found in individuals suffering from CD-associated colitis (Fahlgren *et al.*, 2003). To the best of our knowledge, no data showing an effect of microbial infection on lysozyme production has been published (Mukherjee *et al.*, 2008).

1.6.3.2 | Defensins

Defensins are small peptides (2-6 kDa) that kill bacteria by forming pores across plasma membranes, leading to a loss of membrane potential and cell lysis (Mukherjee and Hooper, 2015). Human defensins are classified into α and β-defensins based on differences in disulphide bond arrangements and spacing of cysteine residues (Selsted and Ouellette, 2005). Whilst enteric α-defensin expression is restricted to Paneth cells (Putsep *et al.*, 2000), β-defensins (hBDs) are expressed by IECs in the small intestine and the colon. Four types of hBDs (hBD1-4) have been identified in colonic IECs, all of which exert microbicidal activity against a range of bacterial species including *E. coli* (Dhople *et al.*, 2006; Estrela *et al.*, 2013; Pazgier *et al.*, 2006).

1.6.3.2.1 | hBD1

hBD1 is constitutively expressed in the small intestine and the colon (Zilbauer *et al.*, 2010) and is not responsive to pro-inflammatory stimuli (Fahlgren *et al.*, 2003). No changes in hBD1 expression have been reported in IECs following infection with *Salmonella* spp., EIEC, *Campylobacter jejuni*, and *Helicobacter pylori* (Chakraborty *et al.*, 2008; Fahlgren *et al.*, 2003; O'Neil *et al.*, 1999; Ou *et al.*, 2009; Zilbauer *et al.*, 2005). However, *Shigella* infection resulted in down-regulation of hBD1 expression in human intestinal biopsies, xenografts and HT-29 cells (Chakraborty *et al.*, 2008; Islam *et al.*, 2001; Sperandio *et al.*, 2008). The same effect has also been observed

following *Vibrio cholerae* and ETEC infection of HT-29 cells (Chakraborty *et al.*, 2008). This likely represents a bacterial survival strategy to avoid hBD1-mediated lysis or immune detection.

1.6.3.2.2 | hBD2

hBD2 is not detected in the intestine of healthy individuals but is expressed in the colonic epithelium of CD and ulcerative colitis (UC) patients (Fahlgren *et al.*, 2003; Zilbauer *et al.*, 2010). Accordingly, hBD2 expression is enhanced by pro-inflammatory stimuli (IL-1α and IL-1β) in Caco-2 and HT-29 cells (Fahlgren *et al.*, 2003; O'Neil *et al.*, 1999; Schlee *et al.*, 2007). hBD2 is also expressed during *in vitro* infection with pathogenic bacteria including EPEC, EIEC, *Salmonella* spp., *Shigella* spp., and *Campylobacter jejuni* (Fahlgren *et al.*, 2003; Hase *et al.*, 2002; Khan *et al.*, 2008; O'Neil *et al.*, 1999; Ogushi *et al.*, 2001; Ou *et al.*, 2009; Sperandio *et al.*, 2008; Zilbauer *et al.*, 2005). Furthermore, commensal bacteria such as *E. coli* Nissle 1917 also trigger an hBD2 response in IECs *in vitro* (Schlee *et al.*, 2007; Wehkamp *et al.*, 2004).

1.6.3.2.3 | hBD3

Similarly to hBD2, hBD3 is expressed in the colonic epithelium of IBD patients but not in healthy individuals (Fahlgren *et al.*, 2003; Zilbauer *et al.*, 2010). The pro-inflammatory cytokines IFN-γ and IL-1β enhance hBD3 expression in LS174T human colonic cells (Fahlgren *et al.*, 2004). Little is known about bacterial regulation of hBD3: whilst *Campylobacter jejuni* induced hBD3 expression in Caco-2 and HT-29 cells (Zilbauer *et al.*, 2005), *Shigella flexneri* triggered both up- and down-regulation depending on the IEC model used (Sperandio *et al.*, 2008). On the other hand, *Salmonella enterica* Serovar Typhimurium infection conferred no effect on hBD3 expression in T84 and Caco-2 cells (Ou *et al.*, 2009).

1.6.3.2.4 | hBD4

hBD4 is expressed in the small intestine and the colon at a relatively low level compared with other defensins (Fahlgren *et al.*, 2004). There is currently no evidence for microbial regulation of hBD4 in the intestine, although expression in lung epithelial cells is induced by infection with *Pseudomonas aeriginosa* and *Streptococcus pneumoniae* (Conejo García *et al.*, 2001). hBD4 does not appear to be responsive to inflammatory stimuli (Conejo García *et al.*, 2001; Fahlgren *et al.*, 2003).

1.6.3.3 | LL-37

LL-37 belongs to the cathelicidin family of AMPs, a structurally heterogeneous group that associates with a 'cathelicidin domain' during intracellular storage (Zanetti, 2004). It is the only cathelicidin currently described in humans, and expression has been localised to the colon but not the small intestine (Hase *et al.*, 2002). LL-37 expression in human colonic cell lines is enhanced upon infection with *Salmonella* spp. and EIEC (Chakraborty *et al.*, 2008; Hase *et al.*, 2002). As with hBD1, LL-37 production is repressed during *Shigella*-infection of human intestinal tissue (Islam *et al.*, 2001; Sperandio *et al.*, 2008) and in cell lines infected with *Vibrio cholerae* and ETEC (Chakraborty *et al.*, 2008). However, it should be noted that the inhibitory effect of *Shigella* spp. on LL-37 expression is not reproduced *in vitro* using cell lines (Hase *et al.*, 2002; Sperandio *et al.*, 2008). LL-37 exerts bactericidal activity against *E. coli*, as well as many other bacterial species (Turner *et al.*, 1998; Yan and Hancock, 2001). Although the mechanism for LL-37-mediated bacterial killing has not been completely defined (Ho *et al.*, 2013), it is known that the peptide can form pores in bacterial membranes (Lee *et al.*, 2011).

1.7 | Models used to study EHEC-IEC interactions

Cultured epithelial cell lines have been widely applied in the study of EHEC infection biology and have played an indispensable role in some of the most seminal

discoveries in the field. In particular, the HeLa cell model has contributed greatly to our understanding of the mechanisms underlying actin pedestal formation during EHEC and EPEC infection. Furthermore, polarised intestinal epithelial cell lines such as T84 and Caco-2 cells have enabled *in vitro* analysis of the innate immune response to infection. However, it is well established that pathogen interactions with cancer-derived cell lines often differ from those taking place on native host tissue (Law *et al.*, 2013). This is due to the fact that cancer-derived cells often carry mutations which change their properties. Cultured IEC monolayers also lack several important factors compared with their *in vivo* counterparts: cross-talk with cells of the surrounding tissue and the immune system, resident microflora, and other specific environmental stimuli that may influence gene regulation. Therefore, it is desirable to confirm results obtained from cell lines by using more physiologically accurate infection models.

One major obstacle in the progression of EHEC research is the lack of a suitable *in vivo* host model. Human infection studies are unethical due to the severity of disease caused by EHEC, and intestinal biopsy samples should not be taken from naturally-infected individuals due to the high risk of perforation (Nataro and Kaper, 1998). Critically, EHEC infection of standard mice leads to low levels of intestinal colonisation and no disease symptoms (Mundy *et al.*, 2006). Germ-free (i.e. no indigenous microflora) and streptomycin-treated (i.e. reduced microflora) are amenable to colonisation by EHEC, although extremely high infectious doses are required to generate disease-like symptoms in these animals (Mohawk and O'Brien, 2011). Furthermore, investigation of EHEC colonisation patterns using these models has yielded conflicting data: whilst infection of streptomycin-treated mice leads to predominantly colonic colonisation (Wadolkowski *et al.*, 1990), infected germ-free mice show signs of colonisation in the ileum and caecum but not the colon (Eaton *et al.*, 2008). Some research has been performed on the natural murine A/E pathogen, *C. rodentium*. *Citrobacter* infection of mice results in loose stools, intestinal inflammation and colonic hyperplasia but does not fully resemble the spectrum of disease caused by EHEC in humans, mainly due to the lack of Stx production (Mohawk and O'Brien, 2011). Mallick and colleagues (2014) have recently constructed an Stxd-producing strain of *C. rodentium*, which induces A/E lesions and Stx-mediated intestinal and kidney damage in mice, and therefore may represent a

more physiologically relevant model of infection than conventional *C. rodentium*. Other investigators have utilised gnotobiotic piglets and infant rabbits as *in vivo* host models for EHEC (Pai *et al.*, 1986; Ritchie *et al.*, 2003; Tzipori *et al.*, 1986). Both animals develop A/E lesions, watery diarrhoea and inflammation upon infection with EHEC. Gnotobiotic piglets also develop neurological HUS-like symptoms, and are therefore currently the best animal model for EHEC infection. However, it is generally difficult to ascertain whether animal infections simulate human infection due to the chance that species-specific interactions may generate confounding data (Fang *et al.*, 2013). Furthermore, the routine use of larger animals is limited by high maintenance requirements and low genetic tractability.

The absence of a suitable *in vivo* model has led to the development of innovative alternatives. *In vitro* organ culture (IVOC) was developed as a means of maintaining human intestinal tissue biopsies in the laboratory, and has also been used for infection studies (Fang *et al.*, 2013). IVOC has a major advantage over cell line models in that the former uses healthy native epithelium that consists of all the major cell types, together with an in-tact mucus layer and microbiota. As such, IVOC has become the gold-standard model for studying EPEC/EHEC-IEC interactions, having contributed to re-evaluation of the role of actin pedestals in A/E pathogenesis (section 1.5.1.3) (Bai *et al.*, 2008; Garmendia *et al.*, 2004; Schüller *et al.*, 2007). A polarised version of the IVOC model (pIVOC) has also been effectively applied to characterise the innate immune response to EPEC infection (Schüller *et al.*, 2009). However, the apparent tissue tropism of EHEC for the distal small intestine and lack of colonic colonisation has been puzzling (Chong *et al.*, 2007; Phillips *et al.*, 2000), and it remains unclear whether this represents the genuine *in vivo* situation (in contrast to data obtained from certain animal studies) or is an artefact arising from experimental factors.

An alternative *ex vivo* model system involves implantation of human foetal intestinal tissue into severe-combined immuno-deficient (SCID) mice. This murine xenograft model has been employed to study EHEC A/E lesion formation (Golan *et al.*, 2011), as well as the role of EHEC flagellin in regulation of IL-8 expression (Miyamoto *et al.*, 2006). Although this model has the potential to greatly contribute to the field, it is currently unclear whether EHEC-xenograft interactions are confounded by murine

factors. Furthermore, this model system lacks resident microbiota and, as with the IVOC model, a functional adaptive immune system. Both IVOC and xenograft models are not suitable for routine laboratory use due to high technical standards and access limitations to human tissues.

1.8 | Summary

EHEC is regarded as a pathogen of great public health importance, causing debilitating and potentially fatal disease for which there is currently no effective treatment. Since its discovery in 1983, great advances in our understanding of EHEC infection biology have been made. The intestinal epithelium plays a crucial role in the events leading to pathogenesis: IECs are the site of EHEC colonisation and A/E lesion pathology; EHEC modulation of IEC signalling leads to diarrhoea; IECs trigger inflammation in response to EHEC proteins, and IECs mediate Stx access to host internal tissue, leading to HC and HUS. Despite a wealth of research in these areas, several of the underlying molecular mechanisms involved in EHEC-IEC interactions remain unclear. Examples include the role of Stx in EHEC adherence and the innate immune reponse, the role of adhesins in initial attachment, and the role of effectors in microvillous effacement. In many cases, our understanding is clouded by the generation of conflicting data, which is a result of the use of artificial experimental model systems. In addition, *ex vivo* analysis of EHEC adherence to IECs using IVOC has thrown into question the clinical relevance of A/E lesions, further prompting the need for new or improved models that allow a closer simulation of the *in vivo* situation. Finally, whilst the discovery of numerous EHEC mechanisms for innate immune suppression has highlighted the importance of immune responses during EHEC infection, the breadth of our knowledge in this area does not extend further than the expression of a few select cytokines. Therefore, it would be beneficial for future EHEC research to focus on (1) further elucidation of processes underlying A/E lesion formation using physiologically relevant infection models and (2) expansion of our knowledge of the innate immune response to EHEC infection.

1.9 | Aims and objectives

The overarching aim of this PhD project was to characterise EHEC interactions with the human colonic epithelium by using physiologically relevant IEC models: human intestinal IVOC and polarised T84 cells. The objectives were to:

1. Determine whether EHEC colonises human colonic IECs and produces A/E lesions
2. Characterise the innate immune response to EHEC infection in human colonic IECs
3. Identify the bacterial factors involved in the interactions specified in objective 1 and 2

CHAPTER TWO

MATERIALS AND METHODS

All reagents were obtained from Sigma-Aldrich, unless otherwise stated. Incubations were conducted at 19-23°C (room temperature), unless indicated otherwise.

2.1 | Bacterial strains and growth conditions

A list of *E. coli* strains used in this study is presented in Table 2.1. All work involving Shiga toxin-producing EHEC strains was conducted at containment level (CL) 3, whilst Stx-negative EHEC and EPEC were handled at CL2. Bacterial stocks were generated from standing overnight cultures grown in 2 mL Luria Bertani Lennox (LB) broth at 37°C. Cryotubes (Sarstedt) were loaded with 700 µL overnight culture and 300 µL autoclaved 50% glycerol (in distilled deionised water (ddH_20), Thermo Fisher Scientific) and stored at -80°C. To initiate a culture, cryotubes were placed on dry ice until a thin upper layer of stock (approximately 1 mm) had thawed. A pipette tip was used to transfer some of the liquid stock onto an LB-agar (Formedium) plate, which was then incubated overnight at 37°C and stored at 4°C for up to 1 month. LB-agar plates were prepared by mixing LB and 1.5% w/v agar with ddH_20. Once autoclaved, approximately 20 mL LB-agar solution was poured into petri dishes and left at room temperature to set and dry. For infection assays, bacteria from several colonies were taken from an agar plate and grown overnight without agitation in 2 mL LB broth at 37°C. Deletion mutants (except EDL933 Δ*espA*) were selected in 50 µg/mL kanamycin or 30 µg/mL chloramphenicol (see Table 2.1). Overnight cultures were spun down at 18,000 x g for 5 minutes and resuspended in serum-free culture medium before inoculation.

2.2 | Cell culture and infection

2.2.1 | General cell culture methods

Cell lines were cultured in 25 cm^2 or 75 cm^2 culture flasks (Sarstedt) at 37°C in a 5% CO_2 atmosphere. Vero African Green Monkey kidney cells (ATCC CCL-81) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4500 mg/L glucose and sodium bicarbonate, supplemented with 4 mM L-glutamine (20 mL/L of 200 mM stock solution) and 10% foetal bovine serum (FBS; non-US origin). Caco-2

Strain	Serotype	Source or reference	Description
EHEC			
EDL933	O157	Riley *et al.*, 1983	isolate from 1982 US outbreak; prototype strain
EDL933 Δ*eae*	O157	Gobert *et al.*, 2008	no expression of intimin, resistant to kanamycin
EDL933 Δ*escN*	O157	Jarvis *et al.*, 1996	no functional T3SS, resistant to kanamycin
EDL933 Δ*espA*	O157	Beltrametti *et al.*, 1999	no functional T3SS
EDL933 Δ*fliC*	O157	Gobert *et al.*, 2008	no expression of flagella, resistant to kanamycin
EDL933 Δ*stx*	O157	Gobert *et al.*, 2007	no expression of Stx, resistant to kanamycin
E135309	O26	G. Smith, Public Health England	isolate from UK
H071840336	O157	G. Smith, Public Health England	isolate from UK
H074620462	O113	G. Smith, Public Health England	isolate from UK
H093740759	O157	G. Smith, Public Health England	isolate from UK
H103540554	O145	G. Smith, Public Health England	isolate from UK
H112180280	O104	G. Smith, Public Health England	isolate from 2011 German outbreak
TUV 93-0	O157	A. Donohue-Rolfe, Tufts University, US	Stx-negative derivative of EDL933
Walla-1	O157	Ostroff *et al.*, 1990	isolate from 1986 US outbreak; prototype strain
85-170	O157	Tzipori *et al.*, 1987	Stx-negative derivative of 84-289
85-170 Δ*hcpA*	O157	Xicohtencatl-Cortes *et al.*, 2007	no expression of HCPA, resistant to kanamycin
85-170 Δ*lpfA1*	O157	Fitzhenry *et al.*, 2006	no expression of LpfA1, resistant to chloramphenicol
EPEC			
E2348/69	O127	Levine *et al.*, 1978	isolate from UK; prototype strain

Table 2.1 | List of *E. coli* strains used in this study. All EHEC strains are Stx-positive unless stated otherwise.

human colonic carcinoma cells (ATCC HTB-37) were cultured in DMEM supplemented with L-glutamine and FBS as described above but with the additional supplement of 1x non-essential amino acids (10 mL/L of 100x stock solution). T84 human colonic carcinoma cells (ATCC CCL-248) were cultured in DMEM/Nutrient F12 Ham medium (1:1 mixture) containing 15mM HEPES and sodium bicarbonate, supplemented with 2.5 mM L-glutamine (12.4 mL/L of 200 mM stock solution) and 10% FBS. Cells cultured in 25 cm^2 flasks were provided with 5 mL culture medium, whilst those in 75 cm^2 flasks were cultured in 15 mL medium. Cells were passaged approximately every 7 days. Culture medium was aspirated from the flask and cells were rinsed with PBS to remove residual medium. Cells were then incubated in 0.5-1 mL 0.25% trypsin-0.02% EDTA at 37°C until they had detached from the base of the culture flask. After vigorous resuspension of cells in culture medium (at least 5 mL to neutralise trypsin activity), cells were transferred into new culture flasks at a dilution ratio of 1:10 (Caco-2), 1:20 (Vero) or 1:5 (T84) relative to the concentration before passage.

2.2.2 | Cryopreservation of cell lines

For long-term storage, cells were cryopreserved in liquid nitrogen. Cells were suspended at a high density (2-4 x 10^6 cells) in 1 mL culture medium containing dimethyl sulphoxide (DMSO, 5% v/v for T84 cells and 10% for all other cell lines) and transferred into cryotubes. To avoid cell damage by formation of ice crystals, cryotubes were placed in a Mr Frosty Freezing Container (Nalgene) containing isopropanol and frozen to -80°C overnight before being transferred to liquid nitrogen storage (vapour phase). To initiate a culture from frozen stocks, cells were thawed at 37°C, diluted in 9 mL warm culture medium and spun at 100 x g for 10 minutes to remove DMSO. Cell pellets were resuspended in 7 mL medium, transferred to a 25 cm^2 culture flask and cultured until confluent. Medium was exchanged the day after thawing.

2.2.3 | Seeding cells for infection assays

For infection assays, cells were seeded into cell culture plates (Sarstedt and Greiner Bio-One) during the passage process. After resuspension of trypsinised cells in culture medium, approximately 50 µL cell suspension was diluted 1:1 with trypan blue solution and 10 µL of this mixture was loaded into a Neubauer cell counting chamber (Hawksley; depth 0.1 mm) and examined using an inverted light microscope (Zeiss Invertoskop ID03). The number of viable cells (distinguishable by their lack of blue staining) within each of the 4 counting areas of the cell counting chamber (Figure 2.1) was determined, and the number of cells per mL (x) was calculated using the following equation:

$$x = (\text{average number of cells counted per counting area} * 2) * 10^4$$

The proportion of dead (blue) cells within the entire cell population was also calculated as an indication of the viability of the culture. A proportion of <10% dead cells was deemed acceptable for use in experiments. The amount of cell suspension needed for seeding (x) was then determined using the following equation:

$$x = \text{total number of cells needed} / \text{cell count per mL}$$

The necessary amount of cell suspension was then transferred into a fresh tube and culture medium was added in order to achieve the correct volume required for seeding. Cells were then administered into wells of a cell culture plate.

2.2.4 | T84 cell infection

T84 cells were used for experiments during passages 49-65. For infection assays on semi-polarised monolayers, cells were seeded out in 24-well plates containing sterile coverslips at a density of 10^5 cells/well and grown for 7 days until fully confluent. Cells were infected with 2 x 10^7 EPEC bacteria or 4 x 10^7 EHEC bacteria for 3 or 5 hours, respectively. To prevent bacterial overgrowth and acidification, media in EHEC-infected cells was exchanged after 3 hours.

For infection assays requiring a fully polarised cell monolayer, cells were seeded out into collagen-coated Transwell permeable filter inserts (12 mm diameter, polyester

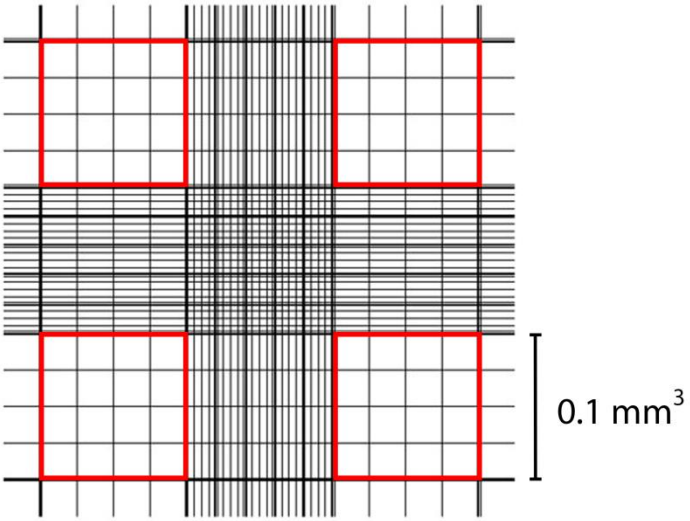

Figure 2.1. Layout of a Neubauer cell counting chamber. Cell counting regions are indicated by red boxes. The volume of cell suspension that fits within each of these regions is 0.1 mm^3, i.e. 10^{-4} mL.

membrane, 0.4 µm pore size, Corning) at a density of 5 x 10^5 cells/insert. Rat tail collagen type I was stored as a stock solution at a concentration of 1.25 mg/mL in 0.1 M acetic acid and working solutions of 50 µg/mL were prepared by diluting the stock by a factor of 1:25 in 62.5% ethanol (in ddH_20). Transwell insert membranes were coated with 200 µL collagen working solution (i.e. 10 µg/insert) and left at room temperature in sterile conditions for 3-4 hours until the liquid had evaporated. Cells were cultured until the transepithelial electrical resistance (TER) of the monolayer exceeded 1500 Ω x cm^2 (7-10 days), which served as an indication that epithelial barrier function had been established. TER was measured using an EVOM2 resistance meter with an STX2 electrode (WPI). Culture medium was exchanged on day 4 post-seeding and every 2 days thereafter. For infection assays, cells were inoculated with 6 x 10^7 EHEC bacteria, 10 ng/ml interleukin (IL)-1β, 25 µM inflammatory signalling inhibitor (MG-132, SB203580, SP600125, PD98059; Calbiochem) or purified H7 flagellin monomers (various concentrations; David Gally, University of Edinburgh, UK) in DMEM/F12 medium without supplements and incubated at 37°C in a 5% CO_2 atmosphere. For infection assays designed for analysis of bacterial colonisation or T84 mRNA expression, bacterial inocula were

left for 3 hours. To prevent bacterial overgrowth and acidification, media were then exchanged hourly for a further 3 hours (colonisation) or up to 9 hours (mRNA). For analysis of protein expression, bacteria were killed 1 hour post-infection by addition of gentamicin (200 µg/ml) to the apical and basal medium and incubations were continued for a further 24 hours. For experiments using IL-1β, inflammatory signalling inhibitors and flagellin, treatments were left on the cells for the duration of the assay with no medium exchanges.

2.2.5 | Vero cell infection

Cells were seeded out into 24-well plates at a density of 1.5 x 10^5 cells/well and cultured overnight. Cells were incubated for 4 hours with 1 x 10^7 EHEC bacteria or 100 ng/ml purified Stx2 (Anne Kane, Tufts Medical Centre).

2.2.6 | Caco-2 cell infection

Cells cultured to passage 8-15 were seeded into 24-well plates containing sterile coverslips (microscopy) or 6-well-plates (protein quantification) at a density of 1.2 x 10^5 or 2 x 10^5 cells/well (respectively) and cultured for 7 days until fully confluent. Cells were then incubated for 24 hours with EHEC (1.2 x 10^8 bacteria in 24-well plates or 5.4 x 10^8 bacteria in 6-well plates) or IL-1β (10 ng/ml). Bacteria were killed 1 hour post-infection using gentamicin (200 µg/ml).

2.3 | *In vitro* organ culture (IVOC) of intestinal biopsies

2.3.1 | Preparation of IVOC medium

IVOC medium was prepared by mixing the following ingredients in 90 mL ddH_20: NCTC-135 (0.94 g), sodium bicarbonate (0.22 g) and D-(+)-Mannose (1 g). This solution was sterilised using a 0.45 µm syringe filter (Sartorius Stedim), after which DMEM containing 4500 mg/L glucose and sodium bicarbonate (90 mL) and newborn calf serum (20 mL) were added. IVOC medium was stored at 4 °C.

2.3.2 | Collection of intestinal biopsies

Biopsy samples were supplied through the Partners in Cancer Research Human Tissue Bank (NRES reference number 10/H0310/21) in collaboration with the Gastroenterology Department, Norfolk and Norwich University Hospital. The study was approved by the University of East Anglia Faculty of Medicine and Health Ethics Committee (reference number 2010/11-030). Recruited patients were undergoing routine colonoscopy for intestinal disorders and gave their written consent for donation of biopsies before the procedure (see Appendix 1 for a copy of The Norwich Biorepository Consent Form version 15).

Up to 8 biopsies were taken from the transverse colon and/or terminal ileum of 35 adult patients aged between 24-77 years and 2 paediatric patients (aged 9 and 13 years) by medically trained staff using grasp capture forceps. Biopsy collection was not pursued in patients who showed macroscopic pathology in the region of interest during colonoscopy. Biopsies were collected in 5 mL plastic bijoux (Ramboldi) containing 2 ml IVOC medium for transportation to the lab and were used immediately upon arrival. Biopsies were examined under a dissecting microscope (Zeiss Stem SV8) and cut in half to yield samples with a mucosal surface area of ~5 mm^2.

2.3.3 | IVOC

IVOC was performed as described previously (Knutton *et al.*, 1987). Biopsies were mounted mucosal-side upwards onto a 1 cm^2 foam support (Simport) placed inside a 12-well culture plate. IVOC medium was added to a level that enabled saturation of the support without submerging the biopsy (~1 mL) to prevent epithelial shedding. For infection, 25 µL bacterial overnight culture or LB alone (i.e. non-infected control) was applied directly to the mucosal surface of the biopsy and the culture plate was placed in a plastic box filled with a 5% CO_2/air mixture (BOC). Samples were then incubated for 8 hours at 37°C on a rocking platform. For a small subset of experiments, samples were incubated with 5% CO_2 in oxygen, rather than air. To prevent bacterial overgrowth, medium was exchanged at 4 and 6 hours post-

inoculation. At the end of infection, biopsies were washed vigorously in 2 mL PBS to remove mucus and non-adherent bacteria.

2.3.4 | Polarised IVOC of colonic biopsies

This protocol is based on the published method for small intestinal pIVOC (Schüller *et al.*, 2009), amended for colonic biopsies. Biopsies were mounted mucosal-side upwards onto a circular Perspex disk (manufactured by the Faculty of Science Mechanical Workshop, University of East Anglia) lined with a nitrocellulose filter (3 µM pore, Whatman), as shown in Figure 2.2. The disks contain a small central aperture (measuring 2 mm in diameter) and the biopsy was positioned in alignment with this. A second 'apical' disk was placed on top of the first 'basal' disk, enclosing the biopsy in a sandwich-like arrangement with the epithelium exposed through the apical aperture. The apical disk was coated in Histoacryl tissue glue (Braun Medical) before placing onto the sandwich in order to fix it onto the biopsy and thereby prevent bacterial access to non-epithelial tissue. The sandwich was then mounted onto a Snapwell support (polycarbonate, Corning), with its apical side facing into the Snapwell chamber. The Snapwell was placed into a 6-well plate and IVOC medium was added to both the apical and basal compartments (180 µL and 3 mL, respectively). Biopsies were then inoculated with 20 µL bacterial overnight culture or LB alone (i.e. non-infected control) and incubated at 37 °C with 5% CO_2/air on a rocking platform.

To prevent bacterial overgrowth, the inoculum was removed after 2 hours and the apical compartment was rinsed once in 200 µL IVOC medium. Two drops (~25 µL) fresh IVOC medium were then added to the edge of the apical compartment, with care taken not to submerge the biopsy, and incubation was resumed for up to 5 hours.

2.4 | Quantification of bacterial colonisation

At the end of infection, cell monolayers were rinsed three times in PBS to remove non-adherent bacteria and lysed through incubation in Triton X-100 (1% in PBS) for 10 minutes. Lysates were then serially diluted in 1 mL PBS and 100 µL of

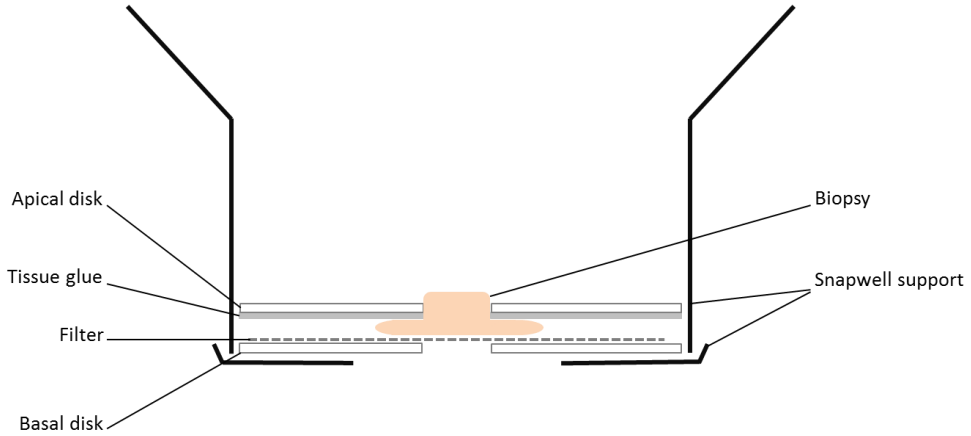

Figure 2.2. pIVOC system (adapted from Schüller *et al*., 2009). Biopsies were sandwiched between 2 perspex disks and installed into Snapwell supports, leaving only the epithelium accessible for experimental treatment.

appropriate dilutions (containing 50-500 colony forming units (CFU)) was plated onto LB agar plates. After overnight incubation at 37°C, the number of CFU was counted. This process was also performed on the bacterial inoculum immediately after inoculation. To calculate the percentage colonisation level, the CFU count for cell lysates was divided by that of the inoculum and multiplied by 100.

2.5 | Immunofluorescence staining

2.5.1 | Cell monolayers and whole biopsies

Cell monolayers and biopsies were rinsed twice in PBS and fixed with 3.7% formaldehyde (in PBS) for 20 minutes. Samples were then permeabilised and blocked by incubation in 1% Triton X-100 and 0.5% bovine serum albumin (BSA) in PBS for 20 minutes.

When staining for occludin, cells were pre-extracted in 0.2% Triton X-100 in PBS for 2 minutes on ice, fixed in 3.7% formaldehyde for 20 minutes, and permeabilised/ blocked in 0.05% Triton X-100 and BSA in PBS for 5 minutes on ice according to the manufacturer's instructions.

Ab name	Host Species	Working conc.	Source
E. coli	Goat	1:200	abcam
EspA	Rabbit	1:500	G. Frankel, Imperial College, UK
hBD2	Rabbit	1:200	abcam
Occludin	Rabbit	1:20	Life Technologies
Stx 204	Rabbit	1:1000	A. Kane, Tufts Medical Centre, USA
Tir	Mouse	1:500	J. Leong, Tufts University, USA

Table 2.2 | List of primary antibodies used for fluorescence microscopy in this study. All antibodies are polyclonal.

Samples were rinsed in PBS before staining. All stains and antibodies were diluted in 0.5% BSA in PBS to reach their working concentrations.

A list of primary antibodies (Abs) used for immunofluorescence staining is presented in Table 2.2. All primary Ab incubations were conducted for 1 hour at room temperature except for anti-hBD2, which was incubated at 4°C overnight. Samples were washed in PBS on a seesaw rocker for 10 minutes between primary and secondary Ab incubation steps. All secondary Abs were Alexa Fluor-conjugated (Life Technologies) and were administered to cells for 30 minutes at a dilution of 1:400. To label cell nuclei, cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Roche), which was administered during the last secondary Ab incubation step at a dilution of 1:5000.

To visualise filamentous actin, samples were incubated with fluorescein isothiocyanate (FITC)-conjugated phalloidin at a dilution of 1:200 for 20 minutes. If used in conjunction with fluorescent antibodies, FITC-phalloidin was administered after the last secondary Ab step.

Samples were washed for 2 x 30 minutes after the final staining step. Cells were mounted onto glass microscope slides (R & L Slaughter) in a drop of Vectashield mounting medium (Vector Laboratories) and covered with a cover slip (Agar Scientific). When mounting intestinal biopsies, cover slips were secured to the

microscope slide by sealing the edges with transparent nail varnish. Samples were imaged using a standard fluorescence (Zeiss Axiovert 200M) or confocal (Zeiss LSM510 Meta) microscope.

2.5.2 | Biopsy cryosections

Biopsies were fixed in 3.7% formaldehyde in PBS for 30 minutes. After washing three times in 2 mL PBS, samples were submerged in 15% sucrose in PBS, chilled to 4°C. Samples were left until fully saturated (approximately 10 minutes), indicated by their sinking to the bottom of the tube. Samples were submerged in chilled 30% sucrose until saturated. After blotting on a paper towel to remove excess moisture, samples were transferred to cryotubes and submerged within a drop of OCT compound (Sakura). Samples were snap frozen by submerging cryotubes in ethanol chilled to -80°C using dry ice and transferred to a -80°C freezer. Frozen samples were cut into 7 µm sections using a Microm HM550 cryostat (Thermo Scientific) and transferred onto poly L-lysine -coated glass slides (Agar Scientific). Sections were left for 30 minutes to adhere to the slide before transferring to a -80°C freezer.

For staining, sections were thawed at room temperature for ~10 minutes and blocked in 0.5% BSA in PBS for 20 minutes. Sections were then stained and imaged as described in section 2.5.1.

2.6 | Scanning electron microscopy (SEM)

At the end of infection, samples were washed twice in 2 mL PBS and fixed in 2.5% glutaraldehyde (Agar Scientific) in PBS at 4°C for at least 12 hours. After 2 further washes in 2 mL PBS, samples were dehydrated through a series of 15 minute incubations in acetone of increasingly higher concentrations (30%, 50%, 70%, 90% and 2 x 100%) in ddH_20 on a rocker. Samples were then dried in tetramethylsilane for 10 minutes on a rocker and air-dried for approximately 10 minutes or until all liquid had disappeared from the sample.

Dried samples were mounted onto aluminium stubs (TAAB Laboratory Equipment Ltd.) under a dissecting microscope. For cell monolayers grown on cover slips or

Transwell membranes, these were placed cell-side up onto stubs lined with adhesive carbon conductive tabs (TAAB Laboratory Equipment Ltd.). Biopsies were mounted onto the stubs in a drop of silver paint (Agar Scientific), taking care not to submerge the epithelium. Mounted samples were then sputter-coated with gold (Polaron SC7640 sputter coater, Quorum Technologies) and imaged with a JEOL JSM 4900 LV or Zeiss Supra 55 VP FEG scanning electron microscope.

To quantify bacterial colonisation of biopsies by SEM, biopsy epithelia were apportioned into fields of view (~250 per sample), and the presence or absence of bacteria within each was noted. The number of bacteria-positive fields was divided by the total number fields counted to calculate the colonisation level, expressed as a percentage. Quantification was conducted at 2700x magnification.

2.7 | Transmission electron microscopy

Samples were fixed in 2.5% glutaraldehyde in 0.1M PIPES buffer at 4°C for at least 12 hours and sent to Kathryn Cross (Electron Microscopy Facility, IFR) for processing and imaging, as described below. Samples were post-fixed in 1% aqueous osmium tetroxide for 1 hour and dehydrated through a graded ethanol series (10, 20, 30, 40, 50, 60, 70, 80, 90 and 3 x 100% in ddH_20) with 15-minute incubations. Embedding then took place through incubation in increasing concentrations of LR White medium grade resin (1 hour in 50%, 66%, 75%, 100%; 2 x 8 hours in 100%) in ethanol. Samples were then mounted in embedding capsules with fresh resin and allowed to polymerise overnight at 60°C. Semi-thin tissue sections (1 µM) were cut using an ultramicrotome (Ultracut E, Reichert-Jung) with a glass knife and collected on glass slides. Sections were stained with toluidine blue and imaged using an Olympus BX60 light microscope to locate adherent bacteria. Areas of interest were cut into 90 nm ultra-thin sections, mounted onto film/carbon copper grids, and stained sequentially with uranyl acetate and lead citrate. Sections were imaged with a Tecnai G2 20 Twin transmission electron microscope (FEI) at 200 kV.

2.8 | Gene expression analysis

2.8.1 | RNA isolation and DNase treatment

Total RNA was isolated from cells and biopsies using the RNeasy Mini kit (Qiagen). Cell and biopsies were lysed in buffer RLT supplemented with 1% β-mercaptoethanol. To homogenise cell monolayers, buffer RLT was flushed over the monolayer until cells had detached from the membrane, transferred to an RNase-free microcentrifuge tube (Sarstedt) and vortexed for 10 seconds. To homogenise biopsies, samples were transferred into a microcentrifuge tube and broken down using a homogenising pestle (Kimble Chase) before being vortexed for 45 seconds. Samples were immediately frozen at -80°C for at least 12 hours. After thawing on ice, lysates were transferred into Qiagen spin columns, and RNA was isolated according to the manufacturer's instructions (section entitled 'Purification of Total RNA from Animal Cells using Spin Technology'). Genomic DNA was degraded by on-column digestion with RNase-free DNase (Qiagen), according to the manufacturer's instructions.

RNA quantity and purity were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Samples were considered pure within acceptable limits if the 260/230 nm ratio (measures presence of salt contaminants) ranged between 1.5 and 2.0, and the 260/280 nm ratio (measures presence of protein contaminants) was ≥1.8.

RNA integrity was analysed by agarose gel electrophoresis. RNA was labelled with DNA nontox dye (PanReac AppliChem) and samples were separated on a 1.5% agarose gel in Tris-borate-EDTA (TBE). TBE stock solution (10x) was prepared by adding 108 g Tris, 55 g boric acid and 40 mL 0.5 M EDTA pH 8.0 and adjusting to a final volume of 1 L with ddH_20. A 1x working solution was prepared by diluting 1:10 in ddH_20. Gels were run at 100 V (constant) for ~45 minutes (Consort E863) and RNA was visualised under UV light using a U:Genius gel imager (Syngene). RNA integrity was considered acceptable if two distinct bands were visible (indicating the presence of 28S and 18S rRNA) with the former appearing stronger than the latter (Figure 2.3).

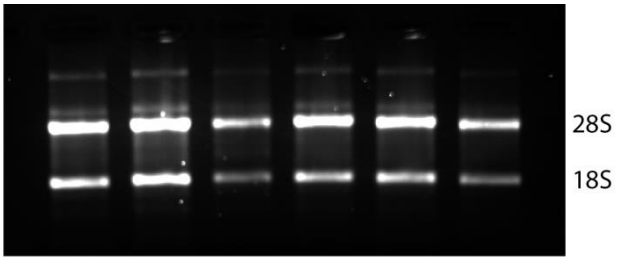

Figure 2.3. Representative image of intact RNA, as determined by agarose gel electrophoresis. Each column represents an individual sample.

2.8.2 | cDNA synthesis

RNA was converted to cDNA by using the qScript cDNA supermix (Quanta Biosciences). Reaction mixes were prepared by mixing 4 µL supermix with 1 µg RNA (volume dependent on concentration of RNA solution) and water up to a total volume of 20 µL. To control for the presence of residual DNA in the RNA samples, which could interfere with downstream applications, a reaction mix was prepared without addition of qScript cDNA supermix (RT-control) for each cDNA synthesis procedure.

cDNA synthesis was conducted according to qScript instructions using a thermal cycler (Biometra Professional Trio) set to the following conditions: 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C. cDNA was diluted 1:2 in filter-sterilised milli-Q water and stored short-term at 4°C or long-term at -20°C.

2.8.3 | Quantitative PCR

Quantitative PCR (qPCR) reaction mixtures were prepared by mixing 5 µL 2x SYBR Green Jumpstart Taq Readymix, 0.1 µL internal reference dye, 1 µL forward/reverse primer (10 µM), cDNA (1 µL for cells and 2 µL for biopsies) and water (3 µL for cells and 2 µL for biopsies) up to a total volume of 10.1 µL. For analysis of hBD2 or LL-37 mRNA levels, 4 µL cDNA and no water was added to reaction mixes. Reaction mixes were also prepared using the RT-negative control sample. To ensure that none of the reagents were contaminated with DNA, no template (NT) control reaction mixes were prepared containing water in place of cDNA for each primer

pair. Reaction mixes were loaded in duplicate into 96-well PCR plates (Sarstedt) on ice. Cling film was placed beneath the plates whilst on ice to prevent accumulation of water and dirt. Plates were sealed with transparent sealing film (Sarstedt) and spun in a swing rotor centrifuge at 2000 x g for 1 minute to collect reagents at the base of the wells. Plates were loaded into an ABI 7500 qPCR machine and subjected to the following cycling program:

Stage 1: 2 minutes at 95°C

Stage 2: 30 seconds at 95°C, 30 seconds at 60°C, 35 seconds at

72°C (repeat for 40 cycles)

Stage 3: 5 minutes at 72°C

Dissociation stage: 15 seconds at 95°C, 60 seconds at 60°C,

15 seconds at 95°C, 15 seconds at 60°C

At the end of the run, cycle threshold (Ct) values and dissociation curves were generated using ABI7500 software and exported for further analysis.

