ORCA – Online Research @ Cardiff

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository:https://orca.cardiff.ac.uk/id/eprint/152123/

This is the author’s version of a work that was submitted to / accepted for publication.

Citation for final published version:

Griffiths, Gareth, Gruenberg, Jean, Marsh, Mark, Wohlmann, Jens, Jones, Arwyn T. and Parton, Robert G. 2022. Nanoparticle entry into cells; the cell biology weak link. Advanced Drug Delivery Reviews 188 , 114403. 10.1016/j.addr.2022.114403

Publishers page: http://dx.doi.org/10.1016/j.addr.2022.114403

Please note:
Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher’s version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See http://orca.cf.ac.uk/policies.html for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.

Nanoparticle entry into cells; the cell biology weak link

Gareth Griffiths[1], Jean Gruenberg[2], Mark Marsh[3], Jens Wohlmann[1], Arwyn T. Jones[4] and Robert G. Parton[5]

[1,4] Department Biosciences, University of Oslo, Blindernveien 31, PO Box 1041,0316 Oslo, Norway, .[2]Department of Biochemistry, University of Geneva, 30 quai E. Ansermet, 1211-Geneva-4, Switzerland, [3] MRC-Laboratory for Molecular Cell Biology, University College London, Gower Street, WC1E 6BT, UK [4] School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Redwood Building, Cardiff, Wales, CF103NB, UK [5]Institute for Molecular Bioscience and Centre for Microscopy and Microanalysis, The University of Queensland, Qld 4072, Australia

ABSTRACT

Nanoparticles (NP) are attractive options for the therapeutic delivery of active pharmaceutical drugs, proteins and nucleic acids into cells, tissues and organs of interest. Research into the development and application of NP most often starts with a diverse group of scientists, including chemists, bioengineers and material and pharmaceutical scientists, who design, fabricate and characterize NP *in vitro* (Stage 1). The next step (Stage 2) generally investigates cell toxicity as well as the processes by which NP bind, are internalized and deliver their cargo to appropriate model tissue culture cells. Subsequently, in Stage 3, selected NP are tested in appropriate animal systems, mostly mouse. Whereas the chemistry-based development and analysis in Stage 1 is increasingly sophisticated, the investigations in Stage 2 are not what could be regarded as 'state-of-the-art' for the cell biology field and the quality of research into NP interactions with cells is often sub-standard. In this review we describe our current understanding of the mechanisms by which particles gain entry into mammalian cells via endocytosis. We summarize the most important areas for concern, highlight some of the most common mis-conceptions, and identify areas where NP scientists could engage with trained cell biologists. Our survey of the different mechanisms of uptake into cells makes us suspect that claims for roles for caveolae, as well as macropinocytosis, in NP uptake into cells have been over-estimated, whereas phagocytosis has been under-appreciated.

1) INTRODUCTION

Over a hundred years ago, Paul Ehrlich coined the term ‘magic bullet’ to convey the idea of drugs that selectively kill pathogens or regulate gene or protein expression locally without affecting healthy tissues [12]. Since the 1950s, when the first nano-sized[1] liposomes became available for delivering drugs to cells, the field of nanomedicine has grown exponentially and now involves thousands of academic labs and many pharmaceutical companies all aiming to realize Ehrlich’s vision by, for example, encapsulating chemotherapeutic drugs in small ‘magic bullets’ that can be selectively delivered to tumors or other diseased sites, thereby enhancing drug efficacy and avoiding side-effects seen with conventional chemotherapy. Although the potential remains, the approach has yet to deliver the anticipated return on investment as evoked in the title of a 2013 review ‘Cancer Nanomedicine: so many papers and so few drugs’ [18] See also [19]!

Failings aside, it must be emphasized that nanomedicine researchers aiming to bring a successful NP to the clinic, face immense challenges. For an NP to deliver a cytostatic drug to a tumor, for example, a particle must 1) be administered to patients either intravenously or by routes that feed into the vascular system (e.g. pulmonary, intramuscular, subcutaneous); 2) be able to cross an endothelial boundary and find a way through a basement membrane and tissues peripheral to the tumor, and 3) should ideally have ligands enabling its binding and internalization into specific cells, where its cargo will be released in an appropriate intracellular or extracellular locations. At least in the context of

[1] *Nano is sometimes defined as 100 nm or less. In this review, we take the broader definition, Nano = 1000 nm or less. Particles >1000 nm are defined as Microparticles (MP).*

cancer (where most activity has been focused) no NP has been yet designed that comes close to satisfying all these conditions [2] [20] [21] [22, 23]. Notwithstanding, there have been successes. In the field of vaccinology for example, where NP-based SARS-CoV-2 vaccines are proving highly effective [24] [25], and lipid-based NP are proving valuable for some *in vivo* CRISPR/Cas9-based gene editing applications [26]. This approach is also considered highly promising for vaccines against cancer [27] [28].

In efforts to improve NP efficacy, many studies have focused on understanding the cell biological mechanisms by which NP enter diseased cells using tissue culture models. Uptake from the extracellular space by membrane invaginations (endocytosis) provides an effective way for cells to capture and internalize NP, though the challenge remains that the cargo still needs to cross the limiting membrane of an endocytic vesicle to mediate its effect within the cell. Here we focus on these specific aspects of NP research, and we contend that the quality of much of the cell biology in this field leaves significant room for improvement. Our comments expand on similar critiques and warnings made earlier [30-32] [34].

The goal of this review is therefore to provide a framework for understanding the different endocytic pathways in detail and to point out the many misconceptions embedded in the NP literature. To this end, we first summarize the mechanisms by which materials are internalized by cells. For the sake of clarity, we restrict our discussion to four main uptake mechanisms; 1. Phagocytosis, 2. Macropinocytosis, 3. Clathrin-mediated endocytosis, 4. Caveolae and a further fifth section in which we briefly summarize other potential, but less extensively characterized mechanisms.

The first process, phagocytosis, deserves special mention because this mechanism is hardly considered in the NP field. The reason for this can be blamed on cell biologists who have long argued that this process is restricted to particles 500 nm or larger in diameter. Since most NP being studied are smaller than this, NP chemists can be forgiven for ignoring this process as a potential mechanism for taking NP into cells. However, we make the case that there is little published evidence for a 500 nm cut off size; it is an emperor without clothes. Indeed, we have assembled evidence supporting the notion that phagocytosis can operate on particles of 100 nm diameter, or less, and should be considered more often in the NP field. Interactions of NPs with the cell surface and their subsequent entry into cells can to some extent be modulated by changes in NP physico-chemical or biological properties (size, shape, stiffness, valence, targeted receptor) or in the biological response of the target cell (drugs that affect signalling or trafficking). For example, increased knowledge of ways to manipulate endocytic pathways can potentially lead to modulation of entry [35, 36]. However, regardless of the nature of NPs and the design of an experiment, in the analysis of the data and in the interpretation of the experiments, we encourage scientists to always keep in mind the concepts that will be discussed below.

The second mechanism of interest is macropinocytosis which is very popular in the NP field. Surprisingly, this process has almost exclusively been studied in *in vitro* cell cultures; only a couple of publications have described macropinocytosis *in vivo*. Significantly, the conditions used to stimulate macropinocytosis in cell culture, such as serum deprivation followed by adding back serum, have an explosive effect - turning on the process of forming large vesicles that take in anything that happens to be in the vicinity - like krill into the mouth of a whale. Under these conditions it is no surprise that NP floating in the medium go in by macropinocytosis. The key question is – is this observation really relevant for the NP uptake *in vivo*?

The third mechanism is clathrin-mediated endocytosis (CME). In terms of molecular details, CME is the most extensively characterized endocytic process, nevertheless aspects of how exactly clathrin-coated vesicles (CCV) form and how their formation is regulated remain controversial. Moreover,

an unexpected link to phagocytosis muddles the water. If a NP is seen to enter a forming CCV, can one be sure that this vesicle will really go into the cell?

The fourth mechanism involves the enigmatic caveolae that hundreds of papers conclude are responsible for carrying NP into cells. A general problem with many of these studies is the reliance on inhibitors that lack the specificity required to identify a role for caveolae. Other methods that might support roles for caveolae, such as advanced electron microscopy (EM), are rarely used in NP research. One of the important take home messages here is that EM is an important, albeit neglected, tool in the NP field. We emphasize this point with a series of electron micrographs that illustrate endocytic pathways (Figs 2-8).

We discuss the main characteristics of these pathways, as well as the less well-characterized CLIC/GEEC and Fast Endophilin-Mediated (FEME) pathways. We critically evaluate current research on NP uptake and the problems in the field. We discuss the crucial step of endosomal escape, describe how this is being studied, and what we can learn from infectious agents such as viruses that have evolved a variety of mechanisms to enter cells via different endocytic pathways and to deliver their nucleic acid cargo to the cytoplasm or nucleoplasm of specific host cells. It may not be generally known in the NP field that a significant body of our knowledge of endocytic pathways is underpinned by work on viruses (reviewed in [40] [42]).

For a summary of the main points see **Box 1**.

2) ENDOCYTIC MECHANISMS (FIG 1) (Definitions Box 2)

2A) PROCESS 1: PHAGOCYTOSIS

An ancient process

Particles as large as multi-micron eukaryotic cells can be taken up by cells via the process of phagocytosis – or 'cell eating' (see **Figs 2,3**). Mechanistically, phagocytosis is intimately associated with actin that, along with clathrin, is considered evolutionarily to be one of the oldest proteins involved in the different uptake mechanisms associated with eukaryotic cells; both proteins are thought to have been present in the last universal precursor of eukaryotes [43, 44]. It is widely believed that phagocytosis was a defining feature of early eukaryote cells that enabled them to take in bacteria for nutrition. According to the endosymbiont hypothesis [47] this process also led to the acquisition of mitochondria and chloroplasts. Although some cells may have lost the ability to phagocytose over evolutionary time, the relevant argument here is that the capacity for phagocytosis is still widespread in modern cells from protozoa, including amoebae that use phagocytosis to ingest bacteria, to many different metazoan cell types.

In mammals, phagocytosis is an especially prominent function of three types of immune cells, macrophages, neutrophils and dendritic cells (DC), which are often referred to as 'professional' phagocytes [48]. However, a wide range of other (non-professional) cells also have some capacity for phagocytosis. If, for now, we accept the widely accepted dogma that only particles of 500 nm or above can be phagocytosed, we can cite many examples of cells that have this capacity. For example, fibroblasts [49, 50], B lymphocytes [51], T lymphocytes [52], keratinocytes [53], neurons [54], endothelial cells [55], smooth muscle cells [56], and hepatocytes [57] can all internalize large particles such as apoptotic cells or latex beads. Even epithelial cells, the most abundant nucleated cells in the body, which are often considered non-phagocytic, can be induced to internalize bacterial pathogens such as Listeria and Yersinia via their apical surfaces, showing that the machinery for phagocytosis, though rarely used, is present in these cells [58]. In some cases, epithelial cell basolateral surfaces are also able to phagocytose bacteria [59, 60]. A direct comparison between professional and non-professional cells for phagocytosis of apoptotic cells *in vitro* and *in vivo* showed

that the professional phagocytes (macrophages), which have a wider variety of phagocytic receptors, responded quicker and were overall more efficient in phagocytosis than non-professional cells [4]. Nevertheless, an important conclusion for the NP field is that most cells that are used in NP research, including cancer cells [61, 62] and the widely used HeLa cells [63], have some capacity for phagocytosis of large particles.

The mechanism of phagocytosis

A key feature of phagocytosis is that it entails *multivalent binding of ligands on the particle surface to a threshold number of receptors on the cell surface* (see above and [64]).This engagement triggers a series of reactions that drive the plasma membrane, in a zipper-like fashion, around the particle to eventually engulf it [65]. The particle is then enclosed within a phagocytic vesicle, or phagosome, within the cytoplasm of the cell.

The first step in this process is the localized activation of a complex series of trans-membrane signaling reactions involving molecules (mostly lipids and proteins) on the cytoplasmic aspect of the area of plasma membrane to which the particle is bound. By far the most prominent of these trans-membrane signaling events is that which induces the transient, membrane-associated assembly of actin filaments; in many studies this assembly peaks at around 10 seconds after the initial contact, and is followed by actin depolymerization, and often a second longer period of actin assembly [66, 67] [68] [69]. In this remarkable, and still poorly understood process, actin monomers insert at the membrane surface and form short filaments with the fast-growing, plus or barbed ends at the membrane and the opposite, minus ends, growing outwards from the membrane. This actin filament assembly drives the outward growth of plasma membrane. The crucial role of actin is indicated by the fact that phagocytosis is acutely sensitive to the widely used specific inhibitor cytochalasin D that binds to the plus (fast-growing) ends of actin filaments and inhibits filament growth [70, 71] (**Fig 3B**). Alternative widely used drugs include latrunculin A, which binds and sequesters actin monomers thereby blocking actin polymerization as well as accelerating depolymerisation of existing filaments [72].

A related process involving rapid trans-membrane signaling linked to actin assembly is seen in the process of chemotaxis of *Dictyostelium* amoebae; studies by Condeelis's group described the process of membrane-catalyzed actin filament assembly in much more detail than has been undertaken in a study of phagocytosis. In response to the chemoattractant cAMP, that binds to cell surface cAMP receptors on this amoeba, an increase in F-actin was detected in only 3 seconds. These authors showed that an initial total of 1500 F-actin filaments per cell increased about 10-fold in response to signaling [70, 73]. The average length of the filaments was estimated to be 0.3 μm both before and after stimulation [70]. In other words, in this system, stimulation triggers the assembly of many short actin filaments on the cytoplasmic face of the plasma membrane (see also [69].

Based on what is generally known about membrane-catalyzed actin assembly, the initial nucleation of filament assembly is likely to be dependent on membrane-associated formins [74]. These proteins are able to nucleate actin filaments and insert actin monomers at the plus end of growing actin filaments close to the membrane, pushing the minus ends away from the membrane [66, 75]. On these filaments, the seven-protein complex known as ARP2/3, in association with N-WASP and other proteins, binds and nucleates the secondary assembly and branching of new actin filaments. In this case, the plus end of these filaments grow away from the branch points by adding actin monomers at the tips of the growing filaments [76]. However, it should be noted that this assembly of actin as it occurs during phagocytosis, or any other membrane-catalyzed event, has not been visualized in detail at ultrastructural resolution and is often referred to by vague terms such as actin 'rearrangement' or 'remodeling'. We speculate that, in addition to involving the force for manipulating membranes, rapid polymerization and de-polymerization of actin may serve to generate a large surface of actin filaments to provide a temporary platform for the binding and organization of signaling complexes in the

vicinity of the cytoplasmic domains of plasma membrane receptor molecules. Upon actin depolymerization the signaling complexes would be disassembled.

In parallel to the actin filament growth and breakdown, a large number of molecules that are components of signaling complexes operate in phagocytosis [77]. These include 1) lipids, e.g., cholesterol, sphingolipids, phospholipids, phosphoinositides (PIPs), diacyl glycerol (DAG), sphingosine, phosphatidic acid; 2) enzymes, including protein kinase A (PKA), protein kinase C (PKC), phospholipase C (PLC) phospholipase D (PLD) and the actin-associated GTPases Rho and Rac; and 3) structural proteins, such as actin and actin binding proteins, including formins, Arp2/3, gelsolin, profilin, N-Wasp and myosins. Downstream of the plasma membrane-associated signaling events, signaling molecules also enter the nucleus to regulate gene expression [77-80]. As far as we can tell, this specific regulation of gene expression in response to the uptake process is predominantly if not exclusively a phenomenon associated with phagocytosis and not with the other internalization mechanisms discussed in this article.

During phagocytosis one can see evidence of active signaling at the whole cell level, visible as ruffling, at sites distant from the phagocytic event, a phenomenon that is much more prominent during macropinocytosis and is discussed in more detail in part 3 [81]. Nevertheless, even non-professional cells can show ruffling when phagocytosing particles [4].

Although many reviews show cartoons of signaling pathways involved in phagocytosis, the fact that rarely are two schemes the same suggests that these maps are really no more than educated guesses based on known interactions between specific components (e.g., phospholipase C converts PI (4,5)P_2 to diacylglycerol and inositol triphosphate). As for most transmembrane signaling events, there are a vast number of components involved, with fast reaction kinetics that all too often have limited experimental accessibility. This is especially true for the many signaling lipids within the membrane; we are currently a long way from a holistic understanding of such processes. Another important issue is the mistaken belief that cell membranes can be equated with a simple lipid bilayer, as in a liposome. Whereas the latter involves at most a handful of different lipids, cellular membranes contain over a thousand different lipid species [82] and many thousands of proteins that function at a level of complexity that is still poorly understood. It should also be realized that the standard cartoon schemes illustrating signaling cascades emanating from the membrane show many components free in the cytoplasm when in actual fact the active forms of these molecules are intimately associated with the cytoplasmic surface of the plasma membrane; this is true for example for phosphoinositides, phospholipases, GTPases such as Rho family GTPases and Src family non-receptor tyrosine kinases. Many of these factors, such as the Rho family GTPases, are assumed to be upstream of membrane-receptor-dependent actin assembly. As discussed below the available evidence suggests that their activation occurs after actin assembly.

Ligands that signal to inhibit phagocytosis.

A number of pathogens have developed survival strategies whereby they bind to cells such as macrophages but they then block phagocytosis and then grow and multiply in an extracellular environment. Prominent examples are *Enteropathogenic E.coli* (EPEC), *Pseudomonas aeruginosa, Yersinia pseudotuberculosis*, *Staphylococcus aureus* and *Clostridium difficile* [83-86]. Many of these pathogens take advantage of injection systems that allow them to secrete toxins into the host cells that inhibits some aspect of phagocytosis. A well-characterized non-pathogen system that inhibits phagocytosis involves the interactions of the two integral membrane proteins CD47 on phagocytic target cells (such as cancer cells and erythrocytes) and SIRPα on phagocytic cells such as macrophages or dendritic cells. CD47 is expressed on the surface of most cells but it is overexpressed in cancer cells. CD47 has been referred to as a ''don't eat me'' signal when it binds SIRPα during the binding of cancer cells to macrophages; the interaction between SIRPα and CD47 signals the phagocytic cell to inhibit phagocytosis. This endows cancer cells with the ability

to avoid been killed by phagocytic cells. Antibodies against CD47 are able to overcome the phagocytosis block and are now being tested in clinical trials against cancer ([87, 88]. Surprisingly, interaction between CD47 and SIRPα does not affect actin polymerization at the contact site arguing that the first recognizable step is a positive signal for phagocytosis [89]. Subsequently the cytoplasmic domain of SIRPα binds to two protein tyrosine phosphatases SHP-1 and SHP-2 and the dephosphorylation of SIRPα and other molecules is thought to initiate a signaling cascade that inhibits phagocytosis [90]. These results argue that after initial binding of a target cell to activate signaling towards phagocytosis the signaling route can be diverted to block the process.

Other physical parameters affecting phagocytosis

For NP or macroparticles (MP), a number of physical features can affect the efficiency of phagocytosis, in particular particle size [91-93], charge [94], shape [91, 95], hydrophobicity/hydrophilicity [3] and elasticity/hardness [96] have been well-documented. Nevertheless, it is difficult to make simple, general conclusions about the importance of these parameters.

What is the minimum size for phagocytosis?

The minimum size for particles taken up by phagocytosis is almost invariably claimed to be 500 nm [80], though evidence for this is difficult to find in the primary literature. As discussed below, this issue may be crucial for NP uptake since most NP are smaller than 500 nm. A series of older publications provide evidence that the mechanism responsible for the contact-mediated, or receptor-mediated, uptake of particles >500 nm is similar if not identical to that responsible for the uptake of much smaller particles. Our interpretation of these studies, which we have grouped into five sets of observations, is that particles as small as 50 nm diameter may also be taken up by receptor-mediated phagocytosis.

1. Roberts and Quastel (1963) [97] found that the total mass (or volume) of polystyrene latex particles of different sizes between 260 nm and 3 μm diameter ingested by neutrophils over a 20 min period was almost identical, whereas 88 nm particles were taken up at a much slower rate, presumably by a different uptake mechanism. The simplest conclusion from these studies is that between 264 nm and 3 μm the cells took up particles by a similar mechanism, presumably phagocytosis, and that they could sense when they became 'full'. They could ingest only a few large particles or many smaller ones, but the volume/mass of polymer internalized was quantitatively the same [67, 97-99]. Eating themselves full is a behavior often seen with professional phagocytes.

2. Weisman and Korn applied the approach developed by Roberts and Quastel to analyze a unicellular amoeba *Acanthamoeba*. In this model, the initial binding and uptake of polystyrene particles into cells was analyzed mathematically and found to be similar to enzyme reaction kinetics [99]. An amoeba could internalize a single bead of 2.8μm diameter or, in the same time, 4000 beads of 126 nm diameter. Importantly, using large beads made of polyvinyl toluene and small beads of polystyrene, that could be distinguished from each other spectrophotometrically, they showed that preincubating cells with small beads could efficiently block the uptake of large beads (the 2.8 μm diameter beads were much less effective at competing with the smaller beads). The authors hypothesized that the particles were binding a finite number of the same (unknown) receptors on the cell surface. When 126nm beads mixed with radioactively labeled bovine serum albumin (BSA), which enters cells by fluid phase endocytosis, were offered to the amoebae, the beads were concentrated 300-fold relative to the BSA. These experiments established that even particles devoid of a specific ligand could enter cells by receptor-mediated phagocytosis, and that this uptake was extremely efficient when compared to (non-receptor mediated) fluid phase endocytosis, i.e. when phagocytosis occurs it is highly efficient, perhaps the most efficient cellular uptake process.

3. Many older studies have monitored the distribution of particles of different sizes and composition after intravenous injection into different mammalian models, including mice, rats, guinea pigs and Rabbits. A consistent finding has been that particles such as latex beads, liposomes or silica in the size range from 50 nm to 2 μm, were detectable in the liver and spleen within seconds, and 50-90% were cleared from the blood in 2 minutes or less. A consensus that emerges for all the different sized particles is that the majority of uptake (~70%) is carried out by liver (Kupffer) macrophages, with a second, smaller pool entering spleen macrophages and, under some conditions, macrophages in the lung. Moreover, many other *in vivo* and *in vitro* studies also found that particles around 50-60 nm were efficiently cleared by macrophages with *the same kinetics* as larger particles [100-105]; for a detailed, direct comparison of 50 and 500nm latex beads see [106]. Within these publications, EM images can be found of Kupffer macrophages *in vivo* that were loaded full with 60 nm or 350 nm latex particles [103] [107]. Evidence that larger MP (up to 2 μm) are rapidly cleared from the blood and enter Kupffer cells within minutes is also evident in EM studies [108] and [109]. In the latter study, 0.2 and 2 μm latex particles were co-administered and collected efficiently in the same macrophages. Another study showed uptake of Listeria bacteria into Kupffer cells that were identified by immunohistochemistry [110]. As phagocytosis is the most likely mechanism known to take up 2 μm particles, the simplest interpretation of these data is that smaller particles can also enter cells via phagocytosis. For additional studies consistent with phagocytosis of sub-100nm particles see [71, 99, 111-113].

It should be noted that these older studies did not address how the NP crossed the endothelial barrier nor the potential role of opsonization (Discussed below). However, these issues were addressed in more recent studies using intravital microscopy in mouse or rat. A range of different fluorescently labelled NP have been injected intravenously and followed in live rodents after different surgical interventions necessary for two-photon or multi- photon intravital imaging. A consensus has emerged that many NP are taken up within seconds after injection by sinusoidal endothelial cells before extravasating and appearing a minute or two later in the liver Kupffer cells. NP were not detected in the most abundant cells, the hepatocytes; in contrast when free dyes were injected they appeared rapidly in hepatocytes and were then cleared into the bile [114-116] (Reviewed by [117]. In one study of calciprotein NP the presumed uptake via phagocytosis was associated with a pro-inflammatory release of TNF-α and IL-1β and was dependent on Scavenger receptor A (discussed below) [114].

4. A detailed study by [50] compared the uptake of fibronectin-coated beads of 85 nm, 300 nm, 760 nm and 1μm diameter into baby hamster kidney (BHK) fibroblasts. Fibronectin is a large molecule with a molecular weight of 440 kDa. All beads had the same surface density of ligands, which meant that the 85nm beads had less than 100 fibronectin molecules whereas the 1μm particles had over 15,000 fibronectin molecules per particle; a density for both particles of about one ligand per 300 nm^2 of bead surface. This density corresponded to a suggested optimal spacing of clustered ligands on the bead surface for efficient phagocytosis of 18nm. Using elaborate differential binding and uptake assays with radiolabeled fibronectin it was shown that, irrespective of size, the same surface area of beads was taken into cells at a similar rate and efficiency, presumably by phagocytosis. So sub-500 nm NP (76 and 300 nm) behaved the same as the larger particles. This elegant study was consistent with the 'zipper hypothesis', a Velcro-like process of sequential binding of ligands on the particles to receptors on the phagocytic cells [65]. The value for the ligand spacing of 18nm is interesting to compare with a recent sophisticated study by Kern and colleagues to quantify the optimal density of Fc receptor ligands on 5 μm beads for efficient phagocytosis by Fc receptors on macrophages [64]. This group developed a DNA origami system for controlling the interactions between the number and spacing of ligands on the beads and the Fc receptors on the cell surface. In addition to monitoring bead uptake, they assayed downstream signaling events, namely actin assembly, receptor phosphorylation and the synthesis of PIP3. Importantly, all these events were enhanced when the ligands were tightly clustered compared to the same number of more dispersed ligands. The most efficient phagocytosis occurred when the ligands were spaced around 7 nm apart;

this is not so different to the 18nm value obtained by McAbee and Grinnell in 1983 [50] in a completely different system.

5. Different lines of evidence have suggested that ~200 nm diameter spherical Herpes simplex virus particles enter cells by (receptor-mediated) phagocytosis [118].

Although particles in the size range 50-200 nm are very common in the field of synthetic NP, there has been little consideration of phagocytosis as a mechanism for their uptake into cells. A recent review even ruled out phagocytosis as having any role in NP uptake - entirely on the basis of particle size [119]. Here, we argue that the notion of 500 nm as a lower size limit for phagocytosis has been interpreted too strictly and, as a consequence, a role for phagocytosis in the uptake of smaller NP may have been overlooked.

Fig 3A shows transmission EM images of 100 and 500 nm latex nanoparticles entering macrophages after only 15 min via a mechanism suggestive of phagocytosis; colloidal gold-BSA NP were internalized with a pulse-chase protocol to label LE and lysosomes. In this short uptake period only a small amount of gold entered phagosomes. Entry of both latex particles into the cell is completely blocked by the actin polymerization inhibitor cytochalasin D although particle binding and early stages of phagosome cup formation appears normal **(Fig 3B).**

Opsonization versus non-opsonization routes for phagocytosis
There are two fundamentally different receptor mechanisms for initiating phagocytosis

i) Non-opsonization phagocytosis:- is dependent on cellular receptors on the phagocytic cell surface recognizing and binding directly to molecular determinants on the surface of particles, independent of host factors (opsonins) such as antibodies or complement [120, 121].[120, 121].

ii) Opsonization phagocytosis: - involves the initial binding of host proteins, opsonins, to the particle surface. Opsonins then engage receptors, such as Fc or complement receptors, on the phagocyte surface [80]. According to Owens and Peppas [122] the process of opsonization is one of the most important biological barriers to controlled drug delivery via NP. Within seconds of exposure to blood (non-stealth, discussed below) NP will bind proteins, with the initial binding being to the most abundant proteins in blood, especially albumin. The complete plasma proteome was estimated to have 3700 proteins, of which approximately 50 had been identified in association with various NP [123]
. The pattern of bound proteins is dynamic and varies over time, with less abundant, high affinity proteins replacing the first wave of bound proteins. The bound proteins are collectively referred to as the '*corona*' whereas those proteins which bind to specific receptors on phagocytic cells of the reticuloendothelial system are the opsonins. Opsonins are by definition any blood serum protein that has a partner receptor on the surface of phagocytes that aids the process of phagocytic recognition. In this process complement proteins such as C3, C4, and C5, immunoglobulins and fibrinogen are typically the most common [123]. A consequence of this interaction is the rapid removal of opsonized NP from the circulation by phagocytes [124] [125]. When recognition by the mononuclear phagocytic system (MPS) occurs, up to 90% of the injected dose is taken up by the liver Kupffer macrophages [126, 127]. When particles "survive" these first 5 min, prolonged blood circulation was found [102]. Therefore, 'the protein adsorption pattern acquired in the first 5 min is the most important one, determining MPS recognition or MPS escape' [128]. Unless the phagocytes are the ultimate target of the NP, this process of being cleared by phagocytes is highly undesirable and considerable effort has been spent to avoid it by engineering 'stealth' particles that hinder binding of opsonins (see below). For an extensive list of different types of NP that have been documented to attach to complement factors, IgG and other serum proteins see [129].

There has been considerable interest in the NP field in the concept of a protein corona, the non-specific adsorption of proteins from the blood to the NP surface [21, 129-131]. Some of the interest is focused on desirable selective binding in the blood of proteins that can facilitate targeting of NP to cells and tissues of interest [132] [133, 134]. However, the binding of proteins is highly dynamic and affected by all the properties of the NP such as size [135], charge [136] and hydrophobicity [137] [138]. Consequently, the pattern of the corona on any NP is complex and unpredictable, and the knowledge invariably based on *in vitro* interactions of the NP with plasma or serum, not on the real *in vivo* situation.

The attachments of serum proteins to NP in the blood is not inevitable. An elegant study of the uptake of (negatively charged) mercaptosuccinic acid capped telluride/cadmium sulfide quantum dots in rats [115] used fluorescence lifetime imaging microscopy (FLIM) to show this. Fluorescence lifetime is a sensitive indicator of bound proteins on the NP surface. Even though these NP in this study were not modified for stealth (e.g., with PEG, see below), they failed to bind any proteins in a three hour incubation with rat blood.

In our opinion when considering that the ultimate aim of developing NP is to fabricate a product that behaves reproducibly in patients, the corona is something that should be avoided. If adsorption of proteins such as albumin to the NP surface is considered beneficial, it makes more sense to add this to the NP in a controlled way (e.g., by covalent binding) during the formulation process. A recent study using different stealth components describes the fabrication of polymeric NP that can be incubated for long periods with blood plasma without the formation of a corona [139]. The stealth concept is discussed in more detail below.

Preventing phagocytosis- The stealth concept

In *in vivo* experiments with NP containing drugs against cancer there is a lot of interest in keeping the NP in circulation as long as possible. This means avoiding undesirable uptake by sentinel cells, such as macrophages, which can reduce the circulation time of NP. Manipulating NP to minimize their interactions with phagocytes (so-called 'stealth' function) can potentially extend their time in the circulation.

The most common approach for equipping a particle for stealth is to attach to its surface polyethylene glycol (PEG), a flexible hydrophilic polymer [124, 140, 141]. There are a number of variables in efficiently covalently coating the NP surface with PEG, in particular the molecular weight of the polymer and the density of the brush-like PEG layer. It is widely assumed in the NP field that the *only* effect of PEG is to prevent opsonins and protein that form the corona (defined above) from binding to the NP, thereby avoiding opsonin-dependent phagocytosis [142]. We showed that (0.5-1μm) latex beads coated with PEG can be manipulated with optical tweezers and brought to the surface of macrophages. In the absence of serum opsonins, and despite forcing contact with the cells, the stealth particles were unable to bind. In other words, PEG *directly* prevented the particles from binding the phagocytic receptors needed for entry; this argues that stealth functions prevent both opsonization and the direct binding of particles to cells. In the absence of PEG the particles attached strongly to the cells within seconds of being brought into contact with the macrophage surface, which exhibited ruffling and phagocytosis of the bound particles [51]. Conversely, in some conditions PEG has been found to stimulate phagocytosis by neutrophils [143]. There are alternative stealth polymers which may be preferred over PEG, such as polysarcosine [38, 144] or polyoxamine [104]. Recent studies show that a stealth coat of either PEG, polysarcosine or poly(*N*-2-hydroxypropylmethacrylamide) (pHPMA) can be very efficient in avoiding non-specific binding on proteins from plasma on core cross-linked polymer NP [139](see also [145]. The authors calculated that with optimal stealth conditions less than one protein molecule bound per nanoparticle indicating that such NP were devoid of a protein corona.

Role of clathrin in phagocytosis

A surprising finding in the phagocytosis field was an apparent role for clathrin in phagocytic events. Aggeler and Werb first showed platinum replica and thin section EM images of clathrin lattices, as well as clathrin coated vesicles (CCV), that were seemingly budding from plasma membranes domains involved in phagocytosis of latex beads by macrophages [146]; See also [147](**see Fig 3A-M**). Subsequent studies showed that clathrin coats also form on phagosomes formed by Listeria bacteria during their entry into macrophages or epithelial cells. Knockdown experiments using RNAi revealed that when clathrin or other proteins involved in (receptor-mediated) phagocytosis were knocked down, phagocytosis was inhibited [148, 149]. In a later publication this group showed that bacterial binding led to tyrosine phosphorylation of the clathrin heavy chain and recruitment of the actin binding protein Hip1R. This linking of clathrin phosphorylation to actin assembly was essential for bacterial internalization [150]. Clathrin was later found to be essential for phagocytosis of apoptotic cells in *C. elegans* [15].Veiga and Cossart also confirmed an earlier study [100] that showed that the GTPase dynamin, which is essential for the pinching off of clathrin-coated (and other types of) vesicles from the plasma membrane, is also necessary for phagocytosis. While the presence of clathrin on forming phagosomes may be the exception rather than the rule ([151] (Griffiths, Unpublished data), the merging of the mechanisms of phagocytosis and clathrin-mediated endocytosis has relevance for the NP field in that knockdown of clathrin and dynamin may also affect phagocytosis (as well as some other pathways) and should not necessarily be regarded as a specific indicator of the clathrin-mediated endocytosis pathway.

Scavenger receptors and uptake of non-biological particles

There is a wealth of evidence that scavenger receptors are involved in the uptake of NP by many cell types. Scavenger receptors (SR) were first described in 1979 by Brown and Goldstein who, a few years earlier, had characterized the receptor for Low Density Lipoprotein (LDL) as the key molecule for the highly efficient uptake of LDL-cholesterol into fibroblasts via clathrin-mediated endocytosis (CME)[152]. By contrast to LDL receptor-mediated uptake, when LDL particles were oxidized they formed aggregates that bound to, and were efficiently internalized by macrophages via a different receptor at a rate 20 times greater than that of native LDL [153]. The receptor that efficiently internalized these oxidized LDL aggregates by phagocytosis became known as the scavenger receptor (SR) [154]. Subsequent studies revealed that SR comprise a large, highly diverse super-family of cell surface transmembrane glycoproteins, expressed primarily on myeloid cells, including macrophages and DCs. SR bind a variety of non-self and altered-self ligands, ranging from oxidized LDL to apoptotic cells, bacteria and other cellular pathogens, including viruses, via pathogen associated molecular patterns (PAMPs) [155].

SR have been classified into several distinct classes, based on their membrane topology (type I, type II or multiple membrane spanning domain proteins) and the nature of their ligand-binding elements (e.g., C type lectin-like domains, collagen-like domains and extracellular matrix (ECM) binding domains). These latter elements are all located within larger extracellular domains, that comprise the bulk of the proteins, and are connected to the plasma membrane by one or more transmembrane domains that link to smaller cytoplasmic domains. The cytoplasmic domains contain trafficking and/or signaling information that is crucial for their functional activities in phagocytosis, adhesion and pathogen clearance. Significantly, a number of these receptors have been shown to interact with some innate immune system Toll-Like Receptors (TLR) upon engagement with selected bacterial pathogens [156-158].

Macrophages have evolved the capacity to clear foreign particles from the body, from bacteria to 'inert' material, and SR play a key role in these processes. Due to their capacity for multi-valent binding, especially to poly-anionic structures, SR can bind to a range of non-biological, amorphous particles, such as carbon, titanium, silica, monosodium ureate, hydroxyapatite and latex with relatively high avidity. Moreover, SR can also bind to crystalline NP containing for example,

cholesterol, silica or asbestos [159, 160]. With respect to these latter materials, a number of experiments have identified two members of the so-called class A SR family (in the original classification) as being especially important for binding non-biological particles, specifically SR-A1 (or CD204) and MARCO (macrophage receptor with collagenous structure, or SR-A6). These experiments combined single and double receptor knockouts, the use of specific monoclonal antibodies targeting the ecto-domain of the receptors to block particle binding, and knock-in transfections to render the normally non-phagocytic COS or CHO cells competent to phagocytose diverse particles via the SR [161, 162].

What is less clear is how these receptors are able to recognize such a broad array of ligands, especially surfaces devoid of distinct structure, such as latex or amorphous silica. A possibility is that most, but not all, of these particles are negatively charged, so they would be expected to bind positively charged receptor domains. MARCO, a 210 kD trimer of a type II single-spanning membrane protein, contains a 110 amino acid C-terminal scavenger receptor cysteine-rich (SRCR) domain, which has a positively charged region that is important for ligand binding. Cell surface proteins with SRCR domains and diverse functions (not related to SR) are found widely across the animal kingdom, but a common function for SRCR domains has yet to be determined [163]. The X-ray structure of the SRCR domain of murine MARCO revealed two highly conserved arginine residues (432 and 434) in the extracellular domain that are crucial for ligand binding [164, 165]. Significantly, different regions of this SRCR domain can bind different ligands: thus, titanium dioxide (TiO_2) NP bind residues 420-431 [162], whereas silica NP bind residues 443-520 [159, 160, 166]. Nevertheless, when positive charged, negative charged or neutral charged 3 µm latex beads were directly compared, the rate and extent of binding and phagocytosis by macrophages was the same [167]. The structural basis for the ability of MARCO and/or other SR to bind so many different ligands thus remains an open question.

Phagocytosis of small (< 500nm diameter) NP and the role of signaling

As discussed above, there is increasing evidence that phagocytic mechanisms can mediate the uptake of particles below the 'official' size cutoff for phagocytosis (500 nm). Experiments using alveolar and other macrophages have revealed that when amorphous or crystalline silica NP in the range 10-150 nm diameter enter cells, they leave potent signaling signatures that have many of the hallmarks of phagocytosis. However, they show little similarity to the clathrin-mediated or caveolin-associated vesicle uptake that one might expect to be involved based simply on considerations of size. First, there is a robust assembly of actin filaments, evident from the effect of cytochalasin D which efficiently blocks both NP uptake and downstream signaling events [168-170](see **Fig 3B**). This actin inhibitor has little, if any, effect on the clathrin- or caveolin-associated pathways, despite the fact that actin has been implicated in uptake by both mechanisms [171, 172]. Nevertheless, in polarized epithelial cells it has been shown to selectively inhibit clathrin-dependent uptake from the apical surface without affecting clathrin uptake from the basolateral surface [173].

Though there are many studies that show NP inside cells, there are few ultrastructural studies showing how small (around 50-100 nm) NP enter cells. One potentially important observation was made by [99] in their comparison of uptake by Acanthamoeba of latex beads ranging from 88 nm to 3 µm. Whereas beads bigger than 500 nm entered cells individually in single phagosomes, 88 - 500 nm beads were 'collected' into groups of particles that entered into the cell together, forming phagosomes with multiple beads. Similarly, in an ultrastructural analysis of silica uptake [174], focused on a variety of cell lines and used high pressure freezing and freeze substitution (a procedure that gives specimen preparation closer to the native structure than standard fixation). Their study revealed that 7nm, 12nm and 22nm amorphous silica NP all entered cells by vesicular pathways, with the details differing between the three different sizes. However, whereas the 22nm NP entered individually the 7nm and 12nm NP entered cells in groups. Korn and Weisman had earlier speculated that this aggregation of small particles on the plasma membrane before phagocytosis may occur through an

active cellular process; the particles were not aggregated before cell contact [99]. Clearly, further studies are needed to address this issue.

As discussed above, Kusaka and colleagues speculated that the uptake of relatively small (50 nm) silica particles was a type of phagocytosis [168] (see also [160]. Downstream signaling events from this uptake process included activation of MAP kinases, production of reactive oxygen species (ROS), and NfkB-mediated expression of the pro-inflammatory cytokines TNF-α, IL-Iβ and IL-18; for IL-Iβ maturation of caspase 1 must also be activated via inflammasomes [168-170, 175, 176]. This robust signaling is preceded by actin assembly, similar to that which occurs during phagocytosis or macropinocytosis and is completely blocked by cytochalasin D [168, 177]. All these phenomena occur in the apparent absence of opsonins, which argues that the silica binds directly to endogenous receptors; this is however a difficult issue to prove.

There appears to be a consensus that the *smaller* the silica particles, the more potent the inflammatory signaling and the greater the extent of apoptosis or other mechanisms of cell death [168, 170, 178]. A study by [179] found potent inflammatory induction in macrophages that was similar for all amorphous silica NP with sizes of 7, 10, 16, 50, 150 and 300nm. All particles had similar cytotoxicity and a detailed comparison of the 10nm and 500nm particles, administered such that they exposed the same total surface area, induced similar patterns of gene expression that could be classified into different functional categories such as transcription, inflammatory response, apoptosis and cell cycle. Similar results were seen in a comparison of 50 and 500 nm amorphous silica particles in lung epithelial cells by [176] who quantified cytokine expression and secretion. Other publications showed the ability of sub-100 nm amorphous silica NP to induce more severe lung inflammation in a mouse model than did 3μm MP [168, 174, 178]. Collectively, these results are not consistent with the notion that only 500nm particles are taken up by phagocytosis, nor with the idea that sub-100 nm particles would enter cells by a non-phagocytic route, for example clathrin-mediated endocytosis. The simplest, albeit radical interpretation is that both types of particles enter cells by phagocytosis.

More evidence that sub-500 nm particles enter cells by phagocytosis and regulate gene expression comes from a study by [180] monitoring newly synthesized cytokine production by human peripheral blood mononuclear cells stimulated with ssRNA–protamine particles. This was cited in the excellent review by [181]: 'The size of a phagocytosed particle can also influence the types of inflammatory cytokines that are produced'. [180] observed interferon-α (IFNα) production in response to 220 nm and 500 nm particles, but not 1200 nm particles. By contrast, 220 nm particles failed to induce TNF-α, but 500 nm and 1200 nm particles both induced robust TNF-α production. Other cytokines, such as IL-1β and IL-6, were induced equivalently regardless of particle size'. Evidently, each particle size can behave differently to the others, but the 220 nm particles appear to enter cells via the same phagocytosis process as the bigger particles.

In the same study another relevant observation supporting our arguments came from comparing the levels of TNF-α secreted by monocytes in response to 220 nm versus 1200 nm particles. One 1200 nm particle contains the same mass as about 100 of the 220 nm ones. Relative to a single large particle per cell, at low concentration of the 220nm NP the small beads stimulated much less TNF-α than the large bead. The authors asked 'whether a high load of NP could mimic the activity of microparticles'. By increasing the relative fraction of small beads, the levels of cytokine produced became equivalent for the two particles. If phagocytosis was occurring with the 1200 nm particles it must surely be the same mechanism for the 220nm NP with a similar mass of RNA particles stimulating an equivalent level of transcription.

