Pathogen entry mechanisms and endocytic responses to plasma membrane damage

Lars Nygård Skalman
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ABSTRACT

Endocytosis is a fundamental cellular process by which cells transport material from the outside to the inside of the cell through the formation of membrane invaginations that bud off from the plasma membrane. This process is important for nutrient uptake, regulating cell surface receptors and the overall plasma membrane composition. Cells have several different types of endocytic pathways where clathrin-mediated endocytosis is the most studied. Importantly, pathogens and secreted virulence factors bind to cell surface receptors and hijack the endocytic pathways in order to enter host cells. Depending on their size and molecular composition, pathogens and virulence factors are thought to make use of distinct endocytic pathways into the cell. This thesis focuses on early host cell interactions with virus, bacterial membrane vesicles and a pore-forming toxin, with a particular emphasis on endocytic mechanisms and plasma membrane repair.

During entry of pathogens, it is thought that interactions with specific cell surface molecules drive the recruitment of endocytic proteins to the plasma membrane. Viruses possess a very defined molecular composition and architecture, which facilitate specificity to these interactions. We found that Adenovirus 37, a human ocular pathogen, binds to αVβ1 and α3β1 integrins on human corneal epithelial cells and that this interaction is important for infection. In contrast to viruses, membrane vesicles shed from Helicobacter pylori are heterogeneous in size and molecular composition. These vesicles harbour various adhesins and toxins that may facilitate binding to the cell surface and recruitment of different endocytic pathways. We developed a quantitative internalization assay and showed that the H. pylori vesicles were internalized mainly via clathrin-mediated endocytosis but were also capable of exploiting other endocytic pathways.

Damage to the plasma membrane disrupts cellular homeostasis and can lead to cell death if not repaired immediately. Although endocytic mechanisms have been shown to be important for plasma membrane repair, little is known about their specific role. Listeriolysin O (LLO) is a bacterial toxin that can form pores in the plasma membrane and disrupt cellular homeostasis. We developed a reporter system for real-time imaging of the endocytic response to LLO pore formation. We found that two clathrin-independent endocytic pathways were important for plasma membrane repair. However, they were not directly involved in removing LLO pores from the plasma membrane. Our data suggests that these endocytic systems might rather influence membrane repair by their ability to regulate the plasma membrane composition, shape and tension.
In conclusion, this thesis describes how pathogens and their virulence factors make use of specific mechanisms to enter host cells as well as revealing new insights on the role of the endocytic pathways in plasma membrane repair.
LIST OF PAPERS

Paper I

Uptake of *Helicobacter pylori* vesicles is facilitated by clathrin-dependent and clathrin-independent endocytic pathways.
Olofsson A*, Nygård Skalman L*, Obi I, Lundmark R, Arnqvist A.

Paper II

Human Adenovirus type 37 uses αVβ1 and α3β1 integrins for infection of human corneal cells.

Paper III

Endocytic response to the pore-forming toxin listeriolysin O
Nygård Skalman L, Holst MR, Lundmark RL. *Manuscript*

Papers not included in the thesis

Six1 regulates proliferation of Pax7-positive muscle progenitors in zebrafish
Nord H, Nygård Skalman L, von Hofsten J.

A novel role of *Listeria monocytogenes* membrane vesicles in inhibition of autophagy and cell death

* These authors contributed equally to this work

Paper I and paper II are both open access publications
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INTRODUCTION

The plasma membrane is a barrier that surrounds and separates the cell interior from the outside world. It serves as a protective physical barrier but at the same time allows for specific transport of molecules in and out of the cell by passive and active processes. Pathogenic microorganisms have developed effective mechanisms to manipulate these processes in order to promote their own survival. Viruses and bacteria constitute a major threat and are a direct cause of millions of deaths world wide each year [1]. A detailed understanding of how different pathogens interact with host cells is of great importance in order to combat infectious diseases. In addition, pathogens are valuable tools that can be used to provide new insights regarding basic cell biology, information that can be used to develop new treatments against many different types of diseases.

The plasma membrane

The plasma membrane is composed of a lipid bilayer and membrane proteins, where proteins make up about half of the mass of the plasma membrane [2]. The lipid bilayer of the plasma membrane has been estimated to comprise several hundred different lipid species [3]. The most abundant lipids are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin and cholesterol [4]. The lipid species are not equally distributed between the inner and outer leaflet, for example, sphingomyelin, phosphatidylcholine and glycolipids are located in the outer leaflet while phosphatidylserine and phosphatidylethanolamine are enriched in the inner leaflet [5, 6]. The asymmetry is maintained by specific enzymes and provides specificity for many important cellular processes [2]. The abundance of certain lipids plays a significant role since the individual properties of the specific lipids affect the packing and fluidity of the membrane [7]. For example, cholesterol can constitute up to 40 % of the membrane lipid content and thus significantly influences the fluidity of the plasma membrane [2]. Cholesterol generally makes the membrane more rigid but at high concentrations it can also help to prevent phospholipid crystallisation [8].

Previously it was thought that proteins and lipids are randomly distributed in a fluid plasma membrane [9]. However, today it is believed to be heterogeneous in its lipid and protein distribution, consisting of ordered and disordered domains [10]. Figure 1a and b shows an illustration of the composition and organisation of the plasma membrane. Lipid rafts are membrane microdomains enriched in saturated phospholipids,
sphingolipids and cholesterol that pack together tightly. These raft domains serve to organise the localisation of membrane proteins, for example, glycosylphosphatidylinositol anchored proteins (GPI-AP) and other specific proteins localise to and cluster in these domains [11]. Lipid rafts have been suggested to be important in cell signalling and membrane trafficking [12, 13]. Another important factor for the generation of localised membrane domains is the connection between the plasma membrane and the actin cytoskeleton. These interactions can have large effects on the lateral movement of lipids and proteins in the membrane [3, 11].

Figure 1. The cell surface. a) Illustration of the organisation and composition of the plasma membrane. b) 3D view of the plasma membrane showing different raft-like domains (a and b are reprinted with permission from [11]). c) Scanning electron micrograph of two human corneal epithelial cells. Notice the blebs on the lower cell.

Numerous plasma membrane proteins are glycosylated on the external side of the membrane, and together with a large amount of glycolipids this leads to a cell surface that is covered with carbohydrates [2]. The plasma membrane contains many different cell surface receptors that facilitate communication with the outside world, and regulate a wide range of cellular functions. An important group of plasma membrane proteins are cell adhesion proteins, which can also function as receptors. One example is the family of integrins that serve as adhesion molecules and bi-directional receptors. Integrins are transmembrane heterodimers that anchors the cell to the extracellular matrix (ECM) on the extracellular part and to the
cytoskeleton on the intracellular part. The heterodimers consist of non-covalently bound α and β subunits, where 18 α and 8 β subunits have been identified so far. These subunits can make 24 unique heterodimers and are important for many cellular functions as well as in infection [14].

**The cytoskeleton**

The cellular cytoskeleton is made of three different types of polymers, actin filaments, intermediate filaments and microtubules, which are all important for the structural organisation and mechanical properties of the cell. Cortical actin, where actin filaments are lined up beneath and cross-linked to the plasma membrane, is crucial for regulating the shape and composition of the membrane [3]. By polymerising and depolymerising, the actin filaments can rapidly alter the shape of the plasma membrane, which is important in cellular migration, polarisation, division and endocytosis [15]. These are highly coordinated events where Rho GTPases such as cdc42, RhoA and Rac1 play a significant role as major regulators [16].