2.8.4 | Primer design and validation

Primers used are presented in Table 2.3. Primer sequences were either obtained from published studies or designed using PrimerBLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Whole gene sequences were obtained from ENSEMBL (http://www.ensembl.org/index.html). Primers were selected only if they met the following parameters: the GC content ranged between 30% and 70%, the self-complementarity score was <8, and the amplified sequence was 60-150 base pairs (bp) in length and spanned an intron. All primer melting temperatures were ~60°C. Lyophilised primers were purchased from Sigma-Genosys, reconstituted in 10 mM Tris (pH 8.0) to obtain a 100 µM stock solution, and stored at -20°C. Working solutions were prepared by diluting stock solution 1:10 in filter-sterilised milliQ water.

Target	Primer sequence	Product length (bp)	Reference or source
hBD1	Fw: 5'-CTGCTGTTTACTCTCTGCTTACTTTT-3' Rev: 5'-CCTCCACTGCTGACGCA-3'	107	Fahlgren et al., 2003
hBD2	Fw: 5'-CTCGTTCCTCTTCATATTCCTGA-3' Rev: 5'-CTAGGGCAAAAGACTGGATGAC-3'	111	Fahlgren et al., 2003
hBD3	Fw: 5'-TGAAGCCTAGCAGCTATGAGGATC-3' Rev: 5'-CCGCCTCTGACTCTGCAATAA-3'	128	Fahlgren et al., 2004
hBD4	Fw: 5'-CCCAGCATTATGCAGAGACTT-3' Rev: 5'-ACCACATATTCTGTCCAATTCAAAT-3'	102	Fahlgren et al., 2004
IL-8	Fw: 5'-TTGAGAGTGGACCACACTGC-3' Rev: 5'-TGCACCCAGTTTTCCTTGG-3'	98	Ou *et al.*, 2009
GAPDH	Fw: 5'-AGGTCGGAGTCAACGGATTT-3' Rev: 5'-TGGAAGATGGTGATGGGATTT-3'	220	Schüller *et al.*, 2009
LL-37	Fw: 5'-GTGCCCCAGGACGACACAGC-3' Rev: 5'-CCCCTGGCCTGGTTGAGGGT-3'	120	This study
Lysozyme	Fw: 5'-AAAACCCCAGGAGCAGTTAAT-3' Rev: 5'-CAACCCTCTTTGCACAAGCT-3'	94	Fahlgren et al., 2003
POLR2A	Fw: 5'-GATGGGCAAAAGAGTGGACTT-3' Rev: 5'-GGGTACTGACTGTTCCCCCT-3'	180	Schüller *et al.*, 2009

Table 2.3 | List of primers used in this study

To confirm primer specificity, dissociation curve analysis of qPCR products was performed (single peak) and qPCR product size was determined by agarose gel electrophoresis. To test amplification efficiency, two-fold serial dilutions of cDNA were prepared and run with each primer. After the run, the Ct values for each dilution were log transformed and plotted onto a scatter graph. The slope of the graph was used to calculate primer efficiency, according to the following formula: (10^(-1/slope)-1)*100. Primer efficiencies ranging between 95 and 130% were considered acceptable.

2.8.5 | Relative quantification

To confirm that Ct values represented levels of converted mRNA (i.e cDNA) and not contaminant DNA, values for the NT- and RT-controls were compared with those for standard samples. The amount of contaminating DNA was considered acceptably low if control samples yielded Ct values that were >5 higher than standard samples (equivalent to a >32-fold difference in transcript levels).

Ct values for the gene of interest were normalised by subtracting the geometric mean Ct for two housekeeper genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and RNA polymerase II (POLR2A). Fold expression values for the gene of interest were calculated in treated samples relative to matched untreated samples using the formula $2^{-\Delta Ct}$, where ΔCt represents the product of subtracting the normalised Ct value of the untreated sample from that of the treated sample.

2.9 | Enzyme-linked immunosorbent assay (ELISA)

All ELISAs were performed in Nunc Maxisorb immunoassay 96-well plates (Thermo Fisher Scientific). Washes were conducted using 200 µL PBS/0.05% Tween. Optical density (OD) readings were taken using a Benchmark Plus Microplate Spectrophotometer (BioRad) and standard curves generated using Microplate Manager 5.2.1 software.

2.9.1 | Sample collection

Culture medium was collected in pre-chilled microcentrifuge tubes and spun at 18,000 x g for 5 minutes at 4°C to pellet cell debris and bacteria. To harvest protein located within cells, T84 monolayers or biopsies were lysed in 1% ice-cold Triton X-100 in PBS supplemented with protease inhibitor cocktail (0.5%). Cells were left to lyse for 5 minutes on ice before being disrupted with a pipette tip (T84 cells) or homogenizing pestle (biopsies), transferred to a microcentrifuge tube, and spun at 18,000 x g for 15 minutes at 4°C to pellet insoluble proteins. Supernatants were stored at -80°C until required.

When preparing samples for hBD2 ELISA, all centrifuge steps were conducted in LoBind microcentrifuge tubes (Eppendorf). To concentrate hBD2, samples were loaded into pre-chilled Amicon ultra centrifugal filter units with a molecular weight cut-off of 3 kDa (Millipore) and spun at 2,000 x g at 4 °C until sample volume had reduced to the desired amount.

2.9.2 | ELISA for Stx2

Plates were coated in 100 µL Stx binding agent diluted in a coating buffer (1.59 g/L sodium carbonate, 2.93 g/L sodium bicarbonate and 0.2 g/L sodium azide in water) for 3 hours. Either P1 glycoprotein or polyclonal rabbit α-Stx 204 (Anne Kane, Tufts Medical Centre), NIH, USA) was used as a binding agent at a dilution of 1:5000 or 1:500, respectively. This was followed by a 2-hour blocking step in 300 µL powdered milk (5% in coating buffer). After 5 washes in PBS/Tween, duplicate wells were loaded with 100 µL sample (diluted 1:5 in DMEM/F12) or DMEM/F12 alone (blank wells). Two-fold serial dilutions of purified Stx2 standard (Anne Kane, Tufts Medical Centre), ranging from 40 to 0.31 ng/ml, were also added to wells in triplicate.Wells were incubated for 1 hour, washed 5 times in PBS-Tween, and subjected to the following incubation steps (interspersed with 5x washing): 20 minutes in monoclonal mouse α-Stx2 NR846 (1:2500 in PBS/Tween, BEI resources), 20 minutes in goat α-mouse IgG conjugated with alkaline phosphatase (1:2000 in PBS/Tween), and 30 minutes in phosphatase substrate dissolved in diethanolamine buffer (prepared in 11 mL batches by mixing 9.7 mL diethanolamine, 1 mL 50 mM

magnesium chloride, 0.2 g sodium azide and adjusted to pH 9.8 using concentrated hydrochloric acid). The OD at wavelength 405 nm (OD_{405}) was then measured, with baseline wavelength set at 605 nm. To remove any signal caused by non-specific binding of antibodies, OD_{405} readings from the blank wells were subtracted from those containing samples. Stx2 concentrations were calculated from the standard curve. Stx levels released by each bacterial strain were normalised according to the level of bacterial growth within the sample (measured by OD_{600}) using the following formula: ng/ml ÷ OD_{600} 0.1.

2.9.3 | ELISA for IL-8 and hBD2

IL-8 and hBD2 were quantified using commercial human ELISA kits (PeproTech), according to the manufacturer's instructions. When using the hBD2 ELISA kit, the following amendments were made: the blocking step was increased to 2 hours, the sample incubation step was increased to 2.5 hours, and the detection antibody step was reduced to 1.5 hours. For biopsies, IL-8 levels were normalised against total tissue protein. Total tissue protein was measured using the DC protein assay (Bio-Rad). The DC protein assay was performed according to manufacturer's instructions, except that the volume of sample used was 40 µl rather than 100 µl. A 2-fold dilution of BSA (in lysis buffer) was used to construct the standard curve, ranging from 94 to 1500 µg/ml.

2.10 | Statistical analyses

Statistical analysis was performed using GraphPad Prism Version 6 software. For comparison of two groups, parametric students *t*-test (cell line experiments) or non-parametric Mann-Whitney test (biopsy experiments) was used. Comparisons of three or more groups were performed using parametric analysis of variance (ANOVA) with Tukey's post-hoc test (cell lines) or non-parametric Kruskal-Wallis with Dunn's post-hoc test (biopsies). All gene expression data were log_{10} transformed before statistical analysis was performed. A P value of <0.05 was considered significant. Degrees of statistical significance are presented as follows: *=$P<0.05$, **=$P<0.01$, ***=$P<0.001$.

CHAPTER THREE

EHEC COLONISATION AND A/E LESION FORMATION

Based on publication:

Lewis, S.B., Cook, V., Tighe, R. and Schüller, S. (2015) Enterohemorrhagic *Escherichia coli* colonization of human colonic epithelium. *Infection and Immunity* **83**, 942-949 (see Appendix 2)

Collaborative work:

Biopsies provided by Vivienne Cook, Richard Tighe and Graham Briars

TEM performed by Kathryn Cross

SEM support provided by Bertrand Lézé

3.1 | Introduction

EHEC colonisation of IECs is considered critical for pathogenesis in humans (Croxen *et al.*, 2013). Colonisation is associated with the formation of attaching and effacing (A/E) lesions, which are characterised by intimate bacterial attachment to the IEC surface, localised microvillous effacement, and polymerised actin beneath the site of attachment (Lai *et al.*, 2013). The mechanisms underlying EHEC adherence to IECs have been characterised in detail using *in vitro* infection models based on cultured epithelial cell lines, and the following sequence of events has been established (Figure 1.4). Initially, EHEC bacteria loosely adhere to the host cell surface using one or more adhesins, including fimbriae, pili and flagella (Table 1.1). Subsequently, the bacterium inserts a type III secretion system (T3SS) into the host cell (Ebel *et al.*, 1998), through which effector proteins such as the translocated intimin receptor (Tir) are transported (DeVinney *et al.*, 1999). Tir inserts into the host plasma membrane, where it binds to and clusters with intimin on the bacterial outer membrane, thereby conferring an intimate attachment (Touzé *et al.*, 2004). Tir then triggers a signalling cascade that leads to recruitment of polymerised actin to the attachment site (DeVinney *et al.*, 1999), which collects to form a pedestal-like structure beneath the bacterium (Knutton *et al.*, 1989). The adherence process also leads to the localised effacement of microvilli, although the direct cause of this phenomenon remains unclear.

EHEC adherence to the intestinal epithelium has also been examined *in vivo* using animal models. Infection of gnotobiotic piglets, infant rabbits and neonatal calves leads to A/E lesion formation in the small intestine and the colon (Dean-Nystrom *et al.*, 1997; Francis *et al.*, 1986; Pai *et al.*, 1986; Tzipori *et al.*, 1986). However, examination of intestinal biopsy tissue from EHEC-infected humans has revealed no signs of bacterial colonisation, despite showing damage to colonic mucosa (Griffin *et al.*, 1990; Kelly *et al.*, 1987; Malyukova *et al.*, 2009; Shigeno *et al.*, 2002). This has cast doubt on whether EHEC colonises the human intestinal epithelium *in vivo*, although clinical samples are generally taken at a progressed stage of disease at which point colonisation may have diminished or be obscured by damaged tissue. EHEC adherence to human intestinal tissue has been investigated experimentally using *in vitro* organ culture (IVOC), a model that involves *ex vivo* infection of human

endoscopic biopsies. Human IVOC studies using Stx-negative EHEC strains have detected A/E lesions on terminal ileal but not colonic IECs (Chong *et al.*, 2007; Phillips *et al.*, 2000). Therefore, it remains unclear whether EHEC colonise human colonic epithelium and whether colonisation has any relevance for onset of colonic pathology during infection of humans.

In this study, we re-examined EHEC colonisation of human colonic epithelium *ex vivo* using wild-type EHEC strains. Previous studies have shown that Shiga toxin (Stx) produced by EHEC enhances adherence to HeLa cells and colonisation in mice (Robinson *et al.*, 2006). Therefore, we hypothesised that Stx produced by wild-type EHEC strains may facilitate adherence to human colonic biopsy epithelium. In addition, we also characterised EHEC colonisation of T84 human colonic carcinoma cells, which are widely used as an *in vitro* model of EHEC infection.

The objectives of this study were to:

1. Determine whether EHEC colonise human colonic epithelium *ex vivo*
2. Characterise the adherence phenotype *ex vivo* (if any) and *in vitro*
3. Identify bacterial factors influencing colonisation *ex vivo* (if any) and *in vitro*

3.2 | Results

3.2.1 | EHEC adherence phenotype on T84 human colon carcinoma cells is dependent on polarisation status

T84 human colonic carcinoma cells were used to characterise EHEC adherence to colonic epithelial cells *in vitro*. These cells resemble colonic crypt cells and form a polarised cell monolayer in culture, evidenced by their columnar shape, apical brush border, high transepithelial electrical resistance (TER), and tight junctions between cells (Madara *et al.*, 1987; Tran *et al.*, 2014). Unlike most other carcinoma cell lines, T84 cells do not express the Shiga toxin (Stx) receptor globotriaosylceramide (Gb3) on their surface and are therefore resistant to Stx cytotoxicity, which mimics the situation in the human intestine (Björk *et al.*, 1987; Miyamoto *et al.*, 2006; Philpott *et al.*, 1997; Schüller *et al.*, 2004). This makes T84 cells a suitable model for studying adherence of EHEC. Cells were seeded onto coverslips at a low cell density and cultured for 7 days to promote growth of a confluent monolayer (Gobert *et al.*, 2007). Monolayers were infected with Stx-negative EHEC strains TUV 93-0, 85-170 and Sakai for 5 hours, and cell morphology was visualised by immunofluorescence staining and scanning electron microscopy (SEM).

Upon examination of T84 monolayers, it was noted that cells exhibited two types of morphology: cells in central areas showed signs of polarisation such as an actin-rich brush border, whereas those in marginal areas appeared non-polarised with few microvilli (Figure 3.1; data shown for TUV 93-0). The majority of EHEC bacteria were found on non-polarised cells, with a relatively small number on polarised cells. EHEC bound to non-polarised cells were associated with polymerised actin beneath attachment sites (Figure 3.1A), indicating that actin recruitment had taken place. In contrast, actin was not observed beneath bacteria on polarised cells. Consistent with these findings, SEM analysis revealed formation of actin pedestals beneath bacteria attached to non-polarised, but not polarised, cells (Figure 3.1B). Interestingly, polarised cells displayed microvillous effacement around bacterial attachment sites, whilst microvillous effacement on non-polarised cells could not be examined due to the lack of a thick brush border on these cells (Figure 3.1B).

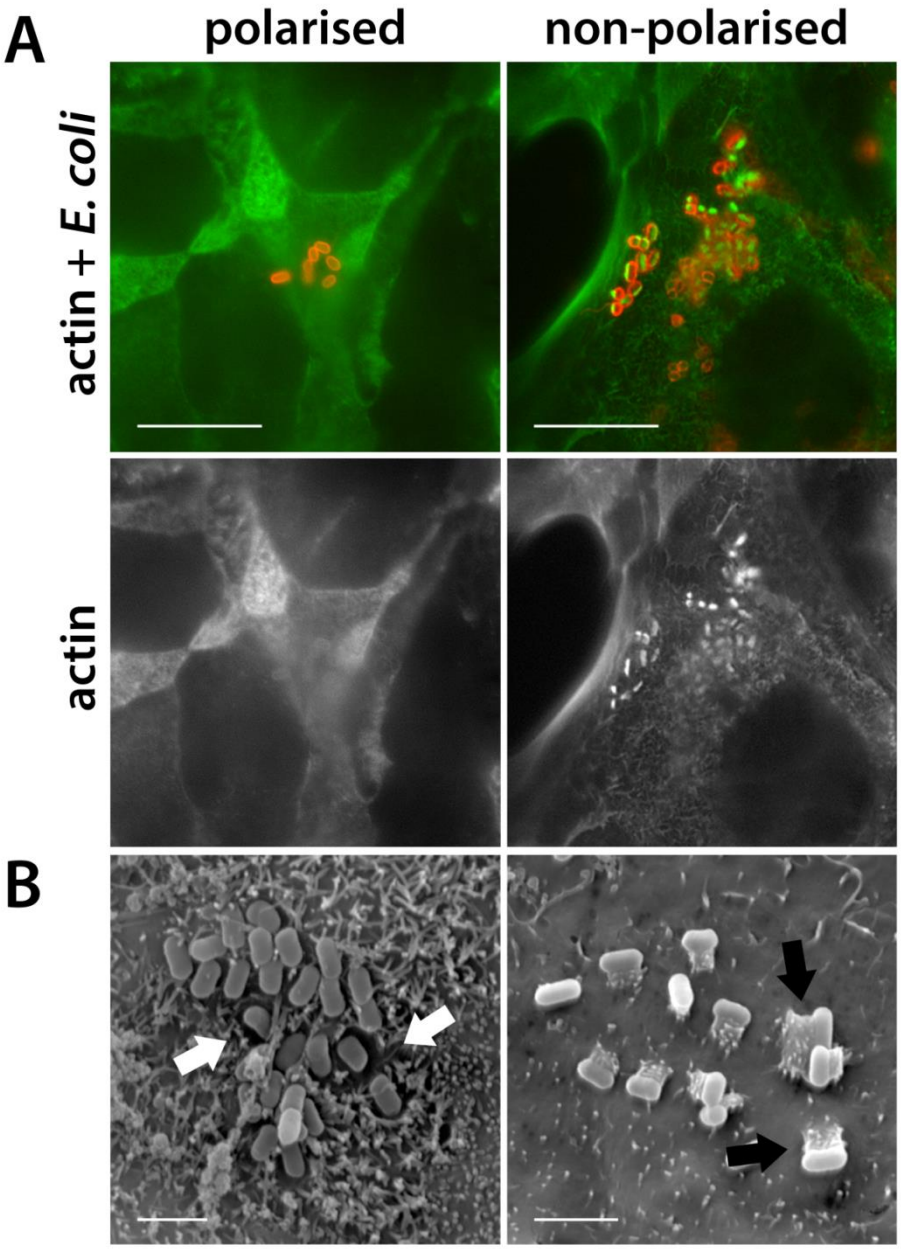

Figure 3.1. EHEC adherence phenotype differs on polarised vs. non-polarised T84 cells. Confluent cells on coverslips were infected with EHEC strain TUV 93-0 for 5 hours. (**A**) Immunofluorescence staining for actin (green) and *E. coli* (red). Top images show merged channels; bottom images show actin channel alone. Scale bars = 10 µm. (**B**) Scanning electron micrographs showing EHEC-associated microvillous effacement (white arrows) on polarised cells and actin pedestal formation (black arrows) on non-polarised cells. Images represent those from two independent experiments performed in duplicate. Scale bars = 2 µm

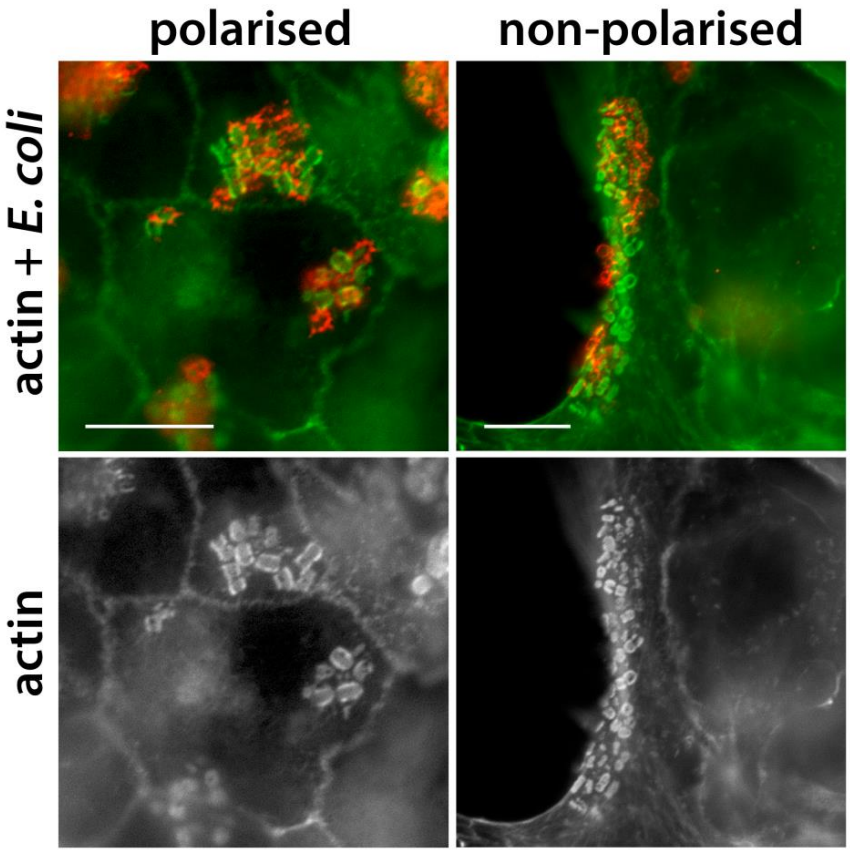

Figure 3.2. EPEC induce actin recruitment in polarised and non-polarised T84 cells. Confluent cells on coverslips were infected with EPEC strain E2348/69 for 3 hours and immunostained for actin (green) and *E. coli* (red). Top images show merged channels; bottom images show actin channel alone. Images represent those from two independent experiments performed in duplicate. Scale bars = 10 µm.

To determine whether actin recruitment in polarised cells was impaired or obscured by the actin-rich brush border, infections were performed using the related A/E pathogen, EPEC. Flourescent actin staining (FAS) revealed the presence of polymerised actin beneath EPEC adhered to both polarised and non-polarised cells, as shown in Figure 3.2.

Put together, this data shows that confluent monolayers of T84 cells seeded out on coverslips were displaying different stages of polarisation. In cells that appeared polarised, EHEC triggered microvillous effacement but no actin polymerisation. This suggests that EHEC may not induce typical A/E lesions in polarised T84 cells.

3.2.2 | Establishment of fully polarised T84 cell culture

In order to obtain fully polarised T84 cell monolayers for EHEC adherence studies, a modified cell culture method was adopted using collagen-coated permeable Transwell inserts (Madara *et al.*, 1987). Cells were seeded onto inserts at a high cell density (5 x 10^5 cells/insert) and cultured for an extended period (up to 14 days), whilst monitoring the TER at regular intervals. By day 7-10 post-seeding, monolayers had developed a TER of above 1500 Ω x cm^2 and displayed other hallmarks of polarisation when examined by microscopy, such as column-shaped morphology, apical brush border and tight junctions between adjacent cells (Figure 3.3). In addition, these cells appeared morphologically identical to the centrally-located cells previously grown on coverslips (Figure 3.4). These monolayers were considered fully polarised, and the Transwell culture method was used for all subsequent T84 cell experiments.

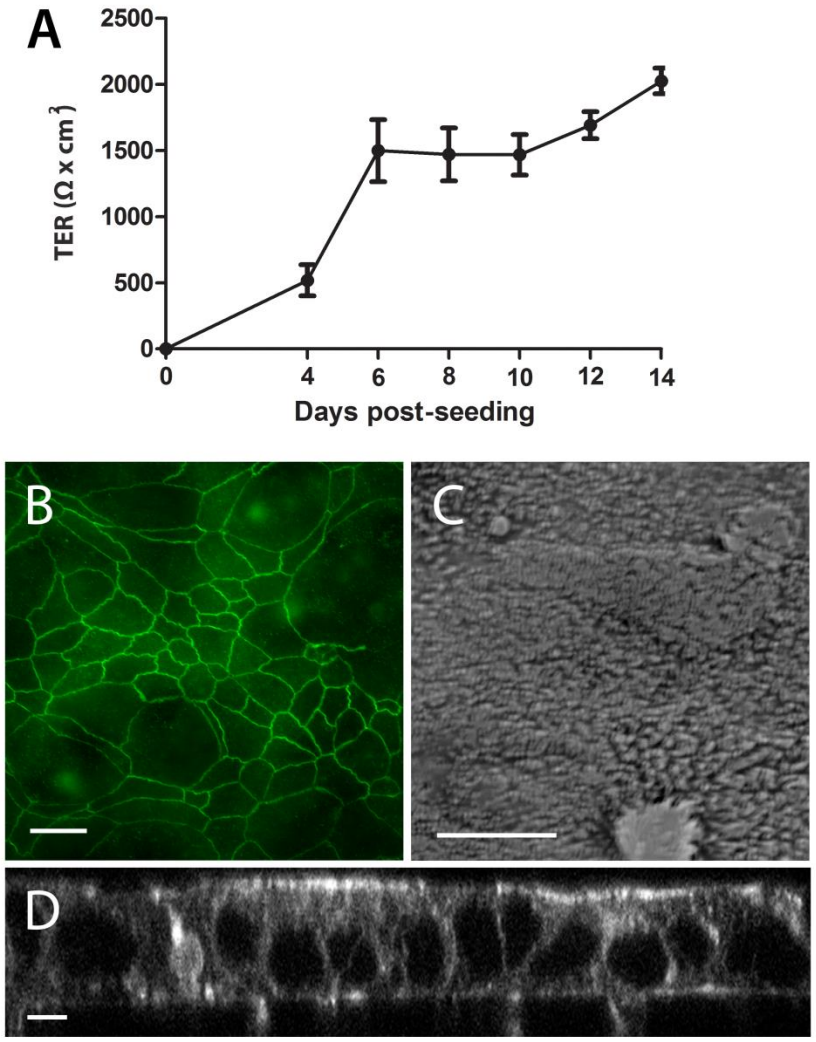

Figure 3.3. T84 cells exhibiting hallmarks of polarisation. Cells were cultured in Transwell inserts for up to 14 days. (**A**) Transepithelial electrical resistance (TER) was monitored every two days from day 4 post-seeding. Data are shown as means ± SE of four experiments performed in triplicate. (**B**) Immunofluorescence image showing the tight junction protein occludin in between cells. (**C**) Scanning electron micrograph showing a dense microvillous brush border on the apical cell surface. (**D**) XZ-scan confocal image of actin-stained cells exhibiting a column-shaped morphology. (**B**, **C**, **D**) Images represent those from at least two independent experiments performed in duplicate. Scale bars = 10 µm.

3.2.3 | EHEC do not induce typical A/E lesions on polarised T84 cells

In order to examine the adherence phenotype of EHEC on fully polarised T84 cells, infections were repeated using cells grown in Transwell inserts. Infection assays were extended to include wild-type strains EDL933 and Walla-1, as well as Stx-negative TUV 93-0. No signs of actin recruitment in polarised T84 cells were observed beneath adherent bacteria following infection for 5-9 hours with any of the three strains (Figure 3.4), indicating that neither wild-type nor Stx-negative EHEC strains recruit actin during colonisation of polarised T84 cells. However, actin recruitment was detected beneath bacteria bound to extruding cells that had lost polarity, and bacterial colonisation of these cells was high in comparison to non-extruding cells (data not shown).

Actin recruitment is mediated by Tir upon its translocation into the host cell *via* the T3SS (DeVinney *et al.*, 1999). Tir insertion into the plasma membrane and clustering by intimin is indicative of intimate bacterial attachment, another A/E lesion hallmark. To determine whether EHEC translocates Tir into polarised T84 cells, monolayers were immunostained for Tir and EspA (component of the T3S translocation tube) following infection with EDL933 and Walla-1 for 6 hours. Figure 3.5A shows that Tir was not detected beneath bacteria adhered to polarised T84 cells. This was not attributable to the absence of a functional T3SS, as EspA-rich translocation tubes were seen protruding the surface of adherent EHEC (Figure 3.5C). Furthermore, Tir translocation was evident beneath EHEC bound to extruding cells that appeared to undergo apoptotic cell death (Figure 3.5B). Put together, these findings indicate that EHEC adherence to polarised T84 cells does not involve actin recruitment or intimate attachment, and therefore does not lead to formation of typical A/E lesions.

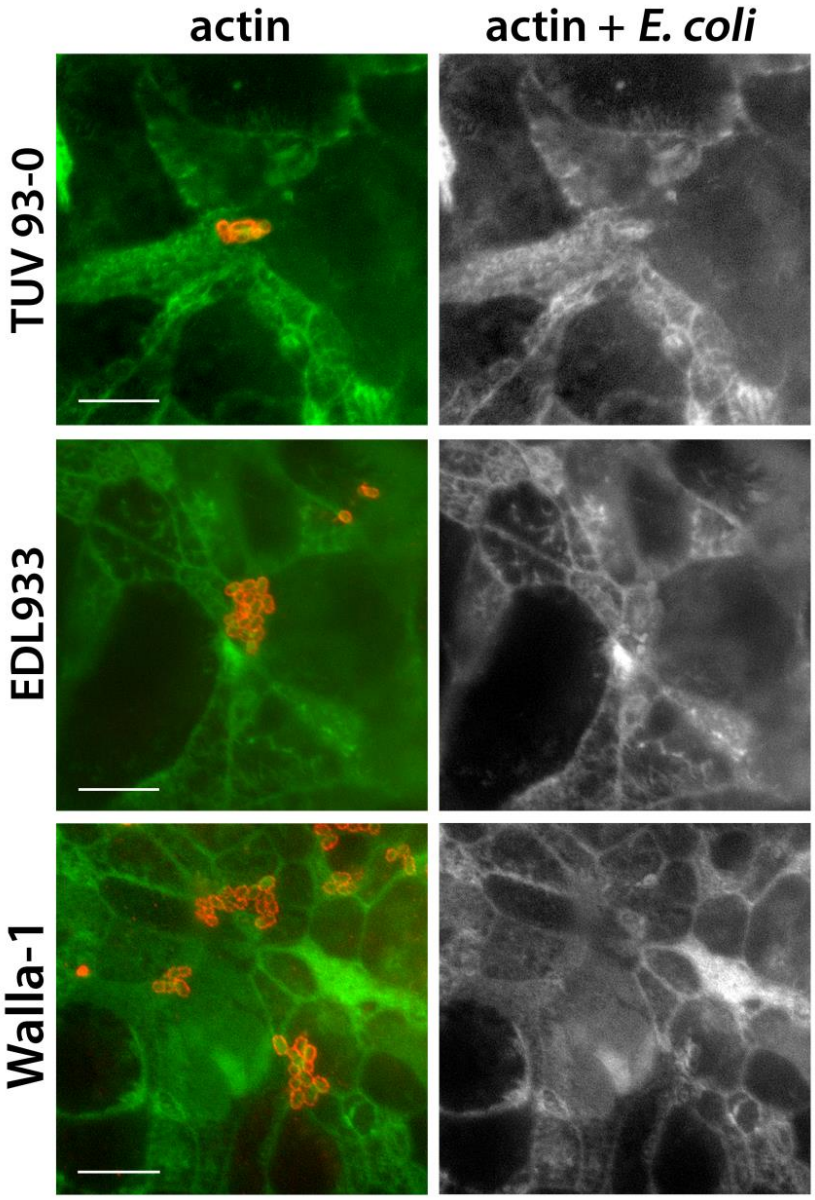

Figure 3.4. EHEC do not induce actin recruitment in polarised T84 cells. Polarised cells were infected with EHEC strains TUV 93-0, EDL933 and Walla-1 for 9 hours and immunostained for actin (green) and *E. coli* (red). Left images show merged channels; right images show actin channel alone. Images represent those from two independent experiments performed in duplicate. Scale bars = 10 µm.

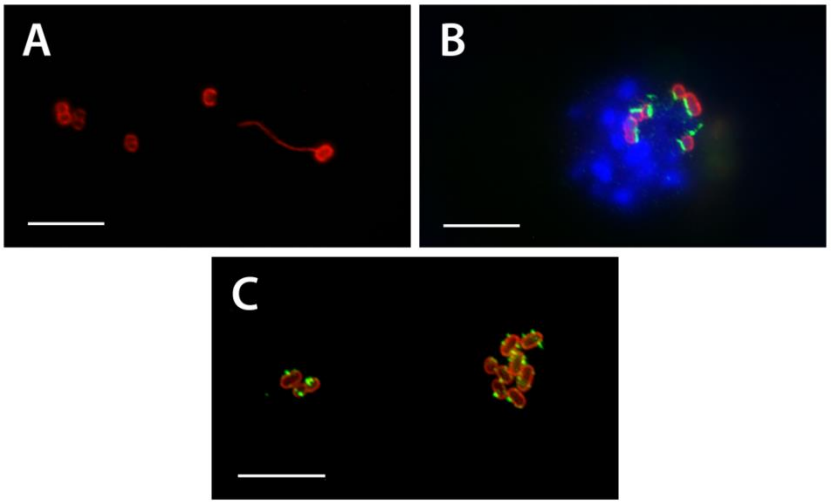

Figure 3.5. EHEC adhered to polarised T84 cells do not associate with translocated Tir. Cells were infected with EHEC strain EDL933 for 6 hours and immunostained for Tir (top; green), EspA (bottom; green) and *E. coli* (red). (**A**) Bacteria adhered to polarised cells with no visible signs of Tir. (**B**) Bacteria adhered to extruding cell (indicated by fragmented nucleus shown after staining with DAPI (blue)) with Tir visible beneath attachment site. (**C**) Adherent bacteria with EspA filaments protruding from their surface. Images represent those from two independent experiments performed in duplicate. Scale bars = 5 µm.

3.2.4 | EHEC colonise human colonic biopsies and induce A/E lesions

In vitro organ culture (IVOC) enables the study of EHEC adherence to human intestinal epithelium *ex vivo*. Previous IVOC infection studies using Stx-negative EHEC strains have reported adherence to terminal ileal but not colonic biopsy epithelium (Chong *et al.*, 2007; Phillips *et al.*, 2000). We utilised the IVOC model in order to characterise adherence of wild-type EHEC strains to human intestinal epithelium. Biopsies taken from the terminal ileum and transverse colon of adults were infected with EHEC strains EDL933, Walla-1 and H07184 for 8 hours and colonisation was examined by scanning electron microscopy (SEM).

All three EHEC strains extensively colonised both ileal and colonic biopsy epithelium (Figure 3.6; data shown for EDL933). In agreement with published

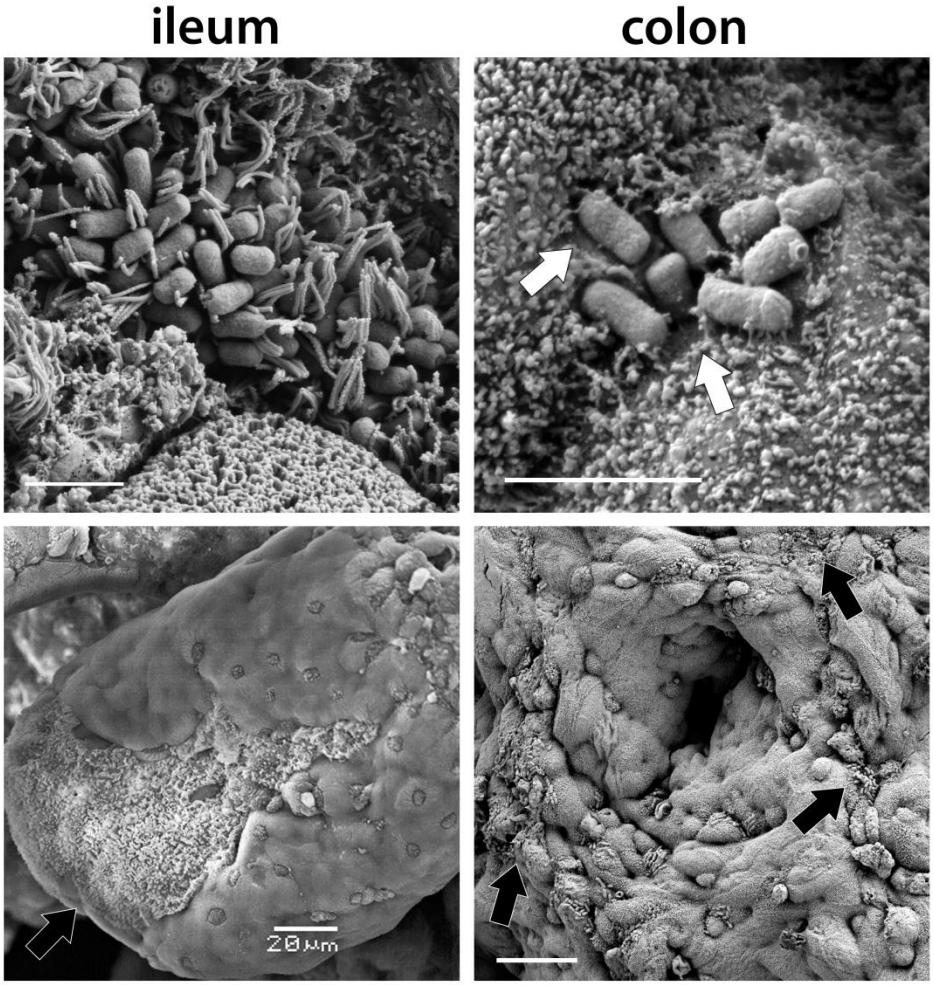

Figure 3.6. EHEC colonise human ileal and colonic biopsies. Endoscopic biopsies from the terminal ileum and transverse colon were infected with EHEC strain EDL933 for 8 hours and adherence was examined by scanning electron microscopy. (Top) EHEC adhered to ileum were surrounded by elongated microvilli, whereas those bound to the colon were associated with a zone of microvillous effacement (white arrows). (Bottom) Bacteria on terminal ileal and colonic biopsies localised around villus tips and crypt openings, respectively (black arrows). Images represent those from three independent experiments performed in duplicate. Scale bars = 2 µm (Top) or 20 µm (Bottom)

findings (Fitzhenry *et al.*, 2002; Phillips *et al.*, 2000), terminal ileal biopsies harboured elongated microvilli at sites of bacterial adherence. No signs of microvillous elongation were found on colonic biopsies but a zone of microvillous

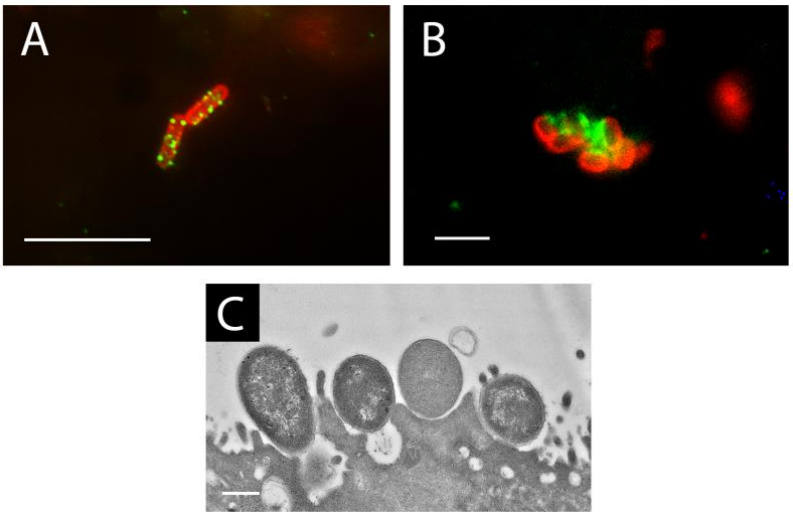

Figure 3.7. EHEC form A/E lesions on human colonic biopsies. Biopsies were infected with EHEC strain EDL933 for 8 hours. Immunostaining was performed for EspA (A) and Tir (B) in green and *E. coli* in red. (**C**) Transmission electron micrograph showing intimate attachment of bacteria to host cell membrane and microvillous effacement. Images represent those from two independent experiments performed in duplicate. Scale bars = 2 µm (A, B) and 0.5 µm (C).

effacement was evident around adherent bacteria, similar to that observed in polarised T84 cells. Colonisation on ileal samples was predominantly localised towards the tips of villi, whilst bacteria on colonic biopsies were most often found on the surface epithelium located between crypts (Figure 3.6).

To further characterise the adherence phenotype of EHEC bound to colonic epithelium, biopsies were immunostained for Tir and EspA following infection with EDL933 and Walla-1 strains. In contrast to findings from T84 cell infections, EHEC on colonic biopsies were associated with translocated Tir beneath sites of attachment (Figure 3.7B; data shown for EDL933). EspA filaments were also detected on adherent bacteria (Figure 3.7A). Transmission electron microscopy (TEM) revealed adherent bacteria in close contact with the IEC membrane and surrounded by a zone of microvillous effacement (Figure 3.7C). Put together, these studies demonstrate that EHEC colonise the human colonic epithelium *ex vivo* and induce formation of

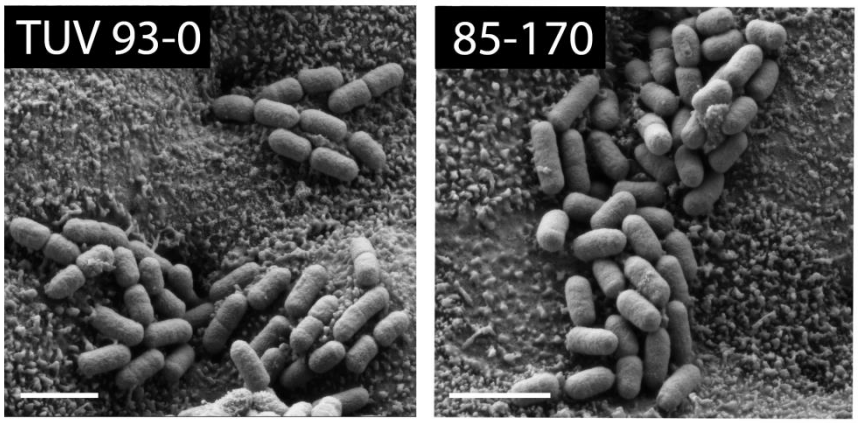

Figure 3.8. Stx-negative EHEC colonise human colonic biopsies. Colonic biopsies were infected with EHEC strains TUV 93-0 and 85-170 for 8 hours and colonisation was examined by scanning electron microscopy. Images represent those from two independent experiments performed in duplicate. Scale bars = 2 μm.

typical A/E lesions, characterised by Tir translocation, intimate attachment and effacement of microvilli.

