Initial steps of murine norovirus infection *in vivo* and *in vitro*

by

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To all living beings (human and non-human) that helped me to get here.

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Acknowledging all the people who have helped me to get me where I am is impossible. After all, I am 36 years old and have been doing science since I was 20... But I will do my best and try to thank the main "players", the ones who directly helped me to finish my PhD and contributed to the conclusion of this phase of my career. I apologize beforehand for those who are left out, but after 30 the memory starts fading away...

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Abstract

Human noroviruses are the leading cause of acute gastroenteritis worldwide. Despite their major impact on public health and economy, very little is known about how human noroviruses interact with their host during infection. Murine norovirus (MNV) is genetically related to human norovirus and is commonly used to study norovirus-host interactions and basic aspects of norovirus biology in a native host. MNV grows well in cell culture and infects a small animal. In this dissertation, we aimed to characterize the initial steps of MNV infection at the host and cellular levels.

Microfold (M) cells are highly specialized intestinal epithelial cells for transporting lumenal antigens across the epithelial barrier by a process known as transcytosis. We are particularly interested in the role M cells play in MNV infection *in vivo*. Previous data from our lab showed that oral MNV infection is significantly reduced or absent in wild-type BALB/c mice depleted of M cells following the injection of an antibody against RANKL (receptor activator of nuclear factor-κB ligand) and in BALB/c $Rag2^{null}/\gamma c^{null}$ mice, respectively. To confirm previous findings and expand the study on the role of M cells in MNV infection, we used another mouse model: a conditional knockout mouse (on a C57BL/6 background) that is deficient for M cells (called M-less mice). We tested whether MNV could orally infect M-less mice and littermate controls with two strains of MNV, MNV-1 and MNV.CR3. Unlike our previous findings, M-less mice and littermates had similar viral yields at both 12 and 24 hpi, suggesting M cells are not required during MNV infection of these C57BL/6 transgenic mice. However, M-less mice infected with MNV-1 had significantly reduced viral titers in the MLN at 24 hpi, which suggests that trafficking of MNV-1 from the intestinal lumen to the MLN is M cell-dependent. Thus, the presence of mature M cells is necessary for optimal dissemination of MNV-1 to the local draining lymph node (MLN) under these experimental conditions.

A key determinant of noroviruses' cell tropism and strict species-specificity is expression of host cellular attachment and entry receptors. 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. Using virus overlay protein binding assay followed by tandem mass spectrometry analysis in two permissive cell lines, RAW264.7 (murine macrophages) and SRDC (murine dendritic cells), four cellular membrane proteins were identified 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, CD98 may play a role in post-binding stages of MNV-1 infection. Our studies demonstrated that multiple membrane proteins can promote efficient MNV-1 infection in a cell type-specific manner.

Our findings improved the current understanding on how MNV crosses the intestinal epithelial barrier to gain access to its target cells, and how it subsequently binds to and enters host target cells.

Chapter 1. Introduction

1.1 Noroviruses

Noroviruses (NoVs) belong to the genus *Norovirus* within the *Caliciviridae* family. Caliciviruses additionally comprise four other genera: *Vesivirus*, *Lagovirus*, *Sapovirus* and *Nebovirus*. Calicivirus virions are small (27-40 nm in diameter), non-enveloped, and of icosahedral symmetry with 32 cup-shaped depressions observed at the five-fold and three-fold axes of symmetry [1]. Members of this family have been isolated from many different animal species, and the diseases they cause vary greatly. *Vesivirus* and *Lagovirus* cause systemic diseases in animals, including upper respiratory tract disease (feline calicivirus) and systemic hemorrhage and massive necrosis of the liver (rabbit hemorrhagic disease virus), while *Norovirus*, *Sapovirus* and *Nebovirus* cause gastroenteritis in humans and/or other animals [1,2]. Noroviruses infect mostly humans but can also infect other mammals such as mice, cattle, pigs, dogs and lions [3-8]. .

1.1.1 *Norovirus* genomic organization and proteins

The *Norovirus* genome is a ~7.4 kb long, linear, single-stranded, positive-sense RNA molecule, which is covalently attached to the VPg protein (viral protein, genome-linked) at the 5′-terminus, polyadenylated at the 3′-terminus, and encodes three to four open reading frames (ORF) [8] (Fig. 1.1). ORF1 is located in the 5′ half of the genome, while ORFs 2-4 are located in the 3′-end. ORF1 encodes the non-structural (NS) genes, which are translated as a large polyprotein that is co-translationally cleaved into at least six proteins: NS1/2 or N-term (N-terminal protein); NS3 or nucleoside triphosphatase (NTPase); NS4 or 3A-like protein; NS5 or VPg; NS6 or protease (Pro); and NS7 or polymerase (Pol) [9-12]. NS1-2, the N-terminal protein, lacks significant sequence similarity to other known proteins. The NS1-2 protein from the human norovirus (HuNoV) Norwalk virus interacts with the Golgi apparatus, disrupts intracellular protein trafficking and may play a role in the induction of intracellular membrane rearrangement required during positive-strand RNA virus replication [13,14]. Similarly, the NS1-2 protein from

murine norovirus (MNV, a model system to study HuNoV), has been suggested to mediate recruitment of endoplasmic reticulum (ER) membranes to the viral replication complex [15]. MNV NS1-2 is also involved in determining tissue tropism and whether the infection is acute or persistent. MNV-1, the genogroup V prototype strain, causes acute infection and preferentially infects the small intestine of mice. However, its tropism is shifted to the colon and it becomes a persistent strain when the NS1-2 residue 94 is changed from an aspartic acid to a glutamic acid [16]. Additionally, the highly divergent N-terminus of MNV NS1-2 has been identified as an inherently disordered region and able to multimerize, which provides the protein structural flexibility and wide binding ability to many different targets. These features allow a single protein to have multiple roles and correlate with the known functions of NS1-2 in MNV infection.[17] NS3 is the viral NTPase, and bacterially expressed NS3 from the HuNoV Southampton virus is able to bind and hydrolyze nucleoside triphosphates [18]. NS4 blocks ER-to-Golgi trafficking leading to Golgi disassembly and inhibition of cellular protein secretion [15,19,20]. Due to the subcellular localization at the Golgi apparatus and endosomes, respectively, a model has been proposed in which NS1-2 and NS4 recruit organellar membranes to the site of MNV replication [15]. VPg (NS5) is covalently linked to the 5′-terminus of the NoV genome and binds to host cellular translation initiation factors to initiate translation of the viral RNA [21-24]. Additionally, VPg functions as the protein primer for replication [25-27]. NS6 is the chymotrypsin-like cysteine protease [28,29] that co-translationally processes the large NS polyprotein and cleaves poly(A)-binding protein, resulting in inhibition of cellular translation [30]. NS7 is the RNA-dependent RNA polymerase [25-27,31,32].

Figure 1.1. *Norovirus* genome. The *Norovirus* genome is made up of a linear single-stranded, positive-sense RNA molecule covalently linked to VPg on its 5′-terminus and polyadenylated on the 3′-terminus. ORF1 (in blue) is about 5kb long and encodes a large (~200kDa) non-structural (NS) polyprotein. A virus-encoded protease co-translationally processes the NS polyprotein yielding the mature forms: N-term, N-terminal protein; NTPase, nucleoside triphosphatase; 3A, 3A-like protein; VPg, virus protein, genome-linked; Pro, protease; Pol, polymerase. ORF2 (in purple) is about 1.6kb long and encodes the major structural capsid protein VP1 (~58kDa). ORF3 (in gray) is about 0.6kb long and encodes the minor capsid protein, VP2 (~22kDa). Murine norovirus has a fourth ORF, ORF4 (in burgundy), which encodes the virulence factor 1 (VF1).

ORF2 and 3 encode VP1, the major capsid protein, and VP2, the minor capsid protein, respectively (Fig. 1.1). Each capsid is composed of 180 copies of VP1 arranged in 90 dimers [33,34]. VP1 is divided into an N-terminal arm, shell (S) and C-terminal protruding (P) domains, which are connected by a flexible hinge. The S domain is highly conserved and forms the interior shell of the viral capsid, surrounding the viral genome (Fig. 1.2a). The P domain forms protrusions that arise from the S domain, forming the arch-like structures that confer the cup-shape appearance of caliciviruses when they are observed by electron microscopy (Fig. 1.2b). The P domain is further subdivided into the P1 subdomain, the stem region that connects the S and P domains, and the P2 subdomain, which is a globular head region that forms the most exposed region of the capsid and is the least conserved among NoVs (Fig. 1.2a). Determinants of virus binding and entry into susceptible cells, antigenicity and immune-driven evolution reside within the P domain [35-43].

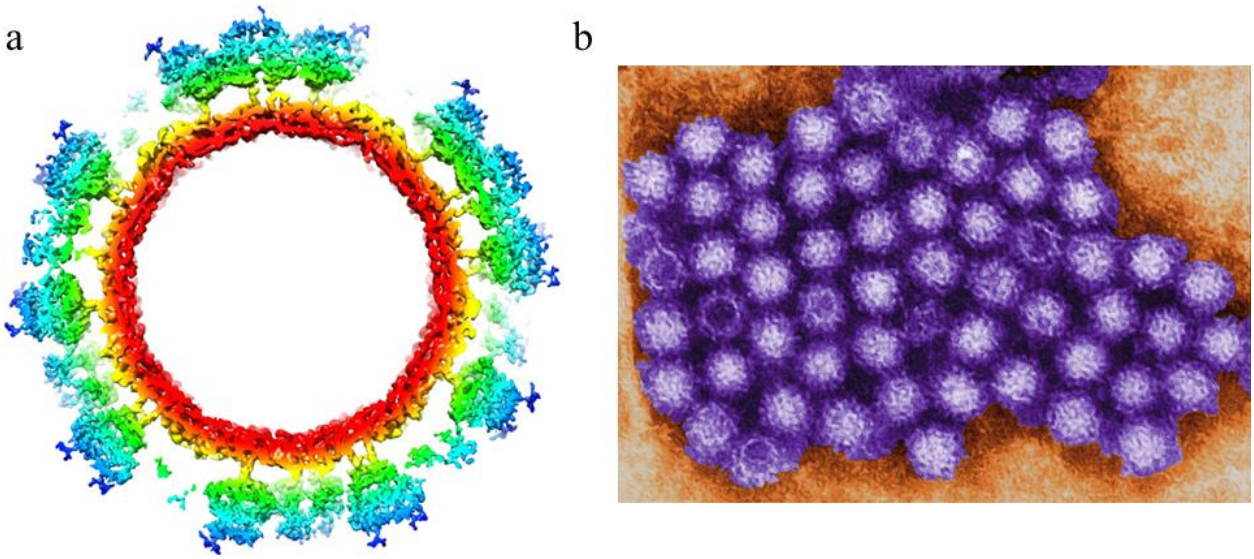

Figure 1.2. Noroviruses capsid ultrastructure morphology. (a) Cryo-electron microscopy image reconstruction of murine norovirus 1 (MNV-1). Central section of MNV-1 shows protruding (P) domains rise well above the capsid shell. P2 subdomain, P1 subdomain, and shell domain are colored blue, green and yellow/red, respectively. Courtesy of Thomas Smith, UTMB Galveston. (b) Digitally-colored transmission electron micrograph of human norovirus virions. Source: CDC Public Health Image Library (ID#: 10708), CDC/Charles D. Humphrey.

The P domain of HuNoV contains histo-blood group antigen binding sites, and epitopes recognized by a monoclonal antibody that blocks HuNoV-cell interactions map to the P2 subdomain [36,44-47]. For MNV, carbohydrate binding sites have been identified within the P2 subdomain by mutagenesis [48], and the Fab fragment of an antibody thought to neutralize

infection by blocking capsid-receptor interactions binds to P2 [38]. Recent evidence suggests that flexibility in surface exposed loops located in the P2 subdomain mediates escape from antibody neutralization by indirectly affecting the antibody binding site [43]. Additionally, VP1 has a regulatory role in the viral replication cycle, increasing Pol activity in a concentration-dependent manner [49]. The minor capsid protein VP2 has a highly basic nature; it is thought to be involved in genome encapsidation and particle assembly [50]; and its absence is known to decrease virus-like particle stability when expressed in insect cells [51]. Yet, only recently were the function and localization of VP2 within the particle elucidated. VP2 associates with the VP1 S domain at the interior surface of the capsid [52], which corroborates the hypothesis that this protein is involved in genome encapsidation and particle assembly.

Unlike other members of the *Norovirus* genus, MNV encodes a fourth ORF, which overlaps with ORF2 but is expressed in a different reading frame (Fig. 1.1). ORF4 encodes the virulence factor 1 (VF1), which is important during MNV infection of cells in culture and in mice by antagonizing the innate immune response [12].

1.1.2 *Norovirus* classification

Based on VP1 amino acid sequence similarity, NoVs are classified into five distinct genogroups (G), which are further divided into at least thirty genotypes (Fig. 1.3) [53,54]. The amino acid divergence within a genogroup ranges between 45 and 61.4%, while within a genotype it is 0-14.1%. GI is divided into at least eight genotypes (GI.1-G1.8), with all strains within this genogroup infecting humans [53]. GII comprises at least 21 genotypes, including both human and porcine strains, while GIV has two genotypes, with human and canine strains [5,7,53,54]. GIII includes three genotypes and causes infection in ruminants, while GV is represented by a single genotype which infects mice [53-55]. Recently, a unified NoV nomenclature and genotyping system has been described [56]. It proposes the use of dual nomenclature using both ORF1 and VP1 sequences and will be especially useful for classification of newly emerging NoVs and recombinant strains. Animal NoVs are genetically closely related to HuNoVs, particularly strains within GII and GIV. This raises the possibility that certain NoV strains cause zoonotic infections, although interspecies transmission has not been documented thus far.

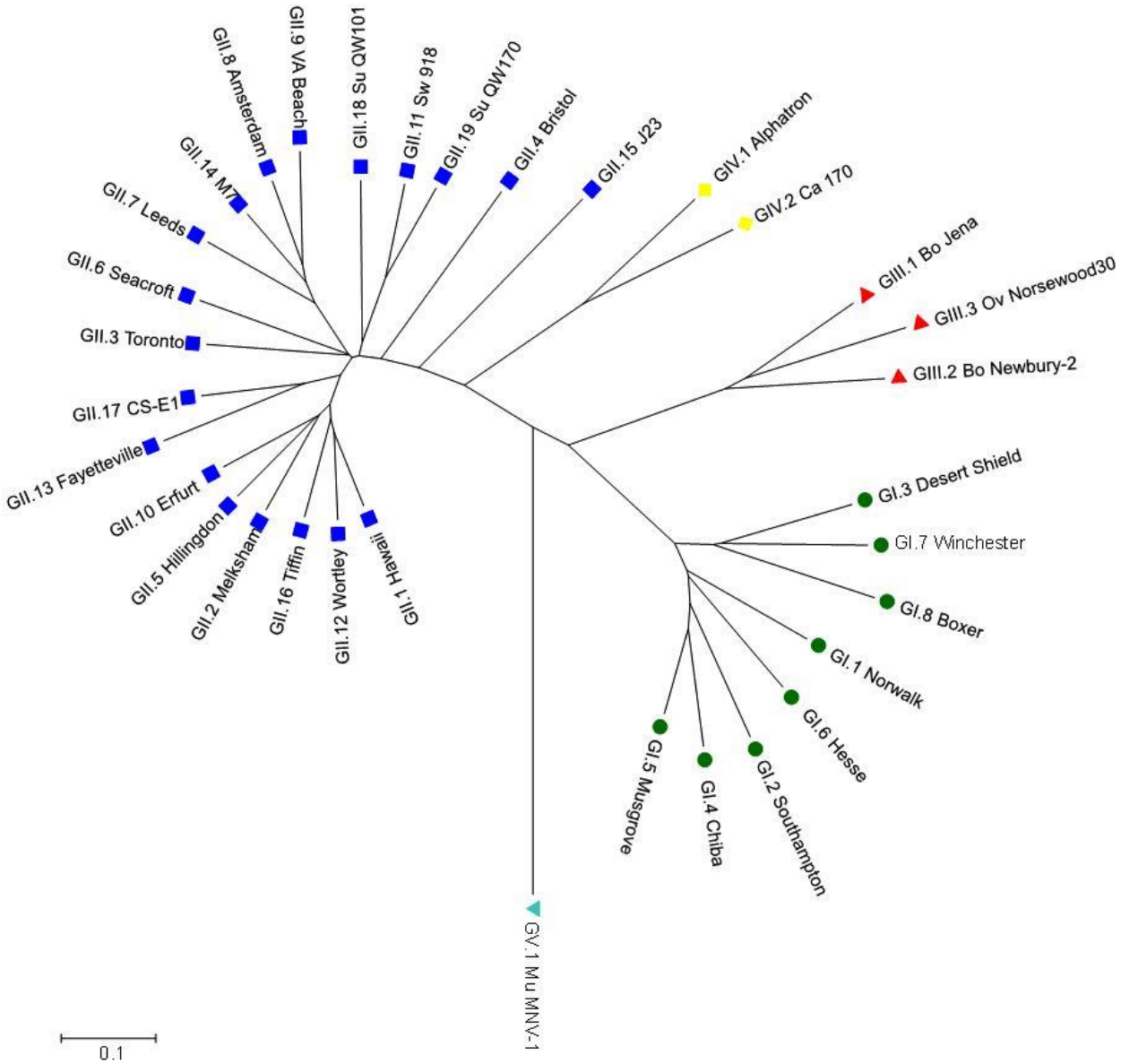

Figure 1.3. *Norovirus* classification. Noroviruses are classified into five genogroups (GI - GV) which are further divided into genotypes based on the complete amino acid sequence of VP1. Representative genotype strains within each genogroup are shown in the phylogenetic tree. The scale bar of 0.1 represents distance expressed as amino acid substitutions per site. GI, green circles; GII, blue squares; GIII, red triangles; GIV, yellow diamonds; GV, cyan inverted triangles. Tree was constructed by the Neighbor-Joining method and Poisson correction model (2,000 bootstrap replicates) using Mega v5 software [57].

