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Escherichia coli K1 interactions with human brain microvascular endothelial cells, a primary step in the development of neonatal meningitis

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This thesis is submitted for the degree of Doctor of Philosophy, University of London

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University of London

December 2011
PAGE NUMBERS CUT OFF IN ORIGINAL
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Abstract

*Escherichia coli* (E. coli) K1 is one of the commonest Gram negative bacteria causing neonatal bacterial meningitis in both developed and developing countries. Haematogenous spread is a key step in *E. coli* K1 meningitis; however, it is not clear how bacteria cross the brain endothelium to gain entry into the central nervous system. Previous studies have focussed mainly on the identification of bacterial virulence factors, as well as the signalling pathways that are activated for the recruitment of actin cytoskeleton to the bacterial adhesion site on the apical surface of human brain microvascular endothelial cells (HBMEC) and finally lead to bacterial uptake. However, the cellular requirements and mechanisms of post-invasion events are poorly understood.

This study aims to further characterize *E. coli* K1 entry, intracellular trafficking and the associated molecular mechanisms. To achieve this, a virulent fluorescent protein-expressing *E. coli* K1 strain was constructed. In a previous study, caveolin-1, a lipid raft marker associated with clathrin-independent endocytosis, was found associated with invading and intracellular bacteria in HBMEC. To further study the effect of caveolin-1 on the bacterial entry, different caveolin-1 mutants were applied here. Overexpression of caveolin-1 Y14A mutant and caveolin-1β, which is non-phosphorylatable, did not block *E. coli* K1 invasion of HBMEC. Furthermore, *E. coli* K1 invasion of caveolin-1 knockout mouse lung endothelial cells (MLEC) was not blocked, which suggested that caveolin-1 was not required for *E. coli* K1 invasion of endothelial cells. The role of dynamin, a large GTPase that has been implicated in the membrane fission of caveolae buds, was also investigated. Based on quantitative microscopy scoring, no evidence of any inhibitory effect on the bacterial invasion was observed in cells overexpressing green fluorescent protein- (GFP) tagged dominant negative dynamin 2 [Dyn2(aa)K44A] and dominant negative dynamin 1 (Dyn1K44A). The experimental evidence from this study therefore
suggests that *E. coli* K1 might invade HBMEC via a caveolae- and dynamin-independent endocytic pathway.

To further explore the endocytosis pathway that the bacteria use to invade HBMEC, immunofluorescence staining of *E. coli* K1 infected HBMEC revealed colocalization of the bacteria with flotillin 1, another lipid raft marker associated with clathrin-independent endocytosis. However, *E. coli* K1 infection of flotillin 1 knockout MLEC demonstrated a significantly increased bacterial uptake. This observation suggests that *E. coli* K1 uptake does not require flotillin 1. In parallel, the number of intracellular non-pathogenic *E. coli* K-12 recovered from the lysates of flotillin 1 knockout MLEC was also significantly higher than that recovered from the lysates of wild type MLEC. Further, overexpression of GFP-tagged flotillin 1 and flotillin 2 in HBMEC inhibited *E. coli* K1 invasion, which suggest flotillin might have a role as a regulatory cell barrier in host defence.
Acknowledgements

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<tr>
<td>AEE</td>
<td>Apical early endosomes</td>
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<td>APS</td>
<td>Ammonium persulphate</td>
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<td>ARE</td>
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<td>BBB</td>
<td>Blood-brain barrier</td>
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<td>Brain microvascular endothelial cells</td>
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<td>Ethylenediamine tetraacetic acid</td>
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<td>Green fluorescent protein</td>
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<td>Human dermal microvascular endothelial cells</td>
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<td>HEPES</td>
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<tr>
<td>mins</td>
<td>Minutes</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MLEC</td>
<td>Mouse lung endothelial cells</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>Manganese chloride</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>N.B.</td>
<td>Note</td>
</tr>
<tr>
<td>n.s.</td>
<td>Not statistically significant</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>Sodium carbonate</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Disodium hydrogen phosphate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NBM</td>
<td>Neonatal bacterial meningitis</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>Ammonium chloride</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>NMEC</td>
<td>Neonatal meningitis <em>E. coli</em></td>
</tr>
<tr>
<td>NPC1L1</td>
<td>Niemann-Pick C1-like 1</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OmpA</td>
<td>Outer membrane protein A</td>
</tr>
<tr>
<td>OriM</td>
<td>Origin of replication</td>
</tr>
<tr>
<td>p.i.</td>
<td>Post infection</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated protein kinase</td>
</tr>
<tr>
<td>palm</td>
<td>Palmitoylation</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBSA</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Pfu</td>
<td>Pyrococcus furiosus</td>
</tr>
<tr>
<td>pH</td>
<td>Hydrogen potential</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PK1A-GFP</td>
<td>Endosialidase–GFP fusion protein</td>
</tr>
<tr>
<td>PKCa</td>
<td>Protein Kinase Ca</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomol</td>
</tr>
<tr>
<td>PolySia</td>
<td>Polysialic acid</td>
</tr>
<tr>
<td>PtdIns(3)P</td>
<td>Phosphatidylinositol-3-phosphate</td>
</tr>
<tr>
<td>PtdIns(3,5)P₂</td>
<td>Phosphatidylinositol-3,5-biphosphate</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>PYG</td>
<td>Proteose peptone, yeast extract, and glucose media</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
</tr>
<tr>
<td>RILP</td>
<td>Rab7-interacting lysosomal protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SCV</td>
<td>Salmonella-containing vacuoles</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>secs</td>
<td>Seconds</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>sp.</td>
<td>Species</td>
</tr>
<tr>
<td>SPI</td>
<td>Salmonella pathogenicity island</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Stat3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>T3SS</td>
<td>Type 3 secretion system</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate EDTA</td>
</tr>
<tr>
<td>TEER</td>
<td>Transendothelial electrical resistance</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>ter&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Tetracycline resistance gene</td>
</tr>
<tr>
<td>TfR</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junction</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>UPEC</td>
<td>Uropathogenic <em>E. coli</em></td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infections</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VF</td>
<td>Virulence factors</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 Neonatal bacterial meningitis (NBM)

Neonatal bacterial meningitis (NBM) is caused by inflammation of the meninges, a result of bacterial infection during the first month of life. In developing countries, of 125000 diagnosed cases about 50000 newborns die of meningitis annually (mortality rate of 40%) (Mulholland, 1998). Comparatively, in developed countries, the NBM mortality rate has declined from about 50% in the 1970s to about 10% in the late 1990s, although the morbidity (0.22 per 1000 births) remains very similar in the past two decades (Holt et al., 2001; Gaschignard et al., 2011). A better understanding of the disease pathogenesis and pathophysiology would improve the morbidity and mortality of the disease, despite the advances in chemotherapy and supportive care (Polin and Harris, 2001; Bonacorsi and Bingen, 2005).

The clinical presentation of NBM is non-specific, including apnea, temperature instability, hypotension, bradycardia, tachycardia, lethargy or irritability, and abdominal distention or feeding intolerance (de Louvois, 1994; Wynn and Levy, 2010). The prevalence of the causative organisms isolated from early- (occurring between day 0 and day 4 of life) and late-onset (occurring between day 5 and day 28 of life) NBM is different, and it varies in different countries (Osrin et al., 2004; Wu et al., 2009; Zaidi et al., 2009; Talbert et al., 2010; Gaschignard et al., 2011). E. coli and group B Streptococci (GBS) are the commonest bacteria isolated from NBM cases; however, the mortality rate of E. coli associated cases is relatively higher than GBS infection in some countries (Holt et al., 2001; Stoll et al., 2002; May et al., 2005; Wu et al., 2009), and about half of the E. coli-associated NBM survivors develop neurological disability (Unhanand et al., 1993: Harvey et al., 1999; Dellagrammaticas et al., 2000).
Following the implementation of intrapartum antibiotic prophylaxis (IAP) treatment in mothers who carry GBS from screening at 35 to 37 weeks of gestation since the mid-1990s in developed countries, the number of GBS-associated NBM cases have declined significantly, while non-GBS NBM cases are either stable (Baltimore et al., 2001) or on the increase (Levine et al., 1999). Notably, increased ampicillin resistance has been found in E. coli strains isolated from E. coli-NBM in the current IAP era (Levine et al., 1999; Baltimore et al., 2001; Stoll et al., 2002; Alarcon et al., 2004; Cordero et al., 2004; Bizzarro et al., 2008).

1.2 *Escherichia coli* (*E. coli*)

*E. coli* is a member of *Enterobacteriaceae*. It is a facultative anaerobic Gram negative bacillus, which colonizes the human neonatal gastrointestinal tract within hours of birth, remains as a commensal and provides a lifetime of benefits to the host from intruding pathogens, including competition for space or binding sites, competition for nutrients, and regulate immune homeostasis (Kaper et al., 2004; Willing et al., 2011). However, some *E. coli* strains have acquired specific virulence factors, and are major community and hospital-acquired pathogens, and veterinary pathogens. The resultant diseases can be categorized into enteric/diarrhoeal disease, urinary tract infections (UTI), and sepsis/meningitis (Kaper et al., 2004; Bonacorsi and Bingen, 2005). There are eight main pathogenic *E. coli* pathovars, with six diarrhoeagenic: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC): two extraintestinal infections: uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC). In addition to the major pathovars, other pathovars have also been identified (Kaper et al., 2004; Croxen and Finlay, 2010).
The *E. coli* species has 170 O lipopolysaccharides antigens or LPS, 103 capsular or K antigens, and 56 flagellar or H antigens, and strains are categorised on the basis of their antigen configuration (Robbins *et al.*, 1974; Kaper *et al.*, 2004).

Strains possessing the K1 capsular polysaccharide predominate the majority of NBM cases (Robbins *et al.*, 1974). Interestingly, only a few O antigens (e.g. O18, O7, O1, O16) are associated with most of the K1 strains isolated from the cerebrospinal fluid (CSF) (Sarff *et al.*, 1975; Pluschke *et al.*, 1983).

1.3 Pathogenesis of *E. coli* K1 neonatal meningitis

In most cases of *E. coli* neonatal meningitis, the infants are believed to acquire the pathogen from the mother’s delivery canal or from the environment, leading to colonization of the infant’s intestinal tract (Robbins *et al.*, 1974; Sarff *et al.*, 1975; Headings and Overall, 1977; Raymond *et al.*, 2008). The bacteria are normally confined to the intestinal tract by the gut mucosal barrier, where they form part of the normal commensal flora. The gut barrier is formed of epithelial cells held closely together by tight junctions, and has major roles in regulating the movement of ions and molecules between cellular spaces, as well as in protecting the host from invading pathogens (Daneman and Rescigno, 2009). In *E. coli* K1 meningitis, the bacteria are able to traverse the gut barrier and enter the bloodstream. The interaction of *E. coli* K1 with gut barrier has not been studied extensively; however, an *in vitro* study with human intestinal epithelial cell lines, Caco-2 and T84, showed that *E. coli* K1 breached the epithelial barrier by disrupting tight junctions, evidenced by a decrease in the transendothelial electrical resistance (TEER) value and the increased passage of non-invasive *E. coli* strain through the monolayer on Transwell® insert in co-infection experiments (Burns *et al.*, 2001).

Once the bacteria have crossed gut mucosal barrier, the bacteria gain access to the bloodstream and must overcome the host defense mechanisms to survive intravascularly.
The bacterial polysaccharide K1 capsule has been shown to possess anti-phagocytic properties (Bortolussi et al., 1979; Mushtaq et al., 2005) and provides resistance to classic complement-mediated bactericidal activity (Cross et al., 1986; Mushtaq et al., 2004). In addition, the neonatal host defense mechanism is less able to deal with an intravascular infection when compared to an adult’s immune system. Neonatal macrophages and neutrophils have been found to be inefficient in phagocytosis (Filias et al., 2011), as well as in the production and secretion of reactive oxygen species (ROS) (Levy, 2007; Wynn and Levy, 2010). Neonatal leukocytes also expresses low levels of surface CR3, which functions as a pathogen sensor and recruits complement (Wynn and Levy, 2010). Moreover, neonates are found to express low levels of C9 and other components of the complement system (C1, C3, C4, C5) (Wilfert, 1978; Lassiter et al., 1992). Bacterial intravascular survival might be further aided by the deficiency of pro-inflammatory and T\(_{H1}\)-polarizing cytokines production by neonatal antigen-presenting cells, which aim to avoid triggering excessive inflammation during colonization of gut commensal flora (Wynn and Levy, 2010). As a consequence of the deficiencies in neonatal immunity, the bacterial bloodstream survival is promoted; hence, the bacteria are able to multiply to a high level of bacteraemia, and finally invade into the cerebrospinal fluid (CSF) (Glode et al., 1977; Pluschke et al., 1983; Kim et al., 1992; Zelmer et al., 2008). It has been documented that patients with bacteraemia of \(>10^3\) colony forming units per ml of blood are highly likely to develop meningitis (Dietzman et al., 1974).

The precise sites and mechanisms that \textit{E. coli} K1 and other meningitis-causing extracellular bacterial pathogens, such as \textit{Streptococcus pneumonia}, \textit{Haemophilus influenza}, and \textit{Neisseria meningitidis}, enter into CSF are still unclear and in many cases controversial. \textit{Haemophilus influenza} and \textit{Neisseria meningitidis} are believed to invade CSF via the blood-cerebrospinal fluid barrier (BCSFB) at choroid plexus based on histological examinations of brain tissue, which found the presence of the bacteria in the
endothelium of choroid plexus and meninges (Smith, 1987; Pron et al., 1997). However, if BCSFB is the bacterial portal of entry, meningitis should be theoretically linked to ventriculitis, which has never been observed in post-mortem specimens (Join-Lambert et al., 2010). The cerebral microvascular endothelial cells, which form the BBB, have been proposed as an alternative bacterial entry site (Quagliarello et al., 1986; Parkkinen et al., 1988), but meningitis-causing extracellular bacteria do not cause encephalitis, and there is no evidence that these bacteria are able to multiply in the brain parenchyma (Join-Lambert et al., 2010). Nevertheless, at later stages of the infection, the increased permeability of BBB, resulting from increased pinocytosis, leads to the disruption of the intercellular junctions of the cerebral capillaries in BBB (Quagliarello et al., 1986). Intriguingly, post-mortem examination of a patient who died of fulminant meningococcal meningitis revealed that the bacteria adhered to cerebral capillary (Mairey et al., 2006), suggesting that meningitis-causing extracellular bacteria do interact with BBB during infection. In the case of *E. coli* K1, bacteria have been found in the perivascular area of the subarachnoid space of histological brain sections of subcutaneously-infected 5-day-old rats (Kim et al., 1992). However a decade later, bacteria were observed in the choroid plexus of histological brain sections of *E. coli* K1-infected 2-day-old rats, where the animals were infected orally (Zelmer et al., 2008). Despite the controversies on the bacterial portal of entry into CSF, *in vitro* studies with cerebral microvascular endothelial cells have allowed the identification of bacterial virulence factors that are essential for endothelial cell adherent and invasion, as reviewed in section 1.8.1. Future studies will hopefully elucidate the precise mechanism by which entry into the CSF is achieved by these pathogens.

Once in the subarachnoid space, the bacteria take advantage of the immune privilege of the CNS and replicate and induce inflammatory cytokines production (Simberkoff et al., 1980). In the CSF, the concentrations of immunoglobulin and complement components are low, which leads to inefficient removal of the pathogens
The bacterial cell wall components, but not the bacterial capsule, have been shown to be potent inducers for tumor necrosis factor (TNF) and interleukin-1 (IL-1) production, which promote the adherence of neutrophils to endothelium, meningeal inflammation, and BBB breakdown (Dinarello, 1989; Waage et al., 1989; Ramilo et al., 1990; Quagliarello et al., 1991). The hallmark of bacterial meningitis is the recruitment of neutrophils into CSF; however, the neutrophils are not efficient killers in the CSF (Ernst et al., 1983). Conversely, neutrophils recruitment appears to have a negative effect and may contribute to the pathogenesis of the disease. When the recruitment of neutrophils was blocked during the infection, brain edema, intracranial pressure, and neuronal apoptosis in the hippocampus of infected animals were reduced (Tuomanen et al., 1989; Braun et al., 1999).

1.4 Blood-brain barrier and blood-cerebrospinal fluid barrier, and in vitro models

The BBB separates the circulating blood from the elements of CNS. The capillary BBB, which is localized in cerebral cortex, is formed by endothelial cells that are connected via a network of junctional complexes, and have very few endocytic vesicles (Figure 1.1A) (Rubin and Staddon, 1999). The junctional complexes are composed of adheren junctions and tight junctions, which restrict the movement of hydrophilic macromolecules and small ions between the blood and the brain, and result in high endothelial electric resistance in the brain capillaries (Abbott et al., 2006). The major molecules of the tight junctions present in brain capillary endothelium are occludin, claudin-1, claudin-5, ZO-1, and ZO-2 (Rubin and Staddon, 1999; Abbott et al., 2006). The tight junctional complexes are further stabilized by adheren junctions, which are mainly composed of cadherin, and the cytoplasmic tail of which is associated with catenins (Rubin and Staddon, 1999). The endothelial cells are also in close contact with astrocytes, the paracrine factors produced by these astrocytes might also contribute to the endothelial cells...
tight junction complexes formation (Arthur et al., 1987). Other perivascular cell types, including pericytes, microglial cells, and perivascular macrophages, form the CNS perivascular immunological barrier to protect the CNS from invading pathogens (Join-Lambert et al., 2010).

In addition to the intracerebral capillaries, the brain post-capillary venules (Figure 1.1B) are also part of the BBB. These venules have very similar properties as intracerebral capillaries, but they do not form as tight barrier as that of intracerebral capillaries (Abbott et al., 2006; Nachman and Rafii, 2008). Ultrastructural studies showed that these venules have discontinuous and less complex junctional complexes than that of intracerebral capillaries (Nagy et al., 1984; AlIt and Lawrenson, 1997). There is no electrophysiological data to demonstrate the resistance of these post-capillary venules, although rat pial venules have been shown to have lower resistance than that of pial arterioles (Butt and Jones, 1992). Furthermore, when the capillaries turn into venules and veins, the contact between endothelial cells and astrocyte processes is gradually reduced as they are separated by the increasing Virchow-Robin perivascular spaces (Join-Lambert et al., 2010).

Primary brain microvascular endothelium (BMEC) of various origins can be isolated and used as a BBB in vitro model; however, the application of the primary cells is limited by the costly cell isolation and maintenance, rapid loss of the differentiated cell phenotypes at later passages, and failure to form tight junctions (Arthur et al., 1987; Bourdoulous et al., 2002). Several groups have immortalized the isolated BMEC by transfecting the cells with SV40 large T antigen, and the immortalized BMEC are shown to express specific brain endothelial properties, cell surface adhesion molecules, and junctional markers (Blasig et al., 2001; Stins et al., 2001; Weksler et al., 2005). Importantly, these BMEC lines are able to form integral layer on Transwell® insert with high endothelial electrical resistance when they are cultured in the absence of astrocytes either in static or in flow-based system (Stins et al., 2001; Cucullo et al., 2008). As in vivo
studies of *E. coli* K1 interactions with BBB in newborn rats do not allow high resolution imaging at cellular level, thus do not allow studies of the cell biology aspect of the infection, the stable properties and indefinite growth potential of these endothelial lines provide an alternative approach, and allow experiments to be conducted with same cell source. Despite the ambiguity of extracellular bacterial pathogens utilize capillary BBB for invading into CSF, the BMEC lines have benefited studies on the interactions of these pathogens with the endothelial cells (Stins *et al.*, 2001; Doran *et al.*, 2005; Coureuil *et al.*, 2009; Orihuela *et al.*, 2009; Untucht *et al.*, 2011).

Mouse brain endothelioma cell line (bEnd5) is another BBB *in vitro* model. However, this endothelial line is not suitable for studying the interactions of meningitis-causing bacterial pathogens with endothelial cells, as the cells showed defects in tight junctional protein expression, and they fail to form intact monolayer on Transwell® insert (Steiner *et al.*, 2011).
Figure 1.1. Vascular arrangement in the cerebral cortex. The illustration shows the arrangement of intracerebral blood vessels in the brain. The illustration was adapted from (Abbott et al., 1999). (A) The capillary BBB. The intracerebral capillaries are composed of endothelial cells that form tight junctions, and they are in contact with foot processes of astrocytes, and pericytes. A thin basement provides both mechanical support and a barrier function. (B) The brain post-capillary venules. In contrast to the capillary BBB, the brain post-capillary venules, which form the venous BBB, do not form as tight barrier as that of the capillary BBB, although they have very similar properties. The presence of Virchow-Robin perivascular space prevents close contact of the endothelial cells with the foot processes of astrocytes.

BCSFB forms the barrier that separates blood components from CSF. CSF is actively produced by the ependymal cells of choroid plexus in brain ventricles. From the choroid plexus, the CSF is drained into the subarachnoid space over brain and spinal cord, and is reabsorbed into blood through arachnoid villi of the dura mater venous sinuses (Join-Lambert et al., 2010).

In contrast to the blood vessels of BBB, the endothelial cells of BCSFB are fenestrated. The ependymal cells that actively produce CSF are linked together by tight junctions (Figure 1.2). Primary choroidal epithelium is available as a BCSFB in vitro model and has been used for studying Streptococcus suis infection (Tenenbaum et al., 2009). In addition to the primary choroidal epithelium, several choroid plexus epithelial cell lines are available. Z310 and TR-CSFB3 are immortalized rat choroid plexus epithelial cell lines carrying the simian virus 40 (SV40) large T antigen gene (Kitazawa et al., 2001; Zheng and Zhao, 2002); while the CPC-2 cell line was derived from a human choroid plexus carcinoma (Kumabe et al., 1996). Although all the cell lines provide lower cost and easier maintenance than primary choroidal epithelium, they vary in the expression of junctional proteins, and the protein distribution for some of these junctional proteins are altered after immortalization (Szmydynger-Chodobska et al., 2007).
Since both BBB and BCSFB are tightly regulated barriers, pathogens need to cross the endothelium (for BBB) or epithelium (for BCSFB) via transcellular (Figure 1.3A), paracellular (Figure 1.3B), or trojan-horse mechanism (Figure 1.3C). For the transcellular traversal route, a pathogen invades the host cell, survives intracellularly, traverses through the cell, and manipulates host cell exocytosis mechanisms for cell egress (Nassif et al., 2002; Kim, 2008). Several pathogens, such as E. coli K1 (Stins et al., 2001), UPEC (Chassin et al., 2008), Streptococcus iniae (Eyngor et al., 2007), Streptococcus pneumoniae (Ring et al., 1998), Streptococcus suis (Tenenbaum et al., 2009), and Neisseria gonorrhoeae (Wang et al., 1998), have been demonstrated to traverse transcellularly in infected cells. For the paracellular traversal route, the pathogen crosses between host cells, with or without disrupting tight junctions (Nassif et al., 2002; Kim, 2008). Trypanosoma sp. have been suggested to cross the BBB via paracellular route (Grab et al., 2004). Mycobacterium tuberculosis (Jain et al., 2006), and Listeria monocytogenes (Greiffenberg et al., 1998), have been suggested to cross a BBB via trojan-horse mechanism, which involves transmigration of an infected phagocyte across the polarized HBMEC (Kim, 2008).
Figure 1.3. Models of pathogen traversal of polarized epithelia or endothelia. (A) Transcellular traversal. (B) Paracellular traversal. (C) Trojan-horse mechanism.

1.5 Plasma membranes structures

Plasma membranes are barriers that protect cell components from external environment. They are composed of dynamic lipid bilayers, which are spanned by or
associated with proteins at either side of the lipid bilayers, and the proteins have roles in membrane trafficking and signalling (Simons and Gerl, 2010; Simons and Sampaio, 2011). The lipid molecules (sphingolipids, and sterols) on the plasma membranes have been proposed to be able to assemble into specialized microdomains, termed lipid rafts, which are usually stabilized by specific proteins (Lingwood and Simons, 2010). They have several significant biological functions, including T cell signalling, viral assembly, ER-to-Golgi trafficking, and endocytosis (Simons and Gerl, 2010).

1.6 Endocytosis

The homeostasis between the cytosol and extracellular environment is maintained by regulating the entry and exit of fluids, solutes, and particles through the membrane lipid bilayer. Small molecules, such as amino acids, sugars, and ions, enter the cell through membrane pumps or ion channels; large molecules enter the non-phagocytic cell by endocytosis (Conner and Schmid, 2003). Various pathogens have also been documented to hijack endocytic pathways for invading non-phagocytic cells (Table 1.1). Endocytic pathways are generally identified by the coat proteins in endocytosis. The major endocytic pathways (Figure 1.4) includes macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis (Conner and Schmid, 2003).

![Figure 1.4. Endocytic routes. The illustration shows various endocytic routes in non-phagocytic cell and the size of the endocytic vesicles (Conner and Schmid, 2003).](image-url)
These endocytic routes are classified by their dependence on large GTPase, dynamin (Figure 1.5), which is recruited to the neck of the budding vesicle on plasma membrane for vesicle scission (Iversen et al., 2003), and it is able to assemble into spirals and form helical structures on lipid nanotubes in vitro (Stowell et al., 1999). In mammals, there are three dynamin isoforms identified. Dynamin 1 is found specifically in neuronal tissue (Scaife and Margolis, 1990); dynamin 2 is ubiquitously expressed (Nakata et al., 1993; Cook et al., 1994); and dynamin 3 is found in testis, lung, and neurons (Nakata et al., 1993). Despite the GTPase domain, dynamin contains a middle domain, a pleckstrin-homology domain, a GTPase effector domain, and a C-terminal proline-rich domain. In addition to vesicle scission, dynamin also functions in mitochondria and chloroplast division and fusion, and cytokinesis, as reviewed in (Praefcke and McMahon, 2004).

![Figure 1.5. Domain structure of a dynamin.](image)

**Figure 1.5. Domain structure of a dynamin.** Protein domains of a dynamin.

The catalytic mechanism of dynamin is very similar as other Ras-family GTPases, although it has lower affinity for GTP and higher rates of GTP hydrolysis than the other Ras superfamily GTPases (Damke et al., 2001). In general, the conversion between GDP- and GTP-bound forms of Ras GTPase is catalyzed by Ras specific guanine-nucleotide-exchange factors (GEF) (Figure 1.6A), while the conversion from the GTP- to the GDP-bound form occurs through GTP hydrolysis catalyzed by GTPase-activating proteins (GAP) (Stenmark, 2009). In the GTP-bound (active) form, Ras activates or binds with high
affinity to other cytoplasmic targets or effector proteins and lead to activation of signalling cascades.

To elucidate the functions of Ras proteins in cells, dominant negative Ras mutants have been widely applied (Figure 1.6B). The dominant negative Ras mutants function by competing with normal Ras for binding to GEF, and form “dead-end” complexes, which are unable to interact with downstream effectors (Feig, 1999). The dominant negative Ras mutants have also been shown to have higher affinities for GDP than for GTP in NIH3T3 cells overexpressing dominant negative Ras mutant, RasS17N (Feig and Cooper, 1988).

![Ras-GTPase cycle](image)

**Figure 1.6. The Ras-GTPase cycle.** (A) The illustration shows the biochemical conversion of GDP-bound Ras to GTP-bound. GEF, guanine-nucleotide-exchange factors; GAP, GTPase-activating protein; PO₄⁻, inorganic phosphate. (B) The illustration shows the mechanism that dominant negative Ras mutants (Ras-DN) compete with normal Ras for GEF in cells expressing the dominant negative Ras.
Based on the understanding of Ras-family GTPases and by referring to the Ras-GTPase structure, dominant negative dynamin mutants have been constructed and the GTPase activities in vitro have been studied (Herskovits et al., 1993; Damke et al., 1994; Marks et al., 2001). It was found that overexpression of K44A or S45N dynamin mutant, which demonstrated weak guanosine triphosphate (GTP) affinity in vitro, inhibits ligand uptake via dynamin-dependent endocytic routes (Herskovits et al., 1993; Damke et al., 1994; Marks et al., 2001; Pelkmans et al., 2002).

1.6.1 Clathrin-mediated endocytosis

Clathrin was the first discovered coat protein, which it forms a triskelion shape, composed of three clathrin heavy chains and three light chains (Pearse, 1976). Clathrin role in mediating endocytosis has been well-studied, and it is the classical receptor-mediated endocytosis pathway (Pearse, 1976). Clathrin-mediated endocytosis has several physiological functions, including cholesterol, iron internalization, and synaptic vesicle recycling (Anderson et al., 1977; Pearse, 1982; Sato et al., 2009).

After the binding of extracellular cargoes on specific receptors on the plasma membrane with cargo-specific adaptor proteins, such as AP2, clathrin triskelions are recruited to the plasma membrane from the cytosol, which leads to the formation of a polyhedral clathrin cage (Henne et al., 2010). In addition to AP2, other adaptor proteins, such as epidermal growth factor substrate 15 (Eps15) and Eps15 interaction protein (epsin), are recruited to the clathrin assembly sites to stabilize the structure on phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂)-rich plasma membrane (Gaidarov et al., 1996; Gaidarov and Keen, 1999; Benmerah and Lamaze, 2007; McMahon and Boucrot, 2011). At the later stage of vesicle formation, dynamin is recruited to the neck of the coated pits by BAR domain-containing proteins, such as amphiphysin, endophilin, and sorting nexin 9 (SNX9), and eventually releases the vesicle from plasma membrane
The requirement of actin polymerization during vesicle scission from the plasma membrane varies with species, and these variations are believed to be due to membrane rigidity, intracellular pressure, and cargo size (McMahon and Boucrot, 2011). In instances where actin is recruited to clathrin vesicles, the role of actin is to contribute energy for vesicle scission and movement (Engqvist-Goldstein and Drubin, 2003; Kaksonen et al., 2005; Aghamohammadzadeh and Ayscough, 2009).

Once the clathrin vesicle is released into the cytosol, clathrin coat is uncoated, and the clathrin triskelia recruited in another cycle of clathrin vesicle assembly. Clathrin uncoating is facilitated by auxilin that binds to the terminal domain and ankle of clathrin triskelia, and then recruits heat shock cognate 70 (HSC70) to uncoat the clathrin coat (Schlossman et al., 1984; Ungewickell et al., 1995).

After the vesicle is released into the cytosol, the endocytosed cargo is sorted in the endosome, and it is either recycled back via recycling endosome to the plasma membrane or targeted to late endosomal compartment, which is increasingly acidified, and eventually degraded in lysosomes. (Figure 1.7) (Idrissi et al., 2008; Kinchen and Ravichandran, 2008). Both early and late endosomes are distinctly different in their protein and lipid composition (Zerial and McBride, 2001; Gruenberg and van der Goot, 2006). The most distinctive markers that distinguish early endosome (EE) are the presence of the small GTPase Rab5, early endosomal antigen-1 (EEA1), and phosphatidylinositol-3-phosphate (PtdIns(3)P) (Clague, 1998; Gruenberg and van der Goot, 2006). Rab7, Rab9, lysosomal-associated membrane protein-1 (Lamp1), lysobisphosphatidic acid (LBPA), phosphatidylinositol-3,5-biphosphate (PtdIns(3,5)P2), are present on late endosomes (LE) (Clague, 1998; Efe et al., 2005; Michell et al., 2006). The presence of Rab7 has been shown to be necessary for trafficking the cargo from late endosome to lysosome (Vanlandingham and Ceresa, 2009), while vesicle transportation from late endosome to
Golgi complex as well as newly synthesized lysosomal enzyme transportation from Golgi to lysosome are mediated by Rab9 (Riederer et al., 1994; Barbero et al., 2002).

**Figure 1.7. Clathrin-mediated endocytic pathway.** The illustration shows the clathrin-mediated endocytosis and the endosome trafficking pathway in the mammalian cell. EE, early endosome; LE, late endosome; RE, recycling endosome; Lys, lysosome; TGN, trans golgi network.

### 1.6.2 Caveolae-mediated endocytosis

Caveolae are membrane microdomains enriched with caveolins, sphingolipid and cholesterol, and act as clathrin-independent sites of endocytosis of a different subset of cargo, such as albumin, and cholera toxin B. Caveolae appear as flask-shaped invaginations of 50 – 80 nm diameter on plasma membrane, and are present in specific
cells types, such as endothelial cells, fibroblasts, type I pneumocytes, and adipocytes (Figure 1.8A) (Pelkmans and Helenius, 2002; Frank et al., 2003; Parton and Simons, 2007).

![Caveolae](image)

**Figure 1.8. Caveolae and caveolin structure.** (A) The electron micrograph shows caveolae on cell surface. (B) and (C) show the insertion of caveolin into caveolar membrane and its structural domains (Parton and Simons, 2007).

Caveolins are abundant in caveolae, and they consist of three subtypes, caveolin-1, caveolin-2, and caveolin-3. Caveolin-1 and caveolin-2 are expressed in non-skeletal cells, whereas caveolin-3 is found in skeletal muscle and cardiac muscle (Parton, 2003; Kumari et al., 2010). Both caveolin-1 and caveolin-3 have been shown to be essential for the
formation of caveolae on the plasma membrane, while loss of caveolin-2 has no effect on
caveolar formation (Parton and Simons, 2007). Furthermore, in the absence of caveolin-1,
caveolin-2 forms low molecular weight oligomers and is retained in the Golgi (Parolini et
al., 1999).

Caveolin shows an atypical topology in the cell, with the protein N and C termini in
the cytoplasm and a hydrophobic hairpin transmembrane domain inserted into lipid
bilayers in a wedge-like manner (Figure 1.8B and C) (Parton and Simons, 2007). The
protein is palmitoylated at the cysteine residue 133, 143, and 156 at the C terminus
(Dietzen et al., 1995). Cysteine palmitoylation is required for cholesterol binding and
chaperone complex formation for cholesterol transport to caveolae, but it is not required
for the protein’s localization to caveolae (Dietzen et al., 1995; Uittenbogaard and Smart,
2000). In addition to palmitoylation, caveolin is also phosphorylated at the tyrosine residue
14 by Src tyrosine kinases, which is essential for caveolar vesiculation (Li et al., 1996).

