Murine norovirus-1 cell entry is mediated through a non-clathrin-, non-caveolae-, dynamin- and cholesterol-dependent pathway

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For many viruses, endocytosis and exposure to the low pH within acidic endosomes is essential for infection. It has previously been reported that feline calcivirus uses clathrin-mediated endocytosis for entry into mammalian cells. Here, we report that infection of RAW264.7 macrophages by the closely related murine norovirus-1 (MNV-1) does not require the clathrin pathway, as infection was not inhibited by expression of dominant-negative Eps15 or by knockdown of the adaptin-2 complex. Further, infection was not inhibited by reagents that raise endosomal pH. RAW264.7 macrophages were shown not to express caveolin, and flotillin depletion did not inhibit infection, suggesting that caveolae and the flotillin pathway are not required for cell entry. However, MNV-1 infection was inhibited by methyl-β-cyclodextrin and the dynamin inhibitor, dynasore. Addition of these drugs to the cells after a period of virus internalization did not inhibit infection, suggesting the involvement of cholesterol-sensitive lipid rafts and dynamin in the entry mechanism. Macropinocytosis (MPC) was shown to be active in RAW264.7 macrophages (as indicated by uptake of dextran) and could be blocked by 5-(N-ethyl-N-isopropyl) amiloride (EIPA), which is reported to inhibit this pathway. However, infection was enhanced in the presence of EIPA. Similarly, actin disruption, which also inhibits MPC, resulted in enhanced infection. These results suggest that MPC could contribute to virus degradation or that inhibition of MPC could lead to the upregulation of other endocytic pathways of virus uptake.

INTRODUCTION

The family Caliciviridae is divided into four genera: Vesivirus, Lagovirus, Sapovirus and Norovirus. The human noroviruses are the most common cause of acute viral gastroenteritis, especially in industrialized countries (Lopman et al., 2003); there is currently no treatment or vaccine for these viruses. In addition, there is still no routine tissue culture system for the propagation of human noroviruses, but the recent discovery of murine norovirus-1 (MNV-1) has provided an efficient model system for the study of norovirus replication, as this virus replicates efficiently in murine macrophages and dendritic cells (DCs) (Karst et al., 2003; Wobus et al., 2004). MNV-1 is highly prevalent in laboratory mice and has been shown to be lethal to mice with impaired innate immunity (Hsu et al., 2006; Karst et al., 2003; Muller et al., 2007).

Noroviruses are non-enveloped and contain a single-stranded RNA genome of about 7.3 kb, which is linked to the viral VPg protein at the 5’ end and is polyadenylated at the 3’ end (Karst et al., 2003; Wobus et al., 2004). The genome is organized into four open reading frames (ORF-1–4). ORF-1 encodes the non-structural proteins, whereas ORF-2 and ORF-3 encode structural proteins (Sosnovtsev et al., 2006). ORF-4 was recently identified within the MNV-1 genome and encodes a single protein of unknown function (Thackray et al., 2007).

For many viruses, infection requires virus uptake by endocytosis. A number of different endocytic pathways have been identified in mammalian cells. Broadly, these are divided into clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIDE) pathways that include caveolin-dependent endocytosis (or caveola-mediated endocytosis) and a number of caveolin-independent pathways such as macropinocytosis (MPC), and the flotillin-dependent and CLIC/GEEC (clathrin-independent cargos/GPI-AP-enriched early endosomal compartment) pathways (Glebov et al., 2006; Marsh & Helenius, 2006; Miaczynska & Stenmark, 2008). CIDE pathways show different requirements...
for cellular proteins such as dynamin, flotillin and small GTPases (Glebov et al., 2006; Marsh & Helenius, 2006; Miaczynska & Stenmark, 2008). Although each endocytic pathway was originally thought of as being distinct, it is now clear that they can overlap considerably and vesicles derived from CIDE can fuse with vesicles derived from the clathrin-dependent pathway (Doherty & McMahon, 2009).

