OPEN

Clinical & Translational Immunology (2016) 5, e71; doi:10.1038/cti.2016.15

www.nature.com/cti

REVIEW

The cell surface environment for pathogen recognition and entry

Jennifer L Stow and Nicholas D Condon

The surface of mammalian cells offers an interface between the cell interior and its surrounding milieu. As part of the innate immune system, macrophages have cell surface features optimised for probing and sampling as they patrol our tissues for pathogens, debris or dead cells. Their highly dynamic and constantly moving cell surface has extensions such as lamellipodia, filopodia and dorsal ruffles that help detect pathogens. Dorsal ruffles give rise to macropinosomes for rapid, high volume non-selective fluid sampling, receptor internalisation and plasma membrane turnover. Ruffles can also generate phagocytic cups for the receptor-mediated uptake of pathogens or particles. The membrane lipids, actin cytoskeleton, receptors and signalling proteins that constitute these cell surface domains are discussed. Although the cell surface is designed to counteract pathogens, many bacteria, viruses and other pathogens have evolved to circumvent or hijack these cell structures and their underlying machinery for entry and survival. Nevertheless, these features offer important potential for developing vaccines, drugs and preventative measures to help fight infection.
Clinical & Translational Immunology (2016) **5,** e71; doi:10.1038/cti.2016.15; published online 15 April 2016

Cells of the innate immune system are charged with the task of detecting and responding to pathogens and other danger signals as part of the body's front-line defence against infection and disease. Macrophages are key amongst the innate immune cell types in their role as sentinels, where they are positioned throughout organ systems and interstitial spaces, in the vasculature and at body surfaces.[1] Macrophages are adapted for detecting, responding to and destroying foreign cells or particles, and they must also alert the rest of the immune system to imminent danger. The cell surfaces of macrophages and other immune cells are designed to maximise interactions with the surrounding milieu and potential pathogens. Indeed among the earliest microscopic studies, the painstaking tracking of fluid-phase uptake and membranes by Steinman *et al.*[2] revealed that the cell surface of a macrophage turns over approximately every 30 min, representing perhaps the most active surface exchange of any cell type. A constant flow of endocytic and exocytic trafficking is required to support this membrane turnover, which is accompanied by high capacity for cargo transport in and out of these cells.

The extreme dynamism of macrophage and lymphocyte cell surfaces is now confirmed using the recently developed lattice light sheet microscope for live, rapid and three-dimensional imaging.[3] It is now evident that in both motile and recumbent cells, that the whole cell surface is in constant motion, ruffling, reaching and invaginating. The cortical actin cytoskeleton in immune cells is geared for rapid, localised and transient actin polymerisation to support the formation of membrane extensions like lamellipodia for cell migration, the filopodia that act as probing fingers and the dorsal ruffles that act as signalling centres and for engulfment.[4–7] Indeed, in the face of a rapidly moving and high turnover plasma membrane, these actin-supported protrusions become important, transiently stable membrane platforms for juxtaposing receptors, integrins, signalling machinery and other molecules that engage with the external milieu.

Cell surface features such as filopodia and ruffles are also essential for engaging and responding to pathogens, and many pathogens have evolved to subvert or hijack our cell surface structures and molecules to enhance their own infectivity and survival.[8–11] Thus, understanding the cell surface and the ways in which it can either prevent, or unwittingly support, pathogenic infections is of prime importance at a time when combating infectious diseases continues to be a major and a global challenge. The membrane protrusions, subdomains and compartments associated with the cell surface can offer new targets and opportunities to develop vaccines, drugs, diagnostics and biological solutions for medical intervention in the prevention or treatment of infections. This review will explore some of the cell protrusions, invaginations, membrane domains, pathways and molecular machinery that help to regulate the volatile but essential realm of the cell surface in innate immune cells.

FILOPODIA AND LAMELLIPODIA

Macrophages are endowed with highly dynamic projections of their cell surface, allowing them to move and to survey the extracellular milieu with extensions such as filopodia, lamellipodia and dorsal ruffles. Cell migration is a well-studied process, and lamellipodia and filopodia are essential for migration and chemotaxis of immune cells.

IMB Centre for Inflammation and Disease Research, Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland, Australia
Correspondence: Professor JL Stow, IMB Centre for Inflammation and Disease Research, IMB, The University of Queensland, 306 Carmody Road, Brisbane, Queensland 4072, Australia.
E-mail: j.stow@imb.uq.edu.au
Received 17 February 2016; revised 4 March 2016; accepted 8 March 2016