Caveolae-mediated endocytosis is not very well-characterized. Caveolin-1 is
relatively immobile on the plasma membrane in vivo (Thomsen et al., 2002); however,
caveolae is able to bud off and is responsible for the uptake of various agents and virus,
such as albumin (Schnitzer et al., 1994), cholera toxin B (Shogomori and Futerman, 2001),
and SV40 (Pelkmans et al., 2001). As in clathrin-mediated endocytosis, the caveolar
pathway requires the recruitment of dynamin and actin polymerization to the endocytic site
(Henley et al., 1998; Oh et al., 1998; Pelkmans et al., 2001; Pelkmans and Helenius, 2002;
Pelkmans et al., 2002; Yao et al., 2005). The released vesicle either fuses with tubular
membrane organelles with neutral pH, termed caveosomes (Pelkmans et al., 2001), or is
directed to regular trafficking pathways as in clathrin-mediated endocytosis (Mayor and
Pagano, 2007). Because of the route to caveosomes, caveolae-mediated endocytosis has
been suggested as a safer cellular route of pathogen entry to avoid lysosomal degradation
(Pelkmans et al., 2001; Parton and Simons, 2007).
In caveolin-1 knockout mice, caveolae are absent on the plasma membrane and albumin uptake is abrogated, but transferrin (a ligand for clathrin-mediated endocytosis) uptake is unaffected (Razani et al., 2001). However, SV40 infection of primary embryonic fibroblasts isolated from caveolin-1 knockout mice is unaffected (Damm et al., 2005). There is also some evidence that caveolin-1 might negatively regulate cargo uptake, as, for example, uptake of Neisseria gonorrhoeae and Staphylococcus aureus into cells overexpressing nonphosphorylatable caveolin-1, or in caveolin-1 knockout cells, is enhanced (Boettcher et al., 2010; Hoffmann et al., 2010). Boettcher et al. (2002) showed that inhibition of Neisseria gonorrhoeae uptake in caveolin-1 expressing cells is due to activation of Vav2- and RhoA-mediated cytoskeletal rearrangement as a result of caveolin-1 phosphorylation. It is still unclear which pathway is implicated in caveolin-1 knockout cells, as caveolin-1 seems to interact with other signalling molecules. Recently, caveolin-1 was shown to regulate endothelial permeability and endothelial nitric oxide synthase (eNOS) pathway as well as nitric oxide (NO) and peroxynitrite production in cells isolated from caveolin-1 knockout mice (Siddiqui et al., 2011).

1.6.3 Clathrin- and caveolae-independent endocytosis

Several endocytic routes that are clathrin- and caveolae-independent have been described lately, and the understandings of these pathways are still very superficial. Most of these pathways are predominantly dynamin-independent, but it is unclear how vesicle scission occurs at the plasma membrane in the absence of dynamin. The following sections briefly discussed some of the clathrin- and caveolae-independent endocytosis.

1.6.3.1 Flotillin-mediated endocytosis

Flotillins are widely expressed in all tissues (Volonte et al., 1999). The proteins form microdomains distinct from caveolae on the plasma membrane (Glebov et al., 2006;
Frick et al., 2007), and have been implicated in a variety of different cellular processes, such as cell adhesion, endocytosis, and cell signalling events (Hoehne et al., 2005; Glebov et al., 2006; Langhorst et al., 2007; Ludwig et al., 2010).

There are two flotillin proteins, flotillin 1 and flotillin 2 (also known as reggie 2 and reggie 1, respectively). Both proteins have an SPFH domain (stomatins, prohibitins, flotillins, HflK/C), also known as prohibitin homology domain (PHB), at the N terminal, which has been shown to interact with F-actin (Figure 1.9) (Langhorst et al., 2007). The C terminal of flotillins contains flotillin repeats that are required for the formation of flotillin homo- and hetero-tetramers on the plasma membrane (Solis et al., 2007). Myristoylation at glycine 2 and palmitoylation at cysteines 4, 19, and 20 are essential for membrane association of flotillin 2 (Neumann-Giesen et al., 2004); whereas flotillin 1 is localized to membrane raft and plasma membrane via the hydrophobic regions at the N terminal (Liu et al., 2005).

![Figure 1.9. Structure of flotillin 1 and flotillin 2.](image)

The illustration shows the protein domains of flotillin 1 and 2. Both flotillins have SPFH/PHB domain at the N terminus, and flotillin repeats are found at the C-terminus. Palmitoylation, myristoylation, and tyrosine residues are indicated (Babuke and Tikkanen, 2007).
Flotillin budding from the plasma membrane is rare (Frick et al., 2007). Hence, the role of the flotillin domain as endocytic carriers is still not clear, and the lack of flotillin-specific ligands further hampers the study of this pathway. Cholera toxin B and glycosyl phosphatidylinositol (GPI)-linked proteins are endocytosed into COS-7 and NIH3T3 cells via a flotillin 1 and 2-mediated, but dynamin-independent, pathway initiated by phosphorylation of Fyn kinase (Glebov et al., 2006; Riento et al., 2009); however, another study found that flotillin 2 was not involved in endocytosis of a GPI-anchored protein into HeLa cells (Langhorst et al., 2008b). Flotillin 2 was also shown to promote clustering of amyloid precursor protein (APP) at the cell surface for internalization via a clathrin-mediated-like endocytosis, which is cholesterol and AP-2 dependent but epsin 1-independent (Schneider et al., 2008). Despite being considered an endocytic coat, flotillin has also been found on late endosomal membranes, evidenced by co-localization of the proteins with LAMP1 and Lysotracker (Dermine et al., 2001; Langhorst et al., 2008b).

1.6.3.2 CLIC/GEEC pathway

Some studies have also found that GPI-linked proteins are endocytosed via clathrin-independent carriers (CLIC) into specialized endosomes termed GPI-linked proteins-enriched early endosomal compartments (GEECs), which have a tubulovesicular structure instead of the typical small spherical carriers of clathrin and caveolar pathways (Sabharanjak et al., 2002; Kirkham et al., 2005). The endocytosis process is regulated by the Rho family member Cdc42, as well as Arfl (an ADP-ribosylation factor family protein) by recruiting a RhoGAP domain-containing protein, ARHGAP10, to the plasma membrane, and it is dynamin-independent (Kumari et al., 2008; Kumari and Mayor, 2008). Recently, it was found that the CLIC/GEEC endocytosis pathway is the key endocytic route for cholera toxin B uptake into fibroblasts (Howes et al., 2010), where previously the toxin had always been regarded as internalized via caveolae- and flotillin-mediated
pathway (Shogomori and Futerman, 2001; Glebov et al., 2006). The post-internalization route of the cargo via CLIC/GEEC pathway is unclear, but study of *Helicobacter pylori* VacA toxin showed that trafficking of GEECs to early endosomes and late endosomes additionally requires polymerized actin (Gauthier et al., 2007).

### 1.6.3.3 Arf6 mediated pathway

Arf6, a GTPase involved in actin remodelling, has been implicated as a mode of clathrin-, caveolae-, and dynamin-independent endocytosis for several cargo proteins, such as class I major histocompatibility complex (MHC1), and E-cadherin, into tubular endosomes (Mayor and Pagano, 2007; Donaldson et al., 2009; Kumari et al., 2010). Endosomes in this pathway are enriched with Arf6, PIP2, and cholesterol, and are also coated with actin. At later stages, the endosomes are either directed to late endosomes or recycled back to plasma membrane (Donaldson et al., 2009). Overall, it is not clear whether Arf6 is involved directly in cargo internalization or if it is required for vesicle trafficking to the downstream pathway, since overexpression of GTP-locked Arf6 blocked at a post-internalisation step (Brown et al., 2001).

### 1.6.4 Macropinocytosis

Macropinocytosis is a non-specific cellular uptake pathway that involves formation of transient membrane protrusions on the plasma membrane for internalization of fluid into a large vacuole, termed macropinosome, 0.2 – 10 μm in diameter (Mercer and Helenius, 2009; Kumari et al., 2010). The formation of the membrane protrusions is driven by small GTPases (Rac1, Cdc42, Arf6), and kinases, such as p21-activated kinase 1 (PAK1), protein kinase C (PKC), c-Src, which lead to activation of the actin polymerization cascade (Ridley et al., 1992; Dharmawardhane et al., 2000; Garrett et al., 2000; Lundmark et al., 2008; Mercer and Helenius, 2008). Macropinosomes are formed as a result of the collapse
and fusion of the membrane protrusions onto the plasma membrane. The membrane fission machinery for a macropinosome formation is unknown, but some studies suggested the fission is achieved by either C-terminal binding protein 1 (CtBP-1) or dynamin for some instances (Liberali et al., 2008; Mercer and Helenius, 2009).

Macropinocytosis uptake overlaps with several other endocytic pathways, and there is no specific cellular marker for the pathway. Rab34 was found to co-localize with actin at membrane ruffles, and it also facilitated macropinosome formation in platelet-derived growth factor- or phorbol ester-induced macropinocytosis in mouse 10T1/2 fibroblasts (Sun et al., 2003), but not in HeLa cells (Goldenberg et al., 2007). Currently, one of the main experimental criteria for macropinocytosis of a pathogen is the inhibition of the pathogen uptake by amiloride or ethylisopropyl amiloride (EIPA) treatment (Mercer and Helenius, 2009). Both chemical inhibitors are not specific to macropinocytosis, and other signalling pathways might be altered during the treatment.

1.7 Transcytosis

Transcytosis is a diverse mechanism that plays an important role in the continuous exchange of molecules across the cells (Predescu et al., 2007). To date, the knowledge on transcytosis pathways is mainly from studies of immunoglobulins, and macromolecules transportation in polarized epithelial cells (Ghitescu and Bendayan, 1992; Apodaca, 2001; Tzaban et al., 2009). However, it is unknown if similar membrane trafficking pathways exist in flat endothelial cells.

After ligands are internalized from apical or basolateral surfaces, the generic trafficking pathways that they follow are to either first enter into apical (AEE) or basolateral early endosomes (BEE), respectively (Figure 1.10). Cargoes sorted for recycling to the plasma membrane are transported to common recycling endosomes (CRE) and sorted into apical and basolateral recycling routes, facilitated by Rab11a, Rab25 and
their effector myosin Vb (myoVb) (Golachowska et al., 2010). Some cargo may recycle directly from BEE to the basolateral plasma membrane surface (Sheff et al., 1999). Similarly, apically internalized cargo may be transported to Rab11-positive ARE and recycle back to apical plasma membrane (Leung et al., 2000). Study of the transportation of immunoglobulin G (IgG) by Fc receptor (FcRN) revealed that apical to basolateral transcytosis of the complex is regulated by Rab25 and myoVb, but not Rab11a (Tzaban et al., 2009). While Rab11a, Rab11 family interacting proteins (Rab11-FIPs), Rab25, and myoVb are required for IgG-FcRN and polymeric IgA (pIgA) basolateral to apical transcytosis (Ducharme et al., 2007; Tzaban et al., 2009). On the other hand, a transcytosis route that results from LE fusion with either apical or basolateral plasma membrane surface has been proposed (Lakkaraju and Rodriguez-Boulan, 2008), based on the observation of the release of exosomes from apical and basolateral surface by different epithelial cells, but the molecular details are unknown (Hundorfean et al., 2007; Mallegol et al., 2007).

For endothelial cells, albumin and insulin transportation across the cultured endothelium monolayer on Transwell® insert has been proposed to be via caveolae (Schnitzer et al., 1994). The in vitro observation has also been demonstrated in vivo by employing caveolin-1 knockout mice, where the uptake and transport of radioiodinated albumin from the blood to the interstitium is abolished in the knockout animals (Schubert et al., 2001). Beyond caveolae-mediated transcytosis, the roles of microtubules, cytoskeleton, motor proteins, and GTPases during transcytosis in endothelium are poorly understood.
Figure 1.10. Endocytosis and transcytosis pathway in polarized epithelial cells. The illustration shows the transcytosis routes in polarized epithelial cells. The light blue arrows highlight apical to basolateral transcytosis route based on study of IgG-FcRN transportation in Mardin-Darby canine kidney epithelial cell line (MDCK) (Tzaban et al., 2009).

1.8 Endocytosis of pathogens

The main difference between phagocytic and non-phagocytic cells is that the uptake of molecules by phagocytic cells is an active process, and the cells are able to take up relatively large cargo; whereas non-phagocytic cells would only endocytose small molecules in general, and the uptake process is passive. In the past, endocytosis was
thought to occur independently of actin, while phagocytosis is an actin-dependent cellular uptake pathway (Messick and Rikihisa, 1993). Therefore, bacteria were thought to enter non-phagocytic cells via phagocytosis-like pathway, which required actin remodelling (Veiga and Cossart, 2006). Generally, invasive bacterial entry of non-phagocytic cells is classically categorized into the “zipper” and the “trigger” mechanisms (Finlay and Cossart, 1997; Cossart and Sansonetti, 2004; Veiga and Cossart, 2006; Haglund and Welch, 2011). *Listeria monocytogenes* and *Yersinia pseudotuberculosis* are two well-studied bacteria that invade host cells by the zipper mechanism. Upon interaction of bacterial surface proteins and cellular receptors on the plasma membrane, such as *Listeria* InlA and InlB interact with E-cadherin and Met respectively (Cossart and Sansonetti, 2004), a cascade of signalling molecules is activated that results in actin polymerization and minor membrane extensions that engulf a bacterium into the epithelial cell line, Vero (Cossart and Sansonetti, 2004). For trigger mechanism, bacteria such as *Salmonella* and *Shigella*, inject bacterial effectors via secretory apparatus, such as the type 3 secretion system (T3SS), into eukaryotic cells, triggering massive actin polymerization and membrane ruffling that results in bacterial engulfment (Cossart and Sansonetti, 2004).

The classical dogma of bacterial invasion of non-phagocytic cells was challenged when *Listeria*, whose entry requires actin polymerization and which was at least 10-fold larger than a clathrin coat, was found to invade epithelial cells by hijacking clathrin-mediated endocytic machinery (Veiga and Cossart, 2005). Following this discovery, the involvement of an endocytic coat in bacterial invasion of non-phagocytic cells was established. It was shown that “zippering” bacteria, but not “triggering” bacteria, hijack cellular endocytic machinery for non-phagocytic cell entry. Intriguingly, EPEC, which remains extracellular during an infection, injects bacterial factors that recruit clathrin to form actin-rich pedestal at the bacterial attachment site (Veiga *et al.*, 2007). Table 1 shows
a list of endocytic pathways with pathogens or pathogen-derived toxins that have been identified to hijack respective endocytosis pathways for cell entry.
<table>
<thead>
<tr>
<th>Endocytic mechanism</th>
<th>Dynamin-dependence</th>
<th>Pathogen or toxin</th>
<th>Cell lines/ cells</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td><em>Staphylococcus aureus</em> (Veiga <em>et al.</em>, 2007)</td>
<td>HeLa, BSC1.</td>
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<td><em>Chlamydia trachomatis</em> (Hybiske and Stephens, 2007)</td>
<td>HeLa</td>
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<td></td>
<td></td>
<td>Ebola virus (Bhattacharyya <em>et al.</em>, 2010)</td>
<td>HEK293T, Vero, HeLa, HOS-CD4, human microvascular endothelial cells.</td>
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<td>Severe acute respiratory syndrome coronavirus (SARS-cov) (Inoue <em>et al.</em>, 2007)</td>
<td>Human hepatoma HepG2</td>
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<td></td>
<td>Vesicular stomatitis virus (Cureton <em>et al.</em>, 2009)</td>
<td>BSC1.</td>
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<td></td>
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<td>Cholera toxin (Torgersen <em>et al.</em>, 2001)</td>
<td>BHK, Caco-2, HeLa.</td>
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<td>FimH-expressing <em>E. coli</em> K-12 strain [ORN103(pSH2)] (Shin <em>et al.</em>, 2000)</td>
<td>Mouse bone marrow-derived mast cells.</td>
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<td>Mouse polyoma virus (Richterova <em>et al.</em>, 2001)</td>
<td>NIH 3T6 mouse fibroblasts, normal murine mammary gland (NMuMG) epithelial cells.</td>
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<td>Cell Type</td>
<td>Pathway</td>
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<td>Hippocampal neuron</td>
<td>Flotillin-mediated endocytosis</td>
<td>Cholera toxin B (Shogomori and Futerman, 2001)</td>
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<td>COS-7.</td>
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<td>Cholera toxin B (Glebov et al., 2006)</td>
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<td>HeLa.</td>
<td>Macropinocytosis</td>
<td>Vaccinia virus (Mercer and Helenius, 2008)</td>
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<tr>
<td>HeLa.</td>
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<td>Human rhinovirus type 8 (HRV8) (Khan et al., 2011)</td>
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<td>Rhabdomyosarcoma cells.</td>
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<td>HIV-1 (Liu et al., 2002)</td>
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<td>Primary human BMEC.</td>
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<td>Kaposi’s sarcoma-associated herpesvirus (KSHV) (Raghu et al., 2009)</td>
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<td>Human dermal microvascular endothelial cells,</td>
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<td>Afipia felis (Schneider et al., 2007)</td>
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<td>human umbilical vein endothelial cells.</td>
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<td>Murine macrophage-like cell lines J774E, RAW264.7.</td>
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<tr>
<td>HeLa.</td>
<td>CLIC/GEEC pathway</td>
<td>Helicobacter pylori VacA toxin (Gauthier et al., 2005; Gauthier et al., 2007)</td>
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<td>Cholera toxin B (Howes et al., 2010)</td>
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<tr>
<td>HeLa.</td>
<td>Arf6-dependent endocytosis</td>
<td>unknown</td>
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<td>Fibroblast</td>
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1.8.1 *E. coli* K1 virulence factors and invasion of HBMEC

Although a newborn rat model has long been established for *E. coli* K1 infection (Glode *et al.*, 1977; Moxon *et al.*, 1977), compared to animal models for other bacterial infections, this has not led to much understanding of the bacterial pathogenesis. Since the availability of purified human brain microvascular endothelial cells (HBMEC) as an *in vitro* BBB model, bacterial invasion mechanisms and bacterial virulence factors (VF) contributing to the disease pathophysiology have been studied extensively (Stins *et al.*, 1994; Huang *et al.*, 1995).

The K1 capsule of *E. coli* K1 has long been identified as the primary bacterial virulence factor responsible for bacterial intravascular survival and replication (Wilfert, 1978; Kim *et al.*, 1992; Metkar *et al.*, 2007). The capsule is a homopolymer of N-acetylneuraminic acid with poly(a-2,8) linkages, structurally similar to the polysialic acid (PolySia) moiety on mammalian neural cell adhesion molecule (NCAM) found in the neonatal brain as well as kidney (Finne, 1982; Finne *et al.*, 1983a; Roth *et al.*, 1987), which is important for cell migration and neuronal plasticity (Rutishauser, 2008). The capsule is also immunochemically similar to the *Meningococcus* group B polysaccharides (Finne *et al.*, 1983b). It has also been shown to exist as O-acetylated and non-O-acetylated forms in clinical isolates, but the significance of the capsule acetylation in bacterial virulence is not clear (Frasa *et al.*, 1993; Colino and Outschoorn, 1999). Capsule variation results from slipped-strand DNA mispairing of a microsatellite domain located at the 5′ of *neuO* gene, an acetylase gene (Deszo *et al.*, 2005; Vimr and Steenbergen, 2006). Zelmer *et al.* (2008) showed that the meningitis-causing K1 strain expresses the non-O-acetylated form of PolySia in the gastrointestinal tract and in the blood compartment, but stops expressing PolySia capsule in the meninges, which might suggest that the bacteria regulate their capsule expression in response to unknown molecules secreted from the host immune cells. It is also believed that structural and immunochemical similarity between *E. coli* K1
capsule and mammalian PolySia glycan made the K1 capsule a poor immunogen for the host, to avoid host autoimmune responses to PolySia glycan (Harvey et al., 2001).

Intriguingly, the K1 capsule also demonstrates anti-phagocytic property (Bortolussi et al., 1979) as E. coli K1 with depolymerised capsule (mediated by endosialidase E) show enhanced uptake into peritoneal macrophages isolated from 5 – 10 days old rats (Mushtaq et al., 2005). It has also been shown that the K1 capsule prevents binding of C3 cleavage products and deposition of C9, which is required for the formation of complement membrane attack complex (MAC), on the bacteria (Cross et al., 1986; Mushtaq et al., 2004). Therefore, the PolySia capsule protects the bacterium from the bactericidal action of serum and the host complement system (Cross et al., 1986), which enable the bacteria to survive and replicate intravascularly to high levels of bacteraemia. In addition to the important role for evading host immunity, the PolySia capsule is also important for the bacterial intracellular survival by preventing the bacteria-containing vacuoles from fusing with lysosomes (Hoffman et al., 1999; Kim et al., 2003).

The ability of E. coli K1 to replicate to high level of bacteraemia is insufficient to develop meningitis, but the bacteria must have the ability to bind to the target tissue under high blood flow and pressure in the BBB, followed by cell invasion. It is known that pathogenic E. coli can form filamentous surface appendages known as pili or fimbriae, which function primarily for cell adhesion at the initial stage of infection (Capitani et al., 2006). E. coli is able to express many types of fimbriae, which can be divided according to the structures of specific mammalian cell receptor that they recognise, such as α-D-mannosides (type 1 fimbriae), α-D-Gal-(1,4)-β-D-Gal (P fimbriae), or NeuAcα2,3-galactose (S fimbriae) (Saukkonen et al., 1988; Wang et al., 2004). Fimbrial phase variation has been observed both in vitro and in vivo (Nowicki et al., 1985; Saukkonen et al., 1988). E. coli K1 expressing type S-fimbriae bind to choroid plexus epithelium, the luminal surface of vascular endothelium, and HBMEC in vitro (Parkkinen et al., 1988;
Prasadarao et al., 1993; Stins et al., 1994). It was also found that the S-fimbriae-expressing bacteria predominate in all body fluid and CSF after infection, and it is more virulent than *E. coli* K1 expressing type I-fimbriated form *in vivo* (Saukkonen et al., 1988). Further study with a *pilA- (fimA)*, the gene that codes for the main fimbriae structure, deleted *E. coli* K1 mutant showed no difference in level of bacteraemia and intestinal colonization, but the bacterial oropharyngeal colonization was impaired (Bloch and Orndorff, 1990). In contrast, by using a different *E. coli* K1 clinical isolate, RS218, and its S fimbriae-deleted mutant, another study found that S fimbriae are not major determinants for bacterial binding to HBMEC and for causing meningitis *in vivo* (Wang et al., 2004). Studies with the RS218 strain further found that the bacteria bind to CD48 on HBMEC via FimH of the bacterial type I fimbriae (Teng et al., 2005; Khan et al., 2007), which is a tip component of the fimbriae (Capitani et al., 2006). Interaction of the bacterial FimH with CD48 increases cytosolic free calcium levels and activates RhoA (Khan et al., 2007). In addition to having a major role in bacterial adhesion, purified *E. coli* K1 FimH has also been shown to activate a murine microglial cell line, which results in production of nitric oxide and tumour necrosis factor-α (TNFα) (Lee et al., 2005).

Outer membrane protein A (OmpA) is a 35 kDa bacterial surface transmembrane protein with four extracellular loops. It is highly conserved in many Gram negative bacteria, and is important for efficient binding of *E. coli* K1 to HBMEC (Prasadarao et al., 1996b). The N-terminal loops of the protein interact with N-acetylglucosamine (GlcNAc) epitopes on the gp96 receptor on HBMEC (Prasadarao et al., 1996a; Prasadarao, 2002). gp96 receptor is an endoplasmic reticulum chaperone, which is also expressed on the cell surface of human colonic carcinoma (Caco-2) cell line (Li et al., 2002), and has also been shown to interact with *Listeria Vip* (Cabanés et al., 2005). OmpA-gp96 interaction induces the signal transducer and activator of transcription 3 (stat3) signalling pathway. phosphorylation of the focal adhesion kinase (FAK), activation of phosphatidylinositol-3-
kinase (PI3K), and leads to the phosphorylation of protein kinase Ca (PKCa) (Reddy et al., 2000a; Reddy et al., 2000b; Prasadarao, 2002; Maruvada et al., 2008). The activation of these signalling events results in actin polymerization at the bacterial adhesion site that is necessary for bacterial internalization into HBMEC (Prasadarao et al., 1999).

Several other bacterial determinants have been identified to be crucially important for E. coli K1 invasion of HBMEC, namely IbeA, IbeB, YijP, and cytotoxic necrotizing factor 1 (CNF1) (Huang et al., 1995; Huang et al., 1999; Wang et al., 1999; Khan et al., 2002). A specific HBMEC receptor for CNF1 has been identified as a 37 kDa laminin receptor precursor (37LRP) by screening the cDNA library of HBMEC in a yeast two-hybrid system (Chung et al., 2003), whereas vimentin was identified as the specific HBMEC receptor that interacts with IbeA (Chi et al., 2010). It was found that both CNF1 and OmpA use different signalling pathway for recruiting actin to bacterial adhesion site, where the interaction of the bacterial CNF1 with HBMEC leads to RhoA activation, without activating PI3K (Khan et al., 2002; Khan et al., 2003).

The data from previous studies might suggest that E. coli K1 invasion of HBMEC involved the activation of several signaling pathways by different bacterial VF, with the primary goal in inducing actin cytoskeleton rearrangement for bacterial entry via zippering mechanism (Prasadarao et al., 1999). However, the specific endocytic pathway hijacked by the bacterial invasion of HBMEC is not well-studied. Clathrin was shown to be absent from the bacterial attachment site (Prasadarao et al., 1999), but the bacteria were reported to invade HBMEC via caveolae-mediated endocytosis (Sukumaran et al., 2002).

Studies performed with the BBB in vitro model showed that, following bacterial invasion, E. coli K1 do not replicate intracellularly (Prasadarao et al., 1999). The bacteria are localised in compartments positive for early endosomal markers (Rab5, EEA1, and transferrin receptor) at early time point of infection, and are labelled for late endosomal markers (Rab7 and LAMP1) without the presence of cathepsin D, a lysosomal hydrolytic
enzyme (Kim et al., 2003). The intracellular E. coli K1 were also reported to co-localize with caveolin-1 (Sukumaran et al., 2002).

The transcytosis route of E. coli K1 across BBB is unclear. The bacteria were demonstrated to traverse HBMEC monolayer grown on Transwell insert without affecting monolayer integrity, which suggested the bacteria crossed the monolayer via transcellular route (Stins et al., 2001; Zhang et al., 2002). In contrast, another study reported that OmpA-expressing E. coli K1 transcytosed HBMEC via a paracellular route by activating PKCa, which subsequently disrupted HBMEC tight junctions by dissociating β-catenins from vascular endothelial cadherins (Sukumaran and Prasadarao, 2003). Similar to E. coli K1, the transcytosis of Neisseria meningitidis across brain endothelial cells is unclear and has been debated for a long time (Nassif et al., 2002; Coureuil et al., 2009). Recently, N. Meningitidis were shown to recruit junctional proteins, such as VE-cadherin, β-catenin, claudin-5, ZO-1, to the attachment site of the bacterial colonies via β-arrestin-dependent pathway, subsequently depleted junctional proteins, and resulted with the opening of intercellular junctions, which allowed the bacterial to traverse endothelial cells via paracellular route (Coureuil et al., 2009; Coureuil et al., 2010).

Although E. coli K1 is known to cause meningitis, the gastrointestinal tract has been shown as the primary site of colonization in neonatal animal model (Saukkonen et al., 1988; Bloch and Orndorff, 1990). Therefore, it is likely that the bacteria have to breach gastrointestinal epithelium into the bloodstream. The application of Caco-2, and human colonic adenocarcinoma (T84) cell lines as in vitro gastrointestinal epithelium model showed that E. coli K1 infection of these cell lines is dependent on the cell differentiation and the bacterium disrupted the tight junctions during infection for crossing the epithelium via paracellular route (Burns et al., 2001). The difference in the bacterial traversal of HBMEC and gastrointestinal epithelium model might implicate that different transcytosis routes were manipulated by the bacteria for crossing the cell barriers.
1.9 Project aims

*E. coli* K1 is able to invade HBMEC *in vitro*, and the bacterial entry is receptor-mediated via zippering mechanism. The signalling pathways that lead to actin polymerization to enable the bacterial internalization into HBMEC have been very well-investigated in previous studies, as detailed above. However, the endocytosis pathway that is responsible for the bacterial uptake is not clear. Some studies have shown that bacterial uptake is clathrin-independent (Prasadarao *et al.*, 1999), but caveola-dependent (Sukumaran *et al.*, 2002). Further, the bacteria have been shown to traverse HBMEC via a transcellular route (Stins *et al.*, 2001), but the transcytosis pathway used by the bacterium has not been described.

The major aim of this study was to further investigate the endocytosis pathway that is responsible for *E. coli* K1 invasion of HBMEC, and to describe the transcytosis pathway that the bacterium manipulates for traversal of HBMEC transcellularly. The hypothesis was that the bacterium invaded HBMEC via caveolae-mediated endocytosis and further traversed to the basolateral surface of HBMEC in a caveolin-1-associated compartment.

The first objective was to make a fluorescent protein-expressing *E. coli* K1 strain that could retain bacterial virulence. The fluorescent *E. coli* K1 strain would allow investigation of bacterium-HBMEC interactions under a confocal microscope. The second objective was to optimize HBMEC transfection efficiency with various transfection methods. High HBMEC transfection efficiency would allow investigation of comparative distributions of cellular markers with intracellular bacteria, and also the effect of a cellular marker and its corresponding mutant on *E. coli* K1 invasion/traversal of HBMEC. The third objective was then to utilise the fluorescent *E. coli* K1 strain and the optimized HBMEC transfection protocol to further investigate *E. coli* K1 invasion via caveolae-mediated endocytosis. The final objective of this project was to study the transcytosis
pathway that the bacterium manipulated for crossing HBMEC monolayer. This would give a much clearer picture of the infective behaviour of this pathogenic bacterium.
2. Materials and methods

2.1 Materials

Unless otherwise stated, all reagents and biochemicals were purchased from Sigma-Aldrich, Poole, Dorset, UK.

2.1.1 Bacterial culture and reagents

2.1.1.1 Bacterial strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype or characteristic(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E44 (E. coli K1) [A kind gift from Dr. Naveed Khan (University of Nottingham, Sutton Bonington, UK)].</td>
<td>O18:K1:H7. A spontaneous rifampin resistant mutant of RS218 strain, isolated from the CSF of meningitis patient (Silver et al., 1980; Achtman et al., 1983).</td>
</tr>
<tr>
<td>HB101 (E. coli K-12) (New England Biolabs Inc., Herts, UK)</td>
<td>F-, thi-1, hsdS20 (rB-, mB-), supE44, recA13, ara-14, leuB6, proA2, lacY1, galK2, rpsL20 (str^r), xyl-5, mtl-1</td>
</tr>
<tr>
<td>DH5α (Invitrogen, Paisley, UK)</td>
<td>F- Φ80lacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK-, mK-) phoA supE44 thi-1 gyrA96 relA1 λ'</td>
</tr>
<tr>
<td>K1-Cherry</td>
<td>E44 transformed with pFPV-mCherry.</td>
</tr>
<tr>
<td>K1-GFP</td>
<td>E44 transformed with pFPV25.1.</td>
</tr>
<tr>
<td>K1rpsM'</td>
<td>E44 transformed with rpsM'</td>
</tr>
</tbody>
</table>

2.1.1.2 Bacterial culture media

<table>
<thead>
<tr>
<th>Media</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luria-Bertani (LB) broth</td>
<td>1% (w/v) tryptone (Merck, Nottingham, UK), 0.5% (w/v) yeast extract (DIFCO, Surrey, UK), 0.5% (w/v) NaCl</td>
</tr>
</tbody>
</table>
Luria-Bertani (LB) agar
1% (w/v) tryptone, 0.5% (w/v) yeast extract. 0.5% (w/v) NaCl, 1.5% (w/v) bacteriological agar (Oxoid, available via Thermo Fisher Scientific, Loughborough, UK)

MacConkey agar (Code: CM0007) (Oxoid)
Bought as pre-made mix: 2% (w/v) peptone, 1% (w/v) lactose, 0.5% (w/v) bile salts, 0.5% (w/v) NaCl, 0.0075% (w/v) neutral red, 1.2% (w/v) agar

2.1.2 Mammalian cell propagation reagents

2.1.2.1 Mammalian cell

<table>
<thead>
<tr>
<th>Cell</th>
<th>Descriptions</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human brain microvascular endothelial cells (HBMEC)</td>
<td>Isolated from cerebral cortex specimens obtained from patients (4 – 7-year-old) with seizure disorders or from post-mortem brains. Immortalized with SV40 large T antigen.</td>
<td>Stins et al., 2001. A kind gift from Dr. Naveed Khan.</td>
</tr>
<tr>
<td>Primary mouse lung endothelial cells (MLEC)</td>
<td>Isolated from mouse lung.</td>
<td>Purified by Dr. Grant Otto and was a kind gift from Dr. Ben Nichols (MRC LMB, Cambridge).</td>
</tr>
<tr>
<td>Primary flotillin 1 knockout MLEC</td>
<td>Isolated from flotillin 1 knockout mouse lung.</td>
<td>Purified by Dr. Grant Otto and was a kind gift from Dr. Ben Nichols.</td>
</tr>
<tr>
<td>Primary caveolin 1 knockout MLEC</td>
<td>Isolated from caveolin 1 knockout mouse lung.</td>
<td>Purified by Dr. Grant Otto and was a kind gift from Dr. Ben Nichols.</td>
</tr>
</tbody>
</table>
2.1.2.2 Mammalian cell culture media

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBMEC growth media</td>
<td>RPMI-1640 supplemented with 20% (v/v) fetal bovine serum (FBS) (Biosera, Ringmer, UK), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg/ml streptomycin, 100 units/ml penicillin, non-essential amino acids (Gibco, Invitrogen), and vitamins (Gibco, Invitrogen).</td>
</tr>
<tr>
<td>MLEC growth media</td>
<td>50 ml low glucose Dulbecco’s Modified Eagles Medium (DMEM) and 50 ml Ham’s F12 supplemented with 20% (v/v) FBS, 0.00125% (w/v) heparin, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin, 0.1 mg/ml endothelial cell growth supplement (AbD Serotec, Oxford, UK).</td>
</tr>
<tr>
<td>Experimental media</td>
<td>RPMI-1640 supplemented with 5% (v/v) FBS, and 2 mM L-glutamine.</td>
</tr>
<tr>
<td>Imaging media</td>
<td>RPMI-1640 without phenol red supplemented with 5% (v/v) FBS, 2 mM L-glutamine, 25 mM HEPES (pH 7.4).</td>
</tr>
<tr>
<td>Freezing media</td>
<td>Growth media supplemented with 10% (v/v) DMSO, and 20% (v/v) FCS.</td>
</tr>
</tbody>
</table>
### 2.1.3 Bacterial expression vectors used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Characteristic(s)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCherry1</td>
<td>Bacterial expression vector containing <em>Mycobacterium tuberculosis</em> codon-optimised mCherry gene, hygromycin resistant.</td>
<td>Carroll <em>et al.</em> (2010); A kind gift from Dr. Paul Carroll (Queen Mary, University of London, UK).</td>
</tr>
<tr>
<td>pCherry3</td>
<td>Bacterial expression vector containing <em>Mycobacterium tuberculosis</em> codon-optimised mCherry gene, hygromycin resistant.</td>
<td>Carroll <em>et al.</em> (2010); A kind gift from Dr. Paul Carroll.</td>
</tr>
<tr>
<td>pEnvyl1</td>
<td>Bacterial expression vector containing <em>Mycobacterium tuberculosis</em> codon-optimised GFP gene, hygromycin resistant.</td>
<td>A kind gift from Dr. Paul Carroll.</td>
</tr>
<tr>
<td>pEnvyl2</td>
<td>Bacterial expression vector containing <em>Mycobacterium tuberculosis</em> codon-optimised GFP gene, hygromycin resistant.</td>
<td>A kind gift from Dr. Paul Carroll.</td>
</tr>
<tr>
<td>pFPV25.1</td>
<td>Bacterial expression vector containing <em>gfpmut3a</em> gene downstream of a <em>Salmonella typhimurium</em> promoter, <em>rpsM</em>, ampicillin resistant.</td>
<td>Valdivia and Falkow (1996); A kind gift from Dr. Olivier Marchès (Queen Mary, University of London, UK).</td>
</tr>
<tr>
<td>pRSET-mCherry</td>
<td>Bacterial expression vector containing mCherry, ampicillin resistant.</td>
<td>Shaner <em>et al.</em> (2004); A kind gift from Dr. Roger Tsien (University of California, San Diego).</td>
</tr>
<tr>
<td>pFPV-mCherry</td>
<td>Bacterial expression vector containing mCherry gene downstream.</td>
<td>Constructed in this study.</td>
</tr>
</tbody>
</table>
of a *Salmonella typhimurium* promoter, *rpsM*, ampicillin resistant.