1.1.3 Human noroviruses (HuNoVs)

HuNoVs are the most common cause of acute non-bacterial gastroenteritis in all age groups worldwide [58,59]. In the USA, HuNoVs are estimated to cause 19-21 million cases of illness with an estimated cost of $2 billion/year [60,61]. They represent 58% of all food-borne

disease outbreaks and, recently HuNoVs were identified as the leading cause of severe acute gastroenteritis in children, surpassing rotaviruses [54,62,63]. Globally, HuNoVs are estimated to cause $60.3 billion/year in societal costs (e.g. productivity losses due to acute illness) [64]. In general, HuNoVs cause a self-limiting disease, which typically lasts one to three days. Common clinical manifestations include nausea, diarrhea, vomiting and abdominal cramps; fever and myalgia may be present [8,54]. Asymptomatic cases are common and account for up to one third of HuNoV infections [65-68]. Furthermore, HuNoVs may cause severe disease characterized by pronounced dehydration, which may lead to hospitalization and death. In the US alone, HuNoVs are estimated to cause 800 deaths/year and over 70,000 hospitalizations [69,70]. The elderly, young children, and immune-compromised individuals are at greater risk of developing the more severe form of HuNoV gastroenteritis [54].

Noroviruses are highly transmissible pathogens. This is mainly due to their environmental stability, relative resistance to certain disinfectants (e.g. mild detergents, phenolic compounds) [1,54] and low infectious dose [71,72]. They are shed in the feces of infected individuals, although particles are also found in vomitus. The primary mode of transmission is via the fecal-oral route, mainly by direct contact with an infected person or contaminated surfaces, or by consumption of contaminated food or water [8,54]. Ingestion of HuNoV particles present on aerosolized vomitus represents an alternative route of infection, which is associated with outbreaks in enclosed settings such as aircrafts and restaurants [73,74]. There are several other key features of HuNoV biology that contribute to their highly efficient transmission and environmental spread. For instance, fecal viral shedding precedes disease onset and persists after clinical remission [65,68,75]. Additionally, the presence of pre-existing antibodies against HuNoV does not correlate with protection against a challenge with the virus, and can even make an individual more susceptible to that challenge than those with no or lower levels of antibodies [68,76-79]. Short-term immune protection exists against HuNoV, yet it is mostly against homologous strains [76,77,80-82]. Like any typical RNA virus, HuNoV mutation rates are high, allowing for great antigenic diversity, which coupled to limited cross-protection and long-term immunity, may lead to repeated infections [59,83-85]. Lastly, HuNoVs have high evolutionary rates, and appearance of new/recombinant epidemic strains has been observed throughout the world [54,59,86].

As HuNoV gastroenteritis has emerged as a global problem, GII.4 strains are described as the most common cause of NoV outbreaks worldwide [59]. HuNoVs evolve and persist in the human population by still unknown mechanisms, although particularly for GII.4 epidemic strains, a model of molecular evolution has been proposed [83]. These strains persist over time in the human population by introducing mutations in the attachment receptor (carbohydrates) binding sites, specifically in surface-exposed residues within the P2 subdomain. These changes occur in response to human herd immunity and result in novel receptor-binding phenotypes. Additionally, mutations accumulate in regions surrounding the attachment receptor binding sites, which results in viral immune escape [83]. Therefore, HuNoV vaccines will likely need periodical reformulation based on current circulating strains [41,83].

Despite HuNoV's major impact on human health and economy, currently, there is no specific drug or commercially available vaccine to treat or prevent infection. Only recently a mouse model and a cell culture system for HuNoVs were established [87,88], and they will greatly aid in developing of anti-HuNoV therapies. However, the study of HuNoVs in a laboratory setting still remains challenging, and basic aspects of the HuNoV biology remain largely unknown. In order to overcome this barrier, the genetically related murine norovirus (MNV) is widely used as a model system to study diverse aspects of NoV biology in a natural host [89-91].

1.1.4 Murine norovirus (MNV)

MNV is the only member of the *Norovirus* genus that replicates well in cell culture, especially in murine macrophages and dendritic cells [92], although it can also replicate in murine B cells [87]. MNV is genetically related and biologically similar to HuNoVs, being a highly infectious enteric pathogen transmitted by the fecal-oral route [93-95]. The MNV-1 strain was originally isolated from the brain of severely immunocompromised $RAG2^{null}$ $STAT1^{null}$ mice [95] and is the genogroup V prototype strain [53]. MNV causes asymptomatic infection in wild-type mice, and viral RNA can be detected in different tissues, as well as for different lengths of time, depending on which virus strain is used to infect and which mouse strain is being challenged [95,96]. Similar to HuNoVs, MNV recombination events have been observed within ORF2, particularly in the ORF1-ORF2 overlap, which is considered a hot spot for NoV

recombination [97,98], suggesting that MNV would be a useful model for NoV recombination studies.

MNV differs from HuNoV in respect to genetic and antigenic diversity. MNV strains present limited sequence divergence, representing a single genotype/serotype within genogroup V in the *Norovirus* genus. However, despite their high genetic identity (13% divergence or less at the nucleotide level), MNV strains differ greatly in their biological properties [98]. For example, MNV-1 causes acute infection in wild-type mice and is cleared by 7 days post infection (dpi) [98]. On the other hand, MNV strain CR3 (MNV.CR3), a field strain isolated from feces, causes persistent infection, with detectable infectious virus loads in the ileum and viral RNA in the feces up to at least 35 dpi [98]. Additionally, the two strains differ in their attachment receptor requirements: MNV-1 uses terminal sialic acids on the ganglioside GD1a and on N- and O-linked glycoproteins to bind macrophages *in vitro*, while MNV.CR3 uses N-linked glycoproteins [48,99].

Like HuNoV, MNV is a ubiquitous pathogen shed in the feces of infected animals, causing it to be the most prevalent endemic virus in mice research facilities [94]. In a seroprevalence study conducted in the USA and Canada, out of 12,639 tested animals, 22.1% were seropositive for MNV-1 [93]. In a recent review, Hsu *et al.* summarized the histological findings of several studies that assessed the influence of MNV in mouse models of disease [100]. MNV experimental infection causes minimal histological changes in wild-type mice. On the other hand, immunodeficient mice commonly have detectable lesions that were typically inflammatory [100]. MNV has also been identified in wild rodents in the UK and Japan [101,102]. Since infectious agents can adversely affect research by altering biological processes in animal models [100,103], the high prevalence of MNV suggests it may act as a confounding factor when mouse models of disease are being investigated [89,91,93,100]. Indeed, MNV has been shown to impact mouse models of IBD and atherosclerosis [104,105], among others. In addition, co-infection of MNV and mouse parvovirus, another mouse pathogen of research colonies, resulted in increased duration of shedding [106], highlighting the multiple effects MNV may have in research mice. Interestingly, MNV has a protective effect in mice with acute lung injury caused by *Pseudomonas aeruginosa*. Co-infection of MNV and *P. aeruginosa* results in increased survival and decreased pro-inflammatory cytokine production [107], which shows that

MNV infection may alter experiments' results and lead to incorrect interpretation of data obtained from mouse models of disease that are not performed with MNV-free animals.

Not surprisingly, MNV eradication from research colonies has proven difficult. The high environmental resistance, the lack of overt clinical signs in many mouse strains, and the wide distribution of MNV in mouse research colonies represent formidable challenges to the effective control and eradication of MNV. Complete depopulation and subsequent facility decontamination has been shown to successfully eradicate MNV, while selective testing and culling of MNV-positive animals was unsuccessful [108]. Furthermore, embryo rederivation [109] and caesarean section [110] are effective in eliminating MNV from mouse colonies, while cross-fostering has been met with varying degrees of success [111,112].

Despite the recent development of long-needed cell culture system and mouse model to study HuNoV in the laboratory setting [88,113], these models have intrinsic limitations [91]. The high cost posed by animal-based studies in association with the need of mice bearing gene deletions to support HuNoV infection limit the use of the mouse model for certain applications. Also, these animals do not become infected by the oral route, the natural route of NoV infection. Therefore the intraperitoneal route must be used [88,114]. Additionally, the cell culture system provides only a modest increase in viral titer, presents high variability in results between experiments, and only one strain of HuNoV has been successfully grown in culture [113]. Thus, the MNV model system remains a powerful tool to study NoV biology *in vivo* and in cell culture. Much has been learned in the past decade about basic aspects of NoV biology, such as binding, internalization, host innate and adaptive immune responses to NoV infection, amongst many more areas of research. Recently, MNV-based studies have provided important information on the crucial role type III interferon (interferon λ) response plays in NoV ability to establish persistent infection [115,116] and how MNV has a beneficial role in intestinal homeostasis and mucosal immunity, similar to the role played by commensal bacteria [117].

The fact that MNV infects a small animal and can be readily propagated in cell culture [92], combined with the genetic tractability of mice [89] and the presence of several robust reverse genetic systems [118-123] underscores the utility of MNV as a model system to study diverse aspects of NoV biology. MNV-based studies will continue to contribute greatly to a better understanding of the fields of NoV and enteric virus biology.

1.2 The intestinal barrier

The intestinal barrier is a physical and functional barrier that regulates molecular transport and serves as the first line of defense between the host interior and the lumenal environment [124-127]. It comprises the mucus layer and the epithelial layer, which act in synergy to maintain physiological and immunological homeostasis of the gastrointestinal tract [125,128] (Fig. 1.4). Enterically transmitted pathogens must breach the intestinal barrier in order to gain access to their site of colonization or to target cells (in the epithelium, lamina propria, or extra-intestinal milieu).

1.2.1 The mucus layer

The mucus layer is a thick and viscous, complex, gel layer that covers the entire gastrointestinal tract, from the stomach to the rectum. It can be separated into: i) an outer layer, which is colonized by commensal bacteria and continuously replaced; and ii) an inner layer, which is firmly attached to the intestinal epithelial cells, is relatively devoid of microorganisms, and like the outer layer, it is constantly renewed. Several biologically active molecules comprise the mucus layer and together they form a barrier that helps defend the host against injuries and/or infection. Mucins glycoproteins, anti-microbial peptides (e.g. defensins, collectins, lysozyme), antibodies (secretory IgA), and trefoil factors are some components of this complex fluid physicochemical barrier [125,128,129] (Fig. 1.4).

1.2.2 The epithelial layer

Underlying the mucus layer is the intestinal epithelial layer, which is responsible for the synthesis and secretion of the mucus layer's components, along with lamina propria leukocytes [128,129]. Intestinal epithelial cells form a contiguous single layer of columnar epithelial cells connected by tight and adherens junctions, acting as a barrier between the lumen and host interior [124,125,128-130] (Fig. 1.4). Additionally, the intestinal epithelium is responsible for production of digestive enzymes, absorption of nutrients, sampling of antigens, and initiation and guidance of mucosal immune responses [125,128,129]. To exert these diverse roles, several distinct cell lineages comprise the intestinal epithelial layer. Each has unique contributions to barrier integrity, nutrient absorption, and mucosal immunity [125,131]. Multipotent stem cells

present in the intestinal crypts give rise to the different intestinal epithelial cell types [125,130-132].

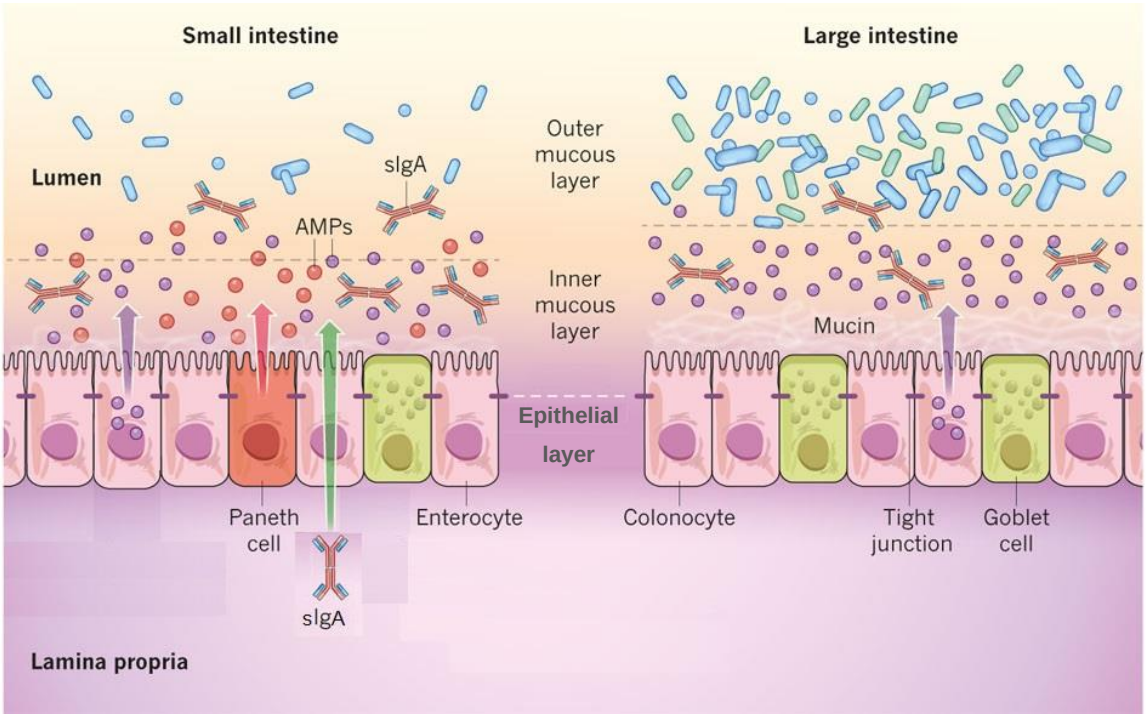

Figure 1.4. The intestinal barrier divides the lumenal environment from the host interior (the lamina propria). The mucus layer is the outer most part of the intestinal barrier and is divided into an outer layer, and an inner layer. The intestinal epithelial layer lies underneath the inner mucus layer. It comprises distinct cell types that form a contiguous single layer of columnar intestinal epithelial cells that is divided by tight junctions into apical (facing the lumen) and basolateral (facing the host interior) regions. These cells are involved in mucus production, intestinal homeostasis, and continuous sensing of the microbiota to induce the production of antimicrobial peptides (AMPs). Additionally, intestinal epithelial cells are important for shuttling secretory IgA (sIgA) across the epithelial layer to promote adequate immune responses to commensals, pathogens and food antigens. Figure modified from Maynard *et al*, 2012 [133].

