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Select membrane proteins modulate MNV-1 infection of macrophages and dendritic cells in a cell type-specific manner

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Abstract

Noroviruses cause gastroenteritis in humans and other animals, are shed in the feces, and spread through the fecal-oral route. Host cellular expression of attachment and entry receptors for noroviruses is thought to be a key determinant of cell tropism and the strict species-specificity. However, to date, only carbohydrates have been identified as attachment receptors for noroviruses. Thus, we investigated whether host cellular proteins play a role during the early steps of norovirus infection. We used murine norovirus (MNV) as a representative norovirus, since MNV grows well in tissue culture and is a frequently used model to study basic aspects of norovirus biology. Virus overlay protein binding assay followed by tandem mass spectrometry analysis was performed in two permissive cell lines, RAW264.7 (murine macrophages) and SRDC (murine dendritic cells) to identify four cellular membrane proteins as candidates. Loss-of-function studies revealed that CD36 and CD44 promoted MNV-1 binding to primary dendritic cells, while CD98 heavy chain (CD98) and transferrin receptor 1 (TfRc) facilitated MNV-1 binding to RAW 264.7 cells. Furthermore, the VP1 protruding domain of MNV-1 interacted directly with the extracellular domains of recombinant murine CD36, CD98 and TfRc by ELISA. Additionally, MNV-1 infection of RAW 264.7 cells was enhanced by soluble rCD98 extracellular domain. These studies demonstrate that multiple membrane proteins can promote efficient MNV-1 infection in a cell type-specific manner. Future studies are needed to determine the molecular mechanisms by which each of these proteins affect the MNV-1 infectious cycle.

Keywords

Norovirus; infection; CD36; CD44; CD98 heavy chain; transferrin receptor 1

1. Introduction

Noroviruses belong to the *Norovirus* genus in the *Caliciviridae* family. They are small, non-enveloped, single-stranded, positive-sense RNA viruses that cause gastroenteritis in humans and other animals (Green, 2013). Human noroviruses are the main cause of acute

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gastroenteritis worldwide, infecting people of all ages (Koo et al., 2010). In the US, these viruses are estimated to cause 19–21 million cases of illness with an estimated cost of \$2 billion/year (Belliot et al., 2014; Hall et al., 2013). Despite the major impact on human health and the economy, little is known about the early events of norovirus infection. The study of human noroviruses in a laboratory setting still remains challenging, and basic aspects of the norovirus infectious cycle, such as cellular tropism and receptor usage during virus infection, have not been studied extensively. Murine norovirus (MNV) is the only member of the *Norovirus* genus that replicates efficiently in cell culture, and it does so in murine macrophages and dendritic cells (Wobus et al., 2004). Like its human counterpart, MNV is an enteric pathogen transmitted by the fecal-oral route. Thus, it is widely used as a model system to study diverse aspects of norovirus biology (Karst and Wobus, 2015; Wobus et al., 2006).

Viruses often use multiple receptors for attachment and internalization (Grove and Marsh, 2011; Mercer et al., 2010; Smith and Helenius, 2004). The identity and distribution of these receptors can determine the extent to which a given virus infects specific cell types, tissues, and hosts (Smith and Helenius, 2004). Noroviruses use carbohydrates as attachment receptors (Karst and Wobus, 2015). Specifically, MNV-1 uses terminal sialic acid moieties on the ganglioside GDI a, N- and/or O-linked glycoproteins as attachment receptors in primary and cultured macrophages (Taube et al., 2012; Taube et al., 2009). However, expression of these sugar moieties on the cell surface is not sufficient for productive infection (Taube et al., 2009). Receptor binding is mediated by the major capsid protein VP1. VP1 is divided into an N-terminal arm, shell (S) and C-terminal protruding (P) domains (Katpally et al., 2010; Prasad, 1999). The P domain (Pd) forms the most exposed region of the capsid and is the least conserved among noroviruses. The MNV Pd contains residues important for carbohydrate binding and escape from neutralizing antibodies (Kolawole et al., 2014; Taube et al., 2012). Following binding, MNV-1 is internalized by a dynamin II- and cholesterol-dependent mechanism (Gerondopoulos et al., 2010; Perry and Wobus, 2010).