**pEGFP**  
Bacterial expression vector encodes a red-shifted variant of wild type GFP, ampicillin resistant.  
Clontech, BD Biosciences, Oxford, UK; A kind gift from Dr. John Raynes (London School of Hygiene and Tropical Medicine).

**rpsM**  
pFPV25.1 with *rpsM* deleted, ampicillin resistant.  
Constructed in this study.

### 2.1.4 Mammalian expression vectors used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Characteristic(s)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pN1-EGFP</td>
<td>Mammalian expression vector encodes a red-shifted variant of wild type GFP, kanamycin resistant.</td>
<td>Clontech, BD Biosciences.</td>
</tr>
<tr>
<td>Cav1-GFP</td>
<td>pN1-EGFP vector containing wild type canine caveolin-1 gene, kanamycin resistant.</td>
<td>Pelkmans <em>et al.</em> (2001); A kind gift from Dr. Ben Nichols.</td>
</tr>
<tr>
<td>Cav1-YFP</td>
<td>Mammalian expression vector carrying EYFP tagged wild type canine caveolin-1 gene, cloned by replacing EGFP gene in Cav1-GFP, kanamycin resistant.</td>
<td>Constructed in this study.</td>
</tr>
</tbody>
</table>
GFP-Rab7WT  pC1-EGFP vector containing wild type canine Rab7 gene, kanamycin resistant. (Bucci et al., 2000); A kind gift from Professor Albert Haas (University of Bonn, Germany) and Dr. Bianca Schneider (Research Center Borstel, Germany).

GFP-Rab7T22N  pC1-EGFP vector containing canine Rab7 T22N mutant gene, kanamycin resistant. Bucci et al. (2000); A kind gift from Professor Albert Haas and Dr. Bianca Schneider.

GFP-Rab7Q67L  pC1-EGFP vector containing canine Rab7 Q67L mutant gene, kanamycin resistant. Bucci et al. (2000); A kind gift from Professor Albert Haas and Dr. Bianca Schneider.

CFP-Rab7WT  Mammalian expression vector carrying ECFP tagged wild type canine Rab7 gene, cloned by replacing EGFP gene in GFP-Rab7, kanamycin resistant. Constructed in this study.

Dyn2(aa)WT GFP  pN1-EGFP vector containing wild type rat dynamin 2 gene, kanamycin resistant. Cao et al. (1998); A kind gift from Dr. Mark McNiven (Mayo Clinic, Rochester, MN).

Dyn2(aa)K44A GFP  pN1-EGFP vector containing rat dynamin 2 K44A mutant gene, kanamycin resistant. Cao et al. (1998); A kind gift from Dr. Mark McNiven.

Dyn1WT GFP  Unknown EGFP mammalian expression vector containing dynamin 1 gene, ampicillin resistant. A kind gift from Dr. Emmanuel Boucrot and Dr. Harvey McMahon (MRC LMB, Cambridge, UK).
Dyn1K44A GFP  Unknown EGFP mammalian expression vector containing dynamin 1 K44A mutant gene, ampicillin resistant. A kind gift from Dr. Emmanuel Boucrot and Dr. Harvey McMahon.

Cav1-palmit-GFP  pN1-EGFP vector containing human caveolin-1 palmitoylation mutant gene, kanamycin resistant. Parat et al. (2003); A kind gift from Dr. Marie-Odile Parat (University of Queensland, Australia).

Cav1-Tyr14-GFP  pN1-EGFP vector containing human caveolin-1 T14A mutant gene, kanamycin resistant. Parat et al. (2003); A kind gift from Dr. Marie-Odile Parat.

Cav1β-GFP  pN1-EGFP vector containing human caveolin-1β gene, kanamycin resistant. Parat et al. (2003); A kind gift from Dr. Marie-Odile Parat.

Flotillin 1-GFP  pN1-EGFP vector containing wild type murine flotillin 1 gene, kanamycin resistant. Glebov et al. (2006); A kind gift from Dr. Ben Nichols.

Flotillin 2-GFP  pN1-EGFP vector containing wild type rat flotillin 2 gene, kanamycin resistant. (Neumann-Giesen et al., 2004); A kind gift from Dr. Ben Nichols.

GFP-Rab5WT  pCl-EGFP vector containing wild type murine Rab5 gene, kanamycin resistant. Nichols et al. (2001); A kind gift from Dr. Ben Nichols.

GFP-Rab5S34N  pCl-EGFP vector containing murine Rab5 S34N mutant gene, kanamycin resistant. Nichols et al. (2001); A kind gift from Dr. Ben Nichols.
GFP-Rab5Q79L  pC1-EGFP vector containing murine Rab5 Q79L mutant gene, kanamycin resistant.

GFP-Rab11aWT  pC1-EGFP vector containing wild type human Rab11a gene, kanamycin resistant.

GFP-Rab11aS25N pC1-EGFP vector containing human Rab11a S25N mutant gene, kanamycin resistant.

GFP-Rab11aQ70L pC1-EGFP vector containing human Rab11a Q70L mutant gene, kanamycin resistant.

LDLR-A18-GFP pN1-EGFP vector containing recycling-deficient mutant of the low-density-lipoprotein receptor gene, kanamycin resistant.

GFP-Rab9WT  pC1-EGFP vector containing wild type murine Rab9 gene, kanamycin resistant.

GFP-Rab9Q66L pC1-EGFP vector containing murine Rab9 Q66L mutant gene, kanamycin resistant.

GFP-Rab9S21N pC1-EGFP vector containing murine Rab9S21N mutant gene, kanamycin resistant.

Nichols et al. (2001); A kind gift from Dr. Ben Nichols.

(Knodler et al., 2010); A kind gift from Dr. Wei Guo (University of Pennsylvania, Philadelphia, PA).

Knodler et al. (2010); A kind gift from Dr. Wei Guo.

Knodler et al. (2010); A kind gift from Dr. Wei Guo.

(Kreitzer et al., 2003); A kind gift from Dr. Geri Kreitzer (University of Cornell).

(Seaman, 2004); A kind gift from Dr. Matthew Seaman (CIMR, Cambridge).

A kind gift from Dr. Matthew Seaman.

A kind gift from Dr. Matthew Seaman.
kanamycin resistant.

LAMP1-GFP  pN1-EGFP vector containing wild type rat lgp120 gene, kanamycin resistant. (Patterson and Lippincott-Schwartz, 2002); A kind gift from Dr. George Patterson.

2.1.5 Primary antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Monoclonal or polyclonal</th>
<th>Fixation</th>
<th>Concentrations used</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-caveolin-1</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Methanol, or formaldehyde</td>
<td>1:200 (IF)</td>
<td>BD Biosciences. A kind gift from Dr. Ben Nichols.</td>
</tr>
<tr>
<td>Anti-caveolin-1</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>Methanol</td>
<td>1:200 (IF)</td>
<td>BD Biosciences. A kind gift from Dr. Ben Nichols.</td>
</tr>
<tr>
<td>Anti-flotillin 1</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>Methanol</td>
<td>1:200 (IF)</td>
<td>BD Biosciences. A kind gift from Dr. Ben Nichols.</td>
</tr>
<tr>
<td>Anti-flotillin 2</td>
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<td>1:200 (IF)</td>
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<tr>
<td>Anti-β-Actin (Clone Ac-15)</td>
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<td>Monoclonal</td>
<td>-</td>
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<td>Sigma-Aldrich.</td>
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<tr>
<td>Antibody</td>
<td>Species</td>
<td>Type</td>
<td>Staining Medium</td>
<td>Dilution</td>
<td>Source</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>---------------</td>
<td>----------------</td>
<td>-----------------------</td>
<td>----------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>Anti- <em>E. coli</em> O18</td>
<td>Rabbit</td>
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<td>Denka-Seiken, Coventry, UK.</td>
</tr>
<tr>
<td>Anti-GFP</td>
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<td>Polyclonal</td>
<td></td>
<td>1:1000 (WB)</td>
<td>Invitrogen.</td>
</tr>
<tr>
<td>Anti-GFP (Clone 3E6)</td>
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<td>Monoclonal</td>
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<td>1:500 (IF)</td>
<td>Invitrogen.</td>
</tr>
<tr>
<td>Anti-EEA1 (Clone 14/EEA1)</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>Formaldehyde</td>
<td>1:200 (IF)</td>
<td>BD Biosciences.</td>
</tr>
<tr>
<td>Anti-pan cadherin (Clone CH-19)</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>Methanol, formaldehyde</td>
<td>1:200 (IF)</td>
<td>Abcam, Cambridge, UK.</td>
</tr>
<tr>
<td>Anti-LAMP2 (Clone ABL-93)</td>
<td>Rat</td>
<td>Monoclonal</td>
<td>Methanol, formaldehyde</td>
<td>1:500 (IF)</td>
<td>Abcam. A kind gift from Dr. Sharon Tooze and Dr. Minoo Razi (Cancer Research UK, Lincoln’s Inn Field, London)</td>
</tr>
</tbody>
</table>
### 2.1.6 Secondary antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Monoclonal or polyclonal</th>
<th>Concentrations used</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>FluoProbes®-546 anti-rabbit</td>
<td>Donkey</td>
<td>Polyclonal</td>
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<td>Interchim, Cheshire Science Ltd., Chester, UK</td>
</tr>
<tr>
<td>FluoProbes®-488 anti-mouse</td>
<td>Donkey</td>
<td>Polyclonal</td>
<td>1:200</td>
<td>Interchim.</td>
</tr>
<tr>
<td>FluoProbes®-642 anti-mouse</td>
<td>Donkey</td>
<td>Polyclonal</td>
<td>1:200</td>
<td>Interchim.</td>
</tr>
<tr>
<td>FluoProbes®-642 anti-rabbit</td>
<td>Donkey</td>
<td>Polyclonal</td>
<td>1:200</td>
<td>Interchim.</td>
</tr>
<tr>
<td>Cy2 anti-rabbit</td>
<td>Goat</td>
<td>Polyclonal</td>
<td>1:200</td>
<td>A kind gift from Professor Ulrich Schaible (Research Center Borstel, Germany).</td>
</tr>
<tr>
<td>Cy2 anti-rat</td>
<td>Goat</td>
<td>Polyclonal</td>
<td>1:200</td>
<td>A kind gift from Professor Ulrich Schaible.</td>
</tr>
<tr>
<td>ECL anti-rabbit IgG, HRP-linked whole antibody</td>
<td>Donkey</td>
<td>Polyclonal</td>
<td>1:3000</td>
<td>GE Healthcare, Buckinghamshire, UK.</td>
</tr>
<tr>
<td>ECL anti-mouse IgG, HRP-linked whole antibody</td>
<td>Sheep</td>
<td>Polyclonal</td>
<td>1:3000</td>
<td>GE Healthcare, Buckinghamshire, UK.</td>
</tr>
</tbody>
</table>
2.1.7 Cell imaging reagents

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>4',6-diamidino-2-phenylindole, dihydrochloride (DAPI)</td>
<td>Molecular Probes, Invitrogen.</td>
</tr>
<tr>
<td>Endosialidase–GFP fusion protein (PK1A-GFP)</td>
<td>Jokilammi et al. (2004): A kind gift from Professor Jukka Finne (University of Turku, Finland).</td>
</tr>
<tr>
<td>Fluorescein isothiocyanate (FITC)</td>
<td>Sigma-Aldrich.</td>
</tr>
<tr>
<td>Phalloidin-TRITC</td>
<td>Sigma-Aldrich.</td>
</tr>
</tbody>
</table>

2.1.8 Reagents used in the transfection of mammalian cells

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>jetPRIME™</td>
<td>Polyplus-transfection SA, Illkirch, France.</td>
</tr>
<tr>
<td>jetPEI™</td>
<td>Polyplus-transfection SA.</td>
</tr>
<tr>
<td>jetPEI™-HUVEC</td>
<td>Polyplus-transfection SA.</td>
</tr>
<tr>
<td>FuGENE® HD</td>
<td>Promega, Southampton, UK.</td>
</tr>
<tr>
<td>Lipofectamine™ 2000</td>
<td>Invitrogen.</td>
</tr>
<tr>
<td>PLUS™ Reagent</td>
<td>Invitrogen.</td>
</tr>
</tbody>
</table>

2.1.9 Primers for constructing pFPV-mCherry

Oligonucleotides were synthesized by Eurofins MWG Synthesis, Germany. The primers were dissolved to 100 pmol/μl in sterile MilliQ water and stored at -20°C.

1. mCherry-FXba          | TGCTCTAGATTTAAGAAGGAGATATACATATATGGTGAGC |
                        | AAGGGCGAGGAG                                         |
2. mCherry-RSph

CATGATGCTTACTTTGACGCTCGTCAT

2.1.10 Buffers and solutions

TBE Buffer, 50x
Dissolve 108 g Tris base (Trizma), 55 g boric acid and 40 ml 0.5 M EDTA [pH 8.0]. Make up to 1000 ml with water.

Agarose Loading Dye, 5x
0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF in 40% (w/v) sucrose solution.

Ampicillin (100 mg/ml) (1000x)
Dissolve 1 g ampicillin in 5 ml sterile MilliQ water, and add 5 ml absolute ethanol to the solution. Store at -20°C, protected from light.

Kanamycin (30 mg/ml) (1000x)
Dissolve 0.3 g kanamycin in 10 ml sterile MilliQ water. Store at -20°C.

Chloramphenicol (34 mg/ml) (1000x)
Dissolve 0.34 g chloramphenicol in 10 ml absolute ethanol. Store at -20°C, protected from light.

10% (w/v) sodium dodecyl sulphate (SDS)
Dissolve 10 g SDS in 100 ml MilliQ water.

CCMB buffer [10 mM KAc, 10% (v/v) glycerol, 80 mM CaCl₂·2H₂O, 10 mM MgCl₂·6H₂O, 20 mM MnCl₂·4H₂O, pH 6.4]
Dissolve 0.1 g KAc, 10 ml glycerol, 1.18 g CaCl₂·2H₂O, 0.2 g MgCl₂·6H₂O, 0.4 g MnCl₂·4H₂O in 50 ml MilliQ water. Adjust pH to 6.4. Top up with MilliQ water to 100 ml, and filter the solution with 0.22 μm syringe filter. Store at 4°C.

10x Tris-Glycine buffer
Dissolve 15 g Tris, and 72 g Glycine in 500 ml MilliQ water. Store at 4°C.

4x resolving buffer [1.5 M Tris, 0.4% (w/v) SDS, pH 8.8]
Dissolve 18.17 g Tris and 4 ml 10% (w/v) SDS in 100 ml MilliQ water. Adjust pH to 8.8. Store at 4°C.
<table>
<thead>
<tr>
<th>Buffer/Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x stacking buffer</td>
<td>Dissolve 6.06 g Tris and 4 ml 10% (w/v) SDS in 100 ml MilliQ water. Adjust pH to 6.8. Store at 4°C.</td>
</tr>
<tr>
<td>1x SDS-PAGE running buffer</td>
<td>50 ml 10x Tris-Glycine buffer and 5 ml 10% (w/v) SDS. Top up to 500 ml with MilliQ water.</td>
</tr>
<tr>
<td>Blotting buffer</td>
<td>100 ml 10x Tris-Glycine buffer and 200 ml methanol. Top up to 1000 ml with MilliQ water.</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulphate (APS)</td>
<td>Dissolve 0.05 g APS in 0.5 ml MilliQ water. Made fresh each time.</td>
</tr>
<tr>
<td>6x SDS sample buffer</td>
<td>Dissolve 1 g SDS, 1.2 mg bromophenol blue, 3 ml glycerol in 7 ml 4x resolving buffer. Store at room temperature. Prior to usage, add 6 µl 2-Mercaptoethanol to 94 µl the solution.</td>
</tr>
<tr>
<td>1x Dulbecco’s phosphate buffered saline (PBSA)</td>
<td>Dissolve 0.2 g KCl, 0.2 g KH₂PO₄, 8 g NaCl, and 1.74 g Na₂HPO₄·7H₂O in 900 ml MilliQ water. Adjust pH to 7.2. Top up to 1000 ml with MilliQ water. Sterilize by autoclaving.</td>
</tr>
<tr>
<td>10x Phosphate buffered saline (PBS)</td>
<td>Dissolve 80 g NaCl, 2 g KCl, 11.5 g Na₂HPO₄·7H₂O, and 2 g KH₂PO₄ in 900 ml MilliQ water. Adjust pH to 7.2. Top up with MilliQ water to 1000 ml. Sterilize by autoclaving.</td>
</tr>
<tr>
<td>0.2 mg/ml hydrocortisone</td>
<td>5 mg/ml hydrocortisone: Dissolve 50 mg hydrocortisone in 10 ml absolute ethanol. Store solution at -20°C protected from light.</td>
</tr>
<tr>
<td></td>
<td>0.2 mg/ml hydrocortisone: Add 0.4 ml 5 mg/ml hydrocortisone to 9.6 ml sterile complete growth medium. Aliquot into 1 ml aliquots. Store at -20°C protected from light.</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 PCR amplification of mCherry gene from pRSET-B mCherry

50 µl PCR reactions were carried out using 1x Pfu reaction buffer (Roche, West Sussex, UK), 0.3 µM primers (see section 2.10), 0.2 mM dNTPs (an equimolar mixture of dATP, dCTP, dGTP, and dTTP to an overall concentration of 0.2 mM), 1 U Pfu DNA polymerase (Roche), and 10 ng pRSET-B mCherry plasmid DNA.

200 µl thin-walled reaction tubes were used in a thermal cycler (MJ-Research, Hertfordshire, UK). The reactions were heated to 95°C for 5 mins before 25 cycles of: 94°C for 45 secs (denaturation), 55°C for 1 min (primer-template annealing), and 72°C for 1 min (polymerisation); followed by 72°C for 10 mins and 4°C indefinitely. The resulting products were analysed on a 0.7% (w/v) agarose gel.

2.2.2 DNA analysis by agarose gel electrophoresis

Agarose gel electrophoresis was used to check restriction digested plasmid DNA products, to visualize PCR products, and to isolate specific DNA fragments for vector and insert preparation.

Typically, 0.7% (w/v) agarose gels were casted by dissolving 0.7 g agarose in 100 ml 1x TBE buffer. When it had cooled to a temperature of around 55°C, 1 µl 10 mg/ml ethidium bromide (Invitrogen) was added. The molten agarose was poured into the gel frame and allowed to set before adding enough 1x TBE buffer to cover the gel.

Prior to loading, 2 µl loading dye was added to the DNA sample and carefully pipetted into the wells. The gel was run at a 5V/cm until the bromophenol blue front had migrated at a sufficient distance. Gels were then visualized using a UV trans-illuminator (Syngene Bio imaging, Synoptics Ltd., UK) and then photographed if required.
2.2.3 DNA fragment purification

For PCR reactions that resulted in a single PCR product on an agarose gel, QIAquick PCR Purification kit (Qiagen, West Sussex, UK) was used according to the manufacturer’s instructions to purify the PCR product from excess primers, nucleotides, polymerases, and salts, before the PCR product was used for setting up a restriction endonuclease digestion. PCR product was eluted in 30 μl buffer EB (10 mM Tris-Cl, pH8.5).

In this study, DNA from post-enzymatic manipulations for vector and insert preparation was purified from agarose gel by using QIAEX II gel extraction kit (Qiagen) according to manufacturer’s instructions. The DNA was eluted by adding 20 μl Tris buffer to the air-dried pellet and incubated at 50°C for 5 mins.

2.2.4 Restriction endonuclease digestion of DNA

Usually, 5 μg DNA was digested in a volume of 50 μl. To this, 5 μl 10x reaction buffer was added, plus enzyme to a concentration of 2 U/μg DNA (typically 1 - 2 μl) and 100 μg/ml BSA. The mixture was mixed and incubated at 37°C for 2 h, or longer if complete digestion was required. At the end of the reaction, the enzyme was inactivated by heating at 65°C for 20 mins or higher temperatures as appropriate.

2.2.5 Ligation of DNA fragments

The reaction that allows for the insertion of the desired gene into a vector is catalyzed by T4 DNA ligase and involves the ATP-dependent formation of a phosphodiester bond between the 3’hydroxyl end of a double-stranded DNA fragment and the 5’phosphate end of the same or another DNA fragment. The reaction conditions for ligation of DNA fragments are shown in Table 2.1.
Table 2.1. Components and conditions used for a ligation reaction.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x T4 DNA ligase buffer</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Insert DNA</td>
<td></td>
</tr>
<tr>
<td>Vector DNA</td>
<td></td>
</tr>
<tr>
<td>T4 DNA ligase (Fermentas, UK)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Final Reaction volume</td>
<td>10.0 μl</td>
</tr>
<tr>
<td>Ligation condition</td>
<td></td>
</tr>
</tbody>
</table>

Depending on the ratio of insert DNA to vector DNA used. Generally 3:1 molar equivalent insert to vector.

The ligation reaction was performed in an autoclaved 0.5 ml centrifuge tube and centrifuged briefly to bring all the contents to the bottom of the tube.

2.2.6 Extraction and purification of plasmid DNA

Plasmids are generally prepared from bacterial cultures grown between 16 - 18 h in the presence of selective agent such as an antibiotic. For routine molecular biology works, small scale plasmid DNA purification was performed with Wizard® Plus SV Minipreps DNA Purification System (Promega); whereas for mammalian cell transfection, medium scale plasmid DNA purification was performed by PureYield™ Plasmid Midiprep System (Promega) according to the manufacturer's instructions.

Both plasmid DNA purification kits uses alkaline conditions to lyse the bacterial cells, which denatures chromosomal and plasmid DNA, as well as proteins. Denatured chromosomal DNA and proteins are precipitated after the addition of acidic buffer for neutralization, while circular plasmid DNA are covalently closed. Plasmid DNA in the solution are bound to an anion-exchange column under appropriate low salt and pH conditions. RNA, proteins and other contaminants are removed by a medium-salt wash. The plasmid DNA was eluted in 10 mM Tris-Cl, pH8.5 buffer and stored at -20 °C.
2.2.7 Determination of DNA concentration and purity definitely in DNA techniques

Determination of DNA concentration was carried out using a spectrophotometer model ND-1000 (NanoDrop®) to measure absorption of DNA solutions at OD\textsubscript{260} and OD\textsubscript{280}. A figure of between 1.8-1.9 indicates pure DNA.

2.2.8 Bacterial culture conditions

\textit{E. coli} were grown at 37°C in Luria-Bertani (LB) broth overnight in a shaker incubator. For transformed bacteria, media was supplemented with ampicillin (100 µg/ml), kanamycin (30 µg/ml) or hygromycin B (50 µg/ml) as appropriate.

2.2.9 Making electroporation competent \textit{E. coli} K1 and transformation of plasmid DNA encoding fluorescent protein into \textit{E. coli} K1 by electroporation

To enable fluorescence visualisation of the bacteria under a confocal microscope, a plasmid DNA encoding fluorescent protein was transformed into the bacteria. The transformation of plasmid DNA into \textit{E. coli} K1 was performed using electroporation according to Seidman \textit{et al.}, 2001, since plasmid DNA transformation using heat shock of chemically-competent cells was very inefficient for this strain.

Briefly, a starter culture of \textit{E. coli} K1 was grown overnight at 37°C in LB broth with shaking. The overnight bacterial culture was inoculated into 500 ml LB broth at 50 times dilution and was grown at 37°C for approximately 1.5 h with shaking, or until the OD\textsubscript{595nm} reached between 0.5 – 0.7. Bacteria were harvested (4200 rpm in Beckman J2-13.1, at 4°C for 20 mins), washed twice in pre-chilled sterile distilled water and once in 10% (v/v) glycerol, suspended in 0.5 ml 10% (v/v) glycerol, and stored at -80°C in 50 µl aliquots. Plasmid DNA (approximately 1.0 µg in maximum 5 µl volume) was added to competent bacteria, pulsed at 2.5 kV (Biorad Gene Pulser Xcell electroporation system) with an electrode gap of 0.2 cm. 250 µl LB broth was added immediately to the bacteria,
and incubated at 37°C for 1.5 h with shaking. The culture was plated onto an LB agar plate supplemented with the appropriate antibiotic to select for transformed bacteria and was incubated at 37°C overnight. Colonies were picked the following day and grown up.

To screen for transformed bacteria that fluoresced, 5 µl of the overnight bacterial culture was spotted onto a glass slide and a cover slip was laid onto the culture carefully to avoid air bubble formation. The transformed bacteria were checked for fluorescence using a Zeiss Axioplan 2 fluorescence microscope with CCD or a Zeiss LSM510 confocal microscope.

2.2.10 Making chemical competent E. coli cells and transformation of plasmid DNA into E. coli by heat shock

To maintain plasmid in bacterial stocks, all plasmid DNA were initially propagated in E. coli DH5α strain. This strain, and E. coli K-12 (HB101) that was used as a non-pathogenic E. coli control in all bacterial assays, are compatible with heat-shock transformation and were prepared by chemical competency (Seidman et al., 2001).

To make competent bacteria, a starter culture of E. coli K-12 or DH5α was grown overnight at 37°C in LB broth with shaking. The overnight bacterial culture was inoculated into 500 ml LB broth at 50 times dilution and was grown at 37°C for 3 h with shaking to the OD595nm reached between 0.5 – 0.7. Bacteria were harvested (4200 rpm in Beckman JS-13.1 at 4°C for 15 mins), washed once in pre-chilled CCMB buffer, and incubated on ice for 1 h. The bacterial suspension was harvested as previously described, the bacterial pellet was re-suspended in 5 ml CCMB buffer, and stored at -80°C in 50 µl aliquots. Plasmid DNA (approximately 1.0 µg in maximum 10% bacterial volume) was added to competent bacteria, mixed and incubated on ice for 30 mins. Each transformation reaction was heat-pulsed at 42°C for 30 s and immediately incubated on ice for 2 mins. 250 µl LB broth was added to the bacteria, and incubated at 37°C for 1.5 h with shaking. The culture was plated
onto an LB agar plate supplemented with the appropriate antibiotic to select for transformed bacteria and was incubated at 37°C overnight. Colonies were picked the following day and grown up in LB supplemented with the appropriate antibiotic.

When making fluorescent E. coli K-12, the transformed bacteria were checked for fluorescence with a Zeiss Axioplan 2 fluorescence microscope with CCD or a Zeiss LSM510 confocal microscope, as described in section 2.2.9.

### 2.2.11 Making bacterial frozen stocks

400 μl log phase bacterial culture was added to 600 μl sterile 75% (v/v) glycerol in a sterile microcentrifuge tube. The bacteria-glycerol mixture was vortexed and stored immediately in -80°C. To recover the bacteria, the bacterial stock was retrieved from -80°C on dry ice, and the frozen surface of the culture was scraped with a sterile inoculation loop. The bacteria on the loop were streaked immediately onto LB agar plate containing appropriate antibiotics, if required. The LB agar plate was incubated at 37°C for 16 – 18 h.

### 2.2.12 Bacterial growth kinetics

A starter culture of the bacteria was grown overnight at 37°C in LB broth with shaking. The overnight culture was inoculated into fresh LB broth at 50 times dilution. The optical density (OD₅₉₅) of the culture was read using a spectrophotometer (WPALightware®, Cambridge, UK). The culture was then incubated at 37°C in a shaker incubator with shaking at 200 rpm for 120 mins. At various time points (30, 60, and 120 mins), 1 ml of the culture was sampled and the OD₅₉₅ was read with the spectrophotometer.
2.2.13 HBMEC and primary mouse lung endothelium (MLEC) culture maintenance

2.2.13.1 HBMEC and MLEC propagation

HBMEC, and MLEC cultures were maintained in a 75cm² vented tissue culture flasks in growth media at 37°C, 5% CO₂ in a CO₂ incubator. Cells were split at approximately a 1:10 ratio every 5 days using the following protocol. Medium was removed and flasks were rinsed with 2 ml 0.25% trypsin, 0.53 mM EDTA solution. An additional 3 ml trypsin-EDTA was added and the cells were incubated at 37°C for 10 mins. 7 ml growth media was added and re-suspended. 9 ml of the trypsin/cell mixture was removed and growth medium was added to the cells. For in vitro infection experiments, 1 x 10⁵ cells/ml HBMEC or MLEC are plated in 24-well tissue culture dishes 3 days prior to experiment; for time-lapse live-cell imaging, 5 x 10⁴ cells/ml HBMEC are plated in LabTek 8-well glass cover slip chambers (Nalge Nunc, VWR) 3 days prior to experiment.

2.2.13.2 Cryopreservation of HBMEC and MLEC

HBMEC and MLEC were trypsinised as above and centrifuged at 1000 rpm for 10 mins. The supernatant was removed. The cell pellet was then re-suspended in cryopreservation media to cell density of 1 x 10⁶ cells/ml and dispensed as 1 ml aliquots into cryotubes. The aliquots were frozen at -80°C in an isopropanol freezing container for 2 – 3 days and then transferred to liquid nitrogen. For recovery from liquid nitrogen the cells were warmed in a water bath for 1 – 2 mins, and then the cells were carefully added dropwise to 10 ml pre-warmed growth media in a 50 ml tube. The cells were centrifuged at 1000 rpm for 5 mins. The media was aspirated and 1 ml fresh pre-warmed growth media was added to the cell pellet and re-suspended gently. The cell suspension was added to 9 ml pre-warmed growth media in a 75 cm² vented flask. The cells were allowed to adhere in an incubator at 37°C with 5% CO₂.
2.2.14 HBMEC transfection

For plasmid DNA transfection into HBMEC cells, 1 x $10^5$ cells/ml HBMEC were seeded onto 70% ethanol cleaned microscope glass cover slips (No. 1 13mm diameter) (VWR, Lutterworth, UK) in a 24-well tissue plate 2 days prior to experiment, in order to achieve approximately 80% confluency for transfection. Various cationic polymer and lipid-based transfection reagents as well as Nucleofection kits were tested.

2.2.14.1 Transfection with jetPRIME™ transfection reagent

On the day of transfection, cells were replenished with 0.5 ml fresh HBMEC growth medium. 1.0 μg plasmid DNA was diluted in 50 μl jetPRIME™ buffer, mixed the tube gently by tapping. 2 μl jetPRIME™ transfection reagent was added to the diluted DNA, and mixed by tapping immediately. The DNA-lipid complex mixture was incubated at room temperature for 10 mins. The DNA-lipid complex was added dropwise onto the cells, and repeatedly pipetted up and down with a P200 micropipette. The cells were incubated with the DNA-lipid complex in a tissue culture incubator at 37°C, 5% CO₂. 5 - 6 hours post-incubation, the medium containing DNA-lipid complex was removed, and cells were washed once with pre-warmed unsupplemented RPMI-1640. Cells were replenished with fresh HBMEC growth medium, were further incubated in a tissue culture incubator for 20 – 24 hours.

2.2.14.2 Transfection with Lipofectamine™ 2000 transfection reagent

On the day of transfection, cells were replenished with 0.5 ml fresh HBMEC growth medium. 0.5 μg of plasmid DNA was diluted in 100 μl Opti-MEM® reduced serum medium (Invitrogen), mixed the tube by tapping. 1 μl PLUS™ reagent was added directly to the diluted DNA, and was mixed by tapping. The reaction was incubated for 5 mins at room temperature. 3 μl Lipofectamine™ 2000 transfection reagent was added to
the diluted DNA, and mixed by tapping immediately. The DNA-lipid complex mixture was incubated at room temperature for 30 mins. The DNA-lipid complex was added dropwise onto the cells, and repeatedly pipetted up and down with a P200 micropipette. The cells were incubated with the DNA-lipid complex in a tissue culture incubator at 37°C, 5% CO₂. 5 - 6 hours post-incubation, the medium containing DNA-lipid complex was removed, and cells were washed once with pre-warmed unsupplemented RPMI-1640. Cells were replenished with fresh HBMEC growth medium, were further incubated in a tissue culture incubator for 20 – 24 hours.