Latex beads and other particles as model particles for phagocytosis

During phagocytosis, it is clear that there is intimate contact between ligand(s) on the particle and receptors in the cell plasma membrane. While for many phagocytic substrates, e.g., bacteria or dead

cells, this might involve multiple receptor-ligand types, a powerful way to simplify the interaction is to use latex beads with one specific ligand on their surface, such as mannose or IgG. This ligand then binds its cognate receptor which, in turn, initiates a specific receptor-dependent signaling program. Using J774 murine macrophages, we compared 1µm latex beads coated with mannose, LPS, IgG, and avidin ([182] see also [183], monitoring (1) phagocytic uptake rates in association with the presence of an actin coat around the forming phagosome, (2) the kinetics of fusion of phagosomes with lysosomal compartments, (3) the gene expression profile initiated by phagocytosis, (4) the protein composition of isolated mature phagosomes, and (5) the time-dependent dynamics of selected protein associations with phagosomes. Latex beads with different coats were internalized at different rates and exhibited distinct kinetics of phago-lysosomal fusion. Furthermore, less than 60% of identified phagosomal proteins and only 10-15% of changes in gene expression were common to all four ligands. Thus, each ligand induced a distinct pattern of gene expression, in conjunction with different rates of phagosome maturation, resulting in different protein composition of (isolated) phagosomes. An elegant complementary study tested beads coated with IgG, mannan, beta glucan, fibronectin and complement factors CIq and iC3b, in bone marrow macrophages [184]. They monitored phagocytic uptake, production of nitric oxide and superoxide, phagosome fusion with lysosomes, cytokine secretion and the ability of the different particles to activate the killing of bacteria when the factors were conjugated to the surface of *E.coli*. As in our study [182], each phagocytic ligand activated a distinct pattern of responses. A more detailed quantitative proteomic analysis using latex beads having IgG (for Fc receptor), complement 3b (complement receptor), LPS, mannan, (pathogen associated molecular pattern TLR's) calreticulin and phosphatidyl serine (PS, for apoptotic cells) and avidin (inert control) was undertaken by [31]. All the ligands induced small but significant unique differences in the pattern of proteins found in the resulting phagosomes. They also showed that beads with PS were anti-inflammatory and repressed the activity of the pro-inflammatory transcription factor NfkB whereas beads with IgG were pro-inflammatory and stimulate translocation of NfkB into the nucleus. In other words, activating a single receptor type can have profound effects on gene expression.

Dykstra et al. also tested the microtubule depolymerizing drug nocodazole and found that only complement factor coated beads require microtubules for their uptake, in agreement with earlier studies by [185] This was also confirmed by [186] who found that non-opsonized 3µm silica NP required microtubules for their phagocytosis. The role of microtubules in phagocytosis is unclear, though it has been suggested they may provide tracks for membrane vesicles that supply the membrane for forming the phagosome [187].

Taken together, these data argue that phagocytic receptor-specific signaling programs direct phagosomes to different physiological states. An interesting but unresolved question is what happens in more realistic situations when two or more ligands on the *same* particle engage different receptors; does one receptor dominate the signaling for example? This can be expected to be the situation when a bacterial pathogen, with multiple surface ligands, binds to the surface of a cell such as a macrophage Early evidence of synergistic interactions between two receptors comes from studies summarized in the classical review by Silverstein et al [188]. Particles, such as erythrocytes coated with sub-optimal levels of complement C3b or with IgG, were not phagocytosed by macrophages or neutrophils. However, particles coated with the same amounts of both proteins were efficiently phagocytosed. With IgG alone, ~6000 molecules per erythrocyte were needed for efficient phagocytosis whereas only 150 molecules were needed for efficient uptake when combined with a few hundred molecules of C3b (or C3d) [189].

More recently, other examples of interactions between two receptors during phagocytosis have been reported. A well-studied example is the synergistic interactions in phagocytic cells between Dectin-1, a pattern recognition receptor that binds specific glucans, such as those found on yeast/ fungi, and Toll-Like receptor (TLR)-2 that binds lipoproteins on fungi and other pathogens [190]. Lin and

colleagues devised a method to manipulate the spatial location of Dectin-1 and TLR-2 ligands on latex beads and to relate their patterns (localized via super-resolution microscopy) to their ability to activate NfkB-dependent transcription, secretion of TNF-α and the production of ROS in macrophages. For optimal synergy, so-called receptor cross talk, they found that the two ligands, and therefore also the two receptors, needed to be separated by a distance of around 500nm; beyond that distance the signaling diminished [191]; it is difficult to compare this study dealing with two different receptors and the study by Kern and colleagues discussed above dealing with only one receptor [64]. In another system, involving interactions between Fc receptors binding IgG opsonized bacteria and TLR's involved in phagocytosis by DCs, the combination of both receptors was needed for transcription and secretion of inflammatory cytokines (TNF-α, IL1B, IL-23 and IL-6). Intriguingly, while binding of both receptors was needed for this response, subsequent phagocytosis was not; moreover, cytochalasin D blocked the internalization process but not trans-membrane signaling to the inflammatory response [190]. Thus, in this case binding of the receptors was sufficient to initiate the signaling events leading to gene expression, independently of continued phagocytosis.

Latex beads for phagosome in vitro actin assembly analysis

The phagocytic uptake and signaling processes are not only incredibly complex, with many players acting in seconds but are also experimentally inaccessible, especially the events that occur on the cytoplasmic side of the membrane. An *in vitro* cell free system that reconstitutes these events could be a powerful tool but appears difficult to conceive at present. However, after they are engulfed, latex beads have physical attributes that enable them to be isolated as very pure phagosome fractions by gradient centrifugation [99, 192]. This has allowed many *in vitro* assays of different functions to be developed [67]. One type of membrane catalyzed actin assembly has been reconstituted on such latex bead phagosomes, where membrane-bound ezrin and newly synthesized phosphoinositides PI(4)P and $PI(4,5)P_2$ were shown to be essential components [193, 194]. This process required ATP but, significantly, not GTP, ruling out the need for GTPases in this system. This assembly could be positively and negatively regulated by many lipids [195, 196]. As in all known membrane-catalyzed nucleation and polymerization of actin filament reactions, the actin monomers are inserted at the membrane surface [193].

2B. PROCESS 2:MACROPINOCYTOSIS

Macropinocytosis is defined as an endocytic process that involves the formation of large, heterogeneously-sized, fluid-filled vesicles, termed macropinosomes, that are visible by conventional light microscopy (above 200 nm in diameter) **(Fig 4).** This process was first described by Warren Lewis in the 1930's who observed macrophages and tumor cells taking in big gulps of the extracellular medium [139, 197, 198]. Although extensively studied in macrophages and immature dendritic cells, macropinocytosis is also a prominent process in a variety of other cells, in particular Dictyostelium amoebae where it functions in nutrient uptake, operating in parallel with the phagocytosis of bacteria [117].

Whereas phagocytic uptake, especially of bacteria, caveolae and clathrin-coated pits/vesicles are structurally distinct and easily identified by EM, both in cells in culture and in tissues, essentially all studies of macropinocytosis, that are morphologically distinct only when viewed in three-dimensions have been carried out in cultured cell models. An exception is a study showing that macropinocyosis can deliver protein nutrients to Ras transformed cancer cells both in culture and in a mouse tumour xenograft model, though macropinocytosis was not directly visualized *in vivo* [199]. In a convincing *in vivo* demonstration, macropinocytosis was seen by two-photon imaging of resident tissue macrophages in the peritoneal serosa of mice ([200]. Another report we could find for macropinocytosis *in vivo* was uptake of fluorescent dextrans by hemocytes in Drosophila embryos [201]. An early EM study of Salmonella uptake into guinea pig intestine also showed evidence suggestive of macropinocytosis, although this was not proposed at the time [202] (Discussed below).

The formation of macropinosomes can best be visualized by adding a fluorescent, fluid phase marker, such as dextran (usually 10-70 KDa) to the cell medium; this label will occupy the content of any macropinosomes that form from the plasma membrane. For short incubation times (15 min) it was claimed that the 70Kd dextran will selectively label macropinosomes but not clathrin-coated vesicles (Canton et al, 2015). However, it is not obvious why a molecule no bigger than 10 nm in diameter [203] cannot enter a CCV, unless it simply reflects the low probability of sufficient fluorescent dextran molecules to be visualized being incorporated into a small vesicle at the concentration used. Forming phagosomes will also not be labelled with these fluorescent compounds due to the tight fit of the membrane around the target that excludes fluid phase markers [204, 205]. Macropinocytosis can occur constitutively in some cells, such as immature DCs and macrophages (discussed below [206, 207] [208], but is usually activated by ligands binding to a selection of specific receptors, often receptor tyrosine kinases such as epidermal growth factor (EGF) [209], insulin, platelet-derived growth factor (PDGF) or colony stimulating factor (CSF) receptors. In addition, macropinocytosis can be activated by expression of oncogenes such as Ras and Src [210] [211]. Pathogens such as *Salmonella typhimurum* release factors that stimulate macropinocytosis to mediate infection in both professional phagocytic cells and cells such as epithelial cells, the bacteria being carried along as collateral with the fluid [212].

In the case of growth factors, the binding of soluble ligands to their cognate receptors initiates trans-membrane signaling events that stimulate actin polymerisation (discussed below) and ruffling that can lead to the formation of large vesicles, especially at the leading edge of cells. Here, there is similarity between macropinocytosis and phagocytosis; however, whereas phagocytosis tends to induce local ruffling adjacent to the particle, macropinocytosis induces a more global response, including the striking formation of circular dorsal ruffles, especially on the top (dorsal) surface of cultured cells, [213, 214]. A recent study in macrophages using lattice light sheet microscopy described the ruffles as being erected and supported by actin filament bundles reminiscent of poles erecting and holding up a tent (Condon 2018). In this model twisting of the hypothetical tent poles facilitated closing of the macropinosome vesicles.

Even though there are no obvious defined zones of macropinocytosis on the plasma membrane, there are evidently membrane boundaries that delineate the ruffling zone. Using a photoactivatable GFP attached to a spanning membrane protein (neuromodulin) in Macrophage-Colony-Stimulating-Factor (CSF) stimulated macrophages, Welliver and colleagues saw that the GFP was restricted to zones co-localizing with circular ruffles [215]. This argued for a diffusion barrier around forming macropinosomes that coincided with the zone of actin assembly and with activated Rac-1. In earlier studies, the activation of Rac-1 and the formation of PIP3 was linked to sealing of the macropinosome vesicles [216].

The process of macropinocytosis can vary between different cell types. [117] described significant differences in the mechanisms and regulation seen in Dictyostelium, DCs, macrophages, epithelial cells and cancer cells. In most cases, within 15 mins of adding a stimulus the majority of macropinosomes move towards the cell center, undergoing sequential fusions with Rab5-positive early endosomes, followed by Rab7-positive late endosomes/lysosomes, where they acquire lysosomal markers, including acid hydrolases and LAMP proteins (see Fig 9C)[217]. Through these fusions, macropinosomes also acquire the proton ATPase complex that acidifies the vacuole lumen [218]. During this maturation, a significant fraction of macropinosomes are converted to tubules, in conjunction with removal of water from their lumen [139, 200, 219]. In contrast, in A431 human epidermal cells, macropinosomes do not fuse with endo-lysosomes, but within minutes of formation they fuse back to the plasma membrane in an apparently futile cycle [220]. A striking example of micropinocytosis was shown by [221] during their investigation of uptake of the bacterial pathogen *Afipia felis* into macrophages. When the bacteria were not opsonized with IgG they entered cells in classical macropinosomes that were convincingly demonstrated by fluorescence microscopy and by

EM. Most of the bacteria did not reach endolysosomes and the bacteria replicated. However, when the bacteria were opsonized with antibody they entered cells in tight-fitting phagosomes and the majority were targeted to endolysosomes where the pathogen was inactivated.

Role of actin, microtubules and signaling in macropinocytosis

Macropinocytosis appears to share many mechanistic similarities with phagocytosis and chemotaxis, in particular the process through which ligand-receptor binding on the cell surface signals actin polymerization on the cytoplasmic surface of the plasma membrane [214] In all three processes there is a consensus that the first peak of actin assembly occurs around 10 seconds after the initial stimulus. Macropinocytosis and phagocytosis are more closely related in that actin polymerization is coordinated with ruffling and the formation of membrane vesicles surrounding a solid particle, in the case of phagocytosis, and engulfing only liquid in the case of macropinocytosis. As with phagocytosis, the assembly of actin appears to be coordinated by formin family members, which nucleate and polymerize straight, unbranched filaments at the membrane surface and the Arp2/3 complex that form branched filaments emanating from the formin-catalyzed filaments [222]. This machinery is regulated by members of the WASP family, and many other proteins, including myosins [223] [224]. Also similar to phagocytosis, macropinocytosis is strongly inhibited by cytochalasin D and other actin inhibitors.

Many signaling molecules are shared between macropinocytosis and phagocytosis. The two processes are rarely studied in parallel and the emphasis on any particular protein or lipid depends on the specific interest of the investigators. The Ras-pathway and phosphoinositides have especially been linked to the core machinery regulating macropinocytosis. Ras-activated PI3-kinases (class-1 PI3-kinase), Ras GTPase itself and the protein kinase B/Akt (which binds phosphatidylinositol 3, 4, 5 tri-phosphate ($PI(3,4,5)P_3$ [PIP3]) are central organizers of macropinocytic cups. A number of additional signaling molecules have been shown to regulate classical macropinocytosis (phosphoinositide 3-kinase (PI3K), phosphoinositide 4,5 bis-phosphate (PIP2), phosphoinositide 3-phosphate (PI(3)P), diacylglycerol (DAG), Rho family proteins. A common scenario is that ligand binding activates receptors that then turn on Ras GTP, which recruits PI3Kinases. These enzymes make the phosphoinositide PIP3 that interacts with Rac and Cdc42, which in turn somehow regulates actin assembly [139, 213, 225]. Most if not all of these signaling molecules are also active during phagocytosis. As with phagocytosis these observations offer only glimpses of the overall mechanism. The key to deciphering molecular differences between phagocytosis and macropinocytosis surely lies with a systems biology-based network analysis [226].

There is a well-entrenched dogma stating that the Rho family GTPases (and other molecules) operate *upstream* of actin assembly in macropinocytosis, phagocytosis and chemotaxis [214] [227]. If so, these GTPases need to be activated *before* around 10 seconds of a phagocytic, macropinocytic or chemotactic signal; moreover, in most signaling schemes there are at least three putative upstream reaction steps before the active Rho protein. However, when quantitative fluorescence microscopy was used to monitor the association of active forms of Rac1, Cdc42, and PIP3 during macropinocytosis, the peak of their activities was detected around 80 sec, that is *after* the initial peak of actin assembly [215, 216]. While this does not rule out that activation of these GTPases starts earlier, this interpretation is consistent with two systems that reconstituted *de novo* assembly of actin on membrane surfaces, latex bead phagosomes [193] and Listeria and Shigella bacteria [228]; in both cases ATP was required but not GTP. What is clear is that despite considerable effort, much remains to be understood in the regulatory mechanisms involved in both macropinocytosis and phagocytosis.

Recently, a role for microtubules was revealed for the initial uptake step of macropinocytosis in a human cancer cell. When microtubules were depolymerized with nocodazole, macropinosome formation and fluid uptake was inhibited. Live cell imaging revealed that the growing tips of microtubules contacted the forming macropinosome precisely at the time when the prominent actin

coat depolymerised. The microtubules, as well as the minus-end motor dynein were needed for both formation of the macropinosomes, and their transport towards the perinuclear region of the cells. A role was also shown for JIP3, a Jun kinase interacting protein that can function as a scaffold for microtubule motors and for Arf 6 GTPase [229].

Constitutive macropinocytosis

Immature DCs and macrophages can take in fluid in large volumes even in the absence of serum or other stimulants [207]. It has recently emerged that this constitutive macropinocytosis is mechanistically quite different from the process that is activated by stimuli such as growth factors. Whereas the latter occurs independently of extracellular calcium, constitutive macropinocytosis is dependent on the presence of calcium in the medium [230]. In myeloid cells a G-protein coupled Calcium Sensing Receptor CaSR was shown to respond to extracellular calcium; when HEK 293 cells were transfected with CaSR they acquired the ability for constitutive macropinocytosis [230]. In this elegant study, it was convincingly shown that macrophages were capable of both types of macropinocytosis. Even in the absence of extracellular calcium they could still respond to the stimulus of macrophage-CSF. The constitutive macropinocytosis was also shown to require a number of factors, including PIP3, Rac1/Cdc42 and phospholipase C. In contrast to mammalian myeloid cells, constitutive macropinocytosis in *Dictyostelium* is not influenced by extracellular calcium [139]

The mammalian cells most active in macropinocytosis appear to be immature DCs; these cells can macropinocytose the equivalent of their cell volume of liquid in about 15 min [208, 231] and can continue this process constitutively, without growth factors, over many days. This ability to undergo macropinocytosis so extensively has been proposed to allow immature DCs to sample large volumes of fluid to capture and process antigens [208]. Consistent with this, these macropinosomes fuse with endosomes and lysosomes, compartments where antigen processing can occur. However, this idea has recently been questioned on the grounds that receptor-mediated mechanisms, such as phagocytosis are much more efficient [207]. The latter group also made an interesting and counter-intuitive observation that pro-inflammatory macrophages (monocytes activated with granulocyte macrophage colony stimulating factor [GM-CSF] followed by interferon gamma and LPS) were inactive in macropinocytosis. In contrast anti-inflammatory macrophages (treated with M-GSF followed by IL-4) were highly active in constitutive macropinocytosis [232]. The significance of these findings remains to be elucidated.

A recent study showed that Rab10 is recruited to forming macropinosomes in myeloid cells competent for constitutive macropinocytosis. Knockdown of this Rab strongly inhibited macropinosome formation but had no effect on either clathrin-mediated endocytosis or phagocytosis of IgG-coated beads [2]. This is one of the few examples of a convincing molecular difference between macropinocytosis and phagocytosis.

Phagocytosis versus macropinocytosis

Although the functions of phagocytosis and macropinocytosis appear to be different, the two processes show similarity in their mechanisms of actin assembly and signaling (Box 3). This makes it very difficult to distinguish between the two processes using inhibitors or by modulating the expression of specific proteins.

One interesting way of comparing phagocytosis and macropinocytosis comes from gene knockout studies in *Dictyostelium*, which actively uses both processes. This analysis confirmed that at least a dozen proteins (including actin) are essential for both phagocytosis and macropinocytosis. However, some molecules, such as myosin VII, are required for phagocytosis but not for macropinocytosis; conversely protein kinase B (Akt) is needed for macropinocytosis but not for phagocytosis [66]. However, it should be noted that this PKB/Akt does play a role in phagocytosis in other cell types [233, 234].

In *Dictyostelium* a striking mechanistic similarity between phagocytosis and macropinocytosis was described by Mercanti *et al.* who showed that two plasma membrane proteins were excluded from both forming phagocytic cups and from macropinosomes early in their assembly [151]. Another two plasma membrane proteins were found at the same concentrations in the forming vesicles and in the rest of the cell surface. That the vesicle forming domains for phagocytosis and macropinocytosis in *Dictyostelium* are similar in mammalian cells is also evident in their similar content of phosphoinositides, most prominently PIP3 [235].

As far as we can see the molecules involved in signaling during phagocytosis [236] are the same as those involved in macropinocytosis [117] [139] [214]. However, while there is extensive literature describing signal-mediated modulation of gene expression, especially of cytokines and chemokines during phagocytosis [181], regulation of gene expression does not appear to be a prominent function of macropinocytosis. One obvious explanation for this difference is that phagocytosis is an extended process. After phagocytosis of a *Staphylococcus aureus* it can take 12-48 h for a macrophage to kill and digest the bacterium [237], while a non-degradable particle such as a latex bead remains indefinitely inside its phagosome. It is now well established that signaling events can continue from within the phagosome [181]. In contrast, ligand-induced macropinocytosis is a much shorter event and is not generally associated with secretion of cytokines and chemokines.

Even when macropinocytosis-associated dorsal ruffling is prominent, it appears that phagocytosis can occur in the same cell at the same time. It had long been believed that the bacterial pathogen *Shigella flexneri* uses a type III secretion system to inject factors into gut epithelial cells to trigger its uptake by macropinocytosis [238]. In elegant light and electron microscopy studies of Shigella entry into epithelial cells, Weiner *et al.* confirmed that macropinosomes were formed and could be labeled by fluorescent dextran in the medium [205]. However, the bacteria were internalized in classical tight-fitting phagosomes that excluded the fluorescent dye. The role of the bacterial effectors is evidently not in entry but in the ruffling process that forms macropinosomes. Subsequently, the bacterial phagosomes fuse with the macropinosomes that are suspected of delivering bacterial effectors that rupture the vacuole (phagosome) and release the bacteria into the cytoplasm to start the cytoplasmic phase of the infection. Rab11 was also shown to be acquired by macropinosomes and essential for the delivery of bacterial effectors to the phagosome. A similar interaction between different pathways was described for the uptake of the non-enveloped virus adenovirus 2 into Hela cells. In this case, binding of virus particles to the cell triggered macropinocytosis, but the virus entered early endosomes via clathrin-mediated endocytosis [239]; in some way the macropinosomes facilitated the escape of the virions from endosomes to the cytoplasm to initiate infection.

Together these observations indicate that it can be difficult to distinguish unequivocally between macropinocytosis and phagocytosis. In attempts to address this issue, many researchers resort to the use of 'selective' inhibitors. As we discuss below, these are of dubious specificity and cannot reliably distinguish one process from the other. Thus, more stringent criteria are crucial in order to show convincingly that macropinocytosis mediates endocytosis of specific cargoes under physiological conditions. We argue that live cell microscopy combined with a fluid marker, such as fluorescent-dextran, is essential to monitor the ruffling process and the formation of macropinosomes over relatively short time periods [198]; many studies claiming uptake of NP by macropinocytosis only monitor uptake after many hours of incubation, often following overnight serum starvation (Discussed below). Scanning EM is also useful in providing a 3D overview of membrane organization [117] **(see Figs 2 and 4).** This approach allows close contact between the cell and target particle to be seen in 3-D during phagocytosis and the absence of intimate contact during macropinocytosis. Thin section transmission EM is less informative for describing macropinocytosis because it is restricted to 2D images that can be difficult to interpret. In the literature there are many unconvincing claims of macropinocytosis based on thin section EM images of NP adjacent to surface filipodia,

however this snap-shot juxta-positioning is far from sufficient to establish macropinocytosis as the mechanism for NP uptake; one cannot be sure of connectivity between membranes in a 2D slice (see **Figs 4 I, J**). In contrast, thin section TEM can be highly informative for characterizing phagocytosis **(Fig 3A, B).**

Macropinocytosis of nanoparticles

Claims that NP are internalized by macropinocytosis need to be stringently scrutinized. For immature DCs, macrophages or other cell types which show constitutive macropinocytosis, it would be expected that NP added to the medium would enter into macropinosomes, simply as content of the extracellular medium. However, it is unclear whether equivalent uptake would also occur *in vivo.* Adding activators like EGF can trigger macropinocytosis; similarly, serum-starving cells followed by addition of NP plus serum would have the same effect [1]. By contrast, if cells are not stimulated, little macropinocytosis is seen unless the material of the NP themselves activate the cells in some way. The question then arises, what are the most natural conditions to test whether or not NP or MP enter cells via macropinocytosis?

As indicated above, to show that a particular NP enter cells by macropinocytosis it is important that the process is documented with live cell microscopy, using a fluorescent fluid phase marker. In our survey of the NP literature claiming uptake by macropinocytosis most of the analyses were carried out on chemically fixed cells, and flow cytometry following trypsin treatment (discussed below) was commonly used to evaluate uptake rather than microscopy. But the evidence actually given the most weight in the NP field invariable derives from experiments using inhibitors, an approach also discussed in detail below. We have found few studies claiming NP uptake by macropinocytosis that could not be just as well explained by phagocytosis.

As an example, we cite the study by [45] who analyzed the uptake of rod shaped and spherical silica NP into HeLa and A-549 cancer cell lines. Using serum starvation before adding NP in serum they found that rod shaped NP were taken in most efficiently by a mechanism that was sensitive to inhibition by amiloride, cytochalasin D, azide and 4°C. These NP stimulated the formation of filopodia, actin polymerization and the activation of small GTP-binding proteins (Rac1, CDC42). They interpreted these data as showing that the uptake process was macropinocytosis but all their criteria could equally well apply to phagocytosis. However, the latter was not seriously considered despite the fact that it is easier to imagine phagocytic binding being more likely to sense shape differences between particles than macropinocytosis.

Macropinocytosis of viruses

Viruses can be considered a special example of NP. As with NP, there are claims that a number of viruses infect cells via macropinocytosis, and again we consider some of these claims questionable. In most of these studies there is, again, a strong emphasis on inhibitors that are supposed to provide evidence for or against specific pathways. The main forms of evidence often considered as being in favor of macropinocytosis are 1. The stimulation of ruffling by the virus is often but not always associated with an increase in fluid phase uptake; 2. Inhibition of uptake by actin inhibitors; and 3. Inhibition by inhibitors of the Na+/H+ exchanger NHE1, such as amiloride, EIPA or other (non-specific) inhibitors of macropinocyosis. Evidence using inhibitors, or expression of proteins affecting signaling that has implicated key molecules, such as Rho family proteins, PKC, PI-3kinase, has been argued to be indicative of macropinocytosis**. *Essentially none of these criteria allow one to distinguish unequivocally between macropinocytosis and phagocytosis*.** For example, in the case of human papilloma virus HPV-16, the proposed macropinocytosis mechanism [240] showed no evidence of ruffling and was not regulated by Rho GTPases. Uptake of this virus appears more consistent with phagocytosis even though the particles are 55 nm diameter [42]; indeed thin section EM analysis showed individual virions in single, non-clathrin invaginations that suggested contact

between the virus and membrane components [240, 241]. When uptake was monitored in the presence of cytochalasin D, the virus particles accumulate in rows on the cell surface, arguing that the virus continues to bind receptors but cannot be internalized in the absence of actin polymerization.

Vaccinia virus (VV) and other large DNA viruses are frequently cited as prototypes for virus infection via micropinocytosis [242] [42] [243]. Vaccinia virions are released from infected cells in two different forms, so called intracellular membrane virus (IMV) and the extracellular enveloped virus (EEV), which differ according to the mode of release and number of surrounding membranes. Both forms of the virus were claimed to enter cells by macropinocytosis, largely based on the use of amiloride, EIPA (see below) that are generally agreed to be inhibitors of dubious specificity. While we agree that the IMV stimulates active signaling and ruffling, in our hands (GG) the EEV (with one additional membrane) fails to activate many signaling events we tested [244]. Extensive EM analysis supports the notion that both viruses fuse at the plasma membrane and release the morphologically distinct nucleocapsid beneath the plasma membrane, including within filipodia projections [244-246]. Such a model is not consistent with uptake during infection by macropinocytosis which should deliver the nucleocapsid to intracellular membranes though we cannot exclude the possibility that the use of different virus strains or cell types may explain these different results'

2C) PROCESS 3: CLATHRIN-MEDIATED ENDOCYTOSIS

After phagocytosis, another well-characterized receptor-dependent mechanism by which eukaryotic cells internalize extracellular material is clathrin-mediated endocytosis (CME), whereby clathrin-coated pits (CCP) assemble on the cytoplasmic side of the plasma membrane, invaginate and pinch off to form clathrin-coated vesicles (CCV; for an excellent historical overview see [88](**See Fig 5).** Whereas in classical phagocytosis the size of the particle determines the size of the phagosome, i.e. the vesicle molds itself around the incoming particle, in CME the architecture of the vesicle, and thus a notional maximum cargo size is determined by the clathrin coat that, in a sense, acts as a vesicle exoskeleton [247, 248]. Plasma membrane-derived CCV are on average around 100 nm in diameter though there may be some flexibility in size, as discussed below [249]. It should be noted that CCV also form on the trans Golgi network [250] and on endosomes [251]; these vesicles are usually smaller (60-80 nm diameter) than those formed at the plasma membrane and will not be further discussed here [252, 253].

While phagocytosis is to a large extent a process for taking in particles such as bacteria, or dead or dying endogenous cells, CME is used by most cells for the selective uptake of smaller molecules and molecular complexes up to ~100 nm diameter. These include nutrients and co-factors (e.g. iron via transferrin; cholesterol via low density lipoproteins), hormones and growth factors and their receptors (e.g. insulin, epidermal growth factor [EGF]) and a variety of other signaling molecules such as G-protein-coupled receptors, [254], as well as viral pathogens and some toxins.

In addition to clathrin, a heterotetrameric adaptor complex, AP-2, is a key component of the coat complex (four other AP complexes, AP-1, -3, -4 and -5, have been implicated in other intracellular trafficking events but are not known to function in CME) [255]**.** Endocytic CCV formation is initiated when the cytoplasmic domains of plasma membrane receptors and PIP2 interact with AP-2 which, in turn, acts to recruit clathrin triskelions. It has also been recently argued that Eps15 and Fcho1/2 initiate endocytosis by forming liquid-like assemblies thereby providing an optimal catalytic platform for endocytosis [131]. Clathrin triskelions are the main component of the bristle-like coat seen in electron micrographs of CCP and CCV (**Fig 5**). As the name suggests, clathrin triskelions are three-legged protein complexes, consisting of three 180 kDa clathrin heavy chains together with three copies of a smaller molecular weight clathrin light chain (CLCA and CLCB). Triskelions can assemble to form polyhedral lattices on the cytoplasmic surface of the plasma membrane. These lattices comprise hexagons, which form flat arrays, and pentagons, which introduce curvature into the flat lattice [88, 247, 248, 256]. The structures, interactions, and arrangements of clathrin and AP2 at the key steps of

coat assembly were recently determined using cryo–electron microscopy, tomography, and subtomogram averaging [257]. Though usually assembled in arrays (CCPs) ~100 up to 150 nm in diameter, that form a single CCV, larger flat clathrin lattice domains are features of the plasma membrane in some cell types [258] [259]).

The mechanism(s) that drive membrane deformation is controversial with two long-standing hypotheses still being debated [260]. According to one model, clathrin triskelions first assemble onto the membrane as a flat hexagonal lattice. Without change in the lattice surface area, curvature is then generated through coat remodeling that introduces pentagons into the lattice. The second model predicts that assembly of intrinsically curved clathrin triskelions onto a forming pit drives the progressive transition from a shallow to a deeply-invaginated pit [261] (see also [139].

Regardless of the specific mechanism, CCP assemble in several well-defined steps. Coated pit assembly involves a plethora of proteins that either form structural components of the CCVs or regulate steps in CCP formation [262]. Assembly is initiated by PIP2-mediated recruitment of AP-2 complexes to the plasma membrane, interactions that are stabilised by binding of endocytosis signals in the cytoplasmic domains of transmembrane receptors, e.g. tyrosine-containing motifs (e.g. YxxØ; where x can be any amino acid and Ø is an amino acid with a large hydrophobic side chain) or dileucine motifs, to recognition sites in the AP-2 mu and alpha subunits, respectively. Subsequently, AP-2 β_2 subunits recruit clathrin triskelions and initiate assembly of the polyhedral clathrin lattice. AP-2 also acts as a scaffold for the recruitment of other clathrin-associated proteins, including Eps15, amphiphysin, CLASP and, in some cases actin, that function to regulate CCV formation and cargo selection [263] [254]. Overall, the interactions of AP-2 with endocytic sorting motifs on cell surface cargo receptors serve to concentrate these proteins in CCVs to facilitate their efficient internalization and the receptor-mediated endocytosis of cognate ligands. By contrast, molecules that lack AP-2 binding signals can be internalized in the CCV lumen, without concentration, and at bulk-flow rates. By contrast, GPI-anchored proteins, and proteins anchored to the actin cortex, are excluded from forming CCPs and undergo relatively slow constitutive endocytosis [264, 265]. For an example of a gold marker that concentrates rapidly in CCP see **Fig.5A**-**E**.

The late stage, highly curved, deeply invaginated CCP cannot pinch off by themselves. Scission is mediated by the GTPase dynamin, which binds to PIP2 in the membrane neck and self-assembles to form a spiral “collar”; coordinated GTP hydrolysis in the dynamin molecules tightens the collar to promote membrane scission, releasing a CCV into the cytoplasm [263]. Dynamin also has Src homology 3 (SH3) proline-rich domains that can bind proteins such as amphiphysin and endophilin; these so-called BAR domain proteins can induce or sense membrane curvature and support dynamin mediated CCV scission [263] [266]. The lifetime of a CCV is in the order of one minute or less; the process of severing a CCV from the plasma membrane is closely followed by disassembly of the clathrin coat, mediated by the chaperone Hsc70, also known as auxilin, and facilitated by synaptojanin, a 5' phosphoinositide phosphatase that acts on PIP_2. After removal of the coat, the naked vesicles fuse with early endosomes (EE), thereby inserting their membrane into the EE membrane and their content into the endosome lumen [249].

Recent work has revealed unexpected complexity in endocytic CCV formation [139]. First, over 60 different proteins [267] [88, 261] and at least four different phosphoinositides [268] have been implicated in CCV assembly, with many of the details still unclear. Moreover, not all plasma membrane CCPs are equal; up to half are short lived (around 20 sec), fail to recruit cargo and spontaneously abort [269]. Additional factors can also regulate CCV formation. For example, some cargo can control CCV formation by regulating receptor ubiquitination; some non-ubiquinated receptors can arrest coated pits after clathrin lattice assembly; dynamin-dependent CCV scission occurs following ubiquitination of lysine residues in the cytoplasmic domain of the receptors, a mechanism that may allow CCP to ‘sense’ their cargo content [270].

Although actin polymerisation is clearly essential for phagocytosis and macropinocytosis, CME was long considered to be independent of actin. This notion was largely based on the inability of actin inhibitors, such as cytochalasin D, to have a significant effect on CME in mammalian cell systems [271]. (Cytochalasin D does inhibit CME from the apical surface of epithelial cells-see above page 11). However, observations in budding yeast showed that local, transient polymerization of actin filaments accompanies CCP/CCV assembly [261, 272]. More recent work in mammalian cells has suggested that actin dynamics can be involved in multiple stages of the CCV cycle, including invagination and the dynamin-associated fission process [171, 262, 273] [274] [117]. However, as in yeast, this actin-dependency appears to be mainly associated with CCV endocytosis from membranes under tension. In the case of polarized epithelial cells, endocytosis from the apical domain, but not the basolateral domain, is actin-dependent, reflecting differences in the tension of the two plasma membrane domains [272]. Nevertheless, in practice actin inhibitors such as cytochalasin D rarely or only moderately impact on CME whereas they generally strongly inhibit phagocytosis and macropinocytosis.

The constitutive formation of CCVs, which occurs throughout much of the cell cycle, drives the highly dynamic turnover of the plasma membrane. CCV formation, from CCP nucleation to CCV scission, takes less than 1 minute. In fibroblasts roughly 0.5-1.6 % of the total plasma membrane surface is involved in CCV formation at any given time [275] [276-278], so that a surface equivalent to the entire plasma membrane is internalized in 1-2 hr. The internal volume of the vesicles so formed is filled with liquid from the surrounding medium, without concentration, and thus contributes to constitutive fluid phase endocytosis (as does macropinocytosis).

Clathrin coated vesicles and NP uptake

In contrast to phagocytosis, there are many claims for NP entering cells via CCVs. It is well documented that many small viruses (≤120 nm diameter), including alpha-, flavi-, adeno-, orthomyxo-, parvo- and picornaviruses, can enter cells in CCVs [40]. The cell surface receptors exploited by viruses normally function to bind physiological ligands or mediate cell-substrate interactions [256] [42, 279]; for example, parvoviruses and the clade B arenaviruses [280] exploit transferrin receptors to enter cells via CME, and vesicular stomatitis virus (VSV) can use LDL receptors [281, 282].

In many respects viruses are excellent models when considering the interaction of NP with cells. As the presence or absence of specific receptors often defines the tropism of viruses for specific cell types, targeting of NP to specific cells and entry pathways can be facilitated by exploiting specific ligand-receptor interactions. Although there are many claims for NP entering cells via CCVs, see e.g. [8, 119, 283-290] [162, 196, 291-294], these conclusions are often based solely on experiments using chemical inhibitors which, as we discuss below, when used alone are unreliable. To clearly establish a role for CME in the uptake of any ligand, multifaceted approaches, using microscopy (live cell fluorescent and EM), as well genetic, biochemical and pharmacological approaches are necessary.

It should be noted that the concept discussed above, that some CCP abort before budding is completed [269], is relevant for claims that NP enter cells by CCV, as association of NP with CCP at the plasma membrane alone may not be sufficient evidence that those pits will be internalized into the cells.

There are convincing EM studies showing uptake of small latex particles and carbon NP that appear to be tightly interacting with the outer surface of CCP (see below). Given that some scavenger receptors can enter cells via CME, for example during uptake of high-density lipoprotein (HDL) [295], and that SR can mediate NP endocytosis via CCV [296-298], it is possible that scavenger receptors (SR) may be mediating these interactions with latex and carbon NP. SR may also be involved in linking particles much larger than 100 nm to giant CCP; [282] showed that clathrin coats

can be extended to accommodate the bullet-shaped VSV particles (70 x 200 nm). In this case, actin filaments appeared to play an active role in facilitating CCP/CCV enlargement.

Fig 5 F shows another example of CCV enlarging to fit a particle bigger than the usual 100nm in diameter. This experiment is from a study where poly-lactic acid (PLA) NP (250-300 nm) were injected into the vasculature of zebrafish embryos. Within 1 minute these particles were found in structures with the typical appearance of (highly enlarged) CCP in the endothelial cells of blood vessels (see [299]. It seems likely that the zebrafish SR, stabilin 2, is involved in targeting the anionic PLA NP to enlarged CCV [300].

It turns out that an earlier EM study described enlarged 'bristle coat' pits and vesicles that were involved in uptake of carbon particles around 30 nm in diameter and latex beads of 111 nm in Rabbit liver and bone marrow endothelium. Intriguingly, these CCP 'collected' multiple carbon or latex NP in large pits of variable sizes (around 200-600 nm) closely resembling CCP. These data argue against the CCP molding itself around a tight-fitting particle but suggests the binding of a group of NP, presumably via a scavenger receptor, signals the pit to expand above normal size [301]; the authors referred to this as 'multiparticle endocytosis'. These observation of multiple small NP (between 88 nm and 550 nm) aggregating into groups adjacent to a large forming endocytic vesicle had been described earlier in *Acanthamoeba* [99] (see above).

2D) PROCESS 4: CAVEOLAE AND ENDOCYTIC UPTAKE

Caveolae are striking flask-like invaginations of the plasma membrane, slightly smaller than CCV (60-80 nm), that are present in many, but not all, cell types and are especially abundant in endothelial cells, adipocytes and muscle (see **Fig 6**). They were first described in EM studies [302] and are now known to be composed of oligomers of a family of integral membrane proteins that has three members, caveolin 1-3 that interact with cholesterol and other membrane lipids [303] [28]. While caveolin 3 is restricted to caveolae in muscle, caveolin 1 and 2 are co-expressed in the majority of adherent mammalian cells.

More recently it emerged that another family of proteins, the cavins (1-4), are also associated with caveolae. Cavin 1 is essential for caveolae formation in muscle and other cells. These peripheral membrane proteins form homo and hetero-oligomers with themselves and interact with caveolins and membrane lipids (particularly phosphatidylserine and PI(4,5)P_2) to form the flask-shaped buds typical of caveolae [28, 304]. In cultured cells caveolae are able to bud from the plasma membrane in a process negatively regulated by the large ATPase, EHD2, and stimulated by lipids [305] [306, 307] [308].

Initially, a number of proteins and infectious agents were proposed to be endocytosed through caveolae, but subsequent work has shown that these pathogens are rarely dependent on caveolae for entry (e.g.[309]). In fact, a consensus is developing that few, if any, surface proteins are dependent on caveolae for their endocytosis [303]. However, caveolar endocytosis appears to be important in fatty acid transport [306]. In addition to budding of caveolae, caveolae can flatten under conditions of stress, releasing the cavins into the cytoplasm and facilitating their interaction with downstream components [310]; [311] [312]. Even though the first observation of caveolae by EM preceded the first description of CCV there are many more open questions about the structure and function of caveolae. Some important features of caveolae relevant to the current article are worthy of mention here. Firstly, relative to many NP, caveolae are small (diameter of the opening estimated to be approximately 50nm [313] [303], restricting access of external agents, yet in many studies this basic consideration is ignored (also see Discussion in [34]). Interestingly, it has been proposed that this limit can be partially overcome by using flexible nanoparticles, expanding the range of particles that could potentially be internalized through caveolae [314]. Secondly, the study of caveolae has been

hindered by the scarcity of caveolar proteins that are exposed to the extracellular milieu and so can be used for labeling by externally administered agents (analogous to the use of transferrin to study uptake via CCVs). In fact, contrary to the conclusions of a number of studies, transmembrane proteins have been shown to be largely excluded from caveolae [315]. An interesting exception is the protein PV1/Plvap, a large transmembrane protein present at the neck of endothelial caveolae in specific tissues *in vivo,* including the lung [316]. The association of PV1/Plap with caveolae *in vivo* has been used to target caveolar endocytic pathways as will be discussed below. A further consideration when studying caveolae in cell culture systems is that they appear to be particularly sensitive to cell culture conditions, as compared to similar cells *in vivo* (see for example [317]), possibly reflecting their ability to respond to environmental conditions such as mechanical stress, flow, and oxidative stress.

The Caveosome

While the fate of internalized caveolae will be addressed below, one important comment is needed. In a publication investigating the uptake of the virus SV40 into cells it was claimed that caveolae fused with distinct endosome-like structures referred to as 'caveosomes'**.** In contrast to early endosomes that have a pH around 6.2, these new organelles had a neutral pH [318]. Almost a decade later, the same group realized that these structures were an artifact caused by over-expression of caveolin tagged with GFP at the C-terminus. They, and others, **advised against using the term 'caveosome'** [319, 320]. Despite these warnings the concept of the caveosome remains very much alive in the NP field e.g. [321-323] [119, 284, 324, 325]; [8, 119, 283, 284, 322, 324, 326-330]. Indeed [330] even claimed that '*Caveosomes are able to bypass lysosomes and therefore protect the contents from hydrolytic enzyme and lysosomal degradation. Hence, pathogens including viruses and bacteria use this entry route to prevent degradation. Since the cargo internalized into the cells by caveolin-dependent mechanism do not end up in the lysosome, this pathway is employed in nanomedicine'*. This is a prime example of important developments in cell biology being mis-understood in the NP field. A quick glance at the majority of endocytic schemes showing NP uptake, e.g. in Google 'Images' for the search phrase 'nanoparticle uptake into cells', reveals the caveosome to be present in a significant number of these diagrams. A similar message was recently published by Skotland and colleagues [331].

Entry of NP via Caveolae: Fact or Fiction

Over 400 papers in PubMed contain the combination of the terms 'nanoparticles' and 'caveolae' and many of these studies claim that NP enter cells via caveolae, e.g. [323]. A large fraction of these papers rely only on inhibitors that the authors consider to be specific for blocking caveolar uptake. The most prominent inhibitor is genistein (e.g. [283, 287, 324, 332, 333]) or filipin, whose incubation with cells. usually followed by cell fluorescence analysis by flow cytometry after trypsin treatment. Another highly questionable practice is to combine results using different inhibitors. If a purported inhibitor of, for example CME, inhibits uptake of an NP by 50% and another inhibitor of caveolae also inhibits 50 %, can one really conclude that half the NP enter via caveolae and the other half via CCV? Proving such a scenario, that is often claimed in NP studies, would demand a serious kinetic analysis by light and electron microscopy, complemented by genetic and biochemical approaches.