**Membrane tension**

Cells need to regulate the membrane tension in order to control shape and maintain structural integrity. This is critical for example in cellular polarisation and migration [17]. Membrane tension is mainly determined by membrane-cytoskeletal interactions and the force actin generates on the plasma membrane in addition to the osmotic and hydrostatic pressures. Membrane tension can be modulated by the addition or removal of membrane, resulting in a decrease or increase in tension, respectively [18]. Therefore, the relationship between membrane area and cell volume is an important factor contributing to membrane tension [19]. Membrane blebbing is a good example of the tension made up by membrane-cytoskeleton interactions where the plasma membrane forms a balloon-like protrusion in order to counteract tension due to the local loss of membrane-cytoskeletal interactions (Fig. 1c) [3, 18].
Endocytosis

Maintaining cellular homeostasis is vital for cell survival and is dependent on the ability of cells to exchange material between the intra- and extracellular space, to regulate the plasma membrane composition and cell surface receptors [20]. This is provided by two functional opposing mechanisms: exocytosis and endocytosis. During exocytosis intracellular vesicles fuse with the plasma membrane in order to transport lipids and proteins to be incorporated into the plasma membrane or expelled to the extracellular space. Endocytosis can be seen as the cells way of eating and drinking and involves both pinocytosis and phagocytosis. During endocytosis small plasma membrane portions are internalised along with its constituents as well as extracellular material such as nutrients, ligands and fluid. The endocytic process can be divided into three basic steps, (1) initiation of a plasma membrane invagination, (2) formation of a bud and (3) scission of the bud to produce a free vesicle [20] (Fig. 2). By internalising receptors, adhesion proteins, nutrients and membrane lipids, endocytosis affects many important cellular processes such as migration, polarisation, mitosis and neuronal signalling. Malfunction in endocytic processes have been linked to several severe diseases such as muscular dystrophies, neurodegenerative diseases and cancer [21, 22]. In addition, endocytic mechanisms are commonly hijacked by pathogens and various virulence factors to gain access to the inside of the cell [23].

There are several different types of endocytic pathways, which are generally defined based on their morphology and by the proteins that regulate them (Fig. 3). They are commonly divided into clathrin-dependent and clathrin-independent endocytic pathways. The different endocytic pathways have preferences for different cargo and play a part in distinct cellular functions.
The proteins involved in the different pathways include specialised proteins capable of bending the membrane into highly curved structures. The activation of different endocytic pathways depends on different specific cues generated at the plasma membrane. These cues can be receptor signalling or clustering, a change in lipid composition, membrane tension or curvature [24, 25]. For example, ligand binding to cell surface receptors can induce clustering and/or signalling that recruits endocytic proteins on the cytoplasmic side of the plasma membrane. This in turn leads to internalisation of the ligand along with the receptor [20, 24]. This process can take a few seconds up to minutes depending on the endocytic pathway [26, 27].

**Figure 3.** Schematic illustration of different proposed endocytic pathways.

**Clathrin-mediated endocytosis**

Clathrin-mediated endocytosis (CME) is the most well studied endocytic pathway and more than 50 proteins have been identified to participate in this mechanism [28, 29]. It is called clathrin-mediated endocytosis since the invaginations formed during CME are surrounded by a protein cage built up by one clathrin light chain and three clathrin heavy chains [29, 30]. Another name commonly used to describe the CME is receptor-mediated endocytosis due to the specific sorting of receptors into these vesicles. CME was first discovered in mosquito oocytes in 1964 by the observation of bristle-coated pits in the plasma membrane as well as coated vesicles just beneath the membrane. Further inside the cell uncoated vesicles were observed, leading to a hypothesis that coated pits are formed in the membrane, released as vesicles and later uncoated [29, 31]. The size of clathrin-coated vesicles is normally around 100 nm and restricted to around 200 nm [32]. In addition to the coat components, the specific uptake of the iron transporting protein...
transferrin and the low-density lipoprotein is often used to define CME. Importantly, certain receptors like the transferrin receptor is constitutively endocytosed while CME of other receptors can be stimulated by ligand binding [32].

The recruitment of clathrin to the membrane is performed by adaptor proteins since clathrin does not bind directly to the membrane [28]. Ligands that are to be internalised via CME bind to their specific cell surface receptor. These receptors have a specific sorting sequence in their cytoplasmic tail that recruits specific adaptor proteins. One important adaptor protein in particular is the heterotetrameric adaptor protein 2 (AP2). AP2 binds to specific sequences on the cytoplasmic side of the receptor and to phosphatidylinositol(4,5)-bisphosphate. This leads to recruitment of clathrin as well as accessory proteins and the subsequent build up of a clathrin-coated pit [28, 29]. Receptors with a sorting sequence accumulate in clathrin-coated pits while proteins without a sorting sequence are excluded [29, 33].

Adaptor proteins and clathrin are not sufficient to drive CME, hence a range of other proteins termed accessory proteins also participate in the process. Proteins with ability to bend membranes such as ENTH and BAR domain containing proteins aid in generating the membrane curvature needed to form the invagination [28]. The BAR domain containing Sorting nexin 9 (SNX9) is recruited to clathrin-coated pits in the late stage of vesicle
formation and may be involved in vesicle release [34-36]. The large GTPase dynamin is essential for scission of the mature vesicle from the membrane. Following vesicle release, the clathrin cage is disassembled in a process mediated by heat shock cognate protein 70 and auxilin. This in turn exposes mediators of vesicular fusion such as the SNAREs, leading to the ability of the vesicles to fuse with endosomes [28, 29]. The basic process of CME is illustrated in figure 4.

CME is an essential cellular mechanism and specific mutations in clathrin, AP2 and dynamin result in embryonic lethality [37-39]. Therefore there is a lack of literature linking these proteins to diseases. A defective CME caused by mutations in other CME related proteins has however been linked to several different types of diseases such as cancer, myopathies and neuropathies, neurodegenerative and psychiatric diseases [40].

Caveolae

Caveolae, "little cave" in Latin, were first observed by electron microscopy already in the 1950s [41-43]. Caveolae are flask shaped invaginations in the plasma membrane with a diameter of around 50-100 nm and are highly enriched in smooth muscle cells, endothelial cells, adipocytes and fibroblasts [44]. Caveolae can also be organised into rosette-like structures with several caveolae next to each other [45]. Similar to lipid rafts, caveolae are high in cholesterol and sphingolipids [45]. Several proteins such as caveolin and the family of cavins are needed for caveolae biogenesis [45]. EH-domain containing 2 (EHD2) is a dynamin like ATPase that localises to the neck of caveolae. Depletion of EHD2 generates a more dynamic caveolae, suggesting that EHD2 stabilises caveolae at the plasma membrane [46, 47]. Similarly to CME, scission of caveolae from the plasma membrane has been proposed to be performed by dynamin [48, 49].

Caveolae are important cellular structures associated with a number of human diseases, such as cancer, respiratory diseases, diabetic cardiomyopathy and muscular dystrophies [50]. The cellular function of caveolae is still debated but caveolae are suggested to involve endocytosis, mechanoprotection, membrane repair, regulation of plasma membrane lipid composition and to serve as signalling platforms [51, 52].

Early studies of endocytosis suggested that caveolae were responsible for the uptake of various cargos, such as the insulin receptor, GPI-anchored proteins and certain toxins and viruses [53, 54]. Later this has been questioned due to that caveolae are generally quite static structures at the cell surface and some of these cargo molecules were found to actually use other routes of uptake.
A role for caveolae in mechanoprotection was first postulated from the observation that caveolae flatten upon mechanical stress [55]. The presence of caveolae has later been shown to be important in several cell types upon mechanical stress on the plasma membrane [51]. This argues for a role of caveolae in mechanoprotection and controlling the lipid homeostasis. Evidence supporting a role of caveolae as major route for uptake or not is still not completely clear. However, caveolae do bud off directly from the plasma membrane without the involvement of other endocytic pathways [56], and therefore it cannot be excluded that caveolae function as a mechanism for specific cargo internalisation.

**CLIC/GEEC**

CLIC/GEEC endocytosis, which stands for clathrin-independent carrier and GPI-AP enriched endosomal compartment, was first described in 2002 when GPI-anchored proteins were found to be taken up in uncoated tubular invaginations (CLICs) with a diameter of around 40 nm. Scission from the plasma membrane of these invaginations resulted in the formation of GPI-AP enriched endosomes (GEECs) [57]. The proposed cargos taken up by CLICs include CD44 and dysferlin [58, 59]. CLIC/GEEC endocytosis is dependent on the small GTPases cdc42 and Arf1, the GTPase Regulator Associated with Focal adhesion kinase-1 (GRAF1), as well as sphingolipids and cholesterol [57, 60-62]. Scission of CLICs from the plasma membrane is dynamin-independent. However, it is not known exactly which proteins that mediate scission [57]. Another protein recently associated with CLIC formation is galectin3. Galectin3 was shown to induce CLIC formation by binding to glycosylated proteins and to interact with glycosphingolipids. Depletion of galectin3 inhibited the uptake of CD44 and the β1 integrin [63], suggesting that its involvement in CLIC/GEEC endocytosis.