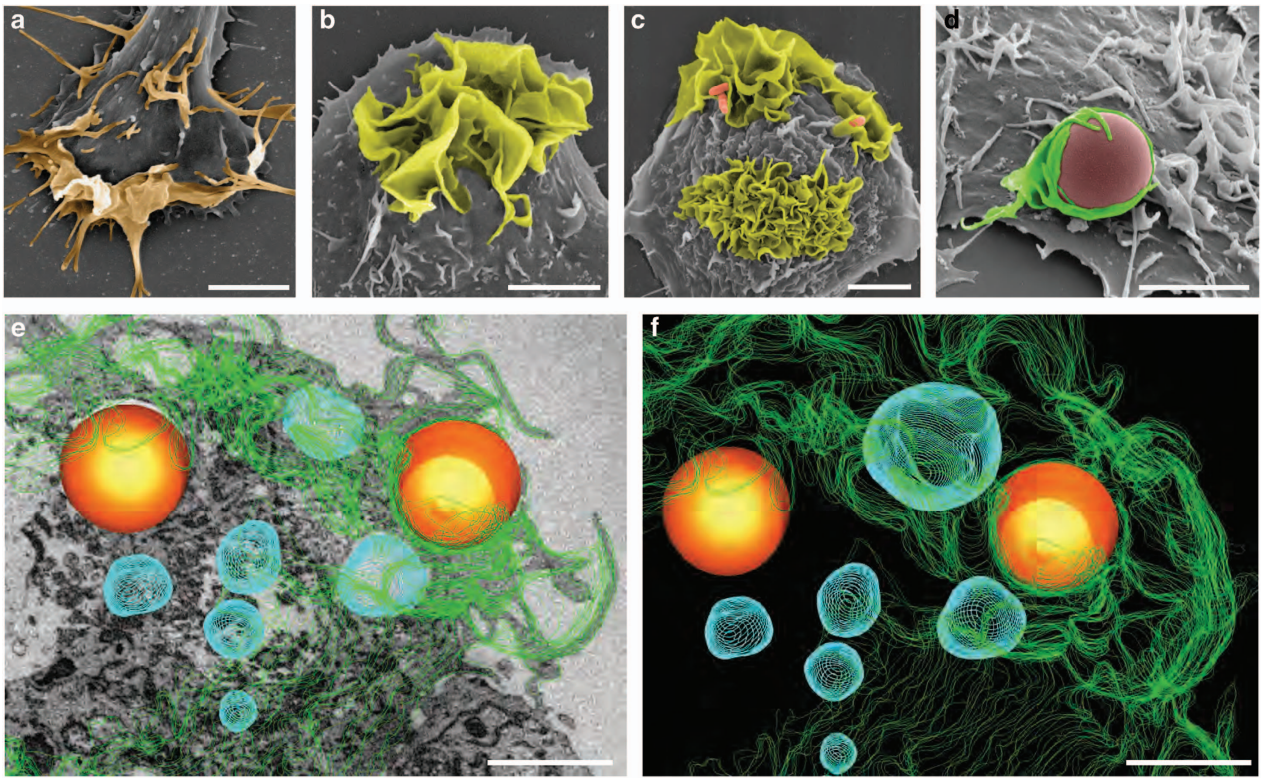

Figure 1 Ultrastructure of the macrophage cell surface and portals for pathogen entry. (**a**–**d**) Scanning electron microscopy of mouse macrophages showing cell surface features pseudocoloured; (**a**) Sheet-like lamellipodia at the leading edge of a migrating cell and long thin filopodia emerging from within the lamellipodia and from elsewhere on the cell surface; (**b**) Constitutive and wave-like dorsal ruffles rising up from the macrophage cell surface; (**c**) *Salmonella typhimurium* (red) enter macrophages by inducing patches of ruffling on the cell surface; (**d**) Ruffles can form into phagocytic cups (green) for the ingestion of pathogens or opsonised latex beads (brown). (**e**–**f**) Serial electron microscope (EM) images (3View, Gatan, Pleasanton, CA, USA) of a mouse macrophage ingesting opsonised latex beads (orange). A single EM image is displayed in (**e**) superimposed with three-dimensional (3D) rendering of 120 sections (colour). In (**f**), the 3D rendered images are shown separately, depicting the ruffled macrophage membranes (green) at the cell surface partially enveloping the beads (orange) as pre-phagosomes and the simultaneous production of macropinosomes (blue) from the ruffles. The macropinosomes undergo maturation, becoming smaller as they move into the cell. Scale bars = 3 μm.

At the leading edge, cell motility is driven by lamellipodia (thin membrane sheets containing a branched actin substructure) and filopodia (elongated membrane fingers (100–300 μm) comprising tight parallel bundles of actin filaments) that both have the growing barbed ends of actin filaments oriented to push the leading edge forward.[4] These structures are abundant on macrophages and their distinctive and recognisable morphologies can be appreciated by viewing the macrophage surface using scanning electron microscopy (Figure 1).

Lamellipodia extend through actin-based polymerisation and branching of actin filaments using machinery including the Arp2/3 and WASP-family verprolin-homologous protein (WAVE) regulatory complexes.[7] The same basic machinery is used to nucleate actin for the actin-based motility of bacterial pathogens, that invade host cells and induce the classical 'actin comet tails' to move around in the cytoplasm.[10]

The molecular composition of filopodia and their formation rely heavily on actin-binding and modifying proteins. Ena/Vasodilator-stimulated phosphoprotein (VASP) proteins prevent capping of actin-barbed ends at the tips of filopodia, formins like Dia2 are involved in producing unbranched actin filaments, fascin for actin bundling and unconventional myosin X motors that can move up and down filopodia, are all well-known elements of these structures.[4] Filopodia can form anywhere on the cell surface, for instance, emerging from lamellipodia at the cell's leading edge during migration (Figure 1a). Filopodia have many and varied cell type dependent functions. Cell adhesion and cell–cell contact can be driven by filopodia loaded with integrins or cadherins at their tips or displayed along their length.[12,13] Macrophages extend many filopodia, commonly using them as 'probes' to explore the surrounding milieu. Macrophages can also use filopodia as tentacles to draw particles towards the cell body[14] and in an extreme form of this process, very long filopodia wrap around and entrap bacteria in the 'coiling phagocytosis' described for lassoing *Legionella* for uptake.[15] Thus, filopodia often precede phagosome formation during contact with pathogens. Filopodia also have cholesterol-rich and highly ordered lipid-raft-enriched membranes[16,17] that are poised to contribute to receptor signalling and membrane trafficking, which in turn are important for the probing functions of these structures in cells like macrophages.

DORSAL RUFFLES

Dorsal ruffles (or hereafter referred to as ruffles) are highly distinctive, sometimes circular (hence the alternative name 'circular dorsal ruffles'), veils of membrane that rise up from the dorsal surface of adherent cells or sometimes occur at peripheral edges of cells, noting however their distinction from the ruffling leading edges of migrating cells. Dorsal ruffles behave in a wave-like manner, rising up and then collapsing back onto the surface, and any fluid, particles or pathogens encircled by the collapsing ruffles are internalised into the cell along with the ruffle membrane.[6,18] The collapsing ruffles can form

macropinosomes for fluid-phase uptake or phagosomes to engulf and internalise larger particulate matter or pathogens.[18] Dorsal ruffles share some morphologically and molecular features with other F-actin-rich surface structures, notably, podosomes and the invadopodia of cancer cells.[5]

Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) stimulate dorsal ruffle formation in fibroblasts and epithelial cells.[19] The respective receptor tyrosine kinases (RTKs) are concentrated in the ruffles, which act as signalling centres and ruffles are enriched in signalling molecules including Ras, Rac, Src, PI3K, Akt, MAPK and PAK1.[6] The formation of dorsal ruffles in fibroblasts also involves crosstalk and co-signalling from integrins and RTKs.[20] The collapse of ruffles into macropinosomes provides for rapid internalisation and downregulation of these clustered RTKs.[5,19] Thus, dorsal ruffles have both positive and negative regulatory roles in growth factor signalling, highlighting their importance to key processes in development and disease.