Most of these studies used immunofluorescence microscopy to claim that NP enter some cells via caveolae; often the images are shown at magnifications too low to see any details and the small size of caveolae, below the diffraction limit of conventional light microscopy, makes it challenging to show convincingly that caveolae containing NP actually pinch off. We argue that one needs EM to convincingly show that 1. NP are actually within surface bound caveolae at early times in the process, and 2. after a finite incubation time the caveolae bud off to form free vesicles as shown in the classical studies of endothelial caveolae using electron dense markers [334]. The current consensus on budding

of caveolae comes from a series of studies using different sophisticated methodologies and it is not sufficient to use just one inhibitor or a single technique. Many details are given in the review by (Rewatkar, 2015)[34]. One example is the use of ascorbic acid to quench surface horseradish peroxidase (HRP) to discriminate between surface-connected and budded caveolae. In these experiments cholera toxin (CT) labeled with HRP is added to cells for short time periods to label the cell surface, including caveolae. After short times of incubation at 37^0C the cells are cooled to 4^0C and the cytochemical diaminobenzidine reaction is performed in the presence of ascorbic acid revealing only the budded caveolae. This method allowed the unequivocal discrimination of surface-connected caveolae, even those with extremely narrow connections to the extracellular milieu, from internal budded caveolae (see for example [335]. This kind of approach is important because in a single thin EM section (as used in the majority of NP studies) many surface-connected caveolae can appear as 'vesicular' profiles (see **Fig 6).**

Another advantage of using EM is that it is easy to identify CCP in the same sections and to quantify whether these are also labeled to any extent. In addition, genetic approaches to remove caveolae (e.g. caveolin-1 or cavin-1 loss of expression) should be used to inhibit uptake. It goes without saying that before one starts with the above experiments, it is important to establish that the cells of interest actually express caveolin, by western blotting and/or immunofluorescence microscopy. We have found few cell culture studies that satisfy most of these criteria, and it remains unclear whether any untargeted NP enter cells by caveolae (see also the discussion by [39]). However, an interesting strategy introduced in recent years has involved using antibodies to specifically target caveolae [336, 337].

2E) OTHER MECHANISMS OF ENDOCYTOSIS

In recent years a number of additional uptake mechanisms have been identified. These are classified by negative, as much as positive criteria, especially with respect to clathrin and dynamin. Thus, the largest class in this category is defined as clathrin-independent endocytosis (CIE). Since (most) phagocytosis, macropinocytosis and caveolae uptake processes do not use clathrin, we restrict this category here to other non-clathrin vesicular pathways [338, 339]. Although the number of different CIE pathways remains unclear, the mechanistic and functional dissection of one pathway, the clathrin-independent carriers/GPI-anchored protein enriched early endosome compartment or CLIC/GEEC pathway, has progressed with the discovery of a number of key molecules and regulators, such as Cdc42 [340], IRSp53 [341] and galectin-3 [342] and the finding that this pathway is functionally linked to mechanical regulation of membrane tension [343]. This CLIC/GEEC pathway can mediate uptake of GPI-anchored plasma membrane proteins that lack a cytoplasmic domain. This feature, and the dependence on cholesterol (inhibition under conditions of cholesterol perturbation where clathrin-dependent endocytosis is unaffected), [344] has led to the suggestion that this CIE pathway is associated with the specific uptake of lipid microdomains, sometimes termed 'lipid rafts'. It is important to note that cholesterol perturbation alone is not sufficient to conclude that a pathway involves caveolae and/or CLIC/GEEC endocytosis (see for example [345]).

The physical characteristics of the proposed transport vesicles, narrow surface tubules [320] might preclude incorporation of NP above 50-100 nm in diameter but one interesting feature of the CLIC/GEEC pathway is worth raising. Multivalent interactions of extracellular lectin-like molecules with membrane proteins and lipids can drive the formation of tubular CLIC/GEEC carriers and incorporation of the cargo [342]. This can be driven by cellular proteins, such as secreted galectin-3 [342], or by other extracellular agents, including the pentameric VP1 protein of the SV40 virus [346]. Whether NP could, in some cases, mimic these interactions to induce the formation of CLIC/GEEC carriers is an intriguing idea. If so, this pathway would blur the boundaries between classical 'zippering' phagocytosis and extracellular clustering driven CLIC/GEEC endocytosis.

Other CIE pathways are an active area of study. A novel ligand-regulated CIE pathway, termed fast endophilin mediated endocytosis (FEME), was described a few years ago [347]. However, the mechanistic understanding of this pathway is only now being established [348-350]. FEME and other CIE pathways are still being debated by specialists and it is currently too early to know whether any NP enter cells via these routes [351]. Interestingly in this context, a recent paper reports that cationic substances (cell-penetrating peptides as well as physiological cationic molecules) enter cells via an unconventional pathway that depends on the small GTPase Rab14, but not on Rab 5 or Rab 7 [352]. Moreover, to date, these alternative pathways have only been identified and characterized in tissue culture cells, it remains unclear where and to what extent they function *in vivo*

2F) TRANSCYTOSIS

Nanoparticles administered to deliver therapeutics may also need to cross cellular barriers such as the endothelium of the blood-brain barrier and epithelial layer of the blood-intestinal barrier. This can involve membrane transport through the cells, a process termed transcellular transport or transcytosis. Transcytosis across the epithelial barrier mainly involves clathrin-mediated endocytosis, delivery to early endosomes and sorting to the opposite pole, and has been mechanistically dissected through studies of model proteins such as the polymeric immunoglobulin receptor. In contrast, the pathways involved in transcytosis across the endothelium are less well-characterized but numerous studies have implicated caveolae in this process [139, 353]. Analysis of transcytosis is outside the scope of this review but readers are directed to recent reviews on transcytosis that cover aspects relating to molecules that have been shown to transcytose and the endocytic processes and proteins involved [354, 355].

3) THE ENDOCYTIC PATHWAY; ALL ENTRY PATHS CONVERGE ON EARLY ENDOSOMES

A surprising fact about all these different entry routes into cells is that they converge on the same pathway to the endocytic system, i.e. there are many different doors to enter the same house, namely the early endosome network. Here, we provide a brief summary of the endocytic pathway with the aim to help NP researchers understand its compartments and the tools available for its study and manipulation, and to point out the boundaries of our current understanding.

The method that is usually first considered to follow the uptake and intracellular trafficking of NP is fluorescence microscopy. This is a powerful approach which, with the help of genetically expressed markers like GFP, can be applied to live cells. Nevertheless, when used by itself it is limited both in resolution and in being restricted to simultaneously visualizing one to three target fluorescent molecules. Importantly, despite the advances of super resolution microscopy, fluorescence microscopy lacks the framework of reference structures of the cell, in the form of membranes, filaments, ribosomes, etc. that is provided by EM at nanometer resolution. However, for analysis of the endocytic pathway by EM two kinds of markers are needed, mostly based on colloidal gold; one to identify the different compartments usually by antibody labeling on sections, after chemical fixation (immuno-gold), the other as a kinetic tracer taken up by cells to identify the different endocytic compartments (e.g. BSA-gold) before chemical fixation. (**See Figs 3A, 7).** Given that EM currently can only be applied to fixed cells, a combination of live imaging light microscopy, followed by EM (correlative light and electron microscopy – CLEM) can provide a compromise and describe the system at different scales [356].

Four distinct compartments have been identified as being transit stations for material, including NP, that enter cells by receptor-mediated pathways or in the fluid phase. Sequentially, these are 1. Early endosomes (EE); 2. Multivesicular body (MVB [also referred to as endosome carrier vesicles (ECV; 3. Late endosomes (LE) and 4. Lysosomes (Lys) (**Figs 1, 7**). In parentheses in Fig 1 we give rough

estimates for the times, after adding a marker to the medium of cultured cells, for the marker to reach the different stations; these times serve only as guides and can vary between different cell types.

EE form a highly dynamic network of inter-connected cisternae, tubules and vesicles located primarily in the peripheral cytoplasm [276, 357-360]. Numerous observations in tissue culture models showed that these membrane networks are highly fusogenic amongst themselves (**Fig 1**). Markers for EE include EEA1, Rab 5 (the latter is also found on other compartments such as plasma membrane and CCVs, and at least in some cells in LE) [251, 361] [362, 363] and APPL1, a Rab5-interacting protein that labels a distinct population of Rab5-positive early endosomal compartments [364] [365] [363]. In addition, a recent quantitative correlative microscopy study suggests that, in contrast to the current view, EEA1, a PI3P-binding protein and Rab 5 effector, is not solely associated with early endosomes, but also found on Rab5-negative late endosomes [366]. The only way to selectively label EE with an endocytic marker is to restrict uptake of extracellular medium to 2 min, maximum 5 min; longer incubations can result in labeling of downstream compartments. The luminal pH of EE is 6.0 to 6.2 due to the activity of the vacuolar proton ATPase [367] [368] [369] [368]. Tubules that pinch off from EE/recycling endosomes are involved in recycling receptors to the plasma membrane, either directly, or indirectly via recycling endosomes or the TGN [370]. However, it was recently proposed that, after acute ionophore treatment to enlarge early endosomes, the formation of recycling endosome (containing SNX1 and Rab11) occurs during endosome maturation and is independent of Rab5-to-Rab7 conversion [371].

MVB/ECV are distinct vesicles of around 0.3-0.5 μm in diameter, often containing small vesicles (intralumenal vesicles [ILV]) that form by inward budding from the outer membrane [372]. Our EM data suggested that these MVB/ECV detach from the EE network but other interpretations are also possible [251, 359, 372]. These organelles are more acidic than the EE, probably acidified down to pH 5.5 by the vacuolar proton ATPase, and can be rendered positive for the pH dye lyso-tracker [251]. They are also likely to be the stage at which Rab 5 is lost and is replaced by Rab 7 ('Rab-conversion') [373] [374]. MVB/ECV can bind to microtubules and can use the minus end microtubule motor, dynein, to move to the perinuclear regions of cells [375-378]. The directional transport of MVB towards the perinuclear area of the cytoplasm is inhibited by the microtubule depolymerizing drug nocodazole. In addition, when cells are incubated at 15-20°C internalized endocytic markers preferentially accumulate in MVB (and some LE) and further transport towards LE is inhibited [379]. A similar block was described in the melanoma cell line Meljuso fed with the protease inhibitor leupeptin [380].

The small vesicles in the lumen of MVB/ECV form by inward budding, a process driven by the ESCRT machinery [372, 381]. Molecules destined for degradation, such as internalized growth factor receptors, are sorted into the membrane of the forming ILV and degraded at a later stage. A consensus is emerging that the ILVs can have different fates: some are delivered to LE/lysosomes where they are degraded, some may be secreted as exosomes (see below), and some may undergo back-fusion with the endosome limiting membrane, a process highjacked by some pathogenic agents to facilitate their entry to the cytoplasm [372, 382, 383] [384], and presumably used by exosomes after delivery to target cells [385].

MVB/ECV deliver their content downstream by fusing with the next compartment of the endocytic pathway, late endosomes (LE) [386]. These pleiomorphic organelles are enriched in hydrolytic enzymes in their lumen which is acidified by the vacuolar proton ATPase down to a pH of 4.5 to 5.5. The outer membrane of LE has high concentrations of two membrane proteins LAMP 1 and 2 that are heavily glycosylated on the luminal side [387] [382, 388](**Fig 7C**). Another useful membrane marker of LE is the tetraspanin CD63 (LAMP-3) [389]. In contrast to LAMP-1 and 2, which are enriched in the outer membrane of LE, of LE and lysosomes, the final compartment of the CD63 labels primarily the internal membranes [390, 391]. Both sets of molecules cycle between endocytic

organelles, the TGN and plasma membrane, so low but variable levels of LAMP molecules can be found in these other sites. LE and ILV also contain an atypical phospholipid, lysobisphosphatidic acid [LBPA; also known as bis (monoacylglycero) phosphate (BMP)], which is not detected in other intracellular organelles [372, 392]. LBPA/BMP plays an important role in controlling the fate of cholesterol and sphingolipids, which are functionally linked in healthy cells [393], and in sphingolipid and cholesterol storage disorders [394]. LBPA/BMP recruits the ESCRT-associated protein ALIX, which in turn binds ESCRT-III on LE – a process responsible for sorting CD63 and other tetraspanins into ILV exosomes [395].

There is a lot of confusion about the definitions of the endocytic pathway. Both the LE and lysosomes are enriched in lysosomal enzymes and in LAMP proteins and most electron microscopists would likely refer to both as 'lysosomes'; in earlier days, before antibody labeling, both sets of structures stained positive for EM cytochemical stains such as acid phosphatase, then the standard way of identifying lysosomes by EM, e.g. [359]. The main differences between LE and Lys are:

1. LE are heterogenous structures with tubular, cisternal and vesicular domains. Like EE, LE elements are able to fuse together and undergo fission. LE tubules are often closely opposed to elements of the TGN and without markers, and the use of EM, elements of these two compartments are difficult to distinguish. In many cell types lysosomes are mostly spherical vesicles (see Marsh et al. 1986), which cannot be selectively labeled by a tracer from the medium (without also labeling the LE) irrespective of the time of incubation, due to their ability to fuse back to the LE; all luminal markers tend to equilibrate between the two compartments [192] [396]. (**Figs 1, 7).**
2. LE are enriched in the cation-independent mannose 6 phosphate receptor (ciMPR) in many, but not all cell types [397]. In some cells, the ciMPR can be more prominently enriched in the TGN [398]. LE are also generally positive for CD63, LBPA and Rab 7 whereas lysosomes are negative for these markers.
3. Both compartments contain lysosomal enzymes. When separated by gradient ultracentrifugation, the lighter LE has roughly 10-20% of the enzyme activities while the heavier lysosomes have the majority of detectable enzyme activity. Despite this, evidence suggests that LE is the main degradative compartment [399].
4. We have suggested that LE function as the 'stomach' of the cell, equivalent to the classical 'lysosome' of De Duve [400], and that lysosomes are storage organelles for (inactive) lysosomal enzymes which can fuse back with LE [386]. The combination of LE and lysosomes has been referred to as a hybrid organelle, the **endolysosome** [192] [401] [396].
5. LE have a pH of 4.5-5.5. There is a dogma that lysosomes in animal cells, much like vacuoles in yeast and plant cells, have a pH equal to or lower than LE. However, this view has been challenged by observations suggesting that the pH of the lysosome is in fact neutral [396] [402, 403]. This topic deserves more research as well as taking consideration of the possibility that the pH of the LE and/or lysosome can fluctuate between acidity and neutrality [404].
6. Both organelles can interact with and move bi-directionally on microtubules. When cells are acidified to pH 6.3 through acidification of the culture medium, the lysosomes as well as latex bead containing phagolysosomes preferentially move to the periphery of the cell using the microtubule plus-end motor kinesin. This effect is reversed when cells are returned to pH 7.4 medium [405-407].

Different endocytic compartments can fuse with the plasma membrane, this was first described in specialized cells such as cytotoxic T cells, osteoclasts, melanocytes and endothelial cells as 'secretory lysosomes' [408]. Under some circumstances the MVB/ECV can exocytose, fusing with the plasma membrane via their outer membrane and releasing their internal vesicles, now referred to as exosomes, which can modulate cellular functions at a distance from the secreting cell [37]. The contents of LE-lysosome hybrid organelles, (endolysosomes) can also be exocytosed from different

cells in a regulated manner [409], and the limiting membrane of these organelles can contribute to repair when the plasma membrane is damaged [327].

3A) Blending of the phagocytic pathway with the endocytic pathway

The phagocytic process and the blending of phagosomes with endocytic compartments is generally the easiest pathway to follow since, in most cases, clearly identifiable particles, such as bacteria or model particles like latex beads with well-defined shapes and sizes, are used as markers. Phagosomes are *de novo* formed vesicles that undergo a dynamic process of fusion and fission with endocytic compartments [386, 410]. This occurs sequentially, first phagosomes acquire markers of EE, such as Rab5 and acidifying to pH 6 to 6.2, but within one hour phagosomes have all the markers of what can best be considered a hybrid of LE and lysosomes [192, 396] and are termed phago-lysosomes. This maturation implies two processes, first depletion of molecules such as the transferrin receptor, presumably by vesicles or tubes that separate by fission from the main vesicles, and second, acquisition of LE markers such as Rab7, from the cytoplasm, and lysosomal enzymes and membrane markers by fusion with LE and lysosomes [411].

Significant insights into phagosome maturation have emerged from experiments using 0.5 to 3 µm latex bead microparticles [67]. These are powerful tools for microscopy, biochemistry and *in vitro* assays. The 1 µm diameter beads have been used extensively to isolate highly purified fractions of phagosomes from cell lysates by flotation on sucrose gradients. These latex bead phagosomes (LBP) were extensively used for mass spectrometry analysis and in *in vitro* assays for monitoring, 1. Phagosome fusion with endocytic organelles, 2. Binding to, and bi-directional motility of LBP along microtubules, 3. Binding to, and assembly of F-actin de novo by LBP membranes, and 4. The acquisition of different newly synthesized lysosomal enzymes by LBP at times ranging from 20 min to 24h [193, 194, 361, 386, 405, 412-414] [415].

3B) Blending of the macropinocytosis pathway with the endocytic pathway

In contrast to phagocytosis, it is technically much more difficult to follow the fate of macropinosomes in cells following their formation. Nevertheless, the available data argue that the main route macropinosomes take is to sequentially fuse with EE, as seen by transient acquisition of Rab5, then with LE/lysosomes when they transiently label with Rab7, as well as Rab20 (that also associates with phagolysosomes) [416], and then acquire ciMPR and lysosomal enzymes. During the maturation process, which occurs over 10-20 min following formation, macropinosomes can transform into tubules as water is removed and move in a centripetal manner along microtubules towards the perinuclear region [139, 417] [218] [418].

A variable fraction of the macropinosomes that form after uptake are recycled back to the plasma membrane [220]. This is especially prominent in some cell types but not others. The fusion of macropinosomes with late endocytic structures requires septins in concert with a low abundance phosphoinositide PI(4,5)P2 [419]. Septins are filamentous heteromeric GTPases that associate with cell membranes and the cytoskeleton [336, 420]. In general, the maturation process for macropinosomes appears very similar to phagosome maturation.

3C) Entry of clathrin coated vesicles and caveolae into the endocytic pathway

It is well-documented that after the dynamin-dependent release of CCV, the free vesicles shed the clathrin coat and within seconds fuse with the pre-existing early endosome network. Fluid-phase markers such as horseradish peroxidase or colloidal gold for example are thereby delivered to EE within one minute of being added to the cell culture medium and can be visualized by EM [275, 276].

In fibroblasts, caveolae can bud from the plasma membrane and fuse with the early endosome in a Rab5-dependent process, though some of these vesicles may fuse back to the plasma membrane [318, 353]. Colocalization of caveolin-1-GFP with internalized cargo (cholera toxin B) in transferrin-negative structures, distinct from EE, has also been described in NIH3T3 cells [315], possibly representing caveolae clusters that have detached from the plasma membrane. On EE, the caveolar domains form a defined structure, that remain positive for both caveolin-1 and cavin-1, and can then bud off EE to fuse back to the plasma membrane [347] [309]. A mutant form of cavin that stabilizes the cavin coat causes accumulation of 'trapped' caveolae on the surface of the endosome and depletes surface caveolae [312]. While definitive evidence for NP uptake via caveolae and delivery to EE is generally lacking in the scientific literature, the targeted uptake of PV1/Plvap-antibody conjugates into the endosomes of lung endothelial cells, coupled with functional assays for efficacy, is an interesting recent approach [337].

4) PROVIDING COMPELLING EVIDENCE FOR A SPECIFIC PATHWAY: THE PROBLEM OF INHIBITORS

The diversity, but also the similarities, between different endocytic mechanisms has made it difficult to understand how exactly these different pathways operate. Many questions remain difficult to answer, e.g., how many pathways are there in a particular cell type of interest, how do these pathways interact with each other, if at all; if one is able to selectively block one pathway, are other routes up-regulated, do pathways observed in tissue culture cells operate in cells *in vivo*? Such questions are already challenging when the molecule of interest, such as EGF, is well defined, but they are much more difficult to address when one is dealing with, for example, non-physiological polymer-based NP. Here we discuss the fact that there has been a serious over-reliance in the NP field in the use of inhibitors of dubious specificity.

Aside from issues of drug specificity, in the vast majority of studies only one or two concentrations of a drug are used and rarely are the necessary positive controls performed, e.g., showing that a drug inhibits the internalization step expected for this pathway. Also, controls showing whether other pathways are affected are especially important because when one pathway is blocked other pathways may be up-regulated (e.g.[421, 422]. Drugs are often used for long periods and in serum-free medium, adding additional problems for interpretation of their effects.

There are a large number of pharmacological-physiological inhibitors that are popular with NP researchers who are convinced that these perturbants block specific pathways. A rigorous analysis, by different authors, has made a compelling case that all of these reagents have effects well beyond what is claimed (e.g. see [30-32, 34, 422-424]. Unfortunately, there is little evidence these warnings have had much impact in the NP field. Applying these drugs is apparently too convenient and too easy and there is a culture of accepting their specificity at face value!

Several of these inhibitors target CME; starting with treatments that have global effects on cells, such as hypertonic sucrose, potassium depletion or cytosol acidification. Then there are drugs that are claimed to be somewhat more selective for CCV such as **chlorpromazine** (used at 10-50µM), **phenylarsine oxide** (10-20µM) and **monodansyl cadaverine**, the latter used at a whopping 5-10 mM. As an example, we can highlight the problems with chlorpromazine. The mechanism by which this cationic molecule, a member of the phenothiazine class of anti-psychotic drugs, inhibits the clathrin pathway is still unclear although there are suggestions it affects dynamin (I and II) [88]. Since dynamin is involved in additional uptake mechanisms besides clathrin (see below) this drug cannot be used as a specific inhibitor of CCV formation. Also, claims that chlorpromazine inhibits clathrin recruitment, or the binding of clathrin to AP-2 are not supported by careful studies [88] [88]. A systematic study in several cell types found that relatively short incubations (2h) with chlorpromazine

could significantly decrease cell viability [424]. These studies also provided evidence that some other endocytic processes were affected by this drug, including phagocytosis in macrophages and neutrophils [425, 426]; this may reflect the observation, discussed above, that clathrin is sometimes essential for phagocytosis.

Dynasore is another popular putative CME inhibitor that is claimed to inhibit the GTPase activity of dynamin, thereby blocking CCV scission [427]. However, as already mentioned, since dynamin is involved in both CCV and caveolae formation it cannot be a specific inhibitor of the clathrin pathway. Dynasore may also inhibit the interactions of dynamin with the actin cytoskeleton [31]. [428] summarized further studies arguing that dynasore also affects cholesterol organization in membranes. A recent study by [429] made a rigorous comparison of three new generation inhibitors of dynamin - dynasore, dyngo 4a and dynole – and came to the conclusion that all three had significant off-target effects. Given that dynamin is involved in multiple pathways this makes it of questionable value to target this molecule for NP uptake analysis.

In a comprehensive and critical evaluation of a range of inhibitors of CME, [422] point out that essentially all the available chemical inhibitors that are claimed to be specific for different endocytic uptake processes are not specific in the sense that most affect more than one pathway. This is certainly the case for hypertonic sucrose, potassium depletion, cytosol acidification, chlorpromazine, monodansylcadaverine and phenylarsine oxide. Considering that clathrin and CCV are also involved in trafficking at the TGN, EE and LE, besides the plasma membrane, and that clathrin is also involved in some phagocytic events (see above), results observed in even complete genetic knockouts of clathrin should be regarded cautiously.

Inhibitors assumed to block caveolae internalization are also riddled with problems. The first of these is genistein, a general inhibitor of tyrosine kinases, which has pleiotropic effects on CME, macropinocytosis and the morphology of the cortical actin cytoskeleton**. Methyl-beta-cyclodextrin** is a compound that extracts cholesterol from membranes and is widely used to inhibit caveolae vesicle uptake. Not surprisingly, this approach is not specific for caveolae; it also inhibits the CLIC/GEEC pathway and the FEME pathway. Acute cholesterol depletion can further inhibit uptake of transferrin, as well as some GPCRs, by CME [430] [345] . Methyl-beta-cyclodextrin extraction of cholesterol also has drastic effects on the actin cytoskeleton [431]. Nystatin, another cholesterol extracting agent, is also used as an inhibitor of caveolae but similarly affects CME [351]. At least for FEME and caveolae, the knockdown or knockout of key structural components (such as endophilins for the FEME pathway, caveolins or cavins for the caveolar pathway) can provide more definitive evidence for the role of these specific pathways in endocytosis.

There are no selective inhibitors of phagocytosis or macropinocytosis (Ivanov 2008). Cytochalasin D (CCD) and latrunculin, which target actin assembly, block both pathways. The sodium-proton exchanger inhibitor, **amiloride** (used in the mM range) and its derivatives such as 5-ethyl-N-isopropyl amiloride (EIPA- used at 50-100 μM), are widely used inhibitors of macropinocytosis. However, whereas amiloride inhibits macropinocytosis in macrophages, it has no effect on this process in dendritic cells [117]. Moreover, both compounds also inhibit phagocytosis [32]. Similarly, PI3kinase inhibitors, such as wortmannin, affect both processes. It has been claimed that the tricyclic anti-depressant drug imipramine is a selective inhibitor of macropinocyosis, and has no effect on phagocytosis or other uptake mechanisms [327] [117]. However, a number of studies have shown that this drug, which has been used since the 1950s, has many pleiotropic effects. It's best characterized effect is to inhibit the reuptake of norepinephrine and serotonin, but it affects a number of other receptors [432] and other diverse functions ranging from acid sphingomyelinase to phagosome function and effects on the actin cytoskeleton [433] [434]. Additional critiques on the use of inhibitors can be found in many reviews [31, 34, 39, 351].

We have made a survey of 22 semi-randomly selected NP papers in which inhibitors have been used to identify different endocytic pathways and realized that the problem of inhibitor use runs much deeper than the simple fact that almost all the commonly used drugs lack specificity (an exception would be cytochalasin D). The poor appreciation of basic cell biology becomes apparent in misconceptions related to the inhibitors (see **Box 4**). For most of the drugs there is not even a consensus as to what process (under ideal conditions) each drug is supposed to inhibit. There is also no consensus as to the range of concentrations at which specific drugs should be used. The minimum and maximum concentrations for some inhibitors can vary up to 20,000 times between studies (Box 3).

5) QUANTIFYING NP ENTRY: THE NEED FOR CAUTION WITH TRYPSIN AND FLOW CYTOMETRY

It is far from trivial to show convincingly that an exogenously applied NP that enters cells does so by a particular pathway. Even before trying to identify a pathway, it is important to be able to distinguish between NP or MP that have truly been internalized versus particles that are still on the cell surface. In most cases particle uptake is quantified by flow cytometry (PubMed has 4385 articles for the combination of NP and flow cytometry). This approach has two potential problems: 1. The trypsin problem and 2. Distinguishing between attached and ingested NPs.

For the trypsin problem cells that are attached to a substrate need to be released prior to the flow cytometry step. Trypsin, a serine protease that can disrupt protein interactions between the cells and their sub-stratum, is routinely used at 37^0C. Often trypsin is combined with EDTA that chelates divalent cations. Obviously, proteins on the cell exterior are susceptible to proteolysis and this has been well documented (e.g., [435]. Clearly, this is a harsh procedure and changes in cell shape are evident for all to see - they round up; when analyzed by thin section EM, trypsin-treated cells appear pathological and are heavily vacuolated, though with time they can recover [436](Our unpublished data). A number of studies have described the formation of prominent cell surface blebs [437, 438]. Trypsin has even been claimed to enter the cytoplasm of cultured cells [439] and in the older literature it has been associated with many pathological changes, including intracellular activities such as incorporation of radioactive thymidine into DNA (See references in [439]. With respect to NP, it seems reasonable to assume that this treatment will remove some surface bound particles, but this will vary for different NP.

Trypsin can inactivate some surface receptors while leaving others unperturbed. Whereas macrophages have receptors that allow them to bind and phagocytose glutaraldehyde-treated erythrocytes, as well as F(ab)2 antigen complexes, following trypsin treatment they lose this capacity. However trypsin had no effect on their ability to bind and phagocytose latex or zymosan particles [188]. A proteomic analysis identified 36 cellular proteins that were affected by trypsin treatment. There was a preferential down-regulation of proteins regulating cell metabolism and growth while proteins controlling apoptosis were up-regulated. Whereas some downregulated proteins recovered their expression levels in a few hours, others failed to recover even after 24h. Changes in protein levels at the cell surface can be expected but many of the proteins whose levels were altered were intracellular proteins, e.g. mitochondrial proteins. The authors concluded that trypsin treatment induced a stress response in the cells [440]. An alternative enzyme one can consider for removing cultured cells more gently is proteinase K on ice. Although this is a more potent protease than trypsin when used at 37^0C, when used at 4^0C the cells maintain their shape and do not round up as occurs with trypsin (see [250] [441].

The second problem is that standard flow cytometry cannot distinguish between surface attached and internalized particles [442]. This is especially a problem when surface particles are located within PM invaginations (for an extreme example see [443] and **Fig 7**. However, techniques are available that can selectively quench a signal due to surface bound NP. For example, trypan blue will

selectively quench fluorescein-labelled cell surface NP when used at 4°C, a temperature that will prevent endocytosis and prevent trypan blue access to intracellular NP [444]. An interesting advance in this area is the development of sensor systems compatible with both flow cytometry and light microscopy for quantitating endocytosis [15, 445]. A feature of these systems is that when used for flow cytometry the quencher is added while the cells are adherent and before addition of trypsin. Unfortunately, the use of such assays still appears to be an exception in the NP field.

6) ESCAPE OF NP FROM ENDOCYTIC ORGANELLES INTO THE CYTOSOL

After entering the endocytic pathway an important question associated with NP uptake is whether the particle, or parts thereof containing the cargo, can gain access to the cytosol? It can be argued that, from the broader perspective of how NP interact with cells, this question is more important than understanding the precise mechanism by which NP enters the cell. Endosomal escape remains a major challenge and bottleneck for many therapeutic cargoes, from relatively small siRNA through to mRNA and plasmid DNA. The same is true with strategies for delivering peptides and proteins targeting intracellular processes in the cytosol or an organelle. For NP that deliver DNA into the cell, the DNA must first enter the cytoplasm and then the nucleus. The process of endosome escape should prefeRably occur without activating innate immune responses [446] [447] [448] and obviously without too much disruption to cell physiology.

A broad spectrum of reagents has been used in conjunction with the cargo-carrying NP to facilitate the escape of the cargo from the endocytic pathway to the cytosol. The best known of these compounds are cationic polymers, fusogenic lipids (discussed below) and cell penetrating peptides (CPP), usually 5-30 amino acid residues that are either cationic or amphiphilic [449, 450]. Other prominent systems include virus like particles (VLP) and bacterial toxins [451]. The mechanisms by which these reagents function in facilitating escape into the cytosol is complex and still actively being deciphered. We will restrict our discussion of these issues here to a few general remarks (below) in order to focus more on the basic cell biology issues.

One key question is whether the NP or its content can cross the membrane of endosomes or lysosomes, and if so, how efficient is this process relative to the total number of NP entering the endocytic pathway [447]? This is a much more difficult question to address than is often realized in the nanomedicine field and many of the published claims are not convincing. Two extreme scenarios can be envisaged. In the first, the intact NP crosses the membrane of the endocytic compartment and the polymer, or other scaffold, is degraded in the cytosol to release the active molecule. In the second, the NP scaffold is broken down in the endocytic pathway and the active molecule has to traverse the endosome or endolysosome membrane barrier.

6A) The complexity of the endocytic organelles

First, we recapitulate the main points about the endocytic pathway with an emphasis on those aspects that are most relevant for 'endosome escape'. This pathway at a minimum comprises early endosomes, including recycling endosomes, endosome carrier vesicles/MVB and the endolysosome compartment, the amalgamation of LE and lysosomes.

NP that have been fully internalized by cells will almost invariably end up first within the lumen of EE (pH 6 -6.2). From there, unless the NP have information (such as transferrin on their surface) that can direct them into a recycling pathway back to the plasma membrane, or the TGN, the default pathway will carry them into the lumen of the late endosomes/lysosomes within 5-20 min of entry [452] [453] [39]. This means that if NP or their active molecules have to escape from EE they only have a few minutes to do so before they are carried downstream to ECV/MVB and then to endolysosomes [276]. The latter compartment has a lower pH, down to 4.5, and can contain in the order of 50 acid hydrolases [454]. The boundary membrane of this compartment has a high density

of the two heavily glycosylated integral membrane proteins LAMP 1 and 2. When NP or their cargo need to escape from this structure, this dense carbohydrate coat presumably acts as an additional barrier besides the membrane to limit escape. As discussed in Section 3, there can be significant heterogeneity in all the endocytic compartments, especially the endolysosomes, that is still poorly understood. Markers such as lysotracker (for low pH), LAMP 1 and 2, CD63 and fluid phase markers that are taken up from the medium do not uniformly label individual organelles or profiles of the same compartment as might be expected [226].

6B) The available tools to monitor endosome escape

In contrast to the serious problem of identifying the mechanisms by which NP enter cells, there are potentially more reliable assays available for monitoring endosome escape. These rigorous assays rely on a signal being registered only when a component in the NP has access to a partner component that is permanently in the cytoplasm or in the case of plasmid delivery, in the nucleus. These assays can be roughly separated into, 1. A fluorescence-based positive signal (or its knockdown), read either by microscopy or by flow cytometry, 2. A biochemical assay, and/or 3. A functional/physiological readout, such as apoptosis. To aid the subsequent discussion, we briefly summarize examples of the first two types of assays. For examples of assay type 3 see [447].

Microscopy assays

Before we briefly discuss recently developed assays, it is important to mention that a large number of studies have used unsatisfactory approaches based on perceived differences in the fluorescence pattern between 'endosomal'(vesicular) and 'cytosolic' signal (discussed below). Other studies rely on assessing dyes such as calcein, a membrane impermeable dye that appears punctate when concentrated in endosomal compartments but diffuse in the cytosol, as an indicator of endosomal escape. Again, assessment of escape appears somewhat subjective and provides only an indirect indication of endosomal leakiness rather than assessing escape of the particle of interest.

Many investigations have used chemically fixed cells for microscopical analysis of endosome escape. In a comprehensive overview, [447] summarized the potential artifacts associated with standard fixation protocols for light microscopy, using paraformaldehyde, or more harsh, methanol. We agree with their statement that 'only live cell imaging should be used to examine subcellular localization of a cargo or molecule of interest'. A striking example of artifacts induced by paraformaldehyde fixation was described in two seminal papers on cell penetrating peptides, delivering either a protein [455] or a fluorophore [456]. Whereas in living cells the fluorescent conjugates localized to an expected pattern for endocytic vesicles, after fixation with paraformaldehyde or methanol and compromising of cell membranes, the signal was seen associated with the nuclei due to the artefactual binding of peptides to DNA [455]. More details are given in [1] and [447].

Notwithstanding, there are a number of interesting systems that allow one to identify the transfer of fluorescent molecules from the lumen of endocytic organelles to the cytosol and also to monitor the response of the cell. In an impressive recent super-resolution microscopy study, Zerial's group was able to clearly resolve different functional domains in the early endosome and to provide evidence that RNA delivered to cells within liposomes was segregated into the recycling tubules of EE from where the RNA crossed into the cytoplasm [363].

Galectins.

Galectins are a family of 15 mammalian proteins expressed in the cytoplasm of many cell types. They function as lectins that bind β-galactoside sugars such as N-acetylglucosamine. These sugars are assembled onto glycoproteins and glycolipids in the lumen of the biosynthetic pathway and are normally not exposed to the cytosol. However, when membranes of endocytic organelles are perturbed, their luminal surfaces can become transiently exposed to the cytosolic milieu and are

available to bind one of these lectins. Using a GFP-tagged galectin, ideally expressed in stable cell lines [457], transient exposure of endosomal β-galactosides on the luminal face of the endosome membrane due to its rupture generates a punctate distribution of fluorescent galectins and is increasingly being used as a marker of endocytic organelle integrity following incubation of cells with NP [458] [459] [460, 461]. Although this can indicate some loss of endosomal integrity, it remains to be determined to what extent membrane damage is needed for galectin recruitment and how this relates to the transfer of NP and NP cargoes out of endocytic organelles.

An impressive example of the use of live cell imaging that combined a galectin with other markers is a study by Wittrup and colleagues [458] in which lipid NP carrying siRNA aimed at downregulating cytosolically expressed GFP were delivered to cells. The passage of Alexa 488 labeled siRNA to the cytosol was linked to redistribution of YFP-galectin 8 to the escape sites, first detected in less than 15 mins after NP entry into cells. Further combinations of GFP family labels revealed that the endosome stage involved could be mapped to a post EEA1, Rab5-positive stage, that preceded the late endosome Rab7-positive stage (**see Fig. 1**). This is one of the few studies where the kinetics of release agree with established kinetics of trafficking through the endocytic pathway and shows that lipid NP and/or their cargo can escape from endosomes relatively early after entry into the cells.

An important caveat: Galectins are also secreted from cells by non-classical secretion across the plasma membrane. They can then be endocytosed into the endocytic pathway, precisely the very sites where should not be localized for the purpose of analyzing endolysosomal membrane integrity [462]. However, another body of evidence argues that galectins can assemble into lattices that prevent their entry into cells. For a comprehensive summary of the literature see [462, 463]. Clearly these findings need to be taken into consideration in studies of NP escape that use galectins as markers of membrane damage.

Split GFP and other proteins

Another elegant assay for endosomal escape takes advantage of the fact that GFP is composed of 11 beta strands that form a barrel structure. When one of the strands (11-16 amino acids, termed M3) is removed, the remaining molecule (usually residues 1-214 and depicted as GFP 1-10) is non-fluorescent. When the two parts (M3 and GFP 1-10) are allowed to interact the fluorescent GFP molecule is reconstituted by self-assembly [449]. In an escape assay, the M3 peptide associated with an NP is delivered to cells expressing GFP 1-10 in the cytosol. If M3 enters the cytosol it will combine with GFP-1-10 to generate a fluorescent protein that can be quantified by microscopy or flow cytometry [449], A similar approach has been applied to split-luciferase and other reporters (see [447] [464]. In the latter publication, the addition of a nuclear localization signal allowed endosome escape and nuclear delivery to be monitored. A recent study described the development and application of an elegant modified split-luciferase approach, termed SLEEQ, to provide quantitative data on endosomal escape relative to cellular uptake [312]. Endosomal escape varied between the cell types studied from <2% of the internalized marker to approximately 7%. In another study aimed at delivering siRNA within liposomes, only 1-2% of the NP could escape into the cytoplasm and only for a limited period [465].

Flow cytometry-based assays

Fluorescence-based flow cytometry assays, as routinely used, cannot distinguish between signal at the cell surface, inside endocytic organelles or in the cytoplasm (unless combined with specific endosomal escape sensors, e.g. [466]. Hence alternative assays are essential.

Biochemical assays

For measuring whole protein delivery at the population level, assays have been developed based on, for example, biotinylation of cytosol-located proteins that are then detected using streptavidin pull-down [467]. One such assay is based on expression in the target cell cytosol of a prokaryotic biotin-

ligase (BirA) that can biotinylate a short 15 amino acid peptide (avi tag). Avi-tagged, NP associated, proteins that gain access to the target cell cytosol will be biotinylated by BirA and can then be quantitated following cell lysis and streptavidin pull-down [447].

6 C) The mechanism of escape

A spectrum of different molecules ranging from highly charged polymers such as polyethylenimin (PEI) [468], fusogenic lipids in liposomes or lipid nanoparticles (LNP), to CPP [321], has been attached in various ways to NP to facilitate escape from endocytic organelles. A popular strategy to facilitate delivery involves fabricating NP with molecules that respond to cellular environmental cues, such as the low pH encountered in endocytic organelles, to become membrane-lytic. Alternatively, NP that can respond to exogenous triggers, such as light [469] or increased temperature [470], have also been developed.

PEI has been the prototype cationic polymer for drug delivery applications and for the 'proton sponge' hypothesis. This posits that the enrichment of secondary and tertiary amines in PEI allows the polymer to buffer protons in endosomes and lysosomes. This is claimed to lead to more protons being pumped in which requires an influx of chloride counterions and water molecules leading to osmotic swelling and rupture of the organelles, thereby allowing the NP/cargo access to the cytosol [471]. This hypothesis has been strongly contested [472] and alternative mechanisms have been proposed that involve direct charge driven interactions of the cationic molecules with membranes that induce lipid reorganization. The idea here is that the overall effect on membranes is a less aggressive disruption than with the proton sponge mechanisms (that predict total lysis) to cause escape through pore formation [473].

Extensive analysis in artificial membrane systems of hundreds of different cell penetrating peptides (CPP) that are able to facilitate endosome escape of cargo such as siRNA, have led to different theoretical models that try to explain how the peptides first interact with the host cell membranes to cause some degree of destabilization leading to pore formation and translocation of the cargo [474]. Importantly, recent studies provide compelling evidence that CPPs increase uptake of target proteins through increased endocytosis, not by enhancing endosomal escape [312]. This study offers a different explanation of endosomal escape in which natural leakiness of endosomes can be exploited for delivery to the cytosol by increasing non-specific adsorption to the plasma membrane and delivery to the endosome. Nevertheless, it is difficult to be completely certain that any individual CPP acts in endosomes and not on the plasma membrane.

NP mixtures of liposomes and polymers have also been described [475]. For nucleic acid delivery, the polymers are often used together with cationic lipids, such as 1,-Dioctadecenoyl-3-trimethylammonium-propane (DOTAP), that promotes encapsulation of negatively charged DNA or RNA [476] [477]. Dioleoylphosphatidylethanolamine (DOPE) is a typical 'helper' lipid which, at low pH, causes destabilization of the liposome and fusion with the organelle membrane to allow release of the cargo into the cytosol. Though extensively characterized in artificial membrane systems, the interaction of these systems with endocytic organelle membranes still needs to be elucidated to identify those features that are critical for transfection. This is extremely challenging as the spatio-temporal environment inside the endocytic organelles is poorly characterized, including the lipid/protein composition of the organelle membrane and their sub-domains. Despite this paucity of information, dynamic simulations have been usefully applied to model endosomal escape of lipoplexes containing, e.g. DOTAP/DOPE, based on a very simple endosomal model [478]. Even in this simplistic model the number of variables that can potentially affect transfection is high and one hesitates to imagine how more complex the situation is in real cells. For recent discussions on CPP see [312, 479-481]

6D) The site of NP escape

In surveying the literature, we identified a number of problems associated with interpreting the available data on NP escape from endocytic organelles:
1. There is a general trend to refer to this process as *endosomal* escape, that is generally interpreted as being *pre-lysosomal* (or pre-endolysosome). The assumption appears to be that NP *must* escape *before* entering low pH, hydrolytic environment of LE/lysosomes; in many cases the escape site is referred to as EE, but there is little evidence to support this claim [131]([482] [449] [483] [484]. This is compounded by the common strategy that involves allowing NP to internalize for many hours, a condition that we expect to fill the endocytic pathway and accumulate the bulk of NP in the LE/lysosomes (see References in Box 3). Only a few studies have investigated the location of NP in endocytic pathways at early time points (i.e. <15-30 mins uptake); for example, of the 22 references cited in Box 3 only three monitor NP uptake in times less than 30 min. In practice, a wide array of particles (especially gold NP) and both fluid phase and receptor-mediated markers added to cells are found to traverse EE in a few minutes and by 15-45 min have reach LE/lysosomes [276, 452, 485-488]. If NP enter cells relatively slowly and inefficiently, it may be necessary to monitor their uptake first by live cell imaging, using assays that allow surface bound NP to be distinguished from NP that are in the cytoplasm [444].