3.2.5 | EHEC colonisation of the human colonic epithelium is not affected by Stx production

Previous IVOC studies using Stx-negative EHEC strains have reported that EHEC is unable to colonise colonic epithelium (Chong *et al.*, 2007; Phillips *et al.*, 2000). As a previous study by Robinson *et al*. (2006) has shown that Stx enhances adherence to HeLa human cervical carcinoma cells and intestinal colonisation in mice, we decided to investigate whether Stx plays a role in EHEC colonisation of human colonic epithelium. To determine whether Stx production is required for EHEC colonisation of colonic biopsies, IVOC was performed using Stx-negative strains TUV 93-0 and 85-170 used in previous studies. As shown in Figure 3.8, both Stx-negative EHEC strains colonised colonic biopsies, displaying a similar adherence phenotype to that observed following wild-type infection. This indicates that Stx is not the determining factor for EHEC colonisation of colonic biopsies during IVOC.

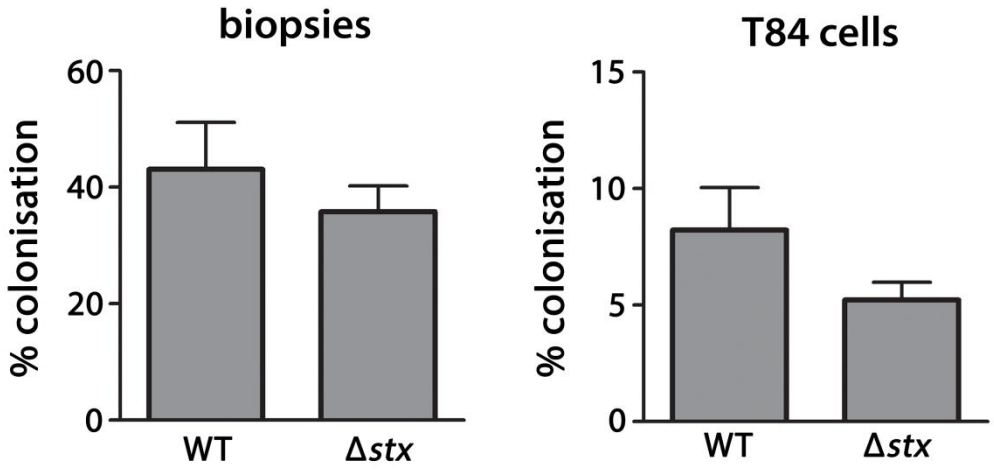

Figure 3.9. Stx production does not affect EHEC colonisation of human colonic epithelial cells. (Left) Human colonic biopsies were infected with wild-type (WT) EHEC strain EDL933 or an isogenic Stx deletion mutant (Δ*stx*) for 8 hours. Samples were viewed by scanning electron microscopy and colonisation was quantified by recording the presence or absence of adherent bacteria in approximately 250 fields of view. Colonisation is expressed as the percentage of fields of view containing adherent bacteria. Data are shown as means ± standard error (SE) from two independent experiments performed in triplicate. (**Right**) Polarised T84 cells were infected with wild-type EDL933 or EDL933 Δ*stx* for 6 hours. Colonisation was quantified by plating serial dilutions of cell lysates and determining the numbers of colony-forming units. Colonisation is expressed as the percentage of adherent bacteria relative to the inoculum. Data are shown as means ± SE from five independent experiments performed in duplicate.

In addition, colonic biopsies and T84 cells were infected with a wild-type (WT) EHEC strain EDL933 or an isogenic Stx deletion mutant (Δ*stx*) for 8 and 6 hours, respectively. SEM-based analysis of biopsies revealed no significant difference in the proportion of epithelium colonised between the two strains (Figure 3.9). Similarly, both strains colonised polarised T84 cells to a similar degree. These data indicate that Stx does not have a significant effect on EHEC colonisation of human colonic epithelium *in vitro* or *ex vivo*.

3.2.6 | Intimin and the T3SS are essential for EHEC colonisation of human colonic biopsies but not T84 cells

Intimin and the T3SS play essential roles in EHEC adherence to HeLa cervical carcinoma cells and colonic epithelium of some animals (Dean-Nystrom *et al.*, 1998b; Donnenberg *et al.*, 1993; Ebel *et al.*, 1998; McKee *et al.*, 1995; Ritchie *et al.*, 2003). To investigate whether intimin and T3S are required for human colonic colonisation by EHEC, biopsies and polarised T84 cells were infected with EHEC strain EDL933 or isogenic mutant strains with deletions in intimin (*eae*), EspA (*espA*) or EscN (*escN*, cytoplasmic ATPase of T3SS). SEM analysis revealed no adherent bacteria on biopsies infected with any of the mutant strains, whilst the wild-type (WT) strain showed good colonisation (Figure 3.10A). Quantification analysis confirmed these observations, with WT colonising 26.0% ± 7.2% of biopsy epithelium, compared to 0% for each of the mutant strains (Figure 3.10B). In contrast, all deletion mutants colonised polarised T84 cells (Figure 3.11) to a level comparable with that of the WT strain. Put together, these data suggest that expression of intimin and T3S are essential for EHEC colonisation of colonic biopsies but not T84 cells.

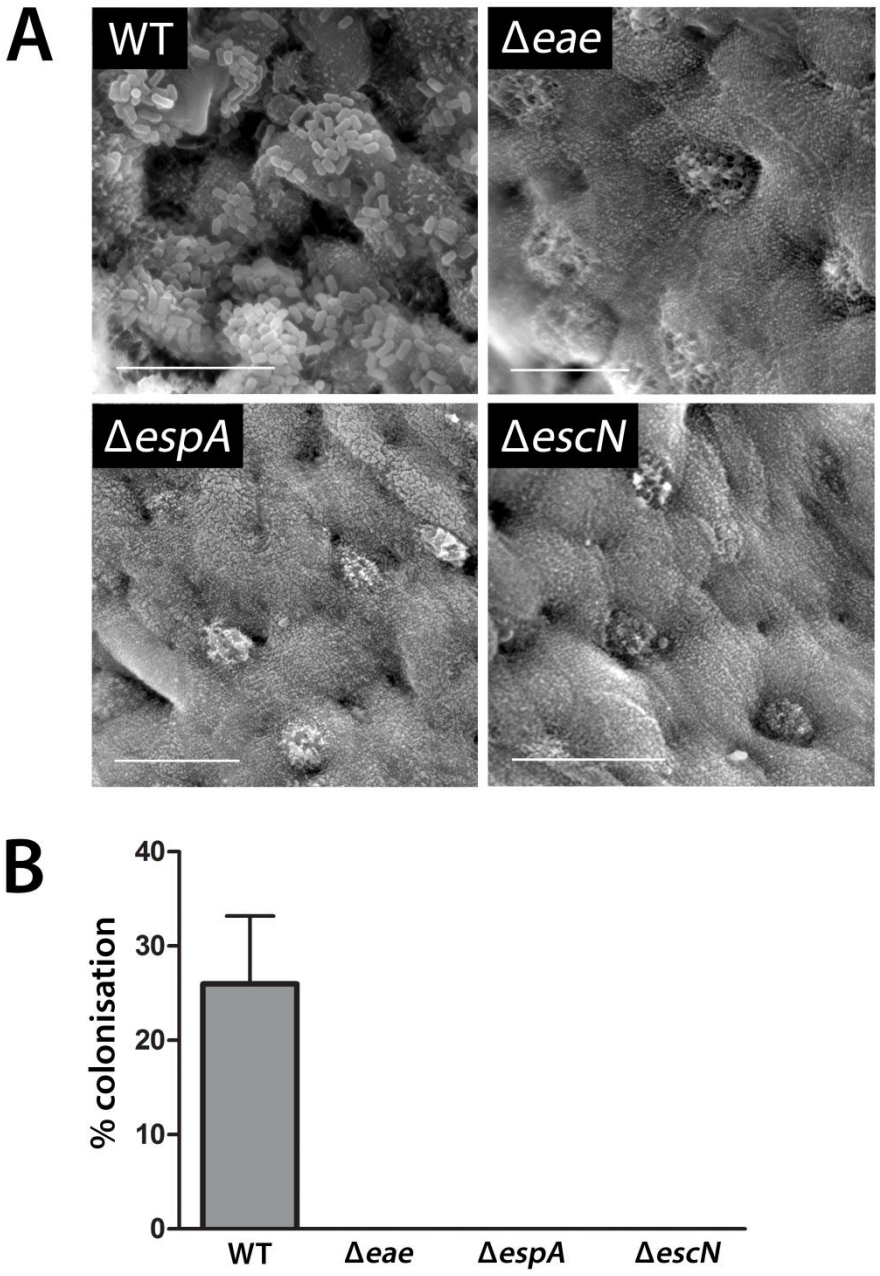

Figure 3.10. EHEC colonisation of human colonic biopsies is dependent on intimin and the T3SS. Colonic biopsies were infected with wild-type (WT) EHEC strain EDL933 or isogenic deletion mutants lacking EspA, EscN or intimin (*eae*) for 8 hours. (**A)** Biopsies were viewed by scanning electron microscopy and (**B**) colonisation was quantified by recording the presence or absence of adherent bacteria in approximately 250 fields of view. Colonisation is expressed as the percentage of fields of view containing adherent bacteria. Data are shown as means ± SE from four independent experiments performed in duplicate. Scale bars = 10 µm.

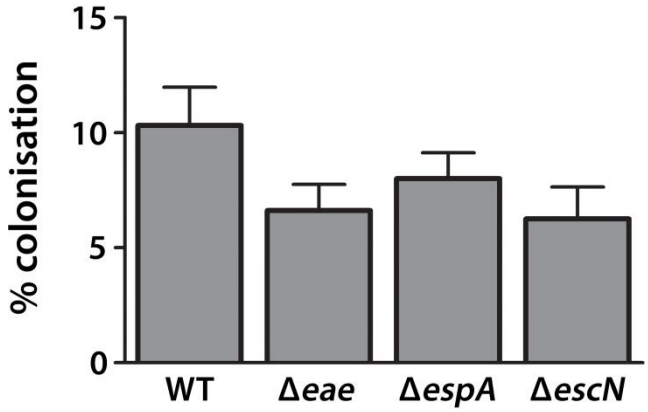

Figure 3.11. EHEC colonisation of polarised T84 cells is not dependent on intimin or T3S. Polarised T84 cells were infected with wild-type EHEC strain EDL933 or isogenic deletion mutants lacking functional EspA, EscN or intimin (*eae*) for 6 hours. Colonisation was quantified by plating serial dilutions of cell lysates and determining the numbers of colony-forming units. Colonisation is expressed as the percentage of adherent bacteria relative to the inoculum. Data are shown as means ± SE from four independent experiments performed in duplicate.

3.2.7 | EHEC colonise human paediatric colonic biopsies

As our data showing EHEC colonisation of colonic biopsies contrasted with those reported previously by Phillips and colleagues (Phillips *et al*., 2000; Chong *et al*., 2007), we compared the experimental methodology employed in each study. It was noted that previous studies had performed IVOC experiments using biopsies from paediatric patients, whilst our study used biopsies from adults. To determine whether EHEC colonic colonisation *ex vivo* was restricted to adult biopsies, a limited set of infections was performed using biopsies obtained from children aged 9 and 13 years. SEM analysis revealed that both EHEC strains EDL933 and Walla-1 colonised paediatric colonic biopsies and exhibited a similar adherence phenotype to bacteria bound to adult biopsies (Figure 3.12). This indicates that age is not a determining factor for EHEC colonisation of human colonic epithelium during IVOC.

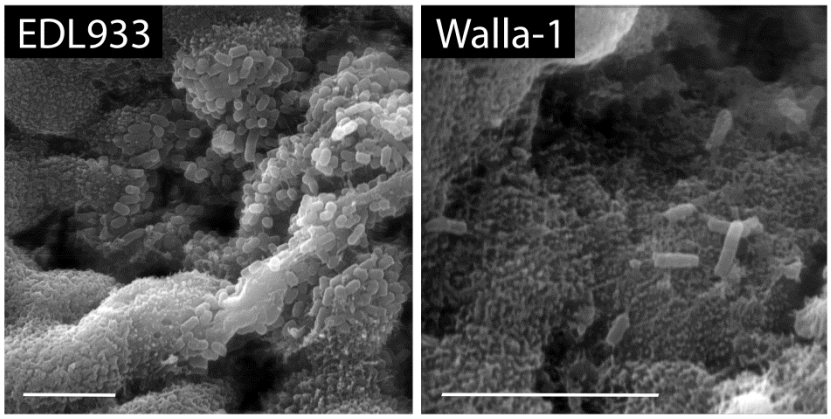

Figure 3.12. EHEC colonise human paediatric colonic biopsies. Paediatric colonic biopsies were infected with EHEC strains EDL933 and Walla-1 for 8 hours, and colonisation was examined by scanning electron microscopy. Images represent those from two independent experiments performed in duplicate. Scale bars = 2 µm

3.2.8 | High oxygen levels suppress EHEC colonisation of colonic biopsies

Another difference between previously published IVOC studies and ours is the oxygen concentration used: whilst Phillips and colleagues performed IVOC at high oxygen concentrations (5% CO_2 + 95% O_2) (Chong *et al.*, 2007; Phillips *et al.*, 2000), the IVOC experiments in our study were performed in atmospheric oxygen concentrations (5% CO_2 + air). As it has recently been reported that EHEC colonisation of T84 cells is enhanced by microaerobiosis in a T3S-dependent manner (Schüller and Phillips, 2010), we decided to test the effect of oxygen on EHEC colonisation of human colonic biopsies during IVOC. Infections with EHEC strain EDL933 were performed under atmospheric (5% CO_2 + air) or oxygen-rich (5% CO_2 + 95% O_2) conditions and colonisation was quantified by SEM. As shown in Figure 3.13, infections performed in 95% oxygen resulted in a significantly reduced level of EHEC colonic colonisation compared with those performed in air (3.1 ± 0.7% vs. 43.1 ± 8.0%), indicating that high oxygen levels suppress EHEC colonic colonisation during IVOC.

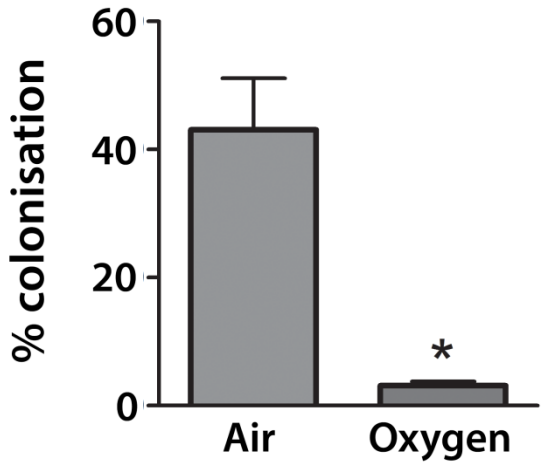

Figure 3.13. EHEC colonisation of human colonic biopsies is inhibited in oxygen-rich conditions. Colonic biopsies were infected with EHEC strain EDL933 and IVOC was performed for 8 hours under high (oxygen) or atmospheric (air) oxygen levels. Samples were viewed by scanning electron microscopy and colonisation was quantified by recording the presence or absence of adherent bacteria in approximately 250 fields of view. Colonisation is expressed as the percentage of fields of view containing adherent bacteria. Data are shown as means ± SE from two independent experiments performed in triplicate. * = $P < 0.05$

3.3 | Discussion

EHEC is considered a colonic pathogen (Croxen and Finlay, 2010). Disease is associated with haemorrhagic colitis (HC), with inflammation and ulcer-like lesions found predominantly in the ascending and transverse colon, respectively (Griffin *et al.*, 1990; Shigeno *et al.*, 2002). Studies using cultured cell lines and animal models have linked pathogenesis with EHEC adherence and A/E lesion formation on IECs (reviewed in Croxen *et al.*, 2013), and it is postulated that EHEC A/E lesions on colonic epithelium contribute to disease during infection of humans. However, clinical evidence of EHEC binding to human intestinal epithelium is lacking (Kelly *et al.*, 1987; Malyukova *et al.*, 2009), and an *ex vivo* model system using human intestinal tissue and Stx-negative EHEC has failed to demonstrate EHEC binding to the colon (Chong *et al.*, 2007; Phillips *et al.*, 2000). In this study, we conducted an in-depth assessment of EHEC adherence to colonic epithelium using physiologically relevant models of human intestinal infection, with the aim of improving our understanding of EHEC-IEC interactions in the colon.

3.3.1 | A/E lesion formation *in vitro*

The T84 human colonic carcinoma cell line has been widely used in the study of EHEC as it forms a polarised monolayer of colonic crypt-like cells in culture (Madara *et al.*, 1987) that do not express the Stx receptor Gb3 on their surface, in line with the situation *in vivo* (Schüller et al., 2004). We used this cell line to examine EHEC adherence to human colonic epithelial cells *in vitro*.

In this study, we found that EHEC did not induce typical A/E lesions on polarised T84 cells. Although bacteria colonised these cells and induced microvillous effacement, adherence sites were not associated with actin pedestal formation or Tir translocation into the plasma membrane. This is consistent with previous studies, which have reported EHEC adherence to polarised T84 cells with no discernible recruitment of actin (Ismaili *et al.*, 1995; Li *et al.*, 1999). Since actin recruitment is dependent on the action of Tir within the host cell (reviewed in Frankel and Phillips, 2008), it seems likely that the observed lack of pedestals occurs as a direct result of the failure of EHEC to translocate Tir and/or failure of Tir to insert into the host cell

membrane. Interestingly, previous studies have found that EHEC bacteria adhered to T84 cells are able to subvert host cell signal processes such as epithelial barrier function and ion transport (Ismaili *et al.*, 1995; Li *et al.*, 1999; Philpott *et al.*, 1998). Modulation of barrier function and ion transport by EHEC and EPEC has been attributed to the action of bacterial effector proteins such as EspF, $EspF_U$, Map, Tir and NleA (Dean *et al.*, 2006; Hodges *et al.*, 2008; McNamara *et al.*, 2001; Viswanathan *et al.*, 2004), suggesting that bacteria adhered to polarised T84 cells are able to secrete effectors into the host cytosol. In agreement with this, we found EspA filaments protruding from adherent bacteria, indicating expression of the T3SS. Put together, these findings suggest that EHEC are able to adhere to polarised T84 cells, translocate effector proteins and modulate host cell signal transduction pathways independently of actin pedestal formation and Tir insertion into the plasma membrane.

Despite their failure to translocate Tir or recruit actin, adherent EHEC bacteria did induce microvillous effacement. This is in agreement with transmission electron micrographs presented by Li *et al*. (1999), which show a lack of microvilli on T84 cells loosely associated with EHEC bacteria at the plasma membrane. Furthermore, studies on EPEC adherence to human biopsies *ex vivo* have reported the presence of partial A/E lesions displaying two or fewer of its hallmarks: *ex vivo* infection of human intestinal biopsies with an intimin-deficient EPEC mutant caused microvillous effacement with no signs of intimate adherence (Shaw *et al.*, 2005), and EPEC strains of the pathogenic serotype O125:H6 have been shown to induce microvillous effacement and Tir translocation on biopsies without recruiting actin (Bai *et al.*, 2008). Our findings, together with published data, suggest that microvillous effacement occurs as the result of a distinct subversive pathway from that conferring intimate attachment and actin pedestal formation, highlighting the multiplicity of subversive elements involved in A/E lesion formation.

The bacterial factors responsible for microvillous effacement during EHEC infection are unknown, and studies with EPEC Tir have yielded conflicting results depending on the model system used. Whilst EPEC-associated microvillous effacement appears to be dependent on the Tir-intimin interaction in Caco-2 cells (Dean *et al.*, 2006; Dean *et al.*, 2013), Tir was not required for the same phenotype following human or

porcine IVOC (Girard *et al.*, 2005; Shaw *et al.*, 2005). Our data on EHEC suggest that Tir insertion into the plasma membrane is not required for microvillous effacement, although we cannot rule out the possibility that Tir is present at low levels in the cytosol and able to induce microvillous effacement from this location. It would be necessary to confirm this by comparing microvillous effacement in T84 cells following infection with wild-type and Tir-deficient mutant EHEC strains. EspF, Map and EspH have also been implicated in EPEC-mediated microvillous effacement (Dean *et al.*, 2006; Shaw *et al.*, 2005), and so it would be interesting to examine the role of these proteins during EHEC infection.

In contrast to what was seen on polarised T84 cells, EHEC formed actin pedestals and translocated Tir on non-polarised cells located in marginal areas or extruding from monolayers. We also observed a greater number of adherent bacteria on these cells compared with polarised cells, suggesting that EHEC preferentially binds to non-polarised T84 cells. Consistent with our findings, Li *et al*. (1999) also observed actin recruitment by EHEC only on T84 cells in marginal areas of the monolayer that did not appear polarised. The reason for this polarisation-dependency may lie in differences in the availability of binding receptors on the plasma membrane. For example, EHEC has been shown to preferentially bind to the phospholipid phosphatidylethanolamine (PE) (Barnett Foster *et al.*, 1999), which becomes available in epithelial (including T84) cell membranes during apoptosis and cell shedding (Barnett Foster *et al.*, 2000; Cotton, 1972). It is therefore possible that PE acts as a receptor for EHEC adherence to non-polarised, but not polarised, T84 cells; thereby promoting formation of A/E lesions. This could be tested by infecting T84 cells with EHEC following treatment with a PE inhibitor and assessing bacterial adherence to non-polarised cells. It would also be useful to compare PE levels and distribution on the surface of polarised vs. non-polarised cells in our model system. To date, it is not known which EHEC protein is responsible for the observed interaction with PE, but it could be one of several putative adhesins that have been identified (see section 3.9.3).

Unlike EHEC, EPEC readily colonised polarised T84 cells and induced actin pedestals. This was also observed in Li *et al*.'s study (1999) and suggests that these two pathotypes use different receptors for initial binding. EPEC is known to produce

two adhesins not found in EHEC: bundle-forming pili (Bfp), which bind to PE; and type I fimbriae, which bind to D-mannose-containing glycoproteins (Girón *et al.*, 1993; Giron *et al.*, 1991; Roe *et al.*, 2001). As PE also acts as an EHEC adherence receptor (Barnett Foster *et al.*, 2000; Barnett Foster *et al.*, 1999), it is unlikely that Bfp is responsible for the difference in adherence phenotype observed between EPEC and EHEC. It has been postulated that EHEC may interact with D-mannose-containing glycolipids *via* its long polar fimbriae (Lpf) (Farfan *et al.*, 2011), which show some sequence homology to EPEC type I fimbriae (Torres *et al.*, 2005b). However, no effect of D-mannose on Lpf-mediated EHEC adherence to HeLa cells was demonstrated in a recent study (Torres *et al.*, 2008). Therefore, it is possible that EPEC may adhere to polarised T84 cells through binding of its type I fimbriae to D-mannose-containing glycolipids. Alternatively, adherence may be mediated by some as-yet unidentified adhesin-receptor interaction.

3.3.2 | A/E lesion formation *ex vivo*

In vitro organ culture (IVOC) of human intestinal biopsies represents the current gold standard method for studying EHEC-IEC interactions. A major advantage of IVOC over cell lines is that the former uses healthy native epithelium comprising all of the major cell types (e.g. enterocytes, goblet cells, autoendocrine cells), together with an in-tact mucus layer and microbiota (reviewed in Fang *et al.*, 2013). The IVOC model has been used by Phillips and colleagues to examine EHEC colonisation of paediatric intestinal epithelium using Stx-negative EHEC strains (Chong *et al.*, 2007; Phillips *et al.*, 2000). They reported EHEC adherence to IECs on terminal ileal but not colonic biopsies. Similarly, examination of intestinal tissue from EHEC-infected patients has not revealed evidence of bacterial adherence, despite finding severe colonic histopathology (Kelly *et al.*, 1987). In contrast, *in vivo* and *ex vivo* infection of gnotobiotic piglets, infant rabbits and neonatal calves resulted in colonisation of the colonic epithelium as well as other intestinal sites (Dean-Nystrom *et al.*, 1997; Francis *et al.*, 1986; Pai *et al.*, 1986; Tzipori *et al.*, 1986), creating a disparity between studies using human and animal tissue. These studies have called into question the role of A/E lesion formation in inducing colonic pathology during EHEC infection of humans.

As previous IVOC studies were conducted using Stx-negative EHEC strains (Chong *et al.*, 2007; Phillips *et al.*, 2000), and because Stx has been implicated in EHEC adherence (Robinson *et al.*, 2006), we decided to re-investigate EHEC adherence to human colonic epithelium using wild-type EHEC. All strains colonised both terminal ileal and colonic epithelia, indicating that EHEC is able to adhere to human colonic IECs and likely does so *in vivo*. The reason why clinical samples from infected patients have failed to show EHEC colonic adherence is unclear, but it may be due to the fact that infections have often reached an advanced stage by the time colonoscopy takes place, by which time colonisation may have diminished or become obscured by the damaged mucosa. Collection of biopsy samples from infected patients at earlier time-points would clarify this point; however, such studies are ethically unsound due to the risk of perforation associated with performing endoscopy on HC sufferers (Nataro and Kaper, 1998).

It was noted that the distribution of EHEC colonies on the epithelium was not equal. Bacteria appeared to be predominantly localised to the surface epithelium between crypt openings on colonic samples and at villus tips on ileal samples. Bacteria were rarely observed deep within colonic crypts or on the lower half of ileal villi. These more luminal regions may confer the advantage of being positioned furthest away from antimicrobial peptides, which are present at high concentrations in crypts (reviewed in Cunliffe and Mahida, 2004).

EHEC adhered to colonic biopsies were associated with typical A/E lesions, characterised by intimate attachment, microvillous effacement and Tir translocation. This contrasts with our *in vitro* data on polarised T84 cells (Section 3.9.1), indicating that distinct interactions between EHEC and colonic IECs are taking place in each model. A similar disparity between *in vitro* and *ex vivo* model systems was reported during an EPEC study, in which Tir phosphorylation mutants were able to recruit actin in human intestinal biopsies but not in HeLa cells (Schüller *et al.*, 2007). This data highlights the risk associated with relying on cell culture models only.

While terminal ileal IECs displayed elongated microvilli at EHEC adherence sites, as shown previously (Chong *et al.*, 2007; Phillips *et al.*, 2000), colonic IECs did not harbour this phenotype. This has also been observed following colonic bovine IVOC with EHEC (Girard *et al.*, 2007) and porcine IVOC with EPEC (Mundy *et al.*, 2007),

and most likely reflects differences in brush border composition between intestinal sites. Put together, our data indicate that EHEC adhered to human colonic epithelium subvert host processes and form A/E lesions *ex vivo*, which likely leads to the onset of colonic pathology during infection of humans.

3.3.3 | Role of bacterial proteins in colonisation

A previous study by Robinson and colleagues identified a role for Stx in EHEC colonisation (Robinson *et al.*, 2006). EHEC adherence to HeLa cells and colonisation of mice was significantly lower following infection with Stx-negative EHEC compared with wild-type, which was attributed to Stx-mediated enhancement in expression of surface nucleolin, a host-derived intimin-receptor. As previous human IVOC studies using Stx-negative EHEC failed to demonstrate EHEC adherence to colonic biopsies (Chong *et al.*, 2007; Phillips *et al.*, 2000), we tested the hypothesis that Stx is required for EHEC adherence to human colonic epithelium. Infection of both colonic biopsies and polarised T84 cells with an Stx deletion mutant resulted in comparable colonisation to that of the wild-type strain, suggesting that Stx does not influence EHEC colonisation of colonic IECs *in vitro* or *ex vivo*. The reason why Stx appears to influence EHEC colonisation of mice and HeLa cells but not T84 cells or human biopsy epithelium is unclear, but it should be noted that an enhancing effect of Stx on intestinal epithelial nucleolin surface levels has not yet been reported. It would therefore be interesting to investigate the effect of Stx on nucleolin redistribution in HeLa vs. T84 cells and/or intestinal biopsies in order to clarify the role of this phenomenon in EHEC adherence.

Intimin was the first bacterial protein shown to play a role in A/E bacterial adherence, with the discovery that deletion of the gene encoding intimin (*eae*) inhibited EPEC attachment and pedestal formation on HeLa cells (Jerse *et al.*, 1990). Intimin mediates adherence *via* binding to Tir, which is translocated into the host cell through the T3SS (reviewed in Frankel and Phillips, 2008). EHEC adherence to HeLa cells has been shown to be dependent on expression of intimin, Tir and the T3SS (DeVinney *et al.*, 1999; Ebel *et al.*, 1998; McKee *et al.*, 1995), and an essential role for intimin during *in vivo* infection of infant rabbits, gnotobiotic piglets and neonatal calves has also been demonstrated (Dean-Nystrom *et al.*, 1998b;

Donnenberg *et al.*, 1993; Ritchie *et al.*, 2003). Furthermore, human IVOC studies have shown that intimin and Tir are essential for EHEC binding to small intestinal epithelium (Fitzhenry *et al.*, 2002; Schüller *et al.*, 2007). We therefore determined the role of intimin and T3S in EHEC colonisation of human colonic biopsies and polarised T84 cells. Colonic biopsies infected with mutant EHEC strains carrying deletions in *eae*, *espA* or *escN* showed no evidence of bacterial adherence or A/E lesions, confirming that intimin and T3S are essential for EHEC colonisation of human colonic epithelium.

In addition to Tir, it has been shown that two host-derived receptors also interact with intimin *in vitro*. EPEC intimin binds β1-integrin (Frankel *et al.*, 1996), which is usually restricted to the basolateral membrane of IECs but is redistributed apically in T84 cells during infection-mediated disruption of epithelial barrier function (Muza-Moons *et al.*, 2003). The second host molecule, nucleolin, has been shown to bind EHEC intimin, and competitive inhibition of nucleolin activity reduces EHEC adherence to HeLa cells (Sinclair and O'Brien, 2002). Although we did not look specifically at the role of host-derived intimin receptors in our IVOC model, the fact that the T3SS is required for adherence and that Tir is present beneath adherent bacteria suggests that intimin-dependent EHEC adherence occurs through binding to a translocated bacterial receptor (i.e. Tir) rather than a host receptor.

In contrast to what was found *ex vivo*, colonisation of polarised T84 cells was unaffected in EHEC strains lacking functional intimin and the T3SS apparatus, compared with that of the wild type. This suggests that EHEC adhere to polarised T84 cells *via* a different mechanism to that used during colonisation of colonic biopsies; one which does not involve intimin or T3S and does not lead to the formation of typical A/E lesions (see section 3.9.1). It is possible that T84 cells express a receptor on their surface that is not expressed on colonic IECs *in vivo* and confers adherence *via* an adhesin other than intimin. A number of putative adhesins have been described in EHEC O157:H7, which are listed in Table 1.1. Although the identity of host-derived receptors for most of these adhesins is currently unknown, binding to extracellular matrix proteins has been demonstrated for Lpf1, ELF, HCP, F9, EhaB and EhaJ (Easton *et al.*, 2011; Farfan *et al.*, 2011; Low *et al.*, 2006; Samadder *et al.*, 2009; Wells *et al.*, 2009; Xicohtencatl-Cortes *et al.*, 2009).

However, the role of these putative adhesins in EHEC adherence to polarised T84 cells has not been investigated.

3.3.4 | Role of host age and oxygen on colonisation

Since the use of Stx-positive vs. Stx-negative EHEC strains did not explain the discrepancy in colonic colonisation seen between previous human IVOC studies (Chong *et al.*, 2007; Phillips *et al.*, 2000) and our own, we sought to ascertain which other factors might be responsible. Whereas Phillips and colleagues performed IVOC on biopsies from paediatric patients and in 95% oxygen, our IVOCs used adult biopsies and atmospheric oxygen concentrations. We first examined the role of host age on EHEC colonic colonisation during IVOC. Infection of paediatric colonic biopsies with strains EDL933 and Walla-1 resulted in colonisation, with an adherence phenotype indistinguishable from that observed following IVOC of adult biopsies. This is consistent with a published IVOC study using EPEC, which reported no difference in bacterial colonisation on adult vs. paediatric biopsies (Humphries *et al.*, 2009). Our findings indicate that host age was not the determining factor for EHEC colonisation in our model system.

IVOC is traditionally performed in an oxygen-rich atmosphere (95%) in order to facilitate gas exchange in the tissue and thereby maximise survival (Deschner *et al.*, 1963; Fang *et al.*, 2013). However, oxygen concentrations in the intestinal lumen are much lower, with levels estimated at 1.4% of atmospheric pressure in the mouse colon (He *et al.*, 1999). A recent bovine IVOC study found that EHEC infections performed at atmospheric oxygen concentrations (21%) yielded colonisation with no detrimental effects on tissue integrity, whilst no colonisation was observed following IVOC at 95% oxygen concentrations (Girard *et al.*, 2007). We tested the effect of oxygen in our human IVOC model by performing EHEC infections in either 95% oxygen or air and comparing the degree of colonisation. We found a significant reduction in colonisation during IVOCs performed in 95% oxygen compared with air, with the former condition almost completely suppressing colonisation. These data indicate that oxygen inhibits EHEC adherence to colonic biopsies, and implicate high oxygen levels as the inhibitory factor for EHEC colonisation in past IVOC studies (Chong *et al.*, 2007; Phillips *et al.*, 2000).

Oxygen has also been shown to affect EHEC adherence to colonic IECs *in vitro*. By performing infections in a vertical diffusion chamber system, which enables the generation of separate apical and basolateral environments, Schüller and Phillips (2010) demonstrated that microaerobiosis (1-1.7% oxygen) enhanced EHEC colonisation of T84 cells compared with aerobiosis. Adherent bacteria produced higher amounts of Tir and EspA under microaerobic conditions compared with aerobic. Similarly, Ando and colleagues (2007) have shown that EHEC grown in anaerobic conditions secrete higher levels of Tir and EspB compared with those grown in aerobic conditions. Therefore, it seems likely that microaerobic conditions in the gut favour EHEC colonisation of colonic IECs by promoting T3S and A/E lesion formation. Unfortunately, it is not possible to investigate EHEC adherence to colonic biopsies in microaerobic conditions as epithelial cell integrity is compromised by the lack of oxygenation (Schüller, personal communication). To date, the molecular mechanisms underlying oxygen-mediated inhibition of adherence have not been investigated. Expression of LEE genes (involved in A/E lesion formation) are known to be controlled by a number of regulatory systems, including the Pch proteins, QS signalling proteins, and the acid stress regulator GadE (Connolly *et al.*, 2015; Figure 1.9). These systems are responsive to external conditions such as pH and the presence of host hormones and bacterial metabolites. Therefore, it would be interesting to determine whether any of these regulatory systems are involved in oxygen-mediated inhibition of EHEC adherence.

Interestingly, previous IVOC studies using high oxygen levels reported EHEC colonisation of distal small intestinal biopsies but not the colon (Chong *et al.*, 2007; Phillips *et al.*, 2000), suggesting that the inhibitory effects of oxygen are not felt or are less potent in the former compared with the latter. Colonisation was largely restricted to the follicular associated epithelium (FAE) of Peyer's patches (Fitzhenry *et al.*, 2002; Phillips *et al.*, 2000), although additional colonisation of villi adjacent to Peyer's patches was seen in one study (Chong *et al.*, 2007). In contrast, our IVOC experiments using atmospheric oxygen levels resulted in extensive colonisation of villi regardless of their proximity to FAE, suggesting that oxygen does suppress adherence to small intestinal biopsies, albeit not as efficiently as to colonic biopsies. One possible explanation is that EHEC colonises FAE *via* a mechanism distinct from that used in other regions, one that is unaffected by oxygen. Alternatively, the small

intestinal epithelium may be easier to colonise than the colon, perhaps due to the thinner mucus layer and less microbiota, which may permit bacterial adherence even in the presence of inhibitory oxygen levels.

3.3.5 | Summary

In this study, we have demonstrated for the first time that EHEC colonises human colonic biopsies *ex vivo* and forms A/E lesions, which likely contribute to colonic pathogenesis during *in vivo* infection. Colonisation was dependent on intimin and T3S, and was suppressed by high oxygen levels traditionally used in IVOC. In contrast, EHEC adherence to polarised T84 cells did not lead to Tir translocation into the host cell membrane and was mediated by factors other than T3S and intimin. Our findings highlight the disparities that can arise between experiments on cultured cell lines and human tissue, and emphasise the importance of using physiologically relevant model systems such as IVOC to study EHEC-IEC interactions.

CHAPTER FOUR

INNATE IMMUNE RESPONSE TO EHEC INFECTION

Collaborative work:

Biopsies provided by Alison Prior, Vivienne Cook, Simon Chan and William Gelson

qPCR support provided by Nigel Belshaw and Giles Elliott

SEM support provided by Bertrand Lézé

A manuscript containing work from this chapter is currently under revision with Infection and Immunity

4.1 | Introduction

EHEC infection is associated with a moderate inflammatory response in the intestinal mucosa, characterised by recruitment of neutrophils to the site of infection (Griffin *et al.*, 1990; Kelly *et al.*, 1990; Kelly *et al.*, 1987). In addition, infected patients suffering from haemolytic uraemic syndrome (HUS) have been shown to harbour elevated circulatory levels of neutrophil chemoattractant interleukin-8 (IL-8) compared with healthy controls (Fitzpatrick *et al.*, 1992; Murata *et al.*, 1998), suggesting that this cytokine plays an important role in the innate immune response to infection. Accordingly, *in vitro* studies have demonstrated that intestinal carcinoma cells express IL-8 in response to EHEC infection (Berin *et al.*, 2002; Dahan *et al.*, 2002). This response has been shown to depend primarily on expression of flagellin (Berin *et al.*, 2002; Zhou *et al.*, 2003), although other bacterial factors such as Shiga toxins (Stxs), long polar fimbriae (Lpf) and haemorrhagic coli pili (HCP) have also been implicated (Farfan *et al.*, 2013; Ledesma *et al.*, 2010; Thorpe *et al.*, 2001; Yamasaki *et al.*, 1999). To date, the IL-8 response to EHEC infection has been studied only in carcinoma cell lines and not in human colonic epithelium.

In addition to secretion of cytokines, intestinal epithelial cells (IECs) are also known to produce antimicrobial peptides (AMPs) and proteins (e.g. lysozyme). These short cationic peptides constitute an important part of the innate immune system, performing direct bactericidal functions as well as regulating various inflammatory processes (Muniz *et al.*, 2012). AMPs known to be produced by colonic epithelium, the site of EHEC colonisation, include human β-defensins (hBDs) 1-4, the cathelicidin LL-37, and lysozyme (Fahlgren *et al.*, 2003, 2004; Hase *et al.*, 2002). Expression of several AMPs appears to be modulated during infection with enteric bacteria and/or inflammation. hBD2-4 and lysozyme expression is enhanced in inflammatory bowel disease (IBD) sufferers compared with healthy controls, and hBD2 is also stimulated upon infection with invasive and non-invasive bacteria including *Salmonella*, *Campylobacter*, *Shigella*, and enteropathogenic and enteroinvasive *E. coli* (EPEC; EIEC) (Hase *et al.*, 2002; Khan *et al.*, 2008; O'Neil *et al.*, 1999; Ogushi *et al.*, 2001; Zilbauer *et al.*, 2005). Conversely, expression of hBD1 and LL-37 has been shown to decrease following infection with *Shigella* spp., enterotoxigenic *E. coli* (ETEC) and *Vibrio cholerae*, which is thought to represent a

bacterial attempt to dampen the innate immune response and thereby increase the chance of survival (Chakraborty *et al.*, 2008; Islam *et al.*, 2001; Sperandio *et al.*, 2008). As yet, the influence of EHEC infection on AMP expression in human colonic epithelium has not been examined.

In this study, we sought to characterise the innate immune response to EHEC infection in human colonic epithelium using physiologically relevant IEC models. In our previous study, we demonstrated that EHEC colonise human colonic epithelium during IVOC (Chapter 3; Lewis *et al.*, 2015). Schuller *et al*. (2009) have previously developed a polarised version of the IVOC model (pIVOC) to study the IL-8 response to enteropathogenic *E. coli* (EPEC) in human duodenal biopsy epithelium. pIVOC restricts bacterial access to the epithelium and away from immune cells in the lamina propria, thus allowing examination of the epithelial immune response in biopsies. Therefore, we adapted this model for use with EHEC and colonic biopsies. We also determined the innate immune response *in vitro* using polarised T84 human colonic carcinoma cells, which are widely used in EHEC infection studies.