The CLIC/GEEC pathway is a high capacity endocytic pathway, responsible for the uptake of a large part of the fluid phase into cells [57]. It has been estimated to be able to turn over the plasma membrane within 12 minutes in fibroblasts [58]. CLIC/GEEC is highly active in protrusions and at the leading edge of a cell, where there is a high turnover of membrane, and it has been shown to play a significant role in cellular migration and polarisation [58, 64, 65].

Exocytosis and endocytosis are important for regulating membrane tension and it is known that exocytosis is promoted by an increase in membrane tension, while endocytosis is induced by a drop in membrane tension [18]. Recently, it was shown that GRAF1 mediates endocytosis of the exocytic protein Rab8 at cellular protrusions. Rab8 accumulated at the plasma
membrane upon an increase in membrane tension while GRAF1 activity was excluded from the membrane. GRAF1 was later recruited to the plasma membrane upon a decrease in membrane tension and internalised Rab8 [65]. These results in combination with the high capacity to internalise membrane suggests that CLIC/GEEC endocytosis has an important role in restoring membrane tension.

Several studies have linked GRAF1 to severe diseases like mental retardation, myeloid leukaemia and gastric cancer [66-71] and it is possible that this is connected to the function of GRAF1 in endocytosis.

**Arf6**

An endocytic pathway dependent on the Arf6 protein has been described and just like the CLIC/GEEC pathway it is cholesterol dependent but dynamin independent [24, 72]. The type of cargo taken up by this pathway is similar to the CLIC/GEEC pathway and since these two pathways resemble each other it is questioned if they are the one and the same pathway or if it is dependent on cell type which of the pathways that is used [24].

**FEME**

A new endocytic pathway that the authors named Fast Endophilin-Mediated Endocytosis (FEME) was recently described in an extensive study [27]. The name is derived from its dependence of endophilin, a protein otherwise involved in CME. This pathway rapidly internalises several ligand-activated receptors such as the β₁-adrenergic receptor and the interleukin-2 receptor. Receptor activation by ligands recruits endophilin to the leading edge of cells after formation of phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P₃). This leads to internalisation of the receptor in small vesicles or tubules. Apart from endophilin and PI(3,4,5)P₃, FEME was shown to be dependent on dynamin, Rac, the PAK1 kinase and actin polymerisation [27]. An important point regarding FEME is that it requires activation by receptor ligands, whereas for example CME can be constitutively active in the uptake of certain receptors [73].

**Flotillins**

Endocytosis mediated by flotillin-1 and -2 is another proposed clathrin and dynamin independent endocytic pathway. However, experimental results are inconclusive whether flotillins represent a specific endocytic pathway or if they act as adaptors for certain cargo to mediate endocytosis through other pathways [24].
Lipid-mediated endocytosis

Certain molecules are thought to be able to cluster on the surface of the plasma membrane and induce inward curvature and tubulation by binding to glycosphingolipid receptors in a certain pattern. These molecules have been shown to tubulate artificial giant unilamellar vesicles by themselves and it is thought that they do the same on the cell surface [25]. Scission of these invaginations is thought to be dependent on dynamin [74].

Macropinocytosis

Macropinocytosis is a growth factor induced type of endocytic mechanism [75]. It involves ruffling and blebbing of the plasma membrane that fold back over and internalise as a large vesicle of around 0.5-10 µm [75]. This is significantly larger than the other endocytic pathways described above, suggesting that macropinocytosis can internalise cargo of a substantial size. This type of endocytosis is not specific for any cargo and will internalise a significant volume of fluid phase, membrane and extracellular molecules. Macropinocytosis is triggered by signalling from receptor tyrosine kinases that change the dynamics of the actin cytoskeleton in order to rearrange the structure of the plasma membrane. The small GTPase Rac1 is very important for this process [75]. Scission of the macropinosome has been linked to the CtBP1/BARS protein and under certain circumstances to dynamin [75, 76].

Phagocytosis

Phagocytosis is a mechanism for uptake of large cargo such as apoptotic bodies and bacteria, and in comparison to macropinocytosis it is a specific uptake mechanism for a defined cargo [77]. Phagocytosis is generally restricted to occur in professional phagocytic cells such as macrophages, which are very efficient at taking up this type of cargo [78]. However, other cell types can also be induced to internalise cargo via phagocytosis [79]. During phagocytosis, ruffles, filopodia or a growing phagocytic cup surround the cargo and eventually seal around the cargo. Following scission the cargo is transported to the inside of the cell in the form of a phagosome [78]. The initial phase of this process is dependent on actin polymerisation while scission is believed to happen via dynamin [78, 80].

Summary - endocytic pathways

Taken together, endocytosis is today described to occur via different pathways. The definition of a pathway is not always clear; some are defined based on their shape, others on cargo preference or the molecular regulators
used [81]. While the CME pathway is a very well characterised event, many of the other pathways still require further clarification. Some of the proposed pathways seem to overlap regarding molecular regulators as well as in morphology and preference for a certain cargo. It is possible that they represent the one and the same mechanism but differ in some specific regulatory protein that is determined by cell type or under the circumstances that something is to be internalised, such as under different types of stress. Also, some of the proposed pathways seem to have other important cellular functions than to serve as major internalisation routes of certain cargo. More research is needed to be able to define if the above mentioned endocytic systems are indeed distinct pathways or if they should rather be viewed as variation of a theme.

**Actin in endocytosis**

The cytoskeleton plays an important role in regulating endocytic mechanisms. Involvement of actin in CME has been a long standing question due to varying results using different actin inhibitors, cell types and the fact that it is essential for CME in yeast [82]. Disruption of actin does not seem to inhibit the initiation step of CME, however, actin is recruited to clathrin-coated pits that stay a long time at the plasma membrane. This suggests that actin is aiding during budding and scission where there is a need for extra force, such as under high membrane tension [32]. Caveolae have been observed to be distributed along actin stress fibers in cells [83]. CLIC/GEEC endocytosis is inhibited by perturbation of actin polymerisation, as is FEME [27, 84]. As mentioned previously, actin also plays a major role in deforming the membrane in both macropinocytosis and phagocytosis. Taken together, actin appears as one of the major regulators of endocytosis acting on most endocytic pathways.

**Intracellular trafficking of endocytic cargo**

Depending on the type of cargo internalised by endocytosis it will be sorted to different compartments in the cell via the endosomal network. The endosomal network is a collection of different membranous compartments (Fig. 5). As an example, vesicles derived from CME fuse with sorting/early endosomes after uncoating, and in these endosomes the first sorting of cargo takes place. Certain receptors and other proteins are recycled back to the plasma membrane either directly via a fast recycling mechanism or via the recycling endosomes [85]. Cargo that is not recycled is transported further to, or matured into, the late endosomes and lysosomes for degradation, or to the trans-golgi network via the retrograde pathway [85]. Specific Rab proteins are important for regulating the transport of cargo between the
different endosomal compartments. Rab5 is critical for early endosome formation while Rab4 and Rab11 are important for recycling. Rab7 regulates the transport between early endosomes and late endosomes/lysosomes. Rab9 is responsible for the transport of cargo from late endosomes to the trans-golgi network [86].

![Diagram of intracellular trafficking after endocytosis]

**Figure 5.** Intracellular trafficking after endocytosis

There is a decreasing pH gradient in the endosomal network. In early endosomes the pH is around 6.2 while lysosomal pH is around 4.5-5.0. The V-ATPase proton pump mediates the acidification of endosomes, and this is very important for the function of endosomes by enabling receptor ligand dissociation and activation of hydrolytic enzymes for degradation of cargo [85, 87]. Vesicles formed from the plasma membrane in CLIC/GEEC and ARF6 endocytosis can also fuse with early endosomes, but can also be recycled back directly to the plasma membrane from GEECs or Arf6 vesicles before fusing with the early endosomes [24]. The phagosome formed by phagocytosis undergoes a similar cycle as for the other endocytic pathways.
where Rab5 and Rab7 are important for the maturation of the phagosome into a phagolysosome in order to degrade the cargo [88].