Viruses can exploit CME and CIDE for entry into cells (Marsh & Helenius, 2006). Further, a number of viruses have

Fig. 1. MNV-1 entry is clathrin-independent. (a) RAW264.7 cells were transiently transfected to express control Eps15 (DIIIΔ2) or DN-Eps15 (EΔ95/295 Esp15) as fusions with eGFP. Transfected cells were identified by eGFP expression. At 12 h post-transfection, cells were infected with MNV-1 (m.o.i. ~2) for 12 h. Infected cells were quantified by confocal microscopy using an antibody to the viral NS7 polymerase protein. Results are shown as the level of infection of the DN-Eps15-expressing cells normalized to the level of infection of the cells expressing the control protein. (b) RAW264.7 cells were transfected to express the control or DN-Eps15 as described in (a). At 12 h post-transfection, Alexa-568-conjugated transferrin (red) was internalized for 10 min and then chased for 20 min in the absence of transferrin. The nuclei were stained (blue) with TO-PRO-3 iodide. Bar, 10 μm. (c) Transferrin internalization was quantified by confocal microscopy. Results are shown as the level of transferrin uptake by the DN-Eps15-expressing cells normalized to the level of uptake by the cells expressing the control protein. (d) RAW264.7 cells were transfected with siRNA targeted against AP-2 or a control siRNA against GFP. At 48 h post-transfection, Alexa-568-conjugated transferrin (red) was internalized for 10 min and then chased for 20 min in the absence of transferrin. The nuclei were stained (blue) with TO-PRO-3 iodide. Bar, 10 μm. (e) RAW264.7 cells were transfected with siRNA targeted against AP-2 or siRNA against GFP and at 48 h post-transfection, cell lysates were analysed by SDS-PAGE and immunoblotting with AP-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antisera. (f) At 48 h post-transfection with the AP-2 and GFP siRNAs, cells were infected with MNV-1 (m.o.i. ~2) for 12 h and infected cells quantified as above. Results are shown as the level of infection of the AP-2 siRNA-transfected cells normalized to the level of infection of the cells transfected with the control GFP siRNA. (g) The siRNA-transfected cells were quantified for transferrin internalization by confocal microscopy. Results are shown as the level of transferrin uptake by the AP-2 siRNA-transfected cells normalized to the level of uptake by the cells transfected with the control siRNA. Results shown in (a), (c), (f) and (g) are means ± SEM of three independent experiments.
been shown to utilize more than one pathway for internalization. For example, influenza virus has been shown to utilize both a CME and a CIDE pathway for infection (Rust et al., 2004) and simian virus 40 can use at least two distinct lipid-raft-mediated endocytic pathways for uptake (Damm et al., 2005; Pelkmans et al., 2001, 2002).

Currently, little is known of the mechanisms used by noroviruses to bind and enter cells. Recently, it was shown that feline calicivirus (FCV) uses junctional adhesion molecule A as an attachment receptor (Makino et al., 2006). In addition, FCV has been shown to bind to 2,6-sialic acid (Stuart & Brown, 2007), which could serve as a co-receptor for infection. Also, FCV is known to be internalized by CME as infection is inhibited by expression of dominant-negative (DN) mutants of proteins (Eps15 and Rab5) that are normally involved in CME (Stuart & Brown, 2006).

MNV-1 replicates efficiently in macrophages and DCs (Wobus et al., 2004) and recently sialic acid was identified as a cellular receptor for MNV-1 on the mouse macrophage cell line, RAW264.7 cells (Taubé et al., 2009). However, in contrast to FCV, a recent report (Perry et al., 2009) showed that MNV-1 infection of RAW264.7 cells and primary DCs is pH-independent, suggesting that the entry pathway is clathrin-independent. Here, we have investigated the cell entry mechanism used by MNV-1 to infect RAW264.7 cells. Our results suggest that MNV-1 entry can occur via a pathway that is clathrin- and caveolae-independent but requires dynamin and cholesterol.