The objectives of this study were to:

1. Determine whether EHEC infection affects expression of AMPs and IL-8 in human colonic epithelium *in vitro* and *ex vivo*
2. Identify which bacterial proteins influence the innate immune response
3. Ascertain which inflammatory signalling pathways are involved
4. Determine the direction of IL-8 and AMP secretion

4.2 | Results

4.2.1 | Selection of EHEC strains

In order to obtain a representative overview of the innate immune response to EHEC, we included several EHEC strains in our study. To aid in the selection of suitable strains, eight wild-type EHEC strains were screened for their ability to colonise epithelial cells and produce Stx. This set of strains included the widely-used prototypes EDL933 and Walla-1, the enteroaggregative/enterohaemorrhagic hybrid strain (H1121) responsible for the 2011 German outbreak, and five other O157:H7 clinical isolates from cases in the UK (Table 2.1). All strains express Stx2(a) only except for EDL933, which also releases Stx1(a) but to a much lesser extent (Tran *et al.*, 2014). Stx2-producing strains were selected due to the fact that Stx2 is more closely associated with severe disease in humans compared with Stx1 (Boerlin *et al.*, 1999; Werber *et al.*, 2003). Infections were performed on cultured Vero African green monkey kidney cells, as these are easily colonised by EHEC and readily internalise Stx (Konowalchuk *et al.*, 1977). Following infection, Stx2 levels in culture medium were quantified by enzyme-linked immunosorbent assay (ELISA), and cells were examined for internalised Stx2 and bacterial colonisation by immunofluorescence staining.

As shown in Figure 4.1A, the amount of Stx2 released into culture medium during infection varied between EHEC strains. Prototype strain EDL933 and H07184 released the highest levels of Stx2, while significantly lower levels were released by all other strains. Infection with H09374 resulted in an intermediate level of Stx2 release, significantly lower than EDL933 and H07184 but higher than other strains. The amount of internalised Stx2 in Vero cells mirrored this pattern, with Stx2 clearly visible inside cells that had been infected with EDL933 and H07184 but not with other strains (Figure 4.1B, data shown for E1353).

Colonisation levels also varied between strains. As shown in Figure 4.2 and Table 4.1, strain H1121 colonised most extensively and displayed an aggregative adherence phenotype distinct from the loose-localised pattern exhibited by the other strains. In addition, H1121 was not associated with polymerised actin beneath the site of attachment, in line with the fact that the genome of this strain is of primarily EAEC origin and does not contain genes necessary for A/E lesion formation (e.g. *eae*)

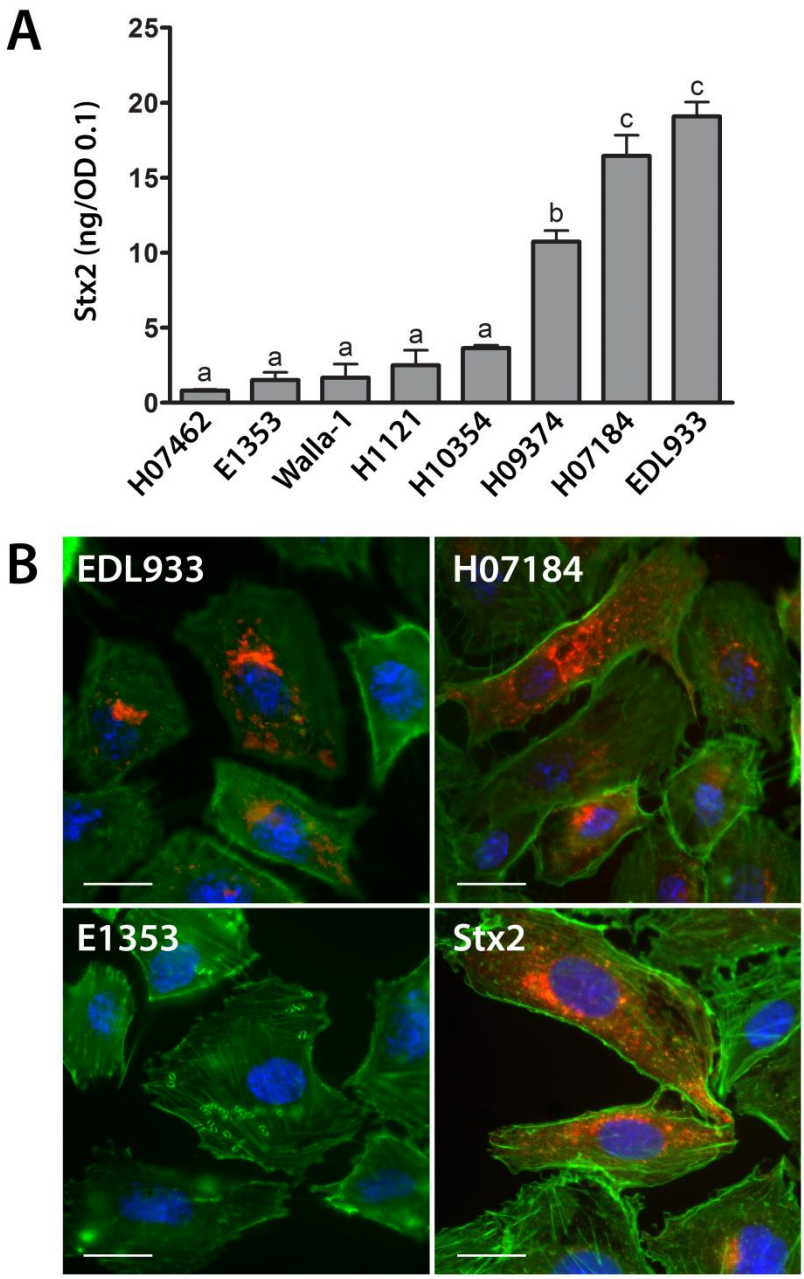

Figure 4.1. Level of Stx2 produced by EHEC differs between strains during infection of Vero cells. Cells on coverslips were infected with EHEC strains or exposed to purified Stx2 for 4 hours. (**A**) Stx protein levels in culture medium were measured by ELISA. Stx levels were normalised relative to bacterial growth, determined by measuring optical density (OD_{600}) of culture medium. Data shown as means ± SE from two independent experiments performed in duplicate. Letters above bars indicate groups of significant differences. (**B**) Cells were immunostained for Stx2 (red), actin (green) and nuclei (blue). Images represent those from two independent experiments performed in duplicate. Scale bars = 5 µm.

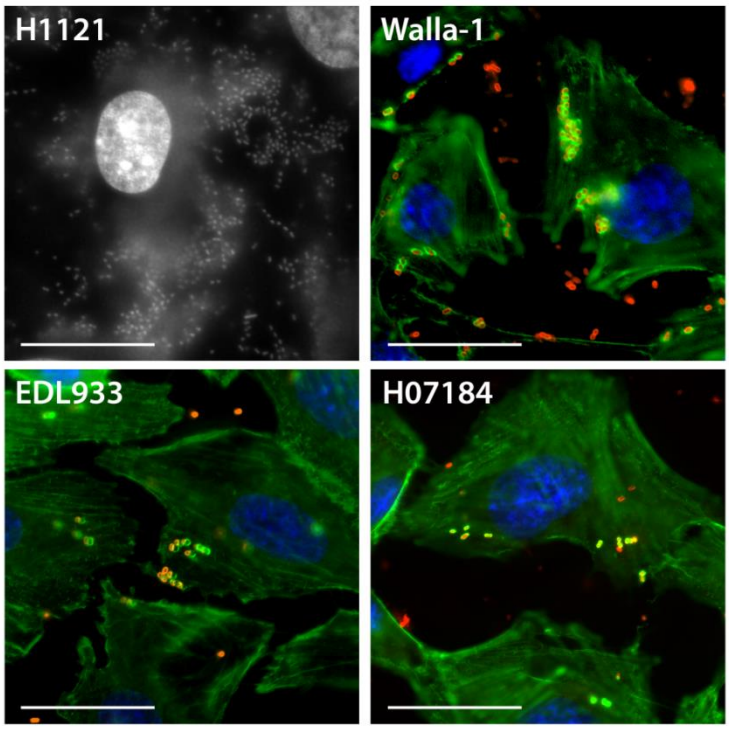

Figure 4.2. EHEC colonisation of Vero cells. Cells on coverslips were infected with EHEC strains for 4 hours. Cells infected with Walla-1, EDL933 and H07184 were immunostained for *E. coli* (red), actin (green) and nuclei (blue), whilst those infected with H1121 were stained for nuclei (blue) only. Images represent those from two independent experiments performed in duplicate. Scale bars = 10 µm.

(Bielaszewska *et al.*, 2011; Rasko *et al.*, 2011). Of the remaining EHEC strains, Walla-1 showed the highest level of colonisation, followed by EDL933. Intermediate levels of colonisation were observed following infection with E1353, H07184 and H07462, whilst very few adherent bacteria were found on cells infected with H10354 and H09374. Interestingly, the relative ability of strains to colonise epithelial cells did not appear to correlate with the ability to produce Stx2, indicating that expression of the two phenotypes is mutually exclusive.

Consequently, three strains were selected for further analysis that together encompassed the various extremes in phenotype expression: EDL933 as a high coloniser and high Stx producer; Walla-1 as a high coloniser and low Stx2 producer, and H07184 as an intermediate coloniser and high Stx2 producer.

Strain	Relative colonisation
H1121	++++++
Walla-1	+++++
EDL933	++++
E1353	+++
H07184	++
H07462	++
H10354	+
H09374	+

Table 4.1. EHEC colonisation of Vero cells differs between strains. Cells on coverslips were infected with EHEC strains for 4 hours. The relative colonisation level of each strain was determined by visually assessing the amount of adherent bacteria following immunostaining for *E. coli*, actin and nuclei (see Figure 4.2 for images). Number of + symbols indicates the extent of bacterial colonisation.

We next examined virulence trait expression during infection of human colonic epithelial cells, the primary target cells of EHEC. Polarised T84 cells grown in Transwell inserts (as described in section 3.2.2) were infected with EDL933, Walla-1 or H07184 for 9 hours. Bacterial overgrowth and loss of epithelial integrity was prevented by removal of the inoculum after 3 hours and subsequent hourly medium exchange. After infection, Stx2 release and colonisation levels were quantified. Release of Stx2 followed a similar pattern to that seen during infection of Vero cells, with EDL933 releasing the highest amount, followed by H07184, then Walla-1 (Figure 4.3A). The ability of strains to colonise T84 cells was also similar to that seen with Vero cell infections, with EDL933 and H07184 showing high and low levels (respectively) (Figure 4.3B). These data indicate that the virulence trait expression profile of each strain is distinct during infection of polarised T84 cells, and therefore these strains were used to investigate the effect of EHEC on AMP expression in human colonic epithelium.

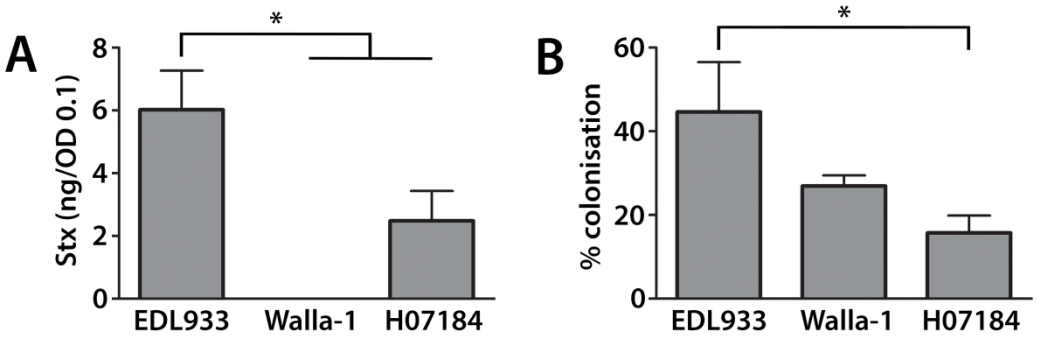

Figure 4.3. Stx2 production and colonisation by EHEC strains during infection of polarised T84 cells. Cells were apically infected with EHEC strains EDL933, Walla-1 and H07184 for 9 hours. (**A**) Stx2 protein levels in culture medium were measured by ELISA and normalised relative to bacterial growth in culture medium, determined by measuring OD600 after 1 hour. (**B**) Colonisation was quantified by plating serial dilutions of cell lysates and determining numbers of colony-forming units. Colonisation is expressed as the percentage of adherent bacteria relative to the inoculum. Data are shown as means ± SE of four (A) or five (B) independent experiments performed in duplicate. Asterisks denote significant differences. * = $P < 0.05$

4.2.2 | Apical EHEC infection of polarised T84 cells induces hBD2 and IL-8 gene expression

To determine whether EHEC influences innate immune gene expression in human colonic epithelium, polarised T84 cells were infected for 9 hours with EHEC strains EDL933, Walla-1 or H07184. To prevent loss of epithelial barrier function due to bacterial overgrowth and acidification of culture medium, medium was exchanged at 3 hours post-infection (PI) and hourly thereafter. Barrier function was monitored by measuring the TER at regular intervals. Following infection, total RNA was harvested and expression of hBD1-4, LL-37, lysozyme and IL-8 in infected monolayers was determined relative to non-infected (NI) controls by quantitative PCR (qPCR) analysis. Primers for each gene of interest (Table 2.3) were validated for specificity and amplification efficiency before use in qPCR assays (Figure 4.4). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and RNA polymerase II (POLR2A) were used to normalise qPCR data, as expression of these genes in T84

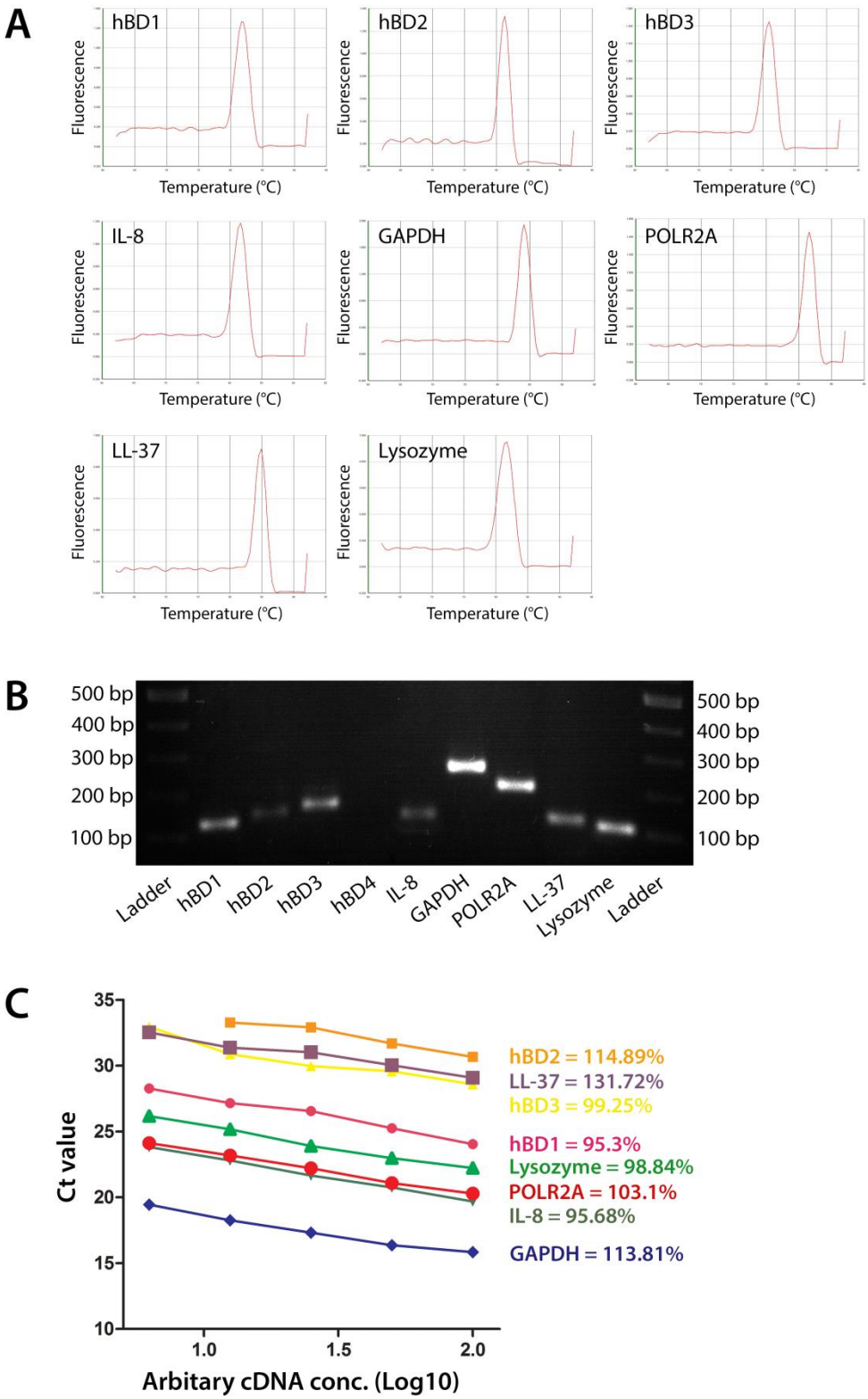

Figure 4.4. Validation of primer specificity and amplification efficiency. (**A**) Dissociation curves were generated for each primer pair. Primers were deemed specific when curves consisted of only one peak. Curves are representative of those generated with every qPCR performed. (**B**) Agarose gel electrophoresis analysis of

qPCR product size. Image is representative of data from two independent experiments. (**C**) Amplification efficiency of each primer pair. Two-fold serial dilutions of cDNA were prepared and run in a qPCR assay with each primer. Cycle threshold (Ct) values were plotted onto a scatter graph and the slope was used to calculate the primer efficiency. Efficiencies ranging from ~95 to 130% were considered acceptable. Data are representative of those from 2 independent experiments.

cells was unaffected by EHEC infection (Figure 4.5B). Figure 4.5A shows that the TER of T84 cell monolayers was not significantly affected by infection with any of the EHEC strains compared with non-infected (NI) cells. As shown in Figure 4.5C, all three EHEC strains induced a significant increase in expression of hBD2 (21.5 ± 9.1 fold for EDL933, 70.4 ± 21.8 fold for Walla-1, and 31.5 ± 2.0 fold for H07184) and IL-8 (6.0 ± 1.6 fold for EDL933, 8.3 ± 2.4 fold for Walla-1, and 8.2 ± 3.9 fold for H07184) mRNA compared with NI controls, whereas no change was observed for hBD1, hBD3, LL-37 or lysozyme. No amplification product was detected for hBD4. No obvious correlation between IL-8/hBD2 expression and colonisation or Stx2 production was observed.

In order to determine the kinetics of hBD2 and IL-8 induction, T84 cells were infected with EHEC strain EDL933 for 3-12 hours. Results in Figure 4.6 show that expression of both hBD2 and IL-8 was significantly induced from 6 hours PI, and this induction increased in magnitude up to 12 hours PI. Although induction levels were highest after 12-hour infections, practical factors favoured a shorter protocol, and so an infection time of 9 hours was selected for subsequent experiments.

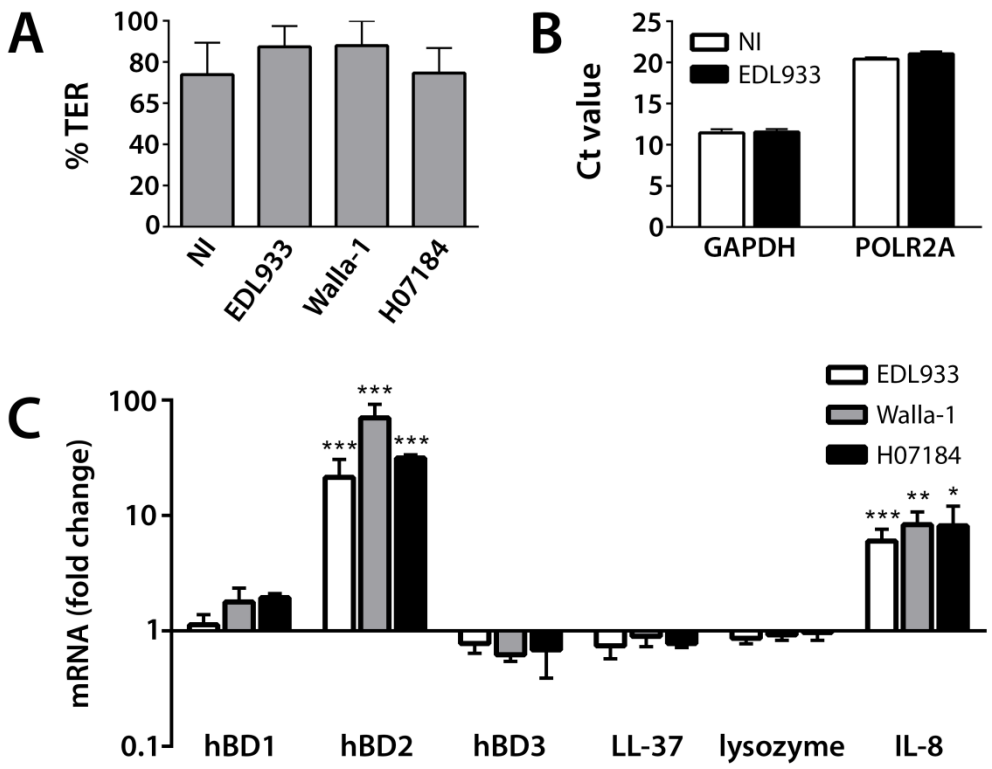

Figure 4.5. Innate immune gene expression in polarised T84 cells following EHEC infection. Cells were apically infected with EHEC or left uninfected (NI) for 9 hours. **(A)** Transepithelial electrical resistance (TER) of monolayers following infection, expressed as a percentage relative to before infection. (**B**) GAPDH and POLR2A mRNA levels in non-infected (NI) and EDL933-infected cells, as determined by qPCR. Data are shown as mean Ct values (arbitrary) ± SE of six biological replicates. (**C**) Fold changes in innate immune gene expression in T84 cells infected with EDL933, Walla-1 or H07184, relative to NI controls, calculated from qPCR data using the ΔΔCt method. Data are shown as means ± SE of four independent experiments performed in duplicate. Asterisks denote significant differences from NI. * = P <0.05; ** = P < 0.01; *** = P < 0.001.

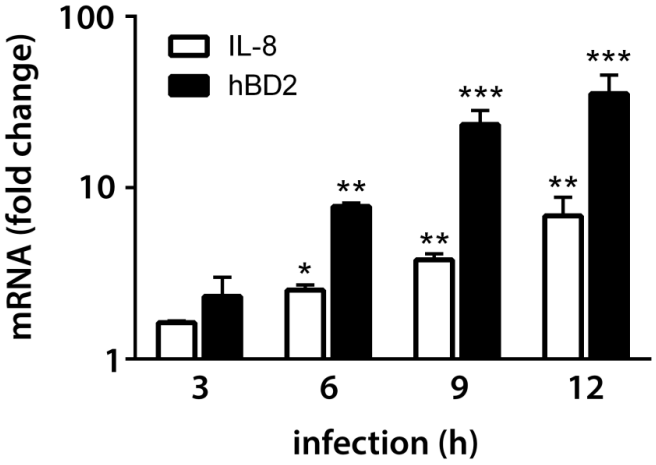

Figure 4.6. Kinetics of hBD2 and IL-8 gene expression in EHEC-infected T84 cells. Cells were apically infected with EHEC strain EDL933 for 3–12 hours, or left NI for 12 hours. Fold mRNA expression in infected cells was quantified relative to NI controls using qPCR. Data represents means ± SE of duplicate samples from a single experiment. Asterisks denote significant differences from non-infected cells. * = P <0.05; ** = P < 0.01; *** = P < 0.001.

4.2.3 | Development of human colonic polarised IVOC

To examine the IL-8 and AMP response to EHEC in human colonic epithelium *ex vivo*, we established a polarised *in vitro* organ culture (pIVOC) model for human colonic biopsy tissue. This method was based on a previously published pIVOC method used to investigate the IL-8 response to EPEC in paediatric duodenal biopsies (Schüller *et al.*, 2009), and is designed to restrict bacterial access to the mucosal surface and away from immune cells in the submucosa. Colonic biopsy samples were sandwiched between two perspex disks with part of the mucosal surface exposed through a central aperture. Samples were then mounted into Snapwell supports (Figure 2.2) and apically infected with EHEC strain EDL933 for either 5 or 7 hours. Samples showing bacterial leakage into the basolateral medium, indicated by increasing acidification and turbidity, were excluded from analysis. After infection, tissue integrity and bacterial colonisation were assessed by scanning electron microscopy (SEM). Figure 4.7 shows preserved epithelium on tissue located within the central aperture at 7 hours PI. Colonisation of epithelium was observed

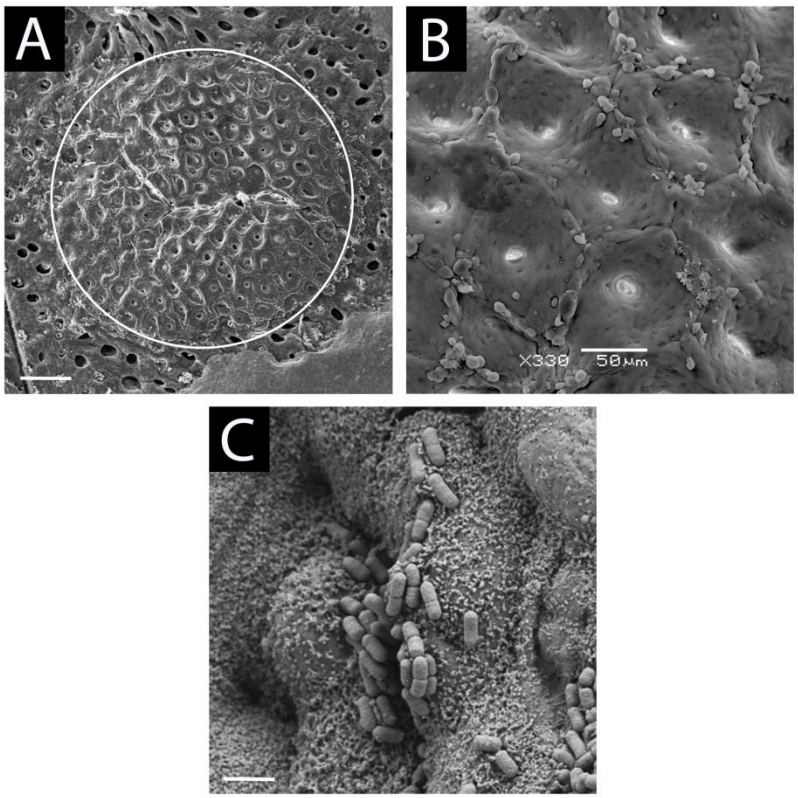

Figure 4.7. Development of colonic pIVOC. Human colonic biopsies were sandwiched between two Perspex disks and mounted into Snapwell supports, restricting bacterial access to the mucosal side. Biopsies were infected with EHEC strains EDL933, Walla-1 or H07184, or given LB alone, for 7 hours and examined by scanning electron microscopy (SEM). (**A**) Biopsy tissue exposed through the central aperture (area within white circle) showed preserved epithelium. Scale bar = 200 µm. (**B**) Intact colonic surface epithelium at higher magnification. Scale bar = 50 µm (**C**) EHEC strain EDL933 colonised epithelium. Scale bar = 2 µm. Images represent those from three independent experiments performed in duplicate.

following infections lasting both 5 and 7 hours, but was most widespread after the latter time point (Figure 4.7C). Therefore, subsequent pIVOC infections were performed for 7 hours.

4.2.4 | Apical EHEC infection of human colonic biopsies results in increased hBD2 and IL-8 gene expression

The influence of EHEC on innate immune gene expression in human colonic epithelium *ex vivo* was investigated using pIVOC. Polarised biopsies were apically infected with EHEC strains EDL933, Walla-1 and H07184 for 7 hours and gene expression was determined by qPCR. As shown in Figure 4.8, EHEC infection resulted in a significant increase in hBD2 (median fold increase = 8.1 for EDL933 and 5.7 for H07184) and IL-8 (median fold increase = 6.6 for EDL933, 5.2 for Walla-1, and 4.8 for H07184) mRNA expression compared with NI controls. Conversely, no effect of infection on hBD1, LL-37 or lysozyme expression was observed. Transcript levels of hBD3 and hBD4 were often below the detection threshold, which precluded analysis of these genes. However, transcripts of corresponding size were detected within some samples, thereby confirming functionality of the qPCR assay (data not shown). In line with results from T84 cell experiments, the level of hBD2 and IL-8 induction by all three EHEC strains was comparable.

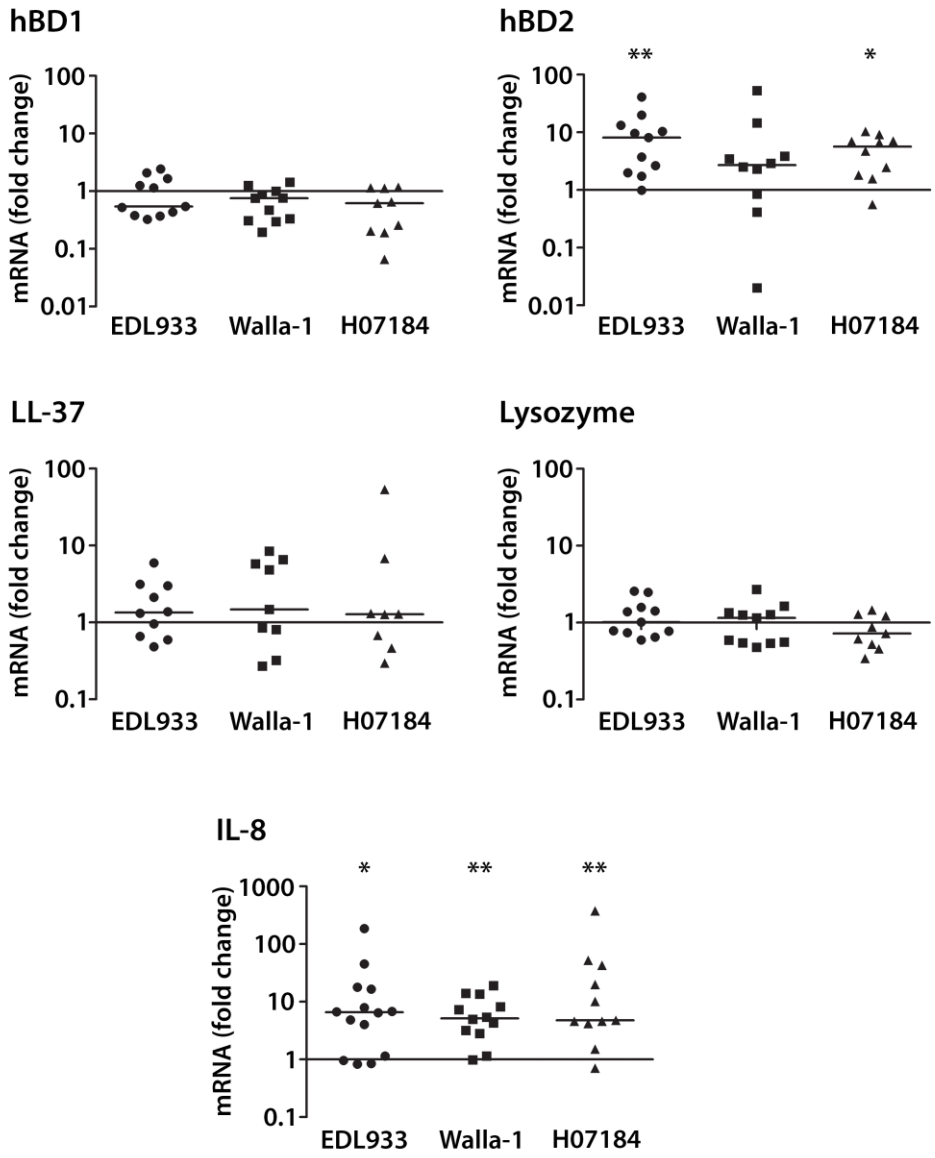

Figure 4.8. Innate immune gene expression in human colonic biopsies following apical EHEC infection. Biopsies were infected with EHEC strains EDL933, Walla-1 and H07184, or given LB alone (NI control), for 7 hours. Fold mRNA expression of select innate immune genes in infected biopsies was quantified relative to NI controls using qPCR. Each data point represents an individual biopsy from a total of six patients. Medians are indicated by a line. Asterisks denote significant differences from NI controls. * = $P < 0.05$, ** = $P < 0.01$.

4.2.5 | EHEC induction of hBD2 and IL-8 gene expression in human colonic IECs is dependent on flagellin

To ascertain which EHEC proteins were responsible for the observed induction of hBD2 and IL-8 in human colonic epithelium, polarised T84 cells were infected with wild-type (WT) EHEC strain EDL933 or isogenic deletion mutants unable to express either *fliC* (encoding flagellin, the main constituent of the flagellum), *escN* (EscN, ATPase of the T3SS) or *stx* (Stx). Infection for 9 hours with these strains did not affect epithelial barrier function, as determined by TER (data not shown). Expression of hBD2 and IL-8 mRNA was then quantified by qPCR. Whilst infection with WT, Δ*escN* and Δ*stx* resulted in significant induction of hBD2 (48.6 ± 12.3 fold for WT, 60.5 ± 15.6 fold for *ΔescN*, and 41.5 ± 8.3 fold for *Δstx*) and IL-8 (5.6 ± 1.3 fold for wt, 7.5 ± 1.2 fold for *ΔescN*, and 4.9 ± 0.3 fold for *Δstx*) expression relative to NI controls, no significant induction was observed following infection with the Δ*fliC* mutant (Figure 4.9A). It has been shown that flagellin plays a role in EHEC binding to bovine intestinal epithelium and mucus (Erdem *et al.*, 2007; Mahajan *et al.*, 2009). To ascertain whether the lack of hBD2 and IL-8 gene induction in the Δ*fliC* mutant was attributable to grossly impaired binding to IECs, immunofluorescence staining was performed on infected T84 cells. As shown in Figure 4.9B, colonisation of T84 cells by the Δ*fliC* mutant was comparable to that of the WT strain.

We then determined the role of EHEC proteins on induction of colonic epithelial hBD2 and IL-8 expression *ex vivo*. Apical infection of human colonic biopsies with EDL933 WT, Δ*escN* and Δ*stx* strains resulted in a significant induction of hBD2 (median fold increase = 15.9 for wt, 14.3 for *ΔescN*, and 17.8 for *Δstx*) and IL-8 (median fold increase = 3.6 for wt, 3.1 for *ΔescN*, and 4.9 for *Δstx*) expression vs NI controls. In contrast, no change in hBD2 and IL-8 expression was observed in Δ*fliC*-infected epithelium (Figure 4.10A). As seen with T84 cells, the *ΔfliC* mutant displayed no marked impairment of colonisation to colonic biopsies when examined using SEM (Figure 4.10B).

As it has been reported that long polar fimbriae (Lpf) and the haemorrhagic coli pilus (HCP) trigger innate immune gene expression in T84 cells during EHEC infection (Farfan *et al.*, 2013; Ledesma *et al.*, 2010), we investigated whether these factors

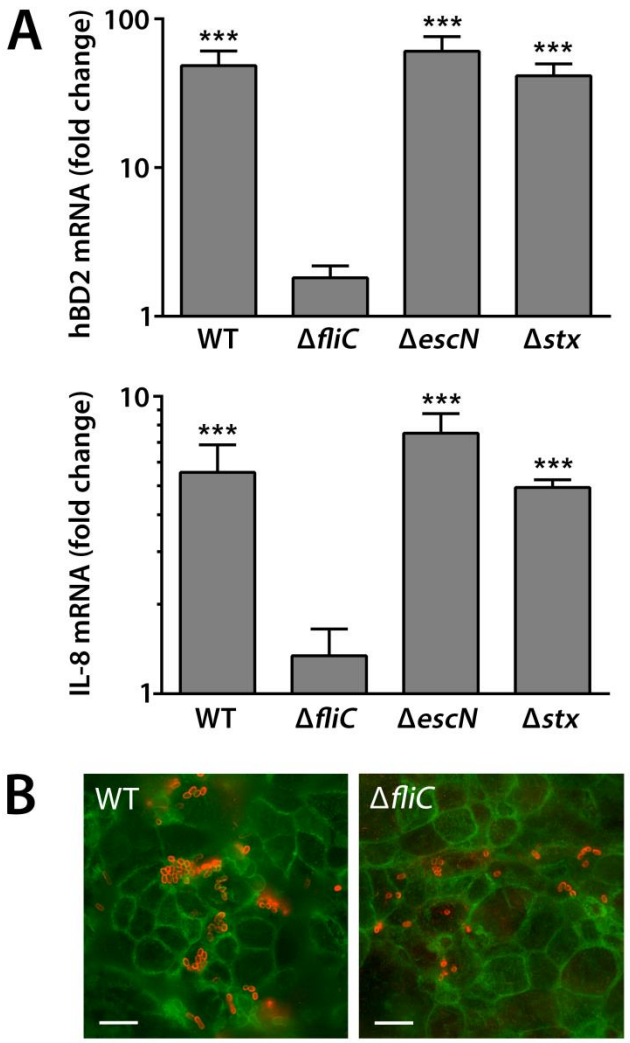

Figure 4.9. EHEC induction of hBD2 and IL-8 gene expression in T84 cells is dependent on flagellin. (A) T84 cells were apically infected for 9 hours with wild-type (WT) EHEC strain EDL933 or isogenic mutants lacking either *fliC*, *escN* or *stx*; or left uninfected. Fold mRNA expression in infected monolayers was quantified relative to uninfected controls using qPCR. Data are presented as means ± SE from three independent experiments performed in duplicate. Asterisks denote significant differences from uninfected cells. *** = $P < 0.001$. (**B**) T84 cells were apically infected with WT EDL933 or an isogenic *fliC* deletion mutant for 6 hours. Colonisation was visualised by immunofluorescence staining for *E. coli* (red) and actin (green). Images represent those from two independent experiments performed in duplicate. Scale bars = 5 μm.

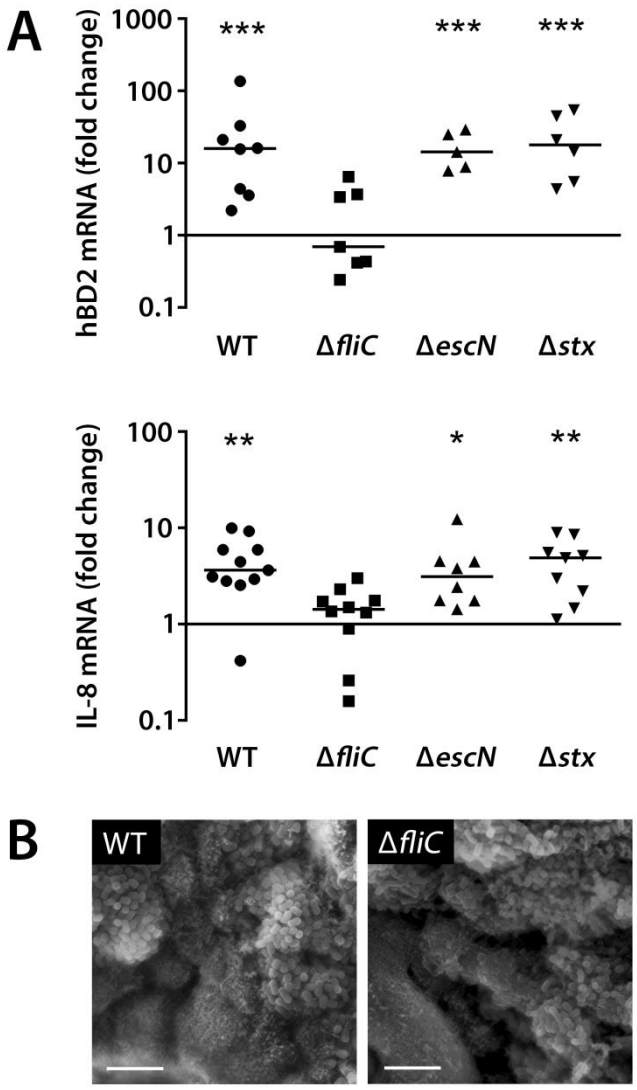

Figure 4.10. Flagellin is required for EHEC induction of hBD2 and IL-8 gene expression in human colonic biopsies. (**A**) Biopsies were infected for 7 hours with wild-type (WT) EHEC strain EDL933 or isogenic mutants lacking either *fliC*, *escN* or *stx*; or given LB alone (NI control). Fold mRNA expression in infected biopsies was quantified relative to NI control biopsies using qPCR. Each data point represents an individual biopsy from a total of 4 patients. Medians are indicated by a line. Asterisks denote significant differences from NI control biopsies. * = $P < 0.05$, ** = $P < 0.01$; *** = $P < 0.001$. (**B**) Biopsies were infected with EHEC strain EDL933 or an isogenic *fliC* deletion mutant for 8 hours and colonisation was visualised by SEM. Images represent those from two independent experiments performed in duplicate. Scale bars = 5 μm.