**Studying endocytosis**

In the last two decades the field of endocytosis has gained increasing attention due to the refinement of mechanisms and proteins that drive internalisation and the development of new techniques to visualise endocytosis in real time. Endocytic events are fast processes and usually occur within a timeframe that is far below the time-resolution of conventional confocal microscopy. The use of fluorescently tagged cellular proteins as well tagged cargo in combination with powerful live-cell microscopy techniques like total internal reflection fluorescence (TIRF) microscopy and spinning disc confocal microscopy has led to many new insights regarding the dynamics of endocytic uptake [26].

TIRF microscopy allows for high resolution imaging in the z-plane of the basal membrane of cells and is ideal for studying the dynamics of endocytic events. In TIRF microscopy a laser is directed at an angle against the glass coverslip that results in light being reflected back from the coverslip. This generates an evanescent field on the other side of the coverslip due to a lower refractive index in the cell culture medium compared to the glass. The evanescent field decreases exponentially with the distance from the glass surface of the coverslip leading to the excitation of fluorophores which are within ~100 nm from the coverslip surface [89].

Spinning disc confocal microscopy is principally a confocal microscope with many pinholes and microlenses arranged on a disc that spins rapidly. This allows for much faster imaging of each confocal plane and lower phototoxicity compared to conventional laser scanning confocal microscopy [26]. Additionally, new techniques for imaging the dynamics of cellular processes are constantly being developed, such as the recent advances on the use off lattice-light sheet to study the dynamics of CME [90].

Much of the literature on which endocytic pathway that is used to take up a specific cargo is based on the use of pharmacological inhibitors, expression of mutant proteins and depletion of proteins siRNA or other means [91-93]. These tools all have their advantages and disadvantages. For example, crosstalk between endocytic pathways has been observed and depletion of one pathway may upregulate other pathways [94].

Pharmacological inhibition of endocytic processes provides a quick and easy way to characterise specific endocytic processes and many different
inhibitors have been used over the years, some more specific than others. An advantage of pharmacological inhibitors is that they generally cause an acute effect on cellular mechanisms, meaning that cells do not have much time to compensate for this effect. The downside is that they often come with adverse side effects [91, 93]. Broad range inhibitors such as actin inhibitors affect a wide range of cellular mechanisms and it is therefore difficult to tell if the phenotype observed is due to direct or indirect effect on a specific endocytic mechanism [91, 93]. Inhibitors affecting the plasma membrane composition of cholesterol are common tools for assessing the role of lipid rafts in endocytosis. These inhibitors affect the composition and structural arrangement of the membrane and with the more recent knowledge on the requirement of cholesterol for different clathrin independent endocytic pathways the results of using cholesterol inhibitors should be interpreted carefully and only in the combination with appropriate controls [91].

Overexpression of dominant-negative or -active mutants of specific proteins to disrupt or enhance certain endocytic processes can be a very efficient and specific method. However, it may also cause severe side effects due to overexpression. Also, differences in expression levels in between cells may also affect the interpretation of the results [91].

Depletion of specific proteins involved in different endocytic mechanisms by siRNA is commonly used and is generally much more specific than pharmacological inhibitors. The downside is that it usually takes days to perform and can lead to upregulation of compensatory mechanisms. Also, the transfection efficiency in cells may vary and there is a potential for off-target effects [91]. Genome editing techniques to specifically edit levels of a protein is a very powerful tool but also very time consuming and can as with siRNA treatment lead to upregulation of other pathways.

Although inhibition of endocytic mechanisms can be unspecific or have side effects, they are still very useful tools if used in combination with proper controls. It is advised to use a combination of pharmacological inhibitors and genetic tools for disrupting endocytic mechanisms when studying uptake of certain cargo [91-93]. By combining temporal and spatial data from advanced microscopy with the use of inhibitors and genetic tools, it is possible to characterise the involvement of cellular factors in the uptake of specific cargo in great detail.
Pathogens

Viruses

Viruses are intracellular parasites that require access to the host cell replication and translation machineries in order to replicate and form new virions. They pack their genome consisting of DNA or RNA into a protein capsid for protection. The capsid can either be naked (non-enveloped) or surrounded by a host cell derived lipid membrane (enveloped). Viruses targeting mammalian cells can range in size from ~20-2000 nm and are often spherical in shape [95]. In order to replicate it is essential for viruses to deliver their genome to the cytoplasm or nucleus, and to do so they need to overcome the physical barrier imposed by host cell membranes [95]. To gain access to the inside of the cell, viruses can either fuse with the plasma membrane or take advantage of the cells endocytic processes. Most viruses are however not able to fuse with the plasma membrane and are therefore dependent on endocytosis for internalisation [96].

Non-enveloped viruses bind to cells via so called fibers or depressions in the viral capsid, while enveloped viruses bind via spike-like glycoproteins that cover the viral membrane envelope [96, 97]. Many viruses attach to the cell surface by binding to specific attachment factors, which are often glycosylated lipids or proteins. This binding serves to concentrate the virus on the cell surface and promotes subsequent interactions with other receptors that can lead to internalisation of the virus [96]. Individual binding events to attachment factors and receptors are very specific but quite weak, however, multivalent interactions with attachment factors and receptors provide avidity and leads to receptor clustering [97]. This in turn may serve to initiate cell signalling and/or internalisation of the virus [96]. Following binding, the process of internalisation can be relatively fast. Single particle tracking of influenza virus showed that the time from binding to the cell surface, to uptake via CME and uncoating of the clathrin-coated vesicle takes approximately 3 minutes. The virus spent about half this time on the cell surface before a clathrin-coated pit started to form [98].

Adenoviruses

Adenovirus is a group of double-stranded DNA viruses consisting of more than 50 different types [99]. They are non-enveloped viruses with a capsid size of around 90-100 nm. The different types of adenovirus are known to cause a variety of diseases but most are related to the airways, eyes and intestines [99]. The viral capsid is made up of three different proteins,
namely, the penton base, the hexon and the fiber [99, 100]. Adenovirus bind via the fiber to cellular attachment factors, while the penton base interacts with other receptors and mediate internalisation into host cells [100]. Most human adenoviruses have a RGD (Arginine-Glycine-Aspartic acid) motif in the penton base that mediates binding to integrins [99]. Different adenoviruses are known to use different endocytic pathways for internalisation into host cells and this is believed to be determined by the interaction with specific attachment factors and integrins [75].

Adenovirus 37 (Ad37), that belong to the type D of human adenoviruses, is one of the major causative agents of epidemic keratoconjunctivitis [101, 102], a highly contagious ocular disease that involves both the conjunctiva as well as the cornea. It is estimated to affect millions of people each year and currently there are no effective treatments available [102].

**Bacteria, membrane vesicles and toxins**

In contrast to viruses, bacteria have their own replication machineries. Although some species are obligate intracellular organisms, most pathogenic bacteria reside, and are able to grow and divide extracellularly. These bacteria are usually around 1-5 µm in length and can secrete various factors in order to promote their own survival [95]. Many pathogenic bacteria secrete virulence factors such as toxins that target host cells by modulating cellular functions or inducing cell death. Several of these bacterial toxins require endocytosis to reach their intracellular target [103, 104].