2.2.14.3 Transfection with jetPEI™ transfection reagent

On the day of transfection, cells were replenished with 1.0 ml fresh HBMEC growth medium. 1.0 μg plasmid DNA was diluted in 50 μl 150 mM NaCl buffer, mixed the tube gently by tapping. 2 or 4 μl jetPEI™ transfection reagent was diluted in 50 μl 150 mM NaCl buffer in a separate microcentrifuge tube, mixed by tapping. The diluted jetPEI™ solution was added to the diluted DNA, and mixed by tapping immediately. The DNA-lipid complex mixture was incubated at room temperature for 30 mins. 100 μl DNA-lipid complex was added dropwise onto the cells, and repeatedly pipetted up and down with a P200 micropipette. The cells were incubated with the DNA-lipid complex in a tissue culture incubator at 37°C, 5% CO₂ for 20 – 24 hours.

2.2.14.4 Transfection with jetPEI™-HUVEC transfection reagent

On the day of transfection, cells were replenished with 1.0 ml fresh experimental medium. 2.0 μg plasmid DNA was diluted in 50 μl 150 mM NaCl buffer, and the tube mixed gently by tapping. 4 μl jetPEI™-HUVEC transfection reagent was diluted in 50 μl 150 mM NaCl buffer in a separate microcentrifuge tube, and mixed by tapping. The diluted jetPEI™-HUVEC solution was added to the diluted DNA, and mixed by tapping
immediately. The DNA-lipid complex mixture was incubated at room temperature for 30 mins. 100 μl the DNA-lipid complex was added dropwise onto the cells, and repeatedly pipetted up and down with a P200 micropipette. The cells were incubated with the DNA-lipid complex in a tissue culture incubator at 37°C, 5% CO₂ for 4 hours, then the medium containing DNA-lipid complex was removed, and cells were washed once with prewarmed unsupplemented RPMI-1640. Cells were replenished with fresh HBMEC growth medium, were further incubated in a tissue culture incubator for 20 – 24 hours.

2.2.14.5 Transfection with FuGENE® HD transfection reagent

On the day of transfection, cells were replenished with 0.5 ml fresh HBMEC growth medium. Before transfection, FuGENE® HD was warmed to room temperature, and mixed briefly by inverting. 0.5 μg plasmid DNA was diluted in 25 μl Opti-MEM® reduced serum medium (Invitrogen), the tube mixed gently by tapping. 1.5 μl FuGENE® HD transfection reagent was added to the diluted DNA, and mixed immediately by repeatedly pipette up and down ≈15 times. The DNA-lipid complex mixture was incubated at room temperature for 15 mins. The DNA-lipid complex was added dropwise onto the cells, and repeatedly pipetted up and down with a P200 micropipette. The cells were incubated with the DNA-lipid complex in a tissue culture incubator at 37°C, 5% CO₂ for 20 – 24 hours.

2.2.14.6 HBMEC Nucleofection with HUVEC Nucleofector Kit-OLD and Amaxa HUVEC Nucleofector Kit

Prior to experiment, Nucleofector solution was thawed to room temperature. HBMEC growth medium was pre-incubated at 37°C, 5% CO₂. 5 x 10⁵ cells/ml HBMEC were prepared and re-suspended in 100 μl Nucleofector solution. 2 μg plasmid DNA was added to the side of the electroporation cuvette and the HBMEC suspension was added
carefully into the cuvette. Cells were electroporated according to the manufacturer’s instructions. 500 µl HBMEC growth medium was added immediately to the electroporated cells and transferred to a tissue culture plate. The cells were incubated in a tissue culture incubator at 37°C, 5% CO₂ for 24 or 48 hours.

2.2.15 HBMEC and MLEC infection

On the day of experiment, 95% confluency or confluent HBMEC or MLEC in 24-well tissue culture plates were washed three times with pre-warmed plain RPMI-1640 media, and replenished with pre-warmed experimental medium. HBMEC were incubated with *E. coli* at MOI of 100. After the addition of bacteria, the plate was centrifuged at 500 x g, 15°C for 5 mins. The plate was incubated at 37°C, 5% CO₂ for 120 mins. The monolayers were washed three times with plain RPMI-1640 medium and further incubated in experimental medium containing 100 µg/ml gentamicin for 60 mins, or 5 and 18 hours for 7 and 20 hours time point respectively, to kill off extracellular bacteria. The monolayers were washed three times with plain RPMI-1640 medium or PBS and lysed with 0.3% (w/v) SDS in PBSA. The serial diluted lysates were plated on LB agar plates, and the plates were incubated overnight at 37°C. The number of bacterial colonies were counted and calculated as cfu/ml = (number of counted colonies / volume of culture plated) x dilution factor.

2.2.16 HBMEC transcytosis assay

5 x 10⁴ cells/ml HBMEC were seeded onto the apical chamber of a 4.67cm² collagen-coated polytetrafluoroethylene (PTFE) Transwell membrane, pore size 0.4 µm (Corning). Transendothelial electrical resistance (TEER) of the HBMEC monolayer on Transwell was measured with a Millicell® ERS volt-ohm meter (Millipore, Watford, UK) according to the manufacturer’s instructions. Briefly, the probe was sterilized by
immersing in 70% ethanol for 30 mins and air-dried. The probe was rinsed with plain RPMI-1640. For measuring TEER, the shorter probe was inserted into the Transwell insert carefully without touching the cells at 90°; while the longer probe was inserted into the medium in the lower chamber. The meter was set to Ohms mode and the resistance was recorded. The resistance of the monolayer was calculated by subtracting the resistance reading obtained from sample well to the resistance reading obtained from blank well. To correct for the area covered by the cell monolayer, the calculated resistance value was multiplied by the effective membrane area of the Transwell insert.

When the cells on the Transwell membrane reached complete confluency, the medium was replaced with HBMEC growth media containing 1.0 μg/ml hydrocortisone to induce intercellular tight junctions formation. The medium was changed on alternate days. 5 days after hydrocortisone treatment, the cells were used for transcytosis assay. The Transwell membranes were washed in plain RPMI-1640 medium and replenished with fresh experimental medium. 0.2 ml bacterial suspension, which contained approximately 1 x 10^8 cfu/ml bacteria, was applied to the apical chamber. At each time point (0, 2, 8, and 12 h incubation), 600 μl medium in the lower chamber was sampled for quantitation of cfu and replaced with 600 μl fresh experimental medium. The recovered organisms were differentiated by lactose fermentation on MacConkey agar.

2.2.17 Cell imaging

2.2.17.1 Fluorescein isothiocyanate (FITC) labelling of E. coli

Bacteria were grown up as above, then pelleted in a microfuge at 13000 rpm for 2 mins and re-suspended in PBS. FITC was added to a final concentration of 0.5 mg/ml in 50 mM Na₂CO₃-100 mM NaCl buffer, pH 8.0. The bacteria were incubated in the dark at room temperature for 10 mins. Free FITC was removed by washing in PBS and centrifugation at 8000 rpm, and the bacteria were used immediately.
2.2.17.2 Cell fixation

HBMEC cells were grown on glass coverslips in a 24-well tissue culture plate until 95% or complete confluent. The cells were incubated with bacteria and were processed for immunofluorescence microscopy. The cells were washed three times with plain RPMI-1640 to remove unbound bacteria prior to fixation. For formaldehyde fixation, cells were fixed with 3% formaldehyde/PBS for 15 mins at room temperature. To quench the autofluorescence signal from formaldehyde, the fixed cells were incubated in 50 mM NH₄Cl for 20 mins at room temperature.

An alternative fixation method to formaldehyde is methanol fixation. Cells on coverslips were incubated with absolute methanol for 10 mins at -20°C. Fixed cells were rehydrated sequentially in the following solutions for 1 min: absolute ethanol, 95% ethanol, 70% ethanol, 50% ethanol, and MilliQ water. After fixation, the cells were washed three times for 5 mins in wash solution (5% FCS/PBS), and immunolabelled as described in section 2.2.19.3.

2.2.17.3 Immunofluorescence staining

For intracellular staining, primary antibody (section 2.1.6) was diluted in wash solution containing 0.2% saponin. 15μl antibody mix was aliquoted onto a piece of Parafilm within a petri dish. The coverslips were inverted onto the antibody drop and incubated for 1 hour at room temperature. The humidity within the petri dish was maintained using a small damp tissue. After 1 hour incubation, the coverslips were washed three times with wash solution. The binding of primary antibodies was visualised with secondary antibodies conjugated to fluorophores (section 2.1.7), by incubating at room temperature in the dark for 1 hour. After washing three times with wash solution, the cells nuclei were stained with 1μg/ml DAPI. The cells were washed once with wash solution.
and once with PBS, cells were mounted on the glass slide with Confocal Matrix (MicroTech-Lab, Austria).

2.2.17.4 Differential antibody staining

To distinguish intracellular from extracellular bacteria, differential antibody staining was performed. For formaldehyde fixed cells, extracellular bacteria were labelled without permeabilization using anti-\textit{E. coli} O18 and FluoProbes®-642 conjugated donkey anti-rabbit. In methanol fixation, the plasma membrane is permeabilized during fixation. Therefore, for methanol fixation, the above staining procedure was performed on ice prior to fixation.

After staining with secondary antibody, cells were permeabilized with 0.1% TritonX-100/PBS for 10 mins, then intracellular bacterial staining using the same primary antibodies, followed by FluoProbes®-546 conjugated donkey anti-rabbit. This staining procedure yields extracellular bacteria with both 642 and 546 signals, while intracellular bacteria only positive for 546 signal only.

2.2.17.5 K1 capsule staining

5 µl sterile PBS was spotted onto a glass cover slip, and bacterial colonies were picked, smeared onto the glass cover slip. The bacterial smear was air-dried, fixed with 2% (v/v) formaldehyde and stained with 10 µg/ml PK1A-GFP probe in PBS without detergent. To differentiate the GFP signal from the probe and intracellular GFP signal from the K1-GFP, the PK1A-GFP-stained K1-GFP was counterstained with mouse anti-GFP clone 3E6 antibody, and followed by Fluoprobes-642 conjugated donkey anti-mouse antibody in PBS without detergent. The anti-GFP antibody only stained the GFP of PK1A-GFP probe, which bound the bacterial surface. The cover slip was mounted onto a glass slide with Confocal Matrix.
2.2.17.6 Confocal microscopy

Fluorescent images were acquired with an inverted confocal microscope (LSM510; Carl Zeiss MicroImaging, Inc.). Argon laser (excitation 488 nm) and a filter set to detect FITC emission (BP505 – 550) for imaging EGFP, FITC, Cy2, and FluoProbes-488; Argon laser (excitation 458 nm) and a filter set to detect CFP emission (BP470 – 500) for imaging ECFP; HeNe1 laser (excitation 543 nm) and a filter set to detect Rhodamine emission (LP585) for imaging mCherry and FluoProbes-546; HeNe1 laser (excitation 514 nm) and a filter set to detect YFP emission (LP530) for imaging EYFP; HeNe2 laser (excitation 633 nm) and a filter set to detect Cy5 emission (LP630) for imaging FluoProbes-642; Laser Diode (excitation 405 nm) and a filter set to detect DAPI emission (BP390 – 420) for imaging DAPI. Cells were observed using a 63x/NA 1.40 plan-Apochromat oil immersion objective with the confocal pinhole set to one Airy unit. Imaging parameters, such as gain and offset levels, and line averaging, are optimised to avoid oversaturation of pixels and to improve signal:noise ratio. Images were acquired using LSM510 software package (Carl Zeiss MicroImaging, Inc.). Image analyses were performed with ImageJ and Adobe Photoshop Elements 8.0.

2.2.17.7 Live cell imaging

For short-duration imaging experiments, HBMEC cells were grown on glass coverslips in a 24-well tissue culture plate, and were infected with *E. coli* K1 expressing fluorescent protein at 37°C with 5% CO₂. Unless otherwise stated, at the end of infection, cells were incubated in experimental medium containing 100 μg/ml gentamicin at 37°C for 1 hour. Coverslips were washed three times with prewarmed unsupplemented RPMI-1640 medium. Coverslips were lifted from the well and mounted onto a low-tech rubber gasket imaging chamber with approximately 35 μl imaging medium (Ward. 2007).
In order to track the intracellular bacterial activity over time, time lapse imaging was set up. For time lapse imaging experiments, HBMEC cells were grown in a LabTek 8-well glass cover slip chamber (Nalge Nunc) and transfected with fluorescent protein-tagged mammalian expression vector. Infection was performed as described above (section 2.2.17), infected cells were replenished with imaging medium containing 100 µg/ml gentamicin, and imaged immediately.

Prior to imaging, the confocal microscope imaging chamber was preheated to 37°C to avoid focus problems resulting from expanding metal components of the stage. If CO₂ chamber was used, CO₂ control was switched on to zero the CO₂ in the system from previous usage, and CO₂ was then supplied to the system approximately 15 mins before imaging. For acquisition of tile-z stack images, tile dimensions \([n(x), n(y)]\) was set in addition to the z-stack parameters. To avoid focus drift during imaging, autofocus function was set up during timelapse imaging.

2.2.17.8 Tile z-stack imaging

For quantification of the effect of cells overexpressing mutant proteins on *E. coli* K1 invasion, tile z-stack imaging was performed. The rationale to acquire tile images was to avoid potential experimental bias by acquiring neighbouring fields over large areas of the cover slip automatically under software control. Imaging parameters were set up as described in section 2.2.19.5, and saved. Z-stack parameters as well as tile dimensions \([n(x), n(y)]\) was set.

2.2.18 Protein gel electrophoresis and Western blotting

Cells were washed once with PBSA. 50µl 6x SDS sample buffer was added to the cells and the cells were scraped off from the tissue culture dish with a cell scraper (Sarstedt, Leicester, UK). The cell lysates were transferred to a 1.5 ml microcentrifuge
tube, and incubated at 98°C for 10 mins, followed by 1 min incubation on ice. The lysates were centrifuged at 13000 rpm for 5 mins, room temperature and supernatants removed to clean tubes. 20 μl samples were resolved in a 10% SDS gel in a Mini-Protean 3 (BioRad) with 2 μl PageRuler prestained protein ladder (Fermentas) in 1x running buffer. Gels were run for approximately 70 mins at constant current 15mA or until the front dye reached the bottom edge of the gel. The separated proteins were then transferred onto PVDF membranes (Millipore, Sigma-Aldrich) using the Mini-protean 3 transfer apparatus (BioRad). Membranes were immersed in methanol for 15 seconds, followed by soaking in distilled water for 2 mins, and then equilibrated in blotting buffer for 5 mins. Proteins were transferred overnight at 4°C at 30V, 90mA. Non-specific binding sites were blocked using PBS/0.1% Tween-20 containing 5% (w/v) dry skimmed milk for 1 hour with shaking at room temperature. To probe for specific proteins, membranes were incubated for 1 hour at room temperature with primary antibodies. After washing three times in PBS/0.1% Tween-20, membranes were incubated with HRP-conjugated secondary antibody at 1:3000 dilution. Antibody-protein complexes were detected using Enhanced chemiluminescent (ECL) Western blotting substrate (Pierce, Fisher Scientific, Leicestershire, UK) and exposure to film (Kodak, Sigma-Aldrich) in the dark for the appropriate amount of time (1 to 20 minutes) depending on signal strength. Film was developed by automatic x-ray film processor (Xograph, Gloucestershire, UK).

2.2.19 Statistical analysis

All experiments were repeated three times and performed in duplicate or triplicate. Data were analysed with GraphPad Prism Version 4.02. For data from two experimental conditions compared with each other, 2-tailed Student’s t test was used to test statistical significance. In instances where multiple experimental conditions were compared with a single control group, statistical significance was tested using one-way ANOVA followed
by Newman-Keuls multiple comparison post-comparison test. A $p$ value less than 0.05 was considered significant.
3. Construction of a fluorescent *E. coli* K1 strain that retains virulence

3.1 Introduction

To date, in published *E. coli* K1 studies involving fluorescence microscopy, the bacteria have either been surface labelled with fluorescent dye or immunolabelled with a bacteria-specific antibody or with an endosialidase-GFP fusion probe (Kim *et al.*, 2003; Sukumaran *et al.*, 2003; Alsam *et al.*, 2005; Zelmer *et al.*, 2008). Whilst these studies have provided great advances in understanding *E. coli* K1 interactions with host cells, the techniques used do have their limitations.

Surface labelling of bacteria with a fluorescent dye, such as fluorescein isothiocyanate (FITC), and NHS Rhodamine, is a rapid and cheap bacterial labelling approach (Hazenbos *et al.*, 1994; Schneider *et al.*, 2000; Steele-Mortimer *et al.*, 2000). However, as the dye only labels the bacterial extracellular surface, the dye will be diluted over each generation during bacterial replication. Furthermore, binding of FITC to the bacterial surface has been demonstrated to interfere with adenylate cyclase toxin activity of *Bordetella pertussis*, which affects bacterial uptake by human neutrophils *in vitro* (Weingart *et al.*, 1999).

Immunolabelling of fixed bacteria with a specific bacterial antibody is another common method widely employed for labelling bacteria in fixed samples. As the sample requires prior fixation, some fixatives, especially aldehydes, generate autofluorescent signal, and some cell structures, such as lipid raft-associated molecules, are not properly preserved by the fixative (Drecktrah *et al.*, 2008; Tanaka *et al.*, 2010). Further, certain bacterial antigens, such as fimbriae (Saukkonen *et al.*, 1988), and *E. coli* K1 capsule (Zelmer *et al.*, 2008), express different forms of their antigens at different stages of infection, therefore selection of antibody requires care. For *E. coli* K1, the endosialidase-GFP probe is an alternative non-antibody probe. It is an engineered noncatalytic *E. coli*
K1-specific bacteriophage-encoded endosialidase tagged with GFP that binds specifically to the K1 capsule in a fixed sample but does not degrade it (Jokilammi et al., 2004).

An alternative bacterial-labelling strategy is cytoplasmic expression of fluorescent proteins in bacterial cells from an introduced plasmid (Weingart et al., 1999; Qazi et al., 2001; Ruthel et al., 2004; Knodler et al., 2005; Drecktrah et al., 2007; Lamberti et al., 2010; Van Engelenburg and Palmer, 2010). This bacterial labelling approach provides stably fluorescent live bacteria for microscopy studies, but some adverse outcomes from the burden of fluorescent protein synthesis to Salmonella pathogenicity have been observed in bacterial uptake into macrophages and epithelial cell lines in vitro as well as in vivo infection (Knodler et al., 2005). In addition to the production of fluorescent proteins, the presence of plasmid, and antibiotic resistance markers, such as tetracycline resistance gene (tet\(^R\)), and chloramphenicol resistance gene (Cm\(^R\)), on the plasmid has also been shown to affect bacterial virulence traits (Abromaitis et al., 2005; Knodler et al., 2005; Clark et al., 2009). However, it has been further demonstrated that by integrating Cm\(^R\) and fluorescent reporter genes into the bacterial chromosome, the fluorescent protein-expressing Salmonella retains its virulence (Clark et al., 2009).

The aim of this project was to study the interactions of E. coli K1 with various cellular and organellar markers in HBMEC by live-cell imaging. Live-cell imaging allows the study of the dynamic interactions of the pathogen with host factors over time (Schroder et al., 2006; Drecktrah et al., 2008). For example, the application of live-cell imaging revealed that Salmonella-induced filaments (Sifs) are highly dynamic and bidirectional, with microtubule-dependent movement (Drecktrah et al., 2008). Therefore, to enable the application of live-cell imaging for studying E. coli K1 interaction with HBMEC, a fluorescent E. coli K1 strain that retains virulence is required. The virulence of the fluorescent protein-expressing E. coli K1 can then be screened with an HBMEC invasion
assay, which has been extensively applied to study *E. coli* K1 pathogenesis, as reviewed in chapter 1.

3.2 Objectives and aims

The aim of this chapter was to construct a fluorescent *E. coli* K1 strain that retained virulence. The specific objectives are:

- To determine the optimal infection conditions for *E. coli* K1 infection of HBMEC by testing different multiplicities of infection (MOI) and varying the incubation time of the cells with the bacteria.
- To transform various bacterial fluorescent reporter constructs into *E. coli* K1 and to screen the virulence of the transformed *E. coli* K1 by the optimised HBMEC invasion assay in comparison to untransformed *E. coli* K1.

3.3 Results

3.3.1 Invasion and intracellular survival of *E. coli* K1 and K-12 in HBMEC

To determine the kinetics of the bacterial invasion of HBMEC, the cells were incubated with the bacteria at MOI of 100 for varying intervals of time (Figure 3.1A). Extracellular bacteria were killed by gentamicin, which is poorly permeable to eukaryotic cell membranes (Barnhart *et al.*, 2002). Therefore, bacteria that have invaded into the host cells are protected from the bactericidal effect of gentamicin. After gentamicin treatment, cells were washed, and the viable intracellular bacteria were quantified as described in chapter 2.

The percentage of recovered intracellular *E. coli* K1 from HBMEC increased with longer incubation times (Figure 3.1B). An incubation of 120 minutes resulted in a significant increase in the percentage of intracellular *E. coli* K1 recovered compared to a 30 and 60 minutes incubation (*p* < 0.05, using *t*-test, one-tailed distribution), which might
be resulted from intracellular bacterial replication. As expected, when the non-invasive *E. coli* K-12 were used, no intracellular bacteria were recovered after 30 or 60 minutes incubation, and only a very low percentage of bacteria (0.00018%) had invaded HBMEC after 120 minutes. These results show that more *E. coli* K1 invade HBMEC with longer incubation times; whereas for *E. coli* K-12, which is a non-invasive laboratory *E. coli* strain, extended incubation time of the bacteria with HBMEC results with no or very low number of bacterial invasion.
Figure 3.1. Invasion of *E. coli* K1 and K-12 into HBMEC. (A) Schematic of experimental design. To determine the optimal infection time, HBMEC were infected with *E. coli* K1 or K-12 at MOI of 100 for different time intervals. The infected cells were washed and incubated in medium containing 100 μg/ml gentamicin for 60 minutes to kill extracellular bacteria at the end of the infection. Cells were washed and lysed. The cell lysates were plated on LB agar to enumerate the number of bacteria. (B) Quantitation of the number of bacteria invaded HBMEC at various time intervals of incubation as indicated. The result represents the percentage of bacteria invaded into HBMEC. Results are from one representative experiment of two independent repeats performed in triplicate and presented as mean of triplicates ± SD.

*E. coli* K1 are able to replicate extracellularly in the blood of infected neonates as well as the neonatal rat model (Glode et al., 1977; Pluschke et al., 1983; Kim et al., 1992; Zelmer et al., 2008). However, the ability of the bacteria to survive intracellularly is unclear. To investigate the ability of *E. coli* K1 to survive intracellularly in HBMEC, the cells were incubated with either K1 or K-12 at MOI of 100 for 120 minutes (Figure 3.2A). Following this, the cells were cultured in experimental medium containing gentamicin to
kill extracellular bacteria (Figure 3.2B). The intracellular bacterial load was then assessed at increasing time intervals to determine bacterial persistence. A minor decrease in the percentages of intracellular *E. coli* K1 was observed at 180 minutes, but the percentage of intracellular *E. coli* K1 increased significantly at the end of the 300 minutes incubation in gentamicin (*p* < 0.05, using *t*-test, one-tailed distribution). For intracellular survival assays involving *E. coli* K-12, a very low percentage of intracellular bacteria (0.0003%) were recovered 60 minutes post-gentamicin treatment, and the percentage of recovered bacteria was further decreased to 0.0000635% at 300 minutes post-gentamicin treatment. The results clearly indicate that *E. coli* K1 are able to survive and replicate intracellularly in HBMEC at least up to 5 hours post infection, as very low number of viable intracellular *E. coli* K1 was recovered after 18 hours incubation in media containing gentamicin (data not shown), which might be due to bacterial killing intracellularly or bacterial egress from the infected HBMEC at later time point. Whereas the non-pathogenic *E. coli* K-12 are not able to survive intracellularly in HBMEC. In addition, these results also demonstrated that the gentamicin did not affect the survival of intracellular K1 bacteria.
Figure 3.2. Intracellular survival of \textit{E. coli} K1 in HBMEC. (A) Schematic of experimental design. To determine the intracellular survival of the bacteria, HBMEC were infected with \textit{E. coli} K1 or K-12 at MOI of 100 for different time intervals. The infected cells were washed and incubated in medium containing 100 \(\mu\)g/ml gentamicin for the time periods indicated. Cells were washed and lysed. The cell lysates were plated on LB agar to enumerate the number of bacteria. (B) Quantitation of the intracellular survival. The result represents the percentage of recovered intracellular bacteria. Results are from one representative experiment of three independent repeats performed in triplicate and presented as mean of triplicates \(\pm\) SD.

3.3.1.1 \textit{E. coli} K1 bearing \textit{M. tuberculosis} codon optimized fluorescent reporter vectors invasion of HBMEC

\textit{E. coli} K1 transformed with \textit{M. tuberculosis} codon optimized fluorescent reporter vectors were initially screened with a cheap \textit{Acanthamoeba} phagocytosis assay and were found to retain virulence when compared to untransformed \textit{E. coli} K1 (data not shown). The final application of the virulent fluorescent \textit{E. coli} K1 strain is for studying the
interactions of the fluorescent bacterial strain with HBMEC, which is a non-phagocytic cell type used as a blood-brain barrier model in vitro. Therefore, the fluorescent E. coli K1 strains, which had demonstrated to be virulent in Acanthamoeba, had to demonstrate similar virulence trait in HBMEC.

K1pmCherry3 and K1pEnvyl were selected for further screening with HBMEC. Invasion assays were performed at MOI of 100 for 60 minutes (Figure 3.3). The percentages of recovered intracellular bacteria for K1pmCherry3, and K1pEnvyl were significantly lower than untransformed K1 (p < 0.01, using t-test, one-tailed distribution). It was also observed that the doubling time for all K1 and K-12 transformed with the Mycobacterium sp. codon-optimised fluorescent reporter constructs were longer than the untransformed K1 and K-12, respectively (data not shown). It appears that although the bacterial strains were equally able to infect Acanthamoeba, the virulence of the bacteria transformed with M. tuberculosis codon optimized fluorescent reporter constructs was impaired in their ability to invade HBMEC. These transformed bacteria were therefore not suitable for use in future experiments.
Figure 3.3. Screening of virulence of *E. coli* K1 expressing fluorescent protein in HBMEC. To determine the virulence of the K1 expressing fluorescent protein, invasion assays were performed by infecting HBMEC with either native *E. coli* K1, K-12 or fluorescent protein expressing *E. coli* K1 strains at MOI of 100 for 60 minutes, followed by incubation in media containing gentamicin for 1 hour. The result represents the percentage of bacteria invaded into HBMEC. Results are from one representative experiment of two independent repeats performed in triplicate and presented as mean of triplicates ± SD.

3.3.1.2 pFPV25.1-transformed *E. coli* K1 invasion of HBMEC

pFPV25.1 plasmid contains *E. coli* codon optimised GFP reporter gene, *gfpmut3a*, fused downstream of a *Salmonella typhimurium* (*S. typhimurium*) promoter, *rpsM* (Figure 3.4A) (Valdivia and Falkow, 1996). This construct had been extensively used in studies involved *S. typhimurium* (Cheminay *et al.*, 2005; Radtke *et al.*, 2007; Gerlach *et al.*, 2008), and enteropathogenic *E. coli* (EPEC) (Unsworth *et al.*, 2007; Marchès *et al.*, 2008); therefore, this plasmid was tested for use in *E. coli* K1.

As with other constructs, pFPV25.1 was transformed into K1 and K-12 as previously described, and the fluorescence stability was confirmed using a confocal microscope (Figure 3.4B). All pFPV25.1-transformed K1 (K1-GFP) fluoresced when observed with a confocal microscope. The growth profile of the K1-GFP was slightly lower when compared to native K1 (Figure 3.4C), with the doubling time of K1-GFP was
approximately 36.3 minutes, and the K1 doubling time was 35 minutes in this specific experiment.

To determine whether the virulence of K1-GFP is retained, HBMEC invasion assays were performed (Figure 3.4D). The percentage of recovered intracellular K1-GFP was roughly 40% lower than that of native K1, however this was shown to be not significant ($p > 0.05$, using $t$-test, one-tailed distribution). The reduction in the K1-GFP invasion efficiency was deemed to be acceptable for further experiments, as the transformant bacteria were still invasive when compared to the non-pathogenic *E. coli* K-12 (Figure 3.4D) and the previous transformed strains (Figure 3.3).
Figure 3.4. Screening of virulence *E. coli* K1 transformed with pFPV25.1 construct in HBMEC. (A) The illustration shows the pFPV25.1 plasmid map. Unique restriction enzyme cutting sites are indicated in blue. (B) The confocal micrograph shows fluorescent *E. coli* K1 transformed with pFPV25.1 (K1-GFP). Scale bar: 10 μm. (C) The growth kinetics of K1-GFP and untransformed *E. coli* K1 in LB broth. The results show the absorbance at 595 nm of the bacterial cultures at the time intervals indicated. (D) To determine the virulence of the transformed K1, invasion assays were performed by incubating HBMEC with either *E. coli* K1, K-12 or K1-GFP at MOI of 100 for 120 minutes, followed by incubation in media containing gentamicin. The result represents the percentage of bacteria invaded into HBMEC. Results are from one representative experiment of two independent repeats performed in triplicate and presented as mean of triplicates ± SD.

It has been shown previously that the bacterial K1 polysialic acid capsule is crucial for bacterial intracellular survival in HBMEC, no defect was observed in the HBMEC invasion by K1 capsule deletion mutant (Kim et al., 2003). Thus, the apparent lower invasion efficiency of K1-GFP seen in Figure 3.4D may be due to impairment in K1
polysialic acid capsule expression, leading to the killing of the intracellular bacteria rather than a reduction in bacterial invasion. To examine the presence of K1 polysialic acid capsule on K1-GFP, the transformed bacteria were smeared onto a cover slip, fixed with formaldehyde, and stained with PK1A-GFP probe, which binds K1 polysialic acid capsule specifically (Jokilammi et al., 2004; Zelmer et al., 2008), in the absence of detergent. As the transformed bacteria already expressed GFP, visualization of the bound PK1A probe was facilitated by counter-staining with a mouse anti-GFP antibody, followed by Fluoprobes 642-conjugated anti-mouse antibody. An experimental control which K1-GFP smear was not stained with the PK1A-GFP probe, but was stained with the anti-GFP antibody, followed by Fluoprobes 642-conjugated anti-mouse antibody, did not detect any signal in the far red channel (Figure 3.5B). This result showed that the intracellular GFP of K1-GFP was not detected by the anti-GFP antibody when staining was performed without permeabilization with detergent. Hence, the staining showed the presence of K1 polysialic acid capsule on the untransformed K1 and K1-GFP (Figure 3.5A and C respectively). Therefore, the reduction in the K1-GFP invasion efficiency was not caused by intracellular bacterial killing as the result of the absence of K1 polysialic acid capsule expression on the transformant, but was probably due to a defect in other bacterial determinants that are essential for HBMEC invasion.
Figure 3.5. The presence of K1 polysialic acid capsule on K1-GFP. To examine the presence of K1 polysialic acid capsule on K1 and K1-GFP, the bacterial culture was smeared on a glass cover slip, air-dried, and fixed with 2% (v/v) formaldehyde. The bacterial smear was stained with 10 µg/ml of PK1A-GFP probe in PBS, then by mouse anti-GFP clone 3E6, and followed by Fluoprobes 642 conjugated donkey anti-mouse antibody without permeabilizing by detergent. As an experimental control (B), K1-GFP was stained with mouse anti-GFP antibody, followed by Fluoprobes 642 conjugated donkey anti-mouse antibody only. (A and C) The images show the presence of the bacterial K1 capsule on K1 and K1-GFP respectively. Contrast of all images was enhanced. GFP signal from intracellular GFP of K1-GFP and from PK1A-GFP probe (green); signal resulted from anti-GFP staining (red). Scale bar: 10 µm (A and C); 5 µm (B).
3.3.1.3 Deletion of \textit{rpsM} promoter from pFPV25.1 and the transformed bacterial invasion of HBMEC

It was previously shown that cytoplasmic fluorescent protein expression affects \textit{Salmonella} virulence (Knodler \textit{et al.}, 2005; Clark \textit{et al.}, 2009). To investigate if GFP expression was the major factor that affects the transformed bacterial invasion and survival in HBMEC, the \textit{rpsM} gene was deleted from pFPV25.1, to construct a promoterless construct that does not express GFP, as illustrated in Figure 3.6.
Figure 3.6. Deletion of \textit{rpsM} from pFPV25.1. The illustration summarizes steps involved in deleting \textit{rpsM} gene in pFPV25.1 using standard cloning techniques. pFPV25.1 was digested with \textit{Smal} and \textit{Xbal}, blunted with Klenow fragment, and re-ligated. The resulting construct was a promoterless bacterial expression vector, \textit{rpsM}. 
The construct was transformed into *E. coli* K1 by electroporation. As the promoter had been deleted, there was no GFP expression in the transformed bacteria, therefore, to additionally confirm the transformed *E. coli* K1 that grew on a ampicillin selection LB agar plate, the plasmid DNA was extracted and linearised by *Hind*III digestion for the expected 4786 bp band compared to the plasmid with intact promoter of 5390 bp (Figure 3.7A). The quality of the plasmid DNA extracted from *E. coli* K1 was poor compared to *E. coli* DH5α strain, a standard *E. coli* cloning strain, possibly due to the presence of nucleases in the pathogenic strain. The growth kinetics of the transformed bacteria, K1rpsM–, was slightly lower than K1, with the doubling time of 32.43 minutes (Figure 3.7B), which is similarly observed in other fluorescent protein-expressing K1.