The goal of the current study was to expand our knowledge regarding the role of host cellular proteins during early steps of MNV infection *in vitro*. Fifty-five proteins were identified as putative MNV-1 interacting proteins by virus overlay protein binding assay (VOPBA) followed by tandem mass spectrometry analysis in both macrophage- and dendritic cell-like cell lines (RAW 264.7 and SRDC, respectively). Four putative MNV-1-interacting proteins were further investigated for their role during MNV-1 infection. Loss-of-function studies demonstrated that CD36 and CD44 are involved in MNV-1 binding to primary dendritic cells, while CD98 heavy chain (CD98) and transferrin receptor 1 (TfRc) are involved in MNV-1 binding to RAW 264.7 cells. MNV-1 bound directly to the extracellular domain of recombinant murine CD36, CD98 and TfRc via its VP1 protruding domain. Moreover, soluble CD98 enhanced MNV-1 infectivity in RAW 264.7 cells. This study broadens our understanding of host cell proteins promoting the initial events in MNV infection, an important step in viral pathogenesis.

2. Material and methods

2.1 Cell culture and mice

RAW 264.7 cells were purchased from ATCC. SRDC cells were a kind gift from Daniel Bout (University of Tours, France). Cells were maintained as previously described (Ruiz et al., 2005). Six- to eight-week old CD44 knockout (005085), CD36 knockout (019006) and matched control mice (C57BL/6J, 000664) were purchased from Jackson Laboratories. Bone marrow-derived macrophages (BMM) and dendritic cells (BMDC) were isolated as previously described (Wobus et al., 2004). All animal procedures were performed in compliance with University of Michigan and federal guidelines and the standards of the NIH Guide for the Care and Use of Laboratory Animals (2011).

2.2. Virus stocks

The plaque-purified MNV-1 clone CW3 (GV/MNV-1/2002/USA) was used at passage 6 for all experiments (Thackray et al., 2007).

2.2. VOPBA and protein identification by tandem mass spectrometry

Isolation of cellular membrane proteins was carried out as previously described (Gaggar et al., 2007). Cellular proteins extract were mixed with Laemmli buffer containing β -mercaptoethanol (SIGMA-ALDRICH), heated for 3 minutes at 95°C, and resolved by SDS-PAGE. Next, proteins were transferred to nitrocellulose membranes (Bio-Rad) and blocked with 5% non-fat dry milk in wash solution (TBS with 0.05% Tween-20) for 2 h at room temperature. Putative MNV-1-interacting proteins were identified by VOPBA as described previously (Gonzalez-Reyes et al., 2009) with minor modifications. Nitrocellulose membranes were incubated with MNV-1 infected cell lysate (2×10^8 PFU) or an equivalent volume of mock-infected lysate overnight at 4°C. Membranes were washed and incubated with an anti-MNV virus-like particle rabbit serum (Wobus et al., 2004) for 2 h at room temperature, followed by a 1 h incubation with a peroxidase-conjugated secondary goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Reactive bands were visualized using Super Signal West Pico Chemiluminescent Substrate (ThermoFisher Scientific) and exposing the membranes to X-ray film (Kodak). Coomassie blue stained gels were performed in parallel to VOPBA and corresponding bands were excised, trypsin digested, and analyzed at the Proteomics Resource Facility (Department of Pathology, University of Michigan) by tandem mass spectrometry.

2.3. Binding assays and growth curves

Binding assays were carried out as previously described (Taube et al., 2009) with minor modifications: 5×10^5 BMM, BMDC, RAW 264.7 and SRDC cells were infected in suspension with MNV-1 (MOI of 2) for 1 h on ice in 500 μ L of medium. Unbound virus was removed by washing cells with ice-cold PBS. Cells were resuspended in 140 μ L of PBS and frozen at -80°C prior to RNA extraction with the QIAmp viral RNA mini kit (QIAGEN), according to the manufacturer's recommendations. Bound virus genome titers were determined by RT-qPCR (Taube et al., 2009), and binding to control cells was set to 100%. For growth curves, 2×10^5 BMM and BMDC were infected in suspension with MNV-1

(MOI of 0.05) for 1 h on ice in 500 μ L of medium. Unbound virus was removed by washing cells with ice-cold PBS. Cells were resuspended in 1 mL of medium, and the infection was allowed to proceed until the indicated time points, when cells were freeze-thawed twice, and viral titers were determined by plaque assay (Gonzalez-Hernandez et al., 2012). RAW 264.7 and SRDC cells (5×10^5) were infected as described for primary cells. Viral titers were determined by one step RT-qPCR as described previously (Taube et al., 2009) and the titer of control cells was set to 100%.