RESULTS

MNV-1 infection of RAW 264.7 cells is clathrin-independent

As FCV is closely related to MNV-1 and has been shown to utilize CME for entry into cultured cells, we determined if MNV-1 also utilizes this same pathway to infect RAW264.7 cells. Eps15 is a molecular scaffold protein that associates with both the adaptin-2 (AP-2) adaptor protein complex (Benmerah et al., 1995, 1996; Iannolo et al., 1997) and epsin 1 (Chen et al., 1998), and is required for CME of transferrin. RAW264.7 cells were transfected to express a DN form of Eps15 that is known to inhibit CME (DN-Eps15 EA95/295; Benmerah et al., 1999), or a control form of Eps15 (DIIIΔ2) that lacks one of the AP-2-binding sites and does not interfere with the clathrin pathway. The transfected cells were identified by confocal microscopy via an enhanced green fluorescent protein (eGFP) tag on the Eps15 proteins (shown in green in Fig. 1). At 12 h post-transfection, the cells were infected with MNV-1 at a low m.o.i. (~2) to favour virus uptake via the most efficient entry pathway. After 12 h, the cells were fixed and labelled using antisera to the viral NS7 polymerase protein to identify infected cells. The Eps15-expressing cells (green) were scored for infection (red) and the number of infected cells in the DN-Eps15-expressing cell population normalized to the level of infection of the cells expressing the control protein (DIIIΔ2). This showed that expression of the DN-Eps15 did not appear to inhibit MNV-1 infection (Fig. 1a), suggesting that CME is not the entry route used by MNV-1 to infect RAW264.7 cells. To confirm that expression of DN-Eps15 inhibited CME, transfected RAW264.7 cells were also incubated with Alexa-labelled transferrin, a commonly used marker for CME (Hinrichsen et al., 2003). As expected, expression of the control Eps15 DIIIΔ2 protein had no effect on transferrin (red) uptake, whereas cells expressing DN-Eps15 showed reduced transferrin uptake (Fig. 1b, c).

To confirm that CME is not required for MNV-1 infection, RAW264.7 cells were transfected with small interfering RNA (siRNA) targeted to the AP-2 adaptor complex. AP-2 is one of the major components of clathrin-coat assembly and plays a central role in formation of clathrin-coated pits (Mills, 2007). Following transfection, immunoblotting was performed to confirm a knockdown of AP-2 (Fig. 1e). Transfection with AP-2 siRNA also inhibited transferrin uptake, confirming that CME was inhibited (Fig. 1d, g). siRNA-transfected cells were also infected with MNV-1 and infection was scored by confocal microscopy, as described above. Although AP-2 levels were greatly reduced and CME was inhibited, knockdown of AP-2 expression had no effect on MNV-1 infection (Fig. 1f). Taken together, these results strongly suggest that MNV-1 does not use CME as the major entry route of RAW 264.7 cells.

Inhibition of endosomal acidification does not affect MNV-1 infection

A recent study has suggested that MNV-1 infection does not require endosome acidification (Perry et al., 2009). To confirm this, we investigated the effect of concanamycin A on MNV-1 infection. Concanamycin A is a potent and specific inhibitor of the vacuolar proton ATPase (Huss et al., 2002) and is commonly used to raise the pH within endosomes. RAW264.7 cells were treated with concanamycin A (or with DMSO as control) for 0.5 h prior to MNV-1 infection for 1 h. The cells were washed and incubated for a further 11 h, and infection quantified using an enzyme-linked immunospot (ELISPOT) assay (Fig. 2a) (Berryman et al., 2005). Treatment of cells with concanamycin A had no effect on MNV-1 infection (Fig. 2b), confirming that MNV-1 infection does not require virus exposure to the low pH within acidic endosomes.

Depletion of cellular cholesterol inhibits MNV-1 endocytosis

Cholesterol depletion affects a number of endocytic pathways, including caveolin-dependent endocytosis (Pelkmans et al., 2001; Smith et al., 2003), as well as other
lipid-raft-mediated pathways (Damm et al., 2005; Vidricaire & Tremblay, 2007). Methyl-β-cyclodextrin (MβCD) depletes cholesterol from the plasma membrane and disrupts lipid rafts and endocytic pathways that involve these structures. RAW264.7 cells were pretreated with MβCD (or DMSO) for 0.5 h and then infected with MNV-1 in the presence or absence (no-drug control) of the drug for 1 h. The virus inoculum and drug were removed by washing and infection continued at 37 °C for a further 11 h, before quantification using the ELISPOT assay. Treatment of cells with MβCD decreased MNV-1 infection by approximately 50% (Fig. 3a). To confirm that the drug affected only an early step in infection, and not subsequent virus replication, the drug was also added immediately after, or 1.5 h after the virus inoculum was removed. Under these conditions, no effect on infection was observed (Fig. 3a), indicating that MβCD inhibited only the cell entry process and not subsequent steps in the replication cycle.