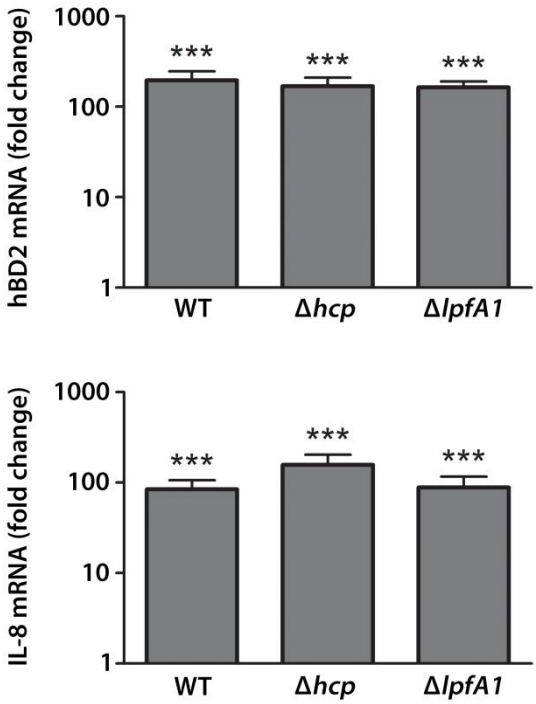

Figure 4.11. Lpf and HCP are not involved in EHEC induction of hBD2 and IL-8 gene expression in T84 cells. T84 cells were apically infected for 9 hours with wild-type (WT) EHEC strain 85-170 or isogenic mutants lacking either *lpfA1* or *hcp*; or left uninfected. Fold mRNA expression in infected monolayers was quantified relative to uninfected controls using qPCR. Data are presented as means ± SE from three independent experiments performed in duplicate. Asterisks denote significant differences from uninfected cells. *** = $P < 0.001$.

played a role in the IL-8 and hBD2 response to EHEC in our system. Polarised T84 cells were infected for 9 hours with wild-type (WT) EHEC strain 85-170 or isogenic deletion mutants unable to express either *lpfA1* or *hcp*. As shown in Figure 4.11, infection with wild type and both mutant strains induced a significant increase in expression of hBD2 (195.9 ± 50.1 fold for WT, 168.6 ± 40.4 fold for *Δhcp*, and 163.4 ± 26.8 fold for *ΔlpfA1*) and IL-8 (84.3 ± 21.8 fold for WT, 157.5 ± 45.7 fold for *Δhcp*, and 88.4 ± 28.0 fold for *ΔlpfA1*) compared with NI controls, suggesting that neither Lpf nor HCP are required for the hBD2 and IL-8 response to EHEC in colonic IECs.

4.2.6 | Purified EHEC flagellin induces hBD2 and IL-8 gene expression in human colonic IECs

To ascertain whether exposure of human colonic IECs to flagellin is sufficient to induce hBD2 and IL-8 gene expression, polarised T84 cells were incubated with EHEC strain EDL933 or various concentrations of purified LPS-free monomeric H7 flagellin (kindly donated by David Gally, University of Edinburgh, UK) for 9 hours. Epithelial barrier function was monitored by measuring TER throughout the procedure and no effect from any condition was observed (data not shown). Analysis of mRNA expression levels by qPCR revealed a dose-dependent hBD2 response to purified flagellin, with induction levels for 1 µg/ml flagellin approximating those observed for infection with EDL933 (101.5 ± 24.0 fold for flagellin and 99.0 ± 23.0 fold for EDL933). In contrast, induction of IL-8 by flagellin was not strictly dose-dependent and did not reach levels observed following infection with EDL933 (Figure 4.12A). To determine whether the IL-8 response to flagellin peaked at an earlier time-point, a kinetic assay was performed whereby cells were incubated with flagellin for 1-9 hours. Results in Figure 4.12B show that, whilst expression of hBD2 was highest after 6-9 hours of exposure to flagellin, IL-8 expression levels peaked much earlier at 1-3 hours post-exposure. Subsequently, IL-8 induction by flagellin was re-examined at the revised time point of 2 hours post-exposure, and a dose-sensitive response was observed with similar induction levels for 1 µg/ml flagellin and EDL933 infection (62.6 ± 8.9 fold for flagellin and 80.8 ± 10.5 fold for EDL933) (Figure 4.12C).

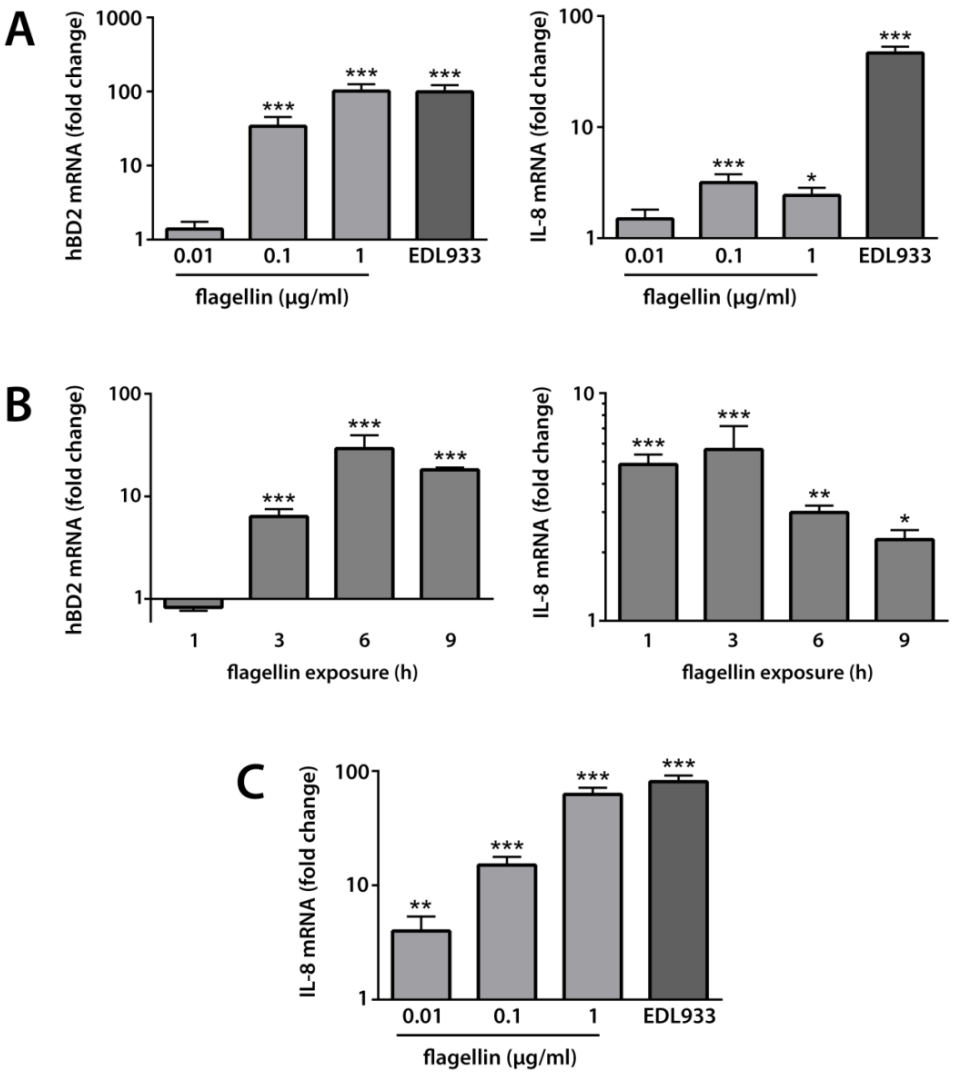

Figure 4.12. Purified flagellin is sufficient to induce hBD2 and IL-8 gene expression in T84 cells. Cells were apically exposed to purified H7 flagellin, infected with EHEC strain EDL933, or left untreated, and mRNA expression was determined by qPCR. (**A**) Incubation with flagellin for 9 hours induced a dose-sensitive increase in hBD2 expression, whereas IL-8 was induced to a lesser extent, and induction was not strictly dose-dependent. Data are shown as means ± SE of three independent experiments performed in duplicate. (**B**) Kinetics of hBD2 and IL-8 induction after exposure to 0.1 µg/ml flagellin. Data are shown as means ± SE of two independent experiments performed in duplicate. (**C**) Dose-sensitive increase in IL-8 expression following exposure to flagellin for 2 hours. Data are shown as means ± SE of two independent experiments performed in duplicate. Asterisks denote significant differences from untreated cells. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

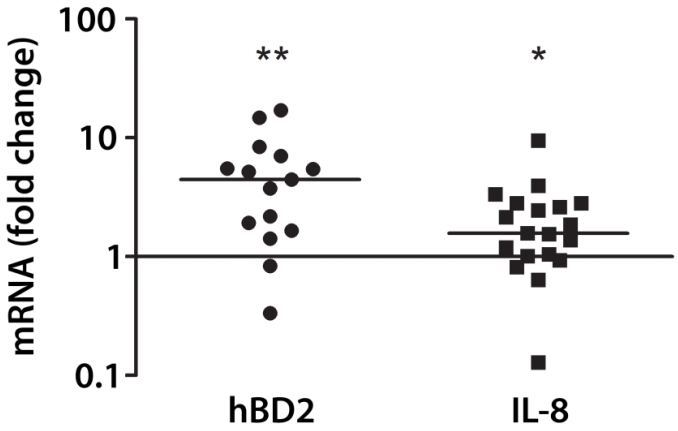

Figure 4.13. Purified flagellin induces hBD2 and IL-8 gene expression in human colonic biopsies. Biopsies were apically exposed to 1 µg/ml purified H7 flagellin or given LB alone (NI control) for 7 hours. Fold mRNA expression in treated vs. NI control samples was determined by qPCR. Each data point represents an individual biopsy from a total of 4 patients. Medians are indicated by a line. Asterisks denote significant differences from untreated cells. * = $P < 0.05$; ** = $P < 0.01$.

We next sought to determine whether flagellin had an effect on hBD2 and IL-8 gene expression in colonic biopsy epithelium *ex vivo*. Following exposure to 1 µg/ml purified flagellin for 7 hours, colonic biopsies expressed significantly higher levels of hBD2 (median fold increase = 5.3) and IL-8 (median fold increase = 2.2) mRNA compared with NI controls (Figure 4.13).

4.2.7 | Involvement of NF-κB and MAPK signalling pathways in the hBD2 and IL-8 response to EHEC

To elucidate the regulatory mechanisms underlying the hBD2 and IL-8 response to EHEC in colonic IECs, NF-κB and mitogen-activated protein kinase (MAPK) pathways (Figure 1.14) were inhibited in T84 cells prior to treatment with purified H7 flagellin. This was achieved by using chemical inhibitors targeting NF-κB (MG-132) and the MAPKs p38 (SB20358), JNK (SP600125), and ERK1/2 (PD98059). TER was measured before and after the procedure, and expression of hBD2 and IL-8 was quantified using qPCR. As shown in Figure 4.14A, cells treated with the NF-κB

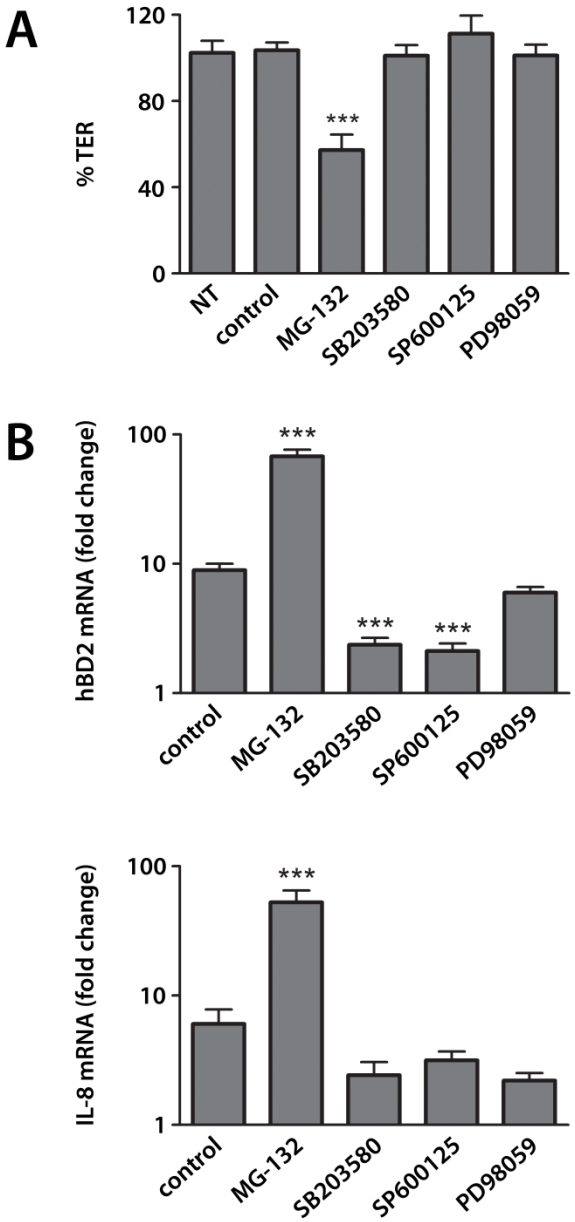

Figure 4.14. Involvement of NF-κB and MAPK signalling pathways in the hBD2 and IL-8 response to purified flagellin. T84 cells were apically and basolaterally exposed to 25 µM MG-132 (NF-κB inhibitor), SB203580 (p38 kinase inhibitor), SP600125 (JNK inhibitor) or PD98059 (ERK1/2 inhibitor), or left untreated (non-treated (NT) and control) for 1 hour. Purified H7 flagellin (0.1 µg/mL) was then added apically to all wells except NT for 6 hours. (**A**) TER of cell monolayers following treatment, expressed as a percentage relative to TER before treatment. Asterisks denote significant differences before vs. after treatment. (**B**) Fold mRNA expression in treated monolayers was quantified relative to NT cells using qPCR. Data are presented as means ± SE from three independent experiments performed in

duplicate. Asterisks denote significant differences from control cells. *** = $P < 0.001$.

inhibitor exhibited a significant reduction in TER, whilst the other inhibitors had no detectable effect. Figure 4.14B shows that flagellin-treated cells expressed significantly higher amounts of hBD2 (757.2 ± 97.7% increase) and IL-8 (873.3 ± 202.6% increase) mRNA when pre-exposed to the NF-κB inhibitor compared with non-treated controls. In contrast, pre-exposure to p38 and JNK inhibitors significantly attenuated the hBD2 response to flagellin (73.5 ± 3.5% decrease for p38, 76.3 ± 3.4% decrease for JNK), and inhibition of ERK1/2 caused a less-pronounced suppressive effect (33.1 ± 7.1% decrease). Similarly, IL-8 expression was markedly reduced in flagellin-treated cells pre-exposed to all three MAPK inhibitors (59.9 ± 10.7% decrease for p38, 47.7 ± 8.8% decrease for JNK, 63.6 ± 5.2% decrease for ERK1/2), although this did not reach significance (Figure 4.14B). These data suggest that MAPK signalling, particularly the p38 and JNK pathways, is involved in the hBD2 response to EHEC infection.

4.2.8 | Effect of EHEC on IL-8 protein expression in T84 cells

Having demonstrated EHEC induction of IL-8 mRNA expression, we next sought to determine whether this effect extended to the protein level. Polarised T84 cells were apically infected with EHEC strain EDL933 or incubated with the IL-8 inducer interleukin (IL)-1β (10 ng/ml) for 24 hours. To prevent cell death and loss of epithelial barrier function due to bacterial overgrowth and acidification of culture medium, gentamicin (200 μg/ml) was added to the culture medium after 1 hour. This treatment reduced the rate of bacterial growth to a level that did not compromise epithelial barrier function after 24 hours, as determined by TER (data not shown). At the end of the incubation, IL-8 protein levels were determined in cell lysates and in apical and basolateral culture media by ELISA. As shown in Figure 4.15A, apical EDL933 infection was associated with a significant increase in IL-8 levels in apical media (240.9 ± 27.4 vs 32.0 ± 6.1 pg/well in NI controls) and to a lesser extent in cell lysates (88.9 ± 21.5 vs 16.6 ± 1.4 pg/well in NI controls) but not in basolateral

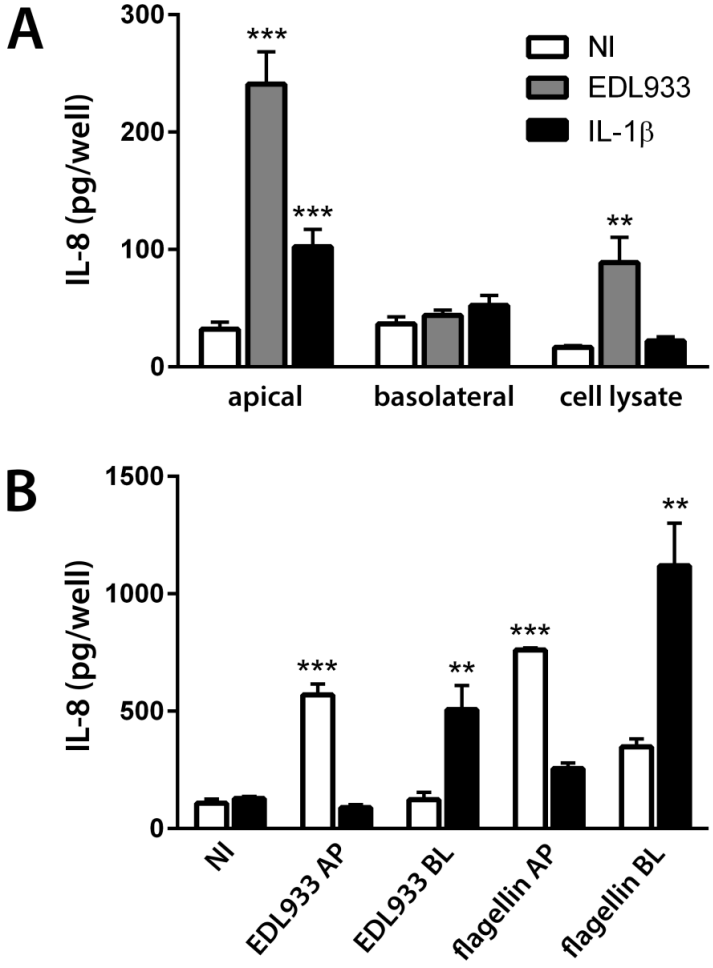

Figure 4.15. IL-8 protein secretion in T84 cells following EHEC infection. (A) Cells were apically exposed to EHEC strain EDL933, IL-1β (10 ng/ml), or left untreated (NI), for 24 hours. Protein levels in apical and basolateral media, and in cell lysates, were determined by ELISA. Data are shown as means ± SE of three independent experiments performed in duplicate. (**B**) Cells were exposed apically (AP) or basolaterally (BL) to EHEC strain EDL933, purified H7 flagellin (1 µg/ml), or left untreated (NI), for 24 hours. Protein levels in apical (white columns) and basolateral (black bars) media were determined by ELISA. Data are shown as means ± SE of two independent experiments performed in triplicate. Asterisks denote significance differences from NI samples. ** = $P < 0.05$; *** = $P < 0.001$.

media. IL-1β treatment also enhanced IL-8 levels in apical media (102.3 ± 14.8 vs 32 ± 6.1 pg/well in NI controls) but not in cell lysates or basolateral media.

As previous studies have reported basolateral IL-8 secretion by polarised T84 cells following basolateral stimulation (Ruchaud-Sparagano *et al.*, 2007; Zhou *et al.*, 2003), our experiments were extended to include basolateral stimulation. Polarised monolayers were incubated with EDL933 or purified flagellin (1 µg/ml) on the apical or basolateral side for 24 hours and IL-8 protein levels in apical and basolateral media were quantified. Results in Figure 4.16B show that apical exposure of T84 monolayers to EDL933 and flagellin significantly induced IL-8 secretion into apical media (569.5 ± 46.2 or 7612 ± 8.5 vs 108.2 ± 16.8 pg/well in NI controls, respectively) but did not affect IL-8 secretion into basolateral media. Conversely, basolateral exposure to EDL933 and flagellin resulted in significantly enhanced secretion into basolateral media (506.6 ± 103.2 or 1119 ± 181.4 vs 128.6 ± 8.05 pg/well in NI controls, respectively) but not apical media. These data indicate that EHEC-induced secretion of IL-8 in polarised T84 cells is both vectorial and directed towards the side of exposure.

4.2.9 | Effect of EHEC on hBD2 peptide expression in T84 cells

To determine the influence of EHEC on hBD2 peptide expression *in vitro*, polarised T84 monolayers were apically incubated with EDL933, purified flagellin (1 µg/ml) or the hBD2 inducer IL-1β (Schlee *et al.*, 2007) (10 ng/ml) for 24 hours, and hBD2 peptide in cell lysates, apical and basolateral media was measured using ELISA. No hBD2 was detected in cell lysates or in culture media (data not shown). This lack of hBD2 detection was not attributable to low functionality of the ELISA, as hBD2 was detected in culture medium of Caco-2 cells following incubation with IL-1β (Schlee *et al.*, 2007) for 24 hours (Figure 4.16). Suspecting that hBD2 peptide secreted by T84 cells may have been below the level required for detection, larger-scale experiments were conducted using a 20-fold greater number of cells per sample. Cell monolayers cultured to confluency in 75cm^2 culture flasks were incubated with IL-1β for 24 and 48 hours. Culture medium and cell lysates were then collected and proteins were concentrated 40-fold using Amicon ultra filtration units. No hBD2 was detected in any of the concentrated samples at either time-point tested (data not shown). We then changed the detection method to immunofluorescence staining in an attempt to visualise hBD2 protein within cells. Returning to the initial infection

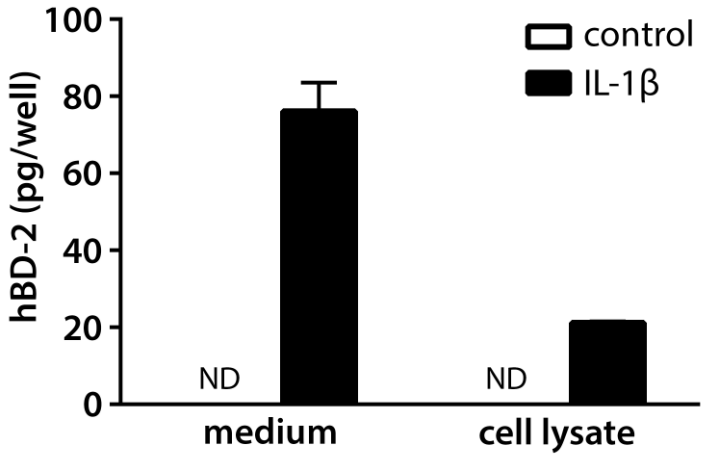

Figure 4.16. hBD2 peptide secretion in Caco-2 cells. Cells grown in 6-well plates were incubated with IL-1β (10 ng/ml) or left untreated (control) for 24 hours and hBD2 peptide levels in culture medium and cell lysates were quantified by ELISA. Data are shown as means ± SE of duplicate samples. ND = none detected.

protocol, polarised T84 cells grown on permeable Transwell inserts were incubated with EDL933, purified flagellin or IL-1β for 24 hours. As shown in Figure 4.17, positive staining for hBD2 was detected in a subset of T84 cells. No difference in hBD2 levels within cells was observed between treatments, indicating that hBD2 protein expression was not influenced by EHEC infection or exposure to flagellin and IL-1β. In order to confirm specificity of the hBD2 antibody, immunofluorescence staining was also performed on Caco-2 cells following incubation with EDL933 and IL-1β for 24 hours. Images in Figure 4.18 show a marked induction of hBD2 protein within cells treated with IL-1β and to a lesser extent with EDL933, compared with NI control cells.

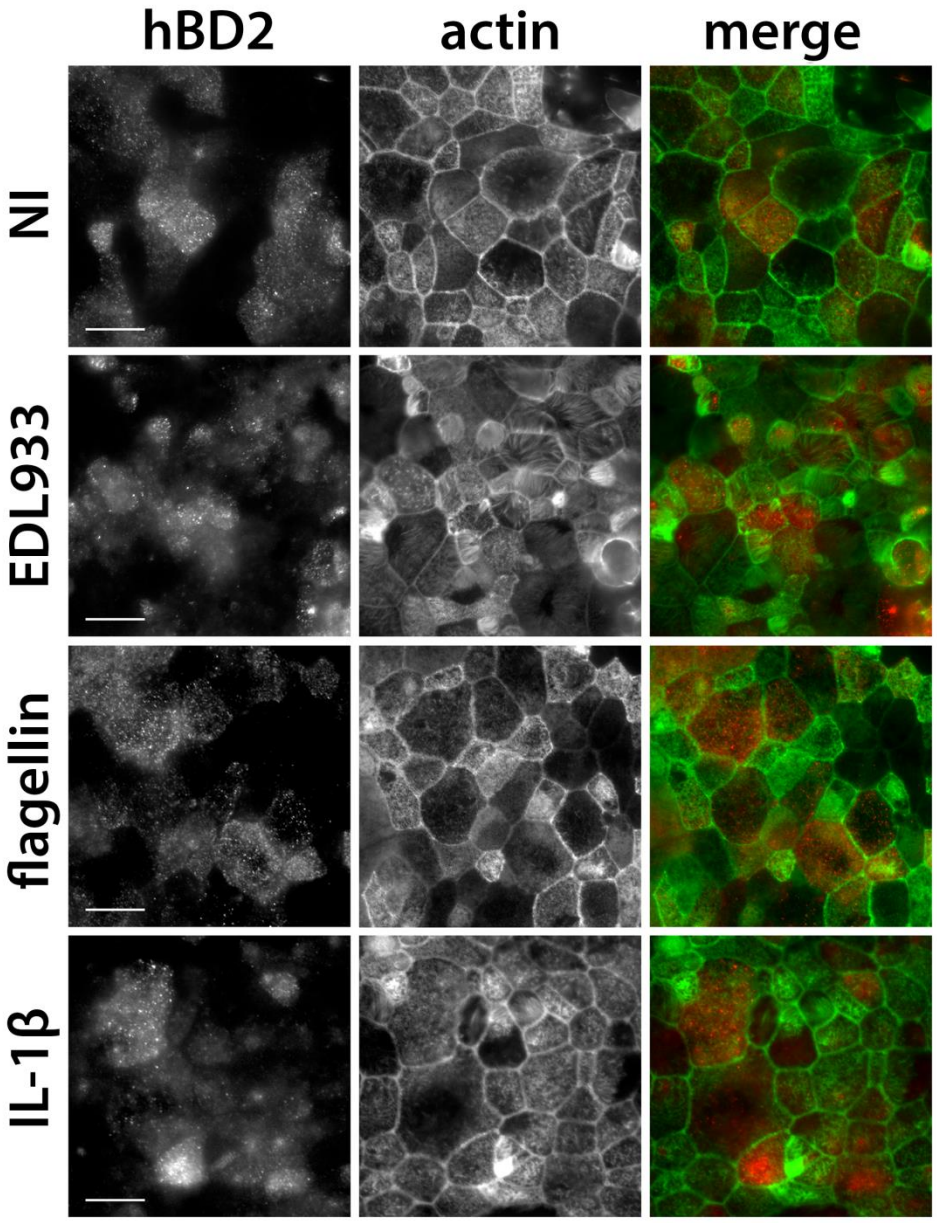

Figure 4.17. hBD2 peptide expression in T84 cells is unaffected by EHEC infection. Cells were apically exposed to EHEC strain EDL933, purified H7 flagellin (1µg/ml), IL-1β (10 ng/ml), or left untreated (NI), for 24 hours. Immunofluorescence staining was performed to visualise hBD2 (red) and actin (green). Right images show merged channels; left and central images show hBD2 and actin channels, respectively. Images represent those from two independent experiments, performed in duplicate. Scale Bars = 10 µm.

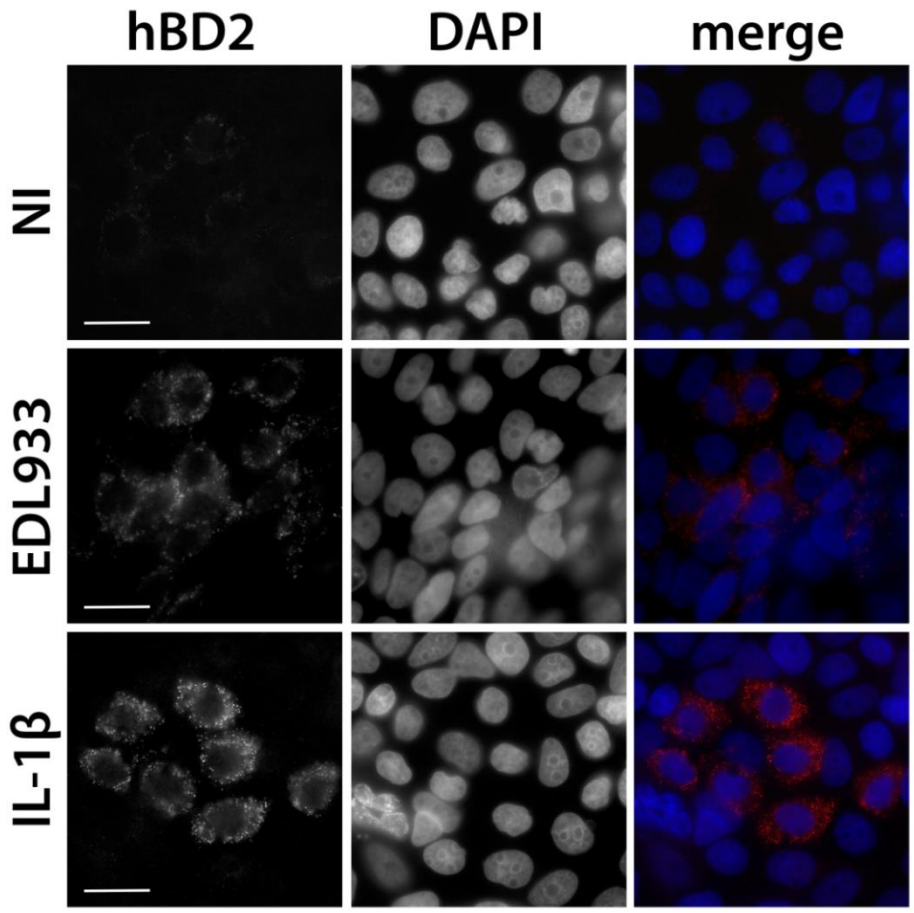

Figure 4.18. hBD2 peptide production in Caco-2 cells. Cells grown on coverslips were exposed to EHEC strain EDL933, IL-1β (10 ng/ml), or left untreated (NI), for 24 hours. Immunofluorescence staining was performed to visualise hBD2 (red) and cell nuclei (DAPI; blue). Right images show merged channels; left and central images show hBD2 and DAPI channels, respectively. Images represent those from duplicate samples. Scale Bars = 10 µm.

4.2.10 | Effect of EHEC on hBD2 and IL-8 protein expression in human colonic biopsies

To determine the influence of EHEC on hBD2 and IL-8 protein expression *ex vivo*, pIVOC was performed on human colonic biopsies incubated with EDL933 or purified flagellin (1 µg/ml) for 7 hours. After the procedure, hBD2 peptide expression within cells was analysed by immunofluorescence staining. Images in Figure 4.19 show that, whilst hBD2 peptide expression was low in non-infected

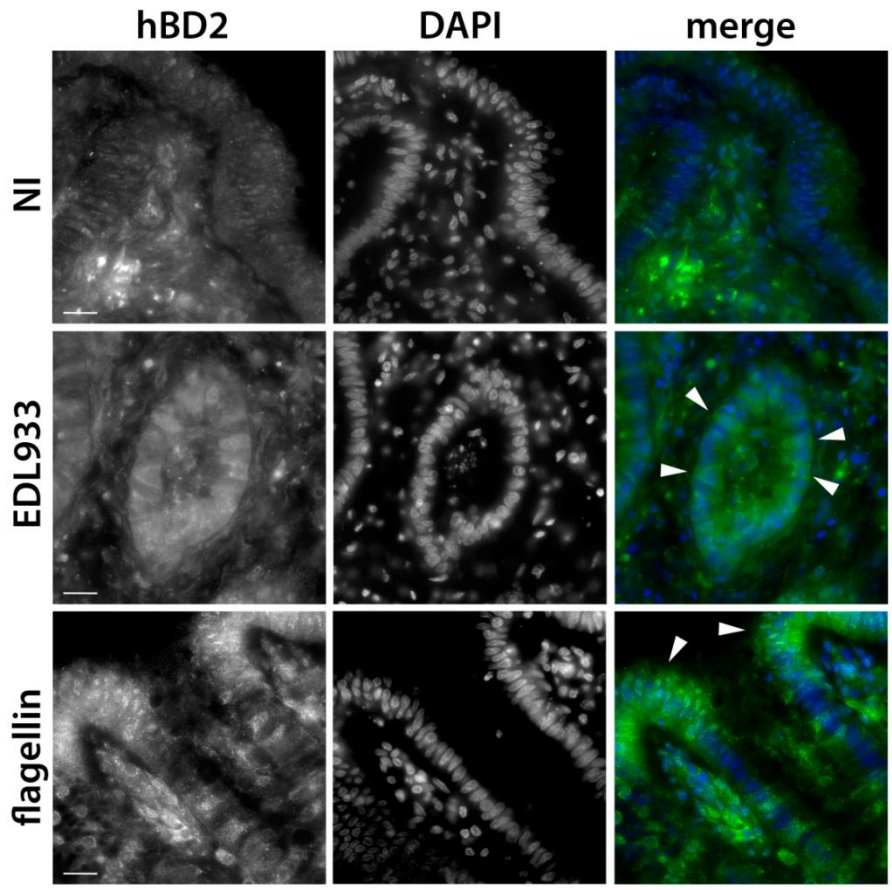

Figure 4.19. hBD2 peptide expression in human colonic biopsies is up-regulated during EHEC infection. Biopsies were apically exposed to EHEC strain EDL933, purified H7 flagellin (1 µg/ml), IL-1β (10 ng/ml), or given LB alone (NI), for 8 hours. Cryosections were stained with anti-hBD2 (green) and counterstained with DAPI (blue) to label cell nuclei. Right images show merged channels; left and central images show hBD2 and DAPI channels, respectively. Images represent those from two independent experiments performed in duplicate. Scale bars = 10 µm.

biopsies, considerably higher hBD2 expression was evident in epithelium from biopsies treated with EDL933 or flagellin. This indicates that exposure to EHEC and flagellin enhances hBD2 peptide expression in human colonic biopsy epithelium, in contrast to T84 cells. IL-8 protein levels in biopsy lysates were also analysed by ELISA. As shown in Figure 4.20, no effect of EDL933 infection or flagellin on IL-8 protein levels was observed relative to NI controls, suggesting that EHEC does not enhance IL-8 protein expression in human colonic biopsies at this time point.

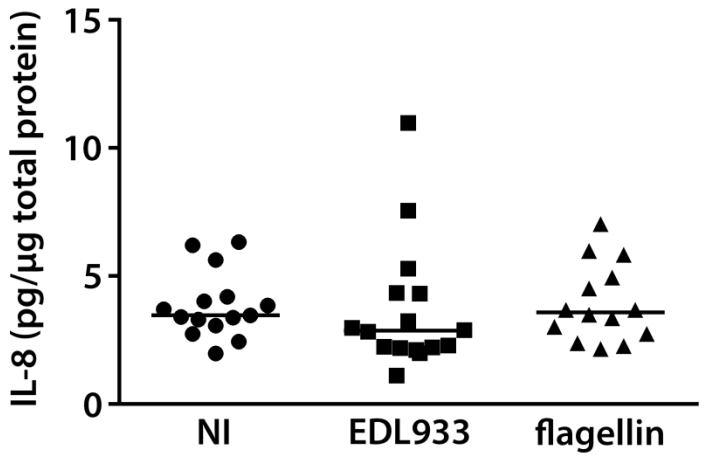

Figure 4.20. IL-8 protein expression in human colonic biopsies. Biopsies were apically exposed to EHEC strain EDL933, purified H7 flagellin (1 µg/ml), or given LB alone (NI), for 7 hours. Protein levels in biopsy lysates were determined by ELISA. Each data point represents an individual biopsy from a total of 3 patients. Medians are indicated by a line.

4.3 | Discussion

The intestinal epithelium is the first line of defence against enteric pathogens, together with the mucus layer and resident microbiota. In addition to forming a physical barrier between the external environment and the internal milieu, IECs secrete antimicrobial peptides (AMPs), which directly kill bacteria and recruit various immune cells to the site of infection (Dommett *et al.*, 2005; Muniz *et al.*, 2012). Despite the important role for AMPs in epithelial defence, expression of these peptides by IECs during EHEC infection has not been examined. In addition to AMPs, IECs are also able to secrete cytokines that trigger inflammation. Studies using T84 and Caco-2 human intestinal carcinoma cell lines have shown that EHEC induces epithelial secretion of IL-8 (Berin *et al.*, 2002; Dahan *et al.*, 2002), a chemokine that promotes infiltration of neutrophils into the intestinal mucosa. However, the influence of EHEC on IL-8 expression by normal human colonic epithelium has not been investigated. In this study, we developed a polarised IVOC model for human colonic biopsies in order to determine the AMP and IL-8 response to EHEC infection in human colonic epithelium *ex vivo*. As previous studies of EHEC-immune interactions have been performed *in vitro* using intestinal carcinoma cell lines, we extended our investigation to include polarised T84 human colonic carcinoma cells.

4.3.1 | Influence of EHEC infection on AMP expression

In the human colon, IECs have been shown to secrete hBD1-4, LL-37 and lysozyme (Fahlgren *et al.*, 2003, 2004; Hase *et al.*, 2002; Zilbauer *et al.*, 2010), and so we tested whether EHEC infection influences expression of these AMPs. Our results show that infection of both T84 cells and human colonic biopsies with EHEC resulted in increased expression of hBD2. The extent of hBD2 induction was comparable for all EHEC strains tested, despite these strains exhibiting differences in levels of Stx production and colonisation. This indicates that hBD2 expression in response to EHEC infection does not appear to be influenced by the amount of Stx produced or extent of colonisation. hBD2 is capable of directly killing *E. coli* by breaking down the bacterial cell membrane (Estrela *et al.*, 2013). In addition, hBD2 has pro-inflammatory properties, acting as a chemoattractant for neutrophils,

immature dendritic cells (DCs) and T cells *via* binding to CC-chemokine receptor 6 (CCR6) (Niyonsaba *et al.*, 2004; Vongsa *et al.*, 2009; Yang *et al.*, 1999); for monocytes by binding to CCL2 (Röhrl *et al.*, 2010); and for mast cells *via* an as-yet unidentified receptor (Niyonsaba *et al.*, 2002; Niyonsaba *et al.*, 2001). Accordingly, intestinal hBD2 expression is elevated in inflammatory bowel disease (IBD) patients vs. healthy individuals (Fahlgren *et al.*, 2003; Wehkamp *et al.*, 2002; Zilbauer *et al.*, 2010), and inflamed tissue contains higher levels of hBD2 compared with non-inflamed areas in ulcerative colitis (UC) sufferers (Wehkamp *et al.*, 2003). Thus, hBD2 is of great clinical relevance to intestinal health and may have the potential to influence the outcome of EHEC infection if used as a therapeutic agent. Future work in this area should include an analysis of whether EHEC colonisation and pathogenesis is reduced in the presence of hBD2, before assessing its potential contribution to therapies.

Several other enteric species have been shown to stimulate hBD2 expression by human intestinal epithelial cells. These include enteroinvasive *Salmonella* spp., *S. flexneri* and *dysenterae*, EIEC and *C. jejuni* (Hase *et al.*, 2002; O'Neil *et al.*, 1999; Ogushi *et al.*, 2001; Zilbauer *et al.*, 2005); non-invasive EPEC (Khan *et al.*, 2008); and even probiotic *E. coli* Nissle 1917 (Schlee *et al.*, 2007; Wehkamp *et al.*, 2004). However, it should be noted that the majority of these findings were garnered by performing infections on human carcinoma cell lines such as Caco-2 and HT-29, with very few studies utilising native human intestinal tissue. Recently, a model for growing human intestinal xenografts in mice has been developed, enabling the study of the innate immune response *in vivo*. Using this model system, Sperandio and colleagues (2008) reported no change in hBD2 expression by human intestinal xenograft tissue inoculated with *S. flexneri* compared with NI controls, despite noting significant up-regulation of hBD2 in infected HT-29 and TC7 carcinoma cell lines. This suggests that the hBD2 response to infection may differ depending on the model system used. To the best of our knowledge, our study is the first to examine the AMP response to bacterial infection using IVOC of intestinal tissue explanted from humans. We found that, although both human colonic biopsies and polarised T84 cells showed enhanced hBD2 mRNA expression following EHEC infection, a concurrent increase in hBD2 peptide expression was detected only in the former model system and not the latter. It is unclear why hBD2 was induced at the mRNA

but not protein level in T84 cells. One possible explanation is that translation of hBD2 was inhibited in T84 cells, perhaps by the action of microRNAs, which target mRNA for degradation (Cai *et al.*, 2009). Alternatively, hBD2 may have undergone a post-translational modification, such as ubiquitylation, that resulted in its degradation or a change in its antigenic properties (Popovic *et al.*, 2014), thereby rendering it undetectable using antibody-based methods. In any case, the observed differences in results obtained from colonic biopsies vs. T84 cells highlights the importance of confirming findings in physiologically relevant model systems.