Dorsal ruffles are dependent on the transient production of F-actin, and inhibitors of F-actin polymerisation, such as cytochalasin D, abolish their formation.[5] Classical F-actin assembly complexes are localised to sites of dorsal ruffle formation. Through their proline-rich domains, Neural Wiskott–Aldrich syndrome protein, WAVE1 and WAVE2 can bind to SH3-domain containing proteins, which include Arp2/3 and membrane phospholipids to help concentrate relevant actin machinery in the dynamic ruffle environment.[21,22] Rho GTPases are also a feature of the ruffle machinery, with both Rac1 and Rac2 implicated in specialised ruffle actin-associated complexes and signalling cascades, along with other Rho family members including RhoA and CDC42.[22–24] Active Rac is needed for the development of pre-ruffle protrusions and for ruffle formation in macrophages.[25,26]

In macrophages and immature dendritic cells, membrane ruffling occurs constitutively,[27] and the perpetual cycles of F-actin polymerisation are promoted by membrane phospholipids at the ruffle sites. Phosphatidic acid in the pre-ruffle membrane helps to generate F-actin, via either recruiting and activating Rac or by activating phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P_2), to support continual actin polymerisation at sites of ruffling.[27,28] Overall, ruffles are highly enriched in phospholipids, which support the charge-based recruitment and attachment of proteins.[28] The inner leaflet of the plasma membrane has the highest pool of PtdIns(4,5)P_2 that supports protein and cytoskeletal interactions for a large suite of host processes including signalling, endocytosis, cell movement and enzyme activation.[26] In ruffles, this PtdIns(4,5)P_2 is also a substrate for the local production of PtdIns(3,4,5)P_3, which is important for recruiting signalling proteins and also potentially for closure of the dorsal ruffle.[6] The PtdIns(3,4,5)P_3 produced in ruffles then persists after closure and through the early stages of formation of macropinosomes and phagocytic cups.[18] These phosphoinositide transitions can be observed using probes such as the pleckstrin homology domain of protein kinase B (AKT).[29,30] The phosphoinositides also recruit effector proteins to the membrane such as transporter associated with antigen processing 1 and Lowe oculocerebrorenal syndrome protein, which can influence the dynamics of the cytoskeleton and subsequent compartment maturation.[22]

The dorsal ruffles are also replete with molecular machinery and families of regulators such as GTPases (Rhos, Rabs, Arfs and Ras) that participate in signalling and membrane trafficking, although in many cases, specific members of these families have not yet been identified specifically on ruffles in different cell types. Interestingly, the ADP-ribosylation factor-like (Arl) protein, Arl13b, has recently been demonstrated in dorsal ruffles.[31] Arl13b is better known as a key component of primary cilia, specialised organelles for mechanosensory regulation and signal transduction. Related to this, Arl13b is a casual locus mutated in the congenital ciliopathy, Joubert syndrome, associated with severely impaired development.[32] Arl13b associated with F-actin in dorsal ruffles and loss of Arl13b also reduces PDGF-induced dorsal ruffle formation. In this role, and in its other roles in endocytic recycling, the Arl13b shares a non-muscle myosin IIA as an effector.[31,33] It is of emerging interest that Arl13b is necessary for formation of dorsal ruffles and cilia as distinct signal transduction membrane domains.

Ruffles for receptor signalling and pathogen detection

Receptor signalling is also integral to pathogen detection and immune responses. Innate immunity relies on a series of host pattern recognition receptors that detect non-self, danger signals and pathogen-derived molecules. Toll-like receptors (TLRs) are one such receptor family and different TLRs are positioned on the cell surface or endosomes to detect and respond to selected extracellular or intracellular pathogens.[34,35] Although macrophages ruffle constitutively, dorsal ruffling is enhanced by exposing the cells experimentally to lipopolysaccharide (LPS) of Gram-negative bacteria, indicating that ruffling is also a pathogen–inducible cell response.[36–38] LPS is a ligand for TLR4 and activated TLR4 generates signals from the cell surface or from endosomes/macropinosomes[35] using different sets of adaptor molecules to elicit cytokine release and other downstream responses. TLR4 and its cell surface adaptors can be found clustered in dorsal ruffles where they are poised with the relevant machinery for PI3K-AKT signalling.[38] As the ruffles collapse, TLR4 is delivered to macropinosomes,[35] or to phagosomes[39] during pathogen engulfment, where signalling from different adaptors varies the signalling and downstream responses.[34] Thus, in addition to receptor (TLR4) trafficking for degradation or recycling, the collapsing ruffle creates another signalling platform for biasing immune outputs. In addition to TLR4-mediated transcriptional regulation and synthesis of cytokines, the dorsal ruffles and pre-phagosome membranes are also sites for release of cytokines such as TNF. Newly synthesised TNF is delivered to recycling endosomes that feed membrane to the base of the ruffles and pre-phagosomal membranes, simultaneously exporting TNF to the surface at this point for release from the cell.[40]

MACROPINOCYTOSIS

Macropinocytosis is one of the most ancient, prevalent and yet perhaps least well-defined forms of endocytosis. It is the process of fluid gulping practiced at some level by most cells of the body, but scaled up to a high volume highway in macrophages as they perform surveillance of the surrounding tissue. The pathway results in the ingestion of fluid, plus any fluid-phase cargo and a large swathe of plasma membrane with its resident transmembrane proteins and any molecules, particles or pathogens attached to them on the external face of the plasma membrane. Unlike receptor-mediated endocytosis and phagocytosis, macropinocytosis is not driven by receptor-ligand interactions. Macropinocytosis is therefore distinguished by being a large volume, non-selective, actin-dependent pathway for internalisation and this is reflected in the nature of the macropinosomes themselves, which are uncoated, nondescript vesicles varying in size from 0.2 to $>2\,\mu m$ in diameter.[41,42] As discussed below, the non-selective, large volume nature of macropinocytosis means that both inevitably, and by design, it serves for the uptake of pathogens. This is highlighted by an intriguing mechanism reported by Bosedasgupta *et al.*,[43] who show that once macrophages have been activated by contact with pathogens, they upregulate receptor-independent

macropinocytosis to override the less efficient process of receptor-mediated phagocytosis to enhance their capacity to clear the pathogens. This switch they found to be mediated by phosphorylation of the actin-binding protein coronin 1, which is known as a prominent regulator of early actin assembly forming a crown-like ring around newly forming macropinosomes in *Dictyostelium* and other organisms.