Enterocytes are the most abundant intestinal epithelial cell type, present both in the small and large intestines. They are involved in nutrients absorption, antimicrobial peptides secretion, and in mucosal immune responses (e.g., by transporting secretory IgA from the lamina propria into the intestinal lumen) [125,130,131]. Goblet cells are also present in the small and large intestines, with the number of cells increasing from the duodenum (4%) to the descending colon (16% of total intestinal epithelial cells). Goblet cells are responsible for producing and secreting mucins, the major constituent of the mucus layer. They also produce trefoil factors, which are secreted components important for epithelial growth and repair [125,130,131]. Recently, goblet cells have been implicated in antigen uptake (from the intestinal lumen into the host interior) by

a process named goblet cell-associated antigen passage [134]. Enteroendocrine cells are scattered throughout the intestine, comprising about 1% of the intestinal epithelial cell population [125,131]. They secrete peptide hormones that regulate digestion, food intake and intestinal motility [125,130,131]. Common progenitor stem cells differentiate while migrating upwards from the intestinal crypt and rely on diverse signaling pathways to differentiate into enterocytes, goblet cells or enteroendocrine cells [125,130,132]. Once these terminally differentiated cells reach the top of the intestinal villi (or crypt, in the case of the large intestine), they undergo programmed cell death and are extruded from the intestinal epithelial layer into the lumen [125,130]. Paneth cells also differentiate from crypt stem cells, but these migrate downwards to the bottom of the crypt base [125,130]. They are found exclusively in the small intestine and secrete antimicrobial peptides, digestive enzymes and growth factors involved in intestinal epithelial cell proliferation and renewal [130,131].

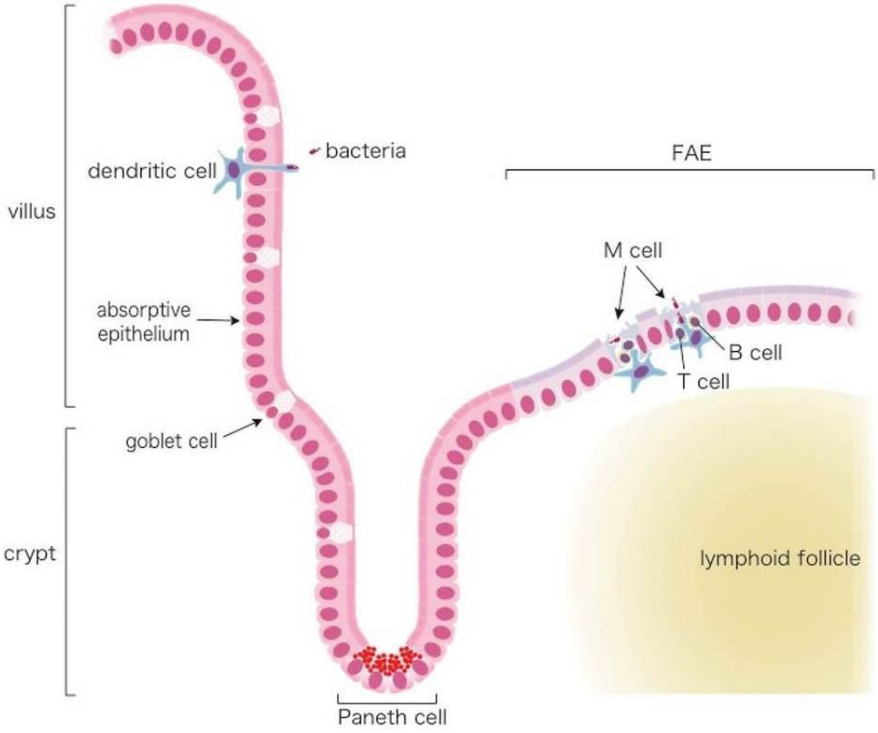

Figure 1.5. The follicle-associated epithelium. The intestinal epithelial barrier overlying gut-associated lymphoid follicles (e.g., Peyer's patches) is called follicle-associated epithelium (FAE) and is formed by M cells and enterocytes. The FAE has unique characteristics that distinguish it from other portions of the intestinal epithelial barrier: reduced glycocalyx and microvilli on M cells apical side and a thinner mucus layer. Figure from Ohno, 2016 [135].

Last, there are microfold (M) cells in the intestinal epithelium, which are the focus in this dissertation. M cells are intestinal epithelial cells specialized for antigen sampling [125,135]. They differentiate from intestinal crypt stem cells that are in close proximity to lymphoid follicles (such as Peyer's patches and cecal patches). The follicle-associated epithelium (FAE)

comprises M cells and enterocytes overlying these secondary lymphoid tissues [125,131] (Fig. 1.5). The intestinal epithelial barrier formed by the FAE is slightly different from the barrier found in other areas of the intestine. This is due to the unique features of FAE, particularly M cells.

Underneath the mucus layer, the intestinal epithelial barrier is covered by glycocalyx, a thick layer of membrane-anchored and secreted glycoproteins [128,129]. M cells have thinner glycocalyx, and because the FAE lacks goblet cells, the mucus layer covering this region is also reduced [128,129,135]. Unlike enterocytes, M cells have reduced microvilli in their apical (lumenal) surface. Another distinctive feature is the pocket-like structure located in the basal membrane (facing the lymphoid follicle), which is occupied by lymphocytes, dendritic cells and macrophages [125,128,135,136]. As a result of this basal pocket, the M cell cytoplasm is very thin [135] (Fig. 1.6).

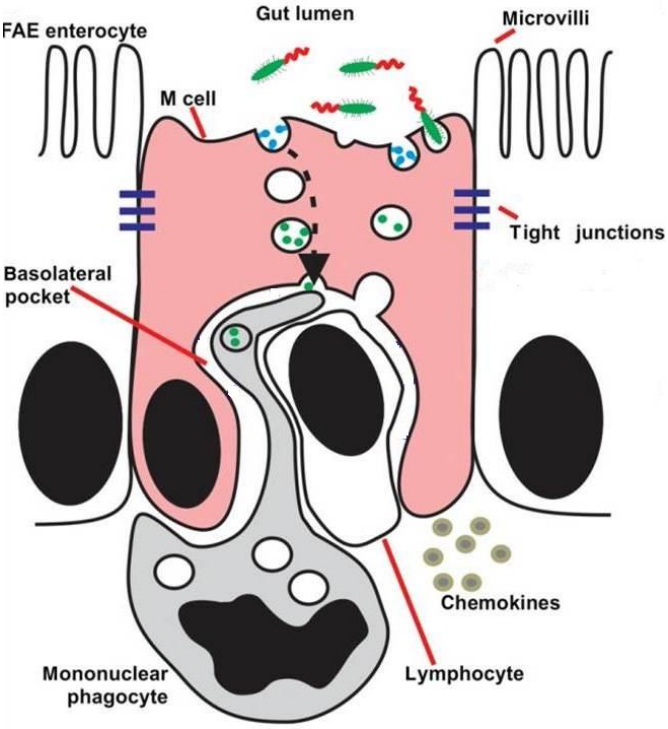

Figure 1.6. The morphology of a microfold (M) cell. M cells have reduced microvilli and glycocalyx (not shown) on their apical side. On their basolateral side, M cells have a basolateral pocket where mono-nuclear phagocytes and lymphocytes acquire transcytosed antigens. Figure modified from Mabbot *et al*, 2013 [137].

M cells are highly specialized epithelial cells. By a process known as transcytosis, M cells perform transepithelial transport of lumenal antigens and intact microorganisms, without disrupting the intestinal epithelial barrier [131,135]. In simple terms, transcytosis can be described as the uptake of macromolecules and their vesicular transport from one side of the cell to the other [138]. The transcytosed cargo exits M cells by exocytosis and is delivered to immune cells located in the M cells' basal pocket. Due to their high transcytotic capacity, M cells are an efficient portal through which lumenal antigens are taken up and presented to the immune cells in the underlying lymphoid follicle. This leads to the induction of tolerance or mucosal and/or systemic immune responses [131,135,137,139].

1.2.3 M cells development and differentiation

The differentiation process of M cells is regulated by interactions between the FAE and cells in the underlying lymphoid follicle. Particularly important to this process are stromal cells located on the subepithelial dome, the region between the FAE and the B cell follicle. These cells are required for differentiation of the common crypt stem cell progenitor into lymphoid follicle-associated M cells [135,137,140]. For the purpose of this dissertation, only Peyer's patch-associated M cells (herein referred to as M cells) and the most common mechanisms of cellular differentiation will be described.

The subepithelial dome stromal cells express high levels of RANKL (receptor activator of nuclear factor-κB ligand), which is an essential molecule for M cell development [135,137,140,141]. RANKL signals via its receptor, RANK, a molecule expressed by epithelial cells throughout the intestine. However, because of RANKL's selective expression by subepithelial dome stromal cells underlying the FAE, M cell differentiation is restricted to the epithelium overlying Peyer's patches [137,141].

Distinct transcription factors regulate the expression of genes that determine the differentiation of the common crypt stem cell progenitor into the different types of terminally differentiated intestinal epithelial cells. This is also the case for terminally differentiated M cells [137,140]. Spi-B, a member of the E26 transformation-specific (Ets) transcription factor family, is induced by RANKL and expressed by M cells. Spi-B is required for commitment to the intestinal M cell lineage [135,137,140]. In addition to RANKL-RANK signaling- and Spi-B-dependent differentiation, M cells rely on leukocytes in the underlying lymphoid follicles to differentiate into functionally mature M cells. For example, B cells promote M cell maturation. However, the precise mechanisms by which B cells (and other hematopoietic cells) influence M cells differentiation/maturation remains to be elucidated [135,137].

M cells express specific molecules that are used to identify them and their stage of maturation [135,137,142]. In mice, some of these M cell-specific markers function as receptors for the uptake of microorganisms/antigens [135,137]. Among intestinal epithelial cells, glycoprotein 2 (GP2), a GPI-anchored protein, is expressed exclusively on the apical membrane surface of functionally mature M cells. GP2 is widely used as an universal mature M cell marker and acts as a bacterial uptake receptor, and it is important for initiating mucosal immune responses against some commensal and pathogenic bacteria [135,137,140,143]. Another

commonly used M cell marker is the lectin UEA-1 (*Ulex europaeus* agglutinin I), which binds to α-1,2 fucosylated residues on the surface of murine M cells [137,140,142]. However, UEA-1 binding is not specific to M cells, because it also binds to other intestinal epithelial cells such as Paneth and goblet cells [143].

1.2.4 M cells are a gateway to pathogen entry in the intestine

M cells are key players in the generation of mucosal immune responses. However, they also represent a weak point in the intestinal epithelium [131]. Their morphological features (reduced microvilli and glycocalyx layer) and transcytosis ability make them a gateway to pathogen entry [125,128,131,137]. Pathogens have developed different mechanisms to cross the intestinal epithelial barrier and productively infect their host, including using the M cells' antigen sampling properties [124,125,131,137,139]. Disruption of intercellular junctions and interaction with epithelial cells receptors (leading to transcellular or paracellular transport) are other examples of mechanisms utilized by pathogens to breach the intestinal epithelial barrier [124].

Bacteria (e.g. *Yersinia enterocolitica*, *Salmonella typhimurium*, *Shigella flexneri*, *Vibrio cholera*), prions, viruses (e.g. HIV-1, murine norovirus [MNV], poliovirus, reovirus) and parasites (e.g. *Cryptosporidium* spp.) are known to use M cells as a portal of entry [128,136,137,144]. Additionally, in the absence of M cells, oral infection by *Y. enterocolitica*, prions, reovirus and murine norovirus is either blocked or reduced [137,144]. Interestingly, some pathogens can modulate the M cells' antigen sampling role. *S. typhimurium* and *Streptococcus pneumoniae* strain R36a are able to induce transdifferentiation of epithelial cells into M cells and/or increase M cell transcytotic activity, thus promoting host colonization and invasion [137,145].

Recently, the role of M cells in norovirus infection has been investigated. Using MNV, our group has started to unveil how noroviruses breach the intestinal barrier and productively infect their host [114,144]. Two distinct MNV strains, MNV-1 and MNV.CR3, use M cells and/or gut-associated lymphoid tissue to productively infect wild-type BALB/c mice and $Rag2^{null}/\gamma c^{null}$ (mice deficient in recombination-activating gene 2 and the common cytokine receptor gamma chain) on a BALB/c background. MNV-1 and MNV.CR3 intestinal infection is decreased in BALB/c mice depleted of M cells following treatment with a neutralizing antibody against RANKL compared to untreated or isotype-treated animals [144]. In another study from

our group, BALB/c Rag2^{null}/γc^{null} mice were used to confirm the role M cells play in MNV intestinal infection. These mice, among many other immune defects, do not have Peyer's patches and other secondary lymphoid follicles. Consequently, they do not have lymphoid follicle-associated M cells and are devoid of *GP2* mRNA [114,146,147]. Oral infection by MNV-1 and MNV.CR3 is completely blocked in these mice [114]. However, both MNV strains are able to infect Rag2^{null}/γc^{null} mice once the intestinal epithelial barrier is bypassed by intraperitoneal infection. Therefore, these studies concluded that Peyer's patch M cells and/or gut-associated lymphoid tissues represent the main route through which MNV breaches the intestinal epithelial barrier to productively infect its host [114,144].

The first aim of my dissertation was to expand the current knowledge about M cells role in MNV infection *in vivo*. Specifically, I investigated the role M cells play on MNV breaching the intestinal barrier in a conditional knockout mouse model of M cell deficiency. These findings are described in chapter two of this dissertation.

1.3 Attachment and internalization: the initial steps of a virus infectious cycle

In addition to studying how norovirus breaches the intestinal barrier to gain access to its target cells, it is essential to study how norovirus subsequently binds to and enters host target cells. These early steps of infection are thought to be key determinants of norovirus' strict species-specificity and cell tropism [23].

A virus infectious cycle comprises several steps: attachment, internalization, uncoating, transcription, translation, genome replication, viral progeny assembly and particles release. The first two initial steps of virus entry are attachment and internalization [148], and the identity and distribution of entry receptors are key determinants of which cell types, tissues, and species viruses can infect [149]. Virus entry into the target cell is a multistep process that begins with binding of the particle to attachment receptors, which helps to concentrate viral particles on the cell surface [148-153]. Subsequent interaction with additional receptor molecules (e.g., functional or entry receptors) actively promotes virus uptake by initiating conformational changes in the virus capsid, activating cellular signaling, promoting endocytosis, or directly driving penetration [150,152]. Several different molecules present on the target cell surface can act as attachment receptors for viruses. The same receptor may be used by different viruses,

while some viruses may utilize multiple (individual) receptors with equivalent roles, or multiple receptors that are each essential for virus entry [150,154].

There is no absolute consensus in the literature about the nomenclature used to identify molecules involved in virus entry. Receptors, coreceptors, attachment factors, entry factors, among other terms, are used, making the nomenclature confusing. Therefore, for the purpose of this dissertation, the following nomenclature will be used throughout the text: i) attachment receptor is any molecule to which viruses bind, but it does not actively promote virus internalization nor trigger cellular signaling pathways; ii) a functional (or entry) receptor is any molecule that binds to viruses (directly or by a bridging molecule), and it is essential for virus uptake and productive infection to occur; iii) a coreceptor is a term used when multiple functional receptors are required and, by convention, the first contacted one is called the functional receptor and the subsequent ones are called coreceptors [155].

1.3.1 Virus attachment

The first step of a virus infectious cycle is the physical interaction of the particle with surface molecules of the target cell. This process is called attachment or binding [148-151,156,157]. In enveloped viruses, the envelope glycoproteins bear the binding sites to cell receptors. In the case of non-enveloped viruses, these are found in projections and indentations on the surface of the capsid protein [149,154]. Binding between viral proteins and cellular attachment receptors generally occurs via electrostatic interactions that are typically of low affinity and may be unspecific [148,150,152,157]. However, the multiple binding sites on virus particles result in high avidity and subsequent concentration of particles on the cell surface [149,151,156].

Viruses commonly use glycoconjugates (glycoproteins, glycolipids, and proteoglycans) as attachment receptors, with the sugar moieties playing a central role in virus binding to the target cell surface [149,150,152,157]. Heparan sulfate is a polysaccharide commonly used by different viruses to attach to the cell surface. Herpes simplex virus, papillomaviruses, paramyxoviruses, and dengue virus, among others, utilize this molecule to bind to the surface of target cells [151,158]. Sialic acid is another carbohydrate moiety that is frequently used by viruses as attachment receptor [148,149,152]. Murine norovirus (MNV), feline calicivirus,

several serotype 3 reovirus strains, and influenza are some examples of viruses that utilize sialic acids to bind to the cell surface [99,159-162].