In addition to hBD2, we determined the influence of EHEC on colonic epithelial expression of several other AMPs: hBD1, 3 and 4; LL-37 and lysozyme. Whilst hBD1 is constitutively expressed by IECs and is largely unresponsive to bacterial or inflammatory stimuli (Fahlgren *et al.*, 2003; O'Neil *et al.*, 1999; Ou *et al.*, 2009; Sperandio *et al.*, 2008; Zilbauer *et al.*, 2010), a few bacterial species such as *Shigella* ssp., ETEC and *V. cholerae* have been shown to down-regulate its expression. (Chakraborty *et al.*, 2008; Islam *et al.*, 2001; Sperandio *et al.*, 2008). Inhibition of LL-37 expression by the same pathogens was also reported in these studies, although up-regulated expression has also been demonstrated following infection with *Salmonella* spp. (Chakraborty *et al.*, 2008; Hase *et al.*, 2002). Inhibition of AMP production by enteric pathogens likely represents a survival strategy to avoid direct harm or immunodetection. Expression of hBD3, hBD4 and lysozyme by colonic epithelium is thought to occur at low levels but is enhanced in IBD patients (Fahlgren *et al.*, 2003, 2004; Zilbauer *et al.*, 2010), suggesting inducible regulation that is linked with the inflammatory response. Little is known about the influence of pathogens on expression of these AMPs, although *C. jejuni* has been shown to enhance hBD3 production in Caco-2 and HT-29 cells (Zilbauer *et al.*, 2005). We found no evidence for EHEC induction of hBD1, hBD3, LL-37 or lysozyme during infection of human colonic epithelium, suggesting that they are not involved in the immune response to EHEC. However, we cannot rule out the possibility that their induction is inhibited by EHEC, as several EHEC effector proteins have been shown to dampen host innate immune pathways (reviewed in Santos and Finlay, 2015). It would be interesting to determine human colonic epithelial expression of these AMPs during infection with a mutant EHEC strain deficient in T3S to ascertain whether EHEC subverts the AMP response.

During our study, we were unable to consistently detect hBD3 and hBD4 mRNA in human colonic biopsies, meaning that we could not determine whether EHEC affects expression of these AMPs *ex vivo*. This is likely due to the fact that hBD3 and 4 are expressed at low levels compared with other AMPs in the colon (Fahlgren *et al.*, 2003, 2004), which were likely below those required for detection by our qPCR assay. This highlights a limitation of the IVOC model system, which requires the use of biopsy tissue that may be insufficiently small for some applications. One possible way to increase the mRNA signal of poorly expressed genes may be to pool biopsy samples during analysis. However, this will impact on the size of experiments due to ethical restrictions on the number of biopsies available from any one patient. Another solution may be to use an alternative model system, such as intestinal xenografts grown on severe combined immuno-deficiency (SCID) mice (Golan *et al.*, 2011; Miyamoto *et al.*, 2006). This model is less restricted by sample size but has other disadvantages such as a lack of human commensal microflora and limited access to tissue, which derives from aborted foetuses.

4.3.2 | Influence of EHEC infection on IL-8 expression

EHEC infection triggers a moderate inflammatory response in the human intestine, characterised by transmigration of neutrophils into the colonic mucosa (Griffin *et al.*, 1990; Kelly *et al.*, 1990; Kelly *et al.*, 1987). Since the discovery of IL-8 as the primary tissue-derived chemoattractant for neutrophils (Baggiolini and Clark-Lewis, 1992), expression of this cytokine in human colonic epithelium has been shown to be responsive to a range of enteric pathogens including *S. typhimurium*, *C. jejuni*, *Vibrio* spp., EPEC and EAEC (Chakraborty *et al.*, 2008; Harrington *et al.*, 2005; McCormick *et al.*, 1993; Zhou *et al.*, 2003; Zilbauer *et al.*, 2005). In addition, studies on EHEC have demonstrated infection-mediated induction of IL-8 in cultured intestinal carcinoma cell lines Caco-2, HT-29 and T84 (Dahan *et al.*, 2002; Jung *et al.*, 1995). In our study, we examined the IL-8 response to EHEC in human colonic epithelium during pIVOC of human colonic biopsies and *in vitro* infection of polarised T84 cells. In line with published findings on non-polarised T84 cells, we found that EHEC stimulated expression of IL-8 by polarised T84 cells. Importantly, *ex vivo* infection of human colonic biopsies with EHEC yielded similar results,

demonstrating for the first time EHEC induction of IL-8 expression in explanted human colonic epithelium.

4.3.3 | Role of bacterial proteins in EHEC induction of hBD2 and IL-8

Several EHEC proteins have been shown the affect innate immune processes in human IECs. Studies examining the IL-8 response to EHEC in carcinoma cell lines and human colonic xenografts have identified flagellin, the main constituent of flagella, as the main factor responsible for induction (Bellmeyer *et al.*, 2009; Berin *et al.*, 2002; Miyamoto *et al.*, 2006; Zhou *et al.*, 2003). However, deletion of *fliC*, the gene encoding flagellin, did not completely abrogate IL-8 induction by EHEC in one study (Bellmeyer *et al.*, 2009), suggesting that flagellin is not solely responsible for the observed response. Aside from flagellin, other factors such HCP and Lpf have been implicated in the IL-8 response to EHEC (Farfan *et al.*, 2013; Ledesma *et al.*, 2010). In particular, the role of Stx has been examined in some detail. Whilst some studies have found that Stx induces IL-8 expression in carcinoma cell lines (Bellmeyer *et al.*, 2009; Thorpe *et al.*, 2001; Yamasaki *et al.*, 1999), others have found no evidence of a stimulatory role for the toxin (Berin *et al.*, 2002; Miyamoto *et al.*, 2006). Conversely, a down-regulatory effect was demonstrated during EHEC infection of T84 cells (Gobert *et al.*, 2007), and so the role of Stx in EHEC induction of IL-8 expression remains controversial. Interestingly, inhibiting the type III secretion system (T3SS) in EHEC and EPEC has been shown to enhance the IL-8 response to infection in HeLa and Caco-2 cells (Hauf and Chakraborty, 2003; Ruchaud-Sparagano *et al.*, 2007; Sharma *et al.*, 2006). Recent functional analysis of secreted effector proteins has revealed that many inhibit inflammatory signal transduction (NleE, NleB, NleH1, NleC, NleD and Tir) (Baruch *et al.*, 2011; Gao *et al.*, 2009; Nadler *et al.*, 2010; Newton *et al.*, 2010; Ruchaud-Sparagano *et al.*, 2011; Sham *et al.*, 2011), whereas only two appear to stimulate it (EspT and NleH2) (Gao *et al.*, 2009; Raymond *et al.*, 2011). Whilst no studies have examined EHEC stimulation of hBD2 expression, it has been demonstrated that the hBD2 response to EPEC in Caco-2 cells can be abrogated or enhanced by deletion of *fliC* or T3S, respectively (Khan *et al.*, 2008).

Based on this research, we sought to determine whether flagellin, T3S, Stx, HCP and Lpf were involved in EHEC stimulation of hBD2 and IL-8 in human colonic epithelium *in vitro* and *ex vivo*. We found that a *fliC*-negative EHEC deletion mutant was unable to stimulate expression of both hBD2 and IL-8 by polarised T84 cells and human colonic biopsies. In contrast, mutant strains deficient in T3S, Stx, HCP and Lpf were able to stimulate comparable hBD2 and IL-8 responses to those observed following infection with the wild-type strain. We also demonstrated that exposure to purified EHEC flagellin was sufficient to induce hBD2 and IL-8 expression in both of our model systems. These data suggest that flagellin is the primary factor responsible for the inflammatory response in human colonic epithelium during EHEC infection, and that T3S and Stx are not involved. Our results are the first to demonstrate a role for flagellin in the colonic epithelial hBD2 response to EHEC infection, and the first to show flagellin-mediated induction of IL-8 during IVOC of human colonic biopsies. Flagellin has also been implicated in the innate immune response to other enteric bacteria: flagellin-dependent hBD2 expression has been reported during infection with EPEC, *S.* serotype Enteritidis and *E. coli* Nissle (Khan *et al.*, 2008; Ogushi *et al.*, 2001; Schlee *et al.*, 2007), whilst induction of IL-8 by EPEC, EAEC, *Salmonella* spp. and commensal *E. coli* has also been attributed to flagellin (Bambou *et al.*, 2004; Gewirtz *et al.*, 2001b; Ruchaud-Sparagano *et al.*, 2007; Steiner *et al.*, 2000; Zhou *et al.*, 2003). Our data on EHEC infection, together with findings from other bacterial infection studies, implicates flagellin as a crucial microbe-associated molecular pattern (MAMP) used in innate immune recognition of a broad range of enteric bacteria.

In addition to triggering inflammation *via* flagellin, it has been shown that EHEC and EPEC dampen the IL-8 response through the action of T3S effector proteins (Hauf and Chakraborty, 2003; Nadler *et al.*, 2010; Sharma *et al.*, 2006; Yen *et al.*, 2010). A similar suppressive effect of the T3SS on hBD2 expression during EPEC infection has also been demonstrated (Khan *et al.*, 2008). These studies were conducted on the cervical HeLa and/or the small intestinal Caco-2 cell line. Interestingly, we did not detect a significant inhibitory effect of the T3SS on IL-8 or hBD2 expression during EHEC infection of polarised T84 cells or human colonic biopsies. Whilst our study is the first to examine the role of the T3SS on innate immune responses in human intestinal tissue, Ruchaud-Sparagano and colleagues have also failed to detect an

inhibitory effect of T3SS on the IL-8 response in T84 but not Caco-2 cells (Ruchaud-Sparagano *et al.*, 2007). Put together, these results suggest that EHEC-mediated suppression of the innate immune response may be delayed or absent in colonic epithelium, in contrast to the small intestine. To clarify this issue, it would be necessary to examine the influence of the T3S on the colonic and small intestinal innate immune response over an extended period of time.

4.3.4 | Flagellin recognition by IECs

Flagellin is recognised by Toll-like receptor-5 (TLR5) on the IEC surface membrane, and blocking TLR5 activity in intestinal carcinoma cell lines inhibits the inflammatory response to flagellin from *S. dublin* and *E. coli* (Bambou *et al.*, 2004; Eaves-Pyles *et al.*, 2011). Therefore, it is likely that the hBD2 and IL-8 response to EHEC infection is triggered by binding of flagellin to TLR5 on the colonic IEC surface.

Given that flagella are a common feature of both pathogenic and commensal bacteria, there is uncertainty as to how detection of flagellin by IECs can serve as an indication of the presence of a pathogen. This problem was first addressed by Gewirtz and co-workers (2001a), who demonstrated that flagellin from *Salmonella* induced an IL-8 response in polarised T84 cells when administered basolaterally but not apically, and that TLR5 was expressed only on the basolateral surface of polarised T84 cells. Based on their findings, the authors postulated that IECs only detect flagellin that has crossed the epithelium and binds to TLR5 on their basolateral surface, which generally only occurs as a result of pathogen activity (i.e. invasion of the epithelium or subversion of epithelial barrier function). However, this hypothesis has been called into question by subsequent studies reporting apical TLR5 localisation and/or flagellin responsiveness in T84, Caco-2 and HCA-7 cells (Berin *et al.*, 2002; Miyamoto *et al.*, 2006; Ruchaud-Sparagano *et al.*, 2007; Zhou *et al.*, 2003). Furthermore, examination of human mucosal tissue by TLR5 immunofluorescence has shown positive staining on the apical and basolateral side of epithelium from duodenum and colon (Cario and Podolsky, 2000; Schüller *et al.*, 2009).

In our study, we found that apical exposure of polarised T84 cells and human colonic biopsies to purified EHEC flagellin was sufficient to induce hBD2 and IL-8 expression. This is in line with a study on EPEC flagellin, which reported apical induction of IL-8 expression in human duodenal biopsies (Schüller *et al.*, 2009). Our attempts to determine TLR5 localisation in T84 cells and colonic biopsies were unsuccessful, hindered by the well-documented lack of high-affinity antibodies (Steiner, 2007). Nevertheless, our data support the theory that basolateral access to flagellin is not required for induction of the innate immune response in human colonic epithelium. It is worth noting that *C. jejuni* flagellin does not induce IL-8 expression in T84 cells (Johanesen and Dwinell, 2006), nor does flagellin from commensal *E. coli* strains ATCC 259922 and JM109 (Schlee *et al.*, 2007). This suggests that IECs do not respond to flagellin from all bacteria indiscriminately, and that variations in flagellar structure or composition may instead be important in distinguishing between pathogenic vs. non-pathogenic bacterial species.

4.3.5 | Regulation of the hBD2 and IL-8 response to EHEC infection

Stimulation of TLR5 on IECs triggers a regulatory cascade that involves both the NF-κB and mitogen-activated protein kinase (MAPK) signal transduction pathways (Figure 1.14) (Steiner, 2007). Accordingly, the IL-8 response to EHEC infection in T84 and Caco-2 cells is abrogated by inhibition of NF-κB and the MAPKs p38 and ERK1/2 (Berin *et al.*, 2002; Dahan *et al.*, 2002). Similarly, we found that inhibition of p38, ERK1/2 and JNK MAPKs in polarised T84 cells suppressed the IL-8 response to flagellin. In addition, the hBD2 response was significantly reduced by inhibition of p38 and JNK, and of ERK1/2 to a lesser extent. These data are in line with published studies showing that the hBD2 response to to *E. coli* Nissle 1917, *Lactobacillus* spp. and IL-1α involves the same MAPK pathways, in addition to NF-κB (O'Neil *et al.*, 1999; Schlee *et al.*, 2008; Schlee *et al.*, 2007). Surprisingly, we found that inhibition of NF-κB signalling using the inhibitor MG-132 resulted in a significant increase in the hBD2 and IL-8 response to flagellin compared with non-treated control cells. This might be explained by the loss of epithelial barrier function incurred after exposure to MG-132, which is likely to permit translocation of

flagellin from the apical to the basolateral surface of IECs and enhance its stimulatory effect on hBD2 and IL-8 expression. Different experimental approaches, such as DNA binding or nuclear translocation assays, would be necessary to determine NF-κB activity after apical flagellin exposure. In this study, we have shown for the first time that the hBD2 response to EHEC infection in human colonic T84 cells involves MAPK signalling. An important next step would be to confirm these findings in colonic pIVOC.

4.3.6 | Vectorial IL-8 secretion

Whilst analysing the influence of EHEC on IL-8 protein expression in polarised T84 cells, we discovered that IL-8 secretion was vectorial: apical exposure to EHEC and flagellin led to secretion of IL-8 in an apical but not basolateral direction, whereas basolateral exposure triggered basolateral but not apical secretion. Whilst IL-8 secretion in polarised T84 cells during EHEC infection has not been examined before, studies using EPEC have reported an increase in basolateral IL-8 secretion when cells were stimulated basolaterally but not apically (Ruchaud-Sparagano *et al.*, 2007; Zhou *et al.*, 2003). Very few studies consider apical IL-8 secretion by IECs during bacterial infection as it is generally assumed that IL-8 is secreted basolaterally into the lamina propria to mediate recruitment of neutrophils to the epithelium. In line with this theory, predominantly basolateral IL-8 secretion has been demonstrated in Caco-2 cells apically infected with EPEC and by HCA-7 cells stimulated with EHEC flagellin (Berin *et al.*, 2002; Ruchaud-Sparagano *et al.*, 2007). In contrast, our data showing apical IL-8 secretion by T84 cells contradict these findings, and indicate that IL-8 may be secreted apically during EHEC infection *in vivo*. This theory is supported by a recent study, which reported apical IL-8 secretion in Caco-2 cells following apical exposure to flagellin (Rossi *et al.*, 2013). Moreover, IL-8 has been detected in luminal contents sampled from the colon of humans and IL-8 levels were higher in UC patients compared with healthy controls (Keshavarzian *et al.*, 1999), further suggesting that IL-8 is secreted into the lumen as part of the inflammatory response. The reason(s) why IL-8 may be secreted into the intestinal lumen is open to conjecture. One possible explanation is that apical IL-8 secretion promotes neutrophil migration across the epithelium and into the lumen

(Keshavarzian *et al.*, 1999). Neutrophils are often found in the lumen of patients suffering from colonic inflammation (Fournier and Parkos, 2012), and their presence at this site likely represents an immune strategy for killing pathogens before they colonise the epithelium. Alternatively, apical IL-8 secretion by IECs could serve a role other than neutrophil recruitment. IL-8 is a ligand for the G-protein-coupled receptor CXCR1, which is expressed on the apical surface of explanted human intestinal epithelium, and so it is possible that IL-8 may perform some as-yet unidentified autocrine function (Rossi *et al*., 2013).

Unfortunately, it was not feasible to determine the direction of IL-8 secretion in colonic biopsies due to the mucus layer and mucosal tissue blocking cytokine diffusion into the medium. However, it was possible to analyse the influence of EHEC on intracellular IL-8 production by measuring protein levels in biopsy lysates. In order to prevent bacterial overinfection and detachment of the biopsy epithelium, the length of infection was restricted to 7 hours. We were unable to detect an effect of EHEC or flagellin on IL-8 production using this infection protocol. Given that protein production in IECs is generally analysed after 18+ hours of infection with A/E pathogens (Farfan *et al.*, 2013; Gobert *et al.*, 2007; Khan *et al.*, 2008), it is probable that our *ex vivo* experiments were not conducted for long enough to allow adequate time for induction of protein expression to take place. Therefore, it is possible that EHEC infection does indeed trigger an increase in colonic biopsy IL-8 protein production, but this takes place at a time-point later than 7 hours. To test this theory, it would be necessary to develop a method for prolonged infection of colonic biopsies that does not compromise tissue integrity and enables analysis of IL-8 protein expression at later time-points. Alternatively, prolonged exposure of colonic biopsies to purified flagellin may be sufficient to induce IL-8 protein expression without affecting tissue preservation.

4.3.7 | Summary

In this study, we have demonstrated that EHEC infection triggers expression of hBD2 and IL-8 in polarised T84 cells and human colonic biopsies. Flagellin was found to be both necessary and sufficient for induction of both genes. Basolateral exposure to EHEC or flagellin was not necessary for induction of this response,

indicating that flagellin is recognised upon contact with the apical surface of IECs. The hBD2 and IL-8 response to flagellin was dependent on p38, JNK and ERK1/2 MAPK activity. Whilst EHEC induction of hBD2 expression extended from gene to protein level in human colonic biopsies, no effect of EHEC was seen on peptide expression in T84 cells. This highlights the importance of using native human tissue rather than cancer-derived cell lines when analysing the innate immune response in IECs. Finally, we found that apical exposure to EHEC and flagellin stimulated apical and not basolateral secretion of IL-8 by polarised T84 cells, suggesting that this cytokine may be secreted into the lumen during *in vivo* infection.

CHAPTER FIVE

CONCLUSION

The aim of this PhD project was to elucidate the interactions between EHEC and the human colonic epithelium. To this end, the first half of the study focussed on EHEC colonisation and attaching and effacing (A/E) lesion formation on intestinal epithelial cells (IECs), whilst the second half characterised the innate immune response to infection. In this section, the main findings from this thesis are summarised, and their contribution to current knowledge is discussed. In addition, important areas for future research are proposed.

5.1 | EHEC colonisation and A/E lesion formation

Despite a wealth of *in vitro* studies indicating the importance of EHEC adherence to IECs in disease pathogenesis, its clinical relevance has been put into question by the lack of evidence for EHEC adherence to human intestinal tissue. Colonic biopsies from EHEC-infected individuals exhibit signs of mucosal damage but not bacterial adherence, and experimental infection of human colonic tissue with EHEC using the *in vitro* organ culture (IVOC) model has not resulted in adherence or A/E lesion formation. In this study, we revisited the IVOC model and successfully demonstrated for the first time that EHEC adhere to human colonic mucosa and form typical A/E lesions at this site. Accordingly, colonisation was dependent on expression of proteins involved in A/E lesion formation (i.e. intimin, EspA, EscN). These data verify the hypothesis that EHEC causes colonic pathology by forming A/E lesions on IECs, and thereby highlights A/E proteins as important therapeutic targets in the search for a treatment for EHEC infection.

Our discovery that EHEC colonises human colonic tissue during IVOC contrasted with previous reports (Chong *et al.*, 2007; Phillips *et al.*, 2000), and this prompted us to investigate the cause of this disparity. We found that IVOC conducted under high oxygen levels, as used in previous studies, severely reduced EHEC colonisation of colonic biopsies compared with atmospheric oxygen concentrations. Therefore, through modification of the existing IVOC protocol to incorporate reduced oxygen levels, we have established a reproducible *ex vivo* model for investigating EHEC interactions with the human colonic mucosa. This optimised IVOC method can now be used to examine other less well-defined aspects of the A/E process, such as the

molecular interactions underlying initial attachment and microvillous effacement, which have so far been confounded by contrasting data from cell line experiments.

Our IVOC data suggest that oxygen may negatively regulate EHEC adherence to human colonic epithelium *in vivo*, which complements a recent study showing that microaerobiosis enhances T3S and A/E lesion formation relative to aerobic conditions (Schüller and Phillips, 2010). Given that oxygen levels in the GI lumen are markedly lower than those in the external environment, it is likely that exposure to lower oxygen concentrations upon entry into the gut serves as an environmental cue for EHEC A/E genes expression and subsequent colonisation of the colonic epithelium. Several regulatory pathways have been shown to modulate A/E gene expression (e.g. quorum sensing, acid resistance, Pch signalling), and it would be interesting to ascertain whether any of these are influenced by oxygen. In addition, other environmental factors (e.g. butyrate, biotin, pH, adrenaline, noradrenaline, AI-3, host cell contact) have been shown to affect EHEC adherence to cultured epithelial cells *in vitro*, and IVOC can now be used to verify their influence *ex vivo*.

We also examined EHEC adherence to the widely-used T84 human colonic epithelial cell line. In contrast to human biopsy tissue, EHEC adherence to polarised T84 cells was not dependent on A/E genes and did not result in formation of typical A/E lesions. This suggests that EHEC adherence to T84 cells is conferred by an A/E-independent mechanism. There are a number of potential EHEC adhesins that may mediate adherence to T84 cells, and this would be an interesting area of research to pursue. It would also be interesting to investigate why Tir is not translocated in polarised T84 cells, as this may lead to development of a method for blocking A/E lesion formation during infection. Importantly, our data demonstrates that EHEC interacts differently with IECs depending on the intestinal model used, and therefore highlights the importance of using physiologically relevant experimental systems.

Whilst published results have indicated a role for Stx in EHEC adherence to HeLa cells and colonisation of mice (Robinson *et al.*, 2006), we did not detect a significant effect of Stx on adherence to polarised T84 cells or human colonic biopsies. This suggests that Stx is not required for EHEC colonisation of human colonic epithelium *in vivo*.

5.2 | Innate immune response to EHEC infection

EHEC infection is associated with moderate colonic inflammation characterised by infiltration of neutrophils. *In vitro* studies have attributed this to production of the neutrophil chemoattractant, interleukin-8 (IL-8), by IECs in response to detection of EHEC flagellin. In addition to recruitment of innate immune cells, IECs have been shown to produce a range of antimicrobial peptides (AMPs) in response to pathogen attack, although this response has not been investigated during EHEC infection. In this part of our study, we developed a polarised colonic IVOC model (pIVOC) and used it to determine the influence of EHEC infection on expression of AMPs and IL-8. The advantage of pIVOC over IVOC is that it restricts bacterial access to the mucosal biopsy surface and thus prevents cross-stimulation of the immune cells in the lamina propria. We found that EHEC induced expression of human β-defensin-2 (hBD2) and IL-8 in a flagellin-dependent manner. This data provides the first example of EHEC regulation of innate immune gene expression in a human intestinal tissue model, and the first evidence of hBD2 induction by EHEC in any model.

Whilst the IL-8 response to EHEC is known to be mediated by the NF-κB and MAPK signal transduction pathways, the mechanisms underlying the hBD2 response to EHEC are unknown. We have shown that EHEC induction of hBD2 in T84 cells is mediated by the p38 and JNK MAPK signalling pathways, and possibly the ERK1/2 MAPK pathway to a lesser extent. An important next step would be to confirm these findings using colonic pIVOC. Our investigation into the role of NF-κB signalling in the hBD2 response to EHEC was inconclusive due to compromised epithelial barrier function caused by the chemical inhibitor. Therefore, it would be necessary to explore this further using different experimental approaches before any conclusions can be made.

hBD2 has potent bactericidal capabilities and is produced in response to a range of bacterial species, indicating an important role for this AMP in innate immune defence. Accordingly, several research groups are investigating the feasibility of utilising hBD2 and other AMPs as therapeutic agents against intestinal pathogens (Mukherjee and Hooper, 2015). Based on our findings, it would seem possible that any such hBD2-based strategy has the potential to successfully combat EHEC infection. In addition to its ability to directly kill bacteria, hBD2 has been shown to

act as a signalling molecule for lymphocytes. Given the prominent role of neutrophil recruitment in EHEC-induced inflammation, future work should focus on determining the influence of hBD2 on neutrophil migration during EHEC infection.

Whilst it has been shown that EHEC dampens the innate immune response in cervical HeLa and small intestinal Caco-2 cell lines (Hauf and Chakraborty, 2003; Khan *et al*., 2008), we did not observe a significant inhibitory effect of the T3SS on IL-8 or hBD2 expression in human colonic tissue or polarised T84 cells. Further work is needed in order to ascertain whether EHEC-mediated suppression of the innate immune response is delayed or absent during infection of human colonic epithelium.

Whilst examining protein production, we discovered that IL-8 is secreted apically by T84 cells following apical stimulation by EHEC or flagellin. This raises the possibility that the cytokine may be secreted into the lumen rather than the lamina propria, as generally assumed. Polarised secretion of cytokines by intestinal cell lines is well-documented in the literature, although most studies show that the direction is basolateral rather than apical. Therefore, at present we cannot rule out the possibility that the secretion pattern observed in T84 cells represents an artefact of the experimental system used, and it is necessary to verify this phenomenon using other intestinal models. Unfortunately, pIVOC could not be utilised for this purpose, as it is technically unfeasible to quantify IL-8 in the mucus and lamina propria. This was a major limitation of our study. Luminal secretion of IL-8 may mediate neutrophil migration across the epithelium, an event for which the causal factors have not been fully defined. In addition, the cellular mechanism for polarised secretion by IECs is currently unknown, and future research should focus on elucidating this process.

Development of the colonic pIVOC system has enabled the study of EHEC-IEC immune interactions *ex vivo*, and other interesting areas of research that may benefit from this model include the effect of EHEC infection on mucus production and the resident microflora.

5.3 | Evaluation of the IVOC model

IVOC/pIVOC can be considered a gold-standard model to study EHEC-IEC interactions, as it is the only human intestinal model that includes a preserved lamina propria, mucus layer and microbiota. However, IVOC does have some inescapable limitations. For example, it is not possible to simulate the microaerobic environment in the gut due to the need for tissue oxygenation. In addition, the use of explant material implies that only localised interactions between EHEC and the host can be studied, thus excluding the adaptive immune response and other systemic manifestations. The short lifespan of biopsy tissue limits the length of IVOC experiments, thus preventing the study of prolonged EHEC-IEC interactions. Finally, different genetic backgrounds of patients may result in high variation of results and require a higher number of experimental repeats compared with cell culture models. This can prove particularly challenging, as practical and ethical restrictions can hinder access to biopsy samples. Despite its limitations, and given the lack of a suitable alternative animal model, IVOC represents a major improvement over the use of cultured cell lines and has the potential to greatly inform our understanding of EHEC infection biology.

One of the most important findings from the colonic IVOC studies presented in this thesis is that, despite the range of adherence factors implicated in EHEC colonisation from studies using cell lines and other models, colonisation during IVOC was completely dependent on a very small set of genes found on the LEE – intimin and those encoding T3SS proteins. It is striking how dependent this bacterium is on a single genetic island for virulence, despite belonging to a species that has evolved to thrive in the human gastrointestinal environment for millions of years. Our findings raise the hope that it will soon be possible to develop a drug or vaccine capable of targeting EHEC-specific colonisation factors to combat infection without harming the gastrointestinal microbiota.

Secondly, our examination of IL-8 and AMP expression during colonic IVOC revealed that the innate immune response to EHEC infection was relatively weak. Although IL-8 and hBD2 expression was upregulated in response to EHEC infection, expression of the other four AMPs tested was unaffected. Furthermore, we saw no evidence for T3SS-mediated suppression of the innate immune response, in contrast

to previous published data showing enhanced responses to EHEC upon disablement of the T3SS (Hauf and Chakraborty, 2003; Khan *et al*., 2008). These findings suggest a less prominent role for the innate immune response than previously thought. It is possible that EHEC possess other non-T3SS-associated mechanisms for evading host immune detection, which could account for the relatively weak response. Alternatively, it may be that EHEC colonisation of the colonic epithelium *per se* does not stimulate a particularly pronounced response, perhaps due to the fact that the bacterium is non-invasive. Further investigation into the molecular interactions between EHEC and primary colonic tissue during IVOC would undoubtedly inform our understanding of the role of the host innate immune system in disease presentation and outcome.

5.4 | Summary

In summary, work from this PhD project has demonstrated for the first time that EHEC colonise the human colonic epithelium *ex vivo* and induces typical A/E lesions dependent on intimin and T3S. This process was inhibited by oxygen and not affected by Stx. In addition, apical EHEC infection induced expression of hBD2 and IL-8 *in vitro* and *ex vivo*, and flagellin was both necessary and sufficient for this response. We also present preliminary evidence of apical IL-8 secretion during infection. Marked differences in EHEC-IEC interactions were evident between the *in vitro* and *ex vivo* system used, which highlights the risks associated with relying solely on cell culture models. The development of the colonic IVOC and pIVOC systems in this study should help to accelerate the progression of EHEC research by providing a human intestinal experimental model that more closely resembles the *in vivo* situation.

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APPENDIX 1

Partners in Cancer Research

Human Tissue Bank consent form

The Norwich Biorepository

The donation, collection, storage and use of samples of tissue and/or fluids and/or other material from an adult for research

Information sheet for patients - Version 15 (21 February 2014)

Thank you for considering giving a sample for biomedical research. This information sheet provides a brief summary to help you to understand what this means and involves.

There is a consent form after the information sheet. It is important that you complete and sign it, if you decide to give us a sample. Please complete all parts of the consent form.

Doctors and other health professionals often take samples (which may be blood, small biopsies, or something else) from patients to help tell us what is wrong and how best to treat it. Larger pieces of tissue or whole organs may be removed by surgeons from some patients as part of their treatment.

Quite often, some of the test sample or surgical tissue is left over at the end. The left-over sample can be used by other doctors or researchers in special experiments to learn more about illness, how it happens and how to treat it, and sometimes to help make new medicines.

We would like to do some experiments on any left-over sample or surgical tissue, once your tests are finished. To do that, we need your permission and signed consent.

Samples donated (given) to the Norwich Biorepository are not:

- Normally used in animal research. It will be made clear to you if animal research is an integral part of the project for which we are seeking a donation.
- Used in cloning experiments. However, the Biorepository would consider the use of donations in non-reproductive cloning experiments based on their scientific value and in the context of prevailing law and ethical standards. It will be made clear to you if cloning experiments are part of the research project for which we are seeking a donation.

If you want to help us, please sign the form at the end of this document.

Continued.....

If you give permission for a sample to be taken –

- The Hospital will own the sample.
- The sample may be stored, usually in a deep freezer, until it is used. The freezer is referred to as a tissue bank in the consent form.
- Nobody involved in the research will know where the sample has come from.
- The sample will be used only in experiments that are ethical and to help other people. Please see the section entitled 'Scientific and ethical approval' below to understand what we mean by *ethical*.
- Your donated sample(s) and any genetic material derived from it (them) may be stored for an indefinite amount of time for future research projects, which may include whole genome sequencing. *Whole genome sequencing* means reading your total DNA code (your genetic blueprint) in a single assessment.
- We might give some of or all the sample to other doctors or researchers for their experiments, if they are ethical and to help other people. Some of these people might work in companies in this country or abroad.
- Data derived from your sample(s) may be placed anonymously in an international database to be used in future research. While we will take all possible steps to maintain your anonymity and protect your privacy, there is a very small risk that genetic information produced in the research and stored on databases could lead to your identification by being linked to other stored information.
- We will keep some facts about you on our Biorepository database.
- Although these facts might be given to the research doctors or scientists to help their experiments, we will NOT tell them your name or other details that would let them know who you are.
- Doctors in the Hospital might also read your hospital records to help them understand what the doctors or scientists find out in the experiments. This is possible because your hospital records can be linked to the anonymous research sample without loss of confidentiality as far as the researchers are concerned. If the research results are important for you, it might be possible using this linkage to feed back the information to your doctor, so that any appropriate action can be considered.

Your treatment will not be affected in any way, if you do not feel able to say yes to our request for a research sample.

The next sections give you some more detailed information. If you have any questions, please do not hesitate to ask the person who is seeking your permission.

WHAT WILL HAPPEN

Tissue, blood or other samples taken from you for diagnosis and/or treatment of your condition will be sent to the Pathology Laboratories, where they will be tested to decide exactly what they are and whether any further treatment will be necessary. This is a standard part of treatment. **Only as much tissue or fluid as is needed will be removed.**

Continued.....

MEDICAL RESEARCH AND WHY THIS PROGRAMME IS IMPORTANT

When all the routine tests have been done, if any samples are left over, with your consent, they could be donated (given) for use in medical research. As part of a research programme which now includes the Norfolk and Norwich University Hospitals NHS Foundation Trust, the James Paget University Hospitals NHS Foundation Trust, the University of East Anglia (UEA), and the Institute of Food Research (IFR), some of the sample or material extracted from it will be stored in the Norwich Biorepository (usually in a special deep freezer) for use by ourselves or by researchers from other centres at a later date. Some of this research may involve an assessment of genetic material (DNA and/or RNA) to help us understand the genetic basis of health and disease.

The purpose of this research is to understand more about human health and disease, and to develop new methods of prevention or new treatments for the benefit of future patient care. Some of these research programmes could lead to the development of new products and processes, which may be developed commercially for the improvement of patient care, in which case there would be no financial benefit to you.

Medically qualified doctors or other suitably qualified staff at the hospital may need to review your hospital records, including case notes, as part of this research in order to relate the research findings to the clinical outcome. It is important to be able see how you progress after the tissue or other samples you have donated (given) have been used in the research project(s). They will not give your name to those doing the research.

The research may also involve training doctors and researchers in scientific medicine, and may lead to higher qualifications for them (e.g., PhD or MD degrees). This is important for future research into diseases and for looking for new, more effective, treatments for them.

LINKS WITH OTHER ORGANISATIONS

If you agree, we may send stored material or products derived from it to other approved tissue banks or companies in this country or abroad to support their research programmes or the research programmes of those companies' clients. Such outside organisations will provide financial support for the Norwich Biorepository (our tissue bank), to help it recover its operating costs. We are not, however, allowed to sell tissue or other samples in order to make any financial profit from these commercial links.

SCIENTIFIC AND ETHICAL APPROVAL

The Norwich Biorepository acts as a custodian of the samples it holds. It releases them only to individuals or organisations that have an acceptable scientific background and work to high ethical standards. We require that all such medical research has been approved by a properly constituted Research Governance Committee before it starts. It must also be approved by a Research Ethics Committee or on behalf of the Research Ethics Committee that oversees the work of the Norwich Biorepository under the terms of the Biorepository's own Research Ethics Committee approval. That committee is the Cambridge East Research Ethics Committee. These committees look particularly at the purpose and validity of the research proposal, the welfare of any participants and issues of consent and confidentiality. We will release samples to commercial companies only if they work to appropriate ethical and scientific standards.

Continued.....

DONATING EXTRA SAMPLES FOR RESEARCH

In certain circumstances you may be asked by the doctor treating you (or by a doctor, research nurse or nurse practitioner working with him/her) to consider donating (giving) tissue or other samples in addition to those to be removed as part of your diagnostic investigation or treatment. **Such extra samples will be taken only if you give your consent and if their removal does not cause you any harmful effects now or in the future.**

YOUR RIGHTS

If your samples are stored, information about your case will be kept on a computer in the Norwich Biorepository. This will help us understand what your illness was like and relate what we find in experiments to what happens to patients. Under the Data Protection Act, you are entitled to ask to see what is recorded about you by applying to the Chairman of the Norwich Biorepository Committee, Norfolk & Norwich University Hospital, c/o Dept. of Cellular Pathology, The Cotman Centre, Colney Lane, Norwich, NR4 7UB. No one other than you has the right to see these records and any information needed for research purposes will be made anonymous before it is given to the researcher.

The researchers will not be able to find out your name or any personal details about you from the information that they receive.

You will have the opportunity to discuss with a doctor issues relating to the possible use of your samples for research purposes. He or she will answer any questions you may have.

MAKING A DONATION (GIFT) OF TISSUE AND/OR OTHER MATERIAL FOR RESEARCH

If you decide that you want your tissue, etc., to be stored in the Norwich Biorepository and used for research purposes, you will be asked to sign a special Consent Form confirming your decision and stating that you have read and understood this sheet. When you sign the form you will give the ownership of the tissue or other samples to the Norfolk & Norwich University Hospitals NHS Foundation Trust. The tissue or other samples will then belong to the Trust, which will store it for an indefinite period of time and will able to decide how it should be used for research. It will also have the right to dispose of unused stored material in an appropriate legal and ethical manner following normal procedures.

If you do not want your tissue to be stored in the Norwich Biorepository, please tell us and do NOT sign the special Consent Form.

If you do not sign this form, the tissue or other samples will still be sent to the laboratory to undergo those tests that are necessary for your care but they will not be used for research purposes. All unused tissue from your procedure will be disposed of using normal hospital methods. We will respect your decision and it will not affect in any way the treatment you receive.

Continued.....

Affix an addressograph label here or complete the following details:

Patient's name.....

Date of birth.....

Hospital no. .....

The Norwich Biorepository

Consent for the collection, storage and release of human samples for research

I agree (Please initial small box) that the following tissue or other material may be used for research, including genetic (DNA and/or RNA) studies and for the possible development of commercial products for the improvement of patient care, from which I would receive no financial benefit:

List samples for research:	

I also agree that (Please initial small boxes, as appropriate):

These samples become the property of the Norfolk & Norwich University Hospitals NHS Foundation Trust ("the Trust") ☐

The Trust may store these samples in a tissue bank / biorepository ☐

The Trust may use these samples at its discretion in properly approved research programmes ☐

The Trust may pass on these samples to other approved tissue banks and/or companies, which may be in this country or abroad, in properly approved research programmes

Yes
No

My genetic material and donated sample(s) may be stored for an indefinite amount of time for future research projects, which may include whole genome sequencing ☐

Information about my case may be kept on the Norwich Biorepository database ☐

Anonymous data derived from my sample(s) may be placed in an international database for future research ☐

Such information may be passed in an anonymous form to persons outside the Trust In connection with research and may be published with any research findings ☐

I agree that appropriately qualified staff employed by the Trust may review my hospital Medical records, including case notes, as appropriate, for the purposes of research using the donated samples ☐

These samples may be used in ethically approved animal research

Yes
No

These samples may be used in ethically approved cloning research

Yes
No

Continued.....

Affix an addressograph label here or complete the following details:

Patient's name.....

Date of birth.....

Hospital no. .....

The Norwich Biorepository

Consent for the collection, storage and release of human samples for research

I confirm that:

1) **I have read and understand the Information Sheet for Patients, Version 15, dated 21 February 2014**
2) **The issues have been explained to me, and that I have had the opportunity to ask questions.**

Signed _____ (Patient) Date _____

I have explained the request for tissue for research purposes and have answered such questions as the patient has asked.

Signed _____ Print name _____
Medical / Nursing Practitioner

Date _____

CONSENT FOR TAKING EXTRA SAMPLES FOR RESEARCH

Please initial the appropriate box for each item:	YES	NO
In addition to the removal of tissue, blood or other fluid samples as a necessary part of my procedure, I also consent to the removal of additional tissue, blood or other fluid samples from the operation site during my procedure PROVIDED THAT SUCH REMOVAL CAUSES ME NO HARM now or in the future, is limited to what I and the doctor treating me (or a research nurse or nurse practitioner delegated by him/her) have discussed and agreed, and which is specified below. * ***Please initial appropriate box***		
***Please list additional samples for research:**		

Signed _____ (Patient) Date _____

I have explained the request for the donation (gift) of extra tissue and/or other samples for research purposes and have answered such questions as the patient has asked.

Signed_____ Print name_____
Medical / Nursing Practitioner

Date _____

APPENDIX 2

Publication containing work from Chapter 3

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Enterohemorrhagic *Escherichia coli* Colonization of Human Colonic Epithelium *In Vitro* and *Ex Vivo*

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Enterohemorrhagic *Escherichia coli* (EHEC) is an important foodborne pathogen causing gastroenteritis and more severe complications, such as hemorrhagic colitis and hemolytic uremic syndrome. Pathology is most pronounced in the colon, but to date there is no direct clinical evidence showing EHEC binding to the colonic epithelium in patients. In this study, we investigated EHEC adherence to the human colon by using *in vitro* organ culture (IVOC) of colonic biopsy samples and polarized T84 colon carcinoma cells. We show for the first time that EHEC colonizes human colonic biopsy samples by forming typical attaching and effacing (A/E) lesions which are dependent on EHEC type III secretion (T3S) and binding of the outer membrane protein intimin to the translocated intimin receptor (Tir). A/E lesion formation was dependent on oxygen levels and suppressed under oxygen-rich culture conditions routinely used for IVOC. In contrast, EHEC adherence to polarized T84 cells occurred independently of T3S and intimin and did not involve Tir translocation into the host cell membrane. Colonization of neither biopsy samples nor T84 cells was significantly affected by expression of Shiga toxins. Our study suggests that EHEC colonizes and forms stable A/E lesions on the human colon, which are likely to contribute to intestinal pathology during infection. Furthermore, care needs to be taken when using cell culture models, as they might not reflect the *in vivo* situation.