The fate of macropinosomes has classically been studied by tracking the uptake of fluid-phase cargo, such as fluorescent dextran, or fluorescently-tagged membrane proteins or lipids in live or fixed cells. These studies routinely show that soon after internalisation, macropinosomes shrink and move towards the centre of the cell as they undergo maturation (Figure 1). Evidence from tracking cargo mixing shows that macropinosomes have fused with other endosomes within 1 min of uptake.[18] Macropinosomes feed some of their contents into recycling pathways. The Rab11-positive recycling endosomal network in macrophages is vast and forms an important repository for plasma membrane and proteins recycling back to the surface, as well as new exocytic traffic, in this high turnover environment.[44] Alternatively, live imaging also reveals that macropinosomes can undergo rapid and extensive tubulation after internalisation, forming a transient network of elongated membranes or tubes that help to sort membrane proteins and fluid-phase cargo into various endosomes, many of which mature into LAMP-1/Rab7 late endosomes/lysosomes where degradation can occur.[41]

The actin polymerisation in dorsal ruffles or at the leading edges of motile cells provides the platform of branched actin filaments needed to assemble the macropinosomes. Much of the same actin polymerising machinery is needed for ruffling and macropinocytosis. Multiple small GTPases contribute to actin regulation during macropinocytosis including the Rho, Rac, Cdc42, Rab, Arf and Ras subfamilies.[45] Arf6 is involved in ruffling and macropinocytosis through roles that include recruiting Rac1 for the production of PI(4,5)P2 or for the activation of phospholipase D (reviewed in[45]). With the expression and manipulation of photoactivatable Rac1, Fujii *et al.*[46] were able to demonstrate the cycles of Rac1 activation needed to initiate ruffling and macropinosome formation and the important deactivation of Rac1 that accompanies closure and maturation. Rab5a is a signature GTPase on macropinosomes, recruited for roles in actin remodelling, signalling and in the closed, maturing macropinosome where PI(3)P has generated the activation of Rab5a and recruitment of its effectors, which may help to stabilise these compartments.[41,45,46] Other Rabs, such as Rab34, Rab21 and Rab31 can be found on macropinosomes serving to recruit a variety of additional effectors.[45]

Phosphoinositide transitions initiated in ruffles carry through to the macropinosomes. The transition of PI(4,5)P2 to PI(3,4,5)P3 to PI(3,4)P2 and then to PI(3)P is conserved during the processes of macropinosome closure and maturation from C.elegans to mammalian cells.[45,47,48] PI3kinases are crucially involved in macropinocytosis and the broad spectrum PI3Kinase inhibitors, LY294002 and wortmannin blocks macropinosome formation, and specifically macropinosome closure, without necessarily affecting the preceding ruffling.[42,49,50] The transient appearance of PI(3,4,5)P3 is accompanied by Rac1 activation and diacylglycerol production, and this is followed by the appearance of PI(3,4)P2 during macropinosome closure.[48] Lipid probes variably show accumulation of PI(3,4,5)P3 on the macropinosome membranes before or just after closure. PI(3,4,5)P3 can then activate phospholipase C and diacylglycerol, as well as protein kinase C and Ras during macropinosome maturation.[48] Accordingly, inhibitors for these enzymes variably block macropinocytosis (Table 1).

The sorting nexins (SNXs) with their signature Phox homology (PX) lipid-binding domains are an important class of phosphoinositide effectors, and multiple SNX family members are associated with, and functionally implicated in, macropinocytosis. SNX5, for instance, is required for macropinocytosis. At the behest of EGFR activation, SNX5 is recruited to PI(3,4)P2-enriched membranes on early macropinosomes and it is present on tubulating macropinosomes, where in both cases, the SNX5 BAR domain may contribute to membrane curvature.[41,42] Macrophages, but interestingly not immature dendritic cells, from SNX5 knockout mice have significantly reduced macropinocytosis and dorsal ruffling.[51]

A diagnostic feature of macropinocytosis, used to distinguish this pathway from clathrin-mediated endocytosis, is its sensitivity to inhibitors of Na^+/H^+ ion exchangers, such as amiloride.[52,53] Although the mode of action of amiloride is controversial, it remains a useful experimental tool for blocking macopinocytic uptake of markers or pathogens.[9,42]

In the immune system, macropinocytosis is an important process for antigen presentation through the environmental sampling performed by antigen-presenting cells such as macrophages and immature dendritic cells. Activated and mature dendritic cells downregulate macropinocytosis in favour of migration to lymph nodes for presentation to T cells.[54] Antigenic peptides derived from internalised material are loaded onto major histocompatibility complex (MHC) molecules for presentation to T cells to invoke adaptive immune responses. Antigens presented by either MHCI, (usually self or endogenous antigens) or MHCII complexes (foreign or extracellular antigens) can come from macropinosomes. Fluid-phase markers ingested by dendritic cells can be delivered to the MHCII compartment, denoted by some late endosome/lysosome markers, for processing and loading. Macropinocytosis can also result in the cross-presentation of extracellular antigens on MHCI after processing of these antigens to peptides in the endosomal system or in the cytoplasm.[55] Inhibition by amiloride implicates macropinocytosis directly in MHC presentation and T-cell activation.[56]

Finally, the non-selective nature of macropinocytosis lends itself to exploitation for the delivery of a variety of particles, soluble drugs, genetic material and biological modifiers into cells. Membrane-penetrating peptides such as trans-activating transcriptional activator (TAT) are taken up into macropinosomes where they can cross the membrane into the cytoplasm providing an important portal for genetic or pharmacologic modifiers.[57] The macropinocytic and ligand-directed uptake of quantum dot nanoparticles can be demonstrated[58]

Table 1 Examples of regulators that can be targeted and agents that can be used to manipulate macropinocytosis and in many cases, the entry of pathogens

Molecular regulators required for macropinocytosis	
Phosphatidic acid	26
Arl13b	33
Snx-5	41,42
PIP_3/PI3ks	45,84
Agents that block macropinocytosis	
Diacylglycerol kinase inhibitor	26
Phospholipase C inhibitors	27
Amiloride	49,52
LY294002	49
Wortmannin	85
Cytochalasin D	

with the potential to target delivery to specific cell types or situations. Recently, the supercharging of macropinocytosis in tumour cells by activating epidermal growth factor receptor (EGFR) and oncogenic Ras provided efficient delivery of engineered cytotoxic exosomes into cells.[59] Macropinocytosis will continue to be an important tool in fighting disease in this and many other ways.