2. When one analyses cells by fluorescence microscopy, using endocytic markers or compartment markers the most prominent labeled structures are *vesicles*. In the NP literature this leads to the erroneous conclusion that the endocytic pathway consists *only* of vesicles, and is often simplified into two classes, endosomes and lysosomes (e.g. [489] [490]. It is a common misconception that endosomes, (assumed to be vesicles) are discrete particles that can be counted. Detailed EM analyses of the endocytic pathway using multiple markers reveals that both EE and LE are an interconnected network of vesicles, cisternae and tubules that can be quantified using stereology, either as surface area or volume, but not number [276]. The tubules, and especially the cisternae are less prominent than the vesicles by fluorescence microscopy and are evident as a diffuse signal. A consequence of this is that, at the light microscopy level, the EE and LE (and the endolysosomes) do not have strictly defined boundaries, as erroneously concluded in many publications. Based on that misconception, many studies have measured endosome escape of fluorescent NP by assuming that after internalization, but before escape, one can quantify the signal by focusing on the ‘vesicular’ signal. Then, after escape, one aims to quantify the ‘diffuse’ fluorescent signal that is supposed to represent the ‘escaped cytosolic pool’ [448]. In contrast to the EE and LE, the ECV/MVB and the ‘real’ lysosomes are predominantly vesicles that can be counted [357] [276, 386] [487] [358, 491].

3. The cytosol represents the cellular space in between cytoplasmic membrane-bound organelles, excluding the nucleus. That the common practice of identifying the cytosol by light microscopy is a fruitless endeavor is illustrated in **Fig 8**. Whereas the boundaries of the cytosol cannot be defined by light microscopy, including super-resolution microscopy, by thin section (~60nm) electron microscopy, where resolution can be better than 5nm, the boundaries imposed by membranes can be defined. This example shows a vascular endothelial cell in a 5 day old zebrafish larva that had a mixture of cationized ferritin (electron dense core, 5 nm) and BSA-gold (10 nm) injected into the caudal vein 2h before fixation and embedding. Both markers enter endothelial cells via clathrin-mediated endocytosis, with BSA-gold entering via receptor-mediated endocytosis (most likely via a scavenger receptor Stab 2; [300] and ferritin that enters cells via fluid phase endocytosis (see **Fig 7**). Both markers label the entire endocytic pathway (**Fig 8A** drawn in red). Other cytoplasmic membrane organelles are colored in blue and the cytosolic compartment appears in green. Imagine the hypothetical situation where a red fluorescent marker has escaped from the endocytic compartments into the cytosol (**Fig 8B**). The green cytoplasm is now indicated in a light red whereas the intensity of the signal in the ‘red’ compartment is slightly lower, consistent with some loss of the signal to the cytosol. If the fluorescence signal could be quantified at this level of resolution it would be an extremely demanding task to estimate the light red signal that is exclusively in the cytosol, especially when (as with fluorescence microscopy) all the (non-labeled) organelles are invisible!

4. The above example assumes a situation where a fluorescent marker uniformly traverses the boundary of all endocytic compartments. In a real experiment it has been estimated that only a few 'events' of escape are visualized over a period of many hours [458, 465]. This makes it even more difficult to quantify the cytosolic pool. For cationic lipid formulations endosomal escape and membrane interaction at the cell surface can most likely coexist.

5. **Fig 8** demonstrates another problem when one restricts the analysis of NP uptake into cells to using light microscopy. In yellow one can see deep invaginations of the plasma membrane of the endothelial cell into the cytoplasmic space. At the level of light microscopy or flow cytometry, a NP within these domains (i.e outside the cytosol) could easily be mistaken to be intracellular.

6. There is a general tendency in the cell biology field, even in the most prominent journals, to publish fluorescence images of cells at magnifications that are too low to recognize details that are described. In the NP field this problem is invariably much more serious and it is very common to see images of arrays of cells to illustrate uptake or escape of NP where each cell is about 1mm or less in diameter and one is expected to recognize intracellular structures!

6E) Bacterial and viral escape from endocytic organelles

There are many intracellular pathogens that enter cells via endocytosis and then access the cytosol by crossing the membrane of an endocytic organelle. These pathogens can provide us with tools and concepts that can be applied to NP to improve cargo delivery to the cytosol. The pathogens range from the size of large macroparticles to the smallest NP [492].

The large particles are bacterial pathogens, best exemplified by *Listeria monocytogenes* and *Shigella flexnieri* that enter cells such as macrophages and epithelial cells by phagocytosis. This leads to temporary residence in a phagosome (Shigella turns out to be more complicated, as discussed under 'Macropinocytosis' above) where the bacteria secrete a cholesterol binding, low pH-activated, membrane permeabilizing protein, exotoxin- Listerolysin O (LLO) in the case of Listeria and Ipa B in the case of Shigella. The detailed mechanisms of lysis of the phagosomal membranes are still being debated but there is no doubt that a significant fraction of, initially phagosomal, pathogens end up in the cytosol where a dramatic next stage in the life cycle is the nucleation of actin filaments on the surface of the bacteria that can propel the pathogens from one cell to the next [492]. Examples using this concept for enhancing NP escape from endosomes in conjunction with liposome carriers or with cationic carriers are described by [489, 493]

Viruses also offer a rich source of tools and concepts for endosome escape. Many viruses contain a membrane, or 'envelope', that encloses and protects the viral genome during the extracellular phase of the viral life cycle. The envelope contains virally encoded fusion proteins that sense cellular cues during engagement with a cell and use these cues to activate membrane fusion whereby the viral membrane fuses with a cellular membrane to deliver the viral genome to the cytosol of the cell. Enveloped viruses, such as the alphavirus, Semliki Forest virus (SFV) or influenza virus (Flu), can enter cell by receptor mediated endocytosis in CCV. Subsequently, the acidic pH in the endocytic pathway can activate fusion, in EE for SFV and in later compartments for influenza. Other enveloped viruses can pick up on different cellular cues. For HIV, co-receptor binding triggers membrane fusion at the plasma membrane or in endosomes; for filoviruses, such as Ebola virus, following endocytosis, proteolytic cleavage of the fusion protein by LE/lysosomal proteases and engagement with a LE/lysosomal membrane protein (NPC1) is required for fusion; while bunyaviruses may be activated by high endosomal potassium levels [494] [40, 492, 495]. Incorporation of viral envelope proteins into liposome membranes can provide a mechanism for NPs to exploit the same pathways and cellular cues to mediate cargo delivery to the cytosol.

Although the endosomal escape mechanisms for non-enveloped viruses are not as well understood as those of enveloped viruses, non-enveloped viruses are nevertheless interesting for the fabrication of NP. Non-enveloped viruses (such as adenoviruses, papillomaviruses and picornaviruses [e.g., poliovirus]), for which the genome is protected within a protein shell, are also internalized by receptor mediated endocytosis, often in CCVs, as described for enveloped viruses [40]. Since viruses such as the adenoviruses or picornaviruses are devoid of a membrane, they cannot fuse with the endosome membrane, and have evolved alternative strategies to escape endosomes [495]. As with enveloped viruses, non-enveloped viruses exploit cellular cues to induce conformational changes in their surface proteins that can result in pore formation, e.g., picornaviruses, calciviruses [496], activation of lytic factors (e.g. adenoviruses [497] and lipid modification [457]. Each virus family appears to have its own mechanism of membrane disruption. For example, parvoviruses, small ~25 nm diameter DNA viruses that include adeno-associated viruses and are frequently used in gene therapy applications, enters EE where one of their two proteins (VP1), which has phospholipase A2 activity, is exposed following a conformational change induced by exposure to the low pH in EE. This PLA2 activity likely induces localized modification of EE lipids that facilitate penetration of the EE membrane. By contrast, the simian polyoma virus SV40 (~45 nm diameter DNA viruses) has information that directs particles from EE to the ER, where disulfide exchange reactions and interaction with the ER-associated degradation machinery have been implicated in escape to the cytosol [498]; see also [499].

6F) Mechanisms of repair of lysosomes/endocytic organelles

In the past decade it has emerged that when the plasma membrane or membrane-containing organelles are compromised, for example through mitosis-associated fragmentation, physical stresses or as a consequence of engagement with bacterial or viral pathogens, cells have evolved sophisticated mechanisms to take care of the damage. Although it is too early to tell, there is a possibility that the major goal of fabricating NP whose cargo can cross cell membranes can be compromised by these membrane repair mechanisms.

For endocytic organelles, cytoplasmic lectins, the galectins (discussed above), play a key role in enabling the cell to identify sites of organelle damage via exposed lumen associated N-linked oligosaccharides to the cytosol [500]. [381, 457, 501, 502]. Recent evidence shows that endosomal membranes can be repaired by the Endosomal Sorting Complex Required for Transport (ESCRT) [503] [504] [505] [395]. This is an evolutionary-conserved machinery composed of four protein complexes (ESCRT-0, -I, -II, -III), and the AAA-ATPase Vps4 and accessory proteins, which drives intracellular membrane deformation processes that occur away from the cytoplasm in a direction opposite to most membrane traffic events that bud into the cytoplasm [501, 502] [457]. Biophysical studies of the yeast ESCRT III protein, Snf7, have identified the principle that ESCRT III proteins can polymerize to form spiral filaments on membrane surfaces that somehow apply a spring-like force to drive membrane bending and fission [506, 507] [508].

There appear to be two different but related strategies that the cell use to deal with damaged membrane organelles [502]. Most of these studies have focused on the terminal compartments of the endocytic pathway, the endolysosomes (EL), but it seems highly likely that the same machinery can also operate earlier in the pathway. A consensus is emerging that when the membrane of these compartments is compromised in a minor way, the cell can ‘patch up’ the ‘leak’ by first releasing calcium from endocytic organelles into the cytosol. [509] focused on three pathogens known to escape from the endocytic pathway into the cytosol, *Listeria monocytogenes, Mycobacterium tuberculosis* and *Candida albicans* and discovered an ESCRT repair mechanism shared by cells infected with these pathogens and the endolysosome damaging di-peptide, L-leucine, L-leucine methyl ester (LLOMe; see below). They identified a kinase, Leucine Rich Repeat Kinase 2 (LRRK2), that is activated under these conditions that recruits and phosphorylates Rab 8A to the damaged membrane sites within minutes of the damage. The recruitment of Rab8A coincides with recruitment

of an ESCRT III component, but precedes the accumulation of galectin 3. In cells lacking LRRK2 the damaged endolysosomes are targeted for destruction by lysophagy, selective autophagy of lysosomes. A similar study showed that ESCRTs are also involved in repairing the damage caused by *Mycobacterium marinum* containing vacuoles in *Dictyostelium discoideum* [505].

This process of repairing membrane damage by the ESCRT machinery is not limited to the endocytic organelles; a similar mechanism has been shown to repair damage to the plasma membrane [510] as well as the nuclear envelope [511] [512].

When damage to an organelle membrane is more extensive the strategy for repair appears to be different, the cell tags the site for removal and digestion [502, 509]. Again, a galectin appears to be an early 'warning signal' presumably involving a more intensive binding to the damaged site than for minor disruptions. This is followed by binding of ubiquitin ligases that tag the site with multiple ubiquitin molecules. One identified ubiquitin ligase is Trim 16 that then leads to recruitment of the molecular machinery, including the common autophagy marker LC3 [504]. An autophagic membrane cisterna then forms that can engulf the tagged membranes in an autophagic vacuole that then fuses with endolysosomes where the damaged membranes are degraded [500, 502]. Trim 16 also binds galectin 3 which gives a hint of how complex and inter-connected these mechanisms are [505].[503].

A number of different particles, such as crystalline silica, appear to induce membrane damage in endocytic organelles, [513], as indicated by loss of acidification and calcium release from endolysosomes, even at otherwise non-toxic levels of silica [200]. Another prominent source of damage is amyloid aggregates that accumulate in endocytic organelles of cells during neurogenerative disease progression, although the mechanism of organelle lysis is poorly understood [457]. Lysosomal disruption can also occur in other patho-physiological conditions, as exemplified by studies of pancreatic ductal adenocarcinoma (PDA) cells. In response to increased endocytic traffic into PDA lysosomes due to elevated autophagy and macropinocytosis, lysosomal repair pathways are upregulated to maintain the function of the organelles [432].

The di-peptide, L-leucine-L-leucine methyl ester (LLOMe) is a powerful research tool for inducing controlled and reversible permeabilization of endosomal membranes. LLOMe is taken into cells by fluid phase endocytosis and condenses in acidic organelles where the action of cathepsin C induces the formation of aggregates that transiently permeabilize the membrane [514, 515]. Within seconds the effect of this compound becomes evident through the loss of the proton gradient, as seen with dyes such as lyso-tracker. With increasing time or concentration lysosomal enzymes are claimed to leak out of the EL, though this could not be confirmed in our recent study [515]. ESCRT proteins can be seen associating with the compromised EL even before the loss of protons is evident and, when the damage is severe, membrane repair can be seen by the re-establishment of a proton gradient and of the expected localization of lyososomal enzymes [502]. Another widely used dipeptide that is similar to LLOMe and can transiently permeabilize late endocytic organelles is the dipeptide glycyl-l-phenylalanine 2-naphthylamide (GPN) [472, 516].

6G) Implications of membrane repair and removal mechanisms for NP endosome release
Although the details of these repair and removal processes are still being elucidated, it seems highly likely that they may be relevant for NP release. This is to be expected because the repair and removal mechanisms do not seem to distinguish between membrane damage induced by pathogens, amyloid aggregates or LLOMe. Moreover, the siRNA-lipid NP loaded endosomes investigated by [458] were rapidly tagged with galectin 8 when damaged and were then targeted for autophagy.
A number of important questions can be raised:

1. In different cell types how much impact does ESCRT-mediated membrane repair and autophagy-based removal of NP-containing endocytic organelles have on the fraction of NP

cargo that manages to reach the cytosol or the nucleus? Are these mechanisms the reason why the efficiency of endosome escape until now has tended to be relatively low [465]? This can be addressed by knocking out key players of the ESCRT or autophagy machinery at the cell culture level.

2. Can NP be fabricated that have a 'burst'-like release *before* the repair or removal mechanisms are switched on? For sure, more detailed kinetic analysis and attention needs to be focused on the precise endocytic stage where endosome escape occurs.

7) FROM CELLS IN CULTURE TO CELLS *IN VIVO*

In the standard strategy for characterizing NP, cell culture studies are usually followed by *in vivo* analysis, most commonly in mouse models. Since so much of what we have discussed at the cell culture level involves imaging methods, it follows that the same or similar methods need to be applied to monitor NP in *in vivo* studies. However, mice are opaque for imaging and one cannot easily put a live mouse under the microscope and monitor the detailed fate of NP. This problem is gradually being overcome with recent improvements in two-photon or multi-photon, intra-vital microscopy [117] [517]. With this approach one can cut open the animal and follow fluorescently labeled NP and cells of interest in selected parts of some tissues for a limited period of time. As this technology develops the results are increasingly impressive and interactions of NP with different cells in the animal are becoming more and more striking - see [117] [114-116] [93, 200, 518].

Nevertheless, this approach is technically demanding, invasive and one still cannot put the whole live mouse under a high resolution microscope. However, this can be done with the embryo of another vertebrate, the zebrafish, that is transparent for imaging. A number of groups are reaching a consensus that this 'see-through' fish has many advantages for visualizing NP and can best be considered as an intermediate model between cultured cells and murine pre-clinical models [463] [38] [2] [200].

Zebrafish eggs are fertilized and develop rapidly outside the mother's body. The embryos are therefore accessible, easy to maintain and available in high numbers. Aside from being transparent for imaging, Zebrafish have a similar genetic structure to humans; they share 70 % of genes with humans while 84% of genes known to be associated with human disease have a zebrafish counterpart [93].

For testing NP, a wide variety of different disease models are available, all of which take advantage of difference fluorescence colors to identify diseased cells and different host cells of interest. For infections with pathogens, fluorescent pathogens can be injected into a choice of different locations where the diseases then develop. We used a zebrafish embryo model of tuberculosis developed by Davis and colleagues [292] to monitor and characterize different types of NP loaded with antibiotics [463, 519].

For cancer models a variety of different fluorescent cancer cells are injected. A number of groups have shown that human cancer cells xenotransplanted into zebrafish embryos exhibit three important features of human solid tumors: rapid cell proliferation, angiogenesis and metastasis [520, 521]. A typical experiment requires only about a week, compared to several weeks to months in the mouse, and experiments are relatively cheap. Only a small number of cancer cells are required and a lack of adaptive immunity in early zebrafish development means that there is no rejection of the transplanted cells. Finally, the ethical barriers to working with zebrafish are relatively low compared to mammalian models [93, 200].

Our group introduced and optimized xenotransplants of mammalian cells in zebrafish to visualize fluorescent and drug-loaded NP [51, 522]. An important innovation was to replace existing sites for injecting cancer cells by the neural tube, the precursor of the central nervous system and a large,

morphologically distinct organ; this allows more robust tumor growth and development of tumor blood vessels, as first shown with mouse melanoma B16 cancer cells. After intravenous injection of NP, this system allowed us to monitor and quantify NP extravasating from the vasculature in the vicinity of the tumor, both inside and outside cancer cells [522].

For characterizing NP, there are four important types of information that are readily available in the zebrafish model simply by imaging the embryos in a basic stereo microscope, namely:

1. Localization. One can rapidly evaluate which cell type has internalized the NP. In our experience with different NP the most common destinations are macrophages, neutrophils and endothelial cells [300] [523] [463, 519].

2. NP circulation time. Again, via imaging, one can quantify the length of time that the NP stay in circulation in the fish [524] [525]. The latter study shows that the results in zebrafish embryos are reasonably predictive of how NP behave in mice.

3. NP toxicity. The zebrafish embryo is exquisitely sensitive to toxins in the water or injected into the embryo [526] [527] [528] [200].

4. Therapy. Two different types of assays can be used to quantify possible therapeutic effects of NP. First the survival of the embryos; the capacity of embryos to die from a disease should be reflected in longer survival times following effective NP treatment. The second method is to image the fluorescent signal in the pathogen or the cancer cells. A successful therapy should result in a reduced total fluorescence in diseased cells [529] [463, 519, 522]. The latter references deal with NP for therapeutic drug delivery. Other studies have shown successful delivery of siRNA in NP to target gene expression in the adult zebrafish heart [530] [531] [532]. See also [2, 533] for applications of siRNA against cancer in zebrafish embryos.

Organoids

An alternative to using model organisms are organoids, simplified *in vitro* 3-D organs based on self-assembly of a limited number of cell types. These models can now be generated for a number of different tissues and species, including human, from tissue explants, biopsy material, or pluripotent stem cells [534] [535]. Organoids for brain, gut, liver, respiratory epithelium and other tissues have been shown to achieve significant degrees of cell diversity and organisation, highly reminiscent of the cognate tissue *in vivo*. Moreover, they can be genetically manipulated using, for example, CRISPR/Cas methods combined with various viral or NP delivery approaches. These systems are highly accessible for NP analysis using a variety of imaging techniques, including live fluorescence microscopy and EM. Indeed, they are particularly amenable to analysis using advance lattice light sheet imaging with the potential for high temporal analysis, combined with high resolution and minimal phototoxicity. Similarly, advanced EM techniques, including 3D volume EM can provide high resolution, and can be coupled with correlative live cell techniques. The use of stem cells can provide highly reproducible experimental material that can be 'bulked-up' for quantitative high through-put approaches [536] [201].

However, a word of caution is required. Poorly constructed and characterized organoids may have few advantages over a cell culture system. Even the most advanced systems that are currently available will be unable to reproduce all the complexities of the *in vivo* environment, meaning that distribution studies using drugs or NP must be carefully interpreted.

8) GENERAL CONCLUSIONS

In this review we have discussed two major aspects of the interactions between NP and cells: 1. NP uptake into the endocytic pathway and 2. NP escape from the endocytic organelles into the cytosol.

Central to this discussion, we have suggested that two different groups of researchers are involved in analyzing these processes: those who are trained to make and characterize NP, whom we refer to as the 'chemists' for simplicity, and scientists trained as cell biologists. We contend that if cell biologists took on the role of 'chemists' and made and characterized their own NP in a manner that ignored the 'standard rules' rigorously developed by the 'chemists', it is unlikely that any manuscript describing their work would be well-received by professional nanomedicine journals. However, these cell biologists would have more of a chance to publish their papers in cell biology journals, where there would be less NP expertise available amongst the commonly used reviewers. We argue that the reverse scenario is precisely what is occurring in the NP field, i.e., 'chemists' are taking on the mantle of cell biologists.

In our review of the literature, it is apparent that while understanding the different routes of entry into cells is complicated for cell biologists, it is seemingly relatively easy for 'chemists', many of whom erroneously believe that there are a number of well-defined, specific inhibitors that allow them not only to distinguish a specific pathway but even to quantify the relative contribution of individual pathways when a combination of routes is used. By now it should be evident that the vast majority of these inhibitors should be used with care and certainly only alongside other more definitive methods.

Given their complexity, we have refrained from trying to proclaim a simple set of rules for distinguishing experimentally between the different uptake mechanisms. In Box 2 we attempt rough definitions and suggest guidelines, though there will be some expert cell biologists who may not agree with all our suggestions (for suggested targets for genetic manipulation of specific pathways see [39]. We propose the use of multiple techniques and tools to test the route of nanoparticle entry.

There is an irony in this situation in that with respect to the main uptake processes 'all roads lead to Rome' in that all the known entry pathways deliver NP into the EE network. Thus, perhaps NP specialists should consider avoiding the issue of identifying specific pathways and focus instead on establishing that internalization occurs, its relative efficiency and showing where in the endocytic pathway internalized NP are located at specific times after endocytosis. The markers, timings, and imaging techniques we discuss above provide a basis for such analysis, which should preferably be done in collaboration with cell biologists who have experience with endocytosis. Another way of articulating this point is to state that nanomedicine is generally accepted as being a multi-disciplinary field, we are simply asking that this should be true in practice – see also [39].

Overall, NP have extraordinary possibilities as vehicles for delivering a variety of cargoes, from small molecules to more complex nucleic acids and proteins, for targeted medical interventions. The success of NP in recently developed RNA vaccines and the promise of similar delivery vehicles in *in vivo* gene editing therapies is indicative of this potential. We believe that more rigorous attention to the cell biology of targeting, uptake and delivery will further improve NP efficiency and specificity and broaden the range of opportunities for their application. We strongly urge NP chemists and the nanomedicine field in general to work closely with expert cell biologists to realize the full medical and therapeutic potential of nanoparticles.

9) ACKNOWLEDGEMENTS

We are extremely grateful to the following experts for their critical evaluation of this manuscript:

John Lucocq, Urska Repnik, Maximilliano Gutierrez, Albert Haas, Matthias Barz, and Angus Johnston. This work was supported by two grants from the Norwegian Research Council (Grant numbers 273319 and 275873) to GG, from the National Health and Medical Research Council of Australia (grants APP1140064 and APP1150083 and fellowship APP1156489 to RGP), the Swiss National Science Foundation Grant No 31003A_159479 (to JG), the National Center of Competence in Research Chemical Biology (to JG) and Lipid X from the Swiss SystemsX.ch Initiative (to JG),

UK Medical Research Council funding to the MRC-UCL Laboratory for Molecular Cell Biology University Unit (MC_UU00012/1 and MC_U12266B) to MM.

10) REFERENCE LIST

[1] M. Al Soraj, L. He, K. Peynshaert, J. Cousaert, D. Vercauteren, K. Braeckmans, S.C. De Smedt, A.T. Jones, siRNA and pharmacological inhibition of endocytic pathways to characterize the differential role of macropinocytosis and the actin cytoskeleton on cellular uptake of dextran and cationic cell penetrating peptides octaarginine (R8) and HIV-Tat, J Control Release, 161 (2012) 132-141.
[2] S. Ashraf, A. Hassan Said, R. Hartmann, M.A. Assmann, N. Feliu, P. Lenz, W.J. Parak, Quantitative Particle Uptake by Cells as Analyzed by Different Methods, Angew Chem Int Ed Engl, 59 (2020) 5438-5453.
[3] M. Bartneck, H.A. Keul, S. Singh, K. Czaja, J. Bornemann, M. Bockstaller, M. Moeller, G. Zwadlo-Klarwasser, J. Groll, Rapid uptake of gold nanorods by primary human blood phagocytes and immunomodulatory effects of surface chemistry, ACS nano, 4 (2010) 3073-3086.
[4] R. Parnaik, M.C. Raff, J. Scholes, Differences between the clearance of apoptotic cells by professional and non-professional phagocytes, Curr Biol, 10 (2000) 857-860.
[5] N. Nagai, F. Ogata, H. Otake, Y. Nakazawa, N. Kawasaki, Energy-dependent endocytosis is responsible for drug transcorneal penetration following the instillation of ophthalmic formulations containing indomethacin nanoparticles, Int J Nanomedicine, 14 (2019) 1213-1227.
[6] I.C. Trindade, G. Pound-Lana, D.G.S. Pereira, L.A.M. de Oliveira, M.S. Andrade, J.M.C. Vilela, B.B. Postacchini, V.C.F. Mosqueira, Mechanisms of interaction of biodegradable polyester nanocapsules with non-phagocytic cells, Eur J Pharm Sci, 124 (2018) 89-104.
[7] I. Youm, J.D. Bazzil, J.W. Otto, A.N. Caruso, J.B. Murowchick, B.B. Youan, Influence of surface chemistry on cytotoxicity and cellular uptake of nanocapsules in breast cancer and phagocytic cells, AAPS J, 16 (2014) 550-567.
[8] R. Huang, G. Zhu, J. Zhang, Y. Lai, Y. Xu, J. He, J. Xie, Betanodavirus-like particles enter host cells via clathrin-mediated endocytosis in a cholesterol-, pH- and cytoskeleton-dependent manner, Vet Res, 48 (2017) 8.
[9] Y. Huang, X. Huang, S. Wang, Y. Yu, S. Ni, Q. Qin, Soft-shelled turtle iridovirus enters cells via cholesterol-dependent, clathrin-mediated endocytosis as well as macropinocytosis, Arch Virol, 163 (2018) 3023-3033.
[10] J. Linares, M.C. Matesanz, M. Vila, M.J. Feito, G. Goncalves, M. Vallet-Regi, P.A. Marques, M.T. Portoles, Endocytic mechanisms of graphene oxide nanosheets in osteoblasts, hepatocytes and macrophages, ACS Appl Mater Interfaces, 6 (2014) 13697-13706.
[11] S.M. Sagnella, H. Duong, A. MacMillan, C. Boyer, R. Whan, J.A. McCarroll, T.P. Davis, M. Kavallaris, Dextran-based doxorubicin nanocarriers with improved tumor penetration, Biomacromolecules, 15 (2014) 262-275.
[12] B. Witkop, Paul Ehrlich and his Magic bullets--revisited, Proc Am Philos Soc, 143 (1999) 540-557.
[13] J. Liu, L. Wang, F. Zhao, S. Tseng, C. Narayanan, L. Shura, S. Willingham, M. Howard, S. Prohaska, J. Volkmer, M. Chao, I.L. Weissman, R. Majeti, Pre-Clinical Development of a Humanized Anti-CD47 Antibody with Anti-Cancer Therapeutic Potential, PloS one, 10 (2015) e0137345.
[14] A. Guo, M. Durymanov, A. Permyakova, S. Sene, C. Serre, J. Reineke, Metal Organic Framework (MOF) Particles as Potential Bacteria-Mimicking Delivery Systems for Infectious Diseases: Characterization and Cellular Internalization in Alveolar Macrophages, Pharm Res, 36 (2019) 53.

[15] D. Chen, Y. Jian, X. Liu, Y. Zhang, J. Liang, X. Qi, H. Du, W. Zou, L. Chen, Y. Chai, G. Ou, L. Miao, Y. Wang, C. Yang, Clathrin and AP2 are required for phagocytic receptor-mediated apoptotic cell clearance in Caenorhabditis elegans, PLoS Genet, 9 (2013) e1003517.
[16] D. Yilmaz, M. Culha, Investigation of the pathway dependent endocytosis of gold nanoparticles by surface-enhanced Raman scattering, Talanta, 225 (2021) 122071.
[17] A. Sipos, K.J. Kim, C. Sioutas, E.D. Crandall, Evidence for Nanoparticle-Induced Lysosomal Dysfunction in Lung Adenocarcinoma (A549) Cells, Int J Mol Sci, 20 (2019).
[18] V.J. Venditto, F.C. Szoka, Jr., Cancer nanomedicines: so many papers and so few drugs!, Adv Drug Deliv Rev, 65 (2013) 80-88.
[19] M.J. Mitchell, M.M. Billingsley, R.M. Haley, M.E. Wechsler, N.A. Peppas, R. Langer, Engineering precision nanoparticles for drug delivery, Nat Rev Drug Discov, 20 (2021) 101-124.
[20] A. Akinc, G. Battaglia, Exploiting endocytosis for nanomedicines, Cold Spring Harb Perspect Biol, 5 (2013) a016980.
[21] S. Behzadi, V. Serpooshan, W. Tao, M.A. Hamaly, M.Y. Alkawareek, E.C. Dreaden, D. Brown, A.M. Alkilany, O.C. Farokhzad, M. Mahmoudi, Cellular uptake of nanoparticles: journey inside the cell, Chem Soc Rev, 46 (2017) 4218-4244.
[22] D. Rosenblum, N. Joshi, W. Tao, J.M. Karp, D. Peer, Progress and challenges towards targeted delivery of cancer therapeutics, Nat Commun, 9 (2018) 1410.
[23] V. Sanna, M. Sechi, Therapeutic Potential of Targeted Nanoparticles and Perspective on Nanotherapies, ACS Med Chem Lett, 11 (2020) 1069-1073.
[24] J. Lopez Bernal, N. Andrews, C. Gower, C. Robertson, J. Stowe, E. Tessier, R. Simmons, S. Cottrell, R. Roberts, M. O'Doherty, K. Brown, C. Cameron, D. Stockton, J. McMenamin, M. Ramsay, Effectiveness of the Pfizer-BioNTech and Oxford-AstraZeneca vaccines on covid-19 related symptoms, hospital admissions, and mortality in older adults in England: test negative case-control study, Bmj, 373 (2021) n1088.
[25] P.T. Heath, E.P. Galiza, D.N. Baxter, M. Boffito, D. Browne, F. Burns, D.R. Chadwick, R. Clark, C. Cosgrove, J. Galloway, A.L. Goodman, A. Heer, A. Higham, S. Iyengar, A. Jamal, C. Jeanes, P.A. Kalra, C. Kyriakidou, D.F. McAuley, A. Meyrick, A.M. Minassian, J. Minton, P. Moore, I. Munsoor, H. Nicholls, O. Osanlou, J. Packham, C.H. Pretswell, A. San Francisco Ramos, D. Saralaya, R.P. Sheridan, R. Smith, R.L. Soiza, P.A. Swift, E.C. Thomson, J. Turner, M.E. Viljoen, G. Albert, I. Cho, F. Dubovsky, G. Glenn, J. Rivers, A. Robertson, K. Smith, S. Toback, Safety and Efficacy of NVX-CoV2373 Covid-19 Vaccine, N Engl J Med, (2021).
[26] K. Musunuru, A.C. Chadwick, T. Mizoguchi, S.P. Garcia, J.E. DeNizio, C.W. Reiss, K. Wang, S. Iyer, C. Dutta, V. Clendaniel, M. Amaonye, A. Beach, K. Berth, S. Biswas, M.C. Braun, H.M. Chen, T.V. Colace, J.D. Ganey, S.A. Gangopadhyay, R. Garrity, L.N. Kasiewicz, J. Lavoie, J.A. Madsen, Y. Matsumoto, A.M. Mazzola, Y.S. Nasrullah, J. Nneji, H. Ren, A. Sanjeev, M. Shay, M.R. Stahley, S.H.Y. Fan, Y.K. Tam, N.M. Gaudelli, G. Ciaramella, L.E. Stolz, P. Malyala, C.J. Cheng, K.G. Rajeev, E. Rohde, A.M. Bellinger, S. Kathiresan, In vivo CRISPR base editing of PCSK9 durably lowers cholesterol in primates, Nature, 593 (2021) 429-434.
[27] F. Pastor, P. Berraondo, I. Etxeberria, J. Frederick, U. Sahin, E. Gilboa, I. Melero, An RNA toolbox for cancer immunotherapy, Nat Rev Drug Discov, 17 (2018) 751-767.
[28] J. Liu, X. Lu, T. Wu, X. Wu, L. Han, B. Ding, Branched Antisense and siRNA Co-Assembled Nanoplatform for Combined Gene Silencing and Tumor Therapy, Angew Chem Int Ed Engl, 60 (2021) 1853-1860.
[29] J. Levican, C. Miranda-Cardenas, R. Soto-Rifo, F. Aguayo, A. Gaggero, O. Leon, Infectious pancreatic necrosis virus enters CHSE-214 cells via macropinocytosis, Sci Rep, 7 (2017) 3068.
[30] T.-G. Iversen, T. Skotland, K. Sandvig, Endocytosis and intracellular transport of nanoparticles: Present knowledge and need for future studies, Nano today, 6 (2011) 176-185.

[31] B.D. Dill, M. Gierlinski, A. Härtlova, A.G. Arandilla, M. Guo, R.G. Clarke, M. Trost, Quantitative proteome analysis of temporally resolved phagosomes following uptake via key phagocytic receptors, Mol Cell Proteomics, 14 (2015) 1334-1349.
[32] A.I. Ivanov, Pharmacological inhibition of endocytic pathways: is it specific enough to be useful?, Methods Mol Biol, 440 (2008) 15-33.
[33] B. Halamoda-Kenzaoui, M. Ceridono, P. Urbán, A. Bogni, J. Ponti, S. Gioria, A. Kinsner-Ovaskainen, The agglomeration state of nanoparticles can influence the mechanism of their cellular internalisation, Journal of Nanobiotechnology, 15 (2017) 48.
[34] P.V. Rewatkar, R.G. Parton, H.S. Parekh, M.-O. Parat, Are caveolae a cellular entry route for non-viral therapeutic delivery systems?, Advanced Drug Delivery Reviews, 91 (2015) 92-108.
[35] S. Muro, M. Mateescu, C. Gajewski, M. Robinson, V.R. Muzykantov, M. Koval, Control of intracellular trafficking of ICAM-1-targeted nanocarriers by endothelial Na+/H+ exchanger proteins, Am J Physiol Lung Cell Mol Physiol, 290 (2006) L809-817.
[36] M. Ansar, D. Serrano, I. Papademetriou, T.K. Bhowmick, S. Muro, Biological functionalization of drug delivery carriers to bypass size restrictions of receptor-mediated endocytosis independently from receptor targeting, ACS nano, 7 (2013) 10597-10611.
[37] N.P. Hessvik, A. Llorente, Current knowledge on exosome biogenesis and release, Cell Mol Life Sci, 75 (2018) 193-208.
[38] S. Bleher, J. Buck, C. Muhl, S. Sieber, S. Barnert, D. Witzigmann, J. Huwyler, M. Barz, R. Süss, Poly(Sarcosine) Surface Modification Imparts Stealth-Like Properties to Liposomes, Small, 15 (2019) e1904716.
[39] J.J. Rennick, A.P.R. Johnston, R.G. Parton, Key principles and methods for studying the endocytosis of biological and nanoparticle therapeutics, Nat Nanotechnol, 16 (2021) 266-276.
[40] M. Marsh, A. Helenius, Virus entry: open sesame, Cell, 124 (2006) 729-740.
[41] E.M. Plummer, M. Manchester, Endocytic uptake pathways utilized by CPMV nanoparticles, Mol Pharm, 10 (2013) 26-32.
[42] J. Mercer, S. Knébel, F.I. Schmidt, J. Crouse, C. Burkard, A. Helenius, Vaccinia virus strains use distinct forms of macropinocytosis for host-cell entry, Proc Natl Acad Sci U S A, 107 (2010) 9346-9351.
[43] N. Yutin, M.Y. Wolf, Y.I. Wolf, E.V. Koonin, The origins of phagocytosis and eukaryogenesis, Biol Direct, 4 (2009) 9.
[44] J.B. Dacks, M.C. Field, Evolution of the eukaryotic membrane-trafficking system: origin, tempo and mode, J Cell Sci, 120 (2007) 2977-2985.
[45] H. Meng, S. Yang, Z. Li, T. Xia, J. Chen, Z. Ji, H. Zhang, X. Wang, S. Lin, C. Huang, Z.H. Zhou, J.I. Zink, A.E. Nel, Aspect ratio determines the quantity of mesoporous silica nanoparticle uptake by a small GTPase-dependent macropinocytosis mechanism, ACS nano, 5 (2011) 4434-4447.
[46] L. Casarrubios, N. Gomez-Cerezo, M.J. Feito, M. Vallet-Regi, D. Arcos, M.T. Portoles, Ipriflavone-Loaded Mesoporous Nanospheres with Potential Applications for Periodontal Treatment, Nanomaterials (Basel), 10 (2020).
[47] L. Sagan, On the origin of mitosing cells, J Theor Biol, 14 (1967) 255-274.
[48] M. Rabinovitch, Professional and non-professional phagocytes: an introduction, Trends Cell Biol, 5 (1995) 85-87.
[49] V. Everts, E. van der Zee, L. Creemers, W. Beertsen, Phagocytosis and intracellular digestion of collagen, its role in turnover and remodelling, Histochem J, 28 (1996) 229-245.
[50] D.D. McAbee, F. Grinnell, Fibronectin-mediated binding and phagocytosis of polystyrene latex beads by baby hamster kidney cells, J Cell Biol, 97 (1983) 1515-1523.
[51] L. Evensen, P.L. Johansen, G. Koster, K. Zhu, L. Herfindal, M. Speth, F. Fenaroli, J. Hildahl, S. Bagherifam, C. Tulotta, L. Prasmickaite, G.M. Mælandsmo, E. Snaar-Jagalska, G. Griffiths, Zebrafish

as a model system for characterization of nanoparticles against cancer, Nanoscale, 8 (2016) 862-877.
[52] Y. Wu, W. Wu, W.M. Wong, E. Ward, A.J. Thrasher, D. Goldblatt, M. Osman, P. Digard, D.H. Canaday, K. Gustafsson, Human gamma delta T cells: a lymphoid lineage cell capable of professional phagocytosis, J Immunol, 183 (2009) 5622-5629.
[53] M.A. Lande, D.E. Birk, M.L. Nagpal, R.L. Rader, Phagocytic properties of human keratocyte cultures, Invest Ophthalmol Vis Sci, 20 (1981) 481-489.
[54] S. Bowen, D.D. Ateh, K. Deinhardt, M.M. Bird, K.M. Price, C.S. Baker, J.C. Robson, M. Swash, W. Shamsuddin, S. Kawar, T. El-Tawil, J. Roos, A. Hoyle, C.D. Nickols, C.H. Knowles, A.H. Pullen, P.J. Luthert, R.O. Weller, M. Hafezparast, R.J. Franklin, T. Revesz, R.H. King, O. Berninghausen, E.M. Fisher, G. Schiavo, J.E. Martin, The phagocytic capacity of neurones, Eur J Neurosci, 25 (2007) 2947-2955.
[55] A.M. Steffan, J.L. Gendrault, R.S. McCuskey, P.A. McCuskey, A. Kirn, Phagocytosis, an unrecognized property of murine endothelial liver cells, Hepatology, 6 (1986) 830-836.
[56] R.E. Garfield, S. Chacko, S. Blose, Phagocytosis by muscle cells, Lab Invest, 33 (1975) 418-427.
[57] T. Soji, Y. Murata, A. Ohira, H. Nishizono, M. Tanaka, D.C. Herbert, Evidence that hepatocytes can phagocytize exogenous substances, Anat Rec, 233 (1992) 543-546.
[58] E. Veiga, J.A. Guttman, M. Bonazzi, E. Boucrot, A. Toledo-Arana, A.E. Lin, J. Enninga, J. Pizarro-Cerdá, B.B. Finlay, T. Kirchhausen, P. Cossart, Invasive and adherent bacterial pathogens co-Opt host clathrin for infection, Cell Host Microbe, 2 (2007) 340-351.
[59] I. Bucior, K. Mostov, J.N. Engel, Pseudomonas aeruginosa-mediated damage requires distinct receptors at the apical and basolateral surfaces of the polarized epithelium, Infect Immun, 78 (2010) 939-953.
[60] J. Mounier, T. Vasselon, R. Hellio, M. Lesourd, P.J. Sansonetti, Shigella flexneri enters human colonic Caco-2 epithelial cells through the basolateral pole, Infect Immun, 60 (1992) 237-248.
[61] G.J. Pinero, R.R. White, Phagocytosis of latex beads by a human gingival epithelial-like cell line in tissue culture, J Dent Res, 56 (1977) 1119-1126.
[62] L. Lugini, F. Lozupone, P. Matarrese, C. Funaro, F. Luciani, W. Malorni, L. Rivoltini, C. Castelli, A. Tinari, A. Piris, G. Parmiani, S. Fais, Potent phagocytic activity discriminates metastatic and primary human malignant melanomas: a key role of ezrin, Lab Invest, 83 (2003) 1555-1567.
[63] C.C. Shepard, Phagocytosis by HeLa cells and their susceptibility to infection by human tubercle bacilli, Proc Soc Exp Biol Med, 90 (1955) 392-396.
[64] N. Kern, R. Dong, S.M. Douglas, R.D. Vale, M.A. Morrissey, Tight nanoscale clustering of Fcγ receptors using DNA origami promotes phagocytosis, eLife, 10 (2021).
[65] F.M. Griffin, Jr., J.A. Griffin, J.E. Leider, S.C. Silverstein, Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane, J Exp Med, 142 (1975) 1263-1282.
[66] J. Cardelli, Phagocytosis and macropinocytosis in Dictyostelium: phosphoinositide-based processes, biochemically distinct, Traffic, 2 (2001) 311-320.
[67] M. Desjardins, G. Griffiths, Phagocytosis: latex leads the way, Curr Opin Cell Biol, 15 (2003) 498-503.
[68] M. Schleicher, A.A. Noegel, Dynamics of the Dictyostelium cytoskeleton during chemotaxis, The New biologist, 4 (1992) 461-472.
[69] L. Chen, C. Janetopoulos, Y.E. Huang, M. Iijima, J. Borleis, P.N. Devreotes, Two phases of actin polymerization display different dependencies on PI(3,4,5)P3 accumulation and have unique roles during chemotaxis, Mol Biol Cell, 14 (2003) 5028-5037.
[70] A.L. Hall, V. Warren, S. Dharmawardhane, J. Condeelis, Identification of actin nucleation activity and polymerization inhibitor in ameboid cells: their regulation by chemotactic stimulation, J Cell Biol, 109 (1989) 2207-2213.