In case of noroviruses, different MNV strains show distinct carbohydrate binding phenotypes [91]. MNV-1, a lab adapted strain [95], binds to terminal α 2,3-linked sialic acid residues on the ganglioside GD1a and on both N- and O-linked glycoproteins [48,99]. MNV strains WU11 (MNV.WU11), and S99 (MNV.S99) are two field isolates that have similar binding phenotypes to MNV-1 [99,163,164]. All three MNV strains use terminal α 2,3-linked sialic acid residues on the ganglioside GD1a as attachment receptors on murine macrophages [99]. In contrast, MNV strain CR3 (MNV.CR3), another field isolate, uses N-linked glycoproteins to infect murine macrophages [48,98]. Yet, MNV.CR3 resistance to neuraminidase treatment remains to be fully characterized. The virus may be binding to yet-to-be-defined carbohydrate moieties on N-linked glycoproteins, such as internal sialic acid moieties, as attachment receptors in murine macrophages [48,91]. Furthermore, most HuNoV strains attach to complex sugar moieties present on ABH and Lewis histo-blood group antigens, and some HuNoV strains can also use sialydated histo-blood group antigens (e.g. sialyl Lewis^{x}) for attachment [44,46,165-171].

Many receptors play a dual role, acting as attachment and functional receptors. These molecules, upon virus binding, are able to induce conformational changes in the viral envelope or capsid (glyco)protein and trigger cellular signaling pathways that culminate in breach of the cellular membrane by diverse mechanisms and virus internalization [149-152,156,157]. An example of a receptor that plays the role of an attachment and functional receptor is poliovirus receptor (PVR or CD155). Poliovirus binds to CD155, and the receptor-virus interaction causes conformational changes in the virus capsid that lead to virus internalization by an unusual endocytic pathway (clathrin-, caveolin-, flotillin-, and microtubule-independent) [157,172-174].

Other viruses exhibit more complex receptor requirements, using at least two different plasma membrane molecules, each of which is essential for virus entry in the target cell. Classic examples of such complex requirements are human immunodeficiency virus type-1 (HIV-1) and hepatitis C virus [150,151]. The HIV-1 envelope glycoprotein (consisting of gp120/gp41 heterodimers) binds to CD4 on the cell surface, and binding leads to conformational changes in the viral protein. A high affinity binding site in gp120 becomes exposed and further interaction with a coreceptor, usually CCR5 or CXCR4, allows structural changes in gp41, the envelope

component that promotes fusion [150,151,156]. In the case of HCV, the virus initially binds to hepatocytes by attachment receptors, such as glycosaminoglycans. Subsequent interaction with at least four surface proteins, likely in a sequential manner, leads to virus entry [175]. The HCV entry receptor complex includes the scavenger receptor class B type I (SR-BI), the tetraspanin CD81, and two tight junction proteins (claudin-1 and occludin). Coexpression of these four proteins confers permissivity for HCV entry [150]. SR-BI and CD81 interact directly with viral envelope glycoprotein E2. The first is involved in virus binding to hepatocytes, but also plays a role in post-binding events, potentially promoting interaction with CD81. The latter may be involved in initiating fusogenic activity of the viral glycoproteins. Claudin-1 and occludin are likely involved in later stages of the virus entry process, during internalization. There is currently no evidence supporting the direct binding of claudin-1 and occludin to HCV virions, and it is thought that they may act by regulating the activity of SR-BI and CD81 [151,175].

Multiple receptor usage is often associated with the need for viruses to overcome barriers imposed by the cell type or tissue they infect [152]. A fascinating example of this intricate mechanism is the entry process of coxsackievirus B (CVB). CVB is transmitted by the fecal-oral and respiratory routes, and it must breach the epithelial barrier in order to initiate infection. CVB needs to gain access to tight junctions of the epithelial cells in order to bind to the coxsackievirus and adenovirus receptor (CAR), which mediates virus internalization. CAR, however, is not expressed on the lumenal (apical) surface of the epithelial cells, therefore it is inaccessible for viruses present in the intestinal/airways lumen [150,176]. CVB overcomes this barrier by first binding to decay-accelerating factor (DAF or CD55), which is expressed on the lumenal surface of epithelial cells [150,152,174,176]. CVB-DAF interaction on the cell surface activates Abl kinase and drives Rac-dependent reorganization of the actin cytoskeleton, which permits the virus to move to tight junctions [176]. CVB is then able to bind to CAR, and the interaction between virus and receptor triggers conformational changes on the viral capsid and leads to virus internalization [148,152,176]. Additionally, CVB-DAF binding activates Fyn kinase, which facilitates CVB endocytosis [150,176].

Lateral movement of viruses along the cell membrane is observed not only for CVB, but for several other viruses [150,151]. For example, HIV-1 and murine leukemia virus bind to filopodia and “surf” on the outside of these projections towards the cell body [177]; adenovirus type 2 has been shown to move to plasma membrane domains proficient for endocytosis [178].

This lateral motion is thought to help viruses encounter molecules necessary for productive infection and/or to position particles in areas where they are more likely to become endocytosed or fuse/penetrate the cell membrane [150].

Another mechanism many families of enveloped viruses have developed to promote infection is called apoptotic mimicry. This lipid-mediated entry mechanism is used by flaviviruses, filoviruses, poxviruses and lentiviruses, among many others [179,180]. The exposed phosphatidylserine (PS) on the viral envelope surface resembles PS present on the surface of host cells undergoing apoptosis. PS exposure on the cell surface is a major hallmark of apoptosis. It engages PS receptors that initiate clearance of apoptotic cells and culminates in engulfment of these cells by phagocytes. Apoptotic mimicry allows viruses to use apoptotic clearance mechanisms to facilitate infection of target cells [179-181]. Two types of PS receptors have been described: those that bind PS directly (e.g. T cell immunoglobulin and mucin receptor (TIM) proteins); and those that use bridging molecules to bind to PS (e.g. Tyro3-Axl-Mer (TAM) family of proteins binds PS via the bridging molecules Gas6 and protein S) [179,182]. Viruses may use one or both types of PS receptors to promote attachment and internalization into the phagocytes involved in apoptotic clearance [150,179,181,183,184].

1.3.2 Virus internalization

After binding to attachment receptors on the surface of the target cell, viruses must interact with a functional receptor(s) to initiate the internalization process [148]. In simple situations, engagement of the functional receptor triggers internalization of the virion-receptor complex by activating the cellular uptake machinery [148,150]. Alternatively, receptor engagement may activate signaling pathways that facilitate virus uptake, or it may directly drive fusion/penetration at the target cell surface [150]. Thus, viruses can be internalized by two main routes: an endocytic route and a non-endocytic route [148,152,156,157,185]. The majority of viruses, though, rely on endocytic internalization; most likely because endocytosis offers several advantages, such as a “free ride” to the cytoplasm and less exposure of viral components on the cellular surface (therefore preventing/delaying the host immune response), among others [149,151,157].

In simple terms, endocytosis can be defined as the uptake of extracellular material (cargo) by invagination of the plasma membrane to form a new, small, intracellular membrane-limited

vesicle [186]. When using an endocytic route, the choice of which endocytic pathway (and, consequently, the intracellular routing) a virus takes is largely dependent on the receptor(s) it interacts with on the surface of the target cell [152,185]. Therefore, viral internalization by endocytosis is variable and depends on trafficking of the functional receptor/coreceptor.

There are several endocytic pathways that viruses can exploit (Fig. 1.7). These are constitutively active or can be induced. Some viruses are internalized by ongoing endocytic activities, which may limit the rate of virus uptake. Thus, many viruses have developed the capacity to induce their own uptake into endocytic vesicles by activating cellular signaling pathways [150,152].

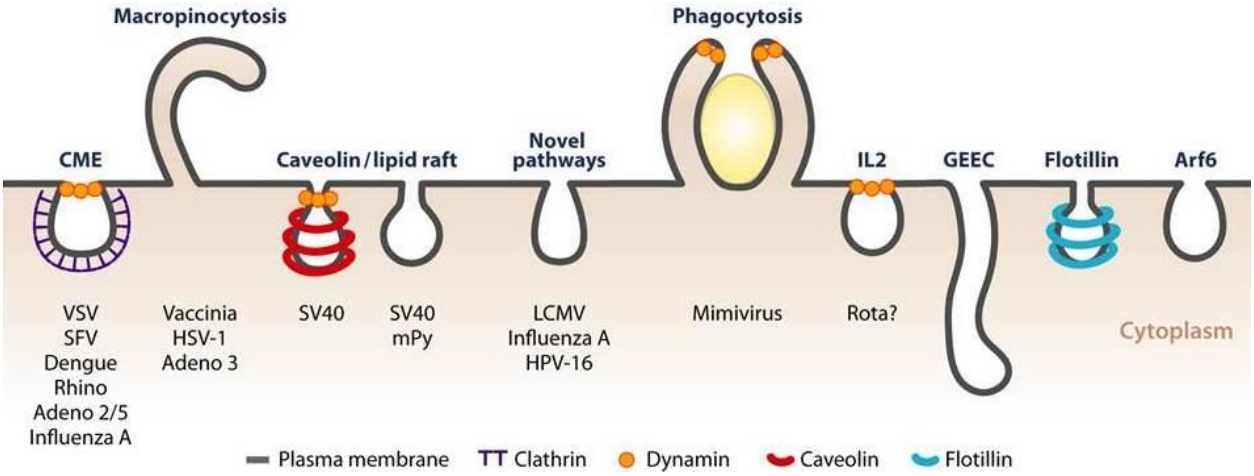

Figure 1.7. Endocytic pathways and viral uptake. Endocytosis in animal cells occurs via several distinct mechanisms, including: clathrin-mediated endocytosis (CME); macropinocytosis, caveolar/raft-dependent-endocytosis; novel pathways (which are unknown to be associated with the other known endocytic pathways); and non-classical, poorly defined pathways, such as interleukin 2(IL-2)/IL-2 receptor-dependent endocytosis, GPI- enriched endocytic compartments (GEEC)-dependent endocytosis, flotillin-dependent endocytosis, and ADP- ribosylation factor 6 (Arf6)/GRAF1-dependent endocytosis. Viruses known to use these mechanisms are listed under each pathway. Phagocytosis is also known to be used by some viruses to infect cells. Abbreviations: Adeno 2/5, adenovirus 2/5; Adeno 3, adenovirus 3; HPV-16, human papillomavirus 16; HSV-1, herpes simplex virus 1; LCMV, lymphocytic choriomeningitis virus; mPy, mouse polyomavirus; SFV, Semliki Forest virus; SV40, simian virus 40; VSV, vesicular stomatitis virus. Figure from Mercer *et al*, 2010 [152].

Although most viruses are taken up either by endocytic or non-endocytic route, some viruses may exhibit flexibility in their internalization mechanism. This plasticity in uptake mechanisms may be advantageous to viruses, because it potentially provides access to a wider range of cell types or makes them less susceptible in the event a given pathway is absent or blocked. Herpesviruses and HIV-1 are examples of viruses that can use endocytosis or may fuse

directly with the plasma membrane, depending on the cell system utilized [150-153]. The following sections will discuss, from a virological perspective, the most commonly used endocytic routes of virus internalization, as well as phagocytosis. Viruses' internalization by endocytic routes is much better understood and characterized than viral direct crossing of the plasma membrane. The latter remains to be studied in fine detail, especially in the case of non-enveloped viruses [149,151], which is the focus of this dissertation and, therefore, it will not be discussed further.

1.3.2.1 Virus internalization by endocytic routes

Clathrin-mediated endocytosis (CME) is the most common endocytic route taken by viruses [151,157]. Endocytosis starts with generation of endocytic vacuoles that are pinched off from the plasma membrane. It is a complex multistep process that involves receptor activation, cargo uptake and sorting, induction of membrane curvature and scission from the plasma membrane [187,188]. The endocytic vacuoles are then targeted to endosomes, and from there, cargo is further sorted to the destination organelles or the cytosol [189].

Clathrin is the principal molecular scaffold for cell membrane reorganization and cargo transport. Sorting (internalization) signals in the receptor cytoplasmic tails initiate cargo selection, inclusion, and recruitment of various adaptor proteins (e.g. AP2). These adaptor proteins bind to the receptor sorting signals, while associating with clathrin. Clathrin then forms a lattice-like coat surrounding the plasma membrane [153,190]. Clathrin-coated pits vary in their composition, with up to 60 different adaptor proteins being recruited to the site of CME. There are increasing numbers described of alternative cofactors, adaptors, tethering proteins, and kinases involved in the formation of clathrin-coated pits/vesicles, and in the regulation of CME [151,153,188]. Budding of coated vesicles from the plasma membrane involves the multidomain GTPase dynamin, and a cycle of oligomerization and GTP hydrolysis. After scission, synaptojanin, auxilin, and HSC70, among other cellular factors, help disassemble the clathrin coat from the vesicle, which is transported to early endosomes [153,190].

CME of viruses is generally a fast process. From virus attachment to receptors and recruitment of clathrin to the membrane and subsequent scission, the endocytic vesicle formation takes about one minute. Subsequent delivery to the early or late endosomes happens within two

to five minutes, or 10-20 minutes, respectively. The changes in pH during the endosomal maturation process culminate with penetration and viral genome uncoating [151-153].

CME occurs constitutively in cells, but cargo such as viruses can induce the formation of clathrin-coated pits [151,153]. For example, CME-internalized influenza A is mainly taken up by clathrin-coated pits assembled underneath the surface of the viral particle. To a lesser extent (about 5%) particles are associated to and internalized by pre-existing clathrin-coated domains in the membrane. However, it is unclear whether clathrin recruitment is triggered by the virus-bound receptor or by a more complex signaling cascade leading to clathrin recruitment [151]. In addition to internalization by CME, influenza virus has also been described to use clathrin-independent routes into the same cell, at the same time [151,153].

Some viruses use clathrin-independent endocytosis pathways for internalization. Of these, the caveolar/raft-dependent pathway is the best studied [151]. Caveolae are invaginations of the plasma membrane with a bulb-like morphology, formed by caveolins and other related proteins known as cavins. They function in endocytosis and transcytosis, in addition to maintaining membrane lipid composition and acting as signaling platforms [191,192]. Caveolar-dependent endocytosis differs from CME in many aspects. Formation of endocytic vesicles depend on the presence of cholesterol, lipid rafts and tyrosine phosphatase/kinase signaling pathways; and cargo internalization is slower. Budding is generally dynamin-2 dependent [151-153,157]. Cargo passes through early and late endosomes, but is commonly transported to the endoplasmic reticulum (ER). After internalization, penetration (through the organelle membrane) and viral genome uncoating may take up 12 hours to occur, depending on the virus and cell type [152,153,185]. Of note, raft-dependent endocytosis is mechanistically similar to caveolae-dependent endocytosis, but it is caveolin-independent [153].

Unlike CME, caveolar-dependent endocytosis is not a constitutive process, but ligand triggered [152,157]. Non-viral ligands include GPI-anchored proteins, insulin receptor, shiga and cholera toxins, cholesterol, albumin, among others [191]. Many viruses that use this internalization pathway make use of glycosphingolipids as their receptors. Among these, polyomaviruses are the best characterized viruses that make use of caveolar-dependent endocytosis [151,152]. However, internalization by caveolae-dependent endocytosis has been reported for other viruses, such as Echovirus 1 (an enterovirus), CVB, and papillomaviruses [149,151].

The polyomavirus SV40 associates with detergent-resistant microdomains in the plasma membrane, binding to its receptor, the ganglioside GM1. Particles enter uncoated, tight fitting indentations and pits, which by electron microscopy, give the impression that virus buds into the cell [150-153,185]. SV40 induces membrane curvature and tyrosine kinase activity, which leads to recruitment of actin and dynamin to virus-containing caveolae. This culminates in scission from the plasma membrane and formation of the caveolae-derived endocytic vesicle [150,152,157]. Although SV40 particles are internalized by caveolae-dependent endocytosis in many cell types, most particles are taken up by a closely related mechanism that is caveolae- and dynamin-independent. This alternative endocytic pathway yields vesicles morphologically indistinguishable from caveolae-containing vesicles. They are also mechanistically similar, both being cholesterol- and tyrosine kinase-dependent [152,153].

MNV-1 is another example of a virus that uses clathrin-independent endocytosis for internalization; however, this mechanism has yet to be fully characterized. Following binding, MNV-1 is taken up by host cells through a mechanism that is dependent on dynamin II and cholesterol, but it is not dependent on clathrin or caveolin [193,194]. Additionally, MNV-1 infection of macrophages and dendritic cells is pH-independent [194,195], although when the endosomal acidification inhibitor chloroquine is present throughout the infection period, MNV-1 titers are reduced in the murine macrophage-like cell line RAW 264.7 cells [196]. These contradictory results suggest that pH may play a role in MNV-1 infection under specific experimental conditions.