PHAGOCYTOSIS

Phagocytosis is a process initiated in macrophages or other professional phagocytes, confronted with a pathogen, a dead cell or large ($>0.5\,\mu m$) opsonised particle. Phagocytosis is a deliberate, receptor-driven process based on recognition of the pathogen or object and engagement with it by receptors on macrophage cell surface ruffles, filopodia or the phagocytic cups that surround and engulf the 'prey' (Figure 1), internalising it into a sealed phagosome. The typical course of phagosome maturation is through fusion with successive endosome and lysosomes, acidification of the lumen and generation of reactive oxygen species, which lead to a destructive phagolysosome designed to kill pathogens and degrade them or any inert particles that have been phagocytosed.[18] Phagocytosis is essential for innate immunity, organ development and maintaining homoeostasis throughout tissues of the body. It contributes, either directly or inadvertently, to a wide range of diseases from bacterial pathogenesis and infection, to the accumulation of aberrant proteins in neurodegenerative diseases, the advancement of atherosclerotic lesions and to cancer and chronic diseases like chronic obstructive pulmonary disease (COPD) and cystic fibrosis.[60–64] Multiple phagocytic receptors are activated during the recognition of pathogens or particles. Pattern recognition receptors (receptors for polysaccharides on yeast, e.g., dectin-1), opsonic receptors (immunoglobulin or complement receptors, e.g., FcγR or CR1) and apoptotic cell or corpse receptors (phosphatidylserine receptors, e.g., TIM-1) are among those that can be co-engaged (reviewed in Flannagan *et al.*[25]). Through receptor crosstalk, signalling from co-activated TLRs, for instance, concomitantly drives cell death/survival and inflammatory responses appropriate for the nature of the pathogen or particle. Receptor signalling takes place in the phospholipid and actin-rich environment of the phagocytic cups where receptor trafficking is also supported by prolific membrane exchange between recycling endosomes or early endosomes and the early phagocytic membranes.

Actin filaments are a feature of only the early stage ruffles, pseudopods and phagocytic cups, leading up to sealing and initial internalisation of the phagosomes whereupon F-actin is rapidly depolymerised and dispersed.[18] The precise timing of this association means that F-actin is a useful experimental marker for early phagosomes. Actin polymerisation is activated by different receptors (through diverse pathways) and engaged for the F-actin and receptor-mediated protrusion of the membranes over the surface of the prey during engulfment. Activated FcγRs recruit Arp2/3, whereas CR3 and integrin-dependent phagocytosis recruits talin and other actin-binding proteins.[65] Multiple lipids contribute to membrane remodelling and other functions associated with phagocytosis. Cholesterol is needed for phagocytic cup formation, and sphingolipids and phospholipids are also extensively engaged.[16,66] The phospholipid transitions that accompany ruffling and macropinocytosis have also been mapped during phagocytosis, beginning with conversion of the PI(4,5)P2 to PI (3,4,5)P3 in presealed phagocytic cups and PI(3)P in fully sealed, internalised phagosomes.[26,67]

Multiple (>20) Rab GTPases, their accessory proteins and varied effectors are especially recruited for phagocytosis, warranted by the intense membrane and protein trafficking, endosome fusion and receptor signalling inherent in the remodelling stages of early phagocytosis, followed throughout the whole process by further trafficking and fusion events.[67] The sequential nature of Rab recruitment also experimentally defines successive stages of phagocytosis.

With its focus as the entrapment and destruction of pathogens, phagocytosis is targeted for disruption by multiple strategies that have evolved in diverse species. Some bacterial virulence factors aim to block phagocytic uptake, whereas for intracellular pathogens, other virulence factors are geared to allow phagocytic entry but subvert its destructive capacity.[68,69] On the other hand, some pathogenic bacteria have developed the ability to actively induce phagocytosis and/or macropinocytosis to gain entry into the host cell.[70] During this type of entry, large extracellular projections and plasma membrane ruffles are evident surrounding the invading bacteria.[71,72]

PATHOGEN ENTRY VIA RUFFLES AND MACROPINOSOMES

Dorsal ruffles and macropinosomes are co-opted or induced by a wide range of human pathogens and infectious agents, from viruses, to bacteria, protozoa and prions, for entry into human cells.[8] *Salmonella* spp. can actively infect almost any cell type through the injection of bacterially-derived effector proteins that modulate a number of host processes such as F-actin polymerisation to induce macropinocytosis.[73] Typically *Salmonella typhimumirum* that causes gasteroenteritis or typhoid fever, invades epithelial cells or macrophages. One of the *Salmonella* effector proteins is SipA, which has been shown to promote the generation of plasma membrane protrusions and ruffles by promoting the polymerisation of F-actin filaments.[74] *Salmonella* induce large localised patches of ruffling on the surface of epithelial cells, whereas on macrophages, large areas of exaggerated ruffling are induced (Figure 1), serving to capture and internalise multiple bacteria near the cell surface. Other bacteria such as *Yersinia* spp. and *Neisseria gonorrhoeae* induce plasma membrane ruffling to gain entry to host cells.[75,76] Additional bacterial effectors hijack cell signalling and the maturation of macropinosomes to avoid detection and destruction, creating protective vacuoles inside host cells for sequestration and proliferation. Many of the *Salmonella* effectors have been characterised, serving to inform us about the roles of various host proteins in normal macropinocytic processes. Bacteria like *Legionella*, which invade macrophages through modifying macropinocytic or phagocytic processes, inject hundreds of effectors, many of which remain to be characterised.[77,78]

Pathogenic viruses commonly infect human cells via macropinocytosis.[9] The highly infectious and deadly Ebola virus and mosquito-borne flaviviruses[79] like Dengue, West Nile, yellow fever and the newly emergent Zika viruses, all rely on macropinocytosis for entry into human cells, where they gain access to intracellular host membranes and machinery for replication. Ebola entry into macrophages is calcium dependent, relying on the class of endosomal two-pore channels, and disrupting the function of these channels either genetically or with pharmacological inhibitors such as Tetrandrine, can prevent virus infection in human macrophages.[80] Vaccinia virus also uses macropinocytosis as a main but possibly not exclusive pathway for entering dendritic cells.[81] In fact, vaccinia can also use an elegant apoptotic mimicry mechanism to enter human cells, enhancing macropinocytic uptake, but also cleverly encasing its infective virions inside a 'flipped' host plasma membrane exposing phosphatidylserine on the outer surface to mimic apoptotic bodies.[82] Virions budding from host cells with this false apoptotic coating are readily taken up by other host cells to spread the infection. HIV infects a variety of cells through cell surface portals and different endocytic mechanisms, and virions can gain entry into macrophages via

macropinosomes followed by evidence of capsid release into the cytoplasm.[83] Cell surface structures are also utilised by viruses to aid their spreading and infection. HIV hijacks cell–cell interactions between dendritic cells and CD4 T cells, by inducing long actin-dependent filopodia loaded at their tips with immature HIV virions, to spread the virus to their target T cells.[11]