Enterohemorrhagic *Escherichia coli* (EHEC) is a major cause of bacterial diarrhea in the developed world, and infections can lead to acute gastroenteritis, hemorrhagic colitis (HC), and systemic hemolytic uremic syndrome (HUS) (1–3). HC and HUS are associated with the release of bacterial Shiga toxins (Stxs), which primarily affect the kidneys and central nervous system, which express large amounts of the Stx glycolipid receptor globotriaosylceramide (Gb3) (4, 5). In contrast, the development of diarrhea is linked to a type III secretion system (T3SS), which enables the bacteria to colonize human intestinal epithelium and modulate host cell signal transduction by injecting bacterial effector proteins (6, 7). Initial events of type III secretion (T3S) comprise the formation of the EspA translocation tube and delivery of the translocated intimin receptor (Tir) into the host cell membrane (8, 9). This is followed by binding of the bacterial outer membrane adhesin intimin to Tir, which initiates formation of attaching and effacing (A/E) lesions (10). EHEC A/E lesion formation has been demonstrated in cultured cell lines and some animal models and is characterized by intimate attachment, microvillous effacement, and actin polymerization beneath adherent bacteria (11–14). Whereas microscopy has demonstrated adherent EHEC in the small intestine and the colon of gnotobiotic piglets, neonatal calves, and infant rabbits (12–14), similar direct evidence of EHEC binding to human colonic epithelium is lacking (15). This is surprising, as EHEC predominantly causes a colonic pathology in humans (15, 16), but the limited numbers of biopsy samples available in the early stages of EHEC disease, before the occurrence of extensive tissue damage, no doubt contribute to the lack of such evidence. *In vitro* organ culture (IVOC) of human endoscopic biopsy samples has been employed to investigate EHEC adherence, and these studies using Stx-negative EHEC strains and oxygen-rich culture conditions have demonstrated A/E lesion formation on the terminal ileum but not the colon (17, 18).

In the present study, we have reexamined EHEC adherence to colonic epithelium using EHEC wild-type strains and atmospheric oxygen levels (i.e., 20% atmospheric pressure). As it has previously been shown that Stxs promote EHEC adherence to HeLa cells and intestinal colonization in mice (19), we sought to determine whether Stx expression would also enable EHEC binding to human colonic epithelium. In addition, IVOC experiments are usually performed under oxygen-rich culture conditions (95% atmospheric pressure) to allow oxygen penetration into deeper tissues, but our earlier studies have demonstrated that oxygen inhibits EHEC T3S and A/E lesion formation (20), which might explain the lack of colonic adherence observed in previous IVOC studies. In addition to investigating EHEC adherence to human colonic explants, we have also included T84 human colon carcinoma cells, which are widely used as an *in vitro* model for colonic EHEC infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. Bacteria were grown while they were standing in LB broth overnight at 37°C. Deletion mutants (except EDL933 Δ*espA*) were selected with kanamycin (50 μg/ml). Bacteria were spun down before infection and suspended in serum-free culture medium.

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TABLE 1 *E. coli* strains used in the study

Name	Description	Source or reference
EDL933	Wild-type EHEC O157:H7	50
EDL933 Δ*eae*	EDL933 *eae* deletion mutant	51
EDL933 Δ*escN*	EDL933 *escN* deletion mutant	52
EDL933 Δ*espA*	EDL933 *espA* deletion mutant	53
EDL933 Δ*stx*	EDL933 stx_1 stx_2 deletion mutant	54
TUV93-0	Stx-negative derivative of EDL933	A. Donohue-Rolfe, Tufts University, USA
85-170	Stx-negative derivative of EHEC O157:H7 84-289	55
Walla-1	EHEC O157:H7	56
H0-7184-0336	EHEC O157:H7	G. Smith, Public Health England
E2348/69	EPEC O127:H6	57

Cell culture and infection. Human colon carcinoma T84 cells (ATCC CCL248) were cultured in Dulbecco's modified Eagle's medium/F-12 nutrient mixture supplemented with 10% fetal bovine serum (Sigma) and used between passages 49 and 65. Cells were seeded out in 24-well plates at a density of 10^5 cells/well and grown for 7 days for full confluence. For Transwell experiments, 5×10^5 cells/insert were seeded on collagen-coated Transwell filter inserts (diameter, 12 mm; pore size, 0.4 μm; Corning Costar). Transepithelial electrical resistance was monitored using an EVOM2 resistance meter with an STX2 electrode (World Precision Instruments), and values above 1,500 $\Omega \cdot cm^2$ after 7 to 10 days of differentiation indicated establishment of epithelial barrier function. Confluent or polarized T84 cells were infected with approximately 2×10^7 or 6×10^7 bacteria, respectively, and incubated for the time periods indicated below. Medium was exchanged at regular intervals to prevent bacterial overgrowth and acidification. Cells were incubated at 37°C in a 5% CO_2 atmosphere. At the end of the experiment, cells were washed twice in phosphate-buffered saline (PBS) to remove nonadherent bacteria and processed according to the need for further analysis.

Quantification of adherent bacteria on polarized T84 cells. Cell monolayers on filters were lysed in 1% Triton X-100 in PBS for 10 min. Serial dilutions of lysates were plated out on LB agar plates, and the numbers of CFU were determined after overnight incubation at 37°C.

***In vitro* organ culture.** This study was performed with approval from the University of East Anglia Faculty of Medicine and Health Ethics Committee (reference 2010/11-030). All samples were provided through the Norwich Biorepository, which has NRES approval (reference 10/H0310/21). Biopsy samples from the terminal ileum or transverse colon were obtained with informed consent during colonoscopy of 14 adult patients (27 to 74 years old) and 2 pediatric patients (9 and 13 years old). Samples were taken from macroscopically normal areas, transported to the laboratory in IVOC medium, and processed within the next hour. IVOC was performed as described previously (21). Briefly, biopsy samples were mounted on foam supports in 12-well plates and incubated with 25 μl of a bacterial overnight culture (approximately 10^7 bacteria). Samples were incubated in air–5% CO_2 or 95% oxygen–5% CO_2 at 37°C on a rocking platform for 8 h. At the end of the experiment, biopsy samples were washed twice in PBS to remove mucus and nonadherent bacteria and processed according to the need for further analysis.

Scanning electron microscopy. Samples were fixed with 2.5% glutaraldehyde in PBS and dehydrated through a graded acetone series. Specimens were dried using tetramethylsilane (Sigma), mounted on aluminum stubs, sputter coated with gold (Polaron SC7640 sputter coater; Quorum Technologies), and viewed with a JEOL JSM 4900 LV or Zeiss Supra 55 VP FEG scanning electron microscope. Bacterial adherence to biopsy sample epithelium was quantified by recording the presence or absence of adherent bacteria within approximately 250 fields of view of 50 by 35 μm^2 covering the whole biopsy sample surface.

Transmission electron microscopy. Biopsy samples were fixed in 2.5% glutaraldehyde in 0.1 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer, postfixed in 1% aqueous osmium tetroxide, and dehydrated through a graded ethanol series. After embedding in LR White medium-grade resin, 1-μm semi-thin sections were cut with an ultramicrotome and stained with toluidine blue to locate adherent bacteria. Ultrathin sections (90 nm) were prepared from areas of interest, stained sequentially with uranyl acetate and lead citrate, and examined in an FEI Tecnai G2 20 Twin transmission electron microscope at 200 kV.

Immunofluorescence staining. Samples were fixed in 3.7% formaldehyde in PBS for 20 min and blocked/permeabilized with 0.1% Triton X-100 and 0.5% bovine serum albumin in PBS for 20 min. Samples were subsequently incubated in primary antibodies (goat anti-*E. coli* from Abcam; rabbit anti-EspA from Gad Frankel, Imperial College London; mouse anti-Tir from John Leong, Tufts University, USA) for 60 min, washed, and incubated in Alexa Fluor-conjugated secondary antibodies (Invitrogen) for 30 min. Filamentous actin was labeled with fluorescein isothiocyanate-conjugated phalloidin (Sigma). Samples were mounted in Vectashield medium (Vector Laboratories) and analyzed using a fluorescence light microscope (Axiovert 200M; Zeiss).

Statistics. All data are shown as means ± standard errors of the means (SEMs). Statistical analysis was performed using GraphPad Prism (version 5) software. Student's *t* test or one-way analysis of variance with Tukey's multiple-comparison test was used to determine differences between two or multiple groups, respectively. A P value of <0.05 was considered significant.

RESULTS

The EHEC adherence phenotype to T84 human colon carcinoma cells is dependent on polarization status. To investigate the adherence of EHEC to T84 cells, confluent cell monolayers grown on coverslips were infected with Stx-negative strain TUV93-0 for 5 h, and the adherence phenotype was investigated by fluorescent actin staining and scanning electron microscopy. It was noted that T84 cells in the center of the monolayer showed signs of polarization, such as an actin-rich microvillous brush border, whereas cells at the margin of the coverslip appeared to be undifferentiated with few microvilli (Fig. 1). While EHEC bacteria adherent to marginal cells formed actin-rich pedestals, the bacteria on central polarized cells were not associated with polymerized actin but displayed signs of microvillous effacement (Fig. 1). Actin pedestal formation in polarized T84 cells was not impaired or obscured due to the high density of actin in the brush border, as T84 cells infected with the related A/E pathogen enteropathogenic *E. coli* (EPEC) showed actin recruitment on both polarized and nonpolarized cells (data not shown). Experiments were extended to EHEC wild-type strains EDL933 and Walla-1 using T84 cells grown on Transwell inserts. In this culture system, T84 cells reached full polarization status, as indicated by a high transepithelial electrical resistance, a column-shaped morphology, an actin-rich microvillous brush border, and the formation of tight junctions (data not shown). Infections were performed for 5 to 9 h, and no actin recruitment was observed for any of the strains tested (Fig. 2).

EHEC colonizes human terminal ileal and colonic biopsy samples. Colonization of human intestinal mucosa by wild-type EHEC was investigated by infecting terminal ileal and transverse colonic biopsy samples, taken from adults during routine endoscopy, with strains EDL933, Walla-1, or H0-7184-0336 for 8 h. Similar to T84 cell infections, IVOC was performed under atmospheric oxygen concentrations (20%). Scanning electron microscopy analysis revealed colonization of ileal and colonic biopsy

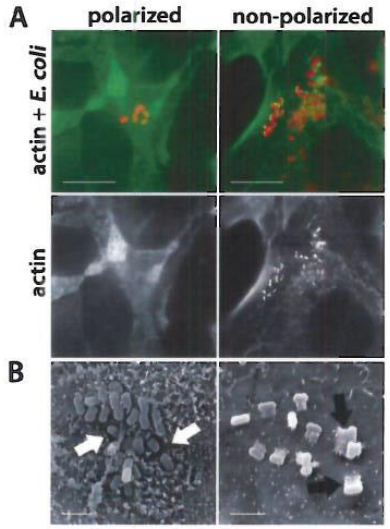

FIG 1 Different adherence phenotypes of EHEC on polarized and nonpolarized T84 cells. Confluent T84 cells on coverslips were infected with strain TUV93-0 for 5 h. Shown are representative images from two independent experiments performed in duplicate. (A) Immunofluorescence staining for actin (green) and *E. coli* (red). (Top) Merged images; (bottom) actin staining as a separate channel. Bars = 10 μm. (B) Scanning electron micrographs showing EHEC-associated microvillous effacement (white arrows) on polarized cells and actin pedestal formation (black arrows) on nonpolarized cells. Bars = 2 μm.

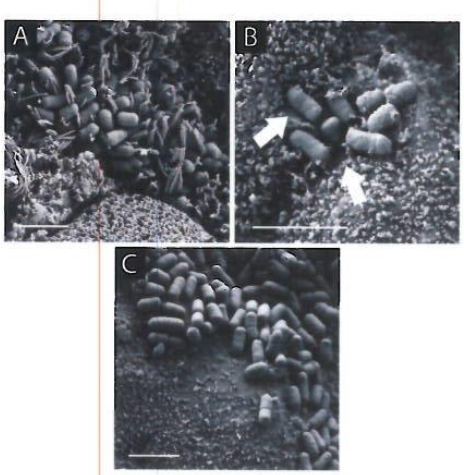

FIG 3 EHEC bacteria colonize human ileal and colonic biopsy samples. Endoscopic biopsy samples from the terminal ileum or transverse colon were infected with EDL933 for 8 h. (A) Scanning electron micrograph showing EHEC bacteria adhering to the terminal ileum and surrounded by elongated microvilli. (B) On the colon, a zone of microvillous effacement (arrows) was evident around adhering bacteria, and adjacent microvilli displayed a normal length. (C) An adherence phenotype similar to that shown in panel B was evident on pediatric colonic biopsy samples. Images are representative of those from three (A and B) and two (C) independent experiments performed in duplicate. Bars = 2 μm.

samples by all strains (Fig. 3A and B; representative images are shown for EDL933). Similar to previous IVOC studies, extensive elongation of microvilli adjacent to adherent EHEC was observed on ileal biopsy samples (Fig. 3A). On colonic biopsy samples, microvillous effacement was apparent next to adhering bacteria, and surrounding microvilli displayed a normal length, which was similar to the phenotype observed on polarized T84 cells (Fig. 3B).

Colonic EHEC colonization has not been observed in previous IVOC studies using pediatric samples (17, 18). To examine whether young donor age was the reason for the lack of EHEC adherence, we also performed IVOC experiments using pediatric biopsy samples. Scanning electron microscopy analysis of EDL933-infected colonic biopsy samples demonstrated EHEC adherence similar to that in adult tissue samples (Fig. 3C).

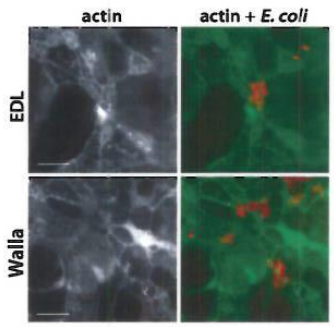

FIG 2 EHEC bacteria do not recruit actin in polarized T84 cells. T84 cells differentiated on Transwell membranes were infected with strain EDL933 or Walla-1 for 5 to 9 h. Immunofluorescence staining for actin (green) and *E. coli* (red). (Right) Merged images; (left) actin staining as a separate channel. Representative images after 9 h of infection from two independent experiments performed in duplicate. Bars = 5 μm.

EHEC colonization of human colonic epithelium is not affected by Shiga toxin production. Previous IVOC studies on Stx-negative EHEC have failed to show the direct colonization of colonic biopsy samples (17, 18). As Stxs have been implicated in EHEC adherence to human epithelial cells and colonization of mouse intestine (19), we investigated whether Stx production was required for colonic binding. IVOC of colonic biopsy samples with Stx-negative strains TUV93-0 and 85-170 (used in previous studies) was performed. As shown in Fig. 4A, both strains showed good colonic colonization with a phenotype similar to that of wild-type EHEC strains.

In addition, adherence of EDL933 and an isogenic Stx-deletion mutant to colonic biopsy samples and polarized T84 cells was quantified. Figures 4B and C show that there was no significant difference in the number of cell-associated bacteria between the two strains ($P = 0.24$ and $P = 0.1236$, respectively).

Involvement of EHEC T3S in colonic adherence. We next determined whether EHEC adherence to colonic epithelium was dependent on T3S or intimin. IVOC of colonic biopsy samples was performed using EDL933 mutants deficient in EspA (the translocation filament), EscN (the cytoplasmic ATPase of the T3SS), or intimin, and colonization was evaluated by scanning electron microscopy. As shown in Fig. 5, all mutants failed to colonize, whereas the wild type showed good adherence. Quantification of

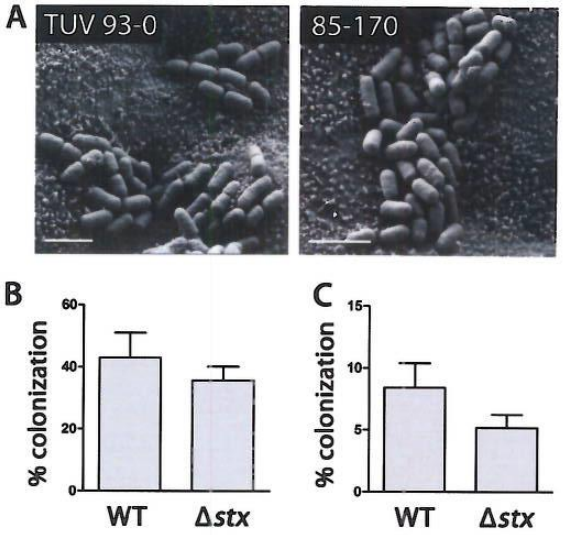

FIG 4 Colonization of colonic epithelium by EHEC is not affected by Stx production. (A) Scanning electron microscopy of biopsy samples from the transverse colon infected with Stx-negative strain TUV93-0 or 85-170 for 8 h. Images are representative of those from two independent experiments performed in duplicate. Bar = 2 μm. (B) Colonic biopsy samples were infected with wild-type (WT) EDL933 or an isogenic Stx deletion mutant (Δ*stx*) for 8 h. Samples were viewed by scanning electron microscopy, and epithelial colonization was quantified by recording the presence or absence of adherent bacteria in approximately 250 fields of view. Colonization is expressed as the percentage of the fields of view containing adherent bacteria. Data are shown as means ± SEMs from two independent experiments performed in triplicate. (C) Polarized T84 cells were infected with wild-type EDL933 or EDL933 Δ*stx* for 6 h. The numbers of adherent bacteria were quantified by plating serial dilutions of cell lysates and determining the numbers of CFU. Colonization is expressed as the percentage of adherent bacteria relative to the inoculum. Data are shown as means ± SEMs from five independent experiments performed in duplicate.

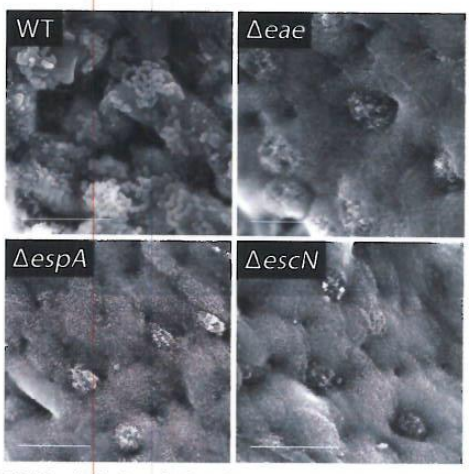

FIG 5 EHEC colonization of colonic biopsy samples is dependent on intimin and T3S. Scanning electron micrographs of biopsy samples from the transverse colon infected with wild-type (WT) EDL933 or isogenic EspA, EscN, or intimin (*eae*) mutants for 8 h. Images are representative of those from four independent experiments performed in duplicate. Bars = 10 μm.

colonized sample areas yielded 25.99% ± 7.19% for the wild type, whereas no areas with adherent bacteria (0%) were detected for any of the mutant strains. Immunofluorescence staining and transmission electron microscopy were subsequently used to evaluate A/E lesion formation. As shown in Fig. 6, adherent EHEC bacteria were associated with EspA filaments and translocated Tir and demonstrated intimate attachment and microvillous effacement. In contrast, adherence of EDL933 to polarized T84 cells was not significantly affected by the absence of EspA, EscN, or intimin (Fig. 7A). Immunofluorescence staining of EDL933-infected polarized T84 cells demonstrated the formation of EspA filaments, but translocated Tir was absent in monolayer-associated cells (Fig. 7B) and detected only in detaching cells which had lost cell polarity (data not shown).

High levels of oxygen suppress EHEC adherence and A/E lesion formation on human colonic biopsy samples. Our previous studies have demonstrated inhibition of EHEC T3S and A/E lesion formation on polarized T84 cells by oxygen (20). To investigate whether oxygen also affected EHEC A/E lesion formation on colonic biopsy samples and might explain the lack of colonization observed in previous studies (17, 18), IVOC was performed under high (95%, as in previous studies [17, 18]) or atmospheric (20%, as in this study) oxygen levels. As shown in Fig. 8, colonization of EDL933 was significantly inhibited under oxygen-rich conditions.

We also performed IVOC under microaerobic (~1.5% oxygen) conditions similar to those in the environment in the human colon but observed severe epithelial cell extrusion even on noninfected samples after 5 h of incubation (data not shown).

DISCUSSION

EHEC is considered a colonic pathogen, and the clinical histopathology of HC is predominantly observed in the ascending and transverse colon (1, 22). However, the intestinal pathogenesis of EHEC has not been well explored, and this is partly due to the lack of suitable animal model systems. Major obstacles include the failure of EHEC to efficiently colonize the mouse or rabbit intes-

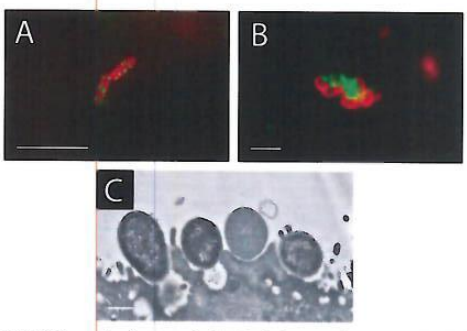

FIG 6 EHEC bacteria form typical A/E lesions on human colonic biopsy samples. Colonic biopsy samples were infected with EDL933 for 8 h. Immunofluorescence staining was performed for EspA (A) or Tir (B) in green and *E. coli* in red. (C) Transmission electron micrograph showing intimate EHEC adherence to host cell membrane and loss of microvilli. Images are representative of those from two independent experiments performed in duplicate. Bars = 2 μm (A, B) or 0.5 μm (C).

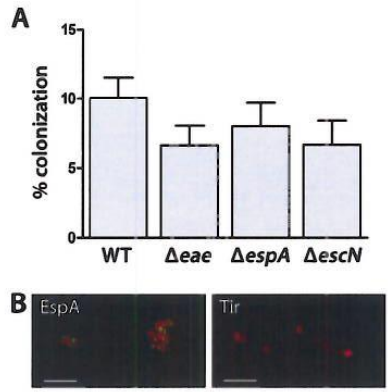

FIG 7 Adherence of EHEC to polarized T84 cells is independent of intimin and T3S and does not involve Tir translocation. (A) Polarized T84 cells were infected with wild-type (WT) EDL933 or isogenic EspA, EscN, or intimin (*eae*) mutants for 6 h. The numbers of adherent bacteria were quantified by plating serial dilutions of cell lysates and determining the number of CFU. Colonization is expressed as the percentage of adherent bacteria relative to the inoculum. Data are shown as means ± SEMs from four independent experiments performed in duplicate. (B) Immunofluorescence staining of polarized T84 cells infected with EDL933 for 6 h. Green, EspA and Tir; red, *E. coli*. Images are representative of those from two independent experiments performed in duplicate. Bars = 5 μm.

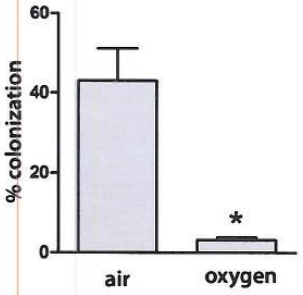

FIG 8 EHEC A/E lesion formation on colonic biopsy samples is suppressed by oxygen-rich conditions. IVOC of colonic biopsy samples with EDL933 was performed for 8 h under high (oxygen) or atmospheric (air) oxygen levels. Samples were viewed by scanning electron microscopy, and epithelial colonization was quantified by recording the presence or absence of adherent bacteria in approximately 250 fields of view. Colonization is expressed as the percentage of fields of view containing adherent bacteria. Data are shown as means ± SEMs from two independent experiments performed in triplicate. *, $P < 0.05$.

tinal tract without prior removal of the resident microflora (23) and the expression of the Stx receptor Gb3 by mouse and rabbit intestinal epithelium, in contrast to the situation in humans (24–26). Therefore, cell culture models have been widely applied, and the T84 human colon carcinoma cell line has been used in many EHEC studies, as it has the structural characteristics of colonic crypt cells (27) and, like human intestinal epithelium, does not express significant amounts of Gb3 and is resistant to Stx cytotoxicity (26).

In our study, we have found that EHEC bacteria adhering to polarized T84 cells do not form typical A/E lesions. While formation of the EspA filament and microvillous effacement were evident, no Tir translocation or actin polymerization was detected in association with adherent bacteria. In addition, EHEC colonization was not significantly affected by the absence of EspA, intimin, or T3S, which suggests the involvement of other adherence factors, such as fimbriae, autotransporters, or flagella (28). These findings are consistent with those of previous studies, which have failed to detect EHEC actin pedestals in confluent T84 cells (29, 30). Interestingly, EHEC bacteria were still able to modulate host cell signal transduction and function, such as intracellular calcium levels, epithelial barrier function, and ion transport, which suggests that T3S into polarized T84 cells can occur independently of the intimin-Tir interaction or actin polymerization (29–32).

It is currently unknown which bacterial factors cause microvillous effacement during EHEC infection, but findings on the related A/E pathogen EPEC appear to be dependent on the model system used, with adherence phenotypes even differing between Caco-2 cell subclones (33). Whereas microvillous effacement and bacterial sinking in Caco-2 cells have been reported to be dependent on intimin and Tir (34), EPEC mutants with mutations in intimin or Tir still cause microvillous effacement and effacing footprints in pediatric duodenal IVOC (35). On the other hand, EPEC microvillous effacement in porcine ileal IVOC appears to be intimin dependent but independent of Tir (36). Our study on polarized T84 cells demonstrates that EHEC effacement can occur independently of Tir translocation into the host cell membrane.

A different EHEC adherence phenotype was apparent on nonpolarized T84 cells at monolayer margins or on detaching cells, where translocated Tir and actin pedestals were observed. This could be due to the availability of phosphatidylethanolamine or other receptors for EHEC binding which become exposed on the cell surface after apoptosis or cell shedding (37, 38). The ability of EPEC to form actin pedestals on polarized T84 cells suggests that this pathogen uses different receptors for initial binding than EHEC and that these receptors are readily expressed on the apical cell membrane. Another possibility for the failure of EHEC to form actin pedestals on polarized T84 cells might be related to particular properties of the apical T84 cell membrane which would prevent proper EHEC Tir insertion or clustering by intimin.

Despite the presence of colonic pathology, it has been controversial whether EHEC can colonize human colonic epithelium *in vivo*, as adherent bacteria have not been reported during clinical infections (15, 39). It has been argued that this may be because of the progressed stage of disease at the time of endoscopy, when bacterial adhesion may have diminished or be difficult to identify due to extensive tissue damage (1, 17). In contrast, EHEC infections in gnotobiotic piglets, infant rabbits, and neonatal calves have shown colonization of the terminal ileum, cecum, and colon (12–14, 40). Adherent bacteria were associated with characteristic A/E lesions accompanied by intimate attachment and loss of microvilli, and adherence was dependent on intimin-Tir interaction (12, 13, 41). Ileal and colonic A/E lesions have also been reproduced in bovine intestinal IVOC and shown to be dependent on Tir (42). In contrast, human IVOC studies using pediatric biopsy samples have demonstrated EHEC binding and A/E lesion formation on terminal ileum but not colon (17, 18). However, some minimal nonintimate adherence to colonic explants was observed after previous incubation of EHEC with terminal ileal biopsy sam-

ples (17). These findings have led to the hypothesis that EHEC initially colonizes the terminal ileum and Peyer's patches, where bacteria are primed for subsequent spread and infection of the colon. Similar colonization dynamics have been described for the mouse A/E pathogen *Citrobacter rodentium*, which demonstrates primary adherence to the lymphoid cecal patch before establishing colonization of the colon (43). Interestingly, a recent study using human intestinal xenografts in mice has reported T3S-dependent EHEC A/E lesion formation on human colon but not on small intestine (44).

In our study, we have found EHEC colonization of human terminal ileum and colon *ex vivo*. Typical A/E lesions similar to those previously described on terminal ileal biopsy samples were observed on colonic explants, demonstrating intimate attachment, microvillous effacement, and Tir translocation beneath adherent bacteria (18, 45). Interestingly, colonic A/E lesions were not accompanied by elongation of the surrounding microvilli, as observed on terminal ileum. This has also been observed on bovine IVOC and human intestinal xenografts and might reflect differences in the organization of the brush border cytoskeleton in the small intestine and colon (42, 44). Similar to previous human intestinal xenograft and animal studies, A/E lesion formation on human colonic explants was dependent on T3S and intimin (13, 41, 44).

In addition to Tir, the host cell protein nucleolin has been described to be a host receptor for intimin (46), and previous studies have shown that Stxs enhance EHEC adherence to HeLa cells and intestinal colonization of mice by inducing surface expression of nucleolin (19). As former human IVOC studies have been performed with Stx-negative EHEC strains (17, 18), we investigated whether Stxs could promote colonic adhesion. Our findings on Stx-negative mutants showed that Stx production did not significantly affect EHEC adherence to human colonic epithelium, which agrees with previous results in infant rabbits, where Stx expression did not alter colonization levels (13).

Other differences from earlier human IVOC studies by Phillips and colleagues (17, 18) which might explain the discrepancies in colonic colonization include the use of adult versus pediatric biopsy samples and lower oxygen concentrations during IVOC. Whereas the influence of age on EHEC colonic infection has not been investigated, IVOC studies with EPEC have demonstrated no significant difference in EPEC binding to adult versus pediatric biopsy samples (47). As we also observed EHEC colonization of pediatric colonic biopsy samples, age was not the determining factor for our findings.

In contrast, we found that the high oxygen levels (95%) commonly used in IVOC to ensure sufficient tissue oxygenation and survival (21, 48) suppressed EHEC adherence to colonic biopsy samples. This is in agreement with the findings of our previous study demonstrating that lower oxygen levels promote EHEC adherence and T3S on polarized T84 cells (20). Similar results have been reported for bovine intestinal IVOC, where the EHEC colonization observed with air (20% oxygen) was improved compared with that achieved with 95% oxygen without compromising tissue integrity (42). Lower oxygen levels are also likely to explain EHEC A/E lesion formation in human colonic xenografts (44). Interestingly, high oxygen levels did not abolish EHEC A/E lesion formation on terminal ileal biopsy samples (17, 18), suggesting higher levels of adherence to the small intestine than to the colon. This might be related to a thinner mucus layer with less microbiota and easier access to the epithelium (49).

In summary, our study demonstrates for the first time that EHEC forms typical A/E lesions on human colon *ex vivo* which are dependent on T3S and intimin. Importantly, A/E lesion formation is dependent on oxygen levels and suppressed by the oxygen-rich culture conditions generally used in IVOC. In contrast, adherence to polarized T84 cells is mediated by factors other than EspA and intimin and does not involve Tir translocation into the host cell membrane. This study emphasizes the difference between cell culture experiments and more relevant model systems, such as IVOC, and suggests that during human infection EHEC forms stable A/E lesions which are likely to contribute to colonic pathology.

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APPENDIX 3

Publication containing work from Chapter 4

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
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Flagellin Induces β-Defensin 2 in Human Colonic *Ex vivo* Infection with Enterohemorrhagic *Escherichia coli*

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Enterohemorrhagic *E.*coli (EHEC) is an important foodborne pathogen in the developed world and can cause life-threatening disease particularly in children. EHEC persists in the human gut by adhering intimately to colonic epithelium and forming characteristic attaching/effacing lesions. In this study, we investigated the innate immune response to EHEC infection with particular focus on antimicrobial peptide and protein expression by colonic epithelium. Using a novel human colonic biopsy model and polarized T84 colon carcinoma cells, we found that EHEC infection induced expression of human β-defensin 2 (hBD2), whereas hBD1, hBD3, LL-37, and lysozyme remained unchanged. Infection with specific EHEC deletion mutants demonstrated that this was dependent on flagellin, and apical exposure to purified flagellin was sufficient to stimulate hBD2 and also interleukin (IL)-8 expression *ex vivo* and *in vitro*. Flagellin-mediated hBD2 induction was significantly reduced by inhibitors of NF-κB, MAP kinase p38 and JNK but not ERK1/2. Interestingly, IL-8 secretion by polarized T84 cells was vectorial depending on the side of stimulation, and apical exposure to EHEC or flagellin resulted in apical IL-8 release. Our results demonstrate that EHEC only induces a modest immune response in human colonic epithelium characterized by flagellin-dependent induction of hBD2 and low levels of IL-8.

Keywords: EHEC, flagellin, colon, β-defensin, interleukin-8, inflammation

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INTRODUCTION

Enterohemorrhagic *E. coli* (EHEC) is a foodborne pathogen of worldwide importance (Croxen et al., 2013). Although infections with EHEC are rare compared with *Campylobacter* and *Salmonella*, they can lead to severe systemic hemolytic uremic syndrome (HUS) resulting in kidney failure and death (Tarr et al., 2005). EHEC mainly affects young children and the elderly, and the predominant serotype in most parts of the world is O157:H7 (Croxen and Finlay, 2010).

EHEC causes diarrhea by adhering to human intestinal epithelium, particularly the colon, and forming attaching and effacing (A/E) lesions (Golan et al., 2011; Lewis et al., 2015). These are characterized by intimate bacterial attachment to the host cell membrane and effacement of underlying microvilli. A/E lesion formation is dependent on a bacterial type III secretion system (T3SS) which enables EHEC to inject effector proteins into the host cell (Jarvis and Kaper, 1996). Around 50 EHEC effector proteins have been identified so far (Tobe et al., 2006), affecting many different host cell functions such as maintenance of epithelial barrier integrity, water and ion transport, and immune response to infection (Viswanathan et al., 2009). In addition to the T3SS, EHEC also produces Shiga

toxins (Stxs) which are linked to HUS and strongly cytotoxic to kidney cells (Obrig and Karpman, 2012). After release into the gut lumen, Stxs transverse the intestinal epithelium and are transported to the kidneys via the bloodstream (Schüller, 2011).

EHEC infection in the human gut is accompanied by neutrophil recruitment to the intestinal mucosa (Griffin et al., 1990; Kelly et al., 1990), and high levels of the neutrophil chemoattractant interleukin (IL)-8 have been detected in HUS patients (Fitzpatrick et al., 1992; Murata et al., 1998) indicating a key role of this cytokine in the innate immune response to EHEC infection. In addition to cytokine production which recruits phagocytes to the site of infection, secretion of antimicrobial peptides and proteins (AMPs) such as defensins, cathelicidins, and lysozyme by intestinal epithelium constitutes an important part in the innate immune defense against intestinal pathogens. Pore-forming AMPs directly kill bacteria and also promote an inflammatory immune response by acting as chemoattractants (Wassing et al., 2014). Human colonic epithelium, the primary target site of EHEC, produces lysozyme and LL-37, which is the only cathelicidin in humans and expressed by upper crypt epithelial cells. In addition, four types of defensins are secreted by human colonic epithelium: While human β-defensin (hBD)1 is constitutively expressed, hBD2-4 are induced by infection and inflammation (Eckmann and Kagnoff, 2005; Muniz et al., 2012).

Previous studies have demonstrated that infection with intestinal pathogens can modulate expression of AMPs and cytokines by the host epithelium, thereby resulting in an induced or suppressed immune response (Islam et al., 2001; Zilbauer et al., 2005; Sharma et al., 2006; Sperandio et al., 2008). In this study, we have investigated the influence of EHEC infection on AMP and IL-8 expression in physiologically relevant colonic biopsy and polarized cell culture models.

MATERIALS AND METHODS

Bacterial Strains and Flagellin

Bacterial strains used in this study are listed in **Table 1**. Bacteria were grown standing in LB broth overnight at 37°C. Deletion mutants were selected with kanamycin (50 μg/ml) or chloramphenicol (30 μg/ml). Bacteria were spun down before infection and suspended in serum-free culture medium. LPS-free purified H7 monomeric flagellin was kindly provided by David Gally, Roslin Institute, Edinburgh.

TABLE 1 | *E. coli* strains used in this study.

Name	Description	Source or references
EDL933	Wild-type EHEC O157:H7	Riley et al., 1983
EDL933 Δ*escN*	EDL933 *escN* deletion mutant	Jarvis and Kaper, 1996
EDL933 Δ*fliC*	EDL933 *fliC* deletion mutant	Gobert et al., 2008
EDL933 Δ*stx*	EDL933 *stx1 stx2* deletion mutant	Gobert et al., 2007
85–170	Stx-negative EHEC O157:H7	Tzipori et al., 1987
85–170 Δ*hcpA*	85–170 *hcpA* deletion mutant	Xicohtencatl-Cortes et al., 2007
85–170 Δ*lpfA1*	85–170 *lpfA1* deletion mutant	Fitzhenry et al., 2006
Walla-1	EHEC O157:H7	Ostroff et al., 1990
H0-7184-0336	EHEC O157:H7	G. Smith, Public Health England

Cell Culture and Infection

Human colon carcinoma T84 cells (ATCC CCL-248) were cultured in DMEM/F-12 mixture supplemented with 10% fetal bovine serum (Sigma) and used between passage 45 and 65. To obtain polarized monolayers, cells were seeded out on collagen-coated Transwell filter inserts (12 mm diameter, 0.4 μm pore; Corning Costar) at a density of 5×10^5 cells/insert. Transepithelial electrical resistance (TER) was monitored using an EVOM2 resistance meter with an STX2 electrode (World Precision Instruments), and values above 1500 $\Omega \times cm^2$ after 7–10 d of differentiation indicated establishment of epithelial barrier function. Polarized T84 cells were infected with approximately 6×10^7 bacteria in plain DMEM/F-12 medium and incubated for 3 h. After that, medium was exchanged every hour to prevent bacterial overgrowth and acidification of the medium. For 24 h infections, gentamicin (200 μg/ml, Sigma) was added 1 h after infection to slow down bacterial growth, and incubations were continued without medium exchange for up to 24 h. For incubations with flagellin and IL-1β (Sigma), no medium exchange was performed. For signal transduction studies, cells were incubated with the chemical inhibitors quinazoline (NF-κB) (28 μM, Sigma), SB203580 (p38), SP600125 (JNK), or PD98059 (ERK 1/2) (25 μM, Merck Millipore) for 1 h before flagellin (0.1 μg/ml) was added for 6 h. Cells were incubated at 37°C in a 5% CO_2 atmosphere. At the end of the experiment, cells were washed twice in PBS to remove non-adherent bacteria and processed according to further analysis.

Polarized *In vitro* Organ Culture (pIVOC)

This study was performed with approval from the University of East Anglia Faculty of Medicine and Health Ethics Committee (ref 2010/11-030). All samples were provided through the Norwich Biorepository, which has NRES approval (ref 08/h0304/85+5). Up to four biopsy samples from the transverse colon were obtained with informed consent during colonoscopy of 23 adult patients (24–77 years old) during routine investigation of potential intestinal disorders. Samples were taken from macroscopically normal areas, transported to the laboratory in IVOC medium and processed within the next hour. Polarized IVOC was performed as described previously (Schüller et al., 2009). Briefly, biopsies were mounted mucosal side upwards on a circular cellulose nitrate filter and sandwiched between two Perspex disks with a 2 mm central aperture (manufactured by the School of Environmental Sciences workshop, University of East Anglia). To prevent bacterial leakage, the apical disk was sealed to the mucosal side of the biopsy with Histoacryl tissue glue (Braun Medical). The sandwich holding the biopsy was then mounted in a Snapwell support (Corning Costar) and inserted in a six-well culture plate. Apical and basal compartments were filled with IVOC medium, and 20 μl of bacterial overnight culture (~10^7 bacteria), purified monomeric H7 flagellin or IL-1β were added apically. A specimen inoculated with medium only was included with each experiment to exclude *in vivo* bacterial colonization.

Samples were incubated in a 5% CO_2 atmosphere at 37°C on a rocking platform for 7 h. Bacterial leakage into the basolateral compartment was assessed by bacterial growth and turbidity of the medium, and respective samples were excluded from analysis. At the end of the experiment, biopsies were removed from the Snapwell support, washed in PBS to remove mucus and non-adherent bacteria and processed for further analysis.

RNA Isolation and Quantitative Real-Time PCR (qPCR) Analysis

Total RNA from cells and biopsies was isolated using the RNeasy Mini kit with on-column DNase digestion (Qiagen) according to the manufacturer's instructions. Biopsy tissue was homogenized with a pestle (Kimble Chase) before extraction. RNA quality was assessed by gel electrophoresis and $OD_{260/280}$ determination. RNA was quantified using a Nanodrop ND-1000 spectrophotometer, and 1 μg RNA was converted to cDNA using the qScript cDNA supermix (Quanta Biosciences) in a 20 μl reaction. Quantitative real-time PCR was performed using an ABI 7500 PCR system (Applied Biosystems). Primers were purchased from Sigma-Genosys. Gene-specific sequences (**Table 2**) were obtained from published studies or designed using PrimerBLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Up to 4 μl cDNA were amplified in a 10 μl reaction containing 0.5 μM of each primer and 5 μl of 2 × SYBR Green JumpStart Taq ReadyMix (Sigma). Cycling parameters were as follows: 2 min at 95°C (initial denaturation); 30 s at 95°C, 30 s at 60°C, 35 s at 72°C (40 cycles); 5 min at 72°C (final elongation); 15 s at 95°C, 60 s at 60°C, 15 s at 95°C, 15 s at 60°C (dissociation for melt curve analysis). PCR product specificity was confirmed by melt curve analysis and agarose gel electrophoresis. Relative quantification of gene expression was performed using the comparative Ct method. Ct values for genes of interest were normalized using the geometric mean Ct of two housekeeper genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and RNA polymerase II polypeptide A (POLR2A). Fold expression levels in treated samples were calculated relative to matched non-treated controls using the formula $2^{-\Delta\Delta Ct}$.

TABLE 2 | Primer sequences used in this study.