[71] M. Koval, K. Preiter, C. Adles, P.D. Stahl, T.H. Steinberg, Size of IgG-opsonized particles determines macrophage response during internalization, Experimental cell research, 242 (1998) 265-273.
[72] I. Fujiwara, M.E. Zweifel, N. Courtemanche, T.D. Pollard, Latrunculin A Accelerates Actin Filament Depolymerization in Addition to Sequestering Actin Monomers, Curr Biol, 28 (2018) 3183-3192.e3182.
[73] R.J. Eddy, J. Han, J.S. Condeelis, Capping protein terminates but does not initiate chemoattractant-induced actin assembly in Dictyostelium, J Cell Biol, 139 (1997) 1243-1253.
[74] D. Vorselen, R.L.D. Labitigan, J.A. Theriot, A mechanical perspective on phagocytic cup formation, Curr Opin Cell Biol, 66 (2020) 112-122.
[75] E. Colucci-Guyon, F. Niedergang, B.J. Wallar, J. Peng, A.S. Alberts, P. Chavrier, A role for mammalian diaphanous-related formins in complement receptor (CR3)-mediated phagocytosis in macrophages, Curr Biol, 15 (2005) 2007-2012.
[76] O. Alekhina, E. Burstein, D.D. Billadeau, Cellular functions of WASP family proteins at a glance, J Cell Sci, 130 (2017) 2235-2241.
[77] C. Rosales, E. Uribe-Querol, Phagocytosis: A Fundamental Process in Immunity, Biomed Res Int, 2017 (2017) 9042851.
[78] D.M. Underhill, A. Ozinsky, Phagocytosis of microbes: complexity in action, Annual review of immunology, 20 (2002) 825-852.
[79] G. Chimini, P. Chavrier, Function of Rho family proteins in actin dynamics during phagocytosis and engulfment, Nat Cell Biol, 2 (2000) E191-196.
[80] A. Aderem, D.M. Underhill, Mechanisms of phagocytosis in macrophages, Annual review of immunology, 17 (1999) 593-623.
[81] D. Schlam, J. Canton, Every day I'm rufflin': Calcium sensing and actin dynamics in the growth factor-independent membrane ruffling of professional phagocytes, Small GTPases, 8 (2017) 65-70.
[82] G. van Meer, D.R. Voelker, G.W. Feigenson, Membrane lipids: where they are and how they behave, Nat Rev Mol Cell Biol, 9 (2008) 112-124.
[83] J.D. Ernst, Bacterial inhibition of phagocytosis, Cell Microbiol, 2 (2000) 379-386.
[84] L.J. Mota, G.R. Cornelis, The bacterial injection kit: type III secretion systems, Ann Med, 37 (2005) 234-249.
[85] D.L. Goosney, S. Gruenheid, B.B. Finlay, Gut feelings: enteropathogenic E. coli (EPEC) interactions with the host, Annu Rev Cell Dev Biol, 16 (2000) 173-189.
[86] R.S. Flannagan, B. Heit, D.E. Heinrichs, Antimicrobial Mechanisms of Macrophages and the Immune Evasion Strategies of Staphylococcus aureus, Pathogens, 4 (2015) 826-868.
[87] C.H. Takimoto, M.P. Chao, C. Gibbs, M.A. McCamish, J. Liu, J.Y. Chen, R. Majeti, I.L. Weissman, The Macrophage 'Do not eat me' signal, CD47, is a clinically validated cancer immunotherapy target, Ann Oncol, 30 (2019) 486-489.
[88] J.A. Daniel, N. Chau, M.K. Abdel-Hamid, L. Hu, L. von Kleist, A. Whiting, S. Krishnan, P. Maamary, S.R. Joseph, F. Simpson, V. Haucke, A. McCluskey, P.J. Robinson, Phenothiazine-derived antipsychotic drugs inhibit dynamin and clathrin-mediated endocytosis, Traffic, 16 (2015) 635-654.
[89] R.K. Tsai, D.E. Discher, Inhibition of "self" engulfment through deactivation of myosin-II at the phagocytic synapse between human cells, J Cell Biol, 180 (2008) 989-1003.
[90] Y. Murata, T. Kotani, H. Ohnishi, T. Matozaki, The CD47-SIRPα signalling system: its physiological roles and therapeutic application, J Biochem, 155 (2014) 335-344.
[91] J.A. Champion, A. Walker, S. Mitragotri, Role of particle size in phagocytosis of polymeric microspheres, Pharm Res, 25 (2008) 1815-1821.
[92] N. Hoshyar, S. Gray, H. Han, G. Bao, The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction, Nanomedicine (Lond), 11 (2016) 673-692.

[93] P.M. Pacheco, B. Le, D. White, T. Sulchek, TUNABLE COMPLEMENT ACTIVATION BY PARTICLES WITH VARIABLE SIZE AND Fc DENSITY, Nano Life, 3 (2013) 1341001.
[94] C.J. van Oss, Phagocytosis as a surface phenomenon, Annual review of microbiology, 32 (1978) 19-39.
[95] G. Sharma, D.T. Valenta, Y. Altman, S. Harvey, H. Xie, S. Mitragotri, J.W. Smith, Polymer particle shape independently influences binding and internalization by macrophages, J Control Release, 147 (2010) 408-412.
[96] K.A. Beningo, Y.L. Wang, Fc-receptor-mediated phagocytosis is regulated by mechanical properties of the target, J Cell Sci, 115 (2002) 849-856.
[97] J. Roberts, J.H. Quastel, PARTICLE UPTAKE BY POLYMORPHONUCLEAR LEUCOCYTES AND EHRLICH ASCITES-CARCINOMA CELLS, Biochem J, 89 (1963) 150-156.
[98] J. Roberts, Quastel, JH, Particle uptake by polymorphonuclear leukocytes and Ehrlich ascites-carcinoma cells, Biochem J, 89 (19663) 150-156.
[99] E.D. Korn, R.A. Weisman, Phagocytosis of latex beads by Acanthamoeba. II. Electron microscopic study of the initial events, J Cell Biol, 34 (1967) 219-227.
[100] J.D. Brain, R.M. Molina, M.M. DeCamp, A.E. Warner, Pulmonary intravascular macrophages: their contribution to the mononuclear phagocyte system in 13 species, Am J Physiol, 276 (1999) L146-154.
[101] L. Illum, S.S. Davis, The organ uptake of intravenously administered colloidal particles can be altered using a non-ionic surfactant (Poloxamer 338), FEBS Lett, 167 (1984) 79-82.
[102] L. Illum, S.S. Davis, R.H. Müller, E. Mak, P. West, The organ distribution and circulation time of intravenously injected colloidal carriers sterically stabilized with a block copolymer--poloxamine 908, Life Sci, 40 (1987) 367-374.
[103] L. Illum, N.W. Thomas, S.S. Davis, Effect of a selected suppression of the reticuloendothelial system on the distribution of model carrier particles, J Pharm Sci, 75 (1986) 16-22.
[104] S.M. Moghimi, C.J. Porter, I.S. Muir, L. Illum, S.S. Davis, Non-phagocytic uptake of intravenously injected microspheres in rat spleen: influence of particle size and hydrophilic coating, Biochem Biophys Res Commun, 177 (1991) 861-866.
[105] V. Lenaerts, J.F. Nagelkerke, T.J. Van Berkel, P. Couvreur, L. Grislain, M. Roland, P. Speiser, In vivo uptake of polyisobutyl cyanoacrylate nanoparticles by rat liver Kupffer, endothelial, and parenchymal cells, J Pharm Sci, 73 (1984) 980-982.
[106] K. Ogawara, M. Yoshida, K. Higaki, T. Kimura, K. Shiraishi, M. Nishikawa, Y. Takakura, M. Hashida, Hepatic uptake of polystyrene microspheres in rats: effect of particle size on intrahepatic distribution, J Control Release, 59 (1999) 15-22.
[107] S.M. Moghimi, L. Parhamifar, D. Ahmadvand, P.P. Wibroe, T.L. Andresen, Z.S. Farhangrazi, A.C. Hunter, Particulate systems for targeting of macrophages: basic and therapeutic concepts, J Innate Immun, 4 (2012) 509-528.
[108] U. Scheffel, B.A. Rhodes, T.K. Natarajan, H.N. Wagner, Jr., Albumin microspheres for study of the reticuloendothelial system, J Nucl Med, 13 (1972) 498-503.
[109] K. Yamashita, H. Fujita, S. Kawamata, Fine structural and cytochemical aspects of granuloma formation derived from Kupffer cells in mice injected with latex particles, Arch Histol Jpn, 48 (1985) 315-326.
[110] S.H. Gregory, L.P. Cousens, N. van Rooijen, E.A. Döpp, T.M. Carlos, E.J. Wing, Complementary adhesion molecules promote neutrophil-Kupffer cell interaction and the elimination of bacteria taken up by the liver, J Immunol, 168 (2002) 308-315.
[111] J.M. Brewer, L. Tetley, J. Richmond, F.Y. Liew, J. Alexander, Lipid vesicle size determines the Th1 or Th2 response to entrapped antigen, J Immunol, 161 (1998) 4000-4007.

[112] M.K. Pratten, J.B. Lloyd, Pinocytosis and phagocytosis: the effect of size of a particulate substrate on its mode of capture by rat peritoneal macrophages cultured in vitro, Biochim Biophys Acta, 881 (1986) 307-313.
[113] J.B. Matthews, A.A. Besong, T.R. Green, M.H. Stone, B.M. Wroblewski, J. Fisher, E. Ingham, Evaluation of the response of primary human peripheral blood mononuclear phagocytes to challenge with in vitro generated clinically relevant UHMWPE particles of known size and dose, Journal of biomedical materials research, 52 (2000) 296-307.
[114] S. Köppert, A. Büscher, A. Babler, A. Ghallab, E.M. Buhl, E. Latz, J.G. Hengstler, E.R. Smith, W. Jahnen-Dechent, Cellular Clearance and Biological Activity of Calciprotein Particles Depend on Their Maturation State and Crystallinity, Front Immunol, 9 (2018) 1991.
[115] X. Liang, J.E. Grice, Y. Zhu, D. Liu, W.Y. Sanchez, Z. Li, D.H. Crawford, D.G. Le Couteur, V.C. Cogger, X. Liu, Z.P. Xu, M.S. Roberts, Intravital multiphoton imaging of the selective uptake of water-dispersible quantum dots into sinusoidal liver cells, Small, 11 (2015) 1711-1720.
[116] W. Li, J. Yan, Y. Yu, Geometrical reorganization of Dectin-1 and TLR2 on single phagosomes alters their synergistic immune signaling, Proc Natl Acad Sci U S A, 116 (2019) 25106-25114.
[117] M. Akamatsu, R. Vasan, D. Serwas, M.A. Ferrin, P. Rangamani, D.G. Drubin, Principles of self-organization and load adaptation by the actin cytoskeleton during clathrin-mediated endocytosis, eLife, 9 (2020).
[118] C. Clement, V. Tiwari, P.M. Scanlan, T. Valyi-Nagy, B.Y. Yue, D. Shukla, A novel role for phagocytosis-like uptake in herpes simplex virus entry, J Cell Biol, 174 (2006) 1009-1021.
[119] D. Manzanares, V. Ceña, Endocytosis: The Nanoparticle and Submicron Nanocompounds Gateway into the Cell, Pharmaceutics, 12 (2020).
[120] I. Ofek, J. Goldhar, Y. Keisari, N. Sharon, Nonopsonic phagocytosis of microorganisms, Annual review of microbiology, 49 (1995) 239-276.
[121] A. Palecanda, J. Paulauskis, E. Al-Mutairi, A. Imrich, G. Qin, H. Suzuki, T. Kodama, K. Tryggvason, H. Koziel, L. Kobzik, Role of the scavenger receptor MARCO in alveolar macrophage binding of unopsonized environmental particles, J Exp Med, 189 (1999) 1497-1506.
[122] D.E. Owens, 3rd, N.A. Peppas, Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles, Int J Pharm, 307 (2006) 93-102.
[123] P. Aggarwal, J.B. Hall, C.B. McLeland, M.A. Dobrovolskaia, S.E. McNeil, Nanoparticle interaction with plasma proteins as it relates to particle biodistribution, biocompatibility and therapeutic efficacy, Adv Drug Deliv Rev, 61 (2009) 428-437.
[124] H.H. Gustafson, D. Holt-Casper, D.W. Grainger, H. Ghandehari, Nanoparticle Uptake: The Phagocyte Problem, Nano today, 10 (2015) 487-510.
[125] N.D. Donahue, H. Acar, S. Wilhelm, Concepts of nanoparticle cellular uptake, intracellular trafficking, and kinetics in nanomedicine, Adv Drug Deliv Rev, 143 (2019) 68-96.
[126] J.E. O'Mullane, P. Artursson, E. Tomlinson, Biopharmaceutics of microparticulate drug carriers, Ann N Y Acad Sci, 507 (1987) 120-140.
[127] E. Liliemark, B. Sjöström, J. Liliemark, C. Peterson, N. Kållberg, B.S. Larsson, Targeting of teniposide to the mononuclear phagocytic system (MPS) by incorporation in liposomes and submicron lipid particles; an autoradiographic study in mice, Leuk Lymphoma, 18 (1995) 113-118.
[128] T.M. Göppert, R.H. Müller, Adsorption kinetics of plasma proteins on solid lipid nanoparticles for drug targeting, Int J Pharm, 302 (2005) 172-186.
[129] E. Papini, R. Tavano, F. Mancin, Opsonins and Dysopsonins of Nanoparticles: Facts, Concepts, and Methodological Guidelines, Front Immunol, 11 (2020) 567365.
[130] C. Fedeli, D. Segat, R. Tavano, L. Bubacco, G. De Franceschi, P.P. de Laureto, E. Lubian, F. Selvestrel, F. Mancin, E. Papini, The functional dissection of the plasma corona of SiO_2-NPs spots histidine rich glycoprotein as a major player able to hamper nanoparticle capture by macrophages, Nanoscale, 7 (2015) 17710-17728.

[131] K.J. Day, G. Kago, L. Wang, J.B. Richter, C.C. Hayden, E.M. Lafer, J.C. Stachowiak, Liquid-like protein interactions catalyse assembly of endocytic vesicles, Nat Cell Biol, 23 (2021) 366-376.
[132] V.H. Nguyen, B.J. Lee, Protein corona: a new approach for nanomedicine design, Int J Nanomedicine, 12 (2017) 3137-3151.
[133] V. Mirshafiee, R. Kim, S. Park, M. Mahmoudi, M.L. Kraft, Impact of protein pre-coating on the protein corona composition and nanoparticle cellular uptake, Biomaterials, 75 (2016) 295-304.
[134] R. Rampado, S. Crotti, P. Caliceti, S. Pucciarelli, M. Agostini, Recent Advances in Understanding the Protein Corona of Nanoparticles and in the Formulation of "Stealthy" Nanomaterials, Front Bioeng Biotechnol, 8 (2020) 166.
[135] D.V. Devine, J.M. Marjan, The role of immunoproteins in the survival of liposomes in the circulation, Crit Rev Ther Drug Carrier Syst, 14 (1997) 105-131.
[136] A. Gessner, A. Lieske, B. Paulke, R. Müller, Influence of surface charge density on protein adsorption on polymeric nanoparticles: analysis by two-dimensional electrophoresis, Eur J Pharm Biopharm, 54 (2002) 165-170.
[137] A. Gessner, R. Waicz, A. Lieske, B. Paulke, K. Mäder, R.H. Müller, Nanoparticles with decreasing surface hydrophobicities: influence on plasma protein adsorption, Int J Pharm, 196 (2000) 245-249.
[138] S. Nagayama, K. Ogawara, Y. Fukuoka, K. Higaki, T. Kimura, Time-dependent changes in opsonin amount associated on nanoparticles alter their hepatic uptake characteristics, Int J Pharm, 342 (2007) 215-221.
[139] I. Alberg, S. Kramer, M. Schinnerer, Q. Hu, C. Seidl, C. Leps, N. Drude, D. Mockel, C. Rijcken, T. Lammers, M. Diken, M. Maskos, S. Morsbach, K. Landfester, S. Tenzer, M. Barz, R. Zentel, Polymeric Nanoparticles with Neglectable Protein Corona, Small, 16 (2020) e1907574.
[140] J.S. Suk, Q. Xu, N. Kim, J. Hanes, L.M. Ensign, PEGylation as a strategy for improving nanoparticle-based drug and gene delivery, Advanced Drug Delivery Reviews, 99 (2016) 28-51.
[141] J.V. Jokerst, T. Lobovkina, R.N. Zare, S.S. Gambhir, Nanoparticle PEGylation for imaging and therapy, Nanomedicine (Lond), 6 (2011) 715-728.
[142] Q. Dai, C. Walkey, W.C. Chan, Polyethylene glycol backfilling mitigates the negative impact of the protein corona on nanoparticle cell targeting, Angew Chem Int Ed Engl, 53 (2014) 5093-5096.
[143] W.J. Kelley, C.A. Fromen, G. Lopez-Cazares, O. Eniola-Adefeso, PEGylation of model drug carriers enhances phagocytosis by primary human neutrophils, Acta Biomater, 79 (2018) 283-293.
[144] A. Birke, J. Ling, M. Barz, Polysarcosine-containing copolymers: Synthesis, characterization, self-assembly, and applications, Progress in Polymer Science, 81 (2018) 163-208.
[145] I. Negwer, A. Best, M. Schinnerer, O. Schäfer, L. Capeloa, M. Wagner, M. Schmidt, V. Mailänder, M. Helm, M. Barz, H.J. Butt, K. Koynov, Monitoring drug nanocarriers in human blood by near-infrared fluorescence correlation spectroscopy, Nat Commun, 9 (2018) 5306.
[146] J. Aggeler, Z. Werb, Initial events during phagocytosis by macrophages viewed from outside and inside the cell: membrane-particle interactions and clathrin, J Cell Biol, 94 (1982) 613-623.
[147] R. Montesano, A. Mossaz, P. Vassalli, L. Orci, Specialization of the macrophage plasma membrane at sites of interaction with opsonized erythrocytes, J Cell Biol, 96 (1983) 1227-1233.
[148] E. Veiga, P. Cossart, Listeria hijacks the clathrin-dependent endocytic machinery to invade mammalian cells, Nat Cell Biol, 7 (2005) 894-900.
[149] P. Cossart, E. Veiga, Non-classical use of clathrin during bacterial infections, J Microsc, 231 (2008) 524-528.
[150] M. Bonazzi, L. Vasudevan, A. Mallet, M. Sachse, A. Sartori, M.C. Prevost, A. Roberts, S.B. Taner, J.D. Wilbur, F.M. Brodsky, P. Cossart, Clathrin phosphorylation is required for actin recruitment at sites of bacterial adhesion and internalization, J Cell Biol, 195 (2011) 525-536.
[151] V. Mercanti, S.J. Charette, N. Bennett, J.J. Ryckewaert, F. Letourneur, P. Cosson, Selective membrane exclusion in phagocytic and macropinocytic cups, J Cell Sci, 119 (2006) 4079-4087.

[152] M.S. Brown, J.L. Goldstein, Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis, Annu Rev Biochem, 52 (1983) 223-261.
[153] D.R. Greaves, S. Gordon, The macrophage scavenger receptor at 30 years of age: current knowledge and future challenges, Journal of lipid research, 50 Suppl (2009) S282-286.
[154] M.S. Brown, J.L. Goldstein, Scientific side trips: six excursions from the beaten path, J Biol Chem, 287 (2012) 22418-22435.
[155] M.R. PrabhuDas, C.L. Baldwin, P.L. Bollyky, D.M.E. Bowdish, K. Drickamer, M. Febbraio, J. Herz, L. Kobzik, M. Krieger, J. Loike, B. McVicker, T.K. Means, S.K. Moestrup, S.R. Post, T. Sawamura, S. Silverstein, R.C. Speth, J.C. Telfer, G.M. Thiele, X.Y. Wang, S.D. Wright, J. El Khoury, A Consensus Definitive Classification of Scavenger Receptors and Their Roles in Health and Disease, J Immunol, 198 (2017) 3775-3789.
[156] J.M. Brown, E.J. Swindle, N.M. Kushnir-Sukhov, A. Holian, D.D. Metcalfe, Silica-directed mast cell activation is enhanced by scavenger receptors, Am J Respir Cell Mol Biol, 36 (2007) 43-52.
[157] A. Murshid, T.J. Borges, B.J. Lang, S.K. Calderwood, The Scavenger Receptor SREC-I Cooperates with Toll-Like Receptors to Trigger Inflammatory Innate Immune Responses, Front Immunol, 7 (2016) 226.
[158] S. Mukhopadhyay, A. Varin, Y. Chen, B. Liu, K. Tryggvason, S. Gordon, SR-A/MARCO-mediated ligand delivery enhances intracellular TLR and NLR function, but ligand scavenging from cell surface limits TLR4 response to pathogens, Blood, 117 (2011) 1319-1328.
[159] S.A. Thakur, R. Hamilton, Jr., T. Pikkarainen, A. Holian, Differential binding of inorganic particles to MARCO, Toxicological sciences : an official journal of the Society of Toxicology, 107 (2009) 238-246.
[160] M. Nakayama, Macrophage Recognition of Crystals and Nanoparticles, Front Immunol, 9 (2018) 103.
[161] A. Palecanda, L. Kobzik, Receptors for unopsonized particles: the role of alveolar macrophage scavenger receptors, Current molecular medicine, 1 (2001) 589-595.
[162] M.S. Arredouani, A. Palecanda, H. Koziel, Y.C. Huang, A. Imrich, T.H. Sulahian, Y.Y. Ning, Z. Yang, T. Pikkarainen, M. Sankala, S.O. Vargas, M. Takeya, K. Tryggvason, L. Kobzik, MARCO is the major binding receptor for unopsonized particles and bacteria on human alveolar macrophages, J Immunol, 175 (2005) 6058-6064.
[163] N.V. Yap, F.J. Whelan, D.M. Bowdish, G.B. Golding, The Evolution of the Scavenger Receptor Cysteine-Rich Domain of the Class A Scavenger Receptors, Front Immunol, 6 (2015) 342.
[164] J.R. Ojala, T. Pikkarainen, A. Tuuttila, T. Sandalova, K. Tryggvason, Crystal structure of the cysteine-rich domain of scavenger receptor MARCO reveals the presence of a basic and an acidic cluster that both contribute to ligand recognition, J Biol Chem, 282 (2007) 16654-16666.
[165] K.E. Novakowski, N.V.L. Yap, C. Yin, K. Sakamoto, B. Heit, G.B. Golding, D.M.E. Bowdish, Human-Specific Mutations and Positively Selected Sites in MARCO Confer Functional Changes, Molecular biology and evolution, 35 (2018) 440-450.
[166] S. Murthy, J.L. Larson-Casey, A.J. Ryan, C. He, L. Kobzik, A.B. Carter, Alternative activation of macrophages and pulmonary fibrosis are modulated by scavenger receptor, macrophage receptor with collagenous structure, FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 29 (2015) 3527-3536.
[167] R.M. Gilberti, G.N. Joshi, D.A. Knecht, The phagocytosis of crystalline silica particles by macrophages, Am J Respir Cell Mol Biol, 39 (2008) 619-627.
[168] T. Kusaka, M. Nakayama, K. Nakamura, M. Ishimiya, E. Furusawa, K. Ogasawara, Effect of silica particle size on macrophage inflammatory responses, PloS one, 9 (2014) e92634.
[169] W.J. Sandberg, M. Låg, J.A. Holme, B. Friede, M. Gualtieri, M. Kruszewski, P.E. Schwarze, T. Skuland, M. Refsnes, Comparison of non-crystalline silica nanoparticles in IL-1β release from macrophages, Part Fibre Toxicol, 9 (2012) 32.

[170] N. Nishijima, T. Hirai, K. Misato, M. Aoyama, E. Kuroda, K.J. Ishii, K. Higashisaka, Y. Yoshioka, Y. Tsutsumi, Human Scavenger Receptor A1-Mediated Inflammatory Response to Silica Particle Exposure Is Size Specific, Front Immunol, 8 (2017) 379.
[171] C.J. Merrifield, D. Perrais, D. Zenisek, Coupling between clathrin-coated-pit invagination, cortactin recruitment, and membrane scission observed in live cells, Cell, 121 (2005) 593-606.
[172] L.M. Fujimoto, R. Roth, J.E. Heuser, S.L. Schmid, Actin assembly plays a variable, but not obligatory role in receptor-mediated endocytosis in mammalian cells, Traffic, 1 (2000) 161-171.
[173] T.A. Gottlieb, I.E. Ivanov, M. Adesnik, D.D. Sabatini, Actin microfilaments play a critical role in endocytosis at the apical but not the basolateral surface of polarized epithelial cells, J Cell Biol, 120 (1993) 695-710.
[174] C. Messerschmidt, D. Hofmann, A. Kroeger, K. Landfester, V. Mailänder, I. Lieberwirth, On the pathway of cellular uptake: new insight into the interaction between the cell membrane and very small nanoparticles, Beilstein J Nanotechnol, 7 (2016) 1296-1311.
[175] M. Låg, T. Skuland, A. Godymchuk, T.H.T. Nguyen, H.L.T. Pham, M. Refsnes, Silica Nanoparticle-induced Cytokine Responses in BEAS-2B and HBEC3-KT Cells: Significance of Particle Size and Signalling Pathways in Different Lung Cell Cultures, Basic & clinical pharmacology & toxicology, 122 (2018) 620-632.
[176] T. Skuland, J. Ovrevik, M. Låg, P. Schwarze, M. Refsnes, Silica nanoparticles induce cytokine responses in lung epithelial cells through activation of a p38/TACE/TGF-α/EGFR-pathway and NF-κB signalling, Toxicology and applied pharmacology, 279 (2014) 76-86.
[177] T. Morishige, Y. Yoshioka, H. Inakura, A. Tanabe, X. Yao, S. Narimatsu, Y. Monobe, T. Imazawa, S. Tsunoda, Y. Tsutsumi, Y. Mukai, N. Okada, S. Nakagawa, The effect of surface modification of amorphous silica particles on NLRP3 inflammasome mediated IL-1beta production, ROS production and endosomal rupture, Biomaterials, 31 (2010) 6833-6842.
[178] R. Merget, T. Bauer, H.U. Küpper, S. Philippou, H.D. Bauer, R. Breitstadt, T. Bruening, Health hazards due to the inhalation of amorphous silica, Archives of toxicology, 75 (2002) 625-634.
[179] K.M. Waters, L.M. Masiello, R.C. Zangar, B.J. Tarasevich, N.J. Karin, R.D. Quesenberry, S. Bandyopadhyay, J.G. Teeguarden, J.G. Pounds, B.D. Thrall, Macrophage responses to silica nanoparticles are highly conserved across particle sizes, Toxicological sciences : an official journal of the Society of Toxicology, 107 (2009) 553-569.
[180] L. Rettig, S.P. Haen, A.G. Bittermann, L. von Boehmer, A. Curioni, S.D. Krämer, A. Knuth, S. Pascolo, Particle size and activation threshold: a new dimension of danger signaling, Blood, 115 (2010) 4533-4541.
[181] D.M. Underhill, H.S. Goodridge, Information processing during phagocytosis, Nature reviews. Immunology, 12 (2012) 492-502.
[182] E. Hoffmann, S. Marion, B.B. Mishra, M. John, R. Kratzke, S.F. Ahmad, D. Holzer, P.K. Anand, D.G. Weiss, G. Griffiths, S.A. Kuznetsov, Initial receptor–ligand interactions modulate gene expression and phagosomal properties during both early and late stages of phagocytosis, European Journal of Cell Biology, 89 (2010) 693-704.
[183] A.M. Pauwels, M. Trost, R. Beyaert, E. Hoffmann, Patterns, Receptors, and Signals: Regulation of Phagosome Maturation, Trends Immunol, 38 (2017) 407-422.
[184] T. Dykstra, O. Utermoehlen, A. Haas, Defined particle ligands trigger specific defense mechanisms of macrophages, Innate Immun, 17 (2011) 388-402.
[185] L.A. Allen, A. Aderem, Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages, J Exp Med, 184 (1996) 627-637.
[186] R.M. Gilberti, D.A. Knecht, Macrophages phagocytose nonopsonized silica particles using a unique microtubule-dependent pathway, Mol Biol Cell, 26 (2015) 518-529.

[187] R.E. Harrison, S. Grinstein, Phagocytosis and the microtubule cytoskeleton, Biochem Cell Biol, 80 (2002) 509-515.
[188] S.C. Silverstein, R.M. Steinman, Z.A. Cohn, Endocytosis, Annu Rev Biochem, 46 (1977) 669-722.
[189] A.G. Ehlenberger, V. Nussenzweig, The role of membrane receptors for C3b and C3d in phagocytosis, J Exp Med, 145 (1977) 357-371.
[190] M. van Egmond, G. Vidarsson, J.E. Bakema, Cross-talk between pathogen recognizing Toll-like receptors and immunoglobulin Fc receptors in immunity, Immunol Rev, 268 (2015) 311-327.
[191] F. Wang, X. Wang, L. Gao, L.Y. Meng, J.M. Xie, J.W. Xiong, Y. Luo, Nanoparticle-mediated delivery of siRNA into zebrafish heart: a cell-level investigation on the biodistribution and gene silencing effects, Nanoscale, 11 (2019) 18052-18064.
[192] M. Desjardins, J.E. Celis, G. van Meer, H. Dieplinger, A. Jahraus, G. Griffiths, L.A. Huber, Molecular characterization of phagosomes, J Biol Chem, 269 (1994) 32194-32200.
[193] H. Defacque, M. Egeberg, A. Habermann, M. Diakonova, C. Roy, P. Mangeat, W. Voelter, G. Marriott, J. Pfannstiel, H. Faulstich, G. Griffiths, Involvement of ezrin/moesin in de novo actin assembly on phagosomal membranes, EMBO J, 19 (2000) 199-212.
[194] H. Defacque, E. Bos, B. Garvalov, C. Barret, C. Roy, P. Mangeat, H.W. Shin, V. Rybin, G. Griffiths, Phosphoinositides regulate membrane-dependent actin assembly by latex bead phagosomes, Mol Biol Cell, 13 (2002) 1190-1202.
[195] M. Kühnel, L.S. Mayorga, T. Dandekar, J. Thakar, R. Schwarz, E. Anes, G. Griffiths, J. Reich, Modelling phagosomal lipid networks that regulate actin assembly, BMC Syst Biol, 2 (2008) 107.
[196] E. Anes, M.P. Kühnel, E. Bos, J. Moniz-Pereira, A. Habermann, G. Griffiths, Selected lipids activate phagosome actin assembly and maturation resulting in killing of pathogenic mycobacteria, Nat Cell Biol, 5 (2003) 793-802.
[197] W.H. Lewis, Pinocytosis by Malignant Cells, The American Journal of Cancer, 29 (1937) 666.
[198] A.T. Jones, Macropinocytosis: searching for an endocytic identity and role in the uptake of cell penetrating peptides, J Cell Mol Med, 11 (2007) 670-684.
[199] C. Commisso, The pervasiveness of macropinocytosis in oncological malignancies, Philos Trans R Soc Lond B Biol Sci, 374 (2019) 20180153.
[200] S. Cassar, I. Adatto, J.L. Freeman, J.T. Gamse, I. Iturria, C. Lawrence, A. Muriana, R.T. Peterson, S. Van Cruchten, L.I. Zon, Use of Zebrafish in Drug Discovery Toxicology, Chem Res Toxicol, 33 (2020) 95-118.
[201] L. Chen, D. Cheng, J. Chu, T. Zhang, Z. Dong, H. Lou, L. Zhu, Y. Liu, A Novel Method to Image Macropinocytosis in Vivo, Front Neurosci, 12 (2018) 324.
[202] A. Takeuchi, Electron microscope studies of experimental Salmonella infection. I. Penetration into the intestinal epithelium by Salmonella typhimurium, Am J Pathol, 50 (1967) 109-136.
[203] J.J. Choi, S. Wang, Y.S. Tung, B. Morrison, 3rd, E.E. Konofagou, Molecules of various pharmacologically-relevant sizes can cross the ultrasound-induced blood-brain barrier opening in vivo, Ultrasound Med Biol, 36 (2010) 58-67.
[204] S. Bosedasgupta, J. Pieters, Inflammatory stimuli reprogram macrophage phagocytosis to macropinocytosis for the rapid elimination of pathogens, PLoS Pathog, 10 (2014) e1003879.
[205] A. Weiner, N. Mellouk, N. Lopez-Montero, Y.Y. Chang, C. Souque, C. Schmitt, J. Enninga, Macropinosomes are Key Players in Early Shigella Invasion and Vacuolar Escape in Epithelial Cells, PLoS Pathog, 12 (2016) e1005602.
[206] A. Veithen, P. Cupers, P. Baudhuin, P.J. Courtoy, v-Src induces constitutive macropinocytosis in rat fibroblasts, J Cell Sci, 109 (Pt 8) (1996) 2005-2012.
[207] J. Canton, Macropinocytosis: New Insights Into Its Underappreciated Role in Innate Immune Cell Surveillance, Front Immunol, 9 (2018) 2286.

[208] F. Sallusto, M. Cella, C. Danieli, A. Lanzavecchia, Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products, J Exp Med, 182 (1995) 389-400.
[209] H.T. Haigler, J.A. McKanna, S. Cohen, Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-431, J Cell Biol, 81 (1979) 382-395.
[210] C. Commisso, S.M. Davidson, R.G. Soydaner-Azeloglu, S.J. Parker, J.J. Kamphorst, S. Hackett, E. Grabocka, M. Nofal, J.A. Drebin, C.B. Thompson, J.D. Rabinowitz, C.M. Metallo, M.G. Vander Heiden, D. Bar-Sagi, Macropinocytosis of protein is an amino acid supply route in Ras-transformed cells, Nature, 497 (2013) 633-637.
[211] A. Veithen, M. Amyere, P. Van Der Smissen, P. Cupers, P.J. Courtoy, Regulation of macropinocytosis in v-Src-transformed fibroblasts: cyclic AMP selectively promotes regurgitation of macropinosomes, J Cell Sci, 111 (Pt 16) (1998) 2329-2335.
[212] S. Kühn, N. Lopez-Montero, Y.Y. Chang, A. Sartori-Rupp, J. Enninga, Imaging macropinosomes during Shigella infections, Methods (San Diego, Calif.), 127 (2017) 12-22.
[213] J.L. Hoon, W.K. Wong, C.G. Koh, Functions and regulation of circular dorsal ruffles, Mol Cell Biol, 32 (2012) 4246-4257.
[214] J.A. Swanson, Shaping cups into phagosomes and macropinosomes, Nat Rev Mol Cell Biol, 9 (2008) 639-649.
[215] T.P. Welliver, S.L. Chang, J.J. Linderman, J.A. Swanson, Ruffles limit diffusion in the plasma membrane during macropinosome formation, J Cell Sci, 124 (2011) 4106-4114.
[216] S. Yoshida, A.D. Hoppe, N. Araki, J.A. Swanson, Sequential signaling in plasma-membrane domains during macropinosome formation in macrophages, J Cell Sci, 122 (2009) 3250-3261.
[217] J.A. Swanson, C. Watts, Macropinocytosis, Trends Cell Biol, 5 (1995) 424-428.
[218] J.G. Donaldson, Macropinosome formation, maturation and membrane recycling: lessons from clathrin-independent endosomal membrane systems, Philos Trans R Soc Lond B Biol Sci, 374 (2019) 20180148.
[219] P.C. Patel, R.E. Harrison, Membrane ruffles capture C3bi-opsonized particles in activated macrophages, Mol Biol Cell, 19 (2008) 4628-4639.
[220] L.J. Hewlett, A.R. Prescott, C. Watts, The coated pit and macropinocytic pathways serve distinct endosome populations, J Cell Biol, 124 (1994) 689-703.
[221] B. Schneider, C. Schueller, O. Utermoehlen, A. Haas, Lipid microdomain-dependent macropinocytosis determines compartmentation of Afipia felis, Traffic, 8 (2007) 226-240.
[222] A. Junemann, V. Filić, M. Winterhoff, B. Nordholz, C. Litschko, H. Schwellenbach, T. Stephan, I. Weber, J. Faix, A Diaphanous-related formin links Ras signaling directly to actin assembly in macropinocytosis and phagocytosis, Proc Natl Acad Sci U S A, 113 (2016) E7464-e7473.
[223] A.J. Davidson, C. Amato, P.A. Thomason, R.H. Insall, WASP family proteins and formins compete in pseudopod- and bleb-based migration, J Cell Biol, 217 (2018) 701-714.
[224] Z. Ujfalusi, M. Kovács, N.T. Nagy, S. Barkó, G. Hild, A. Lukács, M. Nyitrai, B. Bugyi, Myosin and tropomyosin stabilize the conformation of formin-nucleated actin filaments, J Biol Chem, 287 (2012) 31894-31904.
[225] M.C. Kerr, R.D. Teasdale, Defining macropinocytosis, Traffic, 10 (2009) 364-371.
[226] G. Griffiths, On phagosome individuality and membrane signalling networks, Trends Cell Biol, 14 (2004) 343-351.
[227] G. Bloomfield, R.R. Kay, Uses and abuses of macropinocytosis, J Cell Sci, 129 (2016) 2697-2705.
[228] T.P. Loisel, R. Boujemaa, D. Pantaloni, M.F. Carlier, Reconstitution of actin-based motility of Listeria and Shigella using pure proteins, Nature, 401 (1999) 613-616.

[229] C.D. Williamson, J.G. Donaldson, Arf6, JIP3, and dynein shape and mediate macropinocytosis, Mol Biol Cell, 30 (2019) 1477-1489.
[230] J. Canton, D. Schlam, C. Breuer, M. Gütschow, M. Glogauer, S. Grinstein, Calcium-sensing receptors signal constitutive macropinocytosis and facilitate the uptake of NOD2 ligands in macrophages, Nat Commun, 7 (2016) 11284.
[231] C.C. Norbury, Drinking a lot is good for dendritic cells, Immunology, 117 (2006) 443-451.
[232] D.S. Redka, M. Gütschow, S. Grinstein, J. Canton, Differential ability of proinflammatory and anti-inflammatory macrophages to perform macropinocytosis, Mol Biol Cell, 29 (2018) 53-65.
[233] T.P. Kohler, A. Scholz, D. Kiachludis, S. Hammerschmidt, Induction of Central Host Signaling Kinases during Pneumococcal Infection of Human THP-1 Cells, Front Cell Infect Microbiol, 6 (2016) 48.
[234] J. Oviedo-Boyso, R. Cortés-Vieyra, A. Huante-Mendoza, H.B. Yu, J.J. Valdez-Alarcón, A. Bravo-Patiño, M. Cajero-Juárez, B.B. Finlay, V.M. Baizabal-Aguirre, The phosphoinositide-3-kinase-Akt signaling pathway is important for Staphylococcus aureus internalization by endothelial cells, Infect Immun, 79 (2011) 4569-4577.
[235] Y. Lefkir, M. Malbouyres, D. Gotthardt, A. Ozinsky, S. Cornillon, F. Bruckert, A.A. Aderem, T. Soldati, P. Cosson, F. Letourneur, Involvement of the AP-1 adaptor complex in early steps of phagocytosis and macropinocytosis, Mol Biol Cell, 15 (2004) 861-869.
[236] S.A. Freeman, S. Grinstein, Phagocytosis: receptors, signal integration, and the cytoskeleton, Immunol Rev, 262 (2014) 193-215.
[237] R. Baughn, P.F. Bonventre, Phagocytosis and intracellular killing of Staphylococcus aureus by normal mouse peritoneal macrophages, Infect Immun, 12 (1975) 346-352.
[238] P.J. Sansonetti, Rupture, invasion and inflammatory destruction of the intestinal barrier by Shigella, making sense of prokaryote-eukaryote cross-talks, FEMS Microbiol Rev, 25 (2001) 3-14.
[239] O. Meier, K. Boucke, S.V. Hammer, S. Keller, R.P. Stidwill, S. Hemmi, U.F. Greber, Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake, J Cell Biol, 158 (2002) 1119-1131.
[240] J. Mercer, A. Helenius, Gulping rather than sipping: macropinocytosis as a way of virus entry, Curr Opin Microbiol, 15 (2012) 490-499.
[241] M. Schelhaas, B. Shah, M. Holzer, P. Blattmann, L. Kühling, P.M. Day, J.T. Schiller, A. Helenius, Entry of human papillomavirus type 16 by actin-dependent, clathrin- and lipid raft-independent endocytosis, PLoS Pathog, 8 (2012) e1002657.
[242] J. Mercer, A. Helenius, Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells, Science, 320 (2008) 531-535.
[243] E.G. Sánchez, A. Quintas, D. Pérez-Núñez, M. Nogal, S. Barroso, L. Carrascosa Á, Y. Revilla, African swine fever virus uses macropinocytosis to enter host cells, PLoS Pathog, 8 (2012) e1002754.
[244] J.K. Locker, A. Kuehn, S. Schleich, G. Rutter, H. Hohenberg, R. Wepf, G. Griffiths, Entry of the two infectious forms of vaccinia virus at the plasma membane is signaling-dependent for the IMV but not the EEV, Mol Biol Cell, 11 (2000) 2497-2511.
[245] B. Moss, Poxvirus cell entry: how many proteins does it take?, Viruses, 4 (2012) 688-707.
[246] J.A. Armstrong, D.H. Metz, M.R. Young, The mode of entry of vaccinia virus into L cells, J Gen Virol, 21 (1973) 533-537.
[247] E.J. Ungewickell, L. Hinrichsen, Endocytosis: clathrin-mediated membrane budding, Curr Opin Cell Biol, 19 (2007) 417-425.
[248] H.T. McMahon, E. Boucrot, Molecular mechanism and physiological functions of clathrin-mediated endocytosis, Nat Rev Mol Cell Biol, 12 (2011) 517-533.
[249] M. Marsh, H.T. McMahon, The structural era of endocytosis, Science, 285 (1999) 215-220.

[250] J. Green, G.W. Griffiths, D. Louvard, P.S. Quinn, G. Warren, Passage of viral membrane proteins through the Golgi complex, Journal of molecular biology, 152 4 (1981) 663-698.
[251] I. Killisch, P. Steinlein, K. Römisch, R. Hollinshead, H. Beug, G. Griffiths, Characterization of early and late endocytic compartments of the transferrin cycle. Transferrin receptor antibody blocks erythroid differentiation by trapping the receptor in the early endosome, J Cell Sci, 103 (Pt 1) (1992) 211-232.
[252] J.S. Bonifacino, L.M. Traub, Signals for sorting of transmembrane proteins to endosomes and lysosomes, Annu Rev Biochem, 72 (2003) 395-447.
[253] E.A. Latomanski, H.J. Newton, Taming the Triskelion: Bacterial Manipulation of Clathrin, Microbiol Mol Biol Rev, 83 (2019).
[254] A. Sorkin, M. von Zastrow, Endocytosis and signalling: intertwining molecular networks, Nat Rev Mol Cell Biol, 10 (2009) 609-622.
[255] A. Sanger, J. Hirst, A.K. Davies, M.S. Robinson, Adaptor protein complexes and disease at a glance, J Cell Sci, 132 (2019).
[256] J. Grove, M. Marsh, The cell biology of receptor-mediated virus entry, J Cell Biol, 195 (2011) 1071-1082.
[257] O. Kovtun, V.K. Dickson, B.T. Kelly, D.J. Owen, J.A.G. Briggs, Architecture of the AP2/clathrin coat on the membranes of clathrin-coated vesicles, Sci Adv, 6 (2020) eaba8381.
[258] J. Grove, D.J. Metcalf, A.E. Knight, S.T. Wavre-Shapton, T. Sun, E.D. Protonotarios, L.D. Griffin, J. Lippincott-Schwartz, M. Marsh, Flat clathrin lattices: stable features of the plasma membrane, Mol Biol Cell, 25 (2014) 3581-3594.
[259] M. Lampe, S. Vassilopoulos, C. Merrifield, Clathrin coated pits, plaques and adhesion, J Struct Biol, 196 (2016) 48-56.
[260] B. Antonny, C. Burd, P. De Camilli, E. Chen, O. Daumke, K. Faelber, M. Ford, V.A. Frolov, A. Frost, J.E. Hinshaw, T. Kirchhausen, M.M. Kozlov, M. Lenz, H.H. Low, H. McMahon, C. Merrifield, T.D. Pollard, P.J. Robinson, A. Roux, S. Schmid, Membrane fission by dynamin: what we know and what we need to know, EMBO J, 35 (2016) 2270-2284.
[261] M. Kaksonen, A. Roux, Mechanisms of clathrin-mediated endocytosis, Nat Rev Mol Cell Biol, 19 (2018) 313-326.
[262] L.M. Traub, Common principles in clathrin-mediated sorting at the Golgi and the plasma membrane, Biochim Biophys Acta, 1744 (2005) 415-437.
[263] E.M. Schmid, H.T. McMahon, Integrating molecular and network biology to decode endocytosis, Nature, 448 (2007) 883-888.
[264] S.E. Lakhan, S. Sabharanjak, A. De, Endocytosis of glycosylphosphatidylinositol-anchored proteins, J Biomed Sci, 16 (2009) 93.
[265] A. Pelchen-Matthews, J.E. Armes, G. Griffiths, M. Marsh, Differential endocytosis of CD4 in lymphocytic and nonlymphocytic cells, J Exp Med, 173 (1991) 575-587.
[266] M. Singh, H.R. Jadhav, T. Bhatt, Dynamin Functions and Ligands: Classical Mechanisms Behind, Molecular pharmacology, 91 (2017) 123-134.
[267] G.H. Borner, M. Harbour, S. Hester, K.S. Lilley, M.S. Robinson, Comparative proteomics of clathrin-coated vesicles, J Cell Biol, 175 (2006) 571-578.
[268] L.K. Rosselli-Murai, J.A. Yates, S. Yoshida, J. Bourg, K.K.Y. Ho, M. White, J. Prisby, X. Tan, M. Altemus, L. Bao, Z.F. Wu, S.L. Veatch, J.A. Swanson, S.D. Merajver, A.P. Liu, Loss of PTEN promotes formation of signaling-capable clathrin-coated pits, J Cell Sci, 131 (2018).
[269] D. Loerke, M. Mettlen, D. Yarar, K. Jaqaman, H. Jaqaman, G. Danuser, S.L. Schmid, Cargo and dynamin regulate clathrin-coated pit maturation, PLoS Biol, 7 (2009) e57.
[270] A.G. Henry, J.N. Hislop, J. Grove, K. Thorn, M. Marsh, M. von Zastrow, Regulation of endocytic clathrin dynamics by cargo ubiquitination, Dev Cell, 23 (2012) 519-532.