CONCLUSION

Approaches and information emerging from the fields of cell and molecular biology, immunology and biochemistry are adding to our knowledge of the cell surface as morphological and functional 'organelle', an entity that is customised for interacting with the extracellular milieu in many different physiological and patho-physiological situations. Many pathogenic species have already deciphered and learned to exploit aspects of mammalian cell surface behaviour and function to aid infection and their survival. Ever more powerful imaging and microscopy methods will be powerful tools for viewing cell surface behaviour and dysfunction; gene editing techniques, genomics and proteomics are also approaches that are adding to our molecular inventory of cell surface regulators and machinery. Moreover. the knowledge acquired about cell surfaces is essential for devising new preventative measures, new vaccines and new drugs to combat the ever-present threat of common and emerging infectious diseases. Host cell targets for vaccines and drugs offer the potential to avoid or reduce the complications of acquired resistance or rapidly evolving pathogens that thwart our ability to treat and eradicate some of the most prevalent pathogens in our midst.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We wish to thank members of the Stow lab for their help and advice.
We acknowledge the expert assistance of Darren Brown who performed the electron microscopy (SEM and 3view) and prepared the images in Figure 1.
This work was performed in UQ's Centre for Microscopy and Microanalysis.
Funding for this work was provided by grants from the Australian Research Council (DP140101461) and National Health and Medical Research Council of Australia (606788).

1 Gordon S, Plüddemann A, Martinez Estrada F. Macrophage heterogeneity in tissues: phenotypic diversity and functions. *Immunol Rev* 2014; **262**: 36–55.
2 Steinman RM, Brodie SE, Cohn ZA. Membrane flow during pinocytosis. A stereologic analysis. *J Cell Biol* 1976; **68**: 665–687.
3 Chen B-C, Legant WR, Wang K, Shao L, Milkie DE, Davidson MW *et al.* Lattice light-sheet microscopy: imaging molecules to embryos at high spatiotemporal resolution. *Science* 2014; **346**: 1257998.
4 Mattila PK, Lappalainen P. Filopodia: molecular architecture and cellular functions. *Nat Rev Mol Cell Biol* 2008; **9**: 446–454.
5 Buccione R, Orth JD, McNiven MA. Foot and mouth: podosomes, invadopodia and circular dorsal ruffles. *Nat Rev Mol Cell Biol* 2004; **5**: 647–657.
6 Hoon J-L, Wong W-K, Koh C-G. Functions and regulation of circular dorsal ruffles. *Mol Cell Biol* 2012; **32**: 4246–4257.
7 Bisi S, Disanza A, Malinverno C, Frittoli E, Palamidessi A, Scita G. Membrane and actin dynamics interplay at lamellipodia leading edge. *Curr Opin Cell Biol* 2013; **25**: 565–573.
8 de Carvalho TM, Barrias ES, de Souza W. Macropinocytosis: a pathway to protozoan infection. *Front Physiol* 2015; **6**: 106.
9 Mercer J, Helenius A. Gulping rather than sipping: macropinocytosis as a way of virus entry. *Curr Opin Microbiol* 2012; **15**: 490–499.
10 Haglund CM, Welch MD. Pathogens and polymers: microbe–host interactions illuminate the cytoskeleton. *J Cell Biol* 2011; **195**: 7–17.
11 Aggarwal A, Iemma TL, Shih I, Newsome TP, McAllery S, Cunningham AL *et al.* Mobilization of HIV spread by diaphanous 2 dependent filopodia in infected dendritic cells. *PLoS Pathog* 2012; **8**: e1002762.
12 Galbraith CG, Yamada KM, Galbraith JA. Polymerizing actin fibers position integrins primed to probe for adhesion sites. *Science* 2007; **315**: 992–995.
13 Vasioukhin V, Bauer C, Yin M, Fuchs E. Directed actin polymerization is the driving force for epithelial cell–cell adhesion. *Cell* 2000; **100**: 209–219.
14 Kress H, Stelzer EHK, Holzer D, Buss F, Griffiths G, Rohrbach A. Filopodia act as phagocytic tentacles and pull with discrete steps and a load-dependent velocity. *Proc Natl Acad Sci* 2007; **104**: 11633–11638.
15 Horwitz MA. Phagocytosis of the Legionnaires' disease bacterium (*Legionella pneumophila*) occurs by a novel mechanism: engulfment within a pseudopod coil. *Cell* 1984; **36**: 27–33.
16 Kay JG, Murray RZ, Pagan JK, Stow JL. Cytokine secretion via cholesterol-rich lipid raft-associated SNAREs at the phagocytic cup. *J Biol Chem* 2006; **281**: 11949–11954.
17 Gaus K, Gratton E, Kable EPW, Jones AS, Gelissen I, Kritharides L *et al.* Visualizing lipid structure and raft domains in living cells with two-photon microscopy. *Proc Natl Acad Sci* 2003; **100**: 15554–15559.