Another endocytic pathway used by viruses is macropinocytosis. Macropinocytosis is a transient, growth factor induced, actin-dependent endocytic pathway characterized by great actin skeleton rearrangements and cell-wide plasma membrane ruffling. It is usually involved in the uptake of large volumes of extracellular fluid and bulky cargo in large vacuoles called macropinosomes [150,152,153,197]. Macropinosome formation is generally considered to be a nonspecific mechanism for internalization, because it neither relies on ligand binding to a specific receptor, nor is it guided by a cytoplasmic coat [152,157,197]. Macropinocytosis occurs constitutively in professional phagocytes, but in other cell types, it can also be induced by activation of tyrosine kinases [150,153,197]. After macropinosomes form, they move further in the cytoplasm and either are recycled back to the cell surface or feed the endosomal network. This makes macropinocytosis a potential internalization mechanism for a wide variety of viruses

[152,157]. Viruses that are internalized this way are vaccinia virus, Kaposi's sarcoma-associated herpesvirus, herpes simplex virus, and CVB, among others [152,153]. Rubella and species C human adenovirus types 2 and 5 take advantage of macropinocytosis for steps of their infectious cycle other than internalization [153].

Interestingly, macropinocytosis is a major route of entry into dendritic cells and macrophages. Therefore, the role this endocytic pathway plays during infection may prove to be secondary to its importance as a way to present antigens from the invading virus to the host immune system [157].

1.3.2.2 Virus internalization by phagocytosis

Phagocytosis is a mechanism used by specialized cell types, such as macrophages, for the uptake of large particles, such as bacteria [186]. Phagocytosis and macropinocytosis share several features, including the large size of vacuoles formed, transient activation, actin dependency, required cellular factors, and regulatory components. However, phagocytosis is receptor-driven. Attachment of the particle to the cell surface triggers and guides the vacuole formation, which is tightly fit around the particle with little or no fluid uptake [150,152,153,197]. Commonly, phagocytosis is not used for viral entry because of the generally small size of virus particles. However, viruses that have large particles, such as herpes simplex virus, the ameobal pathogen mimivirus, and poxviruses use this endocytic pathway to infect some cell types [150,152,153].

Research on the early steps of viral infectious cycle is fundamental, because it provides important information on host cell-virus interactions and pathogenesis [152]. Furthermore, research on attachment and functional receptors generates important information for fundamental biological processes, in addition to its potential role to develop new therapeutic treatments for viral diseases [151,198]. The second aim of my dissertation was to identify and characterize host cellular proteins that are involved in the early steps of MNV infection *in vitro*. Specifically, I investigated the role of four host cellular proteins during MNV infection of macrophages and dendritic cells. These findings are described in chapter three of this dissertation.

In this dissertation, I aimed to characterize the initial steps of MNV infection at the host and cellular levels. Specifically, my aims were able to improve the current understanding on how MNV crosses the intestinal epithelial barrier and how it binds to and enters host target cells.

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Chapter 2. Intestinal MNV infection is independent of M cells in a conditional knockout mouse model of M cell deficiency

2.1 Introduction

Microfold (M) cells, gatekeepers of the intestine, are highly specialized epithelial cells for antigen transport across the intestinal barrier by transcytosis [1,2]. Transcytosed antigens are delivered to immune cells present in the basal pocket of M cells. Thus, M cells play a crucial role in intestinal immunity, both in the induction of tolerance, as well as mucosal and systemic responses [2-5]. Despite their gatekeeping function, several pathogens highjack M cells' antigen sampling capacity to breach the intestinal barrier and initiate infection [1,3-6].

Noroviruses are small, non-enveloped, single-stranded, positive-sense RNA viruses that cause gastroenteritis in humans and other animals [7]. HuNoVs are the main cause of acute gastroenteritis worldwide [8] but lack easily manipulatable systems for their study. Thus, MNV is a commonly used model system to study norovirus biology in a natural host [9]. It is the only members of the *Norovirus* genus that replicate robustly in cell culture, showing a tropism for macrophages and dendritic cells [10]. Recently, the role of M cells in MNV infection has been investigated in our lab [11,12]. This work showed that M cells and/or gut-associated lymphoid tissues are essential for efficient MNV infection *in vivo*, and that M cells are the main mechanism through which MNV breaches the intestinal epithelial barrier to initiate infection [11,12].

In the current study, we sought to verify the role M cells play during MNV-1 and MNV.CR3 infection in a conditional knockout mouse model of M cell-deficiency. In these mice, RANKL-dependent M cell differentiation is abolished by conditional deletion of the gene encoding RANK (*Tnfrsf11a*) in the intestinal epithelium using the Cre-lox system [13]. These mice, called M-less mice, are devoid of intestinal M cells, although they still have secondary lymphoid follicles such as Peyer's patches and cecal patches [13]. Therefore, M-less mice are ideal for assessing the specific role of M cells during oral MNV infection.

C57BL/6 mice are the parental strain for the M-less mice we used in the current study. We first conducted a viral kinetics assay to identify the earliest time point when we could detect virus in C57BL/6 mice infected with MNV-1 or MNV.CR3. We then infected M cell-deficient mice (M-less mice) and littermate controls orally with MNV-1 or MNV.CR3 for 12 h (the earliest time point virus could be detected) and 24 h. Viral loads in the gastrointestinal (GI) tract were then measured by plaque assay. M-less mice infected with MNV-1 presented significantly decreased virus titer in the mesenteric lymph node (MLN) compared to littermate controls at 24 hpi. Additionally, the initial site of MNV-1 infection was the large intestine (cecum and colon). This finding differs from its parental strain (C57BL/6), whose initial site of MNV-1 infection is the small intestine. All other intestinal segments had comparable virus yield in M-less mice and littermate controls infected with either MNV-1 or MNV.CR3.

Our data suggest that MNV usage of M cells to breach the intestinal epithelial barrier may be mouse-strain specific, and that other mechanisms must exist to allow MNV to cross the barrier and gain access to its target cells in the lamina propria. Additionally, trafficking to the MLN differs between the two virus strains, with MNV-1 getting to the MLN at much earlier time points than MNV.CR3. We also observed a significant decrease in MNV-1 titers in the MLN of M-less mice compared to littermate controls. These two pieces of data suggest that MNV-1 and MNV.CR3 present different (initial) cellular tropism, because virus trafficking to the MLN differed in these two strains and only MNV-1 trafficking was affected by the lack of intestinal M cells. This study broadens our understanding of MNV pathogenesis and early interaction with its natural host. It also raises awareness of the possibility that the use of different mouse strains may impact the outcome of a given study.

2.2 Material and methods

Mice. C57BL/6 mice (000664) were purchased from Jackson Laboratories. Transgenic mice homozygous for a floxed allele of RANK ($RANK^{f/f}$) on a C57BL/6 background were a kind gift from Dr. Ifor Williams (Emory University). Villin-cre mice on a C57BL/6 background (004586) were purchased from Jackson Laboratories and bred to $RANK^{f/f}$ mice to generate mice lacking RANK expression in intestinal epithelial cells, therefore devoid of M cells (herein referred to as M-less mice) [13]. Littermates (Cre negative mice) were used as controls. Six- to eight-week old mice were used for all experiments. Animals were bred and maintained in SPF conditions at the

University of Michigan Medical School. All animal procedures were performed in compliance with local and federal guidelines and the standards of the NIH Guide for the Care and Use of Laboratory Animals [14]. The protocol was approved by the University of Michigan Committee on Use and Care of Animals (PRO00004534).

Viruses stock and plaque assay. Stocks were prepared from the plaque-purified MNV-1 (GV/MNV1/2002/USA) clone CW3 and the fecal isolate MNV.CR3 (GV/CR3/2005/USA) and used at passage 7 for all experiments. Light-sensitive, neutral red-containing MNV-1 and MNV.CR3 stocks were generated as previously described [15] with slight modifications. Briefly, RAW 264.7 cells were infected with MNV-1 or MNV.CR3 (MOI of 0.05) and incubated for 48 h in the presence of 10 μg/mL of neutral red dye (Sigma-Aldrich) in the dark. Cells were freeze-thawed twice to release virus, and virus titers were determined by plaque assay [16]. The neutral red-containing MNV stocks were then used to infect cells as described above, but all activities were carried out in the dark with a red darkroom safelight present. All light-sensitive MNV stocks exhibited a minimum five-log reduction in viral titer upon light exposure compared to the unexposed control.

Infection of mice with light-sensitive MNV. Infection was carried out as previously described [16], with minor modifications. C57BL/6 mice were infected with 1 x 10^5 PFU of light-sensitive MNV-1 or MNV.CR3 by oral gavage in the dark. Intestinal segments (duodenum, jejunum, ileum, cecum, ascending colon and descending colon), feces and MLN were harvested at 9, 12, 18, and 24 hpi in the dark and virus yield was assessed by plaque assay as previously described [12,16]. M-less mice and littermate controls were infected as C57BL/6 mice, using a fivefold higher inoculum: 5 x 10^5 PFU of light-sensitive MNV-1 or MNV.CR3. Tissues were harvested at 12 and 24 hpi in the same manner as described above.

Immunostaining of whole-mounts. Peyer's patches of M-less mice and littermate controls were harvested and identification of M cells was performed by immunostaining using anti-GP2 antibody and rhodamine-labeled UEA-1 as described [12].

***GP2* mRNA qPCR.** Peyer's patches of M-less mice and littermate controls were harvested and quantification of *GP2* mRNA was performed as described [11,13].

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. Data are presented as means ± SEM.

2.3 Results

MNV-1 and MNV.CR3 show different infection kinetics

To verify the role M cells play in MNV crossing the intestinal barrier, we used a transgenic mouse model of M cell deficiency on the C57BL/6 background. To study the early events of MNV-host interaction *in vivo*, it is essential to determine the earliest time point during infection when replicated virus can be detected. Towards that end, we used light-sensitive, neutral red-containing MNV-1 to differentiate replicated from input virus as described [15,16]. C57BL/6 mice were infected with light-sensitive MNV-1 or MNV.CR3 by oral gavage and tissues were harvested at 9, 12, 18 and 24 hpi (Fig. 2.1).

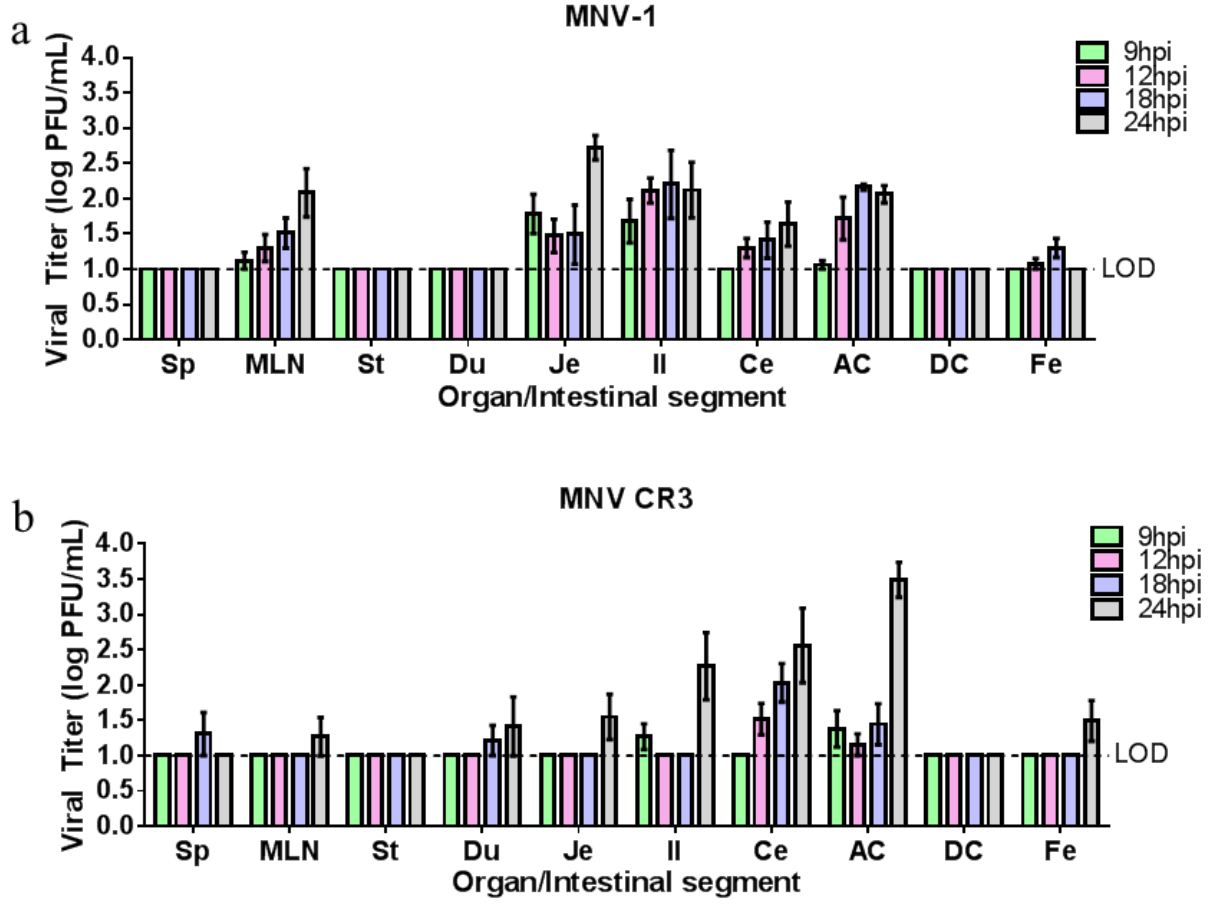

Figure 2.1. MNV-1 and MNV.CR3 have different infection kinetics *in vivo*. C57BL/6 mice were infected with 1 x 10^5 PFU of light-sensitive MNV-1 (a) or MNV.CR3 (b) by oral gavage. Tissues were harvested at indicated time points in the dark. Virus yield was assessed by plaque assay performed after light inactivation of input virus (by exposing samples' dilutions to light). Infection kinetics assay time points are color coded and grouped per organ/segment analyzed. Legend: Sp, spleen; MLN, mesenteric lymph node; St, stomach; Du, duodenum; Je, jejunum; Il, ileum; Ce, cecum; AC, ascending colon; DC, descending colon; Fe, feces; LOD, limit of detection. Five mice were analyzed per group. Results shown are from two independent experiments.

Replicated MNV-1 was detected in the small intestine (jejunum and ileum) as early as 9 hpi (Fig. 2.1a), demonstrating that the initial site of infection was the small intestine in C57BL/6 mice. On the other hand, the earliest time we could consistently detect replicated MNV.CR3 was at 12 hpi in the cecum (Fig. 2.1b). This was similar to findings from BALB/c mice [12]. To our surprise, MNV-1 and MNV.CR3 had different kinetics of trafficking to the MLN. Replicated MNV-1 was detected in the MLN as early as 9 hpi, and virus yield continuously increased throughout time. In contrast, MNV.CR3 trafficking to the MLN was delayed compared to MNV-1, with replicated virus starting to be detected as late as 24 hpi.

Overall, these results indicated that similar to BALB/c mice, MNV.CR3 infection kinetics is delayed compared to MNV-1 in C57BL/6 mice and that the initial site of infection and kinetics of trafficking to the MLN differs between these two MNV strains.

MNV-1 and MNV.CR3 productively infect a transgenic mouse model of M cell deficiency

Previous studies have shown that M cells and/or gut-associated lymphoid tissues are required for MNV infection of the intestine using an M cell antibody-depletion model [12] or $Rag2^{null}/\gamma c^{null}$ mice [11] as models for M cell deficiency. However, both model systems have intrinsic limitations. The depletion model is not able to completely remove M cells. Therefore the question of whether M cells are essential for MNV infection remains unanswered. The other model uses a knockout mouse with severe and extensive immune defects, including the lack of gut-associated lymphoid tissues. Therefore, the complexity of such a system makes it difficult to conclude whether the findings are related to the lack of M cells directly, to the lack of gut-associated lymphoid tissues, to any other immune defects these mice bear, or to a combination of these factors.

To further investigate the role M cells play in MNV breaching the intestinal barrier to productively infect its host, we took advantage of a recently developed conditional knockout mouse model of M cell deficiency based on the Cre-lox system. In these mice, intestinal M cells are absent due to deletion of the RANK gene exclusively in intestinal epithelial cells. Yet, these mice have gut-associated lymphoid tissues [13]. Therefore, this transgenic mouse model of M cell deficiency is ideal for directly assessing the role of M cells in a given biological context, such as enteric virus infection.