2.4. siRNA knockdown

siRNA transfections were carried out according to manufacturer's instructions (Dharmacon). Briefly, RAW 264.7 and SRDC cells were plated at a density of 1×10^5 cells in a 12-well plate and allowed to attach overnight. The following day, cells were transfected with a pool of 4 siRNAs (ON-TARGETplus, Dharmacon) targeting murine CD98 heavy chain (CD98), murine transferrin receptor 1 (TfRc), or a non-targeting (NT) siRNA control pool at a final concentration of 50 nM using 2 μ L of Dharmafect 4 (Dharmacon). Cells were incubated for 48 h, resuspended in culture medium, and infected as described above (section 2.3). In parallel, transfected cells were analyzed by flow cytometry to verify protein knockdown.

2.5. Flow cytometry analysis

To determine the level of protein knockdown, 48 h post transfection, cells were resuspended in culture medium containing the anti-Fc γ R monoclonal antibody 2.4G2, incubated on ice for 15 minutes, and stained with fluorescently labeled anti-CD98 (catalog # ab95686, abcam) or anti-TfRc (catalog # 113813, BioLegend) on ice for 30 minutes. In parallel, cells were stained with the corresponding isotype control antibody or left unstained. Data were acquired on a FACSCanto (BD Immunocytometry Systems) and samples were analyzed using FlowJo software version 10.0.7 (FLOWJO). Protein expression and knockdown levels were calculated based on median fluorescence intensity (MFI) relative to the NT siRNA-transfected cells set to 100% expression.

2.6. ELISA

Binding of MNV-1 or recombinant MNV-1 Pd to the extracellular domain of recombinant murine CD36 (rCD36, catalog # 50422-M08H, SinoBiological), rCD44 (catalog # 6127-CD-050, R&D Systems), rCD98 heavy chain (rCD98, catalog # 50813-M07H, SinoBiological), and recombinant transferrin receptor 1 (rTfRc, catalog # 50741-M07H, Sino biological) was measured by ELISA. 96 well microtiter plates were coated with 1 μ g of recombinant protein or BSA per well overnight at 4°C. After washing and blocking, plates were incubated for 4 h at 37°C with $\sim 1 \times 10^8$ PFU of concentrated MNV-1 lysate or 25 ng of purified recombinant MNV-1 Pd generated as described previously (Hwang et al., 2014; Taube et al., 2010). Equivalent volumes of mock-infected lysate or buffer (used for recombinant MNV-1 Pd purification) were used as negative controls for the MNV-1 and Pd ELISA, respectively. Bound virus and Pd were detected with an anti-MNV virus-like particle rabbit serum (Wobus et al., 2004), followed by peroxidase-conjugated secondary goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories).

2.7. MNV-1 growth curve in the presence of rCD98

MNV-1 was incubated with rCD98 in high (2×10^6 fold) or low (1,000 fold) molar excess (rCD98:MNV-1 VP1) or BSA as a negative control for 1 h at 37°C. Following incubation, RAW 264.7 cells were infected with the rCD98-MNV-1 complex or the BSA-MNV-1 control. A growth curve was performed and viral titers were assessed as described above (section 2.3).