18 Swanson JA. Shaping cups into phagosomes and macropinosomes. *Nat Rev Mol Cell Biol* 2008; **9**: 639–649.
19 Yamazaki T, Zaal K, Hailey D, Presley J, Lippincott-Schwartz J, Samelson LE. Role of Grb2 in EGF-stimulated EGFR internalization. *J Cell Sci* 2002; **115**: 1791–1802.
20 Azimifar SB, Böttcher RT, Zanivan S, Grashoff C, Krüger M, Legate KR *et al.* Induction of membrane circular dorsal ruffles requires co-signalling of integrin–ILK-complex and EGF receptor. *J Cell Sci* 2012; **125**: 435–448.
21 Legg JA, Bompard G, Dawson J, Morris HL, Andrew N, Cooper L *et al.* N-WASP involvement in dorsal ruffle formation in mouse embryonic fibroblasts. *Mol Biol Cell* 2007; **18**: 678–687.
22 Itoh T, Hasegawa J. Mechanistic insights into the regulation of circular dorsal ruffle formation. *J Biochem* 2013; **153**: 21–29.
23 Linder S, Aepfelbacher M. Podosomes: adhesion hot-spots of invasive cells. *Trends Cell Biol* 2003; **13**: 376–385.
24 Mahankali M, Peng H-J, Cox D, Gomez-Cambronero J. The mechanism of cell membrane ruffling relies on a phospholipase D2 (PLD2), Grb2 and Rac2 association. *Cell Signal* 2011; **23**: 1291–1298.
25 Flannagan RS, Jaumouille V, Grinstein S. The cell biology of phagocytosis. *Annu Rev Pathol* 2012; **7**: 61–98.
26 Bohdanowicz M, Grinstein S. Role of phospholipids in endocytosis, phagocytosis, and macropinocytosis. *Physiol Rev* 2013; **93**: 69–106.
27 Bohdanowicz M, Schlam D, Hermansson M, Rizzuti D, Fairn GD, Ueyama T *et al.* Phosphatidic acid is required for the constitutive ruffling and macropinocytosis of phagocytes. *Mol Biol Cell* 2013; **24**: 1700–1712.
28 Heo WD, Inoue T, Park WS, Kim ML, Park BO, Wandless TJ *et al.* PI(3,4,5)P3 and PI(4,5)P2 lipids target proteins with polybasic clusters to the plasma membrane. *Science* 2006; **314**: 1458–1461.
29 Marshall JG, Booth JW, Stambolic V, Mak T, Balla T, Schreiber AD *et al.* Restricted accumulation of phosphatidylinositol 3-kinase products in a plasmalemmal subdomain during Fcγ receptor-mediated phagocytosis. *J Cell Biol* 2001; **153**: 1369–1380.
30 Yeo JC, Wall AA, Luo L, Stow JL. Rab31 and APPL2 enhance FcγR-mediated phagocytosis through PI3K/Akt signaling in macrophages. *Mol Biol Cell* 2015; **26**: 952–965.
31 Casalou C, Seixas C, Portelinha A, Pintado P, Barros M, Ramalho JS *et al.* Arl13b and the non-muscle myosin heavy chain IIA are required for circular dorsal ruffle formation and cell migration. *J Cell Sci* 2014; **127**: 2709–2722.
32 Cantagrel V, Silhavy JL, Bielas SL, Swistun D, Marsh SE, Bertrand JY *et al.* Mutations in the cilia gene *ARL13B* lead to the classical form of Joubert syndrome. *Am J Human Genet* 2008; **83**: 170–179.
33 Barral DC, Garg S, Casalou C, Watts GFM, Sandoval JL, Ramalho JS *et al.* Arl13b regulates endocytic recycling traffic. *Proc Natl Acad Sci* 2012; **109**: 21354–21359.
34 O'Neill LAJ, Bowie AG. The family of five: TIR-domain-containing adaptors in toll-like receptor signalling. *Nat Rev Immunol* 2007; **7**: 353–364.
35 Barton GM, Kagan JC. A cell biological view of toll-like receptor function: regulation through compartmentalization. *Nat Rev Immunol* 2009; **9**: 535–542.
36 Patel PC, Harrison RE. Membrane ruffles capture C3bi-opsonized particles in activated macrophages. *Mol Biol Cell* 2008; **19**: 4628–4639.
37 Williams LM, Ridley AJ. Lipopolysaccharide induces actin reorganization and tyrosine phosphorylation of Pyk2 and Paxillin in monocytes and macrophages. *J Immunol* 2000; **164**: 2028–2036.
38 Luo L, Wall AA, Yeo JC, Condon ND, Norwood SJ, Schoenwaelder S *et al.* Rab8a interacts directly with PI3Kγ to modulate TLR4-driven PI3K and mTOR signalling. *Nat Commun* 2014; **5**: 4407.
39 Husebye H, Aune MH, Stenvik J, Samstad E, Skjeldal F, Halaas O *et al.* The Rab11a GTPase controls toll-like receptor 4-induced activation of interferon regulatory factor-3 on phagosomes. *Immunity* 2010; **33**: 583–596.
40 Murray RZ, Kay JG, Sangermani DG, Stow JL. A role for the phagosome in cytokine secretion. *Science* 2005; **310**: 1492–1495.
41 Kerr MC, Teasdale RD. Defining macropinocytosis. *Traffic* 2009; **10**: 364–371.
42 Lim JP, Gleeson PA. Macropinocytosis: an endocytic pathway for internalising large gulps. *Immunol Cell Biol* 2011; **89**: 836–843.
43 Bosedasgupta S, Pieters J. Inflammatory stimuli reprogram macrophage phagocytosis to macropinocytosis for the rapid elimination of pathogens. *PLoS Pathog* 2014; **10**: e1003879.
44 Murray RZ, Stow JL. Cytokine secretion and phagosome formation are spatially linked in macrophages. *Inflamm Res* 2005; **54**: S93–S93.