To ensure that MβCD did not inactivate the virus itself, virus was incubated with MβCD or DMSO (control) and residual infectivity measured by 50% tissue culture infective dose (TCID₅₀). This analysis showed that MβCD treatment had no effect on MNV-1 infectivity for RAW264.7 cells (data not shown). Addition of soluble cholesterol to RAW264.7 cells after MβCD treatment reversed the inhibitory effect of MβCD on infection (Fig. 3b), further suggesting cholesterol is required for MNV-1 entry.

To confirm that MβCD treatment resulted in inhibition of lipid-raft-dependent endocytosis, we analysed the effect of the drug on cholera toxin B (CTB) uptake. CTB is internalized in a lipid-raft-dependent manner after binding to its receptor GM1 ganglioside (Fujinaga et al., 2003). CTB internalization was blocked when RAW264.7 cells were treated with MβCD, confirming that lipid-raft-dependent endocytosis was inhibited (Fig. 3c). Furthermore, as severe cholesterol depletion has been shown to also inhibit CME (Vela et al., 2007), we confirmed that MβCD treatment did not inhibit uptake of Alexa-568 transferrin (data not shown). These data suggest that MNV-1 infection is cholesterol-sensitive and likely to be mediated by a lipid-raft-dependent pathway.

**Caveolin-1 is not expressed in RAW 264.7 cells**

The above results show that MNV-1 infection is sensitive to cholesterol depletion and may therefore be mediated via lipid rafts. Caveolae-mediated endocytosis is initiated at lipid rafts and inhibited by cholesterol depletion. Therefore, we investigated the role of caveolae in MNV-1 infection. Caveolae formation requires caveolin and therefore caveolae (Cameron et al., 1997; Fra et al., 1994; Gorodinsky & Harris, 1995; Lyden et al., 2002). In order to confirm the absence of caveolin-1 in our RAW264.7 cells, we subjected RAW264.7 cell lysates to immunoblotting. Fig. 3(d) shows that caveolin was detected in HEK293 cells, but not in RAW 264.7 cells (Fig. 3d). This is entirely consistent with previous reports that RAW264.7 cells lack this protein. Thus, the MNV-1 entry pathway in RAW264.7 cells is not dependent on caveolae.

**MNV-1 endocytosis is dynamin-dependent**

Dynamin-2 is a GTPase that mediates vesicle fission from the plasma membrane and is required for both CME and caveolae-mediated endocytosis (Damke et al., 1994; Henley et al., 1998; Oh et al., 1998). To assess if dynamin is involved in MNV-1 infection, we used dynasore, a
small-molecule inhibitor of dynamin (Macia et al., 2006). RAW264.7 cells were treated with dynasore (or DMSO) for 0.5 h, prior to MNV-1 infection. The virus inoculum was removed and the cells were incubated at 37 °C for a further 11 h in the presence or absence (control) of dynasore (as the effects of dynasore are rapidly reversible). The cells were then fixed and infection quantified using the ELISPOT assay. Treatment of RAW264.7 cells with dynasore inhibited infection by 85% (Fig. 4a). Addition of the drug immediately after, or 1.5 h after, the virus inoculum was removed, had no effect on infection, suggesting that the drug only affected entry and not a subsequent intracellular replication step. We also confirmed that dynasore did not inactivate virus in solution using the same approach as described for MJ/CD above (data not shown). As the drug remained present throughout the entire assay, we also assessed any cytotoxic effects on the cells. This analysis showed that only ~5% of cells displayed signs of cytotoxicity after 12 h of treatment with dynasore (data not shown). In order to confirm that dynasore inhibited dynamin-dependent endocytosis, we analysed the effect of the drug on uptake of Alexa-labelled transferrin. As expected, transferrin uptake was inhibited by dynasore (data not shown). These results suggest that dynamin is required for MNV-1 infection of RAW264.7 cells.