Bacteria can also produce membrane vesicles (MVs) that are shed from the surface of the bacteria [105]. These MVs vary in size between 20-300 nm [106] and contain bacterial membrane lipids, proteins, toxins and other bacterial factors such as lipopolysaccharide, peptidoglycan and DNA [107]. Bacterial MVs may serve many different important functions in promoting infection such as immune evasion, modulation of immune cells and as transport vehicles of virulence factors [108, 109]. Bacterial MVs from several different species have been shown to be able to enter host cells via endocytosis or to fuse with the plasma membrane, leading to the delivery of bacterial factors to the interior of the cell [107]. In comparison to viruses which in principal exact copies of each other, bacterial MVs from the same population can vary in size as well as in composition of lipids and proteins [110]. These MVs have a surface covered in different bacterial proteins and lipids, which may mediate interactions with many different cell surface receptors. The association of certain bacterial proteins to MVs has been shown to affect the uptake pathway used by MVs of different species [107, 111, 112].
**Helicobacter pylori membrane vesicles**

*Helicobacter pylori* is a Gram-negative bacterium that chronically infects about half of the world's population. An infection with *H. pylori* results in gastritis and about 15% of infected individuals develop peptic ulcer disease [113]. It is estimated that 1-3% also develop gastric cancer, making it responsible for several hundred thousands deaths per year [114]. *H. pylori* reside in the mucus layer of the stomach where it produces and sheds MVs from its outer membrane. These MVs vary in size from 20-300 nm and contain different toxins and outer membrane proteins, lipids and LPS [110, 115]. For example, the blood group binding adhesin (BabA) and sialic acid binding adhesin (SabA), as well as the vacuolating cytotoxin A (VacA) and the cytotoxin associated gene A (CagA) toxins are all associated with these MVs. Importantly, *H. pylori* MVs can deliver both active CagA and VacA to host cells [111, 116], demonstrating that these MVs can serve as transport vehicles of virulence factors.

**The pore-forming toxin Listerialysin O**

The intracellular pathogen *Listeria monocytogenes* is a Gram-positive bacterium that is found in soil and food. In humans, it can cause a wide range of symptoms, some of them fatal. Immunocompromised and elderly individuals are particularly susceptible as well as pregnant women where an infection can lead to death of the foetus [117]. Listerialysin O (LLO) is a pore-forming toxin (PFT) secreted by *L. monocytogenes*. LLO belongs to the group of cholesterol-dependent cytolysins (CDC) that also includes toxins like streptolysin O (SLO) from *Streptococcus pyogenes* and, perfringolysin O from *Clostridium perfringens*. CDCs bind to the cell surface via cholesterol or a combination of cholesterol and CD59 [118]. CDCs are secreted as monomers, and upon binding to host cell membranes they oligomerise and insert into the membrane and form large pores, usually around 25-40 nm [118]. As is evident from their name, CDCs can function to lyse host cells but they may also have more subtle functions such as inducing cell signalling and allowing the bacteria to gain access to cellular content without killing the cell [118, 119]. LLO is traditionally used by *L. monocytogenes* to escape from intracellular membrane compartments after internalisation [120]. However, it also aids in internalisation of the bacterium into host cells by perforating the plasma membrane to induce changes in plasma membrane dynamics as well as activating signalling cascades [120, 121]. Figure 6 shows an illustration of the type of virus, MVs and toxin studied in this thesis.
Figure 6. Schematic illustration of Ad37, H. pylori MVs and LLO toxin in relation to the plasma membrane.

Endocytosis of viruses, toxins and bacterial membrane vesicles

A great amount of research has been dedicated to identify receptors and endocytic mechanisms used by different pathogens and their virulence factors [22, 95]. Binding to specific protein receptors and lipids, cell type and the size of the cargo is thought to influence which endocytic pathway that will be used for uptake. An important question to ask is if pathogens, toxins and bacterial MVs are actively modulating cellular processes to recruit endocytic mechanisms in order for their own internalisation, or if it is just a consequence of their binding to cell surface molecules that are going to be internalised anyway. Probably, both these events occur. Below are some examples of different pathogens, toxins and bacterial MVs suggested to be internalised by different pathways as well as some examples of how this may be mediated.

CME has been shown to be an important uptake pathway for many viruses, bacterial toxins and MVs. A few examples are dengue virus, hepatitis C virus, adenovirus 2 and 5 [75], MVs from EHEC, H. pylori and Brucella abortus [111, 122, 123] as well as the anthrax toxin [124, 125]. Clathrin can also play a role in uptake of cargo significantly larger than what can normally be internalised via CME. L. monocytogenes enter host cells by a zippering mechanism that involves the interaction of invasin proteins on the surface of the bacteria that bind to host cell receptors. This initiates signalling events that lead to actin recruitment to the plasma membrane and uptake of the bacteria. The clathrin coat plays an important role in this process by forming a platform that recruits the cytoskeleton [126].
The VacA toxin is a PFT that can make small pores in membranes upon oligomerisation and is thought to use sphingomyelin as its main cell surface receptor [127, 128]. Following binding the toxin is taken up via CLIC/GEEC endocytosis [129], however, oligomerisation does not seem to be required for VacA internalisation in cells [130]. Adeno-associated virus 2, a special type of virus that requires co-infection of a helper virus in order to replicate, has also been shown to be internalised via CLIC/GEEC endocytosis, since uptake of the virus was dependent on GRAF1, Arf1 and cdc42 [131].

Ad37 has been shown to be dependent on caveolin for uptake in corneal cells [132]. The SV40 virus binds to the ganglioside GM1 and can be taken up via caveolae-mediated endocytosis [95], but also by CME and lipid-mediated endocytosis [24, 133]. Caveolae-mediated endocytosis has been suggested to be used by several different viruses, bacterial toxins and MVs, however many of these studies are solely based on the dependency of lipid rafts or co-localisation with caveolae [107].

Lipid-mediated endocytosis has been proposed to occur when certain virus and toxins bind to glycolipids, like GM1, in a pentameric pattern. This induces inward membrane curvature and subsequent internalisation in tubular structures. Lipid-mediated endocytosis has also been described for cholera and shiga toxin, and what is common for all these molecules is that their uptake is sensitive to disruption of lipid rafts. [10, 25, 134]. However, the cholera and shiga toxin, as well as the SV40 virus are examples of molecules known to be internalised by multiple pathways, such as CME, CLIC/GEEC and caveolae [95, 135, 136]. It is possible that the induced inward curvature serves to recruit endocytic proteins that can sense this type of curvature. Indeed, one study showed that these molecules do not tubulate the plasma membranes simply by clustering, instead this is done by a microtubuli dependent process [137], while another study showed that endophilin A2, dynamin and actin contribute to the scission of these tubules [74].

Macropinocytosis has been described to be utilised by several different viruses for cellular entry. For example, the highly fatal Ebola virus, which is a long filamentous virus of around 800 - 1000 nm has been shown to use macropinocytosis for cellular entry [138]. Other examples include vaccinia virus and adenovirus 3 [75]. Porphyromonas gingivalis MVs have been seen to be internalised via a Rac1 dependent endocytic mechanism which was independent of dynamin, caveolin and cdc42 [139]. It is possible that this represents uptake of the MVs via macropinocytosis.
Association with lipid microdomains at the plasma membrane, such as lipid rafts, have been shown to be important for a plethora of pathogens and secreted virulence factors [107, 140]. This is not surprising since certain lipids and proteins can cluster in these domains and thereby promote important sites for signalling and initiation of endocytosis [140]. Bacterial MVs from several different species have been suggested to associate with lipid rafts, such as MVs from *Moraxella catarrhalis* [141], *H. pylori* [111, 142], *P. gingivalis* [139] and *H. influenzae* [143].

**Plasma membrane repair**

Maintaining the integrity of the plasma membrane is essential for cells. Damage to the membrane leads to disruption of ion gradients and leakage of cytoplasmic content. Holes in the plasma membrane larger than a few nanometres need to be sealed immediately in order for the cell to survive. Different cells in the body are exposed to factors that rupture the plasma membrane, such as detergents, osmotic stress, mechanical and ischemic stress as well as PFTs [144]. For example, muscle cells that undergo high force eccentric contractions suffer ruptures in the plasma membrane [145] and ischemic membrane injuries occur after a stroke or heart attack [144]. Other mechanical active cells, such as epithelial and endothelial cells are also often exposed to membrane damage [144]. Cells exposed to pathogens secreting PFT may also suffer from plasma membrane damage [146].

During evolution cells have evolved very efficient mechanisms for the repair of damages to the plasma membrane that can seal holes in the membrane in a matter of seconds. The main trigger for initiation of repair, irrespective of the type of damage and cell type, is influx of Ca\(^{2+}\) ions into the cytoplasm [144]. A dramatic rise in intracellular Ca\(^{2+}\) levels activates a wide range of proteins involved in membrane repair. A defective membrane repair response is associated with several different pathologies such as muscle dystrophies and pulmonary diseases [144, 147, 148]. Additionally, an upregulation of plasma membrane repair proteins has been observed in cancer cells [149]. Invasive cancer cells have less rigid plasma membranes than normal cells and are exposed to increased membrane stress when migrating through the extracellular matrix, leading to frequent damages to the plasma membrane [150].