[271] G. Apodaca, Endocytic traffic in polarized epithelial cells: role of the actin and microtubule cytoskeleton, Traffic, 2 (2001) 149-159.
[272] S. Boulant, C. Kural, J.-C. Zeeh, F. Ubelmann, T. Kirchhausen, Actin dynamics counteract membrane tension during clathrin-mediated endocytosis, Nat Cell Biol, 13 (2011) 1124-1131.
[273] C.J. Merrifield, B. Qualmann, M.M. Kessels, W. Almers, Neural Wiskott Aldrich Syndrome Protein (N-WASP) and the Arp2/3 complex are recruited to sites of clathrin-mediated endocytosis in cultured fibroblasts, Eur J Cell Biol, 83 (2004) 13-18.
[274] E. Smythe, K.R. Ayscough, Actin regulation in endocytosis, J Cell Sci, 119 (2006) 4589-4598.
[275] R.G. Anderson, M.S. Brown, J.L. Goldstein, Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts, Cell, 10 (1977) 351-364.
[276] G. Griffiths, R. Back, M. Marsh, A quantitative analysis of the endocytic pathway in baby hamster kidney cells, J Cell Biol, 109 (1989) 2703-2720.
[277] A. Motley, N.A. Bright, M.N. Seaman, M.S. Robinson, Clathrin-mediated endocytosis in AP-2-depleted cells, J Cell Biol, 162 (2003) 909-918.
[278] M. Marsh, A. Helenius, Adsorptive endocytosis of Semliki Forest virus, J Mol Biol, 142 (1980) 439-454.
[279] S.F. Cotmore, P. Tattersall, Parvoviral host range and cell entry mechanisms, Advances in virus research, 70 (2007) 183-232.
[280] J.H. Nunberg, J. York, The curious case of arenavirus entry, and its inhibition, Viruses, 4 (2012) 83-101.
[281] D. Finkelshtein, A. Werman, D. Novick, S. Barak, M. Rubinstein, LDL receptor and its family members serve as the cellular receptors for vesicular stomatitis virus, Proc Natl Acad Sci U S A, 110 (2013) 7306-7311.
[282] D.K. Cureton, R.H. Massol, S.P. Whelan, T. Kirchhausen, The length of vesicular stomatitis virus particles dictates a need for actin assembly during clathrin-dependent endocytosis, PLoS Pathog, 6 (2010) e1001127.
[283] C. Dalal, A. Saha, N.R. Jana, Nanoparticle Multivalency Directed Shifting of Cellular Uptake Mechanism, The Journal of Physical Chemistry C, 120 (2016) 6778-6786.
[284] L. Kou, J. Sun, Y. Zhai, Z. He, The endocytosis and intracellular fate of nanomedicines: Implication for rational design, Asian Journal of Pharmaceutical Sciences, 8 (2013) 1-10.
[285] D.A. Kuhn, D. Vanhecke, B. Michen, F. Blank, P. Gehr, A. Petri-Fink, B. Rothen-Rutishauser, Different endocytotic uptake mechanisms for nanoparticles in epithelial cells and macrophages, Beilstein J Nanotechnol, 5 (2014) 1625-1636.
[286] O. Harush-Frenkel, N. Debotton, S. Benita, Y. Altschuler, Targeting of nanoparticles to the clathrin-mediated endocytic pathway, Biochem Biophys Res Commun, 353 (2007) 26-32.
[287] I.L. Hsiao, A.M. Gramatke, R. Joksimovic, M. Sokolowski, M. Gradzielski, A. Haase, Size and cell type dependent uptake of silica nanoparticles, Journal of nanomedicine & nanotechnology, 5 (2014) 248.
[288] J. Kasper, M.I. Hermanns, C. Bantz, S. Utech, O. Koshkina, M. Maskos, C. Brochhausen, C. Pohl, S. Fuchs, R.E. Unger, C.J. Kirkpatrick, Flotillin-involved uptake of silica nanoparticles and responses of an alveolar-capillary barrier in vitro, Eur J Pharm Biopharm, 84 (2013) 275-287.
[289] L.T.M. Phuc, A. Taniguchi, Epidermal Growth Factor Enhances Cellular Uptake of Polystyrene Nanoparticles by Clathrin-Mediated Endocytosis, Int J Mol Sci, 18 (2017).
[290] N. Reix, A. Parat, E. Seyfritz, R. Van der Werf, V. Epure, N. Ebel, L. Danicher, E. Marchioni, N. Jeandidier, M. Pinget, Y. Frère, S. Sigrist, In vitro uptake evaluation in Caco-2 cells and in vivo results in diabetic rats of insulin-loaded PLGA nanoparticles, Int J Pharm, 437 (2012) 213-220.
[291] S. Huth, J. Lausier, S.W. Gersting, C. Rudolph, C. Plank, U. Welsch, J. Rosenecker, Insights into the mechanism of magnetofection using PEI-based magnetofectins for gene transfer, The journal of gene medicine, 6 (2004) 923-936.

[292] J.M. Davis, H. Clay, J.L. Lewis, N. Ghori, P. Herbomel, L. Ramakrishnan, Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos, Immunity, 17 (2002) 693-702.
[293] J. Panyam, V. Labhasetwar, Dynamics of endocytosis and exocytosis of poly(D,L-lactide-co-glycolide) nanoparticles in vascular smooth muscle cells, Pharm Res, 20 (2003) 212-220.
[294] J. Panyam, W.Z. Zhou, S. Prabha, S.K. Sahoo, V. Labhasetwar, Rapid endo-lysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery, FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 16 (2002) 1217-1226.
[295] E.R. Eckhardt, L. Cai, S. Shetty, Z. Zhao, A. Szanto, N.R. Webb, D.R. Van der Westhuyzen, High density lipoprotein endocytosis by scavenger receptor SR-BII is clathrin-dependent and requires a carboxyl-terminal dileucine motif, J Biol Chem, 281 (2006) 4348-4353.
[296] H. Wang, L. Wu, B.M. Reinhard, Scavenger receptor mediated endocytosis of silver nanoparticles into J774A.1 macrophages is heterogeneous, ACS nano, 6 (2012) 7122-7132.
[297] O. Lunov, T. Syrovets, C. Röcker, K. Tron, G.U. Nienhaus, V. Rasche, V. Mailänder, K. Landfester, T. Simmet, Lysosomal degradation of the carboxydextran shell of coated superparamagnetic iron oxide nanoparticles and the fate of professional phagocytes, Biomaterials, 31 (2010) 9015-9022.
[298] L. Treuel, X. Jiang, G.U. Nienhaus, New views on cellular uptake and trafficking of manufactured nanoparticles, Journal of the Royal Society, Interface, 10 (2013) 20120939.
[299] J. Rességuier, J.P. Levraud, N.K. Dal, F. Fenaroli, C. Primard, J. Wohlmann, G. Carron, G.W. Griffiths, D. Le Guellec, B. Verrier, Biodistribution of surfactant-free poly(lactic-acid) nanoparticles and uptake by endothelial cells and phagocytes in zebrafish: Evidence for endothelium to macrophage transfer, J Control Release, 331 (2021) 228-245.
[300] F. Campbell, F.L. Bos, S. Sieber, G. Arias-Alpizar, B.E. Koch, J. Huwyler, A. Kros, J. Bussmann, Directing Nanoparticle Biodistribution through Evasion and Exploitation of Stab2-Dependent Nanoparticle Uptake, ACS nano, 12 (2018) 2138-2150.
[301] K. Yoshida, H. Nagata, H. Hoshi, Uptake of carbon and polystyrene particles by the sinusoidal endothelium of rabbit bone marrow and liver and rat bone marrow, with special reference to multiparticle-pinocytosis, Arch Histol Jpn, 47 (1984) 303-317.
[302] G.E. Palade, The fine structure of blood capillaries, J Appl Phys, 24 (1953) 1424.
[303] R.G. Parton, M.A. Del Pozo, S. Vassilopoulos, I.R. Nabi, S. Le Lay, R. Lundmark, A.K. Kenworthy, A. Camus, C.M. Blouin, W.C. Sessa, C. Lamaze, Caveolae: The FAQs, Traffic, 21 (2020) 181-185.
[304] O. Kovtun, V.A. Tillu, N. Ariotti, R.G. Parton, B.M. Collins, Cavin family proteins and the assembly of caveolae, J Cell Sci, 128 (2015) 1269-1278.
[305] M. Stoeber, I.K. Stoeck, C. Hänni, C.K.E. Bleck, G. Balistreri, A. Helenius, Oligomers of the ATPase EHD2 confine caveolae to the plasma membrane through association with actin, EMBO J, 31 (2012) 2350-2364.
[306] C. Matthaeus, I. Lahmann, S. Kunz, W. Jonas, A.A. Melo, M. Lehmann, E. Larsson, R. Lundmark, M. Kern, M. Blüher, H. Olschowski, J. Kompa, B. Brügger, D.N. Müller, V. Haucke, A. Schürmann, C. Birchmeier, O. Daumke, EHD2-mediated restriction of caveolar dynamics regulates cellular fatty acid uptake, Proceedings of the National Academy of Sciences, 117 (2020) 7471.
[307] M. Hubert, E. Larsson, N.V.G. Vegesna, M. Ahnlund, A.I. Johansson, L.W. Moodie, R. Lundmark, Lipid accumulation controls the balance between surface connection and scission of caveolae, eLife, 9 (2020).
[308] R. Lundmark, G.J. Doherty, M.T. Howes, K. Cortese, Y. Vallis, R.G. Parton, H.T. McMahon, The GTPase-activating protein GRAF1 regulates the CLIC/GEEC endocytic pathway, Curr Biol, 18 (2008) 1802-1808.

[309] E.M. Damm, L. Pelkmans, J. Kartenbeck, A. Mezzacasa, T. Kurzchalia, A. Helenius, Clathrin- and caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae, J Cell Biol, 168 (2005) 477-488.
[310] B. Sinha, D. Köster, R. Ruez, P. Gonnord, M. Bastiani, D. Abankwa, R.V. Stan, G. Butler-Browne, B. Vedie, L. Johannes, N. Morone, R.G. Parton, G. Raposo, P. Sens, C. Lamaze, P. Nassoy, Cells respond to mechanical stress by rapid disassembly of caveolae, Cell, 144 (2011) 402-413.
[311] K.A. McMahon, Y. Wu, Y. Gambin, E. Sierecki, V.A. Tillu, T. Hall, N. Martel, S. Okano, S.V. Moradi, J.E. Ruelcke, C. Ferguson, A.S. Yap, K. Alexandrov, M.M. Hill, R.G. Parton, Identification of intracellular cavin target proteins reveals cavin-PP1alpha interactions regulate apoptosis, Nat Commun, 10 (2019) 3279.
[312] K.A. McMahon, D.A. Stroud, Y. Gambin, V. Tillu, M. Bastiani, E. Sierecki, M.E. Polinkovsky, T.E. Hall, G.A. Gomez, Y. Wu, M.O. Parat, N. Martel, H.P. Lo, K.K. Khanna, K. Alexandrov, R. Daly, A. Yap, M.T. Ryan, R.G. Parton, Cavin3 released from caveolae interacts with BRCA1 to regulate the cellular stress response, eLife, 10 (2021).
[313] T. Richter, M. Floetenmeyer, C. Ferguson, J. Galea, J. Goh, M.R. Lindsay, G.P. Morgan, B.J. Marsh, R.G. Parton, High-resolution 3D quantitative analysis of caveolar ultrastructure and caveola-cytoskeleton interactions, Traffic, 9 (2008) 893-909.
[314] J.W. Myerson, O. McPherson, K.G. DeFrates, J.H. Towslee, O.A. Marcos-Contreras, V.V. Shuvaev, B. Braender, R.J. Composto, V.R. Muzykantov, D.M. Eckmann, Cross-linker-Modulated Nanogel Flexibility Correlates with Tunable Targeting to a Sterically Impeded Endothelial Marker, ACS nano, 13 (2019) 11409-11421.
[315] E. Shvets, V. Bitsikas, G. Howard, C.G. Hansen, B.J. Nichols, Dynamic caveolae exclude bulk membrane proteins and are required for sorting of excess glycosphingolipids, Nat Commun, 6 (2015) 6867.
[316] Y.X. Li, Y. Wei, R. Zhong, L. Li, H.B. Pang, Transportan Peptide Stimulates the Nanomaterial Internalization into Mammalian Cells in the Bystander Manner through Macropinocytosis, Pharmaceutics, 13 (2021).
[317] E. Durr, J. Yu, K.M. Krasinska, L.A. Carver, J.R. Yates, J.E. Testa, P. Oh, J.E. Schnitzer, Direct proteomic mapping of the lung microvascular endothelial cell surface in vivo and in cell culture, Nat Biotechnol, 22 (2004) 985-992.
[318] L. Pelkmans, J. Kartenbeck, A. Helenius, Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER, Nat Cell Biol, 3 (2001) 473-483.
[319] A. Hayer, M. Stoeber, D. Ritz, S. Engel, H.H. Meyer, A. Helenius, Caveolin-1 is ubiquitinated and targeted to intralumenal vesicles in endolysosomes for degradation, J Cell Biol, 191 (2010) 615-629.
[320] M.T. Howes, M. Kirkham, J. Riches, K. Cortese, P.J. Walser, F. Simpson, M.M. Hill, A. Jones, R. Lundmark, M.R. Lindsay, D.J. Hernandez-Deviez, G. Hadzic, A. McCluskey, R. Bashir, L. Liu, P. Pilch, H. McMahon, P.J. Robinson, J.F. Hancock, S. Mayor, R.G. Parton, Clathrin-independent carriers form a high capacity endocytic sorting system at the leading edge of migrating cells, J Cell Biol, 190 (2010) 675-691.
[321] E. Blanco, H. Shen, M. Ferrari, Principles of nanoparticle design for overcoming biological barriers to drug delivery, Nat Biotechnol, 33 (2015) 941-951.
[322] L.C. Nelemans, L. Gurevich, Drug Delivery with Polymeric Nanocarriers-Cellular Uptake Mechanisms, Materials (Basel, Switzerland), 13 (2020).
[323] G. Sahay, D.Y. Alakhova, A.V. Kabanov, Endocytosis of nanomedicines, J Control Release, 145 (2010) 182-195.
[324] J. Lee, M. Twomey, C. Machado, G. Gomez, M. Doshi, A.J. Gesquiere, J.H. Moon, Caveolae-mediated endocytosis of conjugated polymer nanoparticles, Macromolecular bioscience, 13 (2013) 913-920.

[325] A. Madni, S. Rehman, H. Sultan, M.M. Khan, F. Ahmad, M.R. Raza, N. Rai, F. Parveen, Mechanistic Approaches of Internalization, Subcellular Trafficking, and Cytotoxicity of Nanoparticles for Targeting the Small Intestine, AAPS PharmSciTech, 22 (2020) 3.
[326] V. Ceña, P. Játiva, Nanoparticle crossing of blood-brain barrier: a road to new therapeutic approaches to central nervous system diseases, Nanomedicine (Lond), 13 (2018) 1513-1516.
[327] N.W. Andrews, M. Corrotte, Plasma membrane repair, Curr Biol, 28 (2018) R392-r397.
[328] M. Gedda, P. Babele, K. Zahra, P. Madhukar, Epigenetic Aspects of Engineered Nanomaterials: Is the Collateral Damage Inevitable?, Frontiers in Bioengineering and Biotechnology, (2019) 228.
[329] V. Francia, D. Montizaan, A. Salvati, Interactions at the cell membrane and pathways of internalization of nano-sized materials for nanomedicine, Beilstein J Nanotechnol, 11 (2020) 338-353.
[330] P. Foroozandeh, A.A. Aziz, Insight into Cellular Uptake and Intracellular Trafficking of Nanoparticles, Nanoscale research letters, 13 (2018) 339.
[331] T. Skotland, T.-G. Iversen, K. Sandvig, Cellular uptake of nanoparticles: Involvement of caveolae?, Precis. Nanomed, 4 (2021) 782-786.
[332] T. dos Santos, J. Varela, I. Lynch, A. Salvati, K.A. Dawson, Effects of transport inhibitors on the cellular uptake of carboxylated polystyrene nanoparticles in different cell lines, PloS one, 6 (2011) e24438.
[333] J. Rejman, A. Bragonzi, M. Conese, Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes, Mol Ther, 12 (2005) 468-474.
[334] D. Predescu, M. Simionescu, N. Simionescu, G.E. Palade, Binding and transcytosis of glycoalbumin by the microvascular endothelium of the murine myocardium: evidence that glycoalbumin behaves as a bifunctional ligand, Journal of Cell Biology, 107 (1988) 1729-1738.
[335] M. Kirkham, A. Fujita, R. Chadda, S.J. Nixon, T.V. Kurzchalia, D.K. Sharma, R.E. Pagano, J.F. Hancock, S. Mayor, R.G. Parton, Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles, J Cell Biol, 168 (2005) 465-476.
[336] K.Y. Fung, L. Dai, W.S. Trimble, Cell and molecular biology of septins, International review of cell and molecular biology, 310 (2014) 289-339.
[337] V.V. Shuvaev, M. Khoshnejad, K.W. Pulsipher, R.Y. Kiseleva, E. Arguiri, J.C. Cheung-Lau, K.M. LeFort, M. Christofidou-Solomidou, R.V. Stan, I.J. Dmochowski, V.R. Muzykantov, Spatially controlled assembly of affinity ligand and enzyme cargo enables targeting ferritin nanocarriers to caveolae, Biomaterials, 185 (2018) 348-359.
[338] S. Mayor, R.G. Parton, J.G. Donaldson, Clathrin-independent pathways of endocytosis, Cold Spring Harb Perspect Biol, 6 (2014).
[339] L. Johannes, R.G. Parton, P. Bassereau, S. Mayor, Building endocytic pits without clathrin, Nat Rev Mol Cell Biol, 16 (2015) 311-321.
[340] S. Sabharanjak, P. Sharma, R.G. Parton, S. Mayor, GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway, Dev Cell, 2 (2002) 411-423.
[341] M. Sathe, G. Muthukrishnan, J. Rae, A. Disanza, M. Thattai, G. Scita, R.G. Parton, S. Mayor, Small GTPases and BAR domain proteins regulate branched actin polymerisation for clathrin and dynamin-independent endocytosis, Nat Commun, 9 (2018) 1835.
[342] R. Lakshminarayan, C. Wunder, U. Becken, M.T. Howes, C. Benzing, S. Arumugam, S. Sales, N. Ariotti, V. Chambon, C. Lamaze, D. Loew, A. Shevchenko, K. Gaus, R.G. Parton, L. Johannes, Galectin-3 drives glycosphingolipid-dependent biogenesis of clathrin-independent carriers, Nat Cell Biol, 16 (2014) 595-606.
[343] J.J. Thottacherry, M. Sathe, C. Prabhakara, S. Mayor, Spoiled for Choice: Diverse Endocytic Pathways Function at the Cell Surface, Annu Rev Cell Dev Biol, 35 (2019) 55-84.

[344] N. Chaudhary, G.A. Gomez, M.T. Howes, H.P. Lo, K.A. McMahon, J.A. Rae, N.L. Schieber, M.M. Hill, K. Gaus, A.S. Yap, R.G. Parton, Endocytic crosstalk: cavins, caveolins, and caveolae regulate clathrin-independent endocytosis, PLoS Biol, 12 (2014) e1001832.
[345] N. Signoret, L. Hewlett, S. Wavre, A. Pelchen-Matthews, M. Oppermann, M. Marsh, Agonist-induced endocytosis of CC chemokine receptor 5 is clathrin dependent, Mol Biol Cell, 16 (2005) 902-917.
[346] H. Ewers, W. Romer, A.E. Smith, K. Bacia, S. Dmitrieff, W. Chai, R. Mancini, J. Kartenbeck, V. Chambon, L. Berland, A. Oppenheim, G. Schwarzmann, T. Feizi, P. Schwille, P. Sens, A. Helenius, L. Johannes, GM1 structure determines SV40-induced membrane invagination and infection, Nat Cell Biol, 12 (2010) 11-18; sup pp 11-12.
[347] E. Boucrot, A.P. Ferreira, L. Almeida-Souza, S. Debard, Y. Vallis, G. Howard, L. Bertot, N. Sauvonnet, H.T. McMahon, Endophilin marks and controls a clathrin-independent endocytic pathway, Nature, 517 (2015) 460-465.
[348] A.P.A. Ferreira, E. Boucrot, Mechanisms of Carrier Formation during Clathrin-Independent Endocytosis, Trends Cell Biol, 28 (2018) 188-200.
[349] C.G. Hansen, B.J. Nichols, Molecular mechanisms of clathrin-independent endocytosis, J Cell Sci, 122 (2009) 1713-1721.
[350] A. Hemalatha, S. Mayor, Recent advances in clathrin-independent endocytosis, F1000Res, 8 (2019) F1000 Faculty Rev-1138.
[351] K. Sandvig, S. Kavaliauskiene, T. Skotland, Clathrin-independent endocytosis: an increasing degree of complexity, Histochemistry and cell biology, 150 (2018) 107-118.
[352] E. Trofimenko, Y. Homma, M. Fukuda, C. Widmann, The endocytic pathway taken by cationic substances requires Rab14 but not Rab5 and Rab7, Cell Rep, 37 (2021) 109945.
[353] R.G. Parton, Caveolae: Structure, Function, and Relationship to Disease, Annu Rev Cell Dev Biol, 34 (2018) 111-136.
[354] R. Thuenauer, S.K. Müller, W. Römer, Pathways of protein and lipid receptor-mediated transcytosis in drug delivery, Expert Opin Drug Deliv, 14 (2017) 341-351.
[355] K.Y.Y. Fung, G.D. Fairn, W.L. Lee, Transcellular vesicular transport in epithelial and endothelial cells: Challenges and opportunities, Traffic, 19 (2018) 5-18.
[356] M.R. Russell, T.R. Lerner, J.J. Burden, D.O. Nkwe, A. Pelchen-Matthews, M.C. Domart, J. Durgan, A. Weston, M.L. Jones, C.J. Peddie, R. Carzaniga, O. Florey, M. Marsh, M.G. Gutierrez, L.M. Collinson, 3D correlative light and electron microscopy of cultured cells using serial blockface scanning electron microscopy, J Cell Sci, 130 (2017) 278-291.
[357] M. Marsh, G. Griffiths, G.E. Dean, I. Mellman, A. Helenius, Three-dimensional structure of endosomes in BHK-21 cells, Proc Natl Acad Sci U S A, 83 (1986) 2899-2903.
[358] J. Klumperman, G. Raposo, The complex ultrastructure of the endolysosomal system, Cold Spring Harb Perspect Biol, 6 (2014) a016857-a016857.
[359] J. Gruenberg, G. Griffiths, K.E. Howell, Characterization of the early endosome and putative endocytic carrier vesicles in vivo and with an assay of vesicle fusion in vitro, J Cell Biol, 108 (1989) 1301-1316.
[360] J. Gruenberg, The endocytic pathway: a mosaic of domains, Nat Rev Mol Cell Biol, 2 (2001) 721-730.
[361] V. Claus, A. Jahraus, T. Tjelle, T. Berg, H. Kirschke, H. Faulstich, G. Griffiths, Lysosomal enzyme trafficking between phagosomes, endosomes, and lysosomes in J774 macrophages. Enrichment of cathepsin H in early endosomes, J Biol Chem, 273 (1998) 9842-9851.
[362] K.M. Mayle, A.M. Le, D.T. Kamei, The intracellular trafficking pathway of transferrin, Biochim Biophys Acta, 1820 (2012) 264-281.
[363] P. Paramasivam, C. Franke, M. Stöter, A. Höijer, S. Bartesaghi, A. Sabirsh, L. Lindfors, M.Y. Arteta, A. Dahlén, A. Bak, S. Andersson, Y. Kalaidzidis, M. Bickle, M. Zerial, Endosomal escape of

delivered mRNA from endosomal recycling tubules visualized at the nanoscale, J Cell Biol, 221 (2022).
[364] I. Kalaidzidis, M. Miaczynska, M. Brewińska-Olchowik, A. Hupalowska, C. Ferguson, R.G. Parton, Y. Kalaidzidis, M. Zerial, APPL endosomes are not obligatory endocytic intermediates but act as stable cargo-sorting compartments, J Cell Biol, 211 (2015) 123-144.
[365] A. Urbanska, L. Sadowski, Y. Kalaidzidis, M. Miaczynska, Biochemical characterization of APPL endosomes: the role of annexin A2 in APPL membrane recruitment, Traffic, 12 (2011) 1227-1241.
[366] J. van der Beek, C. de Heus, N. Liv, J. Klumperman, Quantitative correlative microscopy reveals the ultrastructural distribution of endogenous endosomal proteins, Journal of Cell Biology, 221 (2021) e202106044.
[367] C.C. Cain, D.M. Sipe, R.F. Murphy, Regulation of endocytic pH by the Na+,K+-ATPase in living cells, Proc Natl Acad Sci U S A, 86 (1989) 544-548.
[368] C.J. Galloway, G.E. Dean, M. Marsh, G. Rudnick, I. Mellman, Acidification of macrophage and fibroblast endocytic vesicles in vitro, Proc Natl Acad Sci U S A, 80 (1983) 3334-3338.
[369] F.R. Maxfield, D.J. Yamashiro, Endosome acidification and the pathways of receptor-mediated endocytosis, Adv Exp Med Biol, 225 (1987) 189-198.
[370] J.R. van Weering, P.J. Cullen, Membrane-associated cargo recycling by tubule-based endosomal sorting, Semin Cell Dev Biol, 31 (2014) 40-47.
[371] M. Podinovskaia, C. Prescianotto-Baschong, D.P. Buser, A. Spang, A novel live-cell imaging assay reveals regulation of endosome maturation, eLife, 10 (2021).
[372] J. Gruenberg, Life in the lumen: The multivesicular endosome, Traffic, 21 (2020) 76-93.
[373] J. Rink, E. Ghigo, Y. Kalaidzidis, M. Zerial, Rab conversion as a mechanism of progression from early to late endosomes, Cell, 122 (2005) 735-749.
[374] D. Poteryaev, S. Datta, K. Ackema, M. Zerial, A. Spang, Identification of the switch in early-to-late endosome transition, Cell, 141 (2010) 497-508.
[375] R.G. Parton, K. Simons, C.G. Dotti, Axonal and dendritic endocytic pathways in cultured neurons, J Cell Biol, 119 (1992) 123-137.
[376] E. Granger, G. McNee, V. Allan, P. Woodman, The role of the cytoskeleton and molecular motors in endosomal dynamics, Semin Cell Dev Biol, 31 (2014) 20-29.
[377] M. Bomsel, R. Parton, S.A. Kuznetsov, T.A. Schroer, J. Gruenberg, Microtubule- and motor-dependent fusion in vitro between apical and basolateral endocytic vesicles from MDCK cells, Cell, 62 (1990) 719-731.
[378] F. Aniento, N. Emans, G. Griffiths, J. Gruenberg, Cytoplasmic dynein-dependent vesicular transport from early to late endosomes, J Cell Biol, 123 (1993) 1373-1387.
[379] W.A. Dunn, A.L. Hubbard, N.N. Aronson, Jr., Low temperature selectively inhibits fusion between pinocytic vesicles and lysosomes during heterophagy of 125I-asialofetuin by the perfused rat liver, J Biol Chem, 255 (1980) 5971-5978.
[380] S. Zachgo, B. Dobberstein, G. Griffiths, A block in degradation of MHC class II-associated invariant chain correlates with a reduction in transport from endosome carrier vesicles to the prelysosome compartment, J Cell Sci, 103 (Pt 3) (1992) 811-822.
[381] M. Vietri, M. Radulovic, H. Stenmark, The many functions of ESCRTs, Nat Rev Mol Cell Biol, 21 (2020) 25-42.
[382] F.G. van der Goot, J. Gruenberg, Intra-endosomal membrane traffic, Trends Cell Biol, 16 (2006) 514-521.
[383] A.M. Nour, Y. Modis, Endosomal vesicles as vehicles for viral genomes, Trends Cell Biol, 24 (2014) 449-454.
[384] P. Perrin, L. Janssen, H. Janssen, B. van den Broek, L.M. Voortman, D. van Elsland, I. Berlin, J. Neefjes, Retrofusion of intralumenal MVB membranes parallels viral infection and coexists with exosome release, Curr Biol, 31 (2021) 3884-3893.e3884.

[385] G. van Niel, G. D'Angelo, G. Raposo, Shedding light on the cell biology of extracellular vesicles, Nat Rev Mol Cell Biol, 19 (2018) 213-228.
[386] A. Blocker, F.F. Severin, A. Habermann, A.A. Hyman, G. Griffiths, J.K. Burkhardt, Microtubule-associated protein-dependent binding of phagosomes to microtubules, J Biol Chem, 271 (1996) 3803-3811.
[387] V. Lewis, S.A. Green, M. Marsh, P. Vihko, A. Helenius, I. Mellman, Glycoproteins of the lysosomal membrane, J Cell Biol, 100 (1985) 1839-1847.
[388] M. Schwake, B. Schröder, P. Saftig, Lysosomal membrane proteins and their central role in physiology, Traffic, 14 (2013) 739-748.
[389] M.J. Metzelaar, P.L. Wijngaard, P.J. Peters, J.J. Sixma, H.K. Nieuwenhuis, H.C. Clevers, CD63 antigen. A novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells, J Biol Chem, 266 (1991) 3239-3245.
[390] T. Kobayashi, U.M. Vischer, C. Rosnoblet, C. Lebrand, M. Lindsay, R.G. Parton, E.K. Kruithof, J. Gruenberg, The tetraspanin CD63/lamp3 cycles between endocytic and secretory compartments in human endothelial cells, Mol Biol Cell, 11 (2000) 1829-1843.
[391] J.M. Escola, M.J. Kleijmeer, W. Stoorvogel, J.M. Griffith, O. Yoshie, H.J. Geuze, Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes, J Biol Chem, 273 (1998) 20121-20127.
[392] T. Kobayashi, E. Stang, K.S. Fang, P. de Moerloose, R.G. Parton, J. Gruenberg, A lipid associated with the antiphospholipid syndrome regulates endosome structure and function, Nature, 392 (1998) 193-197.
[393] J.T. Hannich, K. Umebayashi, H. Riezman, Distribution and functions of sterols and sphingolipids, Cold Spring Harb Perspect Biol, 3 (2011).
[394] L. Liscum, Niemann-Pick type C mutations cause lipid traffic jam, Traffic, 1 (2000) 218-225.
[395] J. Larios, V. Mercier, A. Roux, J. Gruenberg, ALIX- and ESCRT-III-dependent sorting of tetraspanins to exosomes, J Cell Biol, 219 (2020).
[396] N.A. Bright, L.J. Davis, J.P. Luzio, Endolysosomes Are the Principal Intracellular Sites of Acid Hydrolase Activity, Curr Biol, 26 (2016) 2233-2245.
[397] G. Griffiths, B. Hoflack, K. Simons, I. Mellman, S. Kornfeld, The mannose 6-phosphate receptor and the biogenesis of lysosomes, Cell, 52 (1988) 329-341.
[398] E. van Meel, J. Klumperman, TGN exit of the cation-independent mannose 6-phosphate receptor does not require acid hydrolase binding, Cell Logist, 4 (2014) e954441.
[399] T.E. Tjelle, A. Brech, L.K. Juvet, G. Griffiths, T. Berg, Isolation and characterization of early endosomes, late endosomes and terminal lysosomes: their role in protein degradation, J Cell Sci, 109 (Pt 12) (1996) 2905-2914.
[400] C. de Duve, The lysosome turns fifty, Nat Cell Biol, 7 (2005) 847-849.
[401] J. Huotari, A. Helenius, Endosome maturation, EMBO J, 30 (2011) 3481-3500.
[402] Y. Hirota, N. Masuyama, T. Kuronita, H. Fujita, M. Himeno, Y. Tanaka, Analysis of post-lysosomal compartments, Biochem Biophys Res Commun, 314 (2004) 306-312.
[403] E. Anes, P. Peyron, L. Staali, L. Jordao, M.G. Gutierrez, H. Kress, M. Hagedorn, I. Maridonneau-Parini, M.A. Skinner, A.G. Wildeman, S.A. Kalamidas, M. Kuehnel, G. Griffiths, Dynamic life and death interactions between Mycobacterium smegmatis and J774 macrophages, Cell Microbiol, 8 (2006) 939-960.
[404] C. Butor, G. Griffiths, N.N. Aronson, Jr., A. Varki, Co-localization of hydrolytic enzymes with widely disparate pH optima: implications for the regulation of lysosomal pH, J Cell Sci, 108 (Pt 6) (1995) 2213-2219.
[405] A. Blocker, F.F. Severin, J.K. Burkhardt, J.B. Bingham, H. Yu, J.C. Olivo, T.A. Schroer, A.A. Hyman, G. Griffiths, Molecular requirements for bi-directional movement of phagosomes along microtubules, J Cell Biol, 137 (1997) 113-129.

[406] J. Heuser, Changes in lysosome shape and distribution correlated with changes in cytoplasmic pH, J Cell Biol, 108 (1989) 855-864.
[407] R.G. Parton, C.G. Dotti, R. Bacallao, I. Kurtz, K. Simons, K. Prydz, pH-induced microtubule-dependent redistribution of late endosomes in neuronal and epithelial cells, J Cell Biol, 113 (1991) 261-274.
[408] E.J. Blott, G.M. Griffiths, Secretory lysosomes, Nat Rev Mol Cell Biol, 3 (2002) 122-131.
[409] J.P. Luzio, Y. Hackmann, N.M. Dieckmann, G.M. Griffiths, The biogenesis of lysosomes and lysosome-related organelles, Cold Spring Harb Perspect Biol, 6 (2014) a016840.
[410] A. Haas, The phagosome: compartment with a license to kill, Traffic, 8 (2007) 311-330.
[411] E.L. Racoosin, J.A. Swanson, Macropinosome maturation and fusion with tubular lysosomes in macrophages, J Cell Biol, 121 (1993) 1011-1020.
[412] A. Jahraus, M. Egeberg, B. Hinner, A. Habermann, E. Sackman, A. Pralle, H. Faulstich, V. Rybin, H. Defacque, G. Griffiths, ATP-dependent membrane assembly of F-actin facilitates membrane fusion, Mol Biol Cell, 12 (2001) 155-170.
[413] A. Al-Haddad, M.A. Shonn, B. Redlich, A. Blocker, J.K. Burkhardt, H. Yu, J.A. Hammer, 3rd, D.G. Weiss, W. Steffen, G. Griffiths, S.A. Kuznetsov, Myosin Va bound to phagosomes binds to F-actin and delays microtubule-dependent motility, Mol Biol Cell, 12 (2001) 2742-2755.
[414] R. Kjeken, M. Egeberg, A. Habermann, M. Kuehnel, P. Peyron, M. Floetenmeyer, P. Walther, A. Jahraus, H. Defacque, S.A. Kuznetsov, G. Griffiths, Fusion between phagosomes, early and late endosomes: a role for actin in fusion between late, but not early endocytic organelles, Mol Biol Cell, 15 (2004) 345-358.
[415] U. Becken, A. Jeschke, K. Veltman, A. Haas, Cell-free fusion of bacteria-containing phagosomes with endocytic compartments, Proc Natl Acad Sci U S A, 107 (2010) 20726-20731.
[416] Y. Egami, N. Araki, Spatiotemporal Localization of Rab20 in Live RAW264 Macrophages during Macropinocytosis, Acta Histochem Cytochem, 45 (2012) 317-323.
[417] J.A. Swanson, J.S. King, The breadth of macropinocytosis research, Philos Trans R Soc Lond B Biol Sci, 374 (2019) 20180146.
[418] C.M. Buckley, J.S. King, Drinking problems: mechanisms of macropinosome formation and maturation, FEBS J, 284 (2017) 3778-3790.
[419] L. Dolat, E.T. Spiliotis, Septins promote macropinosome maturation and traffic to the lysosome by facilitating membrane fusion, J Cell Biol, 214 (2016) 517-527.
[420] S. Mostowy, P. Cossart, Septins: the fourth component of the cytoskeleton, Nat Rev Mol Cell Biol, 13 (2012) 183-194.
[421] H. Damke, D.D. Binns, H. Ueda, S.L. Schmid, T. Baba, Dynamin GTPase domain mutants block endocytic vesicle formation at morphologically distinct stages, Mol Biol Cell, 12 (2001) 2578-2589.
[422] D. Dutta, J.G. Donaldson, Search for inhibitors of endocytosis: Intended specificity and unintended consequences, Cell Logist, 2 (2012) 203-208.
[423] A.S. Desai, M.R. Hunter, A.N. Kapustin, Using macropinocytosis for intracellular delivery of therapeutic nucleic acids to tumour cells, Philos Trans R Soc Lond B Biol Sci, 374 (2019) 20180156.
[424] D. Vercauteren, R.E. Vandenbroucke, A.T. Jones, J. Rejman, J. Demeester, S.C. De Smedt, N.N. Sanders, K. Braeckmans, The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls, Mol Ther, 18 (2010) 561-569.
[425] J.G. Elferink, Chlorpromazine inhibits phagocytosis and exocytosis in rabbit polymorphonuclear leukocytes, Biochem Pharmacol, 28 (1979) 965-968.
[426] S. Watanabe, M. Hirose, A. Miyazaki, M. Tomono, M. Takeuchi, T. Kitamura, T. Namihisa, Calmodulin antagonists inhibit the phagocytic activity of cultured Kupffer cells, Lab Invest, 59 (1988) 214-218.
[427] E. Macia, M. Ehrlich, R. Massol, E. Boucrot, C. Brunner, T. Kirchhausen, Dynasore, a cell-permeable inhibitor of dynamin, Dev Cell, 10 (2006) 839-850.

[428] G. Preta, J.G. Cronin, I.M. Sheldon, Dynasore - not just a dynamin inhibitor, Cell communication and signaling : CCS, 13 (2015) 24.
[429] D. Basagiannis, S. Zografou, K. Galanopoulou, S. Christoforidis, Dynasore impairs VEGFR2 signalling in an endocytosis-independent manner, Scientific Reports, 7 (2017) 45035.
[430] S.K. Rodal, G. Skretting, O. Garred, F. Vilhardt, B. van Deurs, K. Sandvig, Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles, Mol Biol Cell, 10 (1999) 961-974.
[431] N. Mundhara, A. Majumder, D. Panda, Methyl-β-cyclodextrin, an actin depolymerizer augments the antiproliferative potential of microtubule-targeting agents, Scientific Reports, 9 (2019) 7638.
[432] R. Fayez, V. Gupta, Imipramine, StatPearls, StatPearls Publishing
Copyright © 2021, StatPearls Publishing LLC., Treasure Island (FL), 2021.
[433] D. Serrano, T. Bhowmick, R. Chadha, C. Garnacho, S. Muro, Intercellular adhesion molecule 1 engagement modulates sphingomyelinase and ceramide, supporting uptake of drug carriers by the vascular endothelium, Arterioscler Thromb Vasc Biol, 32 (2012) 1178-1185.
[434] R. Biswas, K.L. Trout, F. Jessop, J.R. Harkema, A. Holian, Imipramine blocks acute silicosis in a mouse model, Part Fibre Toxicol, 14 (2017) 36.
[435] K. Tsuji, M. Ojima, K. Otabe, M. Horie, H. Koga, I. Sekiya, T. Muneta, Effects of Different Cell-Detaching Methods on the Viability and Cell Surface Antigen Expression of Synovial Mesenchymal Stem Cells, Cell transplantation, 26 (2017) 1089-1102.
[436] M. Masson-Pévet, H.J. Jongsma, J. De Bruijne, Collagenase- and trypsin-dissociated heart cells: a comparative ultrastructural study, Journal of molecular and cellular cardiology, 8 (1976) 747-757.
[437] K.G. Vogel, Effects of hyaluronidase, trypsin, and EDTA on surface composition and topography during detachment of cells in culture, Experimental Cell Research, 113 (1978) 345-357.
[438] L.L. Norman, J. Brugués, K. Sengupta, P. Sens, H. Aranda-Espinoza, Cell blebbing and membrane area homeostasis in spreading and retracting cells, Biophys J, 99 (2010) 1726-1733.
[439] G.M. Hodges, D.C. Livingston, L.M. Franks, The localization of trypsin in cultured mammalian cells, J Cell Sci, 12 (1973) 887-902.
[440] H.L. Huang, H.W. Hsing, T.C. Lai, Y.W. Chen, T.R. Lee, H.T. Chan, P.C. Lyu, C.L. Wu, Y.C. Lu, S.T. Lin, C.W. Lin, C.H. Lai, H.T. Chang, H.C. Chou, H.L. Chan, Trypsin-induced proteome alteration during cell subculture in mammalian cells, J Biomed Sci, 17 (2010) 36.
[441] G. Griffiths, R. Brands, B. Burke, D. Louvard, G. Warren, Viral membrane proteins acquire galactose in trans Golgi cisternae during intracellular transport, J Cell Biol, 95 (1982) 781-792.
[442] M. Claudia, Ö. Kristin, O. Jennifer, R. Eva, F. Eleonore, Comparison of fluorescence-based methods to determine nanoparticle uptake by phagocytes and non-phagocytic cells in vitro, Toxicology, 378 (2017) 25-36.
[443] S. Welsch, O.T. Keppler, A. Habermann, I. Allespach, J. Krijnse-Locker, H.G. Kräusslich, HIV-1 buds predominantly at the plasma membrane of primary human macrophages, PLoS Pathog, 3 (2007) e36.
[444] S. Vranic, N. Boggetto, V. Contremoulins, S. Mornet, N. Reinhardt, F. Marano, A. Baeza-Squiban, S. Boland, Deciphering the mechanisms of cellular uptake of engineered nanoparticles by accurate evaluation of internalization using imaging flow cytometry, Part Fibre Toxicol, 10 (2013) 2.
[445] L.I. Selby, L. Aurelio, D. Yuen, B. Graham, A.P.R. Johnston, Quantifying Cellular Internalization with a Fluorescent Click Sensor, ACS Sens, 3 (2018) 1182-1189.
[446] H.K. Shete, R.H. Prabhu, V.B. Patravale, Endosomal escape: a bottleneck in intracellular delivery, J Nanosci Nanotechnol, 14 (2014) 460-474.