M-less mice and littermate controls were infected with 1 x 10^5 PFU of light-sensitive MNV-1 and tissues were harvested at 9 hpi (Fig. 2.2a). To our surprise, most animals did not have detectable levels of replicated MNV-1 in their GI tract regardless of their phenotype (M-less or littermate controls). We repeated the infection but extended the infection period to 12 h (Fig. 2.2b). About half of the animals of either phenotype had not detectable virus or had virus titers below the limit of detection for the plaque assay. Therefore, we repeated the infection with a fivefold increase in virus inoculum for 12 h. Specifically, M-less mice and littermate controls were infected by oral gavage with 5 x 10^5 PFU of light-sensitive MNV-1 or MNV.CR3 for 12 h (Fig. 2.3). M-less mice infected with MNV-1 had comparable virus yields to littermate controls in all intestinal segments analyzed, as well as in the MLN (Fig. 2.3a). The same was true for MNV.CR3 infected mice, where no difference in virus yields of M-less mice and littermate controls was observed (Fig. 2.3b). In contrast to what was observed in the C57BL/6 infection kinetics assay, MNV-1's initial site of infection was the large intestine, specifically cecum and ascending colon (Fig. 2.3a) of M-less mice and littermate controls.

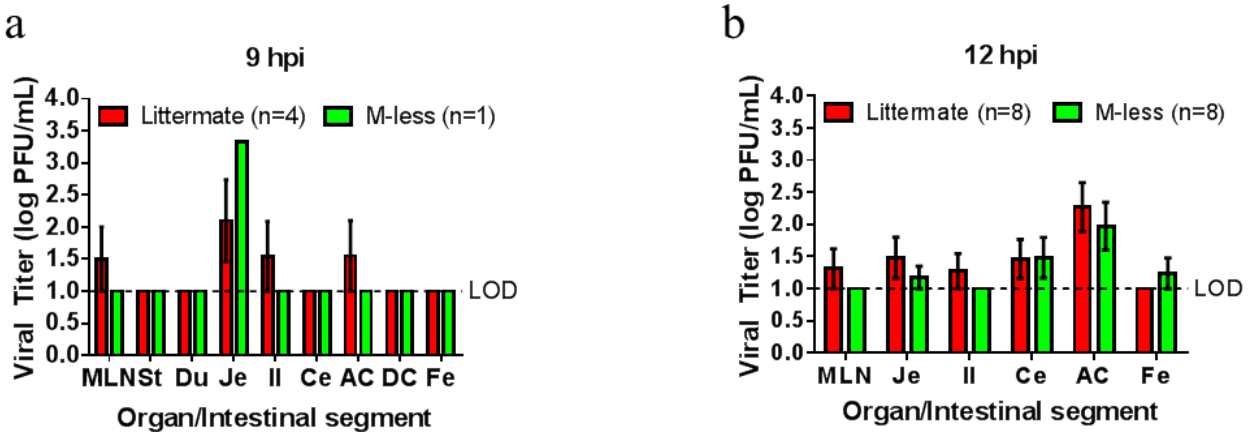

Figure 2.2. MNV-1 infection kinetics of the gastrointestinal tract of M-less mice and littermate controls. M-less mice and littermate controls were infected with 1 x 10^5 PFU of light-sensitive MNV-1 by oral gavage. Tissues were harvested at 9 hpi (a) and 12 hpi (b) in the dark. Virus yield was assessed by plaque assay performed after light inactivation of input virus (by exposing samples' dilutions to light). Legend: MLN, mesenteric lymph node; St, stomach; Du, duodenum; Je, jejunum; Il, ileum; Ce, cecum; AC, ascending colon; DC, descending colon; Fe, feces; LOD, limit of detection. The number of mice analyzed per group is indicated between parentheses. Results shown are from one (a) or three (b) independent experiments.

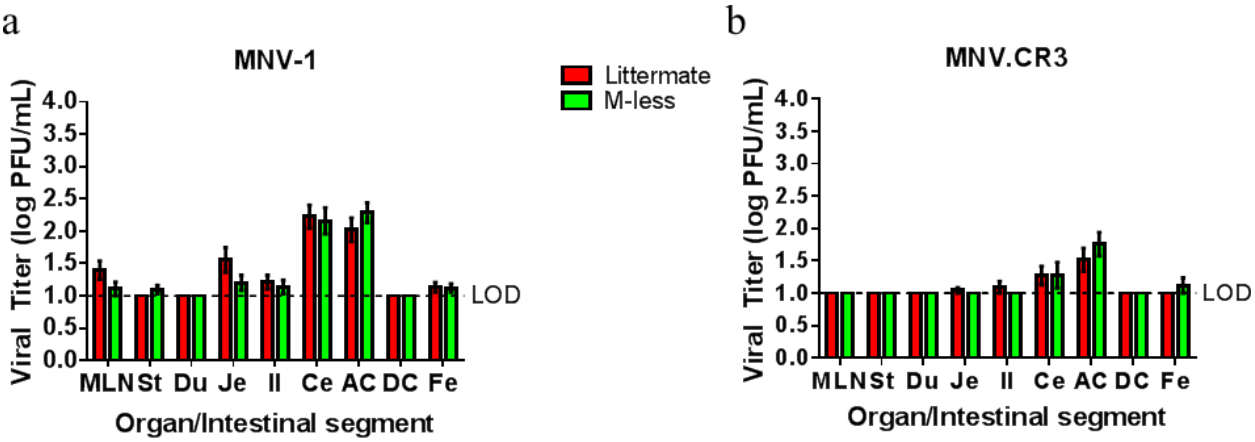

Figure 2.3. Early MNV infection of the gastrointestinal tract is independent of M cells in a conditional knockout mouse model of M cell deficiency. M-less mice and littermate controls were infected with 5 x 10^5 PFU of light-sensitive MNV-1 (a) or MNV.CR3 (b) by oral gavage. Tissues were harvested at 12 hpi in the dark. Virus yield was assessed by plaque assay performed after light inactivation of input virus (by exposing samples' dilutions to light). Legend: MLN, mesenteric lymph node; St, stomach; Du, duodenum; Je, jejunum; Il, ileum; Ce, cecum; AC, ascending colon; DC, descending colon; Fe, feces; LOD, limit of detection. Eight to eleven mice were analyzed per group. Results shown are from at least three independent experiments.

Taken together, these results suggested that MNV-1 and MNV.CR3 infection of the GI tract is independent of M cells in the transgenic mouse model of M cell deficiency used in the current study. Additionally, MNV-1 exhibits different initial tissue tropism in these mice compared to their parental mouse strain (C57BL/6); specifically, an initiation of infection in the large intestine of both M-less mice and littermate controls.

M-less mice lack mature Peyer's patches-associated M cells, and *GP2* mRNA expression is negligible

Unlike previous studies, this study's MNV infection data showed an M cell-independent mechanism of MNV breaching the intestinal epithelial barrier. Therefore, we confirmed that M-less mice were indeed deficient in M cells. We performed immunostaining in Peyer's patch whole-mounts using two M cell markers: GP2 and UEA-1. The first is an M cell-specific marker, a protein expressed only in mature M cells. The second is a lectin that binds to M cells, goblet cells and Paneth cells, among other cell types, in the intestinal epithelium [4]. Littermate control Peyer's patches had crypt cells that were positive only for UEA-1 (e.g. goblet cells, Paneth cells), and the double-positive cells stained both for GP2 and UEA-1 (M cells), were located at the center of the lymphoid follicle (Fig. 2.4a, left panel). On the other hand, M-less mice only

had crypt epithelial cells positive for UEA-1, but no double positive M cells were observed (Fig. 2.4a, right panel).

To confirm these results, we performed RT-qPCR to quantify *GP2* mRNA, a more sensitive technique than immunofluorescence staining. Relative *GP2* mRNA expression in M-less mice was less than 0.1% of littermate controls' expression (which was set to 100%) (Fig. 2.4b). These results confirmed that our M-less mice bred in-house are deficient in GP2-expressing, mature, M cells.

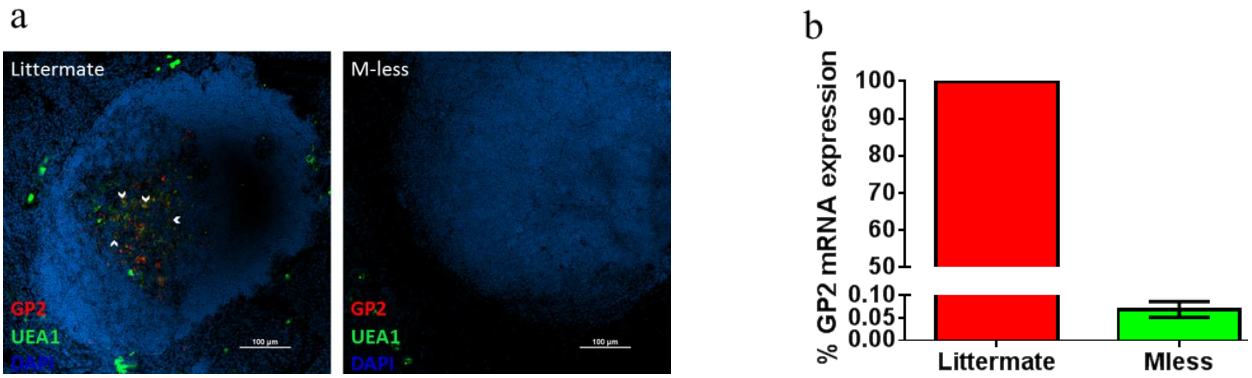

Figure 2.4. M-less mice do not have mature M cells. (a) Peyer's patch whole-mount staining shows absence of M cells in M-less mice. Confocal microscopic images of Peyer's patches stained with M cell markers GP2 (red) and UEA-1 (green) from littermate control (left panel) and M-less mouse (right panel). Some double positive (GP2+ and UEA-1+) cells (M cells) are indicated by arrowheads. Cell nuclei were stained with DAPI (blue). (b) *GP2* mRNA relative expression in Peyer's patches from one littermate control and two M-less mice was determined by RT-qPCR.

M cells are important in MNV-1 trafficking to the MLN at 24 hpi

Since the transgenic mice used in the current study needed a higher inoculum to be infected with MNV than C57BL/6 mice (5×10^5 vs. 1×10^5, respectively), we extended the infection period to 24 h, since several intestinal segments and the MLN of M-less mice and littermate controls had low/no detectable virus titers at 12 hpi. M-less mice and littermate controls were infected with light-sensitive MNV-1 or MNV.CR3 for 24 h (Fig. 2.5). M-less mice infected with MNV-1 had comparable virus yields to littermate controls in all intestinal segments analyzed (Fig. 2.5a). The same was true for MNV.CR3 infected mice, where no difference in virus yields of M-less mice and littermate controls was observed (Fig. 2.5b).

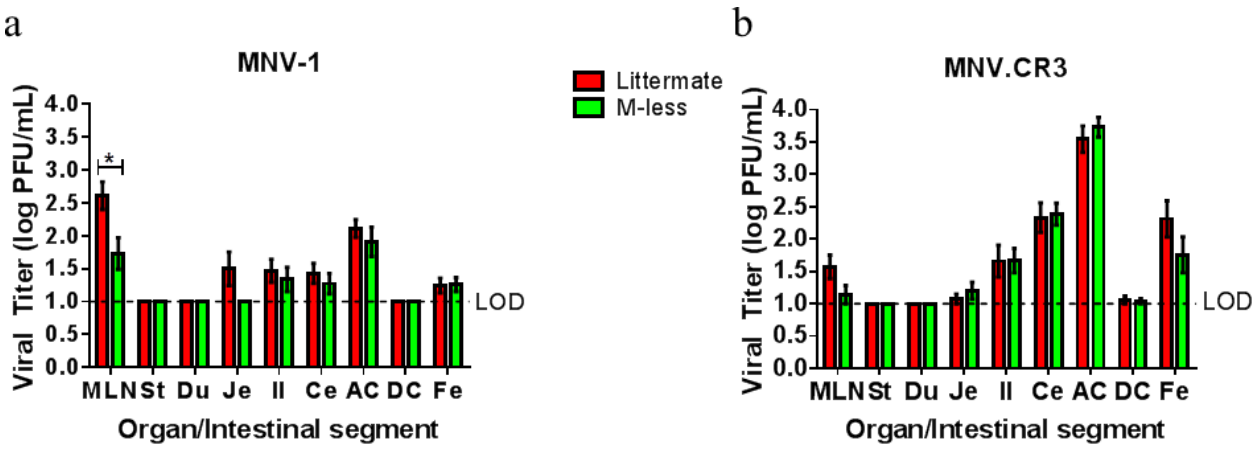

Figure 2.5. MNV-1 trafficking to the MLN is dependent on the presence of M cells at 24 hpi. M-less mice and littermate controls were infected with 5 x 10^5 PFU of light-sensitive MNV-1 (a) or MNV.CR3 (b) by oral gavage. Tissues were harvested at 24 hpi in the dark. Virus yield was assessed by plaque assay performed after light inactivation of input virus (by exposing samples' dilutions to light). Legend: MLN, mesenteric lymph node; St, stomach; Du, duodenum; Je, jejunum; Il, ileum; Ce, cecum; AC, ascending colon; DC, descending colon; Fe, feces; LOD, limit of detection. Eight to ten mice were analyzed per group. Results shown are from at least three independent experiments.

To our surprise, MNV-1-infected M-less mice had a significant lower virus yield in the MLN compared to littermate controls (Fig. 2.5a). Additionally, as observed with C57BL/6 mice, MNV trafficking to the MLN differed between the two virus strains. MNV-1 was detected in the MLN of littermates in high titers, but MNV.CR3 yield in the MLN was modest. These results indicated that MNV-1 trafficking to the MLN is dependent on the presence of mature M cells in the intestine, and it differs between MNV-1 and MNV.CR3.

2.4 Discussion

Understanding the early events of pathogen-host interaction and how enterically transmitted viruses are able to breach the intestinal epithelial barrier to productively infect their host is of paramount importance. Basic physiological mechanisms, such as antigen transcytosis by M cells, may be hijacked and used in favor of the pathogen. This is known to happen with bacteria, prions, viruses and the parasite *Cryptosporidium* spp. [4,12,17,18]. Therefore, by studying the early steps necessary for NoV productive infection, we will have a greater understanding of NoV's pathogenesis. Additionally, these studies will set the ground for the development of successful interventions able to prevent/hamper infection at its very initial stages (e.g. NoV interaction with the intestinal epithelium.).

In the current study, we use MNV to assess the role of M cell transcytosis in the ability of NoVs to breach the intestinal epithelial barrier and cause infection. Prior studies by our group have revealed that oral infection by MNV-1 and MNV.CR3 is dependent on the presence of M cells and/or gut-associated lymphoid tissues [11,12]. However, a model system of complete deficiency of M cells without compromising the gut-associated lymphoid tissue development was still lacking. Therefore, in order to perform a more direct assessment of M cells' role in MNV infection, we took advantage of a recently developed transgenic (conditional knockout) mouse model of M cell deficiency that lacks M cells but retains gut-associated lymphoid tissues [13].

In C57BL/6 mice, MNV-1 initiated infection in the small intestine. However, to our surprise, the initial site of infection of MNV-1 was shifted to the large intestine in both M-less mice and littermate controls. Additionally, in the transgenic mouse model of M cell deficiency, there was no difference in MNV-1 or MNV.CR3 intestinal virus yield between M-less mice or littermate controls. This suggests that, unlike previous studies in wild-type BALB/c mice or transgenic (knockout) mice on a BALB/c background, where MNV infection by the oral route was greatly reduced or absent [11,12], here MNV infection of the intestine was independent of M cells in the transgenic C57BL/6 mice. Although we currently do not have an explanation for the shift in MNV-1tissue tropism or the lack of phenotype observed in M-less mice infected with MNV-1 and MNV.CR3, several possibilities are plausible. Differences in phenotypes of diverse biological processes related to the mouse strain background or transgenic mouse model used in a particular study have been widely reported in the literature. For example, Paneth cell function (e.g., antimicrobial peptides secretion) is largely influenced by the mouse background strain. C57BL/6 mice have more Paneth cells and a less diverse antimicrobial peptide profile compared to 129/SvEv mice [19]. When C57BL/6 and BALB/c mice were compared for fibrosis susceptibility, these two mice strains presented organ-specific differences (e.g., C57BL/6 mice are resistant to hepatic fibrosis but susceptible to pulmonary fibrosis, while the reverse is true for BALB/c mice) [20]. A mouse strain background-dependence is also observed with dextran sulfate sodium-induced colitis [21]. These reports illustrate how widely variable a given biological process (such as the initial site of virus infection in the intestine or MNV use of M cells to breach the intestinal barrier) can be, depending on the mouse strain used. Of note, C57BL/6 mice have fewer mature M cells in the duodenum and ileum than BALB/c mice, and in

the cecum, mature M cells are rarely observed [22]. Therefore, it is possible that MNV infection of C57BL/6 and the transgenic mice used in this study takes advantage of other, potentially more active, pathways to breach the intestinal epithelial barrier. Future studies involving M cell depletion with anti-RANKL neutralizing antibody in C57BL/6 mice, and M-less and littermate controls are necessary to determine whether the M cell-independent phenotype of MNV intestinal infection is a mouse strain-specific finding.