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.02 (GraphPad Software). The unpaired two-tailed Student's t-test was used to determine statistical significance. Error bars represent the SEM, and the number of independent experiments performed is indicated in the figure legends for the respective experiment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Results

3.1. Identification of putative MNV-1-interacting proteins

To identify putative MNV-1-interacting proteins that play a role in MNV-1 infection, we performed VOPBA followed by tandem mass spectrometry analysis. Cell membrane proteins from two highly MNV-permissive cell lines, RAW 264.7 (a macrophage-like cell line) and SRDC (a dendritic cell-like cell line), were used for VOPBA experiments. Proteins of approximately 90 and 110 kDa were reactive with MNV-1 lysate, but not with mock (non-infected cell) lysate (Fig. 1a). The two bands were excised from a Coomassie blue stained gel performed in parallel to the VOPBA, and proteins contained in these bands were identified by tandem mass spectrometry. Proteomic analysis identified 124 and 217 proteins from RAW 264.7 and SRDC cells, respectively. Fifty-five proteins were shared between the two cell lines (Fig. 1b).

Because the goal of this study was to identify cellular proteins that participate in the early steps of MNV-1 infection, we selected proteins for follow-up studies that met the following inclusion criteria: protein expression on the plasma membrane of macrophages and dendritic cells, approximate molecular mass of 90–110 kDa in SDS-PAGE, and relative abundance (based on the number of unique peptides). Of the overlapping proteins, five met the inclusion criteria: HSP90, a chaperone protein involved in folding and stabilization of proteins (Subbarao Sreedhar et al., 2004); the scavenger receptor CD36 (Silverstein and Febbraio, 2009); the hyaluronic acid receptor CD44 (Ponta et al., 2003); the heavy chain of the heterodimeric amino acid transporter CD98 (Deves and Boyd, 2000), and the transferrin receptor 1 (TfRc), involved in cellular uptake of iron (Aisen, 2004) (Fig. 1b). HSP90 is involved in later stages of MNV-1 infection and facilitates stability of the viral major capsid protein VP1 (Vashist et al., 2015). Thus, it was not further analyzed in the current study. Taken together, these results suggested that MNV-1 may interact with multiple cellular proteins localized on the plasma membrane of permissive cells.

3.2. MNV-1 binding to BMDC cells is reduced in the absence of CD36 and CD44

Since CD36 knockout (KO), CD44 KO mice are commercially available, we chose to investigate the role of CD36 and CD44 in MNV-1 binding and infection of primary BMDC and BMM lacking either protein. To measure MNV-1 binding, BMDC and BMM were isolated from CD36 knockout (KO), CD44 KO, and corresponding wild type (WT) control mice, and infected with MNV-1 (MOI of 2) prior to quantifying bound viral genome equivalents by RT-qPCR. A 22% and 50% reduction in MNV-1 binding to CD36 KO and CD44 KO BMDC was observed, respectively, compared to WT control cells (Fig. 2a and 2c). No statistically significant difference in MNV-1 binding was observed in CD36 KO or CD44 KO BMM compared to the WT control cells (Fig. 2b and 2d). To test whether CD36 and CD44 play a role during MNV-1 infection *in vitro*, growth curves were performed in CD36 KO and CD44 KO BMDC and BMM and compared to the respective WT control cells. Cells were infected with MNV-1 (MOI of 0.05) for 0, 12, 24, and 48 h. Similar growth kinetics were observed in CD36 KO and CD44 KO BMDC and BMM compared to their respective WT control cells (data not shown). Overall, these results suggested that CD36 and CD44 facilitate MNV-1 binding to BMDC, but not to BMM, and that MNV-1 infection of BMDC and BMM is independent of CD36 and CD44.