[447] K. Deprey, L. Becker, J. Kritzer, A. Plückthun, Trapped! A Critical Evaluation of Methods for Measuring Total Cellular Uptake versus Cytosolic Localization, Bioconjug Chem, 30 (2019) 1006-1027.
[448] K.I. Cupic, J.J. Rennick, A.P. Johnston, G.K. Such, Controlling endosomal escape using nanoparticle composition: current progress and future perspectives, Nanomedicine (Lond), 14 (2019) 215-223.
[449] F. Anson, B. Liu, P. Kanjilal, P. Wu, J.A. Hardy, S. Thayumanavan, Evaluating Endosomal Escape of Caspase-3-Containing Nanomaterials Using Split GFP, Biomacromolecules, 22 (2021) 1261-1272.
[450] E. Böhmová, D. Machová, M. Pechar, R. Pola, K. Venclíková, O. Janoušková, T. Etrych, Cell-penetrating peptides: a useful tool for the delivery of various cargoes into cells, Physiol Res, 67 (2018) S267-s279.
[451] M.J. Rohovie, M. Nagasawa, J.R. Swartz, Virus-like particles: Next-generation nanoparticles for targeted therapeutic delivery, Bioeng Transl Med, 2 (2017) 43-57.
[452] N.A. Bright, B.J. Reaves, B.M. Mullock, J.P. Luzio, Dense core lysosomes can fuse with late endosomes and are re-formed from the resultant hybrid organelles, J Cell Sci, 110 (Pt 17) (1997) 2027-2040.
[453] R. Kalluru, F. Fenaroli, D. Westmoreland, L. Ulanova, A. Maleki, N. Roos, M. Paulsen Madsen, G. Koster, W. Egge-Jacobsen, S. Wilson, H. Roberg-Larsen, G.K. Khuller, A. Singh, B. Nyström, G. Griffiths, Poly(lactide-co-glycolide)-rifampicin nanoparticles efficiently clear Mycobacterium bovis BCG infection in macrophages and remain membrane-bound in phago-lysosomes, J Cell Sci, 126 (2013) 3043-3054.
[454] J. Alroy, C. Garganta, G. Wiederschain, Secondary biochemical and morphological consequences in lysosomal storage diseases, Biochemistry. Biokhimiia, 79 (2014) 619-636.
[455] M. Lundberg, M. Johansson, Positively charged DNA-binding proteins cause apparent cell membrane translocation, Biochem Biophys Res Commun, 291 (2002) 367-371.
[456] J.P. Richard, K. Melikov, E. Vives, C. Ramos, B. Verbeure, M.J. Gait, L.V. Chernomordik, B. Lebleu, Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake, J Biol Chem, 278 (2003) 585-590.
[457] C.F. Daussy, H. Wodrich, "Repair Me if You Can": Membrane Damage, Response, and Control from the Viral Perspective, Cells, 9 (2020).
[458] A. Wittrup, A. Ai, X. Liu, P. Hamar, R. Trifonova, K. Charisse, M. Manoharan, T. Kirchhausen, J. Lieberman, Visualizing lipid-formulated siRNA release from endosomes and target gene knockdown, Nat Biotechnol, 33 (2015) 870-876.
[459] M.J. Munson, G. O'Driscoll, A.M. Silva, E. Lázaro-Ibáñez, A. Gallud, J.T. Wilson, A. Collén, E.K. Esbjörner, A. Sabirsh, A high-throughput Galectin-9 imaging assay for quantifying nanoparticle uptake, endosomal escape and functional RNA delivery, Commun Biol, 4 (2021) 211.
[460] H. Du Rietz, H. Hedlund, S. Wilhelmson, P. Nordenfelt, A. Wittrup, Imaging small molecule-induced endosomal escape of siRNA, Nat Commun, 11 (2020) 1809.
[461] M. Herrera, J. Kim, Y. Eygeris, A. Jozic, G. Sahay, Illuminating endosomal escape of polymorphic lipid nanoparticles that boost mRNA delivery, Biomater Sci, (2021).
[462] S. Bänfer, D. Schneider, J. Dewes, M.T. Strauss, S.A. Freibert, T. Heimerl, U.G. Maier, H.P. Elsässer, R. Jungmann, R. Jacob, Molecular mechanism to recruit galectin-3 into multivesicular bodies for polarized exosomal secretion, Proc Natl Acad Sci U S A, 115 (2018) E4396-e4405.
[463] F. Fenaroli, U. Repnik, Y. Xu, K. Johann, S. Van Herck, P. Dey, F.M. Skjeldal, D.M. Frei, S. Bagherifam, A. Kocere, R. Haag, B.G. De Geest, M. Barz, D.G. Russell, G. Griffiths, Enhanced Permeability and Retention-like Extravasation of Nanoparticles from the Vasculature into Tuberculosis Granulomas in Zebrafish and Mouse Models, ACS nano, 12 (2018) 8646-8661.

[464] H.Y. Chiu, J.A. Bates, J. Helma, H. Engelke, H. Harz, T. Bein, H. Leonhardt, Nanoparticle mediated delivery and small molecule triggered activation of proteins in the nucleus, Nucleus, 9 (2018) 530-542.
[465] J. Gilleron, W. Querbes, A. Zeigerer, A. Borodovsky, G. Marsico, U. Schubert, K. Manygoats, S. Seifert, C. Andree, M. Stöter, H. Epstein-Barash, L. Zhang, V. Koteliansky, K. Fitzgerald, E. Fava, M. Bickle, Y. Kalaidzidis, A. Akinc, M. Maier, M. Zerial, Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape, Nat Biotechnol, 31 (2013) 638-646.
[466] L.I. FitzGerald, L. Aurelio, M. Chen, D. Yuen, J.J. Rennick, B. Graham, A.P.R. Johnston, A molecular sensor to quantify the localization of proteins, DNA and nanoparticles in cells, Nat Commun, 11 (2020) 4482.
[467] W.P.R. Verdurmen, M. Mazlami, A. Plückthun, A Biotin Ligase-Based Assay for the Quantification of the Cytosolic Delivery of Therapeutic Proteins, Methods Mol Biol, 1575 (2017) 223-236.
[468] D. Routkevitch, D. Sudhakar, M. Conge, M. Varanasi, S.Y. Tzeng, D.R. Wilson, J.J. Green, Efficiency of Cytosolic Delivery with Poly(β-amino ester) Nanoparticles is Dependent on the Effective pK(a) of the Polymer, ACS Biomater Sci Eng, 6 (2020) 3411-3421.
[469] M.S.H. Lim, Y. Nishiyama, T. Ohtsuki, K. Watanabe, H. Kobuchi, K. Kobayashi, E. Matsuura, Lactosome-Conjugated siRNA Nanoparticles for Photo-Enhanced Gene Silencing in Cancer Cells, J Pharm Sci, 110 (2021) 1788-1798.
[470] A. Bordat, T. Boissenot, J. Nicolas, N. Tsapis, Thermoresponsive polymer nanocarriers for biomedical applications, Adv Drug Deliv Rev, 138 (2019) 167-192.
[471] O. Boussif, M.A. Zanta, J.P. Behr, Optimized galenics improve in vitro gene transfer with cationic molecules up to 1000-fold, Gene Ther, 3 (1996) 1074-1080.
[472] P. Atakpa, L.M. van Marrewijk, M. Apta-Smith, S. Chakraborty, C.W. Taylor, GPN does not release lysosomal Ca(2+) but evokes Ca(2+) release from the ER by increasing the cytosolic pH independently of cathepsin C, J Cell Sci, 132 (2019).
[473] T. Bus, A. Traeger, U.S. Schubert, The great escape: how cationic polyplexes overcome the endosomal barrier, J Mater Chem B, 6 (2018) 6904-6918.
[474] K. Matsuzaki, Membrane Permeabilization Mechanisms, Adv Exp Med Biol, 1117 (2019) 9-16.
[475] K. Hadinoto, A. Sundaresan, W.S. Cheow, Lipid-polymer hybrid nanoparticles as a new generation therapeutic delivery platform: a review, Eur J Pharm Biopharm, 85 (2013) 427-443.
[476] X. Cheng, R.J. Lee, The role of helper lipids in lipid nanoparticles (LNPs) designed for oligonucleotide delivery, Adv Drug Deliv Rev, 99 (2016) 129-137.
[477] J.A. Kulkarni, D. Witzigmann, J. Leung, Y.Y.C. Tam, P.R. Cullis, On the role of helper lipids in lipid nanoparticle formulations of siRNA, Nanoscale, 11 (2019) 21733-21739.
[478] B.M. Bruininks, P.C. Souza, H. Ingolfsson, S.J. Marrink, A molecular view on the escape of lipoplexed DNA from the endosome, eLife, 9 (2020).
[479] I. Gessner, I. Neundorf, Nanoparticles Modified with Cell-Penetrating Peptides: Conjugation Mechanisms, Physicochemical Properties, and Application in Cancer Diagnosis and Therapy, Int J Mol Sci, 21 (2020).
[480] A. Borrelli, A.L. Tornesello, M.L. Tornesello, F.M. Buonaguro, Cell Penetrating Peptides as Molecular Carriers for Anti-Cancer Agents, Molecules, 23 (2018).
[481] F. Milletti, Cell-penetrating peptides: classes, origin, and current landscape, Drug Discov Today, 17 (2012) 850-860.
[482] A. Sanghani, K.N. Kafetzis, Y. Sato, S. Elboraie, J. Fajardo-Sanchez, H. Harashima, A.D. Tagalakis, C. Yu-Wai-Man, Novel PEGylated Lipid Nanoparticles Have a High Encapsulation

Efficiency and Effectively Deliver MRTF-B siRNA in Conjunctival Fibroblasts, Pharmaceutics, 13 (2021).
[483] M. Galliani, C. Tremolanti, G. Signore, Nanocarriers for Protein Delivery to the Cytosol: Assessing the Endosomal Escape of Poly(Lactide-co-Glycolide)-Poly(Ethylene Imine) Nanoparticles, Nanomaterials (Basel), 9 (2019).
[484] T. Nakamura, K. Yamada, Y. Fujiwara, Y. Sato, H. Harashima, Reducing the Cytotoxicity of Lipid Nanoparticles Associated with a Fusogenic Cationic Lipid in a Natural Killer Cell Line by Introducing a Polycation-Based siRNA Core, Mol Pharm, 15 (2018) 2142-2150.
[485] J. Thyberg, U. Hedin, K. Stenseth, Endocytic pathways and time sequence of lysosomal transfer of macromolecules in cultured mouse peritoneal macrophages. Double-labeling experiments with horseradish peroxidase and ferritin, Cell Tissue Res, 241 (1985) 299-303.
[486] F.R. Maxfield, Role of endosomes and lysosomes in human disease, Cold Spring Harb Perspect Biol, 6 (2014) a016931.
[487] H.J. Geuze, J.W. Slot, G.J. Strous, H.F. Lodish, A.L. Schwartz, Intracellular site of asialoglycoprotein receptor-ligand uncoupling: double-label immunoelectron microscopy during receptor-mediated endocytosis, Cell, 32 (1983) 277-287.
[488] R.G. Parton, K. Prydz, M. Bomsel, K. Simons, G. Griffiths, Meeting of the apical and basolateral endocytic pathways of the Madin-Darby canine kidney cell in late endosomes, J Cell Biol, 109 (1989) 3259-3272.
[489] A.K. Varkouhi, M. Scholte, G. Storm, H.J. Haisma, Endosomal escape pathways for delivery of biologicals, J Control Release, 151 (2011) 220-228.
[490] A. Sahni, Z. Qian, D. Pei, Cell-Penetrating Peptides Escape the Endosome by Inducing Vesicle Budding and Collapse, ACS Chem Biol, 15 (2020) 2485-2492.
[491] E. van Meel, J. Klumperman, Imaging and imagination: understanding the endo-lysosomal system, Histochemistry and Cell Biology, 129 (2008) 253-266.
[492] P. Cossart, A. Helenius, Endocytosis of viruses and bacteria, Cold Spring Harb Perspect Biol, 6 (2014).
[493] D. Pei, M. Buyanova, Overcoming Endosomal Entrapment in Drug Delivery, Bioconjug Chem, 30 (2019) 273-283.
[494] P. Guardado-Calvo, E.A. Bignon, E. Stettner, S.A. Jeffers, J. Pérez-Vargas, G. Pehau-Arnaudet, M.A. Tortorici, J.L. Jestin, P. England, N.D. Tischler, F.A. Rey, Mechanistic Insight into Bunyavirus-Induced Membrane Fusion from Structure-Function Analyses of the Hantavirus Envelope Glycoprotein Gc, PLoS Pathog, 12 (2016) e1005813.
[495] J. Staring, M. Raaben, T.R. Brummelkamp, Viral escape from endosomes and host detection at a glance, J Cell Sci, 131 (2018).
[496] M.J. Conley, M. McElwee, L. Azmi, M. Gabrielsen, O. Byron, I.G. Goodfellow, D. Bhella, Calicivirus VP2 forms a portal-like assembly following receptor engagement, Nature, 565 (2019) 377-381.
[497] S. Luisoni, U.F. Greber, 2 - Biology of Adenovirus Cell Entry: Receptors, Pathways, Mechanisms, in: D.T. Curiel (Ed.) Adenoviral Vectors for Gene Therapy (Second Edition), Academic Press, San Diego, 2016, pp. 27-58.
[498] M. Schelhaas, J. Malmström, L. Pelkmans, J. Haugstetter, L. Ellgaard, K. Grünewald, A. Helenius, Simian Virus 40 depends on ER protein folding and quality control factors for entry into host cells, Cell, 131 (2007) 516-529.
[499] P. Heller, D. Hobernik, U. Lächelt, M. Schinnerer, B. Weber, M. Schmidt, E. Wagner, M. Bros, M. Barz, Combining reactive triblock copolymers with functional cross-linkers: A versatile pathway to disulfide stabilized-polyplex libraries and their application as pDNA vaccines, J Control Release, 258 (2017) 146-160.

[500] K.P. Bohannon, P.I. Hanson, ESCRT puts its thumb on the nanoscale: Fixing tiny holes in endolysosomes, Curr Opin Cell Biol, 65 (2020) 122-130.
[501] S.T. Cooper, P.L. McNeil, Membrane Repair: Mechanisms and Pathophysiology, Physiol Rev, 95 (2015) 1205-1240.
[502] C. Papadopoulos, B. Kravic, H. Meyer, Repair or Lysophagy: Dealing with Damaged Lysosomes, J Mol Biol, 432 (2020) 231-239.
[503] M.L. Skowyra, P.H. Schlesinger, T.V. Naismith, P.I. Hanson, Triggered recruitment of ESCRT machinery promotes endolysosomal repair, Science, 360 (2018).
[504] M. Radulovic, K.O. Schink, E.M. Wenzel, V. Nähse, A. Bongiovanni, F. Lafont, H. Stenmark, ESCRT-mediated lysosome repair precedes lysophagy and promotes cell survival, EMBO J, 37 (2018).
[505] A.T. López-Jiménez, E. Cardenal-Muñoz, F. Leuba, L. Gerstenmaier, C. Barisch, M. Hagedorn, J.S. King, T. Soldati, The ESCRT and autophagy machineries cooperate to repair ESX-1-dependent damage at the Mycobacterium-containing vacuole but have opposite impact on containing the infection, PLoS Pathog, 14 (2018) e1007501.
[506] N. Chiaruttini, L. Redondo-Morata, A. Colom, F. Humbert, M. Lenz, S. Scheuring, A. Roux, Relaxation of Loaded ESCRT-III Spiral Springs Drives Membrane Deformation, Cell, 163 (2015) 866-879.
[507] N. Chiaruttini, A. Roux, Dynamic and elastic shape transitions in curved ESCRT-III filaments, Curr Opin Cell Biol, 47 (2017) 126-135.
[508] A.K. Pfitzner, V. Mercier, X. Jiang, J. Moser von Filseck, B. Baum, A. Šarić, A. Roux, An ESCRT-III Polymerization Sequence Drives Membrane Deformation and Fission, Cell, 182 (2020) 1140-1155.e1118.
[509] S. Herbst, P. Campbell, J. Harvey, E.M. Bernard, V. Papayannopoulos, N.W. Wood, H.R. Morris, M.G. Gutierrez, LRRK2 activation controls the repair of damaged endomembranes in macrophages, EMBO J, 39 (2020) e104494.
[510] A.J. Jimenez, P. Maiuri, J. Lafaurie-Janvore, S. Divoux, M. Piel, F. Perez, ESCRT machinery is required for plasma membrane repair, Science, 343 (2014) 1247136.
[511] C.M. Denais, R.M. Gilbert, P. Isermann, A.L. McGregor, M. te Lindert, B. Weigelin, P.M. Davidson, P. Friedl, K. Wolf, J. Lammerding, Nuclear envelope rupture and repair during cancer cell migration, Science, 352 (2016) 353-358.
[512] J.H. Hurley, ESCRTs are everywhere, EMBO J, 34 (2015) 2398-2407.
[513] I. Schütz, T. Lopez-Hernandez, Q. Gao, D. Puchkov, S. Jabs, D. Nordmeyer, M. Schmudde, E. Rühl, C.M. Graf, V. Haucke, Lysosomal Dysfunction Caused by Cellular Accumulation of Silica Nanoparticles, J Biol Chem, 291 (2016) 14170-14184.
[514] D.L. Thiele, P.E. Lipsky, Mechanism of L-leucyl-L-leucine methyl ester-mediated killing of cytotoxic lymphocytes: dependence on a lysosomal thiol protease, dipeptidyl peptidase I, that is enriched in these cells, Proc Natl Acad Sci U S A, 87 (1990) 83-87.
[515] U. Repnik, M. Borg Distefano, M.T. Speth, M.Y.W. Ng, C. Progida, B. Hoflack, J. Gruenberg, G. Griffiths, L-leucyl-L-leucine methyl ester does not release cysteine cathepsins to the cytosol but inactivates them in transiently permeabilized lysosomes, J Cell Sci, 130 (2017) 3124-3140.
[516] B.E. Steinberg, K.K. Huynh, A. Brodovitch, S. Jabs, T. Stauber, T.J. Jentsch, S. Grinstein, A cation counterflux supports lysosomal acidification, J Cell Biol, 189 (2010) 1171-1186.
[517] R. Vaghela, A. Arkudas, R.E. Horch, M. Hessenauer, Actually Seeing What Is Going on - Intravital Microscopy in Tissue Engineering, Front Bioeng Biotechnol, 9 (2021) 627462.
[518] Q. Huang, M.A. Cohen, F.C. Alsina, G. Devlin, A. Garrett, J. McKey, P. Havlik, N. Rakhilin, E. Wang, K. Xiang, P. Mathews, L. Wang, C. Bock, V. Ruthig, Y. Wang, M. Negrete, C.W. Wong, P.K.L. Murthy, S. Zhang, A.R. Daniel, D.G. Kirsch, Y. Kang, B. Capel, A. Asokan, D.L. Silver, R. Jaenisch, X. Shen, Intravital imaging of mouse embryos, Science, 368 (2020) 181-186.

[519] F. Fenaroli, D. Westmoreland, J. Benjaminsen, T. Kolstad, F.M. Skjeldal, A.H. Meijer, M. van der Vaart, L. Ulanova, N. Roos, B. Nyström, J. Hildahl, G. Griffiths, Nanoparticles as drug delivery system against tuberculosis in zebrafish embryos: direct visualization and treatment, ACS nano, 8 (2014) 7014-7026.
[520] R. Fior, V. Póvoa, R.V. Mendes, T. Carvalho, A. Gomes, N. Figueiredo, M.G. Ferreira, Single-cell functional and chemosensitive profiling of combinatorial colorectal therapy in zebrafish xenografts, Proc Natl Acad Sci U S A, 114 (2017) E8234-e8243.
[521] Y. Teng, X. Xie, S. Walker, D.T. White, J.S. Mumm, J.K. Cowell, Evaluating human cancer cell metastasis in zebrafish, BMC Cancer, 13 (2013) 453.
[522] N.K. Dal, A. Kocere, J. Wohlmann, S. Van Herck, T.A. Bauer, J. Resseguier, S. Bagherifam, H. Hyldmo, M. Barz, B.G. De Geest, F. Fenaroli, Zebrafish Embryos Allow Prediction of Nanoparticle Circulation Times in Mice and Facilitate Quantification of Nanoparticle-Cell Interactions, Small, 16 (2020) e1906719.
[523] Y. Hayashi, M. Takamiya, P.B. Jensen, I. Ojea-Jiménez, H. Claude, C. Antony, K. Kjaer-Sorensen, C. Grabher, T. Boesen, D. Gilliland, C. Oxvig, U. Strähle, C. Weiss, Differential Nanoparticle Sequestration by Macrophages and Scavenger Endothelial Cells Visualized in Vivo in Real-Time and at Ultrastructural Resolution, ACS nano, 14 (2020) 1665-1681.
[524] S. Sieber, P. Grossen, P. Detampel, S. Siegfried, D. Witzigmann, J. Huwyler, Zebrafish as an early stage screening tool to study the systemic circulation of nanoparticulate drug delivery systems in vivo, J Control Release, 264 (2017) 180-191.
[525] Z. Chen, S.L. Schmid, Evolving models for assembling and shaping clathrin-coated pits, J Cell Biol, 219 (2020).
[526] C.B. Vibe, F. Fenaroli, D. Pires, S.R. Wilson, V. Bogoeva, R. Kalluru, M. Speth, E. Anes, G. Griffiths, J. Hildahl, Thioridazine in PLGA nanoparticles reduces toxicity and improves rifampicin therapy against mycobacterial infection in zebrafish, Nanotoxicology, 10 (2016) 680-688.
[527] N. Palha, F. Guivel-Benhassine, V. Briolat, G. Lutfalla, M. Sourisseau, F. Ellett, C.H. Wang, G.J. Lieschke, P. Herbomel, O. Schwartz, J.P. Levraud, Real-time whole-body visualization of Chikungunya Virus infection and host interferon response in zebrafish, PLoS Pathog, 9 (2013) e1003619.
[528] E. Haque, A.C. Ward, Zebrafish as a Model to Evaluate Nanoparticle Toxicity, Nanomaterials (Basel), 8 (2018).
[529] L.S. Ulanova, M. Pinheiro, C. Vibe, C. Nunes, D. Misaghian, S. Wilson, K. Zhu, F. Fenaroli, H.C. Winther-Larsen, S. Reis, G. Griffiths, Treatment of Francisella infections via PLGA- and lipid-based nanoparticle delivery of antibiotics in a zebrafish model, Diseases of aquatic organisms, 125 (2017) 19-29.
[530] X.Y. Chu, W. Huang, Y.L. Wang, L.W. Meng, L.Q. Chen, M.J. Jin, L. Chen, C.H. Gao, C. Ge, Z.G. Gao, C.S. Gao, Improving antitumor outcomes for palliative intratumoral injection therapy through lecithin- chitosan nanoparticles loading paclitaxel- cholesterol complex, Int J Nanomedicine, 14 (2019) 689-705.
[531] C. Xiao, F. Wang, J. Hou, X. Zhu, Y. Luo, J.W. Xiong, Nanoparticle-mediated siRNA Gene-silencing in Adult Zebrafish Heart, J Vis Exp, (2018).
[532] J. Diao, H. Wang, N. Chang, X.H. Zhou, X. Zhu, J. Wang, J.W. Xiong, PEG-PLA nanoparticles facilitate siRNA knockdown in adult zebrafish heart, Dev Biol, 406 (2015) 196-202.
[533] Y. Shimada, M. Hazekawa, Developing a Model for a siRNA Delivery System by Cancer Implantation into Zebrafish Circulation, Methods Mol Biol, 2174 (2021) 263-275.
[534] J. Kim, B.-K. Koo, J.A. Knoblich, Human organoids: model systems for human biology and medicine, Nature Reviews Molecular Cell Biology, 21 (2020) 571-584.
[535] M. Hofer, M.P. Lutolf, Engineering organoids, Nat Rev Mater, (2021) 1-19.

[536] A.C. Rios, H. Clevers, Imaging organoids: a bright future ahead, Nat Methods, 15 (2018) 24-26.

Box 1. Key points

1. Useful insights from cell biological studies of NP endocytosis are limited due to substandard methodology and interpretation.
2. Studies of NP should make use of state-of-the-art cell biological systems and techniques carried out by cell biologists.
3. Few of the widely used inhibitors in the field are sufficiently specific to be used as agents to test the role of different endocytic pathways.
4. The evidence for involvement of caveolae in endocytosis of nanoparticles is, in general, not convincing
5. The role of phagocytosis as an uptake mechanism for a range of NP, including many less than 500 nm in diameter, has been under-appreciated
6. Studies of endocytosis should be carried out in conjunction with assays of NP efficacy to ascertain how particles reach their site of action

Box 2

Rough definitions and guidelines to distinguish between the four main endocytic pathways

1. **Phagocytosis:** Uptake of particles larger than 50-100nm occur via tight multivalent interactions between ligands on the particle surface and receptors on the plasma membrane of the phagocytic cell. This leads to close contact between particles and enwrapping membrane and strong transient trans-membrane signaling especially to actin assembly (in seconds) and to gene expression (minutes to hours). Phagosomes can maintain their (content) identity for long periods, up to many days depending on the cargo; this is a unique feature of these vesicles (all other vesicles lose their identity in minutes). Fluid phase markers co-administered with the particles are excluded from the newly assembled phagosome vesicle. Significantly inhibited by cytochalasin D.

2. **Macropinocytosis:** Uptake of any sized particles (up to 1µm) via cell ruffling that is either constitutive (macrophages, dendritic cells) or induced by the particles after transient contact with receptors on the plasma membrane or via an inducer such as epidermal growth factor. Signaling leads to potent transient actin assembly. Large vesicles (macropinosomes) form that include the particles in the bulk phase as passengers. Fluid phase markers co-administered with the particles label the spacious macropinosomes. Significantly inhibited by cytochalasin D.

3. **Endocytic clathrin coated vesicles:** Invaginations of the plasma membrane of most cell types that are coated with clathrin triskelions and adaptor proteins that carry and concentrate nutrient and other receptors into the cell. These proteins form a distinct polyhedral, electron dense coat on the cytoplasmic surface of the vesicle seen by electron microscopy. Requires dynamin GTPase to pinch off vesicles that are usually between 50 and 100 nm in diameter but can be up to 300 nm. Takes 30-60 sec to form vesicles that subsequently lose the clathrin coat and fuse with early endosomes. Particles up to 50 nm can enter the vesicles very inefficiently via the fluid phase but can enter efficiently if there are partner receptors in the forming vesicle. Inhibited by genetically manipulating clathrin or adaptor protein 2.

4. **Caveolae:** Bulb-shaped invaginations of the plasma membrane around 60nm in diameter that are devoid of an electron dense coat. Easily identified by electron microscopy. Especially prominent in endothelial cells, adipocytes and smooth muscle. They bud from the cell surface in a process regulated by EHD2 to fuse with early endosomes. Inhibited by genetically manipulating key structural proteins such as caveolin-1 or cavin1.

5. Bulb-shaped invaginations of the plasma membrane around 60nm in diameter that are devoid of an electron dense coat. Easily identified by electron microscopy. Especially prominent in endothelial cells, adipocytes and smooth muscle. They bud from the cell surface in a process regulated by EHD2 to fuse with early endosomes. Inhibited by genetically manipulating key structural proteins such as caveolin-1 or cavin1.

i

BOX 3: Comparison of phagocytosis and macropinocytosis

Different features.

1. Phagocytosis involves receptor-mediated contact and uptake of particles. Receptor contact lasts at least until the particle has been internalized and, with degradable particles, digested; in macropinocytosis there is no persistent contact of particles with receptors.
2. *In vivo,* intravenously injected particles from plasma are cleared by tissue macrophages by phagocytosis. There is no evidence that macropinocytosis can carry out an equivalent clearance of particles.
3. Phagocytosis of specific particles can be competed by co- or pre-incubation with particles that use the same receptors. There is no evidence that such competition occurs with macropinocytosis.
4. When the particles are stealth coated with, for example PEG or poly-sarcosine, phagocytic uptake is strongly reduced. Stealth coating is not expected to have an effect on macropinocytosis.
5. Phagocytosis leads to robust activation of gene expression in response to interaction between particle and cell surface receptors. Such signaling does not appear to occur during macropinocytosis. Many macropinocytosis events are studied after stimulation by growth factors, which themselves modulate gene expression.
6. Macropinocytosis but not phagocytosis is dependent on Rab10 [2]

Shared features

1. Both processes involve dynamic actin filament nucleation on the plasma membrane within seconds of initiation. Both processes are blocked by cytochalasin D and other actin inhibitors
2. After uptake, both macropinosomes (in most cases) and phagosomes usually undergo similar maturation processes involving fusion with endocytic compartments, including lysosomes.
3. Although ruffling is extremely prominent during macropinocytosis it often occurs also during phagocytosis. Even non-professional phagocytic cells can show ruffling when they come in contact with particles [4].
4. Many signaling molecules are shared between the two processes.

BOX 4: The mis-use of inhibitors in NP uptake studies

22 semi randomly selected studies containing the search terms: Nanoparticle, Uptake and Inhibitor between 2010 and 2021. To study uptake mechanisms the authors used a broad range of ***(1): Unsuitable methods***. Many of the inhibitors were used at (***2): An extreme range of concentrations***. Many (***3): interpretations*** bear no relation to known theory, even taking into account the fact that many of the inhibitors are non-specific. There are examples of (***4): the same inhibitor being claimed to affect different pathways*.** The special case of (***5): macropinocytosis***.

1: Unsuitable methods:

We maintain that the best method for studying uptake mechanisms is quantitative (in part, live cell-) fluorescence microscopy- in combination with electron- microscopy, using distinct binding and uptake assays. Of these 22 studies, only one used live cell imaging and none tested the effects of the inhibitor in combination with fluorescence microscopy. Only one study used electron microscopy, albeit at a poor quality and using an unsuitable approach for the quantification. More than half of the studies used flow cytometry; only one [1] of these used methods to separate surface bound and internalised NPs and almost all used trypsin for cell detachment.

Examples of extremely unsuitable methods include:

- "seedless deposition" – a method were gold NPs are internalised, gold enhanced and semi - quantified at the bright field microscopy level by visual inspection [3].
- Using cornea epithelial cells grown on filters, uptake was assessed by a poorly described method evaluating transcytosis from one side of the monolayer to the other, and quantified by HPLC [5].
- Estimating NP uptake by analysis of cell lysates after treatment with detergents (Triton-X-100) [6, 7] or an unspecified lysis buffer [8], using either ELISA[8] or spectrofluorometry [6, 7]. No distinction made between surface bound and internalised NP.

2: Extreme range of inhibitor concentrations used in these studies:

- Amiloride / EIPA 5µM-1mM, (Factor 200) [9, 10]
- Genistein 100nM-400µM, (Factor 4000) [1, 11]
- Methyl-β-cyclodextrin 500nM-10mM, (Factor 20000) [6, 13]
- Sodium azide 1µM-50mM, (Factor 50000) [14, 15]
- Hypotonic sucrose 100µM-500mM, (Factor 5000) [13, 16]

3: Interpretations (based on multiple inhibitors) that make no sense:

- Latex NP taken up by A549 cells "by macropinocytosis or non-endocytic process(es) (e.g., diffusion across and/or poration of apical cell plasma membranes)". [17]
- PLA nanocapsule uptake is based on '' a multifaceted mechanism involving clathrin, caveolin, cytoskeleton, and micropinocytosis in MDA-MB 231 cells''. [7]
- Dextran NP "uptake in SK-NBE(2) cells showed no response to the individual inhibitors, indicating that none of the known major endocytic pathways were responsible for internalization". [11]
- The NP "uptake in THP-1-derived macrophages was only inhibited by NaN3, indicating that the internalization occurred through phagocytosis." [7]
- "40% Inhibition by both latrunculinA and dynasore, implying a role of actin and dynamin, which led to the conclusion the uptake happens by caveolae and clathrin mediated endocytosis".[7]

4: The same inhibitor is claimed to affect different pathways:

- Genistein is variously used for inhibition of caveolae-mediated uptake [10], clathrin independent endocytosis [11], as a general tyrosine kinase inhibitor [29] or unspecific endocytosis inhibitor. [33]
- methyl-β-cyclodextrin is variously used to inhibit micropinocytosis mediated endocytosis [6, 28], clathrin independent endocytosis [11], clathrin- as well as caveolin-mediated endocytosis [37], non-caveola- but cholesterol-dependent endocytosis [9] or caveolae dependent and micropinocytosis [33]. Its uptake inhibition was suggested to work by "affecting the integrity of membrane lipid microdomains or inhibiting clathrin-mediated endocytosis" [9] or the inhibition of lipid rafts. [38, 39]
- Hypotonic sucrose is variously used for the inhibition of clathrin mediated endocytosis [16] or clathrin-mediated, caveolar, and micropinocytosis pathways"[41]
- Wortmannin is variously considered to inhibit: caveolae budding [8], macropinosome development [29] or affecting phagocytosis mechanisms. [10, 45]

5: Macropinocytosis:

- Even though macropinocytosis is described to happen very fast, within 1-15 mins, almost all these studies testing

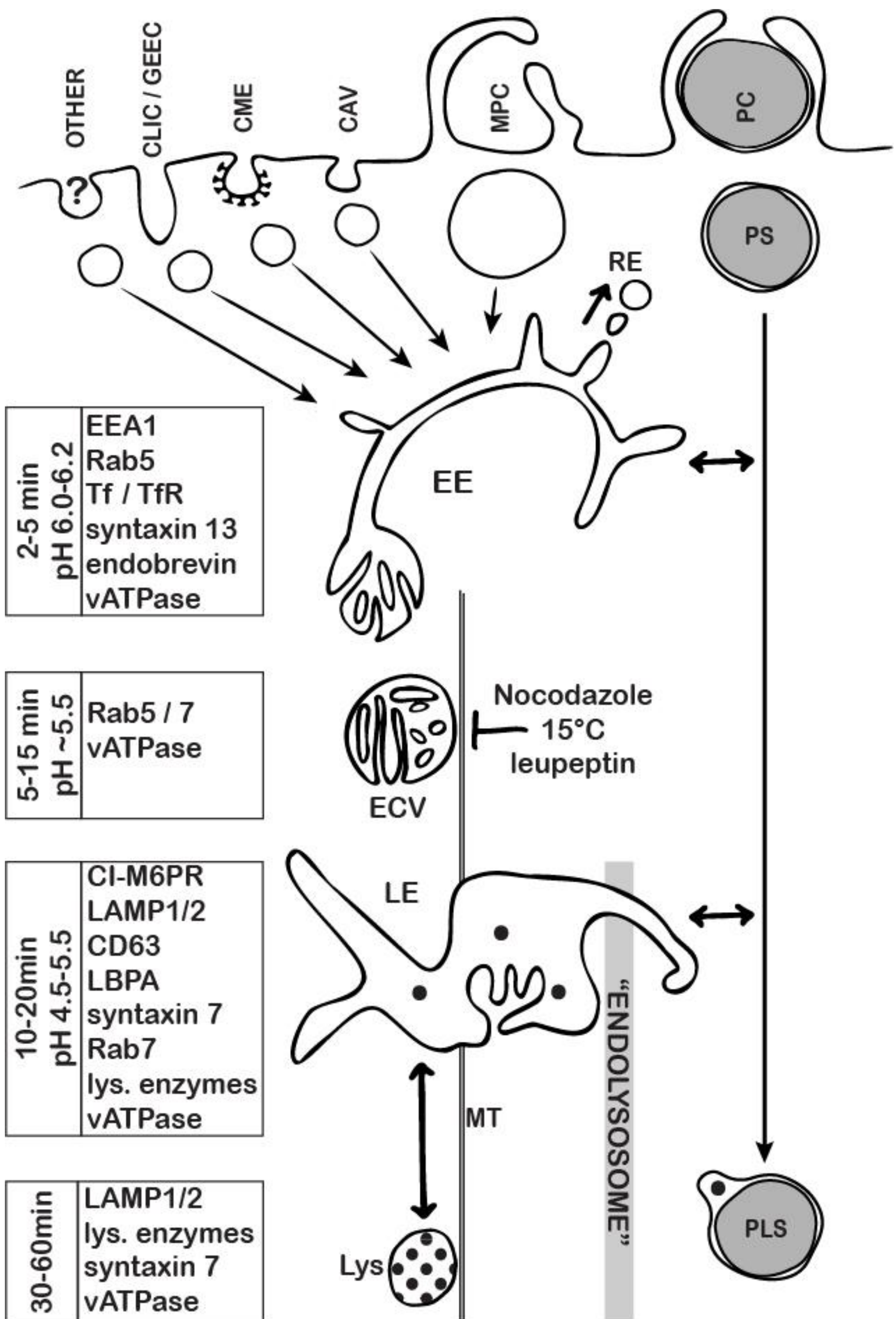
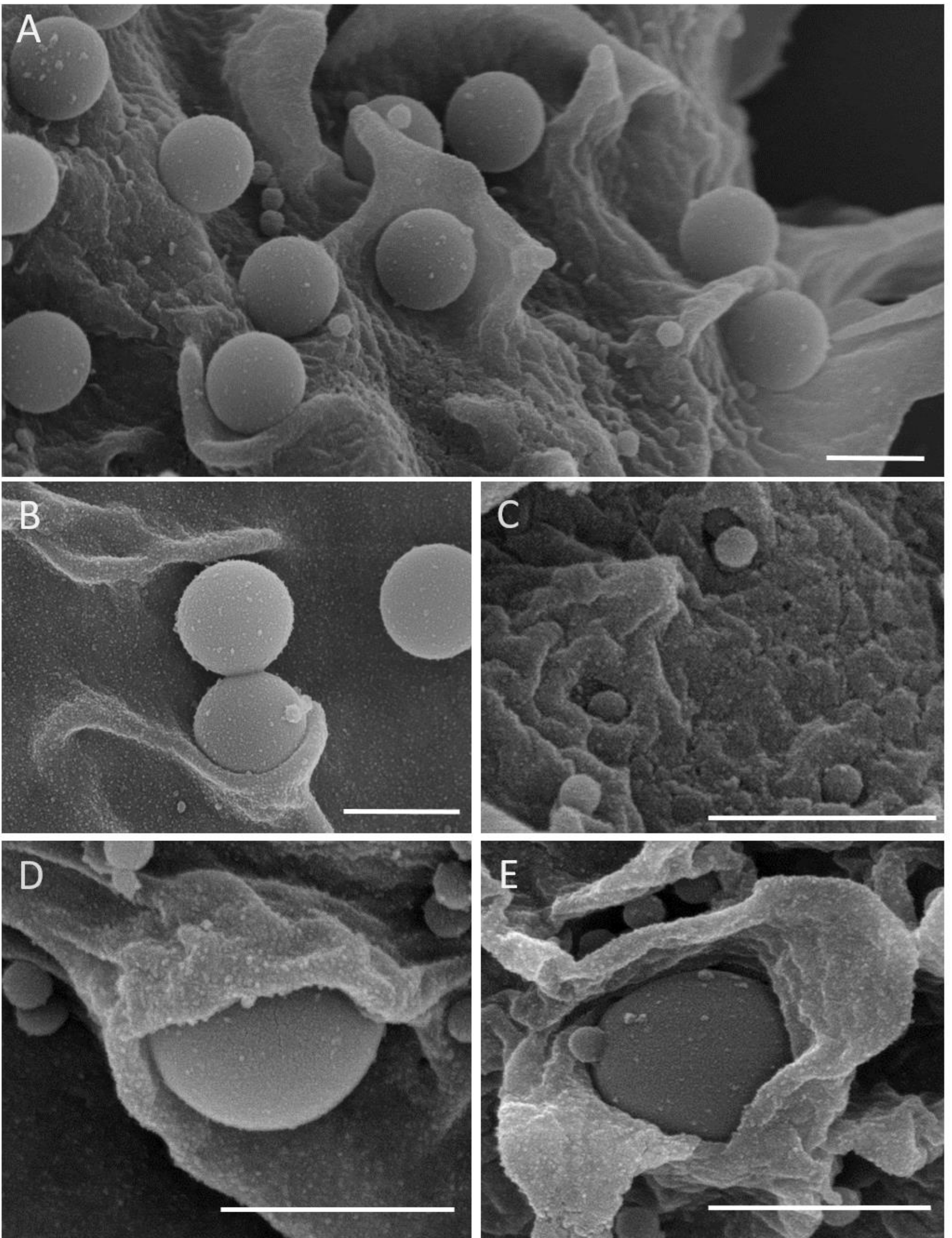

OTHER
CLIC / GEEC
CME
CAV
MPC
PC
?
PS
RE
EE
2-5 min
pH 6.0-6.2
EEA1
Rab5
Tf / TfR
syntaxin 13
endobrevin
vATPase
5-15 min
pH ~5.5
Rab5 / 7
vATPase
ECV
Nocodazole
15°C
leupeptin
10-20min
pH 4.5-5.5
CI-M6PR
LAMP1/2
CD63
LBPA
syntaxin 7
Rab7
lys. enzymes
vATPase
LE
MT
„ENDOLYSOSOME"
30-60min
LAMP1/2
lys. enzymes
syntaxin 7
vATPase
Lys
PLS

Fig.1: Overview of the endocytic pathway

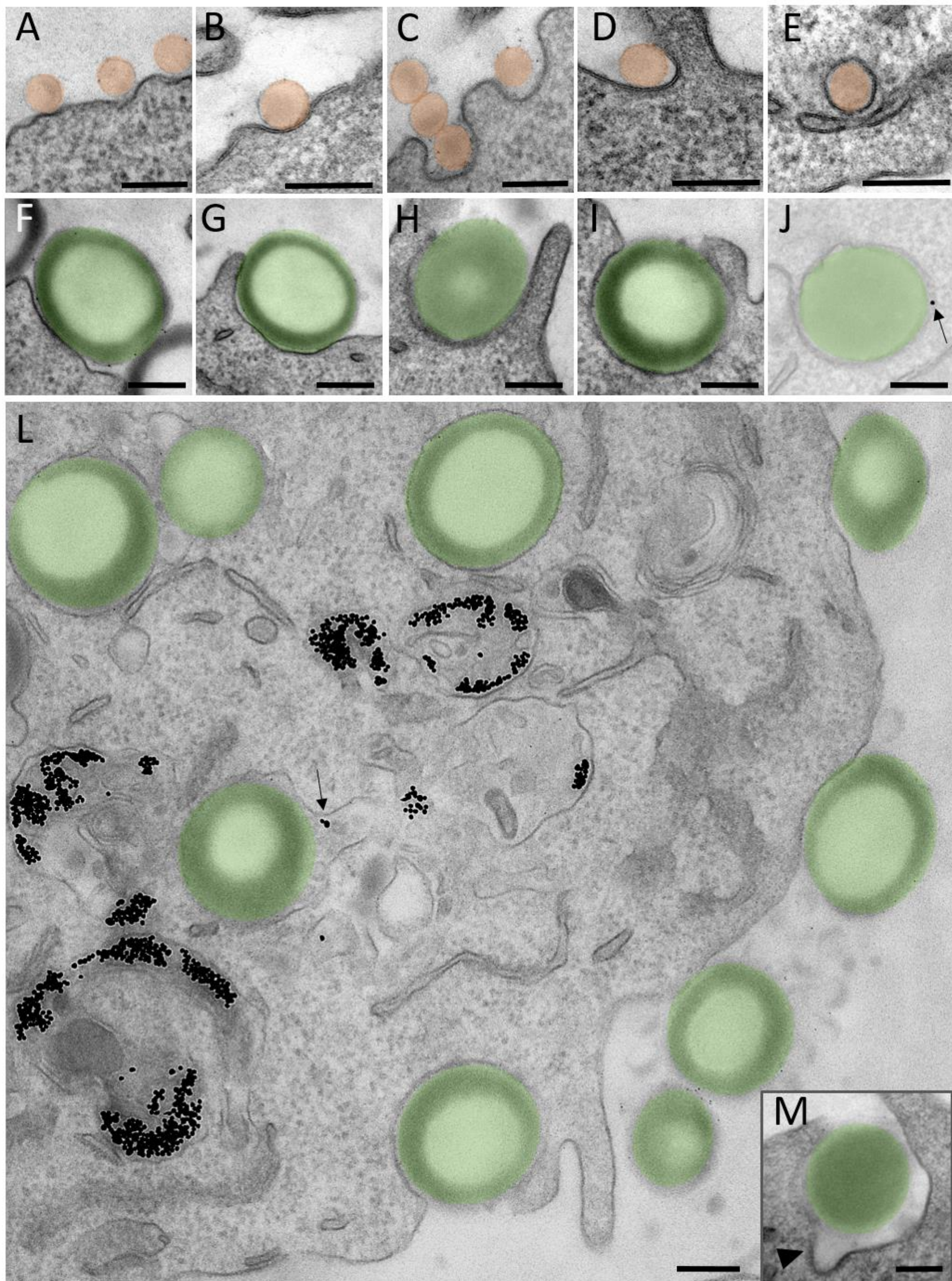
Schematic illustration of the different endocytic processes based on data from mammalian cells in culture: CME-clathrin mediated endocytosis: CAV-caveolae; MPC-macropinocytosis; PC-phagocytosis; RE-recycling endosome; EE-early endosome; ECV-endosome carrier vesicle, also referred to as Multivesicular body; LE-late endosome; LYS- lysosome; PS-phagosome; PLS-phgolysosome. On the left side of the diagram are the typical times spent in each stage. On the right are some of the main marker proteins associated with each compartment. Note that the distinction between LE and LYS is based on the presence of markers such as the cation independent mannose-6- phosphate receptor (CI M6PR) in LE but not in LYS. The pH of LYS has only been estimated in only a few studies that used markers to separate it from LE and found to be neutral (Bright et al 2016)[402] but clearly this needs more investigation in different cell types. The term Endolysosome is increasingly used to refer to the combination of LE and Lys. Based on in vitro experiments with purified endosomes EE can fuse with EE and LE can fuse with LE, indicated by arrows. Nocodazole (which depolymerizes microtubules), reduced temperature (around 15^0C) and leupeptin can inhibit the step from ECV to LE. For more detail and references see the text.