The process of membrane repair is very complex and it is still not known exactly how it is regulated and driven. Different mechanisms have been proposed on how the wounded membrane is actually sealed and it is believed to depend on the type of damage and partly on the cell type [144, 151]. Large mechanically induced wounds spanning several microns have been observed...
to be patched by fusing intracellular cortical vesicles to the wound site in oocytes [152]. In muscle, these types of wounds have been observed to accumulate caveolae around the wound that fuse to each other, leading to a constriction of the wound [52, 153]. Small mechanical lipid-lined wounds could potentially reseal spontaneously after a decrease in membrane tension, induced by a release of folded membrane, exocytosis, leakage of cytoplasmic content and a local loss of membrane-cytoskeleton interactions [151].

PFT induced pores cannot simply be patched or spontaneously reseal since these toxins form stable protein-lined pores [151]. Instead they are thought to be repaired by either (1) caveolae-mediated endocytosis followed by lysosomal degradation of the toxin pores [52], or (2) by shedding of the pore in microvesicles (Fig 7.) [154-156]. Ca\(^{2+}\) influx after membrane damage induces exocytosis of lysosomes in proximity to the wound site [157, 158] and this leads to the release of proteases and acid sphingomyelinase [159, 160]. This was discovered to promote a massive increase in clathrin-independent endocytic activity after both SLO and mechanically induced plasma membrane wounds in HeLa and normal rat kidney cells [161]. Later it was found that the activated endocytic mechanism probably is caveolae-mediated endocytosis [52]. Fluorescently labelled SLO co-localised with caveolin1 and depletion of caveolin in cells showed that cells lacking caveolae were inefficient at repairing both PFT pores and mechanically induced wounds [52].

**Figure 7.** Membrane repair of PFT pores through shedding of microvesicles or endocytosis (adapted from [162]). (ASM = acid sphingomyelinase)
Shedding of SLO toxin has been observed in several independent studies, but also of other PFTs [154-156]. Shedding is suggested to be performed by the endosomal sorting complexes required for transport (ESCRT) machinery, which has been shown to be recruited to the plasma membrane upon treatment with SLO and LLO toxin [156]. The ESCRT machinery is a unique protein machinery that can bend membranes outward away from the cytoplasm, as well as mediate scission of these structures [163]. The opposing results regarding how SLO toxin pores are repaired shows that repair of these types of damages is complex and may involve two functionally different mechanisms.

Several different proteins have been proposed to be important for membrane repair. Annexins are a family of evolutionary conserved proteins that bind to negative phospholipids upon activation by Ca²⁺ ions [164]. There are 12 different annexins and several of them have been shown to be involved in membrane repair [144]. Annexins are recruited to the damaged membrane due to a local increase in Ca²⁺ at the site of the wound to aid in repair of the damaged membrane. The different annexins vary in their Ca²⁺ sensitivity, which leads to a tailored damage response. More sensitive annexins are rapidly recruited to the damaged site when there is a small lesion, while the less sensitive annexins are recruited if the lesion is large [146]. The word, annexin, means to join or attach, and the basic function of annexins is to aggregate and organise membranes [144]. Annexins are thought to help in fusing membranes during repair as well as forming semi-permeable protein arrays that function to plug and stabilise the wounded membrane [144, 153, 165]. Plugging of the hole in the membrane could prevent leakage of cytoplasmic content and give more time for other repair mechanisms to deal with the wound [153]. Other important proteins for membrane repair include dysferlin, MG53, calpains and SNAREs [144]. Endocytic proteins apart from caveolin that has been associated to function in membrane repair include, EHD2, GRAF1 and cdc42 [152, 166].

It is highly likely that a combination of most of the mechanisms described above work in concert to repair damages to the plasma membrane. For example, exocytosis and local loss of cortical cytoskeleton decrease local membrane tension, annexins plug and stabilise the hole, while patching of vesicles, endocytosis, or shedding seals the wound permanently. However, the membrane repair process is not completed when the wound has been sealed, because the plasma membrane becomes significantly altered in composition and shape during the repair process. Restoration of the plasma membrane after injury is also likely to be of great importance, however not much is known about this matter compared to the actual sealing event [167].
AIM OF THE THESIS

The overall aim of this thesis was to study the interaction of viruses and bacterial virulence factors with the host cell surface, and how this affects different endocytic mechanisms.

Specific aims:

I. Establish an assay to measure specific uptake in cultured cells, and use it to study endocytosis of *H. pylori* membrane vesicles in host cells.

II. Identify specific integrins used by adenovirus 37 to cause infection in human corneal cells.

III. Characterise the role of different endocytic pathways in response to plasma membrane damage induced by the pore-forming toxin listeriolysin O from *L. monocytogenes*. 
RESULTS AND DISCUSSION

Paper I

Bacterial MVs are very interesting particles due to their heterogeneity in size as well as protein and lipid content. They harbour a variety of adhesins, toxins and other bacterial substances that can facilitate interactions with many different cell surface receptors. As described earlier, *H. pylori* MVs have been found located inside host cells by electron microscopy [168] and that different endocytic pathways might be used for internalisation [111, 142]. In order to provide a better understanding of how MVs enter host cells we sought to characterise *H. pylori* MV uptake in greater detail.

For our study we chose to use the human gastric adenocarcinoma cell line (AGS) in order characterise the uptake of MVs. At the start of the project we experienced some problems in separating intracellular MVs versus MVs bound to the cell surface by microscopy. To circumvent this issue we made use of labels coupled to cleavable linkers in order to specifically study the intracellular MV localisation and to quantify the uptake of the MVs. For microscopy, we labelled the MVs with biotin-SS-NHS. After cleaving of the extracellular biotin from the MVs with a membrane-impermeable reducing agent, we detected the MVs using fluorescent streptavidin. This allowed us to specifically study the localisation of internalised MVs. We detected MVs in both early endosomes and lysosomes, confirming that the labelled MVs are taken up by endocytosis. To be able to quantify the uptake of MVs in host cells we developed a cell culture plate-based uptake assay that we named qIS (quantification of Internalised Substances). The assay was based on the same principle, with a cleavable linker, but instead of biotin we used a near-infrared fluorescent dye (800CW-SS-NHS) to label the MVs. The cleavable linker coupled to a near-infrared fluorescent dye provides less background and higher signal to noise ratio compared to conventional fluorophores and allows for specific quantification of internalised material. By quantifying the uptake of MVs directly in cell culture plates we could avoid any potential problems arising from detaching cells, which for example is needed for flow cytometry.

By confocal microscopy we found that the MVs co-localised with clathrin, dynamin2 and caveolin1. Using the qIS assay we quantified uptake of MVs after treatment with pharmacological inhibitors and siRNA against common endocytic pathways. As a control for the effect of the inhibitors and siRNA we also measured transferrin uptake in all our quantitative uptake experiments. Dyngo4a, a pharmacological inhibitor that targets all isoforms
of dynamin decreased uptake of the MVs more efficiently than uptake of transferrin. In line with Parker et al., 2010 we found that chlorpromazine, an inhibitor of CME decreased the uptake of MVs but slightly less than transferrin uptake. The fact that Dyngo4a inhibited MV uptake more efficiently than transferrin uptake suggested that MVs might be taken up by other dynamin-dependent pathways different from CME. To confirm the effect of the chemical inhibitors we knocked down AP2 and dynamin2 individually or in combination using siRNA and found that both MV and transferrin uptake was significantly inhibited. However, transferrin uptake was more sensitive to knockdown of both AP2 and dynamin2 than the MVs. These results confirm that the MVs are specifically taken up by CME but that they may also use other endocytic mechanisms.