In order to demonstrate the invasion efficiency of the K1rpsM–, HBMEC invasion assays were performed (Figure 3.9C). Interestingly, the percentage of recovered intracellular K1rpsM– was nearly 2-fold and slightly lower than that of K1 and K1-GFP. The observation might suggest that the reduction in the invasion efficiency of fluorescent protein-expressing K1 is not caused by the expression of fluorescent protein, but the presence of the plasmid DNA alone might be a metabolic burden to the bacteria, hence, the bacterial virulence is affected. Alternatively, the presence of certain genetic components on the expression vector, such as antibiotic resistance gene, contribute to the loss of the bacterial virulence trait (Abromaitis *et al.*, 2005).
Figure 3.7. Screening of E. coli K1 transformed with rpsM- plasmid DNA and the growth kinetics of the transformed E. coli K1. (A) SmaI and XbaI digestion resulted in the deletion of rpsM, the promoter for gfp in pFPV25.1. Therefore, E. coli K1 transformed with rpsM- (K1rpsM-) does not express GFP. To screen for K1rpsM-, plasmid DNA was extracted from the bacteria, and digested with HindIII. The gel photograph shows the products generated from HindIII digestion of the extracted rpsM- plasmid DNA. Lanes: 1, 1 kb DNA ladder; 2, pFPV25.1 digested with HindIII (5390 bp); 3, undigested rpsM extracted from transformed E. coli DH5α (cloning strain); 4, rpsM extracted from E. coli DH5α digested with HindIII (4786 bp); 5, undigested rpsM extracted from transformed E. coli K1; 6, rpsM extracted from E. coli K1 digested with HindIII (4786 bp). (B) The growth kinetics of the transformant in LB broth were compared to the untransformed E. coli K1. The results show the absorbance at 595 nm of the bacterial cultures at the time intervals indicated.

3.3.1.4 Construction of pFPV-mCherry and the mCherry-expressing E. coli K1 invasion of HBMEC

Whilst K1-GFP was shown to fluoresce and to retain the bacterial virulence trait in HBMEC infection, the major aim of this study was to apply the fluorescent bacterial strain for studying the interaction of the bacteria with cellular markers, which the majority of the
available cellular markers containing mammalian expression vectors are also GFP-tagged. Therefore, it was of crucial importance to have an *E. coli* K1 strain expressing other fluorescent proteins, with excitation and emission spectra that did not overlap with GFP, but that still retained virulence.

We chose mCherry as a candidate to test for its known brightness, photostability, and distinct excitation and emission spectra from GFP. The cloning strategy was performed as illustrated in Figure 3.8. All the transformed *E. coli* K1 were found to be fluorescent when they were screened with a confocal microscope (Figure 3.9A). The variability in the fluorescent intensity of the bacteria in Figure 3.9A was due to different focal plane of the bacteria when the image was acquired from live bacteria. The growth kinetics of the transformed bacteria, K1-Cherry, was slightly lower than K1 (Figure 3.9B), the doubling time of K1-Cherry was approximately 36.47 minutes compared to the doubling time of K1, which was 35 minutes in this experiment.

As with other constructs, to determine the virulence of K1-Cherry, HBMEC invasion assays were performed by infecting HBMEC with either *E. coli* K1, K-12, K1-GFP, K1-Cherry or K1rpsM' (Figure 3.9C). The results indicate that K1rpsM', and K1-GFP exhibit a decrease in HBMEC invasion efficiency, however, the decrease was not statistical significant, and they remain significantly more invasive than the non-pathogenic *E. coli* K-12 laboratory strain. In contrast, the invasion efficiency of K1-Cherry was very similar to the native K1 (Figure 3.9C). Therefore, mCherry expression seems to have no adverse effect on *E. coli* K1 virulence.
Figure 3.8. Cloning strategy for constructing a bacterial expression vector carrying mCherry fluorescent protein cDNA. The illustration summarizes steps involved in replacing gfp gene in pFPV25.1. Briefly, mCherry gene was PCR amplified from pRSET-mCherry with forward primer flanked with XbaI restriction site and reverse primer flanked with SphI restriction site. The resulting amplicons were digested with XbaI and SphI and ligated into the corresponding sites of pFPV25.1, thereby replacing the gfp gene.
3.9. Screening of virulence *E. coli* K1 expressing fluorescent protein in HBMEC. (A) A confocal micrograph to show fluorescent K1-Cherry. Scale bar: 5 μm. (B) The growth kinetics of the transformant in LB broth were compared to the untransformed *E. coli* K1. The results show the absorbance at 595 nm of the bacterial cultures at the time intervals indicated. (C) To determine the virulence of the transformed K1, invasion assays were performed by infecting HBMEC with either *E. coli* K1 or K-12 or K1-GFP or K1-Cherry or K1-rpsM at MOI of 100 for 2 hours, followed by incubation in media containing gentamicin for 1 hour. The result represents the percentage of bacteria invaded into HBMEC. Results are from one representative experiment of two independent repeats performed in triplicate and presented as mean of triplicates ± SD.

### 3.3.2 Transcytosis of *E. coli* K1 across HBMEC

During an infection, it is thought that *E. coli* K1 invade and transcytose from the endothelial cells of BBB to the CNS and subsequently trigger the infiltration of immune cells, which results in inflammation of the meninges (Siegel and McCracken, 1981; Kim *et al.*, 1992; Zelmer *et al.*, 2008). To model the BBB *in vitro*, HBMEC, which are polarized
endothelial cells, were grown on collagen-coated Transwell inserts as illustrated in Figure 3.10A. Upon complete confluency, tight junction formation was induced by culturing the cells in the presence of hydrocortisone (Franke et al., 2000). The formation of tight junctions and the integrity of the HBMEC monolayer were evidenced by an increase in transendothelial electrical resistance (TEER) (Figure 3.10B). When the TEER reading reached approximately 20 Ωcm², the monolayer's permeability to 4 kDa FITC-conjugated dextran was assessed (Figure 3.10C). A small amount of fluorescent signal could be detected in the media collected from the basolateral compartment 90 minutes after the introduction of the FITC-conjugated dextran to the apical compartment. However, when the HBMEC containing insert was compared with a cell-free insert (empty insert), the amount of fluorescent signal detected in media collected from basolateral compartment of empty insert was approximately 10-fold higher than that collected from the HBMEC containing insert after 90 minutes of incubation (Figure 3.10C).
Figure 3.10. *in vitro* blood-brain barrier (BBB) model. (A) An illustration of how the BBB *in vitro* relates to the Transwell insert model. (B) To determine the permeability of HBMEC, cells were seeded onto a collagen-coated Transwell insert. When the cells on the Transwell membrane reached complete confluency, the medium was replaced with HBMEC growth media containing 1.0 μg/ml hydrocortisone for stimulating the formation of barrier properties. The transendothelial electrical resistance (TEER) measurements were monitored. The graph shows the TEER measurements (Ωcm²) throughout the culture duration. Results are one representative experiment of 4 independent repeats performed in triplicate and presented as mean of triplicates ± SD. (C) To demonstrate the barrier properties of HBMEC cultured on Transwell insert on day 9, 4 kDa FITC-conjugated dextran (0.1 mg/ml) (Sigma-Aldrich) was added to the apical chamber of cells. After addition of the reagent, medium was sampled at the indicated time intervals. The permeability was quantified as fluorescence intensity of the FITC that passed from the apical chamber to the basolateral chamber.

Although FITC-conjugated dextran transportation through HBMEC monolayer was reduced, the TEER of the HBMEC line used in this study was lower than the reported TEER for other immortalized HBMEC, HCMEC/D3, which ranged from 60 to 80 Ωcm² (Weksler *et al.*, 2005; Cucullo *et al.*, 2008). To confirm that the HBMEC used in this study were able to form intercellular junctions, confluent HBMEC were stained for cadherin, a
transmembrane protein associated with intercellular junctions. The distribution of the staining suggests that the cells are able to form intercellular junctions (Figure 3.11). In addition, F-actin staining also revealed cell periphery localization indicative of intercellular junction formation (Figure 3.11).
Figure 3.11 Immunostaining of cadherin and F-actin of HBMEC. Confluent monolayers of HBMEC cells (passage 15) were fixed with either 2% (v/v) formaldehyde (F-actin) or methanol (cadherin), immunostained for cadherin with mouse anti-pan cadherin, and followed by Fluoprobes 488 conjugated donkey anti-mouse antibody, or stained with 30 μg/ml phalloidin-TRITC. F-actin and cadherin (green), nuclei (blue). Scale bar: 20 μm.

After confirming the successful production of an integral monolayer of HBMEC on the Transwell inserts, a transcytosis assay was performed to study the kinetics of the
bacterial traversal of HBMEC. After the TEER reading of HBMEC on Transwell insert reached at least 20 Ωcm², cells were infected with approximately $1 \times 10^8$ cfu/ml of either K1 or K-12. Samples of media were taken from the basolateral compartment at the indicated times, and these were serially diluted before being plated on LB agar plates to enable bacterial growth and quantification. K1 were detected in the basolateral compartment at 8 hours post-infection (Figure 3.12), while K-12 were absent at all the time intervals studied. These results clearly demonstrate that the non-pathogenic E. coli K-12 are unable to traverse HBMEC, while E. coli K1 are able to invade and subsequently traverse HBMEC.

![Figure 3.12. Transcytosis of E. coli K1 and K-12 through HBMEC.](image)

**Figure 3.12. Transcytosis of E. coli K1 and K-12 through HBMEC.** To study the bacterial transcytosis kinetics, HBMEC on Transwell membranes were incubated with approximately $1 \times 10^8$ cfu/ml of either E. coli K1 or K-12. Media were sampled from the basolateral chamber at the indicated time intervals. The result represents the percentages of bacteria traversed through HBMEC. Results are from one representative experiment of three independent repeats performed in triplicate and presented as mean of triplicates ± SD.

### 3.3.2.1 K1-Cherry transcytosis of HBMEC

Although K1-Cherry had been shown to retain the ability to invade HBMEC at the similar efficiency as the native K1 (Figure 3.9), the transformed bacteria further had to retain the ability to traverse an HBMEC monolayer grown on Transwell filter inserts. To determine whether the mCherry expression affected the ability of K1 to traverse HBMEC, a transcytosis assay was performed by co-infecting HBMEC cultured on Transwell filter
inserts with either *E. coli* K1 and K-12, or K1-Cherry and K-12 (Figure 3.13). As shown in Figure 3.12, K-12 did not traverse HBMEC monolayer on Transwell insert, therefore, K-12 were used as an indicator of the monolayer’s integrity, and K-12 could be differentiated from K1 or K1-Cherry by the distinct small dark pink colony on MacConkey agar. while K1 and K1-Cherry appear as big white or light pink colony on MacConkey agar.

HBMEC monolayers on Transwell filter inserts were incubated with approximately $1 \times 10^8$ cfu/ml of each bacterial strain. Media from the basolateral compartment was collected at various time intervals, and the serially diluted specimens were plated on MacConkey agar. At 8 hours, *E. coli* K1 was detected in the basolateral compartment. and the percentages of the bacteria that traversed the HBMEC monolayer increased significantly from 0.25% at 8 hours to 77.42% at 12 hours post-infection (Figure 3.13A). The absence of *E. coli* K-12 at both intervals indicated that the HBMEC monolayer remained intact during the infection. In contrast, during K1-Cherry co-infection with K-12, K-12 were detected in the basolateral compartment after 12 hours (Figure 3.13B). The percentages of K1-Cherry traversed HBMEC were 0.54% at 8 hours and increased to 40% after 12 hours infection.

In conclusion, although K1-Cherry was able to invade HBMEC as efficiently as native K1, K1-Cherry affects HBMEC monolayer integrity, possibly by deforming the intercellular tight junctions. Therefore, K1-Cherry was suitable for further imaging experiments but not as appropriate for bacterial transcytosis studies.
Figure 3.13. Transcytosis of *E. coli* K1 expressing mCherry through HBMEC. To determine the virulence of the K1-Cherry, transcytosis assays were performed by incubating HBMEC with either K1 and K-12 (A) or K1-Cherry and K-12 (B) with approximately 1 x 10^8 cfu/ml of each bacterial strain. At the time intervals indicated, media were sampled, and plated on MacConkey agar. The results represent the percentages of bacteria traversed through HBMEC. Results are from one representative experiment of two independent repeats performed in triplicate and presented as mean of triplicates ± SD.

3.4 Discussion

The major objective of this study was to construct a fluorescent *E. coli* K1 strain that retains the bacterial virulence trait. To assess the virulence of the fluorescent bacterial strains, an *Acanthamoeba* phagocytosis assay and HBMEC invasion assay were initially employed (data not shown). After various attempts, one of the constructs, pFPV25.1, which contains *E. coli* codon-optimised gfp gene fused downstream of a *S. Typhimurium* promoter, rpsM, retained bacterial invasiveness. Interestingly, the replacement of gfp gene
with a mammalian codon optimised mCherry gene in pFPV25.1 seemed to have no detrimental effect on the bacterial invasiveness, although the intracellular growth of K1-Cherry in comparison to untransformed K1 is not known.

To enable the application of confocal microscopy imaging for studying E. coli K1 interactions with various cellular markers, particularly as the majority of the available mammalian expression constructs encode GFP chimaeric proteins, an E. coli K1 strain expressing mCherry was constructed. mCherry was chosen for the fluorescent protein’s photostability and brightness (Shaner et al., 2005). Further, mCherry is expressed as a monomer, which avoids the potential formation of toxic aggregates in the bacteria as observed in some dimeric or tetrameric fluorescent proteins, such as DsRed, and tdTomato (Shaner et al., 2004; Shaner et al., 2005). mCherry also offers a potential advantage over other fluorescent proteins, as mCherry expression and fluorescence in M. tuberculosis was not affected by hypoxic conditions (Carroll et al., 2010). The long excitation wavelength of mCherry also reduces the phototoxicity effect on live cells, and allows live-cell imaging of the infection (Tsien, 1998). Furthermore, mCherry-expressing E. coli K1 also provide a tool for future deep tissue in vivo imaging experiments, as the long excitation wavelength is able to penetrate tissue efficiently (Carroll et al., 2010).

Previously, an E. coli K1 strain expressing GFP was used to study bacterial interaction with vimentin in HBMEC, but the authors did not demonstrate the virulence of the transformed strain (Chi et al., 2010). Several studies have shown that the production of fluorescent protein and/or the presence of plasmid DNA in Salmonella enterica abrogates bacterial virulence in both epithelial and macrophage cell lines as well as in vivo (Knodler et al., 2005; Clark et al., 2009). In this study, although the growth kinetics of the fluorescent protein-expressing bacteria are slightly lower than the growth kinetics of the native E. coli K1, we found no evidence of correlation in the abrogation of the bacterial virulence and fluorescent protein expression, as bacteria harbouring a promoterless
plasmid DNA, which did not express fluorescent protein, also demonstrated a marginally decreased efficiency in HBMEC invasion. The reduction in the bacterial HBMEC invasion efficiency could be due to the metabolic burden caused by harbouring extra copies of plasmid in the bacteria. Interestingly, we found that expression of a mammalian codon optimised mCherry under the control of a Salmonella promoter, rpsM, did not affect the bacterial invasiveness. In fact, the bacteria invaded HBMEC as efficiently as the native bacteria; whereas a decrease in invasion efficiency was observed for *E. coli* K1 expressing an *E. coli*-codon optimised GFP under the control of rpsM (pFPV25.1).

Although we showed that mammalian codon-optimised mCherry did not affect the reporter’s expression as well as virulence trait in *E. coli* K1, one of our preliminary data additionally observed that expression of *Mycobacterium* sp. (GC-rich organism) codon-optimised fluorescent proteins in both *E. coli* K1 and non-pathogenic *E. coli* K-12 affected the bacterial growth kinetics (data not shown). These fluorescent *E. coli* K1 strains further demonstrated distinct levels of virulence in two different cell types, in which the bacteria were able to infect and to survive in *Acanthamoeba*, which is an active phagocytic cell (data not shown); while they failed to invade non-phagocytic HBMEC. This observation suggests that expression of the *Mycobacterium* sp. codon-optimised fluorescent proteins in *E. coli* K1 might have affected the expression of a set of bacterial virulence factors, main factors essential for HBMEC invasion, but probably not the factors that are required for the bacterial intracellular survival. In order to further understand the effects of the expression of *Mycobacterium* sp. codon-optimised fluorescent proteins on the bacterial virulence factors expression, it would be important to study the expression and the form variations of the bacterial polysialic K1 capsule in both native and transformed bacterial strains, as well as the expression of some of the known bacterial virulence factors that are required for the bacteria adhesion and invasion of HBMEC, such as Type 1 fimbriae, OmpA, and Cnf1.
Additionally, several lines of evidence have demonstrated that the presence of a tetracycline resistance gene \((\text{tet}^R)\), or chloramphenicol resistance gene \((\text{Cm}^R)\), on a cloning vector might have an adverse effect on *Salmonella* survival in macrophages, and could impair the bacterial invasiveness of epithelial cells (Abromaitis et al., 2005; Clark et al., 2009). However, Clark et al. (2009) further demonstrated that the DNA copy number determined the outcome of fluorescent reporter expression in *Salmonella* virulence by showing that chromosomally-encoded GFP, also carrying a \(\text{Cm}^R\) gene, did not affect *Salmonella* invasion. In this study, there was no direct evidence on the correlation of gene copy number and the transformed bacterial virulence, but plasmid copy number might contribute to the loss of virulent traits in transformed *E. coli* K1 which would need to be tested against chromosomal integration. However, the major hindrance for stably integrating fluorescent protein gene into the *E. coli* K1 genome is the lack of access to the bacterial genome data in public databases, although the findings from the sequencing projects have been published (Xie et al., 2006).

Another risk of having a bacterial strain harbouring a fluorescent reporter construct is the stability of the construct in the bacteria. It was shown that plasmids expressing fluorescent proteins from the *Mycobacterium bovis* hsp60 promoter were unstable in *Mycobacterium smegmatis* (Carroll et al., 2010). In this study, it was observed that all K1-Cherry expressed mCherry when the bacteria were cultured for 2 hours without antibiotic selection (data not shown) and upon cell infection (see Chapter 6), bacteria maintained fluorescence for 20 hours or more without antibiotic selection, which suggested that the pFPV-mCherry construct was stably maintained in K1-Cherry.

In this study, the HBMEC demonstrated lower TEER than the TEER reported by Stins et al. (2001), as well as the TEER measurement for other immortalized HBMEC, HCMEC/D3, which ranged from 60 to 80 \(\Omega\text{cm}^2\) (Weksler et al., 2005; Cucullo et al., 2008). The low TEER detected in our system might indicate an inability of these cells to
form a tight monolayer on the Transwell® membrane. Alternatively, the low TEER might be caused by the presence of FBS in the medium during TEER measurement. It has been previously reported that primary brain endothelial cells (PBEC) were less permeable to sucrose under FBS-free condition, and it was also evidenced by an increase in the TEER reading in that experimental condition (Franke et al., 2000). It was also speculated that the content of growth factors in the growth medium used for cell maintainence might also affect the tight junctional proteins expression of these cells, hence, different TEER was observed from Stins et al. (2001). The reduction in FITC-dextran and the non-invasive *E. coli* K-12 translocation in transwells with hydrocortisone-stimulated HBMEC, in combination with the distribution of cadherin and actin suggest that the cells are capable of forming intercellular interactions. However, the expression of several other major tight junctional proteins, such as claudin-5, occludin, ZO-1, and JAM-A, need to be studied, to further characterize the tight junction phenotype of the HBMEC.

Despite the low TEER of the HBMEC on Transwell, it was demonstrated that *E. coli* K1, but not non-pathogenic *E. coli* K-12, could traverse HBMEC monolayer cultured on Transwell insert (Figure 3.12). The result from this study also confirmed the published result by Stins et al. 2001, whereby we demonstrated that the HBMEC monolayer on Transwell insert remained intact during the bacterial infection, as *E. coli* K-12 was absent in the basolateral compartment of the Transwell filter insert when co-infection of both *E. coli* K1 and K-12 was performed. Although *E. coli* K1 expressing mCherry (K1-Cherry) were shown to retain the ability to invade HBMEC, the presence of non-pathogenic *E. coli* K-12 in the basolateral compartment at late time point of K1-Cherry and K-12 co-infection of the HBMEC monolayer on Transwell filter insert might suggest the leakage of the monolayer. Immunofluorescent staining of the K1-Cherry infected HBMEC on Transwell membrane with either fluorophore conjugated-phalloidin (for F-actin) or specific tight junction antibodies, such as occludin, and claudin 5, might be able to confirm the observed
results. F-actin co-localizes with tight junction proteins in intact polarized epithelium and endothelium, and the staining also allows detection of the presence of an intercellular gap, which is an indicator of permeable tissue monolayer (Martins-Green et al., 2008). It might also be interesting to study the bacterial factors that are affected by the fluorescent protein expression, which led to the change in bacterial virulence and disruption of HBMEC monolayer integrity during infection.

The negative effect of the K1-Cherry on the HBMEC monolayer in the Transwell insert was a disappointing discovery. Despite this effect, the fluorescent bacteria were still considered to be a useful tool for studying HBMEC invasion in this study. Alternative approaches include the application of PK1A-GFP and *E. coli* K1 specific antibody to label the bacteria along with antibodies to cellular markers; however, these methods come with their own caveats. Essentially, access to *E. coli* K1-specific and certain cell marker antibodies was limited; as such GFP-tagged cellular markers were the primary tool for studying intracellular markers that *E. coli* K1 may interact with. Although an alternative non-antibody *E. coli* K1 probe, the endosialidase-GFP probe (PK1A-GFP), was available, the application of this probe in studying the bacteria interaction with various GFP-tagged cellular markers would require changing the GFP tag of the cellular proteins to red fluorescent protein (RFP), which frequently changes the conformation of cytosolic protein (Ward, T., personal communication), and the functionality of each construct would thus need to be validated before use. Also, the application of PK1A-GFP and *E. coli* K1-specific antibody for the bacterial staining only works in fixed tissue, but fixation (as well as the application of detergent for cell permeabilization) of GFP-tagged cytosolic and membrane proteins often results with redistribution of the proteins (Tanaka et al., 2010; Schnell et al., 2012). On the other hand, labelling of the bacteria with fluorescent dye was not considered, as one of the preliminary data showed that FITC-labelled, and the labelling buffer-exposed *E. coli* K1 demonstrated lower invasion of HBMEC than that of untreated
E. coli K1 (data not shown). Similar observation was made in Mycobacterium tuberculosis study (Schaible, U., personal communication). Therefore, the availability of K1-Cherry allows live-cell imaging of infected HBMEC overexpressing GFP-tagged cellular markers. Although some caution is needed with the results obtained from this system, important results could be further verified with the use of untransformed bacteria in a fixed tissue system if time and antibody availability allowed.

In conclusion, the level of bacterial virulence can be affected by the presence of plasmids, and cytoplasmic production of fluorescent proteins. An mCherry-expressing E. coli K1 strain that retains bacterial invasiveness was successfully constructed in this study and this can therefore be used in future live-cell imaging experiments for studying the bacterial invasion of HBMEC.
4. Optimization of HBMEC transfection efficiency

4.1 Introduction

Genetic materials (such as DNA, RNA, and siRNA) can be delivered into eukaryotic cell via chemical, lipid, physical, or viral vector-mediated methods (Kingston, 2001). The availability of these gene delivery technologies allows the study of a gene function and expression in a cell (Hawley-Nelson et al., 2008). The technology also allows production of correctly folded protein, especially for proteins that require special post-translational modification, such as glycoprotein, for structural studies or as a therapeutic or diagnostic agent (Chan et al., 2002; Bowden et al., 2008).

Viral vectors, such as retroviral, adenoviral, and baculoviral systems, usually give promising cell transduction efficiency (Kingston, 2001). However, for infection study, the major concern with this transfection approach is the potential cellular changes resulting from viral vector-uptake on bacterial infection of the transduced cells.

Several common non-viral vector transfection approaches, such as calcium phosphate transfection, liposome-mediated transfection, and electroporation, offer alternative approaches for introducing genetic material into mammalian cells. For calcium phosphate-, and liposome-mediated transfection, negatively charged DNA is bound to the positively charged surface of calcium, or cationic lipid. Following the attachment of calcium-DNA or lipid-DNA complexes on the cell surface, uptake of the complex is triggered via uncharacterized pathways (Kingston, 2001; Kingston et al., 2003; Hawley-Nelson et al., 2008).

Electroporation uses an electric field to open up micro-sized pores transiently in the plasma membrane to allow diffusion of macromolecules, such as DNA, into the cells. This approach is applicable to all cell types under optimized experimental settings (Potter, 2003). In the past decade, eukaryotic cell electroporation (particularly specific ‘difficult’ or
primary cell types) has been further improved by the introduction of Nucleofection technology (Lonza, Germany), which involves electroporating cells under optimized electroporation conditions in special proprietary buffers. The technology offers greatly improved transfection efficiency and cell viability to various generic cell lines and primary cells. Endothelial cells, which are notoriously difficult to transfect, have also benefited from this technology (Doulet et al., 2006; Lui-Roberts et al., 2008; Kang et al., 2009).

In previous studies, Lipofectamine™ 2000 was used for transfecting plasmid DNA into HBMEC, and transfected cells were treated with antibiotic for stable clone selection (Khan et al., 2002; Sukumaran and Prasadarao, 2002; Sukumaran et al., 2002; Rudrabhatla et al., 2006). Although stable transfection offers 100% of the selected cells expressing the exogenous protein of interest, the efficiency of the gene incorporation into the genome is low, and typically require 2 – 3 months to generate a stable cell line. One part of this study involved the application of a collection of GFP tagged cellular markers and their mutant forms to screen for their effects on E. coli K1 invasion and intracellular survival in HBMEC. Hence, it is practically unfeasible to generate stable cell lines for all constructs used within the limited time of this study. Further, the majority of constructs used code for peripheral membrane proteins (for example Rab GTPases), which are poorly expressed in stable cells (Ward, personal communication). Therefore, a transient transfection protocol that provides high transfection efficiency is required for this study.

4.2 Objectives and aims

The aim of this chapter was to optimise HBMEC transfection efficiency, with the specific objectives being:

- To optimise nucleofection parameters with Amaxa® Nucleofector® Kit.
- To optimise HBMEC transfection efficiency with different liposomal and non-liposomal based transfection reagents.
4.3 Results

4.3.1 Plasmid DNA transfection with Amaxa Nucleofection kit

Using human umbilical vein endothelial cells (HUVEC) specific Amaxa Nucleofection kit, plasmid DNA transfection rate up to 70% and siRNA transfection rate of 90% have been achieved for HUVEC (Cutler, personal communication; Lui-Roberts et al., 2008). As recommended by the Cutler lab (MRC Laboratory for Molecular Cell Biology, UCL, London), two Amaxa Nucleofection kits, namely HUVEC Nucleofector® Kit-OLD (Old) and Amaxa® HUVEC Nucleofector® Kit (New), were tested for plasmid DNA nucleofection into HBMEC. For pmaxGFP nucleofection performed with New, two alternative nucleofection programs, U-001 and A-034, were applied as suggested by the manufacturer. GFP expression was screened with a confocal microscope at 24 and 48 hours post-nucleofection. The number of GFP-expressing cells and total number of cells in phase images were counted manually, and transfection efficiency was expressed as the percentage of GFP-expressing cells (Figure 4.1). At 24 and 48 hours post-nucleofection, the transfection efficiency was less than 20% for nucleofection performed with both kits (Figure 4.1A). Although nucleofection performed with New by applying program A-034 (New/A-034) gave lower transfection efficiency than the transfection efficiency for nucleofection performed with either Old, or with New by applying program U-001 (New/U-001), more cells were attached to coverslips, and fewer rounded cells were observed (data not shown). Therefore, nucleofection with New/A-034 was chosen for further optimizing nucleofection conditions by altering concentration of pmaxGFP (Figure 4.1B). HBMEC nucleofection with 5μg plasmid DNA successfully brought up the transfection efficiency to approximately a fold higher than the transfection efficiency for nucleofection with 2μg of plasmid DNA, although no difference was observed in the transfection efficiency at both time points.
Figure 4.1. Optimization of HBMEC transfection with Amaza Nucleofector kit. (A) To optimize plasmid DNA nucleofection of HBMEC, HBMEC were nucleofected with 2 μg pmaxGFP by either HUVEC Nucleofector® Kit-OLD (program U-001) (Old) or Amaza® HUVEC Nucleofector® Kit [program U-001 (New/U-001) or A-034 (New/A-034)]. pmaxGFP expression was assessed and imaged with a confocal microscope 24 and 48 hours after nucleofection. Transfection efficiency is expressed as a percentage of maxGFP-expressing cells over total number of cells. Results are from one experiment. (B) Optimization of New/A-034 was performed by nucleofecting HBMEC with either 2 μg or 5 μg pmaxGFP, and transfection efficiency was assessed after 24 and 48 hours. Transfection efficiency is expressed as a percentage of maxGFP-expressing cells over total number of cells. Results are from one experiment.

Nucleofection with PBS was tried as described by Kang et al. (2009), which claimed high transfection efficiency in arterial and venous endothelial cells, but resulted with no transfected cells. The cost of a nucleofection kit is high, but the transfection efficiency of HBMEC was generally unsatisfactory with no more than 80% efficiency which would be very difficult for screening purposes, hence alternative transfection reagents were tried.
4.3.2 Plasmid DNA transfection with liposomal- and non-liposomal-based transfection reagents

In the majority of previous studies, Lipofectamine™ has been widely used for transfecting plasmid DNA into HBMEC (Reddy et al., 2000a; Sukumaran et al., 2002; Prasadara et al., 2003; Sissons et al., 2005). Therefore, different transfection conditions with Lipofectamine™ LTX was applied to HBMEC with different concentrations of pN1-EGFP with or without PLUS reagent in Opti-MEM® reduced serum medium. To avoid experimental bias, tiled images were acquired with a confocal microscope 24 and 48 hours post-transfection. The number of GFP-expressing cells and total number of cells in phase images were counted manually, and transfection efficiency was expressed as the percentage of GFP-expressing cells (Figure 4.2). There was no difference observed in the transfection efficiency between 500 ng and 1000 ng of plasmid DNA used at both time points. However, the application of PLUS reagent in transfection reactions improved the transfection efficiency by at least a fold for both plasmid DNA concentration at both time points. The results show that plasmid DNA can be transfected into HBMEC by Lipofectamine™ LTX, and the transfection efficiency is improved by PLUS reagent to approximately 30%, although this level of transfection efficiency was still unsatisfactory for screening purposes.
Figure 4.2. The effect of PLUS reagent on HBMEC transfection efficiency. To optimize plasmid DNA transfection of HBMEC with Lipofectamine™ LTX, HBMEC were incubated with either 500 ng or 1000 ng of pN1-EGFP-lipid complex in the presence or absence of PLUS reagent. EGFP expression was assessed and tile images were taken with a confocal microscope 24 and 48 hours after transfection. Transfection efficiency is expressed as a percentage of EGFP-expressing cells over total number of cells. The results represent the percentage of EGFP-positive HBMEC. Results are from one experiment.

In the attempt to optimize HBMEC transfection efficiency, various lipid-based and cationic polymers proprietary transfection reagents were applied for transfecting pN1-EGFP into HBMEC (Figure 4.3A). 24 hours post-transfection, the general transfection efficiency for all transfection reagents was below 30%, and the lowest transfection efficiency, below 10%, was obtained for transfection performed with FuGENE® HD. However, for transfection performed with jetPRIME™, HBMEC transfection efficiency was increased significantly to approximately 50% when 1 µg of plasmid DNA and 2 µl of jetPRIME™ were employed (Figure 4.3A). Western blotting confirmed an increase in the GFP protein levels for transfection performed with 1 µg of plasmid DNA and 2 µl of jetPRIME™ compared to the other transfection methods (Figure 4.3B). Some rounded cells, presumably dead cells, were frequently observed (data not shown). In conclusion, HBMEC transient transfection efficiency was significantly improved by increasing plasmid DNA concentration and volume of jetPRIME™ in the transfection mixture.
Figure 4.3. Optimization of HBMEC transfection using different transfection reagents. (A) HBMEC cells were transfected with pN1-EGFP using different transfectants. LPF, Lipofectamine™ LTX and PLUS™ reagent with 0.5 μg plasmid DNA; jPr1, 1 μl jetPRIME™ with 0.5 μg plasmid DNA; jPr2, 2 μl jetPRIME™ with 1 μg plasmid DNA; jPEI, jetPEI™ with 1 μg plasmid DNA; jPEI-H, jetPEI™-HUVEC with 2 μg plasmid DNA; FG, FuGENE® HD with 0.5 μg plasmid DNA. 24 hours post-transfection, GFP expression was assessed by acquiring tiled images with a confocal microscope. Transfection efficiency is expressed as a percentage of EGFP-expressing cells over total number of cells. Results are representative of two independent experiments performed in duplicate and presented as mean ± standard deviation. Statistical significance was evaluated with analysis of variance and Newman-Keuls post hoc analysis. (B) Total GFP protein in the transfected cell lysate was determined by Western blotting analysis, lanes equivalent to chart above.

4.4 Discussion

This study showed that HBMEC can be transfected with plasmid DNA using a Nucleofection kit, although the transfection efficiency was unsatisfactory for screening purposes. It was possibly due to the differences between HUVEC and HBMEC, as studies have reported the heterogeneity of different endothelial cells (Swerlick et al., 1992; Salcedo et al., 2000; Man et al., 2008), and the heterogeneity of microvascular endothelial cells isolated from different anatomical sites (Invernici et al., 2005; Lu et al., 2007).
Hence, the optimized HUVEC nucleofection condition might not be suitable for HBMEC nucleofection, and the HBMEC nucleofection conditions would need extensive optimization.

As the cost for a nucleofection kit is high, but with low efficiency, alternative transfection reagents were explored. Plasmid DNA could be transfected into HBMEC by Lipofectamine™ LTX, and the transfection efficiency was improved by pre-complexing plasmid DNA with PLUS reagent before the addition of Lipofectamine™ reagent. The best transfection efficiency obtained from transfection performed with liposomal-based transfection reagents (Lipofectamine™ LTX, and FuGENE® HD) was approximately 30%. In addition to liposomal-based transfection reagents, cationic polymer (non-liposomal) transfection reagents, including jetPRIME™, jetPEI™, and jetPEI™-HUVEC, were applied. Among these transfection reagents, HBMEC transfection efficiency was significantly increased to approximately 50% by altering plasmid DNA : jetPRIME™ ratios. As observed with HUVEC specific Amaxa Nucleofection kits, the HBMEC transfection efficiency achieved with HUVEC specific jetPEI, jetPEI™-HUVEC, was less satisfactory, probably due to the differences between HUVEC and HBMEC, as discussed above. Despite the significant improvement in the HBMEC transfection efficiency as observed from EGFP expression in pN1-EGFP transfection with jetPRIME™, the transfection efficiency was found to vary for different recombinant constructs used later in the study, presumably due to effects of exogenous protein expression in cells.