Another explanation regarding the shift in tissue tropism observed for MNV-1 in the transgenic mice is the potential differential glycosylation pattern of the intestinal epithelium. MNV uses carbohydrates as attachment receptors [23,24]. If the large intestine of the transgenic mice (M-less and littermates) expresses MNV-1-binding carbohydrates (exclusively or at greater levels than the small intestine), the shift in tissue tropism would be expected, since MNV-1 would now initiate infection where it binds more. Differential intestinal epithelial glycosylation patterns among different mouse strains are not without precedent. BALB/c, C57BL/6 and CBA mice present different carbohydrate composition of their intestinal mucins [25]. Additionally, differences in the innate immune cells in the lamina propria and in the intestinal microbiota cause different glycosylation patterns in the intestinal epithelium [26]. Therefore, if the transgenic mice have differences in their microbiota and/or lamina propria leukocyte populations (compared to their parental strain, C57BL/6), these would potentially explain the shift in tissue tropism observed by the current study.

An alternative explanation for the lack of phenotype we observed in the current study (M cell-independent MNV intestinal infection) is the presence of passenger mutations in the M-less mice. Passenger mutations are embryonic stem cell-derived DNA sequences flanking the targeted transgene that contain mutations. These have been implicated as confounding factors in several transgenic mouse strains, although the issue is often ignored [27]. A classic example is the strong protection observed in caspase 1 knockout mice against lethal lipopolysaccharide treatment [27,28]. The knockout mouse resistance phenotype is mainly due to the presence of an inactivating passenger mutation in the caspase 11 gene. Therefore, this is a precedent for the possibility that M-less mice have passenger mutation(s) that could alter their antigen uptake mechanisms in the intestine (e.g., by enhancing goblet cell-associated antigen transport or paracellular transport), rendering these mice susceptible to MNV infection, despite the lack of M cells.

We also observed previously unreported biological differences between MNV-1 and MNV.CR3. MNV-1 trafficking to the MLN happened early in infection, at 9 hpi (Fig. 2.4a) and was dependent on the presence of M cells (Fig. 2.7a). In contrast, MNV.CR3 trafficking to the MLN was modest, happened later (at 24 hpi, Fig. 2.4b), and was not affected by the lack of intestinal M cells (Fig. 2.7b). These findings led us to hypothesize that MNV-1 and MNV.CR3 exhibit different cellular tropism early in infection.

Trafficking from the intestinal lamina propria to the MLN requires antigens to be carried by migratory dendritic cells (DCs). In the intestinal lamina propria, there is a large population of DCs, with two predominant subsets: migratory DCs and nonmigratory gut-resident DCs [29,30]. The migratory DCs are characterized by expression of CD103 and lack of CX3CR1 ($CD103^+$ $CX3CR1^-$). They present classical DC functions, promoting IgA production, development of regulatory T cells, and imprinting gut homing on lymphocytes [29-33]. The nonmigratory gut-resident DCs are characterized by lack of CD103 and expression of CX3CR1 ($CD103^-$ $CX3CR1^+$). They have macrophage features, serving as a barrier against invading pathogens, modulating immune responses directly in the mucosa, producing tumor necrosis factor alpha, promoting colitis and T_H17 T cell development [29,30,32,33].

Based on the C57BL/6 kinetics assay data, we hypothesize that MNV-1 infects $CD103^+$ $CX3CR1^-$ migratory DCs early in infection. This would explain the early "arrival" of MNV-1 to the MLN and the increase in virus yields throughout time. Migratory DCs would become infected in the lamina propria, migrate to the draining lymph node (MLN), and subsequent rounds of MNV-1 replication would allow neighboring susceptible cells in the MLN to become infected (without the need to migrate back to the intestinal lamina propria to become infected). This mechanism of migratory DC infection is likely dependent on M cell transcytosis of MNV-1 from the lumen into the basal-pocket. This hypothesis is supported by the fact that M-less mice had reduced titers of MNV-1 in the MLN compared to littermates and migratory DCs use M cells to sample antigens from the intestinal lumen [29-31]. On the other hand, MNV.CR3 was only detected in the MLN at 24 hpi, and at low titers. Thus, we hypothesize that nonmigratory gut-resident DCs are the cells preferentially infected by this strain early in infection. These cells extend their dendrites through the intestinal epithelium and actively sample antigens from the intestinal lumen. Additionally, they do not migrate to the draining lymph nodes [29,30,32,33]. Interestingly, in a previous study by our group, migratory DCs were shown to be essential for

trafficking of MNV.CR3 at the MLN at 24 and 48 hpi, but not for infection of intestinal segments [34]. These findings corroborate our hypothesis that migratory DCs are not the preferential cells targeted by MNV.CR3 early in infection. Taken together, the cellular features presented by $CD103^{-}$ $CX3CR1^{+}$ gut-resident DCs would explain the slow "arrival" of MNV.CR3 to the MLN and the M cell-independent mechanism of breaching the intestinal epithelial barrier. Of note, it is unlikely that MNV gets to the MLN by systemic dissemination (via bloodstream), instead of being carried by cells from the lamina propria (via afferent lymphatics). As shown in Fig. 2.4, MNV-1 and MNV.CR3 are not detected in the spleen of C57BL/6 mice early in infection, which suggests that MNV-1 trafficking to the MLN is via permissive cells that migrate to this organ.

In summary, our study demonstrated: i) the presence of mature M cells is necessary for optimal dissemination of MNV-1 to the local draining lymph node (MLN); ii) the two highly genetically similar virus strains, MNV-1 and MNV.CR3, showed different biological properties with regard to trafficking to the MLN; and iii) pathogens may have different mechanisms to breach the intestinal epithelial barrier depending on the mouse strain being used.

Based on our findings and previous work performed in our lab, we propose a working model of MNV infection *in vivo*, in which MNV dependence on M cells is mouse strain-specific (Fig. 2.6). MNV infection of wild-type BALB/c mice is dependent on the presence of M cells (Fig. 2.6, left). Upon M cell depletion by a neutralizing anti-RANKL antibody, MNV intestinal infection of wild-type BALB/c mice is greatly reduced compared to the control group (mice treated with isotype antibody) [12]. In the transgenic mouse model of M cell deficiency used in the current study, MNV intestinal infection is not dependent on the presence of M cells (Fig. 2.6, center). MNV titers in the intestine of M-less mice and littermate controls were comparable. Whether MNV intestinal infection of wild-type C57BL/6 is M cell-dependent remains to be investigated (Fig. 2.6, right).

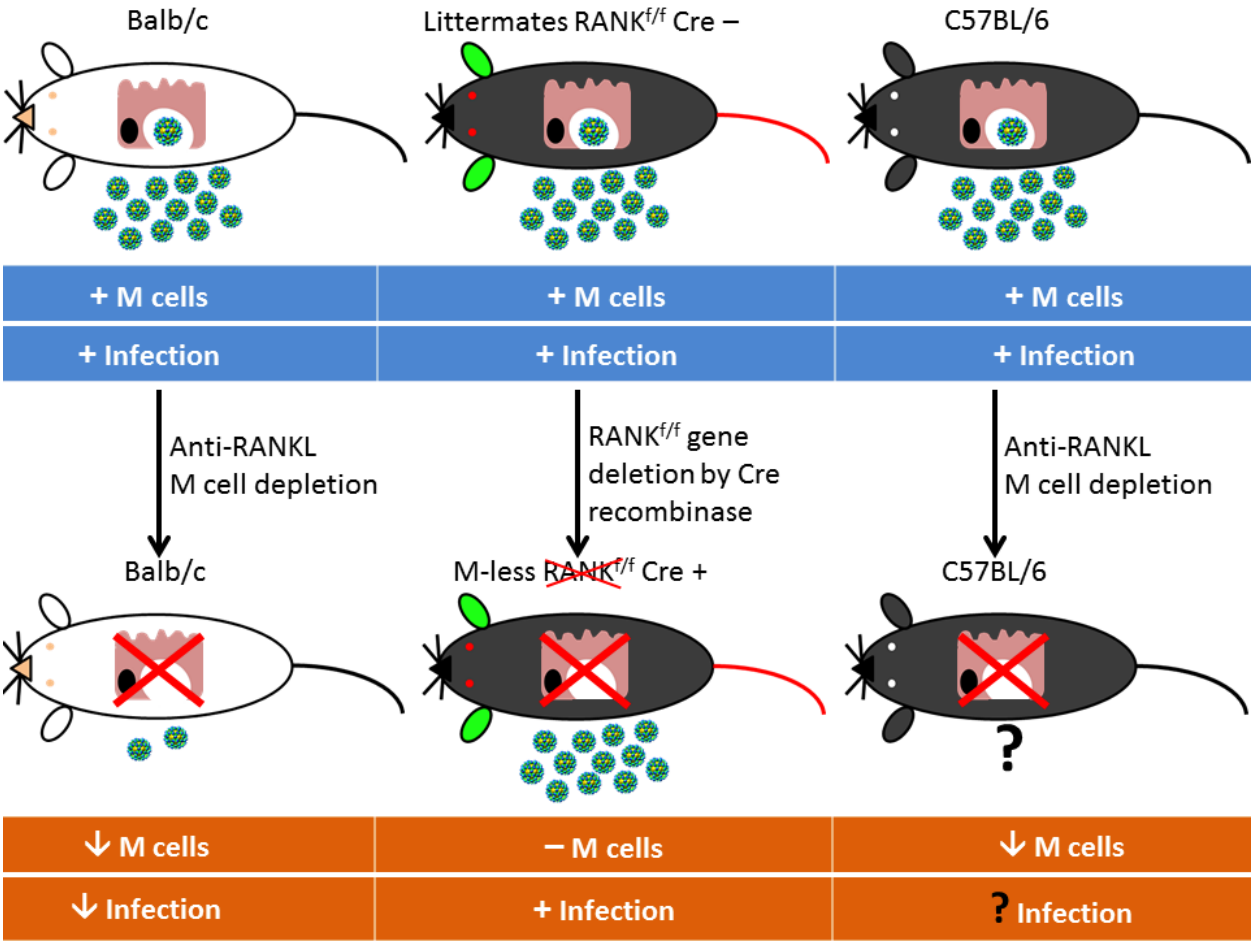

Figure 2.6. Working model of MNV infection *in vivo*. Wild type BALB/c mice (top left), transgenic RANK$^{f/f}$ Cre negative mice (littermates) on C57BL/6 background (top center) and wild type C57BL/6 mice (top right) become orally infected by MNV-1 and MNV.CR3. Upon M cell depletion by a neutralizing anti-RANKL antibody, wild type BALB/c mice show a striking decrease in MNV intestinal infection (bottom left). Transgenic RANK$^{f/f}$ Cre + mice have the RANK$^{f/f}$ gene deleted by Cre recombinase and are deficient in M cells (M-less mice). Despite the absence of M cells, M-less mice have comparable virus yield to littermate controls (bottom center). Whether anti-RANKL M cell depletion of wild type C57BL/6 mice (bottom right) would hinder MNV intestinal infection in a similar manner to what is observed with wild type BALB/c mice is unknown.

Taken together, these findings expand our current knowledge of how enteric pathogens breach the intestinal barrier to successfully infect their host. Additional studies are necessary to confirm the mouse strain-dependent M cell usage during MNV infection and to verify whether MNV-1 and MNV.CR3 have different cellular tropism early in infection *in vivo*. Research on the early steps of pathogen-host interaction may help identify targets for potential anti-viral therapies.

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Chapter 3. Select membrane proteins modulate MNV-1 infection of macrophages and dendritic cells in a cell type-specific manner

Author's note: this chapter is a modified version of the previously published research article: Bragazzi Cunha J and Wobus CE. Select membrane proteins modulate MNV-1 infection of macrophages and dendritic cells in a cell type-specific manner. 2016. Virus Res 222:64-70.

3.1 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 [1]. Human noroviruses are the main cause of acute gastroenteritis worldwide, infecting people of all ages [2]. In the US, these viruses are estimated to cause 19-21 million cases of illness with an estimated cost of $2 billion/year [3,4]. Despite its major impact on human health and economy, little is known about the early events of norovirus infection. Recently, promising advances, including the establishment of a mouse model and cell culture system for human noroviruses, have been reported [5,6]. However, 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 robustly in cell culture, and it does so in murine macrophages and dendritic cells [7]. Like its human counterpart, MNV is an enteric pathogen transmitted by the fecal-oral route. Thus, it is used as a model system to study diverse aspects of norovirus biology in a natural host [8].

Viruses often use multiple receptors for attachment and internalization [9-11]. The identity and distribution of these receptors can determine the extent of cell types, tissues, and hosts a given virus can infect [10]. Norovirus infection is initiated by virus binding to carbohydrate attachment receptors on the target cell surface[12]. Within the *Caliciviridae* family, feline calicivirus was shown to bind to sialic acid and junctional adhesion molecule-A, with

binding to the latter leading to infection [13,14]. To date, only attachment receptors have been identified for noroviruses. Human noroviruses can bind to heparan sulfate [15], gangliosides [16], and to ABH and Lewis histo-blood group antigens (HBGAs) [17-21]. Binding to HBGAs is virus strain-specific, and HBGAs are a genetic susceptibility factor for infection [17,18,20,22]. However, expression of HBGAs does not confer permissiveness [23], suggesting that additional factors are needed. MNV also uses carbohydrates as attachment receptors. Depending on the virus strain, terminal sialic acid moieties on the ganglioside GD1a, N- and/or O-linked glycoproteins can function as attachment receptors in primary and cultured macrophages [24,25]. However, expression of these sugar moieties on the cell surface is not sufficient for productive infection [25]. 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 [26,27]. 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 [24,28]. Following binding, MNV is internalized by a dynamin II- and cholesterol-dependent mechanism [29,30].

The goal of the current study was to expand our knowledge on the role host cellular proteins play during early steps of MNV infection *in vitro*. Towards that end, we focused on the two initial steps of MNV infection: binding and entry, since these are thought to be key factors contributing to the strict tropism of noroviruses [23]. Fifty-five proteins were identified 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, CD98 enhanced MNV-1 infectivity in RAW 264.7 cells and may play a role in post-binding steps of MNV-1 infection *in vitro*. This study broadens our understanding of host cell proteins promoting the initial events in MNV infection, an important step in viral pathogenesis.

3.2 Material and methods

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 [31]. Six- to eight-week old CD44 knockout (*CD44*, 005085), CD36 knockout (*CD36*, 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 [7]. 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 [32].

Virus stocks. A stock produced from the plaque-purified MNV-1 clone CW3 (GV/MNV-1/2002/USA) was used at passage 6 for all experiments [33].

VOPBA and protein identification by tandem mass spectrometry. Isolation of cellular membrane proteins was carried out as previously described [34]. Cellular proteins extract were mixed with Laemmli sample 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 [35] 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 [7] 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.

Binding assays and growth curves. Binding assays were carried out as previously described [25] 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 µL of medium. Unbound virus was removed by washing cells with ice-cold PBS. Cells were resuspended in 140 µL 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 [25], 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 µL 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 [36]. 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 [25] and the titer of control cells was set to 100%.

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 µL of Dharmafect 4 (Dharmacon). Cells were incubated for 48 h, resuspended in culture medium, and infected as previously described [25]. In parallel, transfected cells were analyzed by flow cytometry to verify protein knockdown.

Flow cytometry analysis. To determine the level of protein knockdown, 48 h post transfection, cells were resuspended in culture medium containing the anti-FcγR 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.

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,

SinoBiological) was measured by ELISA. 96-well microtiter plates were coated with 1 µg 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 ~$1x10^8$ PFU of concentrated MNV-1 lysate or 25 ng of purified recombinant MNV-1 Pd generated as described previously [37,38]. 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 [7], followed by peroxidase-conjugated secondary goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories).

MNV-1 growth curve in the presence of rCD98. MNV-1 was incubated with rCD98 in high ($2x10^6$ 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.