3.3. MNV-1 binding to RAW 264.7 cells is reduced in CD98- and TfRc-depleted cells

CD98 and TfRc gene deletions are embryonically lethal (Levy et al., 1999; Tsumura et al., 2003), and no commercially available knockout mice for either gene are available. This precluded us from testing primary bone marrow-derived cells. Therefore, we used a siRNA knockdown-based approach to investigate a role for CD98 and TfRc in MNV-1 binding to RAW 264.7 and SRDC cells. RAW 264.7 cells were transfected with CD98 siRNA, TfRc siRNA, or NT siRNA for 48 h. RAW 264.7 cells were first analyzed by flow cytometry to verify protein knockdown (Fig. 3a). Knockdown levels were 31.9% ($\pm$ 2.3%) for CD98 siRNA-transfected cells (Fig. 3a, left panel) and 68.4% ($\pm$ 2.8%) for TfRc siRNA-transfected cells (Fig. 3a, right panel) compared to NT siRNA-transfected cells. Next, RAW 264.7 cells were infected with MNV-1 (MOI of 2) and bound virus genome equivalents were quantified by RT-qPCR. MNV-1 bound 33% less to CD98 siRNA-transfected cells and 35% less to TfRc siRNA-transfected cells when compared to the NT siRNA-transfected control cells (Fig. 3b). A similar experiment was performed in SRDC cells. CD98 and TfRc were knocked down in SRDCs to comparable levels as seen in RAW 264.7 cells (data not shown). However, MNV-1 binding to CD98 or TfRc siRNA-transfected SRDC cells was similar to NT siRNA-transfected control cells (data not shown). Taken together, our results indicated that CD98 and TfRc facilitate MNV-1 binding to RAW 264.7, but not to SRDC cells. However, we cannot rule out that the modest knockdown obtained for CD98 may be masking a more pronounced role of this protein during MNV-1 infection.

3.4. MNV-1 infection of RAW 264.7 cells is reduced in CD98-depleted cells

To determine whether CD98 and TfRc are involved in post-binding steps of MNV-1 infection, we performed growth curves with CD98 and TfRc siRNA- and NT siRNA-transfected RAW 264.7 cells. Forty eight hours after transfection, cells were infected with MNV-1 (MOI of 0.05) and infection was allowed to proceed for 0 or 8 h. MNV-1 genome

titers were decreased 25% at 0 h post infection (hpi) and 40% at 8 hpi in CD98 siRNA-transfected cells compared to the NT siRNA-transfected control cells (Fig. 3c), while similar growth kinetics were observed in TfRc siRNA-transfected cells and NT siRNA-transfected control cells (Fig. 3c). This raised the possibility that CD98 may also have a role in viral replication. However, when viral RNA was transfected into CD98- or NT siRNA-transfected control cells, similar infectious virus yield were observed 8 hours post vRNA transfection by plaque assay (data not shown). This indicated CD98 depletion did not have a major effect on viral RNA replication in RAW264.7 cells and that CD98 likely acted at an earlier point in the life cycle. In SRDC cells, no difference was observed in MNV-1 infection between CD98 siRNA- and NT siRNA-transfected cells (data not shown). However, MNV-1 infection was increased in TfRc siRNA-transfected cells compared to the NT siRNA-transfected control cells with almost double (~180%) the number of genome equivalents present in TfRc siRNA-transfected cells at 8 hpi (data not shown). Therefore, these results indicated that CD98, but not TfRc, is required for efficient MNV-1 infection of RAW 264.7 cells, and that TfRc may restrict MNV-1 infection in SRDC cells.

3.5. MNV-1 binds to the extracellular domain of recombinant murine CD36, CD98 and TfRc via interaction with VP1 Pd

To corroborate a role of the identified proteins in MNV-1 infection and to determine whether MNV-1 can directly interact with CD36, CD44, CD98, and TfRc, we tested whether MNV-1 virions or MNV-1 Pd bind to recombinant (r) CD36, CD44, CD98, and TfRc by ELISA (Fig. 4). Microtiter plates were coated with the extracellular domains of rCD36, rCD44, rCD98, and rTfRc and their binding to MNV-1 or MNV-1 Pd binding was analyzed. To verify successful coating of proteins, we used rCD98 as a surrogate and confirmed its binding to plates using an anti-CD98 antibody. Importantly, MNV-1 virions bound to rCD36-, rCD98- and rTfRc-coated wells, while no binding was observed to rCD44- or BSA-coated wells, or when proteins were incubated with mock lysate (Fig. 4a).

Given that the Pd is the norovirus receptor binding domain and outer most region of the norovirus major capsid protein VP1, we next tested whether this domain mediates the MNV-1 interaction with rCD36, rCD98, and rTfRc. MNV-1 Pd bound efficiently to all three recombinant proteins (Fig. 4b). No binding was observed of Pd to BSA-coated wells, or when proteins were incubated with buffer only, confirming specificity of the interaction. These results indicated that MNV-1, via its Pd, binds directly to the extracellular domains of rCD36, rCD98, and rTfRc, while it did not bind to amino acids 25–224 of the extracellular amino-terminal domain of rCD44 included in the recombinantly expressed protein.