Viral vectors offer another approach for introducing genetic materials into cells with generally high transduction efficiency. Most of these viral vectors enter into cells via a receptor-specific endocytic pathway, and some of these vectors, especially those originated from retroviruses, stably integrate the transferred gene into the genome of the transduced cells (Ramezani and Hawley, 2002; Lindemann and Schnittler, 2009). In general, viral vector transduction yields high transduction efficiency in most cell lines:
however, the transduction efficiency for endothelial cells varies from 40 – 90% (Shichinohe et al., 2001; Cefai et al., 2005; Anliker et al., 2010). Interestingly, Anliker et al. (2010) constructed measles viral haemagglutinin-pseudotyped lentiviral vectors based on single-chain antibodies recognizing cell-surface antigens, which specifically targeted endothelial cells via CD105, a transmembrane glycoprotein predominantly expressed on endothelial cells (Fonsatti and Maio, 2004). The CD105-targeted viral vector resulted in transduction efficiency of HUVEC of approximately 80%, while the transduction efficiency of human dermal microvascular endothelial cells (HDMEC) was 60.5% (Anliker et al., 2010). This finding further demonstrated the differences of HUVEC from other sources of endothelial cells. Despite the endothelial cells variations, pseudotyped lentiviral vectors might be useful in improving HBMEC transfection efficiency. The major concern for this approach is the triggering of inflammatory responses after exposing the cells with viral vectors, which might affect the transduced cells responses to E. coli K1 infection.

Although common routine transfection methods are used for in vitro studies, researchers in the field of drug or gene delivery have strong interests in developing non-viral-based nano-sized delivery vehicles for efficient delivery into vessels and blood-brain barrier endothelial cells (Dufes et al., 2004; Zhang et al., 2009; Georgieva et al., 2011; Liu et al., 2011). In the efforts of developing delivery vehicles, various transfection methods have been developed, such as a peptide based delivery system targeting caveolae-mediated endocytosis (Liu et al., 2011), glucose-bearing delivery vesicle (Dufes et al., 2004), and DNA intercalating conjugates with a short nuclear localization signal (NLS) peptide (Zhang et al., 2009). However, the transfection efficiency of these alternative vectors remains unsatisfactory for clinical treatment.
5. E. coli K1 invasion of human brain microvascular endothelial cells via a caveolae-, flotillin 1-, and dynamin-independent pathway

5.1 Introduction

Phagocytosis is a process that involves internalization of large particles into specialized cells such as phagocytic protozoa, and phagocytic leukocytes of the immune system. It functions to clear pathogens, such as bacteria or yeast, apoptotic cells, and cell debris (Conner and Schmid, 2003), although some pathogens can piggyback on the phagocytic process and then create a propagatory intracellular niche. The process is an active and regulated process involving specific cell surface receptors and signalling cascades that regulate actin cytoskeleton rearrangement (Conner and Schmid, 2003; Kumari et al., 2010).

As with phagocytic cells, non-phagocytic cells are able to take up fluid and small molecules via pinocytosis, such as clathrin-mediated endocytosis, caveolae-mediated endocytosis, and flotillin-mediated endocytosis, which are classified by the requirement of dynamin during vesicle scission at the plasma membrane (Conner and Schmid, 2003; Doherty and McMahon, 2009). Pathogenic bacteria have also been demonstrated to gain entry into non-phagocytic cells via these endocytic pathways, in addition to the classical classification of bacterial entry based on trigger and zipper mechanisms (Finlay and Cossart, 1997; Cossart and Sansonetti, 2004). It was found that endocytic coats and accessory proteins are recruited during bacterial invasion of non-phagocytic cells via zipper mechanism, but are not recruited to those bacteria, such as Salmonella enterica Typhimurium, and Shigella, which trigger host entry by injecting bacterial effectors and lead to actin cytoskeleton rearrangement (Veiga et al., 2007). The requirement of dynamin and examples of pathogens that utilise specific endocytic pathways for cell entry are summarized in Table 1.1.
Dynamin is a 100 kDa GTPase, which is able to form helical tubes on lipid nanotubes in vitro (Sweitzer and Hinshaw, 1998; Stowell et al., 1999). and assemble at the neck of an endocytic vesicle for vesicle scission (Iversen et al., 2003). In mammalian cells, there are three closely related dynamin isoforms identified. Dynamin 1 (Dyn1) is found specifically in neuronal tissue (Scaife and Margolis, 1990; Sontag et al., 1994), and there are eight splice variants identified (Cao et al., 1998); whereas dynamin 2 (Dyn2), which has four splice variants, is ubiquitously expressed (Nakata et al., 1993; Cook et al., 1994; Sontag et al., 1994; Cao et al., 1998). Dynamin 3 (Dyn3) is expressed predominantly in testes, brain, lung and heart, and has 13 splice variants (Nakata et al., 1993; Cao et al., 1998). It was shown that each splice variant of dynamin isoforms is targeted to distinct cellular locations in Clone 9 cells, a normal rat hepatocyte cell line (Cao et al., 1998), and has different endocytic functions (Cao et al., 2007). Interestingly, the functions of Dyn1 and Dyn2 are indistinguishable in non-polarized epithelial cell line, HeLa; however, in polarized epithelial cell lines, such as MDCK, both dynamins have distinct endocytic functions at the different membrane surfaces (Altschuler et al., 1998). The role of dynamin in endocytosis is often studied using either the chemical inhibitor Dynasore or overexpressing a mutant form of the dynamin protein. Overexpression of the K44A or S45N mutant, which demonstrates weak guanosine triphosphate (GTP) affinity in vitro and thus unable to release the endocytic vesicle from the plasma membrane, is sufficient to inhibit transferrin uptake via clathrin-mediated endocytosis as well as SV40 uptake via caveolae-mediated endocytosis (Marks et al., 2001; Pelkmans et al., 2002; Ferguson and De Camilli, 2012).

Caveolin-1 is a major protein in caveolae, and it has two isoforms, namely caveolin-1α (full length caveolin-1) and caveolin-1β (Parton and Simons, 2007; Parat, 2009). Caveolin-1β is a product of alternate translation, and it lacks 31 amino acids found at the N-terminal of caveolin-1α (Parat, 2009). It has also been proposed that caveolin-1β
is unable to form caveolae in the absence of caveolin-1α (Fujimoto et al., 2000). Caveolin-1 is inserted into plasma membrane lipid bilayers in a wedge-like manner, with its N- and C-termini in the cytoplasm (Figure 1.6) (Parton, 2003). The protein is palmitoylated at the cysteine residue 133, 143, and 156 at the C termini (Dietzen et al., 1995), which has been shown to be essential for cholesterol binding and chaperone complex formation for cholesterol transportation to caveolae, but it is not required for caveolin-1 localization to caveolae (Dietzen et al., 1995; Uittenbogaard and Smart, 2000). The tyrosine residue 14 of caveolin-1 is phosphorylated by cellular Src tyrosine kinase (Li et al., 1996). Abrogation of caveolin-1 phosphorylation by mutating this tyrosine residue to phenylalanine (Cav1 Y14F) might block the downstream effectors, and thus inhibits albumin uptake in rat lung microvascular endothelial cells (RLMEC) via caveolae-mediated endocytosis (Hu et al., 2006). Expression of Cav1 Y14F mutant in caveolin-1 knockout cells also inhibited raft internalization although caveolae was formed on the plasma membrane, suggesting that the non-phosphorylatable caveolin-1 mutant is still recruited to the plasma membrane, but defective in internalisation (del Pozo et al., 2005). As such, Cav Y14F mutant provides a useful tool in studying caveolin-mediated endocytosis.

In addition to clathrin-mediated endocytosis, which requires dynamin for vesicle scission from the plasma membrane, dynamin has also been implicated in caveolae-mediated endocytosis, whereby overexpression of GDP-locked dynamin K44A mutant inhibits caveolar budding and cholera toxin B (a ligand used for studying caveolae-mediated endocytosis) uptake in fibroblasts and endothelial cells (Oh et al., 1998; Shajahan et al., 2004; Yao et al., 2005). The tyrosine residue 231 and 596 of Dyn2 are required for caveolin-1 binding; however, only overexpression of non-phosphorylatable Dyn2 Y597F mutant in rat lung microvascular endothelial cells successfully inhibit albumin and cholera toxin B uptake to the level observed in cells overexpressing the Dyn2 K44A mutant (Shajahan et al., 2004).
There are a number of endocytic pathways that are independent of dynamin, including flotillin-mediated endocytosis (Glebov et al., 2006), Arf6-mediated endocytosis (Donaldson et al., 2009), and the CLIC/GEEC endocytosis pathway (Kumari et al., 2008). These endocytic pathways are still not very well-studied and it is unclear how membrane scission on the plasma membrane occurs in these pathways in the absence of dynamin.

*E. coli* K1 invasion of HBMEC has been proposed to be receptor-dependent, via the zipper mechanism, where actin cytoskeleton rearrangement is induced upon bacterial surface antigen attachment to a specific receptor on the host cell surface (Prasadaraao et al., 1999). Numerous host cell receptors have been identified in parallel with the identification of bacterial virulence determinants required for host cell binding and invasion (Prasadaraao, 2002; Chung et al., 2003; Khan et al., 2007). Upon *E. coli* K1 binding to a cell receptor on HBMEC surface, a cascade of signalling molecules as detailed in 1.6.1, such as the signal transducer and activator of transcription 3 (stat3), focal adhesion kinase (FAK), phosphatidylinositol-3-kinase (PI3K), and protein kinase Cα (PKCa), RhoA, are activated and results in actin polymerization, which leads to bacterial invasion into HBMEC (Prasadaraao et al., 1999; Reddy et al., 2000a; Reddy et al., 2000b; Prasadaraao, 2002; Khan et al., 2003; Maruvada et al., 2008). It has been shown that the bacterial invasion of HBMEC is clathrin-independent (Prasadaraao et al., 1999), but may be caveolae-mediated (Sukumaran et al., 2002). These authors also showed association of intracellular *E. coli* K1 with caveolin-1, and proposed that the caveolin-1 pathway was involved in bacterial transcytosis (Sukumaran et al., 2002). The caveolae-mediated endocytosis pathway is thought to be dynamin-dependent *in vitro* and *in vivo* (Oh et al., 1998; Pelkmans et al., 2002; Yao et al., 2005); however, the role of dynamin and the functional role of endocytic pathway during *E. coli* K1 invasion of HBMEC is not clear.
5.2 Objectives and aims

The aim of this chapter was to investigate the endocytic pathway used by *E. coli* K1 to invade HBMEC. The specific objectives are:

- To study the role of dynamin for *E. coli* K1 invasion of HBMEC.
- To study the requirement of caveolin-1 for *E. coli* K1 invasion of HBMEC.
- To study the requirement and the role of flotillin 1 for *E. coli* K1 invasion of HBMEC.

5.3 Results

5.3.1 *E. coli* K1 invasion of HBMEC is dynamin-independent

The caveolae-mediated endocytic pathway has previously been implicated in *E. coli* K1 invasion of HBMEC (Sukumaran et al., 2002). The authors also reported association of intracellular *E. coli* K1 with caveolin-1, hence, they proposed that caveolin-1 was involved in the bacterial transcytosis. However, the authors did not investigate the involvement of dynamin, which has been found to be required in vesicle membrane fission in caveolae-mediated endocytosis both *in vitro* and *in vivo* (Oh et al., 1998; Pelkmans et al., 2002; Shajahan et al., 2004; Yao et al., 2005), during *E. coli* K1 invasion HBMEC.

Both Dyn1 and Dyn2 have been implicated in caveolae-mediated endocytosis (Oh et al., 1998; Pelkmans et al., 2002; Shajahan et al., 2004; Yao et al., 2005), but the majority of previous endothelial studies have focussed on Dyn2-dependent endocytic mechanisms as this isoform is ubiquitously expressed in mammalian tissues (Cook et al., 1994), whereas Dyn1 is more usually associated with neuronal tissue. Therefore, Dyn2 was initially selected to study its role during *E. coli* K1 invasion of HBMEC. Among the four Dyn2 splice variants (Cao et al., 1998), Dyn2(aa) and Dyn2(ab) have been shown to interact directly with caveolin-1 *in vitro* (Yao et al., 2005), and overexpression of GDP-locked Dyn2(aa)K44A (also termed dominant negative) mutant has been shown to inhibit
cholera toxin B internalization into rat fibroblasts and endothelium (Shajahan et al., 2004; Yao et al., 2005). We hypothesized that overexpression of Dyn2(aa)K44A mutant would inhibit E. coli K1 invasion of HBMEC if the bacteria invaded HBMEC via a caveolae-mediated endocytic pathway. Dynasore, a cell-permeable inhibitor of Dyn1, Dyn2, and dynamin-related protein 1 (Drp1) (Macia et al., 2006), was not applied as HBMEC used in this study are sensitive to a lot of chemical inhibitors (Kim, K.S., personal communication).

To study the role of dynamin during the bacterial invasion quantitatively, HBMEC were transiently transfected with GFP-tagged Dyn2 chimaera, either wild type or dominant negative [Dyn2(aa)K44A GFP]. LDLR A18 GFP, which expressed GFP-tagged recycling-deficient mutant of the low-density-lipoprotein receptor (LDLR) on the plasma membrane, was transfected into HBMEC as a transfection control. After 5 hours incubation of plasmid DNA-jetPRIMETM complex with HBMEC, media was removed and replenished with fresh media, and further incubated for approximately 16 hours. Cells were infected with K1-Cherry for 2 hours, incubated for 1 hour in media containing gentamicin to kill off extracellular bacteria and then imaged on the confocal microscope. Tiled z-stack confocal imaging was performed to acquire a large field and to minimise experimental bias, and GFP-expressing cells were manually scored for the presence of intracellular K1-Cherry (Figure 5.1A). The transfection efficiency of Dyn2(aa)WT, Dyn2(aa)K44A, and LDLR A18 GFP, was 19.5 ± 1.6%, 19.1 ± 2.8%, and 11.7 ± 3.8%, respectively. Quantification of the infected GFP-expressing cells showed no inhibition in bacterial invasion in cells overexpressing Dyn2(aa)K44A GFP. In fact, there was a modest enhancement in E. coli K1 internalization into these cells compared to cells expressing wild type Dyn2, though it was not statistically significant.

As the endothelium specific dynamin isoform is unknown, the role of the neuronal specific Dyn1 on E. coli K1 invasion was studied in similar manner (Figure 5.1B). The
transfection efficiency of Dyn1WT, Dyn1K44A, and LDLR A18 GFP, was 41.4 ± 7.0%, 20.9 ± 6.0%, and 38.3 ± 3.9%, respectively. As with Dyn2, the bacterial entry was not inhibited in cells that overexpressed dominant negative Dyn1 (Dyn1K44A-GFP) and again there was a trend to a slight increase in bacterial uptake in mutant over wild type Dyn1 expression.

Figure 5.1. Overexpression of dominant negative dynamin 1 and 2 do not inhibit *E. coli* K1 invasion of HBMEC. (A) and (B) To study the requirement of dynamin during *E. coli* K1 invasion, HBMEC cells were transiently transfected with GFP tagged Dyn2 or Dyn1 chimaera: either wild type (WT), or dominant negative mutant (K44A). LDLR A18 GFP was transfection control. Cells were infected with K1-Cherry at MOI of 100 for 2 hours, followed by 1 hours incubation in gentamicin containing media, and processed for microscopy. GFP chimaera-expressing cells were scored for the presence of intracellular K1-Cherry. Data shown are the mean ± SEM from three independent experiments; at least 50 GFP-expressing cells were counted per sample.

5.3.2 *E. coli* K1 invasion of HBMEC is caveolin-1-independent

Caveolae-mediated endocytosis has been implicated to be dynamin-dependent (Oh *et al.*, 1998; Pelkmans *et al.*, 2002), however, the data above suggested that overexpression of dominant negative forms of both dynamin 1 and 2 did not inhibit *E. coli* K1 invasion of HBMEC. Hence, the requirement of caveolin-1 during *E. coli* K1 invasion of HBMEC was reassessed. To address the requirement of caveolin-1 during *E. coli* K1 invasion, HBMEC were infected with *E. coli* K1, fixed at various time points post infection,
immunostained for caveolin-1. Caveolin-1 staining revealed bright punctate structures at the cell periphery and at the juxtanuclear position as well as in the cytoplasm (Figure 5.2A). After 30 minutes of infection, invading *E. coli* were found localized in compartments weakly stained for caveolin-1 (Figure 5.2A).

**Figure 5.2.** Association of *E. coli* K1 with caveolin-1 decreases over time of infection. (A) HBMEC were infected with K1-Cherry at MOI of 100 for 30 minutes, and fixed with 2% formaldehyde, and immunostained for caveolin-1. Caveolin-1 is shown in green, bacteria in red, nuclei in blue. The white arrow indicates bacterium localising with caveolin-1; white arrow heads indicate bacteria that do not localise with caveolin-1. A single confocal slice is shown. Scale bar: 5 μm. (B) Quantification of the observed phenotype in (A) at various times p.i. Data shown are the mean ± SEM from two independent experiments; ≥100 bacteria were counted per sample.
Quantification of the observed phenotype in Figure 5.2A showed that only about 4% of the bacteria were closely associated with caveolin-1 after 30 minutes infection, and the percentages reduced to about 1% and 0.5% at 2 and 20 hours post infection (p.i.). Therefore, association of *E. coli* K1 with caveolin-1 seemed to be a rare event which occurred at the early time point when the bacteria were invading the cell. This would therefore suggest that caveolae-mediated uptake is not a major route of invasion for *E. coli* K1 into HBMEC.

To further address the role of caveolin-1 during *E. coli* K1 uptake, various GFP-tagged caveolin-1 mutant constructs were applied. Caveolin-1 phosphorylation has been demonstrated to be required for albumin uptake via caveolae (Hu *et al.*, 2006), and overexpression of caveolin-1 Y14F phosphorylation mutant, which is unable to be phosphorylated by Src family protein kinases (Labrecque *et al.*, 2004), in COS-1 cells partially inhibits *Campylobacter jejuni* invasion (Watson and Galán, 2008). To study the effect of caveolin-1 tyrosine phosphorylation on *E. coli* K1 invasion, GFP-tagged caveolin-1 Y14A mutant (Caveolin-1 Tyr14. N.B. this mutant is also non-phosphorylatable) was transiently overexpressed in HBMEC, and the cells were then infected with K1-Cherry. Quantification of the infected GFP-expressing cells showed no inhibition in the bacterial internalization as compared to cells overexpressing wild type caveolin-1 (Caveolin-1 WT-GFP) (Figure 5.3). Similar results were seen by overexpressing the non-phosphorylatable isoform, caveolin-1β (Caveolin-1β-GFP), which lacks the 31 amino acids of the N-terminal of full length caveolin-1 (caveolin-1α) as a result of alternate initiation during translation.

Caveolin-1 is also post-translationally modified by palmitoylation at the three cysteine residues at the hydrophobic region at the C-terminal. Whilst this is not required for the plasma membrane localization of caveolin-1, it is required for transport of newly synthesized cholesterol to the plasma membrane (Dietzen *et al*., 1995; Uittenbogaard and
Smart, 2000; Parat and Fox, 2001). Previous studies showed that depletion of cholesterol from HBMEC leads to reduction of *E. coli* K1 invasion (Sukumaran et al., 2002; Chi et al., 2010). We hypothesized that the lack of cholesterol on the plasma membrane that results from overexpression of a caveolin-1 mutant that cannot be palmitoylated is able to block *E. coli* K1 invasion of HBMEC if the bacterial entry is cholesterol dependent. To test this hypothesis, HBMEC were transfected with a GFP-tagged caveolin-1 palmitoylation mutant construct, in which the 133, 143, and 156 cysteine residues were mutated to serine (Caveolin-1 palm') (Parat et al., 2003), infected with K1-Cherry, and imaged 3 hours p.i. (Figure 5.3). Quantification of the number of infected GFP-expressing cells revealed no inhibitory effect of the mutant on *E. coli* K1 internalization. These cumulative results strongly indicated that in our infection model, caveolae are not used as a route of invasion for *E. coli* K1 into HBMEC.
Figure 5.3. Overexpression of caveolin-1β isoform and caveolin-1 mutants do not affect *E. coli* K1 invasion of HBMEC. To study the effect of different caveolin-1 mutants and caveolin-1β isoform on *E. coli* K1 invasion of HBMEC, HBMEC were transiently transfected with GFP-tagged caveolin-1 chimaerae: either wild type (WT), caveolin-1β, palmitoylation mutant (palm'), or phosphorylation mutant (Tyr14) as indicated. Cells were infected with K1-Cherry at MOI of 100 and were imaged 3 hours p.i. Data shown are the mean ± SEM from two independent experiments; ≥100 GFP cells were counted per sample and experiment. LDLR A18 was used as transfection control. The transfection efficiency of each construct was: LDLR A18 GFP = 34.24 ± 8.8%; Caveolin-1 WT-GFP = 41.8 ± 13.9%; Caveolin-1β-GFP = 29.8 ± 10.3%; Caveolin-1 palm' = 32.3 ± 12.1%; Caveolin-1 Tyr14 = 43.2 ± 5.8%.

To investigate this further, an invasion assay was performed with mouse lung endothelial cells (MLEC) isolated from wild type (WT) and caveolin-1 knockout (Cavl K/O) mice and cultured as described. MLEC were used instead of brain microvascular endothelial cells (BMEC) due to the technical difficulty to isolate a sufficient quantity of BMEC from mice, and the difficulty in culturing them beyond a single passage (Liebner, S., personal communication). The results from the invasion assay performed in RPMI-1640 media showed that the number of recovered intracellular *E. coli* K1 per cell was approximately 40-fold higher than the number of recovered intracellular non-pathogenic *E. coli* K-12 per cell (Figure 5.4). This data indicated that MLEC could be used as an alternative in vitro model for studying *E. coli* K1 pathogenesis. When the invasion assay was performed with MLEC Cav1 K/O, *E. coli* K1 invasion of MLEC was not inhibited as
would be expected if caveolin were required for bacterial entry. However, the number of recovered intracellular *E. coli* K1 per cell was approximately 2-fold higher than the number of bacteria recovered from MLEC WT, although this observed increase in intracellular bacteria was not statistically significant, while there was no comparable increase in K12 uptake into MLEC Cav1 K/O cells. Overall, our data indicates that in our system *E. coli* K1 invasion of HBMEC is not dependent on caveolae-mediated endocytosis.

![Graph showing bacterial uptake](image)

**Figure 5.4. Caveolin-1 knockout does not inhibit *E. coli* K1 invasion of MLEC.** To confirm the requirement of caveolin-1 during *E. coli* K1 invasion of endothelial cells, MLEC purified from wild type and caveolin-1 knockout mice were infected with either *E. coli* K1 or K-12 at MOI of 100 for 2 hours, followed by 30 minutes incubation in gentamicin containing media. The results represent the mean number of bacteria per cell. Results are from one representative experiment of three independent repeats performed in triplicate and presented as mean of triplicates ± SEM.

### 5.3.3 Flotillin 1 is recruited to invading and intracellular *E. coli* K1

All the evidence from this study suggested that *E. coli* K1 invasion of HBMEC is dynamin- and caveolae-independent. Therefore, other dynamin-independent endocytic pathways were explored. Cholesterol has been shown to be required for *E. coli* K1 invasion of HBMEC by treating the cells with chemical inhibitors, such as filipin, methyl-β-cyclodextrin, and nystatin (Sukumaran *et al.*, 2002; Chi *et al.*, 2010). We therefore investigated the potential role of flotillin 1, which is associated with cholesterol and lipid raft microdomains, and independent of dynamin, during *E. coli* K1 invasion. To address the involvement of flotillin 1 during *E. coli* K1 uptake, HBMEC were infected with *E. coli*
K1. Cells were fixed over a time course from 0.5 hours p.i. to overnight and differential bacterial staining was performed to discriminate between extra- and intracellular bacteria. Flotillin 1 staining revealed bright punctate structures at the cell periphery and in the cytoplasm (Figure 5.5A). After 30 minutes infection, flotillin 1 could be found concentrated at bacterial attachment sites; 2 hours post-infection, strong co-localization of flotillin 1 with intracellular bacteria were observed (Figure 5.5A). In Figure 5.5B, quantification of *E. coli* K1 association with flotillin 1 over time showed that the association of flotillin 1 with extracellular bacteria was approximately 5%, and the percentage decreased to about 1% by 2 hours. For the intracellular bacteria, the percentages of bacteria co-localized with flotillin 1 increased sharply from about 5% at 30 minutes after infection to approximately 30% after 2 hours infection, and this association remained fairly constant at later time points. In comparison, caveolin-1 associated *E. coli* K1 was 4% and 1% at 30 minutes and 2 hours p.i., respectively (Figure 5.2B). These results suggest that flotillin 1 might have a role during *E. coli* K1 invasion of HBMEC.
Figure 5.5. Association of flotillin 1 with extra- and intracellular E. coli K1 over time of infection. (A) HBMEC were infected with E. coli K1 for the times indicated, fixed, and differential bacterial and flotillin 1 staining was performed as described in Chapter 2. Flotillin 1 is shown in green, extracellular bacteria in purple, intracellular bacteria in blue. A single confocal slice is shown. Flotillin 1 is associated with extracellular bacteria on the cell surface (white arrows), and concentrated around intracellular bacteria at the later time point (yellow arrows). Scale bars: 10 μm. (B) Quantification of bacteria-flotillin 1 association as in (A) at various time of infection. Data shown are the mean ± SEM from two independent experiments; ≥100 bacteria were counted per sample.

5.3.4 E. coli K1 invasion of flotillin 1 knockout MLEC is enhanced

To elucidate the role of flotillin 1 during E. coli K1 infection, an invasion assay was performed with flotillin 1 knockout MLEC (MLEC Flot1 K/O) (Figure 5.6A). At 150 minutes p.i., in the absence of flotillin 1, the intracellular bacterial density per cell for both E. coli K1 and non-pathogenic E. coli K-12 was significantly higher than the recovered bacteria from MLEC WT. The mean number of intracellular E. coli K1 per cell recovered from MLEC Flot1 K/O was approximately 25-fold higher than the mean number of the intracellular bacteria per cell recovered from MLEC WT (p < 0.01). In MLEC WT, there was almost no E. coli K-12 recovered; however, in MLEC Flot1 K/O, the mean number of intracellular E. coli K-12 was about 0.02, which was about 60% increased compared to the mean number of E. coli K-12 recovered from MLEC WT (p < 0.0001).

Despite being a plasma membrane protein, flotillin 1 has also been implicated as a late endosomal marker (Dermine et al., 2001; Glebov et al., 2006; Riento et al., 2009). Therefore, the increase in recovered intracellular bacteria from MLEC Flot1 K/O cells
could result either from enhanced bacterial entry into the cells, or from a defective late endosomal/lysosomal pathway due to flotillin 1 knockout, potentially preventing targeting of bacteria to lysosomal destruction. To address this question, both MLEC WT and MLEC Flot1 K/O cells were infected with *E. coli* K1 for 30 minutes, fixed, and differential bacterial staining was performed (Figure 5.6B). There were no intracellular bacteria found in MLEC WT after 30 minutes of infection. In contrast, approximately 8% of Flot1 K/O cells were found to contain intracellular bacteria. This result suggested that the increased number of recovered intracellular bacteria from MLEC Flot1 K/O lysates was due to enhanced bacterial entry.

**Figure 5.6. Flotillin 1 knockout enhances *E. coli* K1 uptake into MLEC.** (A) To study the role of flotillin 1 during *E. coli* K1 invasion of endothelial cells, MLEC purified from wild type and flotillin 1 knockout mice were infected with either *E. coli* K1 or K-12 at MOI of 100 for 2 hours, followed by 30 minutes incubation in gentamicin containing media. The results represent the mean number of bacteria per cell. Results are from one representative experiment of three independent repeats performed in triplicate and presented as mean of triplicates ± SEM. (B) To confirm the enhanced recovered intracellular bacteria was due to increased bacterial invasion, wild type and flotillin 1 knockout MLEC were infected with *E. coli* K1 for 30 minutes, fixed with formaldehyde, and differential bacterial staining was performed. Cells containing intracellular bacteria were counted. Data shown are the mean ± SEM from two independent experiments; ≥40 cells were counted per sample.
5.3.5 Flotillin as cellular regulatory barrier model

Caveolin-1, flotillin 1, and flotillin 2 are lipid raft associated membrane proteins in mammalian cells. Previous studies have shown that knockdown or knockout of either protein leads to the alteration of expression levels of the other proteins in the target cells (Chintagari et al., 2008; Vassilieva et al., 2009; Ludwig et al., 2010). To characterize the knockout MLEC used in this study, cells were stained for caveolin-1, flotillin 1, and flotillin 2 (Figure 5.7). The immunofluorescence micrographs showed that caveolin-1 distribution in both MLEC Flot1 K/O and MLEC WT was similar, where bright punctate structures were observed in the cytoplasm and cell periphery, although the protein expression might be lower in the flotillin 1 knockout cells than the wild type cells (Figure 5.7A. N.B. Contrast has been enhanced in these micrographs.). However, in MLEC Cav1 K/O cells, for cells immunostained for flotillin 1, there was no punctate structure found in the cells (Figure 5.7B). Contrastingly, comparison of the flotillin 2 phenotype in the different MLEC cell lines revealed that in WT cells bright punctate structures were observed (Figure 5.7C). These were also present in MLEC Cav1 K/O, but loss of the phenotype was observed in MLEC Flot1 K/O. These observed phenotypes might suggest that the loss of flotillin 1 and 2 caused the plasma membrane of MLEC Flot1 K/O to become more penetrable to bacteria.
Figure 5.7. Flotillin 1 and flotillin 2 distributions are altered in caveolin-1 and flotillin 1 knockout MLEC. (A) MLEC WT and MLEC Flot1 K/O were stained with anti-caveolin-1 (green). Bright punctate structures were observed in the cytoplasm and cell periphery of both cells. (B) MLEC WT and MLEC Cav1 K/O were stained with anti-flotillin 1 (green). Bright punctate structures were observed in MLEC WT, but absent in MLEC Cav1 K/O. (C) MLEC WT, MLEC Flot1 K/O, and MLEC Cav1 K/O were stained with anti-flotillin 2 (green). Bright punctate structures were observed in MLEC WT and MLEC Cav1 K/O, but absent in MLEC Flot1 K/O. Scale bars: 5 µm.

To test the hypothesis that flotillin may have a barrier function in preventing bacterial invasion, GFP-tagged flotillin 1, flotillin 2, caveolin-1, and LDLR A18 were transfected into HBMEC, infected with KI-Cherry at MOI of 100 for 2 hours, followed by 1 hour incubation in media containing gentamicin, and imaged with a confocal microscope. The number of GFP-expressing HBMEC with intracellular KI-Cherry was scored manually. As an experimental control, infection of untransfected HBMEC was included (Figure 5.8). The percentage of infected HBMEC overexpressing GFP-tagged flotillin 1
after 3 hours infection was about 3-fold lower than the percentage of infected HBMEC overexpressing GFP-tagged caveolin-1 and LDLR A18 at the similar infection time point. In HBMEC overexpressing GFP-tagged flotillin 2, no infected cells were found after 3 hours infection. This preliminary observation suggests that overexpression of flotillin 1 or flotillin 2 has inhibitory effect on E. coli K1 invasion of HBMEC.

Figure 5.8. Overexpression of GFP tagged flotillin 1 and flotillin 2 affect E. coli K1 entry of HBMEC. To study the effect of flotillin 1 and flotillin 2 overexpression in HBMEC on E. coli K1 invasion, HBMEC were transiently transfected with a GFP-tagged constructs as indicated. Untransfected HBMEC was included as experimental control. Cells were infected with K1-Cherry at MOI of 100 for 2 hours, incubated in media containing 100 μg/ml gentamicin for 1 hour, and imaged. Data shown are from one experiment.

5.4 Discussion

E. coli K1 invasion of HBMEC is dynamin- and caveolin-1 independent

Some bacteria, such as Salmonella Typhimurium, invade non-phagocytic cells by injecting bacterial effectors via T3SS, which leads to membrane ruffle formation and results in bacterial uptake (Gerlach et al., 2008). Some E. coli K1 clinical isolates were found to possess T3SS, and this T3SS was shown to be required for bacterial invasion of HBMEC and intracellular survival (Yao et al., 2006; Yao et al., 2009). The E. coli K1 strain used in this study, E44 strain, does not have T3SS, however, it harbours genes for
general secretory pathway (GSP), which are also present in uropathogenic *E. coli* strain CFT073 and other Gram negative bacteria (Welch *et al.*, 2002; Yao *et al.*, 2006). Studies performed with E44 proposed that the K1 bacterial invasion of HBMEC was via receptor-mediated, clathrin-independent “zippering” mechanism (Prasadaraao *et al.*, 1999). Later, Sukumaran *et al.* (2002) reported that *E. coli* K1 hijacked the caveolae-mediated pathway for entry into HBMEC, and the authors also proposed that caveolin-1 was involved in bacterial transcytosis.

Caveolae-mediated endocytosis has been implicated to be dynamin-dependent via a GTP-dependent fission process (Oh *et al.*, 1998; Yao *et al.*, 2005). Further evidence to support the requirement for dynamin in caveolae-mediated endocytosis has been demonstrated by the recruitment of Dyn2 during SV40 internalization into a fibroblast cell line (Pelkmans *et al.*, 2001; Pelkmans *et al.*, 2002), and that phosphorylation of tyrosine 231 and 597 of dynamin 2 is required for caveolin-1 binding, as shown by expression of non-Src-phosphorylatable Dyn2 Y597F mutant in microvessel endothelial cells, which abolished albumin and cholera toxin subunit B uptake (Shajahan *et al.*, 2004). Therefore, we hypothesized that overexpression of GDP-bound Dyn2 K44A mutant, which has also been shown to abolish caveolae-mediated endocytosis in endothelium (Shajahan *et al.*, 2004), might block *E. coli* K1 invasion of HBMEC, if the bacteria utilized the caveolae-mediated pathway for HBMEC entry. However, we did not find any inhibitory effect on the bacterial internalization in cells overexpressing GDP-bound Dyn2 (Figure 5.1A). Different isoforms and splice variants of dynamin exist in mammalian cells (Cao *et al.*, 1998; Hinshaw, 2000), and furthermore different dynamin isoforms regulate receptor-mediated endocytosis at the different membrane surfaces in polarized MDCK cells (Altschuler *et al.*, 1998). Since HBMEC are polarized cells, we repeated the experiments with GDP-bound Dyn1 (Figure 5.1B), but again *E. coli* K1 invasion was not inhibited. As observed by Watson and Galan (2008) in their study of *Campylobacter jejuni* invasion of...
COS-1 cells, minor enhancement in *E. coli* K1 uptake in cells overexpressing GDP-bound dynamin was also observed in this study. These data suggest that dynamin is not required for *E. coli* K1 internalization into HBMEC. However, it should be noted that the functionality of the dominant negative mutants in our system has not been validated. Whilst the phenotype of the cells overexpressing the K44A mutants is similar to that previously published, it is possible that HBMEC dynamin-dependant endocytosis is still functioning. To ensure the observation with *E. coli* K1 was not due to non-functioning GDP-bound dynamin mutants in HBMEC, the inhibitive effect of the mutants on endocytosis should be confirmed by studying the uptake of transferrin, a ligand for clathrin-mediated endocytosis. A functional GDP-bound dynamin mutant is anticipated to inhibit transferrin uptake into HBMEC overexpressed the mutant. Furthermore, since dynamin isoforms seem to have redundant functions (Altschuler *et al.*, 1998), there is a possibility that the other endogenous dynamin isoforms and splice variants might take over the role in cells overexpressing a specific GDP-locked dynamin isoform or splice variant.