45 Egami Y, Taguchi T, Maekawa M, Arai H, Araki N. Small GTPases and phosphoinositides in the regulatory mechanisms of macropinosome formation and maturation. *Front Physiol* 2014; **5**: 374.
46 Fujii M, Kawai K, Egami Y, Araki N. Dissecting the roles of Rac1 activation and deactivation in macropinocytosis using microscopic photo-manipulation. *Sci Rep* 2013; **3**: 2385.
47 Maekawa M, Terasaka S, Mochizuki Y, Kawai K, Ikeda Y, Araki N *et al.* Sequential breakdown of 3-phosphorylated phosphoinositides is essential for the completion of macropinocytosis. *Proc Natl Acad Sci* 2014; **111**: E978–E987.
48 Yoshida S, Gaeta I, Pacitto R, Krienke L, Alge O, Gregorka B *et al.* Differential signaling during macropinocytosis in response to M-CSF and PMA in macrophages. *Front Physiol* 2015; **6**: 8.
49 Araki N, Johnson MT, Swanson JA. A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J Cell Biol* 1996; **135**: 1249–1260.
50 Araki N, Hatae T, Furukawa A, Swanson JA. Phosphoinositide-3-kinase-independent contractile activities associated with Fcγ-receptor-mediated phagocytosis and macropinocytosis in macrophages. *J Cell Sci* 2003; **116**: 247–257.
51 Lim JP, Gosavi P, Mintern JD, Ross EM, Gleeson PA. Sorting nexin 5 selectively regulates dorsal-ruffle-mediated macropinocytosis in primary macrophages. *J Cell Sci* 2015; **128**: 4407–4419.
52 Koivusalo M, Welch C, Hayashi H, Scott CC, Kim M, Alexander T *et al.* Amiloride inhibits macropinocytosis by lowering submembranous pH and preventing Rac1 and Cdc42 signaling. *J Cell Biol* 2010; **188**: 547–563.
53 Amyere M, Payrastre B, Krause U, Smissen PVD, Veithen A, Courtoy PJ. Constitutive macropinocytosis in oncogene-transformed fibroblasts depends on sequential permanent activation of phosphoinositide 3-kinase and phospholipase C. *Mol Biol Cell* 2000; **11**: 3453–3467.
54 Mellman I, Warren G. The road taken: past and future foundations of membrane traffic. *Cell* 2000; **100**: 99–112.
55 Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. *Nat Rev Immunol* 2012; **12**: 557–569.
56 Norbury CC, Hewlett LJ, Prescott AR, Shastri N, Watts C. Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. *Immunity* 1995; **3**: 783–791.
57 Kaplan IM, Wadia JS, Dowdy SF. Cationic TAT peptide transduction domain enters cells by macropinocytosis. *J Control Release* 2005; **102**: 247–253.
58 Iversen TG, Frerker N, Sandvig K. Uptake of ricinB-quantum dot nanoparticles by a macropinocytosis-like mechanism. *J Nanobiotechnology* 2012; **10**: 1–11.
59 Nakase I, Kobayashi NB, Takatani-Nakase T, Yoshida T. Active macropinocytosis induction by stimulation of epidermal growth factor receptor and oncogenic Ras expression potentiates cellular uptake efficacy of exosomes. *Sci Rep* 2015; **5**: 10300.
60 Maiuri MC, Grassia G, Platt AM, Carnuccio R, Ialenti A, Maffia P. Macrophage autophagy in atherosclerosis. *Mediators Inflamm* 2013; **2013**: 14.
61 Ly KT, Casanova JE. Mechanisms of *Salmonella* entry into host cells. *Cell Microbiol* 2007; **9**: 2103–2111.
62 Berenson CS, Kruzel RL, Eberhardt E, Sethi S. Phagocytic dysfunction of human alveolar macrophages and severity of chronic obstructive pulmonary disease. *J Infect Dis* 2013; **208**: 2036–2045.
63 Donnelly LE, Barnes PJ. Defective phagocytosis in airways disease. *Chest* 2012; **141**: 1055–1062.
64 Zeineddine R, Yerbury JJ. The role of macropinocytosis in the propagation of protein aggregation associated with neurodegenerative diseases. *Front Physiol* 2015; **6**: 277.
65 Dupuy AG, Caron E. Integrin-dependent phagocytosis – spreading from microadhesion to new concepts. *J Cell Sci* 2008; **121**: 1773–1783.
66 Yeung T, Grinstein S. Lipid signaling and the modulation of surface charge during phagocytosis. *Immunol Rev* 2007; **219**: 17–36.
67 Yeo JC, Wall AA, Luo L, Stow JL. Sequential recruitment of Rab GTPases during early stages of phagocytosis. *Cell Logist* 2016; **5**: e1140615.
68 March C, Cano V, Moranta D, Llobet E, Pérez-Gutiérrez C, Tomás JM *et al.* Role of bacterial surface structures on the interaction of *Klebsiella pneumoniae* with phagocytes. *PLoS ONE* 2013; **8**: e56847.
69 Bakowski MA, Braun V, Brumell JH. *Salmonella*-containing vacuoles: directing traffic and nesting to grow. *Traffic* 2008; **9**: 2022–2031.
70 Ohl ME, Miller SI. *Salmonella*: a model for bacterial pathogenesis. *Annu Rev Med* 2001; **52**: 259–274.
71 Zhou D, Galan J. *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. *Microbes Infect* 2001; **3**: 1293–1298.
72 Valdez Y, Ferreira RBR, Finlay BB. Molecular mechanisms of Salmonella virulence and host resistance. In: Sasakawa C (ed.), *Molecular Mechanisms of Bacterial Infection via the Gut*. Springer: Berlin, Heidelberg, 2009, pp C93–C127.
73 Galán JE. Molecular genetic bases of *Salmonella* entry into host cells. *Mol Microbiol* 1996; **20**: 263–271.
74 Popp D, Yamamoto A, Iwasa M, Nitanai Y, Maéda Y. Single molecule polymerization, annealing and bundling dynamics of SipA induced actin filaments. *Cell Motil Cytoskeleton* 2008; **65**: 165–177.
75 Wölke S, Ackermann N, Heesemann J. The *Yersinia enterocolitica* type 3 secretion system (T3SS) as toolbox for studying the cell biological effects of bacterial Rho GTPase modulating T3SS effector proteins. *Cell Microbiol* 2011; **13**: 1339–1357.
76 Timmerman MM, Shao JQ, Apicella MA. Ultrastructural analysis of the pathogenesis of *Neisseria gonorrhoeae* endometrial infection. *Cell Microbiol* 2005; **7**: 627–636.
77 King NP, Newton P, Schuelein R, Brown DL, Petru M, Zarsky V *et al.* Soluble NSF attachment protein receptor molecular mimicry by a *Legionella pneumophila* Dot/Icm effector. *Cell Microbiol* 2015; **17**: 767–784.
78 Hubber A, Roy CR. Modulation of host cell function by *Legionella pneumophila* type IV effectors. *Annu Rev Cell Dev Biol* 2010; **26**: 261–283.
79 Weissenböck H, Hubálek Z, Bakonyi T, Nowotny N. Zoonotic mosquito-borne flaviviruses: worldwide presence of agents with proven pathogenicity and potential candidates of future emerging diseases. *Vet Microbiol* 2010; **140**: 271–280.
80 Sakurai Y, Kolokoltsov AA, Chen C-C, Tidwell MW, Bauta WE, Klugbauer N *et al.* Two-pore channels control Ebola virus host cell entry and are drug targets for disease treatment. *Science* 2015; **347**: 995–998.
81 Sandgren KJ, Wilkinson J, Miranda-Saksena M, McInerney GM, Byth-Wilson K, Robinson PJ *et al.* A differential role for macropinocytosis in mediating entry of the two forms of vaccinia virus into dendritic cells. *PLoS Pathog* 2010; **6**: e1000866.
82 Mercer J, Helenius A. Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. *Science* 2008; **320**: 531–535.
83 Maréchal V, Prevost M-C, Petit C, Perret E, Heard J-M, Schwartz O. Human immunodeficiency virus type 1 entry into macrophages mediated by macropinocytosis. *J Virol* 2001; **75**: 11166–11177.
84 Yoshida S, Hoppe AD, Araki N, Swanson JA. Sequential signaling in plasma-membrane domains during macropinosome formation in macrophages. *J Cell Sci* 2009; **122**: 3250–3261.
85 Gould NS, Min E, Day BJ. Macropinocytosis of extracellular glutathione ameliorates tumor necrosis factor α release in activated macrophages. *PLoS ONE* 2011; **6**: e25704.