Target	Primer sequence	References
hBD1	F 5′-CTGCTGTTTACTCTCTGCTTACTTTT-3′	Fahlgren et al., 2003
	R 5′-CCTCCACTGCTGACGCA-3′	
hBD2	F 5′-CTCGTTCCTCTTCATATTCCTGA-3′	Fahlgren et al., 2003
	R 5′-CTAGGGCAAAAGACTGGATGAC-3′	
hBD3	F 5′-TGAAGCCTAGCAGCTATGAGGATC-3′	Fahlgren et al., 2004
	R 5′-CCGCCTCTGACTCTGCAATAA-3′	
hBD4	F 5′-CCCAGCATTATGCAGAGACTT-3′	Fahlgren et al., 2004
	R 5′-ACCACATATTCTGTCCAATTCAAAT-3′	
IL-8	F 5′-TTGAGAGTGGACCACACTGC-3′	Ou et al., 2009
	R 5′-TGCACCCAGTTTTCCTTGG-3′	
GAPDH	F 5′-AGGTCGGAGTCAACGGATTT-3′	Schüller et al., 2009
	R 5′-TGGAAGATGGTGATGGGATTT-3′	
LL-37	F 5′-GTGCCCCAGGACGACACAGC-3′	This study
	R 5′-CCCCTGGCCTGGTTGAGGGT-3′	
Lysozyme	F 5′-AAAACCCCAGGAGCAGTTAAT-3′	Fahlgren et al., 2003
	R 5′-CAACCCTCTTTGCACAAGCT-3′	
POLR2A	F 5′-GATGGGCAAAAGAGTGGACTT-3′	Schüller et al., 2009
	R 5′-GGGTACTGACTGTTCCCCCT-3′	

Forward (F) and reverse (R) primer sequences.

Scanning Electron Microscopy

Samples were fixed with 2.5% glutaraldehyde in PBS and dehydrated through graded acetone series. Specimens were dried using tetramethylsilane (Sigma), mounted on aluminum stubs, sputter-coated with gold (Polaron SC7640 sputter coater, Quorum Technologies), and viewed with a JEOL JSM 4900 LV scanning electron microscope.

Immunofluorescence Staining

Biopsy samples were fixed in 3.7% formaldehyde in PBS for 30 min, cryoprotected in 15 and 30% sucrose in PBS for 10 min, embedded in OCT compound (Sakura), snap-frozen in a dry ice/ethanol bath and stored at −70°C until use. Serial sections of 7 μm were cut with a Microm HM550 cryostat (Thermo Scientific), picked up on poly L-lysine-coated slides and air-dried. Tissue sections were blocked with 0.5% BSA in PBS for 20 min. Cryosections were subsequently incubated in rabbit anti-hBD2 (abcam) overnight at 4°C, washed and incubated in Alexa Fluor 488-conjugated secondary antibody (Life Technologies) for 30 min. Cell nuclei were counterstained with DAPI (Roche).

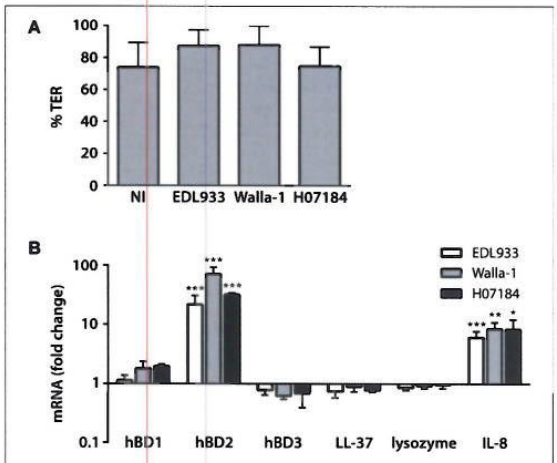

FIGURE 1 | EHEC infection induces hBD2 and IL-8 expression in polarized T84 cells. Polarized T84 cells were apically infected with EHEC strains EDL933, Walla-1, H07184, or left non-infected (NI) for 9 h. **(A)** EHEC infection did not compromise epithelial barrier function. TER after infection is expressed as percentage of TER before infection. **(B)** AMP and IL-8 mRNA levels were quantified by qPCR and expressed as fold changes relative to NI controls. Data are shown as means ± standard errors of the means (SEM) of four independent experiments performed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. NI control.

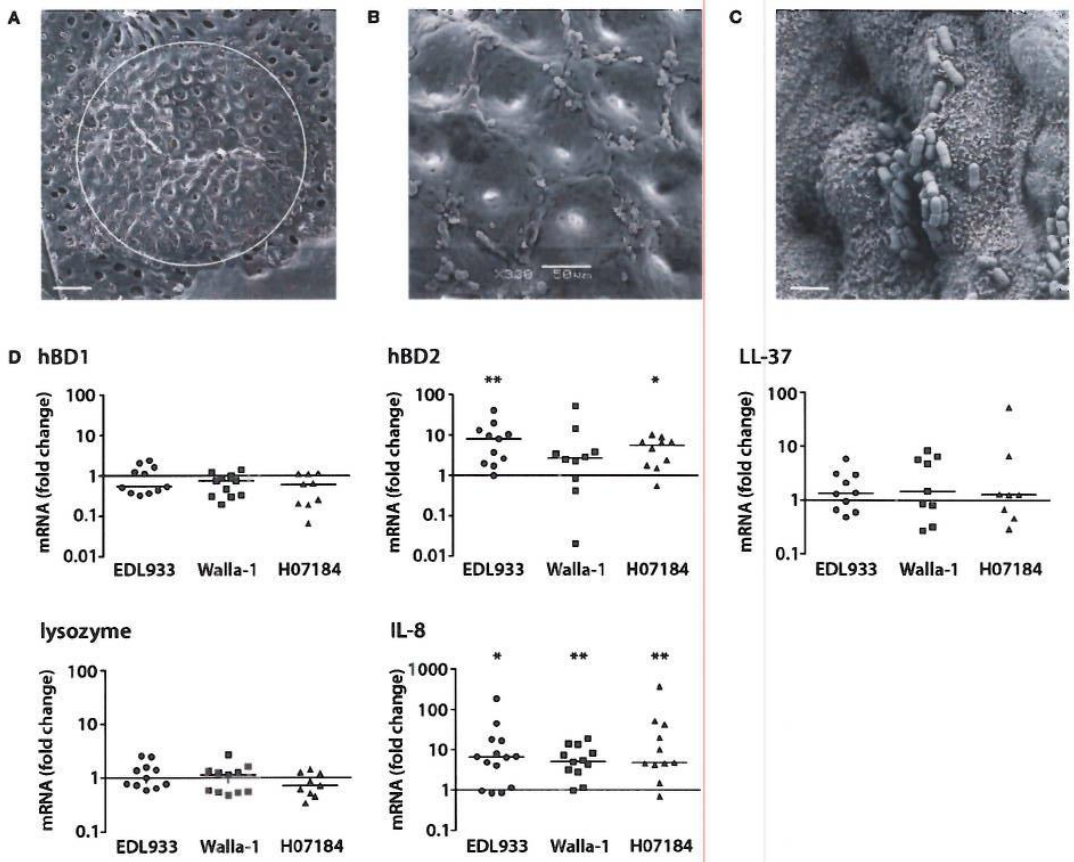

FIGURE 2 | EHEC induction of hBD2 and IL-8 in a polarized human colonic biopsy model. Human colonic biopsy samples were sandwiched between two Perspex disks with a central aperture and mounted in Snapwell supports as described in Experimental Procedures. Mucosal sides of matched biopsies were infected with EHEC strains EDL933, Walla-1, H07184, or left NI for 7 h. **(A)** Scanning electron micrograph of colonic biopsy after 7 h of incubation. The white circle indicates the central aperture of the disk. Bar = 200 μm. **(B)** Intact colonic surface epithelium at higher magnification. Bar = 50 μm. **(C)** EDL933 intimately adhering to colonic biopsy epithelium. Bar = 2 μm. Images are representative of three experiments performed in duplicate. **(D)** AMP and IL-8 mRNA levels were quantified by qPCR and expressed as fold changes relative to matched NI controls. Data points represent individual biopsies from a total of six patients. Medians are indicated by a line. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. NI control.

Samples were mounted in Vectashield (Vector Laboratories) and analyzed using a fluorescence light microscope (Axiovert 200 M, Zeiss).

IL-8 ELISA

Polarized T84 cells were lysed on ice in 1% Triton X-100 and 1μl/200 μl protease inhibitor cocktail (Sigma) in PBS, and Triton-insoluble proteins were removed by centrifugation. IL-8 concentrations in lysates and supernatants were determined using a human IL-8 ELISA kit (PeproTech) according to the manufacturer's instructions.

Statistics

Statistical analysis was performed using GraphPad Prism software (version 5). qPCR data were log transformed before analysis. For parametric T84 cell data, one-way ANOVA with Tukey's multiple comparisons test was used to determine differences between multiple groups. For non-parametric biopsy data, Wilcoxon's signed-rank test or Kruskall–Wallis with Dunn's multiple comparisons test was used to determine differences between two or multiple groups, respectively. A $P < 0.05$ was considered significant.

RESULTS

Apical EHEC Infection of Polarized T84 Cells Induces hBD2 and IL-8 Expression

To determine the influence of EHEC infection on AMP and IL-8 expression by human colonic epithelium, polarized T84 human colon carcinoma cells were infected with EHEC O157:H7

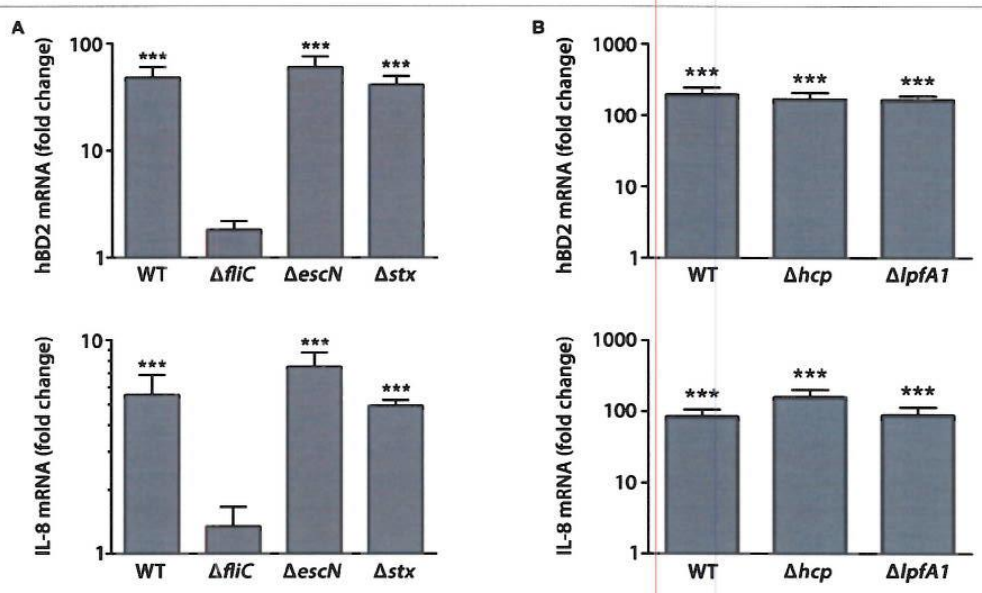

FIGURE 3 | EHEC induction of hBD2 and IL-8 expression in polarized T84 cells is dependent on flagellin. Polarized T84 cells were apically infected with EHEC EDL933 wild-type (WT) and isogenic deletion mutants in *fliC*, *escN*, or *stx* **(A)** or EHEC 85–170 wild-type (WT) and isogenic deletion mutants in *hcp* or *lpfA1* **(B)** for 9 h. IL-8 and hBD2 mRNA levels were quantified by qPCR and expressed as fold changes relative to NI controls. Data are shown as means ± SEM of three independent experiments performed in duplicate. ***$P < 0.001$ vs. NI control.

strains EDL933, Walla-1, and H0-7184-0336 (H07184) on the apical side. Bacterial overgrowth and loss of epithelial integrity was prevented by removal of bacteria after 3 h and subsequent medium exchange at hourly intervals. As shown in **Figure 1A**, this protocol resulted in maintenance of epithelial barrier function for up to 9 h as determined by TER. Expression of hBD1-4, LL-37, lysozyme, and IL-8 was determined by qPCR analysis. Results in **Figure 1B** show that apical EHEC infection resulted in a significant induction of hBD2 (21.5 ± 9.1 fold for EDL933, 70.4 ± 21.8 fold for Walla-1, and 31.5 ± 2.0 fold for H07184) and IL-8 (6.0 ± 1.6 fold for EDL933, 8.3 ± 2.4 fold for Walla-1, and 8.2 ± 3.9 fold for H07184) mRNA expression compared with non-infected (NI) controls whereas no significant change was observed for hBD1, hBD3, LL-37, and lysozyme. No specific amplification product was detected for hBD4. Subsequent kinetic analysis of hBD2 and IL-8 mRNA expression indicated that levels of both transcripts were first significantly induced at 6 h post-infection and showed a continuous increase during a 12 h period of infection (**Supplementary Figure 1**). For practical reasons, infections of polarized T84 cells were performed for 9 h in subsequent experiments.

Apical EHEC Infection Results in hBD2 and IL-8 Induction in a Polarized Human Colonic Biopsy Model

Our recent studies have demonstrated EHEC adherence to human colonic biopsy epithelium by forming typical A/E lesions (Lewis et al., 2015). To investigate the effect of EHEC infection on AMP and IL-8 expression *ex vivo*, a pIVOC model, which restricts bacterial access to the mucosal side of the biopsy, was developed. This was based on the pIVOC system established earlier to investigate the inflammatory response in duodenal biopsies (Schüller et al., 2009). Colonic biopsy samples were mounted in Snapwell supports, infected with EDL933, Walla-1, or H07184 on the mucosal side and incubated for up to 7 h. Tissue preservation and EHEC adherence were evaluated by scanning electron microscopy. As shown in **Figure 2A**, good tissue morphology was observed within the area of the central aperture (white circle) with intact surface epithelium visible at higher magnifications (**Figure 2B**). In addition, EHEC adhered intimately to colonic epithelium as described previously (**Figure 2C**; Lewis et al., 2015). Analysis of AMP and IL-8 expression by qPCR demonstrated a significant increase in hBD2 (median fold increase = 8.1 for EDL933 and 5.7 for H07184) and IL-8 expression (median fold increase = 6.6 for EDL933, 5.2 for Walla-1, and 4.8 for H07184) in EHEC-infected samples compared with non-infected controls. In contrast, transcript levels of hBD1, LL-37, and lysozyme were not significantly affected by EHEC infection (**Figure 2D**). Amplification levels for hBD3 and hBD4 were generally below detection threshold levels. However, amplification products of corresponding sizes were detected in some experiments, thereby confirming primer specificity and functionality of the qPCR assay (data not shown).

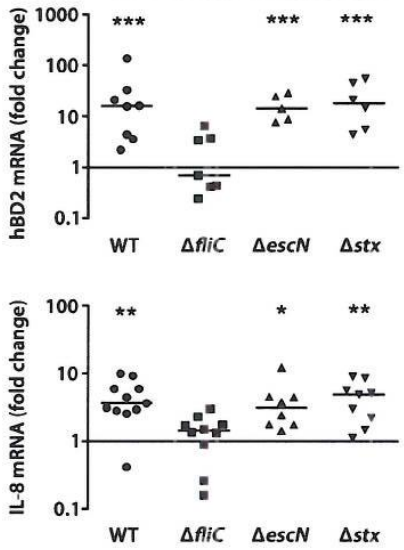

FIGURE 4 | EHEC stimulation of IL-8 and hBD2 expression in colonic biopsies requires flagellin. Colonic biopsies were infected with EHEC EDL933 wild-type (WT), isogenic deletion mutants in *fliC*, *escN*, or *stx* or left NI on the mucosal side, and incubations were performed for 7 h. IL-8 and hBD2 mRNA levels were quantified by qPCR and expressed as fold changes relative to matched NI controls. Data points represent individual biopsies from a total of four patients. Medians are indicated by a line. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. NI control.

EHEC Induction of Colonic Epithelial hBD2 and IL-8 Expression Is Dependent on Flagellin

We next determined which EHEC proteins were involved in hBD2 and IL-8 induction in colonic epithelium. Polarized T84 cells were apically infected with wild-type (wt) EDL933 or isogenic deletion mutants in *fliC* (flagellin, main component of EHEC flagellum), *escN* (cytoplasmic ATPase of T3SS), or *stx* (Stxs) for 9 h. In addition, the contribution of long polar fimbriae (Lpf) and hemorrhagic coli pili (HCP) was examined by using isogenic deletion mutants in *lpfA1* and *hcp* of EHEC strain 85–170. None of the strains significantly compromised epithelial barrier function as determined by TER (**Supplementary Figure 2A**). Expression levels of hBD2 and IL-8 were determined by qPCR. Whereas, infection with EDL933 wt, Δ*escN*, and Δ*stx* showed a significant induction of hBD2 (48.6 ± 12.3 fold for wt, 60.5 ± 15.6 fold for Δ*escN*, and 41.5 ± 8.3 fold for Δ*stx*) and IL-8 (5.6 ± 1.3 fold for wt, 7.5 ± 1.2 fold for Δ*escN*, and 4.9 ± 0.3 fold for Δ*stx*) expression vs. NI controls, no significant effect was observed in EDL933 Δ*fliC*-infected cells (**Figure 3A**). For infections with 85–170, all strains significantly increased expression of hBD2 (195.9 ± 50.1 fold for wt, 168.6 ± 40.4 fold for Δ*hcp*, and 163.4 ± 26.8 fold for Δ*lpfA1*) and IL-8 (84.3 ± 21.8 fold for wt, 157.5 ± 45.7 fold for Δ*hcp*, and 88.4 ± 28.0

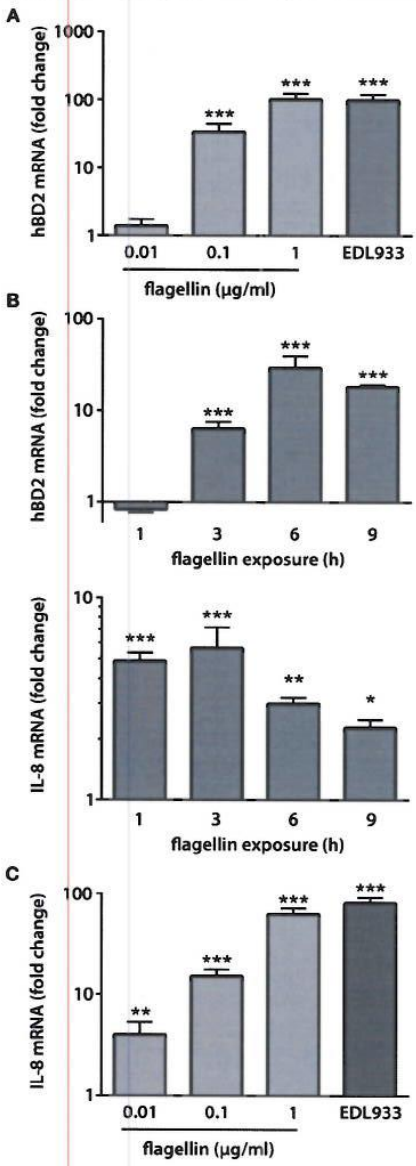

FIGURE 5 | EHEC flagellin is sufficient to induce hBD2 and IL-8 expression in polarized T84 cells. Polarized T84 cells were incubated with EDL933, purified flagellin or left NI on the apical side. IL-8 and hBD2 mRNA levels were quantified by qPCR and expressed as fold changes relative to NI controls. **(A)** Purified flagellin induced a dose-dependent increase in hBD2 mRNA expression after 9 h. Data are shown as means ± SEM of three independent experiments performed in duplicate. **(B)** Kinetics of IL-8 and hBD2 induction by purified flagellin (0.1 μg/ml). Data are shown as means ± SEM of two independent experiments performed in duplicate. **(C)** Purified flagellin induced a dose-dependent increase in IL-8 mRNA expression after 2 h. Data are shown as means ± SEM of two independent experiments performed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. NI control.

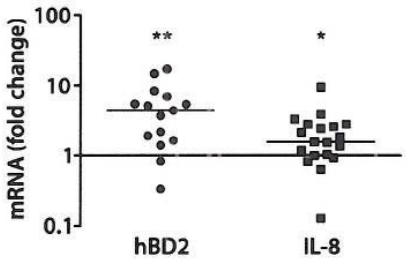

FIGURE 6 | EHEC flagellin induces hBD2 and IL-8 expression in colonic biopsies. Colonic biopsies were incubated with purified flagellin (1 μg/ml) or left NI on the mucosal side for 7 h. IL-8 and hBD2 mRNA levels were quantified by qPCR and expressed as fold changes relative to matched NI controls. Data points represent individual biopsies from a total of four patients. Medians are indicated by a line. *$P < 0.05$, **$P < 0.01$ vs. NI control.

fold for Δ*lpfA1*) vs. NI controls (**Figure 3B**). Notably, induction levels of hBD2 and IL-8 expression by EHEC strain 85–170 were considerably higher compared with EDL933 (**Figure 3**).

We also determined the effect of flagellin, T3S and Stxs on hBD2 and IL-8 expression in colonic pIVOC. As shown in **Figure 4**, EDL933 wt, Δ*escN*, and Δ*stx* significantly enhanced hBD2 (median fold increase = 15.9 for wt, 14.3 for Δ*escN*, and 17.8 for Δ*stx*) and IL-8 (median fold increase = 3.6 for wt, 3.1 for Δ*escN*, and 4.9 for Δ*stx*) expression in colonic biopsies whereas no significant effect was observed for the Δ*fliC* mutant.

Purified Flagellin Induces hBD2 and IL-8 Expression in Colonic Epithelium

To examine whether EHEC flagellin alone was sufficient to increase hBD2 and IL-8 expression, polarized T84 cells were infected with EDL933 or incubated with different concentrations of purified LPS-free monomeric H7 flagellin on the apical side for 9 h. No significant effect on TER was observed under any of the conditions tested (**Supplementary Figure 2A**). As demonstrated in **Figure 5A**, a dose-dependent increase in hBD2 mRNA expression was observed after 9 h of incubation with similar induction levels for 1 μg/ml flagellin and infection with EDL933 (101.5 ± 24.0 fold for flagellin and 99.0 ± 23.0 fold for EDL933). In contrast, only very low induction levels were noted for IL-8 (**Figure 5B**, data shown for 0.1 μg/ml flagellin). Therefore, kinetic analyses were performed, and cells were apically exposed to flagellin for 1–9 h. Whereas, highest induction levels for hBD2 were observed after 6–9 h of flagellin exposure, IL-8 mRNA expression was induced much earlier and peaked at 1–3 h of exposure (**Figure 5B**). IL-8 induction was subsequently examined after 2 h of flagellin exposure, and a dose-dependent response was noted with induction levels for 1 μg/ml flagellin approximating those observed during infection with EDL933 (62.6 ± 8.9 fold for flagellin and 80.8 ± 10.5 fold for EDL933; **Figure 5C**).

Similar to findings in polarized T84 cells, purified flagellin was also sufficient to induce hBD2 (median fold increase = 5.3) and

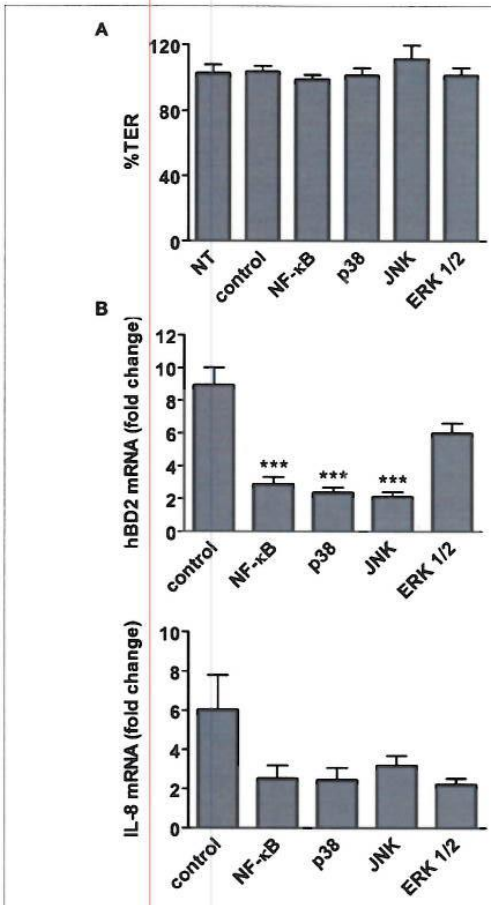

FIGURE 7 | Flagellin-mediated hBD2 induction is dependent on NF-κB, MAPK p38, and JNK. Polarized T84 cells were incubated with specific inhibitors of NF-κB, p38, JNK, ERK1/2, or solvent (control) for 1 h before purified flagellin (0.1 μg/ml) was added for 6 h. Alternatively, T84 cells were left untreated (NT) for 7 h. **(A)** Influence of chemical inhibitors on epithelial barrier function. TER after treatment is expressed as percentage of TER before treatment. **(B)** hBD2 and IL-8 mRNA levels were quantified by qPCR and expressed as fold changes relative to NT samples. ***$P < 0.001$ vs. control. Data are shown as means ± SEM of three independent experiments performed in duplicate.

IL-8 (median fold increase = 2.2) expression in colonic pIVOC (**Figure 6**).

Flagellin-Induced hBD2 Expression Is Mediated by NF-κB, Map Kinases p38 and JNK

We further characterized the signaling pathways involved in flagellin-induced hBD2 and IL-8 expression. To this aim, polarized T84 cells were treated with specific chemical inhibitors before flagellin (0.1 μg/ml) was added. Gene expression was quantified by qPCR. None of the chemical inhibitors

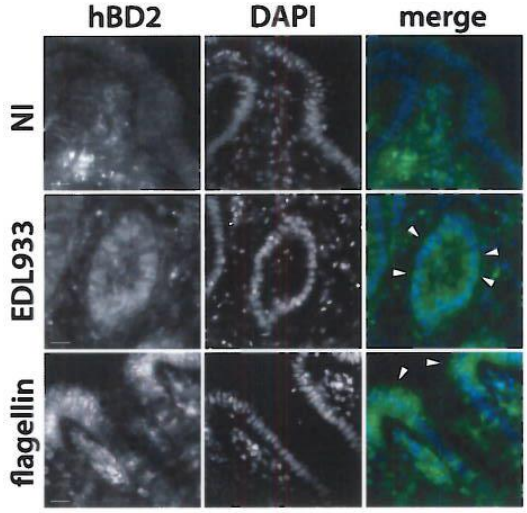

FIGURE 8 | EHEC stimulates hBD2 peptide expression in colonic biopsy epithelium. Colonic biopsies were incubated with EDL933, purified flagellin (1 μg/ml) or left NI for 8 h. Cryosections from snap-frozen tissue were stained with anti-hBD2 (green), and cell nuclei were counterstained with DAPI (blue). Arrowheads show epithelial cells with induced hBD2 expression. Images are displayed as separate monochrome images of each fluorescence channel (hBD2, DAPI) and merged color images from both channels (merge). Shown are representative images from two independent experiments performed in duplicate. Bars = 10 μm.

compromised epithelial barrier function (**Figure 7A**). While inhibition of NF-κB or the MAPKs p38 and JNK significantly reduced hBD2 gene expression, treatment with the ERK1/2 inhibitor did not have any significant effect (**Figure 7B**). For IL-8, all inhibitors reduced gene expression, but this did not reach significance (**Figure 7B**).

EHEC Infection Induces Epithelial hBD2 Peptide Expression in Colonic Biopsies

Having shown that EHEC flagellin induced hBD2 mRNA expression, we investigated whether this also affected hBD2 peptide levels. Colonic biopsies were infected with EDL933 or incubated with purified flagellin (1 μg/ml) on the mucosal side, and hBD-2 peptide expression was assessed by immunofluorescence staining. As shown in **Figure 8**, elevated hBD2 peptide expression was observed in epithelial cells of colonic biopsies incubated with EDL933 or flagellin vs. NI controls.

EHEC-Induced IL-8 Protein Secretion in Polarized T84 Cells Is Directional and Depends on the Side of Exposure

We further investigated EHEC-induced IL-8 expression at the protein level. Polarized T84 cells were apically infected with EDL933 or incubated with the potent IL-8 inducer IL-1β (10 ng/ml) for 24 h. This time point was chosen according to previous IL-8 secretion studies on EHEC and enteropathogenic *E. coli* (EPEC) (Hauf and Chakraborty, 2003; Baruch et al., 2011; Sham et al., 2011). To prevent cell damage and breach of barrier function due to bacterial overgrowth and acidification, EHEC-infected monolayers were treated with gentamicin (200 μg/ml) after 1 h. This treatment did not result in complete killing, but reduced bacterial growth so that infections could be continued for up to 24 h. TER was not significantly affected by any of the treatments (**Supplementary Figure 2B**) indicating maintenance of epithelial barrier function. At the end of the incubation, IL-8 protein levels were determined in cell lysates and apical and basolateral supernatants. As shown in **Figure 9A**, apical EHEC infection significantly increased IL-8 protein levels in apical supernatants (240.9 ± 27.5 vs. 32.0 ± 6.2 pg/well in NI controls) and to a lesser extent in cell lysates (88.9 ± 21.5 vs. 16.6 ± 1.4 pg/well in NI controls) but not in basolateral media. Similarly, apical IL-1β exposure resulted in significantly enhanced amounts of IL-8 in apical supernatants (102.3 ± 14.9 vs. 32.0 ± 6.2 pg/well in NI controls) but not in cell lysates or basolateral media. We further extended these studies and investigated the influence of the side of exposure on the direction of IL-8 release into the media. To this aim, polarized T84 cells were incubated with EDL933 or purified flagellin (1 μg/ml) on the apical or basolateral side for 24 h, and IL-8 levels were evaluated in apical and basolateral supernatants. None of the treatments significantly affected the TER (**Supplementary Figure 2B**). As demonstrated in **Figure 9B**, apical exposure to EDL933 or flagellin led to a significant increase of IL-8 secretion into apical supernatants (569.5 ± 46.2 or 761.2 ± 8.5 vs. 108.2 ± 16.9 pg/well in NI controls, respectively) whereas IL-8 levels in basolateral media were not significantly affected. On the other hand, basolateral exposure of polarized T84 cells to EDL933 or flagellin significantly stimulated IL-8 protein release into basolateral compartments (506.6 ± 103.2 or 1119.0 ± 181.4 vs. 128.6 ± 8.1 pg/well in NI controls, respectively) whereas IL-8 levels in apical media were not significantly affected (**Figure 9B**).

DISCUSSION

The intestinal epithelium is the first line of defense against enteric pathogens, and the expression and release of AMPs represents an important part of the innate immune response during intestinal infections. In the colon, the main target site of EHEC, epithelial cells express hBD1-4, LL-37, and lysozyme. In this study, we have investigated the effect of EHEC infection on AMP expression in two physiologically relevant infection models: human colonic biopsies and polarized T84 cells. As demonstrated by our recent studies, IVOC of human colonic biopsies supports EHEC A/E lesion formation (Lewis et al., 2015). However, standard IVOC is not suitable to study the host immune response as it allows bacterial access and stimulation of the submucosal tissue surface. Therefore, we adopted a polarized IVOC method developed previously for small intestinal biopsy samples (Schüller et al., 2009). In addition to IVOC, we used T84 human colon carcinoma

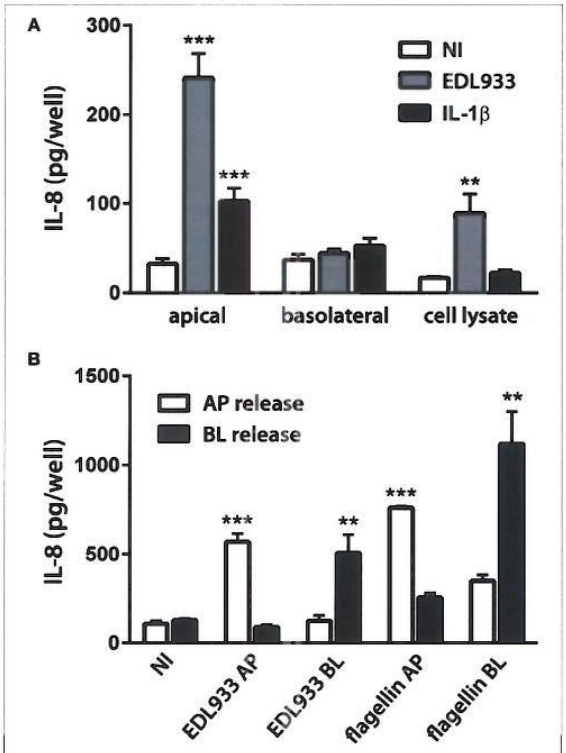

FIGURE 9 | EHEC-induced IL-8 release from polarized T84 cells is directional and depends on the side of exposure. Polarized T84 cells were incubated with EDL933, IL-1β (10 ng/ml), flagellin (1 μg/ml), or left NI on the apical **(A,B)** or basolateral side **(B)** for 24 h. IL-8 protein levels in cell lysates, apical and basolateral supernatants were determined by ELISA. **(A)** Apical exposure to EDL933 or IL-1β stimulated apical IL-8 release. Data are shown as means ± SEM of three independent experiments performed in duplicate. **(B)** Apical (AP) exposure to EDL933 or flagellin induced apical IL-8 release (white columns), whereas basolateral (BL) exposure to EDL933 or flagellin induced basolateral IL-8 release (black columns). Data are shown as means ± SEM of two independent experiments performed in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. NI control.

cells which lack expression of the Stx receptor Gb3 similar to human colonic epithelium (Schüller et al., 2004) and form highly polarized epithelia with high TER when grown on membrane supports (Madara et al., 1987).

Our results show that apical EHEC infection induced hBD2 expression in both colonic biopsies and polarized T84 cells whereas all other AMPs remained unaffected. This is of relevance as hBD2 has been demonstrated to directly kill *E. coli* and also promote the release of immuno-modulating adenosine (Estrela et al., 2013). In contrast to hBD1 which is constitutively expressed in human colonic epithelium and thus confers a baseline protection against bacterial pathogens, hBD2 is undetectable or only expressed at very low levels in healthy children and adults, but elevated in patients with inflammatory bowel disease (Fahlgren et al., 2003; Zilbauer et al., 2010). This might be due to enhanced contact of the gut microbiota with intestinal epithelium, and previous studies have demonstrated induction of hBD2 by both commensal and pathogenic bacteria. Most of these studies were performed using intestinal epithelial cell lines (e.g., Caco-2 and HT-29) and have demonstrated stimulation of hBD2 expression by enteroinvasive pathogens such as *Salmonella* spp., *Shigella flexneri* and *dysenteriae*, enteroinvasive *E. coli*, and *Campylobacter jejuni* (O'Neil et al., 1999; Ogushi et al., 2001; Hase et al., 2002; Zilbauer et al., 2005). In addition, non-invasive EPEC and probiotic *E. coli* Nissle 1917 have also been shown to induce hBD2 expression (Schlee et al., 2007; Khan et al., 2008). In contrast, very few studies have been carried out on human intestinal tissue so far. Whereas experiments using human intestinal xenografts in mice have confirmed hBD2 induction by *Salmonella typhi* (O'Neil et al., 1999), no change in expression was observed after infection with *S. flexneri* (Sperandio et al., 2008). To the best of our knowledge, this is the first study using IVOC of human intestinal biopsies to investigate the AMP response to bacterial infection.

We further investigated the bacterial factors involved in hBD2 induction and found that EHEC flagellin was both necessary and sufficient to stimulate a response *ex vivo* and *in vitro*. This is in agreement with studies on EPEC and *Salmonella enterica* serotype Enteritidis which also demonstrated hBD2 induction mediated by flagellin (Ogushi et al., 2001; Schlee et al., 2007; Khan et al., 2008). Interestingly, the hBD2 response to flagellin from non-pathogenic *E. coli* such as ATCC 259922, JM109, and Nissle 1917 is strain-dependent suggesting differences in binding to a potential receptor (Schlee et al., 2007). We further tested which signal transduction pathways were involved in flagellin-mediated hBD2 induction by using chemical inhibitors of NF-κB and MAPKs. These studies showed that flagellin-induced hBD2 expression was dependent on NF-κB, MAPK p38, and JNK but not ERK1/2. NF-κB dependency of hBD2 induction has been described before in LPS-stimulated macrophages (Tsutsumi-Ishii and Nagaoka, 2002) and intestinal epithelial cells treated with flagellin from *E. coli* Nissle (Wehkamp et al., 2004). The latter study also demonstrated involvement of the MAPKs JNK but not p38 or ERK 1/2. In addition, *Bacteroides fragilis* enterotoxin induced hBD2 by p38 activation (Yoon et al., 2010), and inhibitors of p38, JNK and ERK 1/2 reduced hBD2 induction by probiotic lactobacilli, although this did not reach significance (Schlee et al., 2008).

In addition to studying the AMP response to EHEC infection, we also examined expression of the pro-inflammatory cytokine IL-8 which acts as a chemoattractant and recruits neutrophils to the site of infection (Baggiolini and Clark-Lewis, 1992). Previous studies using non-polarized cells have shown that EHEC infection induces IL-8 release, and that this is dependent on NF-κB and MAP kinases (Dahan et al., 2002; Gobert et al., 2007). While several bacterial factors including long polar fimbriae, hemorrhagic coli pili, and Shiga toxins (Thorpe et al., 2001; Ledesma et al., 2010; Farfan et al., 2013) have been implicated in this response, many studies suggest that flagellin is the principal inducer of IL-8 production during EHEC infection (Berin et al., 2002; Zhou et al., 2003; Miyamoto et al., 2006). This is confirmed by our results on human colonic biopsies and polarized T84

cells and agrees with earlier *ex vivo* studies using human colonic xenografts in mice (Miyamoto et al., 2006).

While flagellin provides the initial stimulus for a pro-inflammatory response, it has been shown that T3S effectors encoded outside the locus of enterocyte effacement dampen down NF-κB activation and IL-8 release at later stages of EHEC and EPEC infection (Hauf and Chakraborty, 2003; Nadler et al., 2010; Baruch et al., 2011). In addition, a similar T3S-dependent suppressive effect has been observed in EPEC-dependent hBD2 expression (Khan et al., 2008). Notably, all of these studies have been performed using cervical HeLa cells or undifferentiated Caco-2 cells. Interestingly, we did not detect any significant inhibitory effect of the T3SS on hBD2 and IL-8 expression during EHEC infection of colonic biopsies and polarized T84 cells. A similar observation has been reported by Ruchaud-Sparagano and colleagues who demonstrated T3S-dependent suppression of IL-8 induction in EPEC-infected polarized Caco-2 but not T84 cells (Ruchaud-Sparagano et al., 2007). As fully differentiated Caco-2 cells display a small intestinal rather than colonic phenotype (Engle et al., 1998), these results might suggest a delayed or absent immunosuppressive effect of EHEC infection in human colonic vs. small intestinal epithelium.

As it was technically not feasible to accurately quantify epithelial IL-8 secretion in colonic biopsies due to the presence and varying thickness of the submucosal tissue, polarized T84 cells were employed for this part of the study. Unexpectedly, we found that IL-8 secretion was vectorial and depended on the direction of the stimulus: Whereas, apical exposure to EHEC or flagellin induced apical IL-8 release, basolateral exposure resulted in IL-8 secretion to the basolateral side. Whilst EHEC-induced IL-8 secretion has not been examined in polarized T84 cells, studies on EPEC and EPEC flagellin have reported increased basolateral IL-8 secretion after basolateral but not apical stimulation (Zhou et al., 2003; Ruchaud-Sparagano et al., 2007). Very few studies have examined apical IL-8 secretion as it is generally assumed that IL-8 is mainly secreted basolaterally to mediate neutrophil recruitment to the epithelium. In line with this theory, predominantly basolateral IL-8 secretion has been shown in polarized HCA-7 cells stimulated with EHEC flagellin and polarized Caco-2 cells apically exposed to EPEC (Berin et al., 2002; Ruchaud-Sparagano et al., 2007). However, similar to our results, stimulation of polarized HT 29/19A or Caco-2 cells with IL-1 or TNF-α resulted in polarized IL-8 secretion dependent on the side of exposure (Lammers et al., 1994; Sonnier et al., 2010). In addition, vectorial IL-8 release has been shown in flagellin-stimulated polarized Caco-2 cells (Rossi et al., 2013). While luminal IL-8 could aid neutrophil transmigration across the epithelium as demonstrated in urinary tract infections (Godaly et al., 2001), autocrine epithelial signaling via apically expressed IL-8 receptors (CXCR1) has also been suggested (Rossi et al., 2013).

Taken together, our study demonstrates that colonic EHEC infection elicits a weak innate immune response with induction of hBD2 but no other AMPs and low levels of IL-8. These results suggest that inflammation plays a minor role in intestinal EHEC pathogenesis.

AUTHOR CONTRIBUTIONS

SL and SS designed the study, analyzed the data, and prepared the manuscript. SL and SE performed the experimental work. AP, VC, SC, and WG selected suitable patients, obtained informed consent, and provided human biopsy samples.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2016.00068

Supplementary Figure 1 | Kinetics of hBD2 and IL-8 gene expression during EHEC infection. Polarized T84 cells were apically infected with EHEC strain EDL933 for 3–12 h, or left NI for 12 h. hBD2 and IL-8 mRNA levels were quantified by qPCR and expressed as fold changes relative to NI controls. Data represent means ± SEM of two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. NI control.

Supplementary Figure 2 | Influence of different treatments on T84 cell barrier function. Polarized T84 cells were apically inoculated with EHEC wild-type EDL933 or 85–170 (WT), deletion mutants (Δ*fliC*, *escN*, *stx*, *hcp*, or *lpfA1*), purified flagellin (1 μg/ml) or left non-infected (NI) for 9 h **(A)** or incubated with EDL933, IL-1β (10 ng/ml), flagellin (1 μg/ml), or left NI on the apical (AP) or basolateral side (BL) for 24 h **(B)**. TER after treatment is expressed as percentage of TER before treatment. Data are shown as means ± SEM of three independent experiments performed in duplicate.

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