3.6. The extracellular domain of recombinant murine CD98 enhances MNV-1 infectivity.

MNV-1 was shown to directly interact with CD98 and reduction in CD98 levels reduced MNV-1 infection. Therefore, next we wondered whether CD98 functions as an entry receptor for MNV-1 in RAW 264.7 cells and investigated the ability of the extracellular domain of recombinant murine CD98 (rCD98) to competitively inhibit MNV-1 infection. rCD98 was incubated with MNV-1 prior to RAW 264.7 cells infection in two ratios; in high molar excess (2×10⁶ fold excess rCD98:MNV-1 VP1, Fig. 5a), and in low molar excess (1,000 fold excess rCD98:MNV-1 VP1, Fig. 5b). Cells were infected with MNV-1 (MOI of

0.05) and infection was allowed to proceed for 0 or 8 h. To our surprise, at 0 hpi, cells incubated with rCD98-MNV-1 complex in high molar excess had significantly increased virus binding compared to cells infected with the BSA-MNV-1 control (Fig. 5a). Increased genome titers in cells infected with rCD98-MNV-1 compared to the control was also observed at 8 hpi, although this difference was not statistically significant. However, the increase in genome titers was lost when the competition was performed with rCD98 in low molar excess (Fig. 5b). These results demonstrated that rCD98 is unable to competitively inhibit MNV-1 infection of RAW 264.7 cells but instead enhances MNV-1 binding and infectivity in a concentration-dependent manner in this cell type.

4. Discussion

The early steps of norovirus infection, prior to viral RNA replication, are thought to be key determinants of species-specificity and cell tropism (Guix et al., 2007). The carbohydrate moieties that mediate MNV attachment, and the cellular mechanisms involved in MNV internalization have been partially characterized (Gerondopoulos et al., 2010; Perry and Wobus, 2010; Taube et al., 2012; Taube et al., 2009). However, specific cellular proteins that play a role during early steps of MNV infection are not yet known. In the current study, we combined a proteomic-based approach with ELISA and loss-of-function assays to identify proteins that facilitated MNV-1 infection of dendritic cells and macrophages *in vitro*. CD36, CD44, CD98 and TfRc mediated MNV-1 attachment to cells in a cell-type specific manner. Consistent with a role as MNV attachment factors is their expression pattern, which is not limited to cell types susceptible to MNV (i.e., macrophages, dendritic cells and B cells), but is also found in other cells, such as intestinal epithelial cells (Chen et al., 2015; Jones et al., 2014; Kennel et al., 1993; Lynes et al., 2011; Nguyen et al., 2010; Wobus et al., 2004).

Specifically, our data showed that two plasma membrane glycoproteins, CD36 and CD44, are important for efficient MNV-1 binding to primary murine BMDC. Direct binding of MNV-1 to rCD36 was mediated by the Pd. No binding was observed to rCD44. The commercially available rCD44 comprises the amino-terminal domain of the native protein (Gln25 - Thr224, accession # NP 033981), which contains the hyaluronic acid binding motifs, but not the stem region, which greatly varies in size between CD44 isoforms (Ponta et al., 2003). Thus, whether MNV-1 binds to CD44's stem region not included in the recombinant protein instead remains to be investigated. Furthermore, efficient binding of MNV-1 to murine macrophage RAW 264.7 cells was dependent on the presence of two other membrane glycoproteins: CD98 and TfRc. Binding of virus to the extracellular domain of both proteins was mediated by the Pd. MNV-1 is known to bind to glycans present on the ganglioside GD1a and on N- and/or O-linked glycoproteins (Taube et al., 2012; Taube et al., 2009). All recombinant proteins used in our experiments were expressed in mammalian cells and are known to be glycosylated. Therefore, future studies are needed to determine whether the MNV-1 Pd interacts with the glycan moieties or the protein backbone. Additionally, the current study was limited to MNV-1, so it will be interesting to test whether other strains of MNV use the same proteins during infection *in vitro*.