Microtubules are involved in MNV-1 infection

Nocodazole interferes with microtubule function and vesicular trafficking through the endosomal pathway (D’Hondt et al., 2000). RAW264.7 cells were treated with nocodazole (or DMSO) for 0.5 h prior to MNV-1 infection and infection was quantified using the ELISPOT assay. Nocodazole treatment inhibited MNV-1 infection by 40–50% (Fig. 4b), suggesting that an early stage of MNV-1 infection requires intact microtubules. Addition of the
drug immediately after the virus inoculum was removed also had a small inhibitory effect on infection, suggesting that microtubules may also be involved in trafficking of the virus post entry. At the concentrations used, nocodazole was shown to disrupt the microtubules, as revealed by indirect immunofluorescence confocal microscopy using anti-tubulin antisera (data not shown).

**Blocking MPC enhances MNV-1 infection**

MPC is used by a number of viruses for infectious entry (Mercer & Helenius, 2008). To determine if MPC is active in RAW264.7 cells, we used uptake of Alexa-labelled dextran (Dharmawardhane et al., 2000). Fig. 5(b) shows that dextran was internalized by RAW264.7 cells, indicating that MPC was active. Next, we investigated the effect of 5-(N-ethyl-N-isopropyl) amiloride (EIPA) on infection. EIPA is an analogue of amiloride and inhibits Na⁺/H⁺ exchangers and MPC without affecting other endocytic pathways such as CME, or caveolae-mediated endocytosis (West et al., 1989). We first confirmed that EIPA blocked MPC in RAW264.7 cells by showing that treatment of the cells with this drug inhibited dextran uptake (Fig. 5b). However, we found that EIPA had an unexpected effect on MNV-1 infection, as treatment of cells with EIPA resulted in enhanced MNV-1 infection (by 47% at 25 μM and 67% at 50 μM) (Fig. 5a). Actin filaments are important for MPC, therefore we investigated the effect of actin disruption on MNV-1 infection using cytochalasin D, which prevents actin polymerization and disrupts the actin cytoskeleton (Brenner & Korn, 1980). Consistent with the effect of EIPA, cytochalasin D treatment of RAW264.7 cells also enhanced MNV-1 infection (Fig. 5c) by about 60%. At the concentrations used, cytochalasin D was shown to disrupt actin filaments, as revealed by indirect immunofluorescence confocal microscopy using Alexa-conjugated phalloidin (Fig. 5d).

**Depletion of flotillin-1 does not inhibit MNV-1 internalization**

We also analysed the effect of flotillin-1 depletion on MNV-1 infection. Flotillin-1 is associated with lipid rafts and was recently identified as a component of a novel CIDE pathway (Glebov et al., 2006; Lang et al., 1998; Volonte et al., 1999). RAW264.7 cells were transfected with siRNA against flotillin-1 and inhibition of flotillin-1 expression confirmed by immunoblotting (Fig. 6a); no effect on flotillin-1 expression was observed when compared with cells transfected with a control siRNA targeted to GFP. This knockdown of flotillin-1 had no effect on MNV-1 infection (Fig. 6b), suggesting that the flotillin-1-dependent pathway is not involved in MNV-1 entry.

**DISCUSSION**

Viruses have been shown to utilize a number of different endocytic pathways to enter and infect cells. CME would appear to be the most commonly used, but it is increasingly clear that a number of CIDE pathways are also used by several different viruses. It has recently been shown that entry of MNV-1 into RAW264.7 cells is pH-independent (Perry et al., 2009). In this study, we have further examined the entry route used by MNV-1 to infect RAW264.7 cells. Initially, we investigated if infection by MNV-1 is dependent on CME, since FCV (which is closely related to MNV-1) is internalized via this pathway (Stuart & Brown, 2006). Our experiments show that DN Eps-15 and AP-2 knockdown inhibited transferrin uptake but had no
Effect on MNV-1 infection, strongly suggesting that the mechanism of MNV-1 entry is clathrin-independent. Similarly, concanamycin A treatment confirmed that a low pH within endosomes is not required for MNV-1 infection, further supporting the conclusion that a clathrin-independent pathway is most likely required for MNV-1 entry.