Although it appears that dynamin is not required for *E. coli* K1 uptake in our system, the observation does not completely eliminate the involvement of caveolae-mediated endocytosis. Recently, it was reported that the uptake of cholera toxin B (CTB), which is a ligand commonly used for studying caveolae-mediated endocytosis, was not inhibited in Dyn2 knockout fibroblasts (Liu *et al.*, 2008). Therefore, the absolute requirement for dynamin in caveolae-mediated endocytosis seems to be unclear and a role for caveolin that might be dynamin-independent was investigated further. Immunofluorescence staining of *E. coli* K1-infected HBMEC showed weak staining of the bacteria with caveolin-1 (Figure 5.2A), as compared to Sukumaran *et al.* (2002) who showed strong staining of the bacteria with caveolin-1. The observed discrepancy could be due to channel cross-talk in their results, because very high intensity of nonspecific background signals was shown in their micrographs, which they indicated as specific...
bacterial staining (using anti-K1 or anti-S-fimbriae antibody). Further, our findings showed that the association of the bacteria with caveolin-1 was a rare event, with only approximately 4% of the bacterial population associated with caveolin-1 after 30 minutes infection (Figure 5.2B), and the percentage of association further decreased to about 1% at the end of 2 hours infection.

Overexpression of tyrosine 14 mutant, caveolin-1 Y14F, in COS-1 cells has been shown previously to partially inhibit *Campylobacter jejuni* invasion (Watson and Galán, 2008), however, in this study overexpression of caveolin-1 Y14A in HBMEC had no inhibitory effect on *E. coli* K1 invasion. This result was further backed up by expressing the GFP-tagged caveolin-1β isoform, which is not phosphorylatable, in HBMEC. Instead of inhibiting *E. coli* K1 internalization into HBMEC, which might be expected if caveolin-1 is required for uptake, a mild increase in the number of cells with intracellular bacteria was observed when overexpressing caveolin-1β. The functionality of caveolin-1 Y14A and caveolin-1β in HBMEC need to be validated by studying the uptake of lactosylceramide (LacCer), which was a specific ligand for caveolae-mediated endocytosis (Singh et al., 2003) to substantiate these observations.

To further support our data that caveolin-1 is not required for *E. coli* K1 infection, an invasion assay was performed with MLEC isolated from either wild type or caveolin-1 knockout mice. The main reason for using lung endothelia was due to the technical difficulty to isolate of brain microvascular endothelium in sufficient quantity from mice and the difficulty to then culture the cells in vitro (Liebner, S., personal communication). Although bacterial invasion efficiency of MLEC is low, MLEC are still a valid in vitro model for studying *E. coli* K1 pathogenesis, as parallel infection with the non-pathogenic *E. coli* K-12 showed that these bacteria did not invade MLEC. Further, it was also shown previously that *E. coli* K1 was able to invade other endothelial cells with lower invasion efficiency (Prasadarao et al., 1996a). Importantly, an invasion assay performed with
caveolin-1 knockout in MLEC did not inhibit *E. coli* K1 invasion (Figure 5.4). Although this evidence, in combination with our data using non-phosphorylatable caveolin-1 mutant expression in HBMECs, suggests that *E. coli* K1 invasion is independent of caveolin-1, the possible effects of alterations in other signalling pathways or receptor expression on the plasma membrane caused by the removal of caveolin-1 in the knockout cells cannot be disregarded (Barth *et al.*, 2007; Matthews *et al.*, 2008). Recently, Siddiqui *et al.* (2011) demonstrated the absence of caveolin-1 led to destabilization of adherens junctions and activation of endothelial nitric oxide synthase (eNOS) in endothelial cells. The eNOS nitrated GTPase activating protein (GAP) p190RhoGAP-A, resulting in impaired GAP and RhoA activation (Siddiqui *et al.*, 2011). Since RhoA was required for *E. coli* K1 invasion of HBMEC (Khan *et al.*, 2002), the upregulation of RhoA in caveolin-1 knockout cells could facilitate the bacteria invasion of caveolin-1 knockout MLEC.

In addition to studying the role of caveolin-1 during *E. coli* K1 invasion of HBMEC, we were also interested in studying the requirement of cholesterol for bacterial invasion of HBMEC. It was previously shown that *E. coli* K1 invasion of HBMEC was blocked by a number of cholesterol inhibitors (Sukumaran *et al.*, 2002; Chi *et al.*, 2010). Caveolin-1 is irreversibly palmitoylated at the three cysteine residues in the hydrophobic region at the C-terminal (Figure 1.6) (Dietzen *et al.*, 1995; Parat and Fox, 2001). Mutation of these cysteine residues affects caveolin-chaperone complex formation and abolishes transport of newly synthesized cholesterol to plasma membrane caveolae (Dietzen *et al.*, 1995; Smart *et al.*, 1996; Uittenbogaard and Smart, 2000; Fu *et al.*, 2004). We found no inhibitory effect of the caveolin-1 palmitoylation mutant on *E. coli* K1 internalization of HBMEC. However, this observation is insufficient to rule out the role of cholesterol during *E. coli* K1 invasion of HBMEC, as caveolin-1 knockout mice displayed normal mRNA and protein levels of cholesterol transport protein Niemann-Pick C1-like 1 (NPC1L1) in intestinal mucosa, and did not show any defect in cholesterol transportation (Valasek *et al.*.
Therefore, overexpression of the caveolin-1 palmitoylation mutant might not have completely abolished the cellular cholesterol trafficking pathway, and cholesterol could be transported or acquired via alternative pathways. This could be more directly tested by depleting cholesterol with β-trimethyl cyclodextrin, which is less toxic than filipin (Schneider, B., personal communication), or by cholesterol inhibiting agents, such as simvastatin, and lovastatin, if the cells can withstand these treatments.

**Loss of flotillin 1 enhanced *E. coli* K1 internalization**

Since cholesterol had been previously implicated in *E. coli* K1 invasion of HBMEC (Sukumaran *et al.*, 2002; Chi *et al.*, 2010), we therefore looked at the requirement of flotillin, which is associated with lipid rafts (Glebov *et al.*, 2006), during the bacterial invasion of HBMEC. Although flotillin endocytosis is relatively rare (Doherty and McMahon, 2009; Riento *et al.*, 2009), it has been shown to endocytose a proportion of cholera toxin B, and this uptake is dynamin-independent (Glebov *et al.*, 2006). Other than the uptake of cholera toxin B, flotillin is required for uptake of plasma membrane components such as glycosyl-phosphatidylinositol (GPI)-linked proteins and glycosphingolipids (Glebov *et al.*, 2006; Frick *et al.*, 2007). Flotillin 1 has been shown to co-localize with lysosomal transmembrane glycoprotein, LAMP1 (Derminé *et al.*, 2001), in addition to its localization on plasma membrane. It was suggested that the presence of flotillin 1 on the phagolysosomal membrane might be involved in actin accumulation and polymerization (Derminé *et al.*, 2001). No known pathogen is documented to invade eukaryotic cells via a flotillin-dependent pathway, although some bacteria have been found to associate with flotillin intracellularly (Li *et al.*, 2008a). For example, *Campylobacter jejuni* and *Brucella abortus* associate with flotillin 1 transiently (Arellano-Reynoso *et al.*, 2005; Watson and Galán, 2008), while in *Mycobacterium marinum* infection of monocytes.
flotillin 1 was found on the bacterial replication phagosomes which lack LAMP1 (Hagedorn and Soldati, 2007).

Quantification of the population of *E. coli* K1 associated with flotillin 1 revealed that a small population of *E. coli* K1 (5%) associated with flotillin 1 after 30 minutes infection, but the bacterial population which associated with flotillin 1 increased to about 30% after 2 hours infection (Figure 5.5A and B). The role of flotillin 1 on intracellular bacteria-containing vacuoles is unknown. In this study, the population of *E. coli* K1 associated with flotillin 1 remained stable at later time points. The low initial colocalisation with flotillin might suggest that *E. coli* K1 do not require flotillin for uptake, but that *E. coli* K1 might hijack a flotillin 1 pathway once inside the cell, and utilize the cytoskeletal machinery for intracellular transportation to the other membrane surface.

To elucidate the role of flotillin 1 during *E. coli* K1 infection, the invasion assay was performed with MLEC isolated from flotillin 1 knockout mice. Instead of an inhibitory effect on the bacterial entry, we observed a 25-fold increase in *E. coli* K1 density recovered from the cell lysate of flotillin 1 knockout MLEC in comparison to wild type MLEC. This trend was also observed for infection performed with non-pathogenic *E. coli* K-12. The observed increase in intracellular bacteria could be due to either increased bacterial uptake, or a non-functioning lysosomal degradation pathway, due to the fact that flotillin 1 has been found on late endosomes (Dermine *et al.*, 2001). However, after 30 minutes infection with *E. coli* K1, which should be insufficient for the bacteria-containing vacuoles to mature to late endosomal stage and to fuse with lysosomes, there was no infected wild type MLEC, while 8% of the flotillin 1 knockout MLEC were infected. This observation strongly supported the hypothesis that the enhanced number of intracellular bacteria recovered from flotillin 1 knockout MLEC was due to increased bacterial uptake rather than an inhibition in lysosomally targeted bacterial lysis. There is a possibility that in these flotillin 1 knockout MLEC, the equilibrium of membrane protein populations are
disrupted following the total absence of flotillin 1 in these cells, hence the bacterial internalization might be shunted to another endocytic pathway. Therefore, this result might need to be confirmed with flotillin 1 knockdown by flotillin 1 specific siRNA in HBMEC, where a small amount of flotillin 1 remains in the knockdown cells, and might maintain the equilibrium of the membrane protein populations. On the other hand, the functionality of the lysosomal degradation pathway in MLEC FlotlKJO can be studied by comparing the number of recovered intracellular *E. coli* K-12, which have been shown to be unable to survive intracellularly (Figure 3.5), in Flot1K/O MLEC at early and extended time points of infection. A significant reduction in the number of recovered intracellular *E. coli* K-12 from Flot1K/O MLEC at the extended time point of infection would be expected if the lysosomal pathway remains functional in the knockout cells. Alternatively, the functionality of the lysosomal degradation pathway can be studied by assessing the long-lived protein degradation as described in (Razi et al., 2009).

**Flotillin as a regulatory cell barrier**

Although caveolae and flotillin form distinct microdomains, their cellular expressions and protein stability are interdependent (Solis *et al.*, 2007; Chintagari *et al.*, 2008; Vassilieva *et al.*, 2009; Ludwig *et al.*, 2010). In conjunction with previous published data on caveolin-1, flotillin 1, and flotillin 2 expression levels in protein knockdown or knockout cells, as well as our immunofluorescence observations on the protein distributions, we propose that both flotillin 1 and flotillin 2 distributions on the plasma membrane might have a role as a regulatory cellular barrier. This is evidenced by the loss of flotillin 2 punctate structures in flotillin 1 knockout MLEC, which showed enhanced bacterial internalization for both *E. coli* strains; whereas in caveolin-1 knockout MLEC, only flotillin 1 distribution was affected, and a mild increase which was not statistically significant in *E. coli* K1 uptake into the knockout MLEC was observed. It is also possible
that the inhibitory effect on *E. coli* K1 uptake into caveolin-1 knockout MLEC is cancelled by the loss of flotillin 1, but some flotillin 2 is still expressed on the surface of these cells. Hence, the increase in bacterial internalization was not as great as in flotillin 1 knockout MLEC.

Previously, Hoffmann *et al.* (2010) observed increased membrane fluidity in caveolin-1 knockout fibroblasts, which was corroborated by strong increased mobility of GFP-GPI in these knockout cells based on FRAP experiment. The increased GFP-GPI mobility correlated with enhanced *Staphylococcus aureus* uptake in caveolin-1 knockout fibroblasts (Hoffmann *et al.*, 2010). Since membrane fluidity is known to be influenced by cholesterol (Spector and Yorek, 1985), the absence of both flotillin isoforms on the plasma membrane might affect the cholesterol content of the plasma membrane, which made the membrane more easily penetrable by the bacteria.

It is known that some plasma membrane rafts interact with cytoskeletal structures (Maxfield, 2002; Meiri, 2005; Langhorst *et al.*, 2007; Sverdlov *et al.*, 2009; Ludwig *et al.*, 2010), and this interaction has been hijacked by certain pathogens, such as SV40, for invading non-phagocytic mammalian cells (Pelkmans *et al.*, 2001; Pelkmans *et al.*, 2002). Rather than facilitating pathogen entry by raft-cytoskeleton interactions, it was found that tyrosine-phosphorylated caveolin-1 induced cytoskeleton rearrangements at *Neisseria gonorrhoeae* attachment sites and blocked the bacterial internalization into a human cervix carcinoma cell line, ME-180 (Boettcher *et al.*, 2010). We speculate that the enhanced *E. coli* K1 into flotillin 1 knockout MLEC could also link to an impact on cytoskeletal structures, as flotillin 2 has been shown to interact with F-actin via its stomatin, prohibitin, flotillin, and HflK/C (SPFH) domain, and the protein lateral mobility is modulated by the actin cytoskeleton (Langhorst *et al.*, 2007; Langhorst *et al.*, 2008a). This model is also supported by our data where bacterial internalization was inhibited in HBMEC overexpressing-GFP-tagged flotillin 1 and flotillin 2 (Figure 5.8). Therefore, we propose
that flotillin might act as cellular regulatory barrier either by regulating plasma membrane cholesterol or by modulating plasma membrane-cytoskeleton interactions and, hence, protect eukaryotic cells from pathogen invasion.
6. Intracellular *E. coli* K1 interacts with various organellar markers, revealing the potential bacterial transcytosis pathway in HBMEC

6.1 Introduction

After entry into host cells, bacteria successfully escape the direct attack from the host immunity, but the intracellular environment is also unfriendly to the bacteria. Indeed, intracellular bacteria face “intracellular immunity” from the host cell degradation mechanism, and the bacteria have to obtain nutrients and biosynthetic precursors, as well as evade detection by the host’s immune system (Bhavsar *et al.*, 2007; Ham *et al.*, 2011).

In order to survive intracellularly, pathogenic bacteria have evolved diverse strategies to modulate host cellular machinery to make a cosy and protected intracellular niche for survival and replication (Brumell and Scidmore, 2007; Ham *et al.*, 2011). To escape from lysosomal degradation, some bacteria, such as *Listeria monocytogenes* (Tweten, 2005; Henry *et al.*, 2006), *Mycobacterium marinum* (Stamm *et al.*, 2003; Hagedorn and Soldati, 2007), *Burkholderia pseudomallei* (Sitthidet *et al.*, 2011), and *Shigella flexneri* (Sansonetti *et al.*, 1986), trigger rupture of the phagosome and escape into the cytosol. *Listeria* is the best-studied pathogen to employ this strategy, whereby the bacterium secretes listeriolysin O for delaying endosomal maturation and for the formation of transmembrane pores on the vacuolar membrane (Tweten, 2005), thereby escaping from the late endosome-like compartment into the cytosol (Henry *et al.*, 2006). The expression of GTP-locked Rab5 (Rab5Q79L) mutant showed co-localization of the bacteria with Rab5Q79L, but did not block the bacterial escape into the cytosol (Henry *et al.*, 2006). In the cytosol, these bacteria replicate and acquire host actin for cell-cell spreading *in vitro* (Hagedorn and Soldati, 2007).

On the other hand, those invasive bacteria that reside and replicate in membrane-bound vesicles must delay or block endosomal maturation to avoid fusion with lysosomes...
by modifying the endosomal membrane (Gruenberg and van der Goot, 2006). To prevent lysosomal fusion, pathogenic Brucella secretes cyclic β-1.2-glucan (CβG) that is structurally similar to cyclodextrins and disrupts lipid rafts (Arellano-Reynoso et al., 2005). Brucella-containing vacuoles eventually fuse with the endoplasmic reticulum (ER) after transient acquisition of late endocytic markers (Celli et al., 2003; Starr et al., 2008).

For Salmonella enterica serovar Typhimurium, once inside the cells, the bacterium secretes bacterial virulence effectors into the cytosol via T3SS, and the Salmonella-containing vacuoles (SCV) undergo standard endosomal maturation to late endosomes, without interacting with mannose-6-phosphate-containing late endosomes or lysosomes (Méresse et al., 1999; Steele-Mortimer et al., 1999). In macrophage cell line, SpiC, one of the bacterial effectors encodes within SPI-2 pathogenicity island, is thought to prevent fusion of SCV with lysosomes (Uchiya et al., 1999). In fact, it was shown that before SCV mature to late endosomal stage, various regulators of endocytic recycling, including Arf6, Rab4, syntaxin 13, and Rab11, are transiently associated with SCV. These recycling regulators are not only required for recycling cell surface protein from SCV, but they are also essential for acquisition of lysosomal glycoproteins to SCV (Smith et al., 2005). SCV also recruit Rab7-interacting lysosomal protein (RILP) to allow migration to the perinuclear region, where the bacterial vacuoles are associated with Golgi (Salcedo and Holden, 2003; Guignot et al., 2004).

The obligate intracellular bacteria Chlamydia species have evolved an unique strategy for the bacterial intracellular survival and replication. Chlamydiae replicate in a specialized compartment that is not acidified and does not fuse with lysosomes (Heinzen et al., 1996), but the bacterium-containing compartment is enriched with host lipids, such as sphingomyelin and cholesterol, which are acquired via golgi-dependent transportation, and the acquisition is a bacterially driven process (Carabeo et al., 2003). Additionally, screening with a panel of GFP-tagged Rab GTPases constructs revealed that the
Chlamydial compartment is also decorated with various Rab GTPases, such as Rab11, Rab4, Rab1, Rab6, and Rab14 (Rzomp et al., 2003). The functional roles of most of these GTPases on the chlamydial compartment are not clear, but the association of Rab11a with the compartment has been shown to regulate the development of the bacterial elementary bodies (an infectious form of the bacteria) by affecting Golgi fragmentation via the golgin-84 dependent pathway (Lipinski et al., 2009). Recently, it was demonstrated that the bacterium secretes a serine protease, chlamydial protease-like activity factor (CPAF), which regulates the membrane integrity of the bacterial compartment, prevents superinfection by degrading early Chlamydia effectors, Tarp, during infection of a pre-infected cell, as well as interfering with the caspase-1 apoptotic pathway of infected cells (Jorgensen et al., 2011).

Although the majority of invasive bacteria survive intracellularly by avoiding fusion with lysosomes, Coxiella burnetii, the etiologic agent that causes Q fever, evolved a radically opposite strategy. Coxiella-containing vacuole (CCV) fuses with the autophagosome before the vacuole fuses with lysosome and its vacuole is decorated with lysosomal glycoproteins, vacuolar H⁺-ATPase, Rab7, cathepsin D, and other lysosomal hydrolytic enzymes (Beron et al., 2002; Brumell and Scidmore, 2007; Romano et al., 2007; Voth and Heinzen, 2007). The acidic environment is required for stimulating the intracellular bacteria to become metabolically active, and also to activate the bacterial type 4 secretion system in order to evade the host innate immune signalling pathways (Newton and Roy, 2011).

In order to establish a successful infection, pathogenic bacteria not only survive intracellularly, but they need to disseminate systemically. However, the host’s body is structured with various barriers of sheets of epithelial and endothelial cells. Therefore, to disseminate to other anatomical sites, the bacteria need to traverse through these polarized barrier cells.
To date, most knowledge on transcytosis pathways in polarized epithelial cells has been contributed by studies of immunoglobulins, and macromolecules, such as albumin, and insulin (Ghitescu and Bendayan, 1992; Predescu and Palade, 1993; Apodaca, 2001; Minshall et al., 2003; Ducharme et al., 2007; Tzaban et al., 2009). However, it is unknown if similar membrane trafficking pathways exist in flat endothelial cells. Several pathogens are known to traverse polarized epithelial or endothelial cells, but bacterial transcytotic pathways are poorly understood (Nikitas et al., 2011), and the majority of previous studies mainly focused on the requirement for microfilaments, motor proteins, and microtubules (Bomsel, 1997; Bras and Ketley, 1999; Eyngor et al., 2007; Hu et al., 2008; Wang et al., 2008). Recently, uropathogenic *E. coli* (UPEC) traslocation across renal medullary collecting duct (MCD) was demonstrated to depend on lipid rafts via a Toll-like receptor 4 (TLR4)-dependent process. The authors correlated the decrease in bacterial translocation across cholesterol-depleted MCD with the downregulation of proinflammatory mediator expression (Chassin et al., 2008).

A previous study has shown that the majority of *E. coli* K1 acquire the early endosomal marker, transferrin receptor (TfR), after 30 minutes of infection (Kim et al., 2003). The association with TfR gradually decreased at later time points of infection, while the population of bacteria associated with late endosomal marker, LAMP1, increased over time of infection (Kim et al., 2003). *E. coli* K1 has been shown to survive intracellularly in a late endosomal-like compartment devoid of lysosomal enzymes (Kim et al., 2003). The bacterium is able to traverse an HBMEC monolayer *in vitro* without affecting the monolayer’s integrity (Stins et al., 2001), but to date the transcytosis pathway has not been identified.
6.2 Objectives and aims

The aim of this chapter was to identify the intracellular fate of *E. coli* K1 and a potential transcytosis pathway in HBMEC. The specific objectives are as below:

- To screen the interactions of *E. coli* K1 with various cellular compartments by transfecting HBMEC with various GFP-tagged chimaerae.
- To study the identified pathway(s) for *E. coli* K1 traversal of HBMEC cells.

6.3 Results

6.3.1 *E. coli* K1 containing compartment is not arrested at early endosomal stage

A previous study showed that *E. coli* K1 did not replicate intracellularly in HBMEC (Prasadarao et al., 1999), but data from our intracellular survival assay showed that *E. coli* K1 could survive and replicate in HBMEC up to 7 hours p.i. (Figure 3.2). Therefore, we were interested to study the host proteins that were present on the bacterial containing compartments. We first analyzed the *E. coli* K1 compartmentation in HBMEC by infecting HBMEC transiently overexpressing GFP-tagged Rab5 chimaerae with K1-Cherry for 2 hours, followed by 1 hour incubation in media containing gentamicin, and processed for live-cell imaging. Wild type Rab5 (Rab5WT) was found diffuse in the cytoplasm with vesicular structures observed at the perinuclear region and some at the cytoplasm (Figure 6.1A). K1-Cherry were found in areas that exclude cytosolic Rab5WT expression and had no membrane accumulation of Rab5 around them. This observation demonstrated that *E. coli* K1 did not arrest endosomal maturation at early endosomal stage, evidenced by the absence of Rab5 accumulation on the *E. coli* K1 containing vacuoles (ECV) 3 hours p.i. in HBMEC transiently overexpressing GFP-tagged Rab5WT. We also looked for co-localization of GFP-Rab5 with K1-Cherry at an early time point (30 minutes p.i.) in fixed cells; however, no co-localization of Rab5 with K1-Cherry observed (data not shown). One possibility is that the interaction of Rab5 and ECV is rapid and transient, or
Rab5 is not involved in the ECV development. We further investigated the interaction of K1-Cherry with early endosome by infecting HBMEC with *E. coli* K1 for 30 minutes, fixed, and stained for early endosomal antigen 1 (EEA1) (Figure 6.1D), which is an effector of Rab5 (Galperin and Sorkin, 2003). EEA1 staining showed bright punctate structures in the cell cytoplasm and some weak signal of EEA1 around the bacteria was observed, which might suggest that ECV did fuse with early endosomes during its maturation, but once again this interaction might be too rapid and transient to be detectable in our assay.

To overcome the possibility that any interactions of *E. coli* K1 were too short to be detected, we investigated if Rab5 mutant chimaerae might have an effect on the protein localization with ECV. Rab5Q79L and Rab5S34N are GTP- and GDP-locked Rab5 mutants, respectively, which the GTPase activity of these mutants has been well-characterized (Stenmark *et al.*, 1994). The defects in these mutants prevent normal vesicle fusion and maturation, as such if *E. coli* K1 did interact with Rab5-associated vesicles, normal vesicle progress would be stalled, and an accumulation of Rab5 and *E. coli* K1 expected. In HBMEC overexpressed Rab5Q79L, large vesicular structures were observed in the cytoplasm, and K1-Cherry was located in an enlarged vacuole (Figure 6.1B). Whereas in HBMEC overexpressed Rab5S34N, diffuse punctate signal was observed (Figure 6.1C), and K1-Cherry was localized in the cytoplasm without any protein accumulation around them.

As overexpression of GDP-locked Rab5 (Rab5S34N) was shown to inhibit *Coxiella burnetii* entry of CHO cells (Romano *et al.*, 2007), to ensure that *E. coli* K1 internalization into HBMEC overexpressing GFP-tagged Rab5S34N was not affected, HBMEC overexpressing GFP-Rab5 chimaerae were infected with K1-Cherry at MOI of 100 for 2 hours, followed by incubation in media containing gentamicin for 1 or 5 hours, and processed for live-cell imaging. The number of GFP-expressing cells with intracellular
K1-Cherry was quantified. We did not find any evidence of the inhibition of *E. coli* K1 invasion in HBMEC overexpressing GDP-locked Rab5 (Rab5S34N) 3 and 7 hours p.i. (Figure 6.1E) as would be expected if Rab5-associated vesicles were involved in the intracellular survival of *E. coli* K1. Therefore, our results suggested that ECV interact transiently with early endosomal markers, and the interaction was only observed in cells overexpressing Rab5Q79L mutant. On the other hand, *E. coli* K1 invasion of HBMEC was not affected in HBMEC overexpressing Rab5 chimaera.
Figure 6.1. The *E. coli* K1-containing vacuole is not arrested at an early endosomal stage. (A) - (C) Representative confocal micrographs of HBMEC cells transiently expressing GFP-Rab5WT (A), GFP-Rab5Q79L (B), and GFP-Rab5S34N (C) that were infected with mCherry-expressing *E. coli* K1 (K1-Cherry) (red) at MOI of 100 for 2 hours, followed by 1 hour incubation in media containing gentamicin, and were imaged with a confocal microscope as described in Chapter 2. The white arrows indicate intracellular K1-Cherry. (D) Representative confocal micrograph of HBMEC infected with *E. coli* K1 (red) at 30 minutes p.i., fixed and immunostained for EEA1 and visualized by FluoProbes®-488-conjugated secondary antibodies (green). Nuclei were stained with DAPI (blue). A single confocal slice is shown. The white arrows indicate bacterium localising with EEA1, and the white arrow heads indicate bacterium that does not localise with EEA1. Scale bars: 5 μm. (E) To investigate overexpression of GFP-tagged Rab5 on K1-Cherry invasion. HBMEC were transiently transfected with GFP-tagged Rab5 chimaeras, infected with K1-Cherry as described above, followed by 1 or 5 hours incubation in media containing gentamicin, and imaged as in (A) – (C). pN1-EGFP was included as the experimental control. GFP-expressing HBMEC with intracellular *E. coli* K1 were scored by confocal microscopy at 3 and 7 hours p.i. Data are mean ± SEM from two independent experiments.

### 6.3.2 *E. coli* K1 does not utilize endosomal recycling pathway

Rab11 is one of the Rab GTPases that regulates endosomal recycling at the juxtanuclear recycling endosome. To determine whether *E. coli* K1 utilizes the Rab11 recycling pathway, HBMEC were transiently transfected with GFP-tagged Rab11 chimaera, either Rab11a wild type (Rab11aWT) (Figure 6.2A), or GTP-locked Rab11a (Rab11aQ70L) (Figure 6.2B), or GDP-locked Rab11a (Rab11aS25N) (Figure 6.2C), infected with K1-Cherry as described above, followed by incubation in media containing gentamicin for 1 or 18 hours, and imaged. Both GDP- and GTP-locked Rab11a mutants were applied in case ECV association with Rab11a was transient. Bright punctate structures were observed in the cytoplasm and juxtanuclear position of HBMEC overexpressing Rab11aWT; while only bright punctate structures were observed in the cytoplasm of HBMEC overexpressing Rab11aQ70L. In HBMEC overexpressing Rab11aS25N, the GFP signal was dispersed in the cytoplasm, no punctate structure was observed. At 3 and 20 hours p.i., none of the Rab11a chimaera localized specifically with intracellular K1-Cherry. No localization of intracellular K1-Cherry with GFP-Rab11aWT, and GFP-Rab11aQ70L suggests that Rab11a is not involved in regulation of the pathway hijacked by intracellular *E. coli* K1 during the bacterial infection of HBMEC.
**Figure 6.2.** *E. coli* K1-containing vacuoles do not acquire Rab11a during endosomal maturation. (A) - (C) Representative confocal micrographs of HBMEC cells transiently expressing GFP-Rab11aWT (A), GFP-Rab11aQ79L (B), and GFP-Rab11aS25N (C) that were infected with K1-Cherry (red) at MOI of 100 for 2 hours, followed by incubation in media containing gentamicin for 1 or 18 hours, and imaged with a confocal microscope as described in Chapter 2. The white arrows indicate intracellular K1-Cherry. Scale bars: 5 μm.

6.3.3 *E. coli* K1 localizes in a late endosomal/lysosomal compartment

Kim et al. (2003) also showed that the K1 capsule prevented the ECV that acquired characteristics of late endosome (positive for Rab7 and LAMP1) from fusion with lysosomes 2 hours p.i., evidenced by the absence of cathepsin D co-localization with ECV. We further investigated the role of Rab7 on ECV development by infecting HBMEC transiently overexpressing wild type Rab7 (Rab7WT) with K1-Cherry as described for 2 hours, and imaged live with a confocal microscope. Two mutant forms of Rab7 GTP-locked Rab7 (Rab7Q67L), or GDP-locked Rab7 (Rab7T22N), were also used in case any association with Rab7 was transient. Vesicular and punctate structures were observed in the cytoplasm and juxtanuclear position of HBMEC expressing Rab7WT (Figure 6.3A), and K1-Cherry was localized in a membrane bound compartment decorated with Rab7 in the cell cytoplasm. It was observed that all the intracellular K1-Cherry were localized in
Rab7WT compartments (data not shown). In HBMEC overexpressing Rab7Q67L (Figure 6.3B), large vesicular structures were observed and K1-Cherry were localized in the mutant protein decorated compartments. In contrast to HBMEC overexpressing Rab7WT and Rab7Q67L, diffuse punctate signals were seen in the cytoplasm of HBMEC overexpressing Rab7T22N (Figure 6.3C). In these Rab7T22N-expressing HBMEC, clumps of K1-Cherry were found localized in areas devoid of cytoplasmic Rab7T22N.

To ensure that the bacterial invasion was not affected by the overexpression of these Rab7 chimaerae in HBMEC, HBMEC expressing GFP-Rab7 chimaerae were infected with K1-Cherry as described in section 6.3.1 and processed for imaging (Figure 6.3D). The number of K1-Cherry infected GFP-Rab7 chimaerae expressing HBMEC were counted. The preliminary data showed that the number of infected Rab7Q67L-expressing HBMEC was lower than the number of infected HBMEC overexpressing either Rab7WT or Rab7T22N; however, the number of infected Rab7Q67L-expressing HBMEC increased sharply to about 10%, which was higher than that of other infected Rab7 chimaerae-expressing HBMEC. This data is inconclusive and the experiment needs to be repeated.

We further asked if ECV-Rab7 association is transient or stable. To address this question, time-lapse imaging of HBMEC transiently overexpressing GFP-tagged wild type Rab7 with K1-Cherry was performed 2 hours p.i. (Figure 6.3E). K1-Cherry was found to remain in Rab7-enriched compartments for prolonged periods of time (here shown approximately 6 hours). Intriguingly, cross-sectional view of the last time point revealed that the bacteria in the Rab7 compartments were localized at the basolateral membrane surface; while at 2 hours p.i., cross-sectional view revealed that the bacteria were very close to the apical surface of the cell (Figure 6.3F).
Figure 6.3. Localization of intracellular *E. coli* K1 in Rab7 compartments. (A) - (C) Representative confocal micrographs of HBMEC cells transiently expressing GFP-Rab7WT (A), GFP-Rab7Q67L (B), and GFP-Rab7T22N (C) that were then infected with K1-Cherry (red) at MOI of 100 for 2 hours. Transfected, infected HBMEC cells were imaged at 2 hours p.i. with a confocal microscope as described in Chapter 2. The white arrows indicate intracellular K1-Cherry. (D) To investigate overexpression of GFP-tagged Rab7 chimaerae on K1-Cherry invasion, HBMEC overexpressing GFP-tagged Rab7 chimaerae were infected with K1-Cherry at MOI of 100 for 2 hours, followed by incubation in media containing gentamicin for 1 or 5 hours, and imaged as in (A) - (C). pN1-EGFP was included as the experimental control. GFP-expressing HBMEC with intracellular *E. coli* K1 were scored by confocal microscopy at 3 and 7 hours p.i. Data are from one experiment. (E) Representative frames of time-lapse imaging of K1-Cherry infected GFP-Rab7WT-expressing HBMEC 2 hours p.i. The elapsed time (hours: minutes) is indicated at the lower left hand corner of each panel. The white arrows indicate intracellular K1-Cherry co-localized with Rab7. Scale bars: 5 μm. The white dotted lines indicate the position of cross-sectional view in (F). (F) The micrographs show the cross-sectional view of infected cells at 2:00 and 5:45 timepoints. Scale bar: 5 μm.
In agreement with Kim et al. (2003), our live-cell imaging results also revealed localization of the bacteria in LAMP1-positive compartments at 3 and 20 hours p.i. (Figure 6.4A and B). We also observed that the intracellular K1-Cherry in LAMP1 compartments appeared intact and brightly fluorescent 20 hours p.i. (Figure 6.4B). Intriguingly, several the bacteria were located in these LAMP1 compartments 20 hours p.i., while individual bacillus was observed in LAMP1 compartments 3 hours p.i. This observation might suggest intracellular bacterial replication in LAMP1 compartment at later time point, which was supported by increased number of recovered intracellular E. coli K1 7 hours p.i. (Figure 3.5)

Figure 6.4. E. coli K1-containing vacuoles acquire the lysosomal membrane glycoprotein, LAMP1. (A) and (B) Representative confocal micrographs of HBMEC cells transiently expressing LAMP1-GFP that were infected with K1-Cherry (red) at MOI of 100, followed by 1 or 18 hours incubation in media containing gentamicin, imaged at 3 (A), and 20 hours p.i. (B) with a confocal microscope. The white arrows indicate intracellular K1-Cherry. Scale bars: 5 μm.