Our findings further demonstrate that MNV-1 uses different cellular proteins in a cell-type specific manner. Different cellular protein requirements to bind to and/or infect distinct

target cell types are not without precedent and have been observed with other viruses. For example, HIV-1 binding to macrophages is mediated by macrophage mannose receptor (CD206), while DC-SIGN (CD209) mediates binding to dendritic cells (Geijtenbeek et al., 2000; Londrigan et al., 2012; Nguyen and Hildreth, 2003). Another example is dengue virus-1, which uses CD209 to infect dendritic cells, while the high-affinity laminin receptor mediates dengue virus-1 infection of liver cells (Navarro-Sanchez et al., 2003; Thepparit and Smith, 2004). Differential protein usage by MNV-1 on different cell types may also be driven by changes in glycosylation patterns since the four glycoproteins analyzed in the current study have multiple glycosylation sites, whose glycosylation pattern can change depending on the cell type (Bartolazzi et al., 1996; Goodman et al., 2010; Hoosdally et al., 2009; Powlesland et al., 2009). Future studies are needed to confirm a role for differential glycosylation in cell-type specific receptor usage by MNV and to identify the cell-type specific function of these proteins during MNV infection.

The findings of reduced MNV binding and infection in CD98 siRNA knocked-down cells suggest that CD98 might have multiple functions during MNV-1 infection. CD98 plays different roles in the infectious cycle for a variety of viruses. It is involved in vaccinia virus endocytosis (Schroeder et al., 2012) and in de-envelopment of herpes simplex 1 virus (Hirohata et al., 2015). Additionally, the light chain xCT of the CD98 amino acid transporter heterodimer is an entry receptor for Kaposi's sarcoma-associated herpesvirus (Chakraborty et al., 2012). Therefore, it is conceivable that CD98 may play additional role(s) in the MNV-1 infectious cycle in addition to mediating viral binding to host cells. However, when we tested whether CD98 functioned as an entry receptor during MNV-1 infection of RAW 264.7 cells, the soluble form of CD98 (i.e., the extracellular domain of recombinant murine CD98) did not competitively inhibit infection. Instead, it enhanced MNV-1's infectivity of RAW 264.7 cells when incubated with virus in high molar excess. Therefore, CD98 plays a role other than as an entry receptor during the MNV-1 infectious cycle. One such possible role may be as a coreceptor, as is observed for other non-enveloped viruses. For example, coxsackievirus A9 uses the MHC class I as an entry receptor and GRP78 as a coreceptor, which is thought to promote virus interaction with the entry receptor (Triantafilou et al., 2002). A role for CD98 as a co-factor that mediates MNV-1 interaction with other cellular proteins responsible for virus binding and entry would also explain our finding that a large excess of soluble rCD98 enhances MNV-1's ability to infect RAW 264.7 cells, instead of inhibiting infection. Enhanced virus binding following incubation with a soluble coreceptor has been reported to occur in some conditions with HIV and soluble CD4 (Clapham et al., 1992). Recently, Ebola virus was shown to have enhanced infectivity *in vitro* when incubated with ficolin-1 prior to infection. Ficolin-1 is thought to function as a bridge molecule in receptor-mediated Ebola virus entry into target cells (Favier et al., 2016). However, whether the enhancement of MNV-1 attachment occurs in a physiological context, remains to be determined in the future. Nevertheless, future studies promise to reveal new role(s) for CD98 during MNV-1 infection.

5 Conclusions

In summary, our study expands the number of attachment molecules elucidated for MNV-1. Specifically, we identified four cellular plasma membrane glycoproteins, CD36, CD44,

CD98 and TfRc, which promoted MNV-1 binding in a cell type specific manner. MNV-1 directly interacted with the extracellular domains of CD36, CD98 and TfRc via its Pd. Thus, the detailed molecular mechanisms by which each of these four glycoproteins affect the viral life cycle await further investigation. Identifying cellular proteins involved in the early stages of infection is crucial to our understanding of MNV tropism, pathogenesis, and norovirus biology in general, and may help identify potential targets for therapy.

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Figure 1.