P

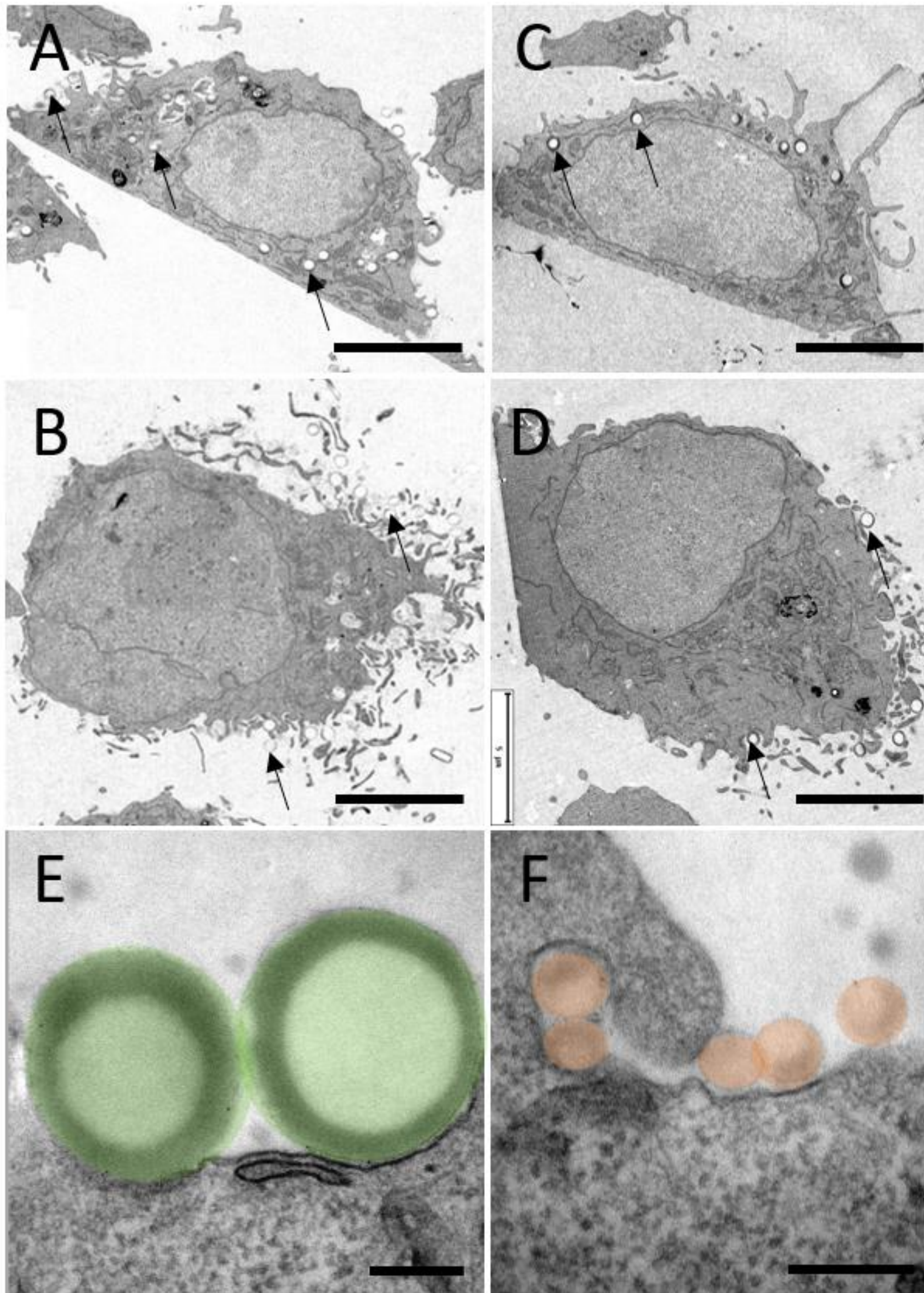
Fig.2: Phagocytosis-SEM

Phagocytosis of 100 nm and 500 nm latex beads (added together) after 15 min by J7774 A1 mouse macrophage cell line seen in different stages of uptake by scanning EM (SEM). Scale bar - 500nm



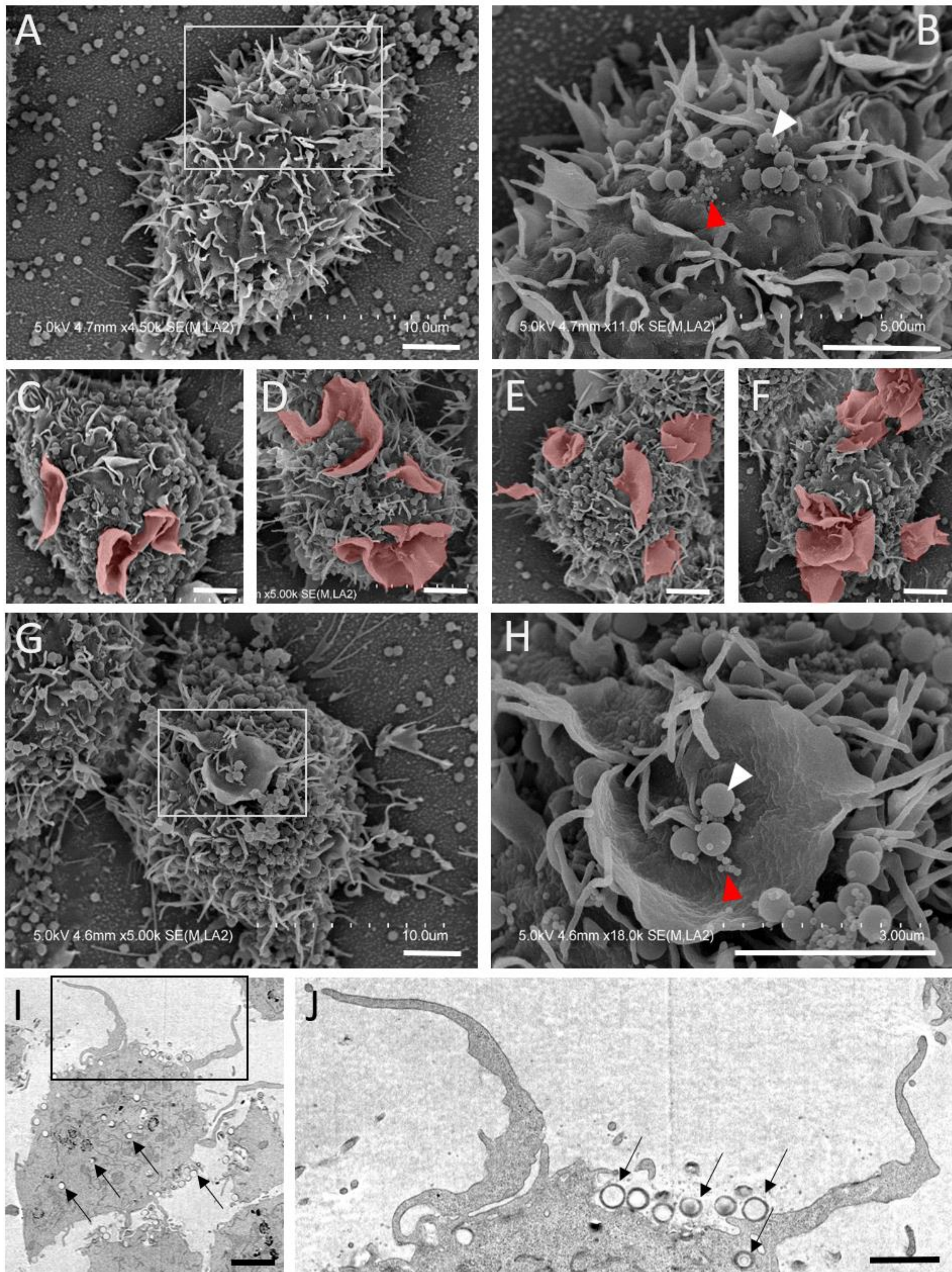
<u>Fig.3A:</u>Phagocytosis TEM

Phagocytosis of 100 nm (A-E- colored in orange) or 500 nm latex beads (F-M-colored in green) after 15 min by J7774 A1 mouse macrophage cell line seen after epoxy resin embedding and sectioning imaged by transmission EM (TEM). The cells were allowed to internalize 15 nm gold - BSA particles for 30min followed by 2h chase in medium free of gold. The gold then serves as a marker to identify both LE and LYS without distinguishing the two compartments. Different stages in the uptake process are shown. J shows a 500 nm bead in the cytoplasm after the initial stage of fusion with LE/Lys- a single gold particle (arrow) is indicated in the phagosome lumen. The arrow in L shows two gold particles next to the large bead and a higher density of gold particles in late endocytic structures can be seen. M shows a putative clathrin coated pit (arrowhead) associated with the phagocytic uptake of a 500 nm latex particle. Scale bar – 200nm.



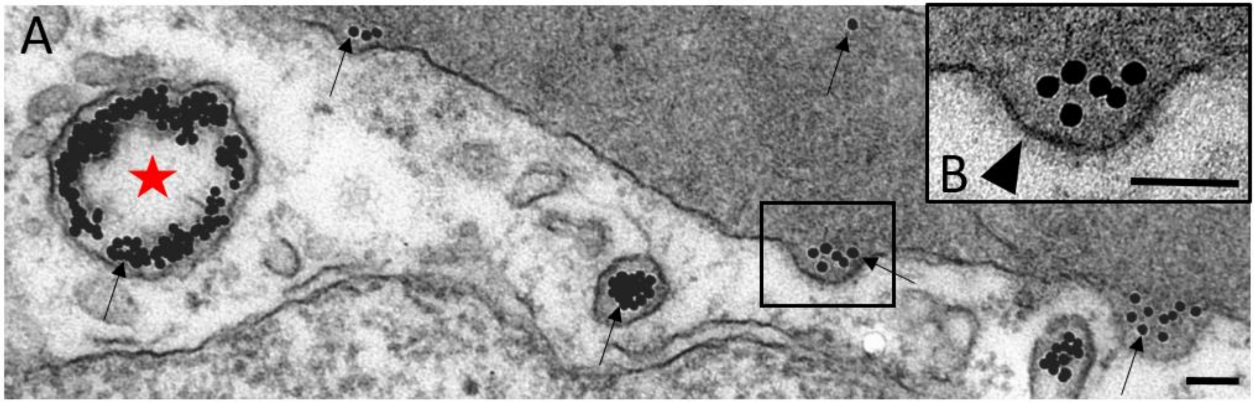
<u>Fig 3B</u>. Phagocytosis-cytochalasin D-TEM

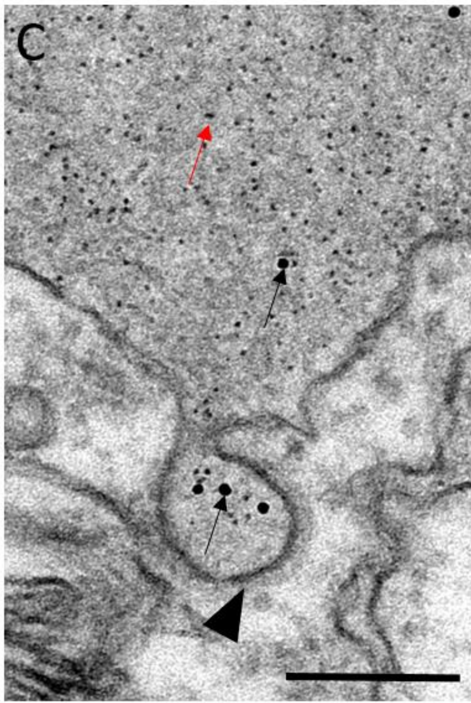
Cytochalasin D blocks phagocytic and macropinocytotic uptake but not binding of large and small nanoparticles. J774 A1 macrophages were either treated with 15µM cytochalasin D (B, D, E, F) or kept untreated (A, C,). Before Cyto D treatment micropinocytosis (MPC) was induced by 1h treatment with serum-free medium (B-F). All samples were incubated with a mixture of serum containing medium containing 100 and 500 nm latex particles for 15 min followed by chemical fixation and TEM preparation. Large beads are indicated by arrows or labelled green in E, small beads are labelled orange in F. The uptake inhibition with CytoD treatment is apparent by the absence of endocytosed beads in the cytoplasm (B, D) in contrast to the cells not treated with the inhibitor (A, C). The binding of both sizes of particles to the plasma membrane was similar with and without the inhibitor. Scale bars 5µm in A-D, 200nm in E and F.

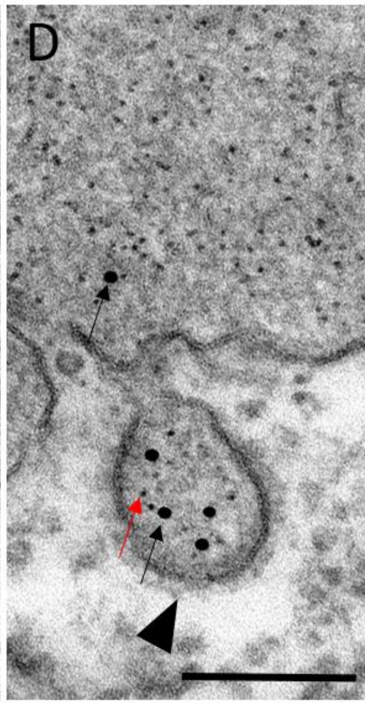


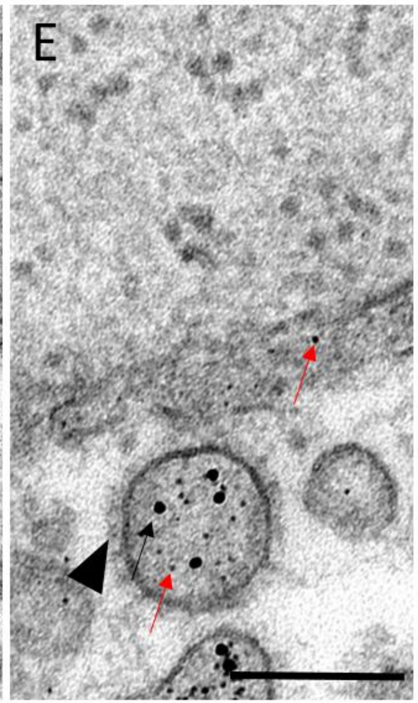
<u>Fig.4: </u>Macropinocytosis SEM and TEM

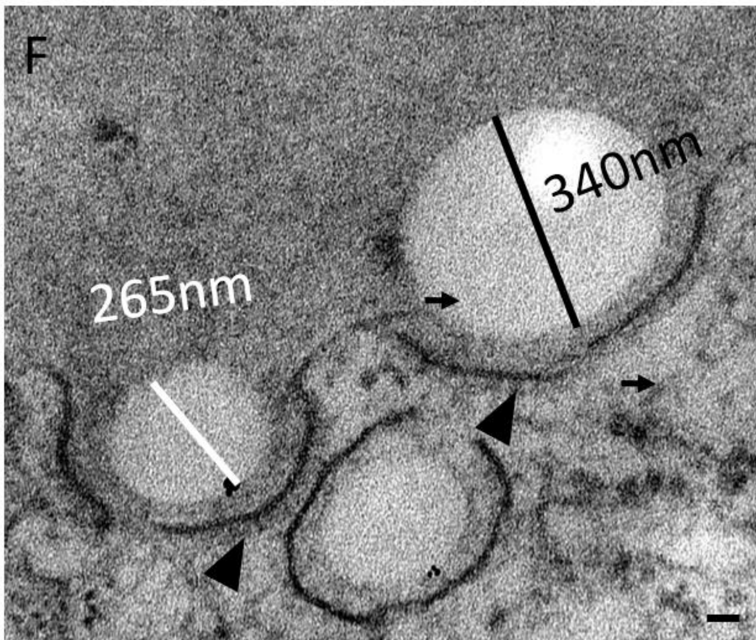
J774 A1 macrophages were left untreated in serum-containing medium (A, B) or stimulated for micropinocytosis (C+D) by 1h with serum-free medium followed by a mixture of serum containing (10%) medium with a. mixture of 100 and 500 nm latex particles for 15 min (A-J). Both specimens were prepared for SEM imaging. Although diverse surface projections are evident in the control cells they lack the striking lamellar structures typical of forming macropinosomes (colored in pink in C to F). In G one macropinosome is seen taking up 100 and 500 nm latex particles- highlighted in H. I and J show the same specimen as C-H prepared for TEM thin sections. Large beads are indicated by arrows. A putative forming macropinosome is shown-the area boxed in I is enlarged in J. This image shows the difficulty in unequivocally identifying the formation of macropinosomes using 2D sections.


A
B


C


D


E


F
340nm
265nm

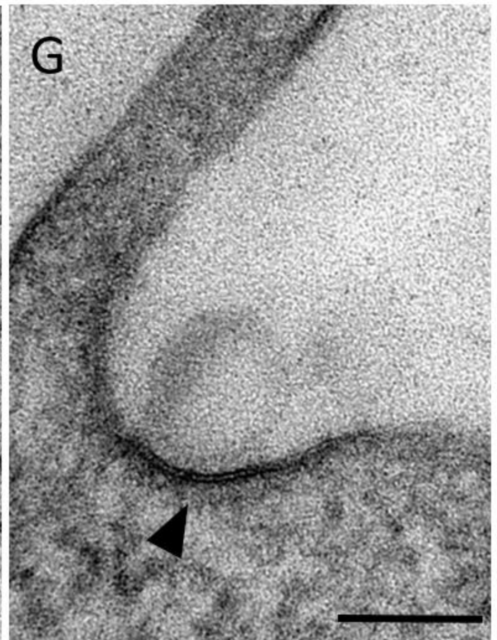
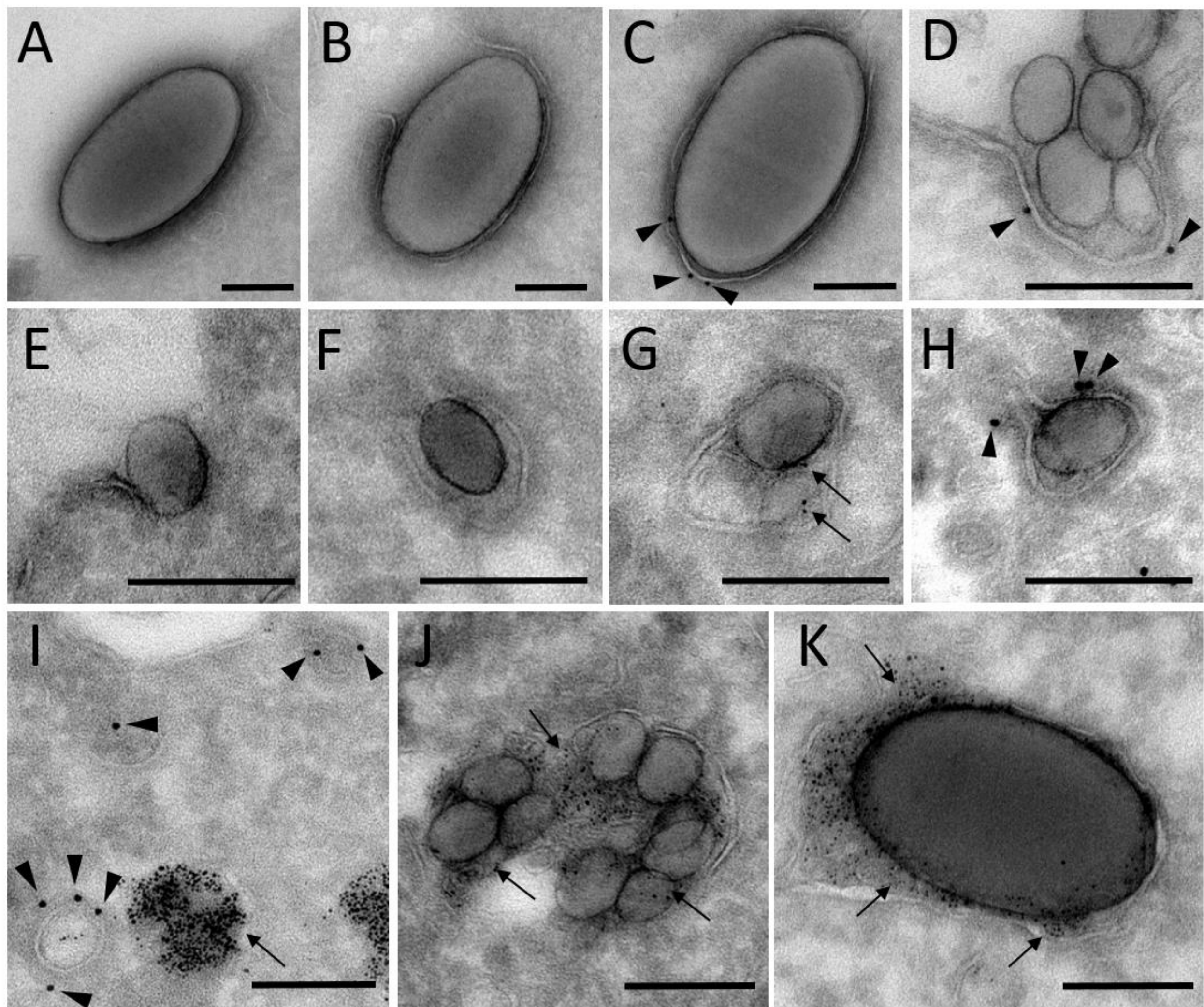

G

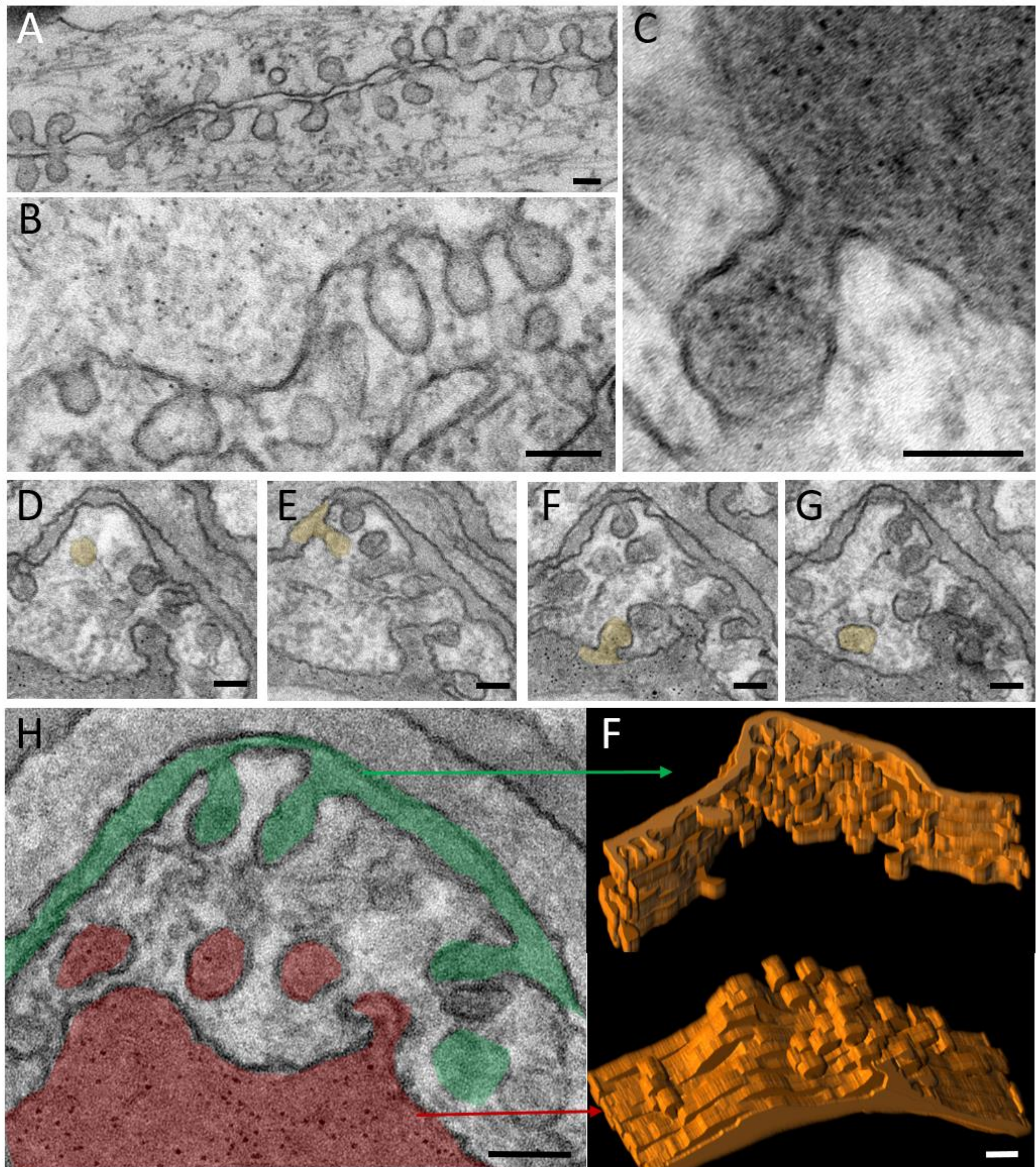
Fig.5: Clathrin coated pits and vesicles

Zebrafish embryos (five day old) were injected via the caudal vein with a mixture of 15 mm gold-BSA and anionic ferritin (A-E). At different times, starting at five minutes the embryos were fixed with glutaraldehyde and prepared for TEM thin section analysis. The focus then was on how the endothelial cells endocytose the 15nm gold (small black arrows) and the 5nm electron dense iron core of the ferritin particles (small red arrows) from the lumen of the blood vessels. It became evident that within minutes of the injection both the gold particles and the ferritin enter the endothelial cells via clathrin coated pits (CCP), with the characteristic electron dense projections on their cytoplasmic surface (arrowheads). However, there is a fundamental difference in the mechanism by which these two markers enter the coated pit. The ferritin appears to enter by bulk phase endocytosis since its concentration in the CCP is similar to that in the blood vessel lumen. In contrast, the gold particles seem to enter by receptor mediated endocytosis, presumably by receptors binding the albumin on the gold surface (A-E). This is evident in the significant concentration of the particles in the CCP/CCV relative to the concentration in the blood vessel and in the concentration of gold relative ferritin in the endocytic compartments (red asterisk in A). F shows an example of particles significantly larger than the standard 100 nm diameter what is usually considered to be the maximum diameter of the internalize particle. Shown are Poly Lactic acid A particles which co-encapsulated gold particles (arrows; made according to [299]); these were injected into the caudal vein of zebrafish larvae and are seen here entering CCV that are bigger than 250-300 nm. **G:** shows a 100 nm latex bead entering via a putative CCP in a J774 A1 macrophage. Scale bars: 100 nm



<u>Fig.5B:</u> Thawed cryo sections labeled with anti-clathrin and immunogold

J774A.1 mouse macrophage cells were incubated with 5nm gold-BSA for 0,5 h followed by a chase in medium free of gold for 1h to pre-label late endosomes and lysosomes (arrows). The cells were then incubated with 500 nm (A;B;C;K) or 100 nm D;E;F;H;G;J) latex beads for 15 min before fixation in 4% paraformaldehyde in PHEM buffer for 20h and preparation of cryo sections. The sections were labeled with a commercial monoclonal rabbit anti clathrin antibody (abcam, ab172958) at 1: 5 dilution. Clathrin labeling indicated by arrowheads.
A and B show 500 nm beads entering the cells without any signs of clathrin while C shows a patch of clathrin labeling. D shows a cluster of 100nm latex beads in a clathrin pit showing the respective morphology and labeling, similar to Yoshida 1984 [317]. E and F show 100nm particles entering cells without clathrin while H shows these small beads entering via a clathrin coated pit. Quantitation showed that only 1.8% of the 100nm beads and 4.3% of the 500nm beads entered cells via clathrin pits. I shows clathrin coated pits/vesicles free of latex beads adjacent to LE/ Lys while J shows 100 nm beads and K 500 nm beads of the minority which had entered LE/Lys after15 min.



<u>Fig.6:-</u>Caveolae

A: High numbers of caveolae are evident on adjacent plasma membranes of two cells of the notochord of a 5 day old zebrafish larva. B-H is from the zebrafish experiment described in the legend to Fig 5. In C a small amount of ferritin but no gold-BSA enters the caveolae of the dorsal aorta endothelium. D-G show sections from a serial section reconstruction of caveolae on the two surfaces of the endothelial cell. C shows a high magnification image of a caveola to show the ferritin. In yellow in D-G are shown putative caveolae that could be interpreted as having h budded to form free vesicles. However, the serial section reconstruction seen in F failed to reveal any free vesicles. Scale bars: 100nm

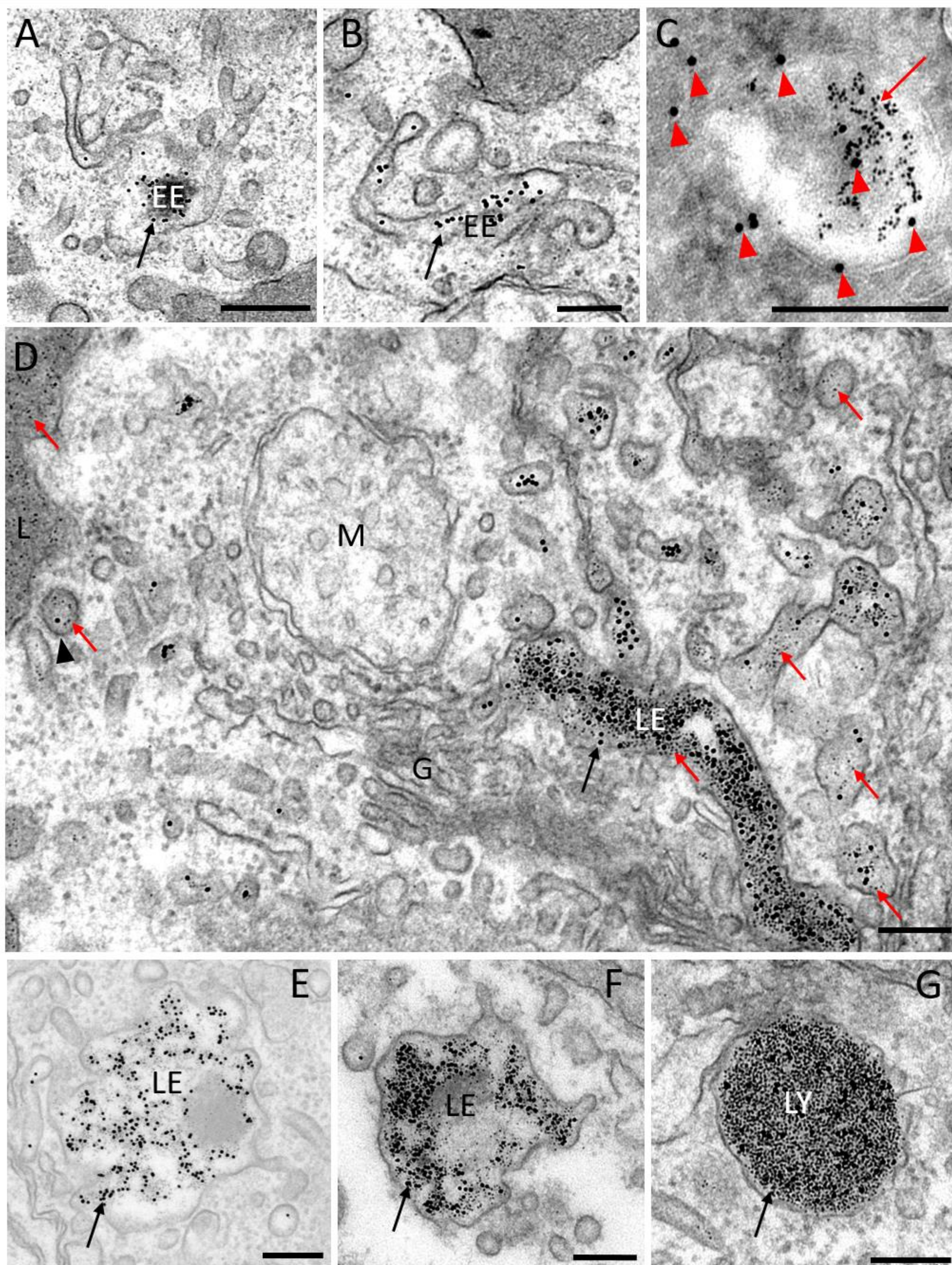
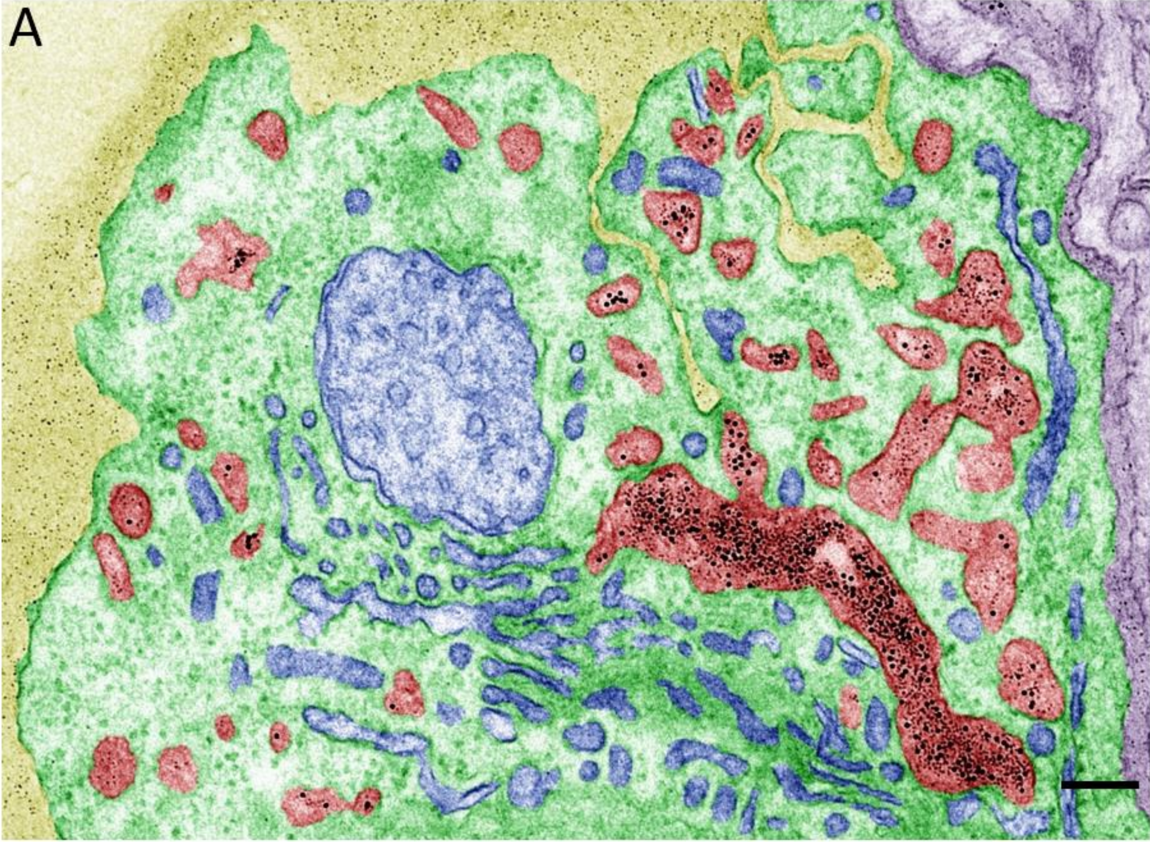

A
EE
B
EE
C
D
L
M
LE
G
E
LE
F
LE
G
LY

Fig.7: Overview of the endocytic pathway

This is from the experiment described in Fig 5 at 2h after injection of 10 nm gold-BSA and ferritin. This shows the caudal vein endothelium labeled with high concentration of gold (black arrow) and much lower concentration of ferritin (arrowheads). All figures except G show Epoxy resin-embedded sections imaged by TEM. A and B show putative early endosomes (EE) that are typically heterogeneous in structure. Putative LE and LYS are indicated in E-G. D shows an overview of the crowded area in the perinuclear region; indicated are Golgi stack (G), mitochondrion (M), CCV (arrowhead), LE, blood vessel lumen (L). Fig G shows a thawed cryo section of a J774 macrophage that had internalized 5 nm gold BSA (red arrow) into the lumen of LE/LYS after a pulse-chase (as in Fig 5). The section was labeled with anti-Lamp 1 and 10 nm gold (red arrowheads). Lamp labels predominantly the peripheral membranes of both LE and LYS. Scale bars: 200nm


A

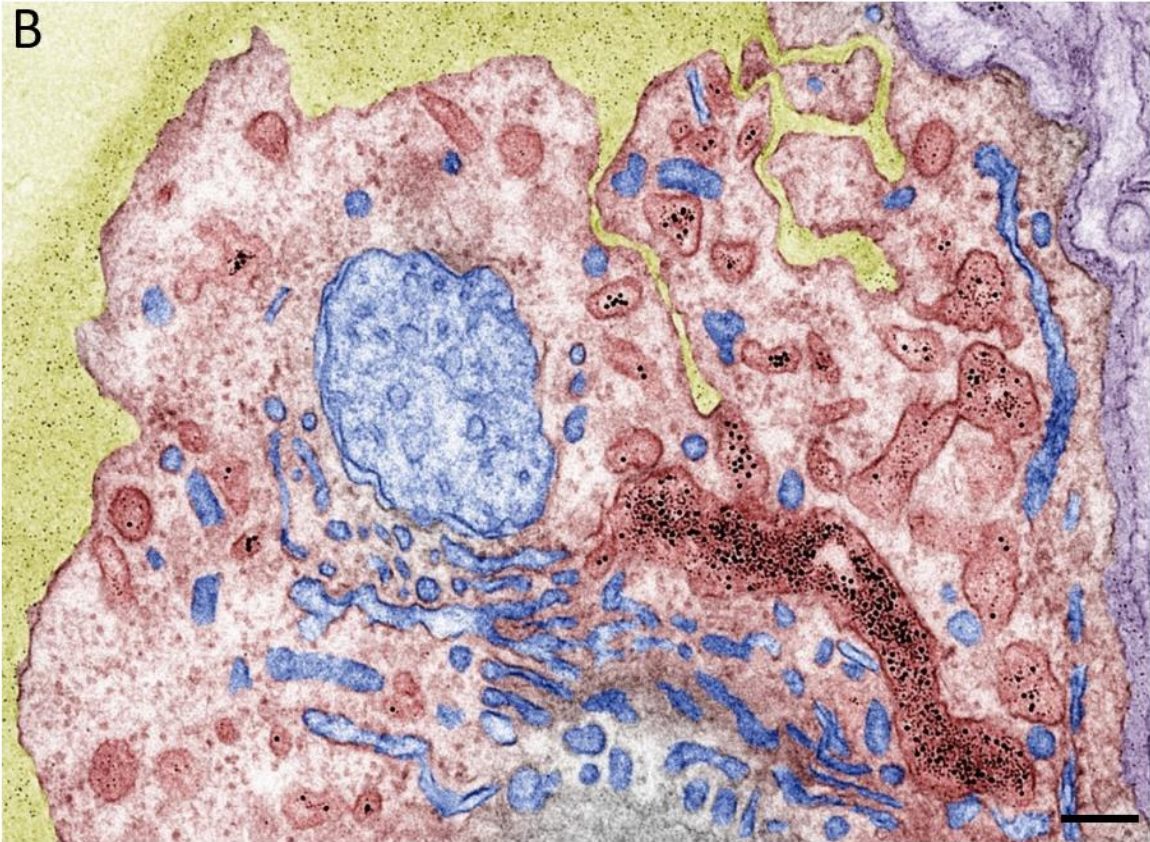

B

Fig 8. The complexity at endocytic compartments as visualized by electron microscopy. Pseudocolored electron micrographs; A) external milieu in yellow, cytosol in green, endocytic compartments containing colloidal gold in red. B) shows a hypothetical situation where some of the red marker has escaped from the endocytic organelles to the cytosol.

See text at page 39, Scale bars: 200nm.

---

ii