We further investigated the interaction of Rab9 chimaerae with E. coli K1. GFP-Rab9 chimaera, either wild type Rab9 (Rab9WT), or GTP-locked Rab9 (Rab9Q66L), or
GDP-locked Rab9 (Rab9S21N), was transfected into HBMEC, and the transfected cells were infected with K1-Cherry as described (Figure 6.5). The cells were imaged with a confocal microscope 3 and 20 hours p.i. Both GDP- and GTP-locked mutants were also used in case any association with Rab9 was transient. In HBMEC overexpressing Rab9WT, vesicular structures were concentrated at the juxtanuclear position as well as in the cytoplasm (Figure 6.5A), whereas in HBMEC overexpressing Rab9Q66L, fluorescent signal was diffuse in the cytoplasm with some large vacuolar structures observed (Figure 6.5B). In HBMEC overexpressing Rab9S21N, fluorescent signal was diffuse in cytoplasm, but some enlarged vacuolar structures were observed, with some of these structures appeared at juxtanuclear position (Figure 6.5C). We found that intracellular K1-Cherry were localized in Rab9WT and Rab9Q66L-enriched compartments at 3 and 20 hours p.i. in HBMEC transiently overexpressing respective GFP-tagged Rab9 chimaerae (Figure 6.5A and B respectively), but not with Rab9S21N (Figure 6.5C). Interestingly, similar with the observation with bacteria localized in LAMPI compartment, several K1-Cherry was found in an individual Rab9WT compartment at 20 hours p.i., which might suggest bacterial replication.

From these results we conclude that ECV development might involve early endosomal markers, in which the interaction might be rapid and transient. ECV was not directed to recycling endosomes which involved Rab11, but the vacuole matured to late endosome-like compartment with Rab7, Rab9, and LAMPI detected on the vacuole.
Figure 6.5. *E. coli* K1-containing vacuoles acquire Rab9 during endosomal maturation. (A) - (C) Representative confocal micrographs of HBMEC cells transiently expressing GFP-Rab9WT (A), GFP-Rab9Q66L (B), and GFP-Rab9S21N (C) that were infected with K1-Cherry (red) at MOI of 100 for 2 hours, followed by incubation in media containing gentamicin for 1 or 18 hours, and imaged at 3, and 20 hours p.i. with a confocal microscope as described in Chapter 2. The white arrows indicate intracellular K1-Cherry. Scale bars: 5 μm.

6.4 Discussion

The *E. coli* K1 invasion pathway of HBMEC has been studied extensively for the past two decades (Prasadarao et al., 1993; Huang et al., 1995; Prasadarao et al., 1996a; Prasadarao et al., 1996b; Reddy et al., 2000a; Reddy et al., 2000b; Khan et al., 2003; Prasadarao et al., 2003; Maruvada et al., 2008; Liu et al., 2010); however, the trafficking pathways involved in bacterial intracellular survival are poorly understood. The classical degradative endocytic pathway to the lysosome is prevented by the presence of the K1 capsule, which is essential for the bacterial intracellular survival by preventing lysosomal fusion with the ECV (Hoffman et al., 1999; Kim et al., 2003).

In this study, we showed that *E. coli* K1 does not arrest endosomal maturation at early endosomal stage and that overexpression of various GFP-tagged Rab5 chimaerae does not affect the bacterial invasion of HBMEC. Intracellular *E. coli* K1 does not localize to the Rab11a recycling compartment, but the bacteria were found to localize in late...
endosomal compartments, which are positive for Rab7, Rab9, and LAMP1. Time-lapse imaging revealed that the intracellular bacteria were associated with Rab7 2 hours p.i. and remain associated throughout the imaging time point.

Numerous pathogenic bacteria, such as Campylobacter jejuni, Coxiella burnetti, and Salmonella enterica serovar Typhimurium, localize in late endosomal-like compartments, but the bacteria-containing vacuoles do not fuse with lysosomes (Méresse et al., 1999; Beron et al., 2002; Watson and Galán, 2008). Although cathepsin D has previously been shown to be absent from ECV (Kim et al., 2003), we were unable to repeat this finding and to demonstrate the absence of cathepsin D from the Rab7/LAMP1/Rab9-associated ECV at later time points of infection, due to reagent compatibility (data not shown). However, intracellular mCherry-expressing E. coli K1 remained intact and brightly fluorescent 20 hours p.i. (Figure 6.4B, 6.5A – C), which might indicate the bacteria were viable (Hagedorn and Soldati, 2007). In vitro study has also demonstrated that fluorescent protein fails to retain in bactericidal compound-killed Mycobacterium tuberculosis, while the protein is stably maintained in bacteriostatic compound-treated bacteria (Carroll et al., 2010). Recently, the application of a fluorescence dilution bacterial reporter system found that a large population of Salmonella Typhimurium does not replicate upon entry into macrophages, but enters a dormant stage, in which the bacteria are unculturable on laboratory medium, but some are able to respond to an extracellular chemical inducer, and equally there is no evidence of their being killed intracellularly (Helaine et al., 2010). The Salmonella findings open up a critical question of the viability and dormancy of intracellular E. coli K1 in various identified cellular compartments in this study, especially at late time points of infection. One of the results from this study showed that E. coli K1 could survive intracellularly 7 hours p.i. (Figure 3.2), but a very low number of viable bacteria were recovered 20 hours p.i. (data not shown), which was possibly due to bacterial dormancy, or bacterial killing, or the majority
of the bacteria having egressed from the infected cells at late time point and by gentamicin in the media.

In the previous chapter, ≥30% intracellular bacteria were found to be associated with flotillin 1, while comparison to more traditional endocytic markers in this chapter additionally found that the intracellular bacteria were localized in late endosomal compartments that are positive for Rab7 (Figure 6.3A and E), Rab9 (Figure 6.5A), and LAMP1 (Figure 6.4A and B). With these observations, we questioned the correlation between flotillin 1 and these late endosomal markers on ECV. Flotillin 1 is considered as a late endosomal marker and co-localizes with LAMP1 (Derrmine et al., 2001), but live intracellular Mycobacterium marinum (M. marinum) are found to replicate in flotillin 1 compartments that are devoid of LAMP1 in human peripheral monocytes (Hagedorn and Soldati, 2007). Therefore, it is important to characterize if those flotillin 1-associated ECV exist as an independent compartment or associate with LAMP1, and which of these compartments provides a safe niche for the bacteria. These experiments are ongoing. Furthermore, to elucidate the specific endosomal stage at which flotillin 1 is recruited to ECV, the presence of flotillin 1 on ECV in cells overexpressing the dominant negative Rab proteins should be studied.

During this study, it was also hoped to identify a potential transcytosis pathway that E. coli K1 hijack for crossing HBMEC, however, our data is so far insufficient to address this question. HBMEC are rich with caveolae, which is rationale to speculate the caveolae as the major endocytic and also transcytotic pathway for the cells (Minshall et al., 2003). However, this study showed that only approximately 4% of E. coli K1 was associated with caveolin-1 at the early infection time point, and this further reduced to less than 1% at later time points of infection. Therefore, it is very unlikely that E. coli K1 traverses to the basolateral surface via a caveolae-mediated pathway. Based on the current data, we speculated that E. coli K1 could potentially harness late/lysosomal, flotillin 1-dependent
pathway for traversing HBMEC (Figure 6.6, model 2), which has been proposed for exosome egress (Lakkaraju and Rodriguez-Boulan, 2008; Thery et al., 2009). Further, the cellular markers present on ECV are typically identified on exosomes (Chertova et al., 2006; Simons and Raposo, 2009; Chen et al., 2011). Alternatively, ECV associated with flotillin 1 (independent of LE markers) might be the route by which the bacterial population traverses and exocytoses from HBMEC (model 1) (Figure 6.6). The presence of flotillin 1 on ECV could have a role in the recruitment of actin and other motor proteins onto the endosome (Dermine et al., 2001), as flotillin binds F-actin via its stomatin, prohibitin, flotillin, and HflK/C (SPFH) domain (Langhorst et al., 2007). In addition to actin and motor proteins recruitment to ECV, the actin binding ability of flotillin could be required for vesicles docking onto the target membrane, and for exocytosis (Nightingale et al., 2011). Overall, other potential transcytotic markers, such as Rab25 and Rab1a, will be investigated in future experiments. Once a potential transcytotic marker is identified on ECV, the endosomal stage which the marker is recruited can be identified with the application of the available Rab dominant negative mutant constructs.
Figure 6.6. Potential *E. coli* K1 transcytotic pathway in HBMEC. *E. coli* K1 enters HBMEC via an actin-dependent process with unknown endocytic pathway. After internalization, the ECV transiently acquires different markers of the classical endocytic pathway. We speculated that *E. coli* K1 might traverse HBMEC via either flotillin 1 (model 1) or late endosomes (LE) with flotillin 1 (model 2) pathway. EE, early endosome; LE, late endosome.
7. General discussion and conclusion

*E. coli* is one of the bacteria predominantly isolated from neonatal bacterial meningitis cases (Osrin *et al.*, 2004; May *et al.*, 2005; Wu *et al.*, 2009; Talbert *et al.*, 2010; Gaschignard *et al.*, 2011), with high mortality rate observed in some countries (May *et al.*, 2005; Wu *et al.*, 2009). The majority of the *E. coli* strains isolated from neonatal meningitis cases possess K1 capsule (Robbins *et al.*, 1974; Sarff *et al.*, 1975), which is structurally homologous to, and immuno-cross-reactive with, the α-2,8-linked polysialic acid (polySia) glycan highly expressed on the developing brain and kidney of newborn mammals (Finne *et al.*, 1983a; Roth *et al.*, 1987; Harvey *et al.*, 2001).

For this study, an immortalized HBMEC line, which was generated by transfecting primary HBMEC isolated from patients aged 4–7-year-old with SV40 large T antigen (Stins *et al.*, 2001), representing the BBB, was applied to study *E. coli* K1 pathogenesis *in vitro*. Early in the study, we tried *E. coli* K1 infection on mouse brain endothelioma cell lines (bEnd5), an *in vitro* BBB model commonly applied for T cell migration studies (Steiner *et al.*, 2011), but this resulted in no intracellular bacteria recovered from infected bEnd5 lysates (data not shown). With the HBMEC model, *E. coli* K1 invasion efficiency was approximately 0.1% of the initial bacterial inoculums (Figure 3.2), which agreed with the published *E. coli* K1 invasion efficiency by Prasadarao *et al.* (1996). In fact, this level of bacterial invasion efficiency is low, and the bacterial invasion is FCS-dependent, which is a major experimental hindrance for studies that involve cholesterol depletion. The low bacterial invasion efficiency leads to some degree of experimental difficulty, especially in the studies of the bacterial entry pathways. Despite various optimization steps that were performed, such as determination of the optimum MOI, bacteria-HBMEC incubation time, total volume of experimental media used during infection, and centrifugation of the bacteria to the cell surface for synchronizing the infection, only minor improvements were
achieved. The observed low bacterial invasion could be an outcome of age-related differences in the HBMEC used, as indirectly evidenced by an earlier study that demonstrated the age-related susceptibility of an infant rat model in developing *Haemophilus influenza* type b-associated meningitis (Moxon *et al.*, 1977), and a similar observation was made with *E. coli* K1 infection *in vivo* (Zelmer, A., personal communication)(Mushtaq *et al.*, 2005). The difference in neonate- and adult-derived mouse brain microvascular endothelial cells (BMEC) was also demonstrated by different level of protease secretion following glutamate induction, though both endothelial cells exhibited similar phenotypes (Legros *et al.*, 2009). Therefore, we hypothesize that *E. coli* K1 would invade neonate-derived HBMEC more efficiently than HBMEC derived from patients aged 4 – 7 years of age such as the HBMEC used in this study. Owing to the involvement of complicated ethical issues in obtaining and utilizing human tissues either from live or dead patients lately (Carmichael, 2011; Marchant, 2011), neonate BMEC could be obtained from other species such as bovine and porcine, as no difference was reported in *E. coli* K1 invasion efficiency of BMEC derived from other species (Prasadarao *et al.*, 1996a).

An additional difficulty in this invasion model is that HBMEC, like other endothelial cells, are also resistance to plasmid DNA transfection, either by lipid- or polymer-based transfectants, or by electroporation (Kovala *et al.*, 2000; Segura *et al.*, 2001; Kang *et al.*, 2009; Anliker *et al.*, 2010). In this study, various transfection reagents were tried, but the transfection efficiency usually ranged between 10 - 30% (Figure 4.2). Transfection efficiency of about 50% was achieved by altering the amount of pN1-EGFP plasmid DNA and jetPRIME™ used per transfection reaction (Figure 4.3) however. with other recombinant constructs this ranged between 20 – 45%. The current transfection efficiency could probably be further improved with gene-specific lentiviral vectors, which were pseudotyped with mutated truncated measles viral haemagglutinin, and displayed a
single-chain variable fragment (scFv) specific to CD105, an endothelial cell specific marker (Anliker et al., 2010). However, it has been documented that the application of lentiviral vector for transducing monocyte-derived dendritic cells resulted in decrease CD1d presentation (Garg et al., 2011). Therefore, there is a possibility that transduced cells might respond differently to infection.

Confocal microscopy imaging was one of the experimental techniques heavily applied in this study. To enable visualization of the bacteria under a confocal microscope, fluorescent *E. coli* K1 were required, either by surface-labelling with fluorescent dye, or by immunostaining with specific antibody, or by cytoplasmic expression of fluorescent protein. There is a limited choice of *E. coli* K1-specific antibodies, and bacterial surface-labelling with FITC dye led to unexpected imaging channel cross-talk, as well as a decrease in bacterial invasion of HBMEC. Therefore, in Chapter 3, we described the making of fluorescent bacteria that retain bacterial virulence by transforming the bacteria with various bacterial expression vectors expressing fluorescent protein. All the transformed bacteria fluoresced under a confocal microscope, but the majority of them demonstrated impairment in bacterial invasion of HBMEC, except *E. coli* K1 transformed with pFPV25.1, which has been widely applied in *Salmonella* (Cheminay et al., 2005; Radtke et al., 2007; Gerlach et al., 2008), and pathogenic *E. coli* studies (Radtke et al., 2007; Marchès et al., 2008). Surprisingly, the invasiveness of transformed *E. coli* K1 bearing pFPV-mCherry construct, in which the *gfpmut3a* gene of pFPV25.1 was replaced with mammalian codon-optimised mCherry cDNA, was closely similar to the untransformed bacteria (Figure 3.19). Even so, our results show that the presence of plasmid DNA and expression of fluorescent proteins could affect bacterial virulence, and the possibility that the fluorescent protein gene or the plasmid DNA copy number might also affect bacterial virulence gene expression must be kept in consideration when interpreting results (Clark et al., 2009; Valdivia, R., personal communication). The
mCherry-expressing *E. coli* K1 not only served as a useful tool for this study, retaining virulence in the assays here, but also might be a valuable tool for future preliminary *in vivo* live animal imaging, in which the application of the longer excitation wavelength needed for mCherry also enables better deep tissue penetration and is less prone to tissue autofluorescence.

*E. coli* K1 entry has been proposed to be receptor-dependent and via zipper mechanism (Prasadara et al., 1999), which was further supported by the absence of T3SS in the RS218 clinical strain or the E44 strain used in all studies by Kim's group for the past two decades (Yao et al., 2006). The molecular events that lead to actin polymerization at the bacterial adhesion site have been very well-studied (Prasadara et al., 1999; Reddy et al., 2000a; Reddy et al., 2000b; Prasadara, 2002; Khan et al., 2003; Maruvada et al., 2008). The endocytic pathway manipulated by the bacteria for HBMEC entry was reported to be clathrin-independent (Prasadara et al., 1999), and caveolae-dependent (Sukumaran et al., 2002). Based on the current knowledge of caveolae-mediated entry, dynamin is required for caveolar fission *in vitro* and *in vivo* (Oh et al., 1998; Pelkmans et al., 2002; Yao et al., 2005), and dynamin might also have a role in actin cytoskeleton recruitment (Lee and De Camilli, 2002). However, previous studies have not investigated the role of dynamin during *E. coli* K1 internalization. Given the proposed entry site of caveolae, we hypothesized that overexpression of GDP-locked dynamin would inhibit the bacterial uptake into HBMEC. To our surprise, we found no inhibitory effect on the *E. coli* K1 entry in HBMEC overexpressing GDP-locked dynamin 1 or 2 (Figure 5.1A and B). Although these constructs have been widely used in previous studies (Cao et al., 1998; Watson and Galán, 2008; Rahn et al., 2011) the uptake of a known specific ligand, such as transferrin (clathrin-mediated endocytosis), into HBMEC overexpressing GDP-locked dynamin need to be assessed to ensure this observation was not due to non-functioning GDP-locked dynamin mutants. Dynamin inhibitors, such as dynasore (Macia et al., 2006), could be
applied for blocking dynamin isoforms in cells and studied their effects on *E. coli* K1 invasion of HBMEC. Since dynasore is a broad spectrum inhibitor, the application of this inhibitor for studying *E. coli* K1 invasion of HBMEC would rule out the possibility that other endogenous dynamin isoforms and splice variants take over roles in cells overexpressing a GDP-locked dynamin isoform.

To confirm the findings by Sukumaran *et al.* (2002), we revisited the role of caveolin-1 during *E. coli* K1 infection of HBMEC, and found that only about 4% of the bacteria were localized in compartments weakly stained for caveolin-1 at 30 minutes p.i., i.e., at a timepoint where we would expect to see bacteria undergoing invasion, and that population of bacteria further reduced to approximately 1% at later timepoints indicating no obvious association with caveolin-1 during uptake or with the intracellular ECV (Figure 5.2A and B). Furthermore, the application of caveolin-1 mutants had no effect on *E. coli* K1 internalization. Importantly, *E. coli* K1 invasion of caveolin-1 knockout MLEC was not inhibited. However, this observation is insufficient to completely rule out the bacterial invasion via non-caveolae-mediated endocytic pathway, as Siddiqui *et al.* (2011) demonstrated the absence of caveolin-1 led to destabilization of adherens junctions, activation of endothelial nitric oxide synthase (eNOS) in endothelial cells, which impaired GTPase activating protein (GAP) p190RhoGAP-A by nitrating tyrosine residue 1105, and led to RhoA activation (Siddiqui *et al.*, 2011). RhoA was shown to be required for *E. coli* K1 invasion of HBMEC (Khan *et al.*, 2002), therefore, the inhibitory effect of bacterial invasion in caveolin-1 knockout MLEC might be cancelled by RhoA. To further elucidate the role of caveolin-1 during *E. coli* K1 invasion, caveolin-1 Y14A mutant, which is non-phosphorylatable and its expression in cells inhibited caveolae-mediated endocytosis (del Pozo *et al.*, 2005; Hu *et al.*, 2006), could be transfected into caveolin-1 knockout MLEC. and infect with the bacteria. It is hypothesized that the bacterial invasion of caveolin-1
Y14A expressing caveolin-1 knockout MLEC would be inhibited if caveolae-mediated endocytosis is exploited by *E. coli* K1 for cell invasion.

At this stage of the study, our findings are contradictory to the Sukumaran et al. (2002) findings, where they reported inhibition in *E. coli* K1 invasion in HBMEC overexpressing dominant negative caveolin-1 (caveolin-1 with two amino acids mutation at the scaffolding domain for phosphor-PKCa interaction), and strong staining of the invading and intracellular bacteria with caveolin-1. As discussed above, further experiments are needed to further substantiate our findings; however, there are also several ambiguities in their findings. First, in the panel of micrographs that was indicated as bacteria-specific staining, very strong non-specific background was observed, and appears to show staining of the entire cell periphery. This strongly suggests that the caveolin-1 signal on the bacteria that they observed could be signal resulting from channel crosstalk with bacterial staining. Secondly, the authors stated that the presented micrographs were from 30 minutes p.i. (MOI unknown, but it was stated that $1 \times 10^7$ *E. coli* K1 were used for infection), and a high number of bacteria was observed in the panels that they claimed to be intracellular. We have never seen such a high number of intracellular bacteria at 30 minutes p.i., a maximum single bacterium per cell is more frequently observed in our hands at the similar time point. We only see clumps of intracellular bacteria at much later timepoints (see below). Thirdly, filipin was applied to deplete cholesterol from HBMEC plasma membrane in their study, and a dose-dependent reduction in recovered intracellular *E. coli* K1 was reported. We tried to repeat this experiment, but 30 minutes incubation with 1 or 4 µg/ml of filipin in FCS-free media resulted in massive cell detachment from the well, probably due to the sensitivity of HBMEC to some chemical inhibitors (Kim, K.S., personal communication).

Since no link was found between *E. coli* K1 invasion and dynamin- and caveolin-1-mediated endocytic uptake, which endocytic pathway that the bacteria manipulated for
invading HBMEC was explored. We chose to focus on the flotillin-mediated pathway, which forms distinct lipid raft microdomains on the plasma membrane and the pathway is dynamin-independent. Both flotillin 1 and flotillin 2 co-localize and co-assemble into microdomains distinct from caveolae microdomains (Frick et al., 2007). It has been shown that knockout of flotillin 1 causes concomitant reduction in flotillin 2 expression (Ludwig et al., 2010), and this study showed that in flotillin 1 knockout MLEC, flotillin 2 distribution was disrupted as evidenced by the absence of flotillin 2 punctate staining. We observed weak flotillin 1 signal accumulated beneath approximately 5% of extracellular bacterial population and 5% of the intracellular bacterial population were associated with flotillin 1 at 30 minutes p.i. (Figure 5.5A and B). The intracellular bacteria that associated with flotillin 1 increased to about 30% at 2 hour p.i. However, in flotillin 1 knockout MLEC, instead of blocking the bacterial internalization as would be expected if flotillin 1 were required for uptake, both *E. coli* K1 and non-pathogenic *E. coli* K-12 intracellular density was significantly enhanced at 150 minutes p.i. (Figure 5.6A). The enhanced bacterial density was caused by enhanced bacterial invasion into flotillin 1 knockout MLEC, and not because of non-functioning lysosomal pathway (Figure 5.6B). These results show that *E. coli* K1 does not require the flotillin 1-mediated pathway for invading HBMEC, and the bacterial invasion should also be flotillin 2 independent since both flotillin 1 and flotillin 2 co-assemble to form microdomains and their expression are interdependent.

The data in this study suggest that *E. coli* K1 endocytosis is dynamin-independent, and the precise endocytic pathway utilized is still unclear. There are three major dynamin-independent endocytic pathways, namely the Arf-6-regulated pathway, the Cdc42-regulated pathway, and macropinocytosis (Mayor and Pagano, 2007; Kumari and Mayor, 2008; Mercer and Helenius, 2009; Howes et al., 2010; Kumari et al., 2010). Arf6 and Cdc42 dominant active and dominant negative constructs have been sourced and will be
tested in HBMEC to study their effects on *E. coli* K1 invasion. To date, there is no singular cellular target to study macropinocytosis, a number of different cellular markers together are needed to imply the activity of the macropinocytic pathway. It is interesting to note that current experimental criteria to implicate macropinocytosis specifically exclude a dependency on the Rho GTPase RhoA (Mercer and Helenius, 2009). Activation of RhoA during *E. coli* K1 invasion of HBMEC has been published previously (Khan *et al.*, 2002; Khan *et al.*, 2003). Furthermore, overexpression of dominant negative RhoA T19N mutant reduces *E. coli* invasion; while overexpression of the dominant positive form (RhoA G14V) increased invasion (Khan *et al.*, 2002). Rho A has also been proposed as a clathrin-independent but dynamin-dependent endocytic pathway for interleukin 2 (IL2) receptor (Lamaze *et al.*, 2001), which is contradictory with the dynamin-independent findings in this study. Since RhoA is one of the key signalling molecules involved in the regulation and dynamics of the actin cytoskeleton, the activation of RhoA seen in *E. coli* K1 infection of HBMEC may be a bystander effect due to the actin rearrangement triggered during infection. Indeed, a dependency of *E. coli* K1 invasion on RhoA has not been shown.

Enhanced intracellular bacterial density was observed in flotillin 1 knockout MLEC, which we believed was a result of enhanced bacterial uptake, evidenced by higher number of infected flotillin 1 knockout cells than the number of infected wild type cells after 30 minutes infection (Figure 5.6B). We hypothesize that the cholesterol content of the plasma membrane might have been depleted in the flotillin 1 knockout cells; hence, the membrane fluidity might be enhanced, and could lead to facilitated penetration by the bacteria. Alternatively, flotillin 1 might have a role in regulating cellular cytoskeletal structures (Langhorst *et al.*, 2008a), that might form a cellular barrier at the bacterial attachment sites, or the absent of flotillin 1 disrupt the cytoskeletal signalling pathway as observed in caveolin-1 knockout endothelial cells (Siddiqui *et al.*, 2011). This cellular barrier hypothesis might explain one of our preliminary data, wherein HBMEC
overexpressing either GFP-tagged flotillin 1 or flotillin 2 showed a lower rate of infection than HBMEC overexpressing other GFP-tagged recombinant constructs at 3 hours p.i. (Figure 5.8).

The general role of flotillin 1 in vivo is not known. Flotillin 1 knockout mice do survive, although a defect in neutrophil and monocyte recruitment in response to chemoattractant has been observed (Ludwig et al., 2010). In addition to have a key role in host defense system, the data from this study as well as the results from others suggest that flotillin 1 might actively maintain integral barrier against external environment at the cellular and systemic level (Li et al., 2008b). Therefore, future study should look into the susceptibility of flotillin 1 knockout animals to infections (particularly enteric and neurotropic pathogens), as well as autoimmune and allergy diseases, such as Crohn’s disease and Asthma.

Another part of this project was to study the E. coli K1 intracellular survival by screening the organellar markers on the bacterial compartments. We found that the post invasion ECV had the characteristics of late endosomes. Intriguingly, at 20 hours p.i., we observed clumps of intact and fluorescent intracellular bacteria localized in these late endosomal compartments, which we believe to result from intracellular bacterial replication, also evidenced from our survival assay data (Figure 3.2). There was no evidence of E. coli K1 clumping in culture based on live microscopy examination (data not shown). Again, this finding is contradictory with previous publication which claimed that E. coli K1 did not replicate intracellularly based on transmission electron micrographs, as TEM micrograph only shows small regions of interest, there is potential bias in figure selection (Prasadarao et al., 1999; Sukumaran and Prasadarao, 2002). This study also found 35% of intracellular bacteria were localized in flotillin 1 compartments at late time points of infection, to understand the distribution of flotillin 1 and late endosomal markers (Rab7 and LAMP1) on the ECV is a high priority. More detailed analysis of the host cell
factors present on the ECV could be achieved by performing a proteome analysis with mass spectrometry of purified ECV as described in (Urwyler et al., 2009).

A previous study showed that the inhibition of lysosomal fusion is achieved by the presence of the K1 capsule on the bacteria (Kim et al., 2003), but the lysosomal inhibition strategy that involves the K1 capsule is unknown. Therefore, other bacterial factors may involve in inhibiting ECV fusion with lysosomes. The bacteria might secrete bacterial virulence factors into the cytosol of infected HBMEC via the bacterial GSP system, which might be the alternative secretion system in the absence of T3SS (Yao et al., 2006), and interfere with host factors (such as GTPases, vATPases, components of homotypic fusion and vacuole protein sorting (HOPS) complex, N-ethylmaleimide sensitive factor (NSF), etc.) required for late endosome-lysosome fusion. Further, the bacteria were shown to contain icmF and icmH in one of the bacterial pathogenicity islands (Xie et al., 2006). The *Legionella* homologues of these proteins encode a type IV secretion system required for *Legionella* species' intracellular survival by avoiding trafficking to lysosomes in human macrophages (VanRheenen et al., 2004). To study if the icmF, icmH, and other potential bacterial secretory proteins are translocated into the cytosol of infected HBMEC, split-GFP technology could be applied, whereby the bacterial proteins are tagged with the 13-amino-acid 11th strand of the GFP β-barrel (GFP11), and the non-fluorescent complementary fragment of the first ten strands of GFP (GFP1-10) is expressed in HBMEC (Van Engelenburg and Palmer, 2010). Fluorescent signal is resulted if the GFP11-tagged bacterial protein is translocated into the cytosol and complemented with GFP1-10 in HBMEC. After the identification of secreted bacterial proteins, gene specific knockout bacterial mutants could be constructed for screening of bacterial factors that are required for inhibiting lysosomal fusion to ECV.

The ability of *E. coli* K1 to traverse HBMEC cultured on a Transwell® insert without disrupting the monolayer's integrity was described a decade ago (Stins et al.,
2001), but the cellular pathway has never been studied. Sukumaran et al. (2002) proposed the bacteria utilized caveolin-1 for HBMEC traversal based on their observation of co-localization between the intracellular bacteria and caveolin-1. However, there are some ambiguities with their findings as discussed previously, and we only found about 1% of bacteria weakly associated with caveolin-1 at later time points of infection. Therefore, we believe that caveolin-1 is unlikely to form the basis of the bacterial transcytosis pathway, unless the 1% population of *E. coli* K1 weakly associated with caveolin-1 is sufficient for the bacteria to egress from HBMEC at the basolateral membrane. Based on our findings in Chapter 6, we speculated that *E. coli* K1 might egress from HBMEC via late/lysosomal [N.B. Lysosomal membrane proteins, such as LAMP1, are actively interacting with plasma membrane, and are involved in membrane repairing (Divangahi et al., 2009).], or a flotillin 1-dependent pathway. The role of flotillin 1 on the intracellular vacuoles might have a role in recruiting motor proteins for intracellular transportation, for docking of the vacuoles to the target membrane surface, or for releasing the cargo. The low transfection efficiency of HBMEC is the major experimental obstacle for studying bacterial transcytosis pathway. An alternative experimental approach would be the application of tannic acid, which only fixes the membrane surface in contact, at the basolateral compartment of Transwell® filter insert to arrest the bacteria at the basolateral membrane surface (Polishchuk et al., 2004), and the infected HBMEC on the Transwell® filter insert is fixed and immunostained for organellar markers.

Although HBMEC has been widely applied for studying the *E. coli* K1 pathogenesis (Huang et al., 1995; Reddy et al., 2000a; Stins et al., 2001; Kim et al., 2003; Khan et al., 2007), the precise bacterial entry site *in vivo* remains controversial. The current model is based on findings by Kim et al. (1992), where, in a newborn (5-day-old) rat model, the authors found the bacteria present in the perivascular area of the subarachnoid space, but not in the choroid plexus, where the BCSFB is found (see section
1.4). The group further showed that the bacteria invaded isolated brain microvascular endothelium more efficiently than endothelial cells isolated from other anatomical sites in vitro (Prasadarao et al., 1996a). More than a decade later, Zelmer et al. (2008) found evidence to support the alternative BCSFB entry site in their newborn (2-day-old) rat model. The observed discrepancy by both groups is complex and multifactorial, where one of the factors could be the age of the animals used, or the different route of bacterial administration in these studies. The precise bacterial entry site in the animal could be elucidated by the application of multiphoton microscopy for deep tissue live animal imaging (Melican and Richter-Dahlfors, 2009; Melican et al., 2011), in conjunction with innovative thin-skull microsurgery procedure (Marker et al., 2010). The main challenge for this experimental approach is to keep the fragile, hypothermia-prone newborn animal alive and physiologically stable during the surgery procedure as well as during imaging. Live animal deep tissue imaging is also important to confirm previous in vitro findings that have arisen from the HBMEC model, as differences in Listeria monocytogenes infection cycle both in vitro and in vivo has been demonstrated recently (Nikitas et al., 2011). Further, an infection is more heterogeneous than merely E. coli K1-HBMEC interactions in culture medium, and would also depend upon a complex interaction between the pathogen and capillary endothelium as well as various tissues, including microglial cells, astrocytes, immune cells, inflammatory factors, etc. It has also been shown that approximately 40% of plasma membrane proteins failed to express on the plasma membrane of purified endothelial cells maintained in vitro (Durr et al., 2004), which might affect the results obtained in vitro and in vivo. All the future in vivo imaging works can be achieved by the mCherry-expressing E. coli K1 constructed in this study.
Conclusion

The purpose of this study was to investigate the interactions of *E. coli* K1 with HBMEC in order to elucidate the pathway by which the bacteria cross the BBB into the CNS. To this end, an mCherry-expressing *E. coli* K1 that retains the bacterial invasiveness of HBMEC was constructed. Our data suggest *E. coli* K1 invade HBMEC via a caveolae-, dynamin-, and flotillin-independent pathway. Once inside the cells, *E. coli* K1 were found to localize in late endosome-like compartment. We speculate that the bacteria may cross BBB via late/lysosomal pathway. Interestingly, when MLEC knockout cells were used to model bacterial infection, the absence of flotillin 1 was found to enhance bacterial invasion compared to wild type. We further suggest flotillin has an essential role in maintaining the cell wall as a barrier to the external environment. In addition, flotillin appears to be an important component in maintaining mucosal membrane integrity, having significant impacts in diseases such as Crohn's disease and asthma (Li et al., 2008b). Thus, the results in this study not only have an impact in the understanding of how *E. coli* K1 traverse the BBB, but are also of interest in relation to the role of flotillin in maintaining cellular barrier integrity.
8. References


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