A number of CIDE pathways originate from lipid rafts. These pathways require cholesterol and can be inhibited by the cholesterol-depleting agent MβCD. We found that treatment of RAW264.7 cells with MβCD inhibited MNV-1 infection, but only when the drug was added during virus entry and not when added after virus uptake. Repletion of cholesterol after MβCD treatment restored infection, further suggesting that cholesterol is required for MNV-1 infection.

Our data suggest that MNV-1 entry into RAW264.7 cells is cholesterol- and hence lipid-raft-dependent. Caveolae-dependent endocytosis also requires intact lipid rafts and hence, is normally inhibited by cholesterol depletion (Murata et al., 1995). Caveolin is a key component of caveolae (Morrow & Parton, 2005); however, a number of previous studies have shown that RAW264.7 cells lack caveolin (Cameron et al., 1997;Fra et al., 1994; Gorodinsky & Harris, 1995; Lyden et al., 2002) and we have confirmed these observations in our RAW264.7 cells. These data do not rule out the possibility that MNV-1 infection may occur through a caveolae-dependent pathway in other cell types that express caveolin. The recently described flotillin-dependent pathway is also raft-associated (Glebov et al., 2006) and may be inhibited by cholesterol depletion; however, our results showed that flotillin-1 depletion had no effect on MNV-1 infection, suggesting that this pathway is also not used for MNV-1 infection.

The role of dynamin in both CME and caveolae-dependent endocytosis is well established, whereas its role in other endocytic pathways is less clear (Marsh & Helenius, 2006; Miaczynska & Stenmark, 2008). Here, we show that inhibition of dynamin using dynasore decreased MNV-1 infection of RAW264.7 cells, but only when added at the entry stage of infection, indicating that MNV-1 entry is dynamin-dependent.

Treatment of RAW.264.7 cells with nocodazole (which disrupts microtubules) also inhibited MNV-1 infection. However, at this stage we cannot be certain if microtubules are required for entry or for post-entry virus trafficking as
Murine norovirus-1 cell entry

Recently, a number of viruses have been shown to use MPC, or MPC-like entry pathways for infection (reviewed by Mercer & Helenius, 2009). MPC is especially active in specialized antigen-presenting cells, such as macrophages and DCs. Therefore, we also investigated a role for MPC in MNV-1 infection. We established that MPC was active in RAW264.7 cells but surprisingly, inhibition of MPC, by either EIPA or cytochalasin D, resulted in an increase in MNV-1 infection. At present, we do not know why inhibiting MPC should lead to an increase in infection. A possible explanation is that MPC is responsible for a large proportion of fluid-phase uptake and inhibition of MPC could result in upregulation of other endocytic pathways (by way of compensation) that could then be used for MNV-1 uptake. Alternatively, it is possible that a proportion of virus enters RAW264.7 cells by MPC and is delivered to lysosomes for destruction. If this is the case then inhibition of MPC may lead to enhanced infection. However, further studies are needed to understand this phenomenon.

In conclusion, we have presented evidence that infection of RAW264.7 cells by MNV-1 is mediated by a clathrin-, caveolae-, flotillin- and pH-independent pathway that requires intact lipid rafts, dynamin and microtubules. Together these studies suggest that different members of the calicivirus family may utilize different cell entry pathways for infection.

**METHODS**

**Cell culture and viruses.** RAW264.7 and HEK293 cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) and modified Eagle’s medium, respectively, supplemented with 10 % fetal bovine serum, non-essential amino acids (1 %), penicillin (100 U ml⁻¹; Invitrogen) and streptomycin (100 mg ml⁻¹; Gibco-BRL) at 37 °C with 5 % CO₂. MNV-1 (strain CW.1) was a gift of Professor Herbert Virgin (Washington University, St Louis, MO, USA) and was propagated in RAW264.7 cells. The virus titre was determined by TCID₅₀ on RAW264.7 cells.