Inhibition of caveolae-mediated endocytosis by siRNA knockdown of cavin1 did not affect the uptake of the MVs, suggesting that caveolae is not a major route of uptake. Interestingly, siRNA knockdown of GRAF1, important for CLIC/GEEC endocytosis [60], increased the uptake of MVs but decreased the uptake of transferrin. The CLIC/GEEC endocytosis is involved in the turnover of the plasma membrane [58], and disabling this pathway may lead to an altered plasma membrane composition and tension, and this could somehow facilitate *H. pylori* MV uptake and inhibit transferrin uptake.

Previous studies have shown different results regarding cholesterol inhibitors on the uptake of *H. pylori* MVs. To gain further insight into whether lipid raft domains are associated with the uptake of MVs we used the cholesterol inhibitors methyl-β-cyclodextrin (MβCD) and filipin. MβCD extracts cholesterol from the plasma membrane while filipin sequester cholesterol into large domains [93, 169]. If used in the right concentration these inhibitors can inhibit several clathrin-independent pathways while CME is not affected [22]. The results showed that extraction of cholesterol, but not sequestration decreased MV uptake. None of the drugs inhibited uptake of transferrin, but filipin reduced the uptake of the cholera toxin B subunit, which is known to associate with lipid rafts [170]. These results are hard to interpret due to the chemical nature of the inhibitors but could indicate that the amount of cholesterol in the plasma membrane influences MV uptake more than transferrin uptake. The effect on uptake by MβCD could depend on changes in membrane fluidity upon cholesterol depletion or miss-localisation of receptors important for MV uptake. MβCD can, apart from removing membrane cholesterol, also remove other lipids from the membrane and this might also affect the results [169]. Interestingly, we saw that a lower concentration of MβCD induced a small increase in MV uptake, which was also observed in a previous study [111]. A moderate cholesterol depletion with MβCD has been suggested to aggregate lipid rafts [171], and it
is possible that we induced lipid raft aggregation which may promote MV uptake. A more detailed analysis of the influence of MβCD on MV uptake is needed to resolve this matter.

Taken together, we could show that *H. pylori* MVs are mainly taken up by CME but that they are also able to use other types of endocytic pathways and we suggest it is due to their heterogeneity. Several studies have implied that MV adherence and uptake is heavily influenced by the specific proteins associated to their surface. For example MVs depleted of VacA have been shown to be inhibited by chlorpromazine to a greater extent than MVs carrying VacA [111]. The BabA and the SabA adhesins, responsible for *H. pylori* adherence to the gastric epithelium [172-174], mediate binding of MVs to gastric tissue sections [110]. Also, MVs carrying the CagA toxin localised near tight junctions in an epithelial monolayer, while MVs devoid of CagA did not [116]. Host cell association of MVs from *V. cholerae* and enterotoxigenic *Escheria coli* have also been shown to be affected by the presence certain proteins [112, 175]. This fact makes it hard to directly compare results from different studies, since the MV content is influenced depending on the method of isolation, bacterial growth phase and the bacterial strain [107]. Another parameter that could potentially affect the endocytic pathway used is the size of the MVs. Further experiments, comparing uptake of the bacterial MVs from different species and separated according to their size would be very interesting to perform in order to address this matter.

Additionally, the qIS assay we developed is a versatile method that can be used for a wide range of substances and it also has the potential to be used in larger screens. We have used it to measure uptake of both proteins and bacterial MVs, and after this paper was published it has also been used to quantify dextran uptake into cultured cells [64].

**Paper II**

Interactions between pathogens and cellular surface proteins are important for pathogen entry into host cells. Identification of such specific interactions could lead to the development of new inhibitors in order to treat infections. Ad37 interacts with the sialic acid containing glycan GD1a for initial attachment to the cell surface [176]. Similar to most human adenoviruses, Ad37 has a RGD motif in the penton base that enables interactions with integrins [101]. It was previously shown that Ad37 can bind more efficiently than other adenoviruses to αVβ5 integrins in an *in vitro* study [177]. However, in a proteomics based screen of human cornea, the β5 integrin was not detected while several other integrins where [178]. Therefore, the aim of
this project was to investigate which integrins that are of importance for Ad37 infection in human corneal cells.

Initially, we examined the expression of different integrins in human corneal tissue as well as in a cultured cell line of human corneal epithelium (HCE). We found the integrins α2, α3, α6, αV, β1 and β4 to be expressed in both corneal tissue and HCE cells. Expression of α4, α5, β3 and β5 was not detected. This suggested that Ad37 use other integrins than αVβ5 to infect corneal cells. In an inhibition experiment where HCE cells were pre-incubated with specific antibodies against different integrins we found that antibodies against α3, αV and β1 inhibited infection to approximately 30% while a combination of both α3 and αV antibodies reduced infection to 50%. Ad37 infection could also be inhibited by pre-incubating cells with peptides containing the RGD motif, as well as with the extracellular matrix proteins laminin and vitronectin. None of the antibodies, peptides or extracellular matrix proteins used in this study did inhibit binding to HCE cells, demonstrating that the integrin interaction is not important for cellular binding by serving as an attachment factor. We investigated if Ad37 co-localised with α3 and αV integrins in HCE cells by incubating the cells at 4°C, or for 15 and 30 min at 37°C using confocal microscopy. We found a 20-40% co-localisation at all time points, however, due to the high abundance of integrins we performed a pixel-shift analysis to verify the significance of these results. The analysis showed that there was only a significant co-localisation of Ad37 with α3 and αV integrins after 30 min of incubation at 37°C. This indicates that internalisation of Ad37 is either slow or that the virus is taken up along with the integrins since viral receptors are known to often follow the virus into the cell [96]. Finally, by growing the HCE cells in a multilayer, that resembled the corneal tissue, and infecting them with Ad37 we could see a correlation between infected cells and the expression pattern of α3, αV and β1 integrins.

Taken together, these results show that α3, αV and β1 integrins are important for Ad37 infection, but not binding, in human cornea and could explain the tissue tropism of the virus. This knowledge could be important for the development of inhibitors against viral keratoconjunctivitis. It is likely that Ad37 can use α3, αV and β1 integrins to enter corneal epithelial cells and/or for endosomal escape since inhibition of this interaction leads to a significantly reduced infection efficiency. Several integrins have been linked to caveolin1-dependent endocytosis [179] and a previous study suggested that Ad37 is internalised via a caveolin1-dependent endocytic pathway in human corneal fibroblasts [132]. Therefore, it would be very interesting to investigate if there is a direct link between the integrin interactions identified with caveolin1-dependent endocytosis. By designing a
live-cell microscopy assay to study the interactions of Ad37, integrins and caveolae under different conditions in real-time one could potentially resolve the mechanism of how Ad37 is internalised in human corneal cells.

**Paper III**

Endocytosis has recently gained more attention in the field of plasma membrane repair and has been suggested to mediate the repair of PFT induced wounds as well as other types of damage [52, 159, 161]. However, internalisation of an active toxin pore has not been observed, and together with the reports that the SLO toxin is shed in microvesicles, questions are being raised whether endocytosis is the responsible mechanism for removing active toxin pores or not [154]. In order to provide a better understanding of the role that different endocytic pathways play in response to PFTs that induce large pores, we treated cells with the active LLO toxin and investigated the importance and dynamics of different endocytic mechanisms in response to the induced damage.

To perform this we knocked down proteins essential for CME, caveolae and CLIC/GEEC endocytosis in HeLa cells and treated them with the LLO toxin. This revealed that cells depleted of proteins involved in CLIC/GEEC and caveolae were more sensitive to LLO than control cells. Cells depleted of clathrin were also sensitive to LLO, but to a smaller small degree. To further characterise the role of caveolae, CLIC/GEEC and CME in response to LLO pores we established a live-cell microscopy based assay. To detect the sites where LLO induced pores appear, we expressed annexin A6 with a fluorescent tag and could confirm that annexin A6 was recruited to the site of damage as fast as 10 seconds after Ca²⁺ influx. We transiently transfected annexin A6-mCherry in HeLa Flp-In TeRex cells expressing the endocytic proteins SNX9, caveolin1, EHD2 or GRAF1 tagged with GFP and investigated if they localised to the site of LLO induced damage. Interestingly, within 1 minute after damage, none of these endocytic proteins where recruited directly to the sites where annexin A6 had accumulated. This suggested that neither CME, caveolae nor CLIC endocytosis function to rapidly internalise LLO pores in order to seal the damaged plasma membrane. We did, however, observe that cells often formed thin protruding spikes filled with annexin A6 upon treatment with LLO, and these structures could last a long time at the plasma membrane. This has also been observed with the SLO toxin and annexin A1 [155] and could represent shedding of microvesicles or a way for the cell to seal off the damaged membrane from the cytosol to give time for other mechanisms to deal with the damaged membrane [153].
Since our knockdown results along with previous studies implied that several endocytic proteins are important for membrane repair we investigated if LLO treatment had any effect on the overall activity of these endocytic proteins. Using live-cell TIRF microscopy we found that the number of structures at the basal membrane of GRAF1 decreased rapidly upon annexin A6 recruitment to the plasma membrane. We also observed a recovery of the number of GRAF1 structures after around 1.5 minutes in some, but not all cells.