Identification of MNV-1-putative interacting proteins by VOPBA followed by tandem mass spectrometry. (a) Representative image of RAW 264.7 cells VOPBA. Membrane proteins were extracted from RAW 264.7 cells, separated by SDS-PAGE, and VOPBA was performed using MNV-1 or mock lysate. Virus bound to cellular proteins was detected by an anti-MNV rabbit serum. (b) Schematic representation of the identified proteins in the two permissive cell lines tested (RAW 264.7 and SRDC cells). Bands observed by VOPBA (indicated by asterisks) were excised from a Coomassie blue stained gel, and proteins were identified by tandem mass spectrometry. Each hit depicted in the figure represents a distinct protein. Five proteins met the inclusion criteria (outlined in results, section 3.1) as candidate MNV-1-interacting proteins.

Figure 2.

MNV-1 binding is reduced in CD36- and CD44-deficient bone marrow-derived dendritic cells (BMDC) but not bone marrow-derived macrophages (BMM). Wild type (WT), CD36 knockout (CD36 KO), or CD44 KO BMDC (a and c) and BMM (b and d) were infected with MNV-1 (MOI of 2). Bound virus was quantified by RT-qPCR. Results from four independent experiments performed in duplicate are shown as percent binding of virus genome equivalents to cells, which were calculated relative to the WT control cells set to 100%. * $p < 0.05$.

Figure 3.

TfRc and CD98 facilitate MNV-1 binding to RAW 264.7 cells, while CD98 is required for efficient virus infection. RAW 264.7 cells were transfected with CD98 siRNA, TfRc siRNA, or non-targeting (NT) siRNA control. At 48 h post transfection, cells were stained with fluorescently labeled anti-CD98 or anti-TfRc (a) or infected with MNV-1 (b and c). (a) Transfected cells were analyzed by flow cytometry to verify protein knockdown. Left panel: representative image showing CD98 expression in RAW 264.7 cells transfected with NT siRNA (open histogram, solid black line) or CD98 siRNA (grey filled histogram). Right panel: representative image showing TfRc expression in RAW 264.7 cells transfected with NT siRNA (open histogram, solid black line) and TfRc siRNA (grey filled histogram). Open histogram, dotted black line: unstained control, (b) CD98- and TfRc-siRNA knockdown significantly reduced MNV-1 binding to RAW 264.7 cells. Transfected cells were infected with MNV-1 (MOI of 2), and bound virus was quantified by RT-qPCR. Results from three independent experiments performed in duplicate are shown as percent binding of virus genome equivalents to cells, which were calculated relative to the NT siRNA-transfected cells set to 100%. (c) CD98- siRNA knockdown significantly reduced MNV-1 infection of RAW 264.7 cells. Transfected cells were infected with MNV-1 (MOI of 0.05), and viral genome titers were assessed by RT- qPCR at 0 and 8 hpi. Results from three independent experiments performed in duplicate are shown as percent infection, which was calculated relative to the NT siRNA-transfected cells set to 100%. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4.

MNV-1 interacts with rCD36, rCD98, and rTfRc via its protruding domain (Pd). MNV-1 and MNV-1 Pd binding to the extracellular domain of recombinant proteins was evaluated by ELISA. (a) Recombinant CD36, CD44, CD98, TfRc or an irrelevant protein (BSA) were coated onto 96 well microtiter plates and incubated with MNV-1 or mock lysate. (b) Recombinant CD36, CD98, TfRc or BSA were coated onto 96 well microtiter plates and incubated with MNV-1 Pd or buffer. Results are represented as the absorbance values measured at 415 nm (A_{415}). The dashed line is drawn at the A_{415} mean value for the BSA-coated wells plus twice the value of the SEM. Values above the line were considered positive. Results are from at least two independent experiments with conditions tested in duplicate.

Figure 5.

Recombinant CD98 enhances MNV-1 infection in a concentration dependent manner. rCD98 was incubated with MNV-1 prior to infection in 2×10^6 fold molar excess (a) or 1,000 fold molar excess (b), or with BSA. RAW 264.7 cells were then infected with MNV-1 (MOI of 0.05) and viral genome titers were assessed by RT-qPCR at 0 and 8 hpi. Results are shown as percent infection, which were calculated relative to the BSA-MNV-1 infected control cells set to 100%. Results are from two independent experiments with conditions tested in duplicate per experiment. **** $p < 0.0001$.