**Antibodies and reagents.** The anti-α-tubulin antisera (DM1A) was from Sigma. Anti-flotillin-1 antiserum (clone 18) was from BD Biosciences. Anti-adaptin-2 (AP-2) monoclonal antibody (sc-55497) and polyclonal anti-caveolin-1 sera (sc-894) were from Santa Cruz Biotechnology. The anti-GAPDH polyclonal antibody (6C5) was from Ambion. The anti-MNV NS7 polymerase polyclonal serum was a gift of Dr Ian Goodfellow (Imperial College, London, UK). Alexa-568 transferrin, Alexa-555 cholera toxin B (CTB) and Alexa-555 dextran were all from Invitrogen, as were the Alexa-Fluor-conjugated secondary antibodies. EIPA, concanamycin A, cytochalasin D, nocodazole, dynasore and MβCD and water-soluble cholesterol (C4951) were from Sigma. Stock solutions of concanamycin A, EIPA, cytochalasin D, dynasore and nocodazole were prepared in DMSO. A stock solution of MβCD was prepared in DMEM. Where appropriate, an equivalent dilution of DMSO was included as the control treatment.

**Quantification of virus infection assays.** To quantify MNV-1 infection, a modification of an ELISPOT assay was used (Berryman et al., 2005). Briefly, 3 × 10⁵ cells were seeded per well in 96-well tissue-culture plates and grown overnight until approximately 80 % confluent. The cells were incubated for 1 h at 37 °C with MNV-1 at an m.o.i. of ~2 p.f.u. per cell. The cells were washed to remove excess virus and incubated in growth medium at 37 °C for a further 11 h. Cells were fixed by the addition of cold 4 % paraformaldehyde (PFA; Sigma) in PBS for 1 h. The cells were then permeabilized with 0.1 % Triton X-100 in PBS for 15 min. Following incubation for 0.5 h in blocking buffer (0.5 % BSA in PBS), the cells were incubated with anti-MNV NS7 polymerase antiserum (1:1500) for 1 h at room temperature. The cells were washed again and incubated with a biotinylated goat anti-rabbit IgG antiserum (1:400; Southern
Biotechnologies) followed by a streptavidin-conjugated alkaline phosphatase (1:1000; Caltag Laboratories) in blocking buffer for 1 h at room temperature. The alkaline phosphatase substrate (Bio-Rad) was added for 10 min, according to the manufacturer’s instructions. The infected cells stained dark blue and were quantified by using an ELISPOT plate reader (Zeiss KS ELISpot). Non-specific labelling was determined by performing the assay on mock-infected cells.

To determine the effect of pharmacological inhibitors of endocytosis on MNV-1 infection, cells were: (i) pretreated with the drug for 0.5 h prior to infection with MNV-1 for 1 h, also in the presence of the drug; or (ii) treated with the drug for 1.5 h immediately after the virus inoculum was removed; or (iii) the drug was added 1.5 h after the virus inoculum was removed. In the case of the dynasore, the drug was present throughout the assay.

To control for effects of the inhibitors on virus in solution, virus was incubated with DMSO alone. Drug-treated and control viruses were then titrated by TCID<sub>50</sub> assay as described previously (Bailey et al., 2008).

Following MβCD treatment, cells were treated with 400 μg water-soluble cholesterol ml<sup>-1</sup> for 15 min at room temperature, before removing the medium, to replete the cholesterol in the plasma membrane. Cells were subsequently infected with MNV-1 as described above and virus infection quantified by confocal microscopy.


**Immunoblotting.** Cell pellets were resuspended in radioimmuno-precipitation buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM PMSF) and centrifuged at 14 000 r.p.m. (Mikro 22 R) for 5 min at 4 °C. Lysates were subjected to SDS-PAGE (12%) and proteins transferred to nitrocellulose membranes. Membranes were probed with the indicated primary antibodies followed by horseradish peroxidase-conjugated species-specific secondary antibodies (1:2000; Dako). Proteins were visualized by chemiluminescence (Pierce).

**Statistical analysis.** GraphPad prism 5 (GraphPad) was used to perform the statistical analysis. One way ANOVA with Dunnett post-hoc test was used to evaluate the differences between treatments. Significance was determined by a P-value of <0.05, and significance is indicated in each figure.

**ACKNOWLEDGEMENTS**

We thank Professor Herbert Virgin (Washington University, St Louis, MO, USA) for MNV-1 and Dr Stephen Berryman (IAH, Pirbright, UK) for technical help with the ELISPOT assay.

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