The disappearance of GRAF1 assemblies after LLO addition to cells was very interesting since knockdown of GRAF1 as well as cdc42 reduced the cells ability to repair LLO induced damage. GRAF1 was previously shown to be important for the repair of mouse muscle cells damaged with a laser and in regulating the localisation of dysferlin to the damaged membrane [166]. In order to further characterise the response of GRAF1 to the LLO toxin we used spinning disc microscopy, which facilitates the visualisation of GRAF1 tubulovesicular structures. The disassembly of GRAF1 structures after LLO addition was even more obvious using this approach and recovery of GRAF1 assemblies in cells was seen to occur directly after the annexin A6 response declined. This effect on GRAF1 is likely to depend on the influx of Ca^{2+} since we could show that by increasing the intracellular Ca^{2+} levels, GRAF1 assemblies disappeared, while depleting intracellular Ca^{2+} levels increased the number of GRAF1 assemblies. We saw a strong induction of GRAF1 assemblies in a fraction of cells just after a decline in annexin A6 signal and this typically lasted a short period of time. This effect could represent GRAF1 functioning to restore the plasma membrane tension and/or composition, after a decline in intracellular Ca^{2+} levels as seen by annexin A6 disappearance.

Caveolae have been suggested to internalise toxin pores after formation of ceramide in the plasma membrane and that this would be essential for repair [180]. Therefore it is surprising that we did not observe caveolae localising to annexin A6 marked sites or any drastic changes on general caveolae activity in cells treated with LLO. This could imply that caveolae could have other roles during membrane repair such as organisers of the plasma membrane and/or in buffering changes to plasma membrane tension. A recent study showed that shedding of the SLO toxin in microvesicles was only induced when functional toxin was added to the cells, while a mutant variant of SLO that is unable to form pores, was instead internalised [154]. This could explain why the SLO toxin has been observed to be internalised via caveolae [52]. The ESCRT machinery has been shown to be recruited to damaged membrane sites and that lysosomal exocytosis occurred distal to these sites, further suggesting that PFT pores are shed and not endocytosed [156]. These
results might rather suggest that the lysosomal exocytosis and subsequent ceramide formation is not involved in acute pore removal but more to remodel the plasma membrane. The massive endocytosis observed upon membrane damage in certain studies could also be dependent on some other endocytic mechanism not studied by us. To address whether LLO monomers or pores are internalised via caveolae or some other mechanism it would be interesting to study uptake of both functional and mutant variants of fluorescently labelled LLO. Also, since annexin overexpression may affect the repair process [162] it would be good if one could have an additional specific marker that is rapidly recruited to the site of damage.

Another proposed model that could explain why we did not see any recruitment of endocytic proteins to the plasma membrane, is that shedding and internalisation could represent two separate repair mechanisms that are activated depending on the amount of damage the cell is exposed to. It has been suggested that low levels of damage induce microvesicle shedding, while a high level of damage induce a massive ceramide-driven internalisation event [155]. By measuring uptake of different cell surface receptors in cells treated with different concentrations of toxin one could perhaps address this matter.

Taken together, our results indicate that both caveolae and CLIC/GEEC endocytosis are important for a functional repair mechanism of LLO induced damage. However, the endocytic pathways studied here are probably not involved in direct internalisation of LLO pores. Instead, their role might be to regulate the localisation of membrane repair proteins, the lipid composition as well as the shape and tension of the plasma membrane. These properties are bound to be important for an efficient repair machinery as well as to restore the membrane to normal after damage.
CONCLUSIONS

Endocytosis is a fundamental process adapted to maintain cellular homeostasis and meet specific demands of cells. However, this process is also crucial for many pathogens in order to promote their own survival [95]. How different endocytic processes are regulated and intertwined is with the development of new technologies now starting to be revealed. Still there are many questions remaining regarding the role of different endocytic pathways as well as how they are triggered. The following section is a reflection on what drives the use of different endocytic pathways and how this could be coupled to infection.

The diverse set of endocytic pathways appear to have different, although to a certain degree overlapping, functions. CME is constitutively active and responsible for many of the cells housekeeping functions and provides an efficient way to take up ligands and the specific receptors under normal conditions [181]. It is therefore not surprising that CME appears to be a common pathway for both viruses and MVs that bind to various cell surface receptors to gain entrance to the cell [95, 107]. Since CME is a relatively slow process and restricted in size it is not very efficient in turning over large amounts of membrane and its constituents. This is rather performed by other pathways such as CLIC/GEEC, FEME and macropinocytosis [18, 181]. These pathways are very efficient when the cell quickly needs to adapt to sudden changes such as during different stresses or after stimulation by different growth factors. For example, the epidermal growth factor receptor (EGFR) is at low EGF concentrations internalised by CME. However, at high EGF concentrations there is a massive signalling from the cell surface and this activates clathrin-independent endocytosis, which rapidly removes EGFR from the cell surface and sends it for degradation in order to prevent overstimulation [182, 183]. This shows that certain stimuli can affect the uptake of receptors and their ligands and this could be important in the context of pathogen entry. It also provides a cautionary note about how the concentrations used during experimental setup can affect which pathway specific molecules are taken up by. Additionally, this shows that the endocytic pathway used to take up a certain cargo is not only dependent on the cell surface molecule it binds to but also that the timeline in which a particular event needs to occur can determine which pathway is utilised.

In contrast to the above mentioned pathways, caveolae are generally relatively static structures with only a small portion undergoing endocytosis during steady state conditions [56]. The uptake of several viruses, MVs and toxins has been linked to caveolae, however much of this has later been
shown to depend on other circumstances, such as high overexpression of caveolin1 or the understanding that other clathrin-independent pathways besides caveolae are also dependent on lipid rafts [51, 107]. Nevertheless, these results do not rule out that certain pathogens could stimulate internalisation of the otherwise stable caveolae.

Even though caveolae does not seem to be a major route of uptake it is known to influence the activity of other endocytic pathways. Several components of caveolae act as negative regulators of CLIC/GEEC endocytosis since an abundance of these proteins inhibit CLIC/GEEC endocytosis, while low levels stimulate CLIC/GEEC endocytosis [94]. It is important to find out how endocytic mechanisms are triggered and how they influence each other in order to better understand how the cell surface is regulated under different conditions. This could provide answers on how pathogens are able to enter cells via different pathways as well as further insight into if certain pathogens merely hijack endocytic events or if they actively modulate them in order to promote their own internalisation.

As an example of how endocytic mechanisms can be actively modulated is the use of membrane repair processes in order for pathogenic substances to enter cells. PFTs are known to promote uptake of macromolecules probably by initiating a membrane repair response [121]. Also, some type C adenoviruses have been shown to take advantage of the same procedure by injuring the plasma membrane with a lytic viral protein that subsequently promotes endocytosis of the virus [184]. This shows that damaging the host cellular membrane could be a common way for pathogens to promote entry into host cells.

By studying the cell surface interactions and uptake of pathogens and substances like adenoviruses, PFTs and bacterial MVs one can gain further insight into events that occur during infection but also regarding basic cell biology. Additionally, the types of viruses and bacterial substances studied in this thesis are all interesting to use as molecular tools ranging from vaccination to gene therapy and delivery of various substances to host cells [99, 107, 185-188].
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