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. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3.3 Results

Identification of putative MNV-1-interacting proteins

To identify putative MNV-1-interacting proteins that play a role in early steps of 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. 3.1a). The two respective 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. 3.1b).

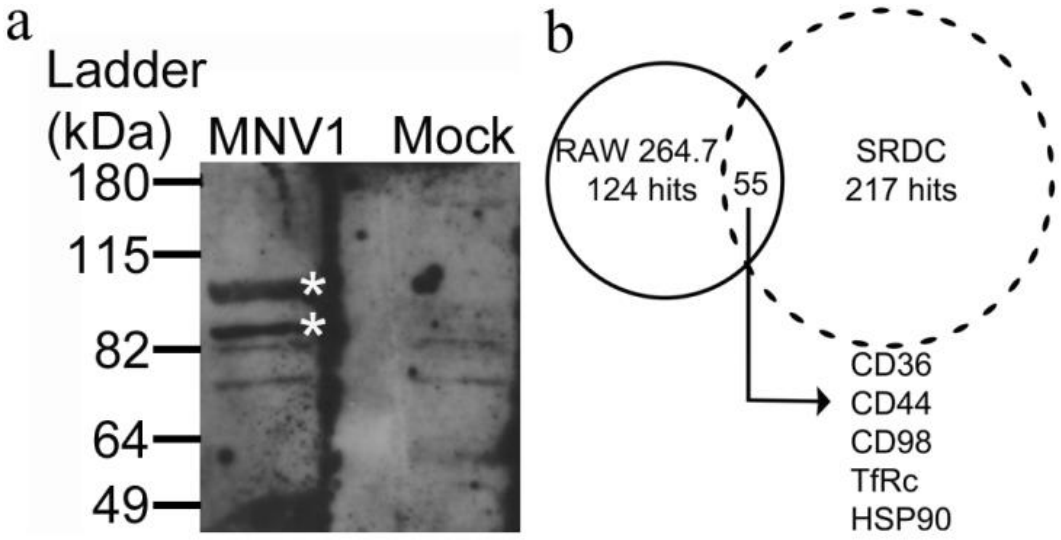

Figure 3.1. Identification of MNV-1-putative interacting proteins by VOPBA followed by tandem mass spectrometry. (a) Representative image of RAW 264.7 cells VOPBA. Membrane proteins were extracted from RAW 264.7 cells, separated by SDS-PAGE, and VOPBA was performed using MNV-1 or mock lysate. Virus bound 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.

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 proteins met the inclusion criteria: HSP90, a chaperone protein involved in folding and stabilization of proteins [39]; the scavenger receptor CD36 [40]; the hyaluronic acid receptor CD44 [41]; the heavy chain of the heterodimeric amino acid transporter CD98 [42], and the transferrin receptor 1 (TfRc), involved in cellular uptake of iron [43] (Fig. 3.1b). HSP90 is involved in later stages of MNV-1 infection and facilitates stability of the viral major capsid protein VP1 [44]. 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.

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

Since CD36 knockout (KO) and CD44 KO mice are commercially available, we used them 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 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. 3.2a and 3.2c). No statistically significant difference in MNV-1 binding was observed in CD36 KO or CD44 KO BMM compared to the WT control cells (Fig. 3.2b and 3.2d).

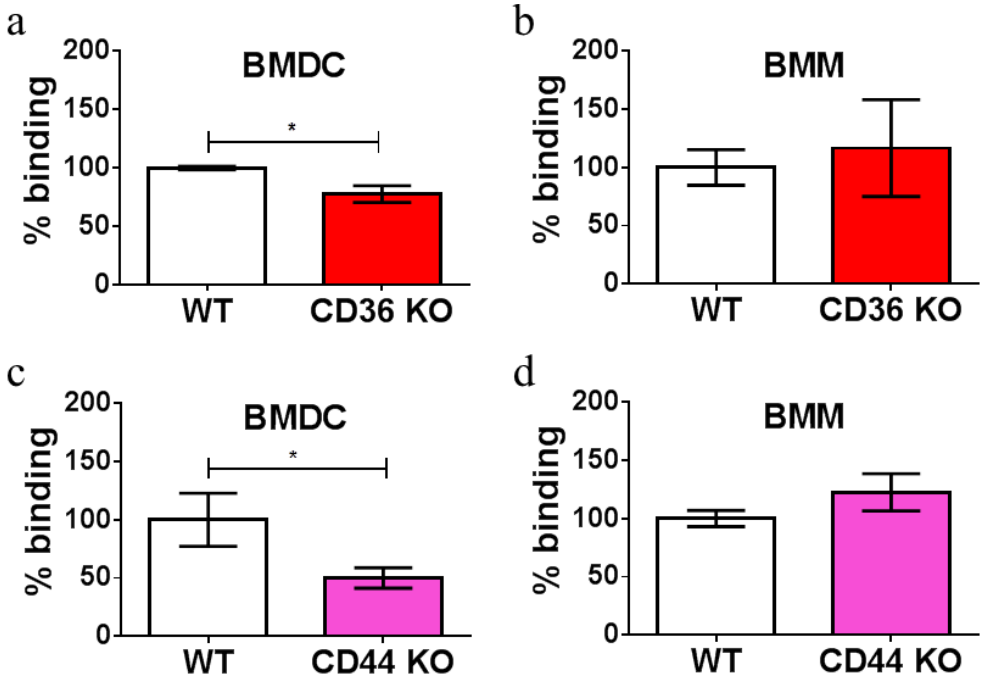

Figure 3.2. MNV-1 binding is reduced in CD36- and CD44-deficient bone marrow-derived dendritic cells (BMDC) but not bone marrow-derived macrophages (BMM). Wild type (WT), CD36 knockout (CD36 KO), or CD44 KO BMDC (a and c) and BMM (b and d) were infected with MNV-1 (MOI of 2). Bound virus was quantified 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%. *$p<0.05$.

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) and infection was allowed to proceed 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 (Fig. 3.3). 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.

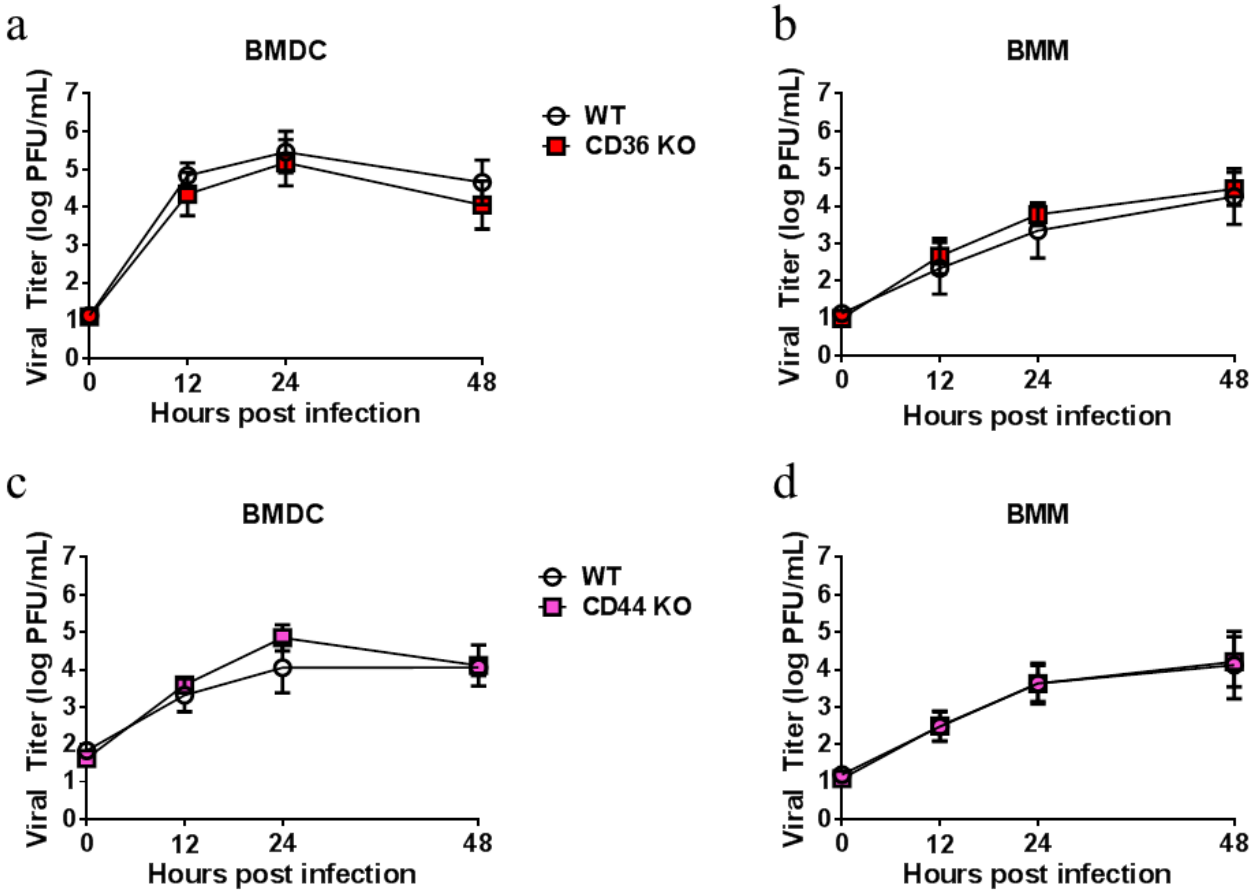

Figure 3.3. MNV-1 infection kinetics in CD36- and CD44-deficient bone marrow-derived dendritic cells (BMDC) and macrophages (BMM) is similar to wild type cells. Wild type (WT), CD36 knockout (CD36 KO), or CD44 KO BMDC (a and c) and BMM (b and d) were infected with MNV-1 (MOI of 0.05) and infection was allowed to proceed for 0, 12, 24, and 48 h. Virus yield was assessed by plaque assay. Results shown are from four independent experiments performed in duplicate.

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

CD98 and TfRc gene deletions are embryonically lethal [45,46], 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. 3.4a).

Knockdown levels were 31.9% (± 2.3%) for CD98 siRNA-transfected cells (Fig. 3.4a, left panel) and 68.4% (± 2.8%) for TfRc siRNA-transfected cells (Fig. 3.4a, 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. 3.4b). 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. However, MNV-1 binding to CD98 or TfRc siRNA-transfected SRDC cells was similar to NT siRNA-transfected control cells (Fig 3.5a).

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.

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 control 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. 3.4c), while similar growth kinetics were observed in TfRc siRNA-transfected cells and NT siRNA-transfected control cells (Fig. 3.4c).

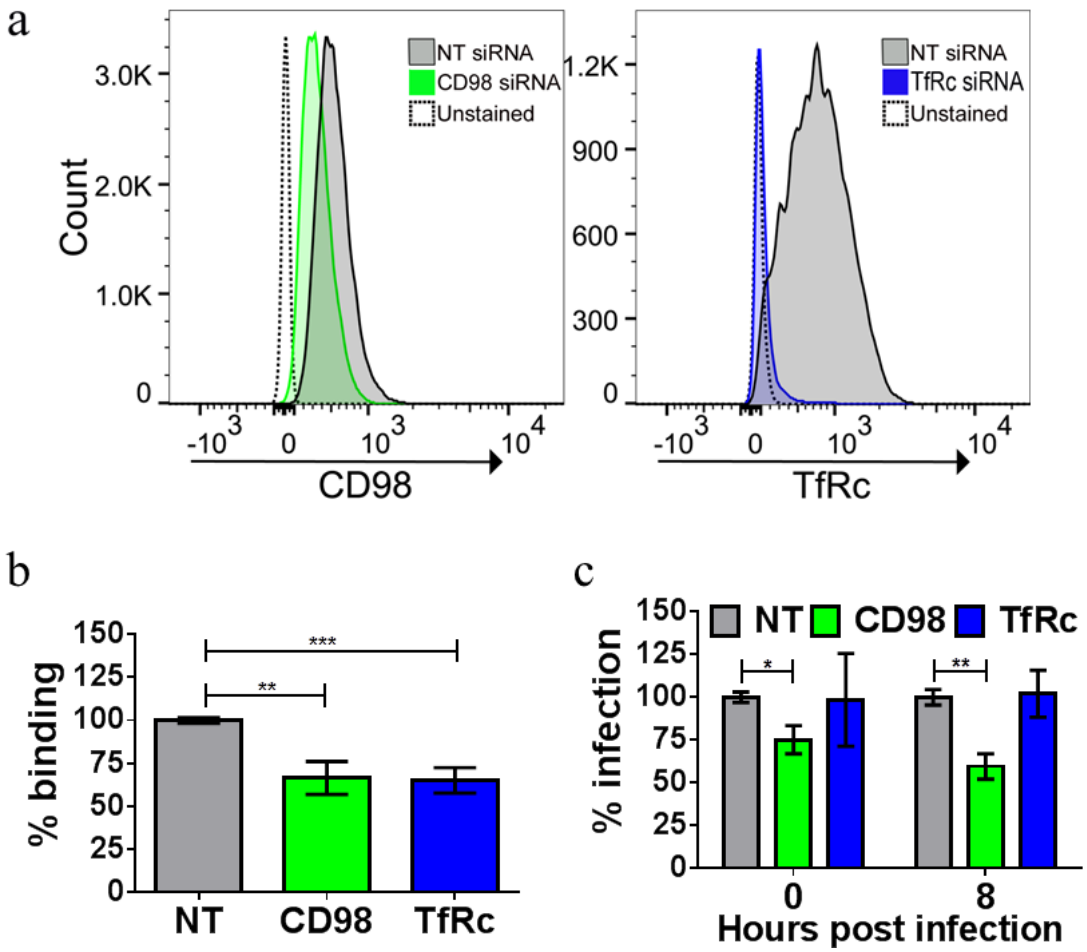

Figure 3.4. TfRc and CD98 facilitate MNV-1 binding to RAW 264.7 cells, while CD98 is required for efficient virus infection. RAW 264.7 cells were transfected with CD98 siRNA, TfRc siRNA, or non-targeting (NT) siRNA control. At 48 h post transfection, cells were stained with fluorescently labeled anti-CD98 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%. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

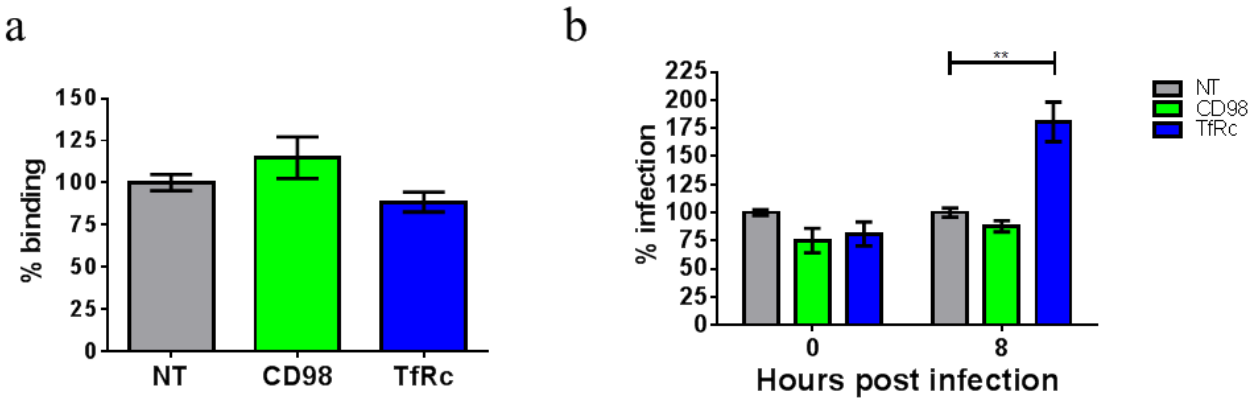

Figure 3.5. Transferrin receptor 1 (TfRc) may restrict MNV-1 infection in SRDC cells. SRCD cells were transfected with CD98 siRNA, TfRc siRNA, or non-targeting (NT) siRNA control. At 48 h post transfection, cells were infected with MNV-1. (a) CD98- andTfRc-siRNA knockdown SRDC cells presented comparable MNV-1 binding to NT-siRNA transfected control 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%. (b) TfRc-siRNA knockdown significantly increased MNV-1 infection of SRDC cells at 8 hpi. 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%. **$p<0.01$

In SRDC cells, no difference was observed in MNV-1 infection between CD98 siRNA- and NT siRNA-transfected cells (Fig. 3.5b). 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 (Fig. 3.5b). 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.

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. 3.6). 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. 3.6a).

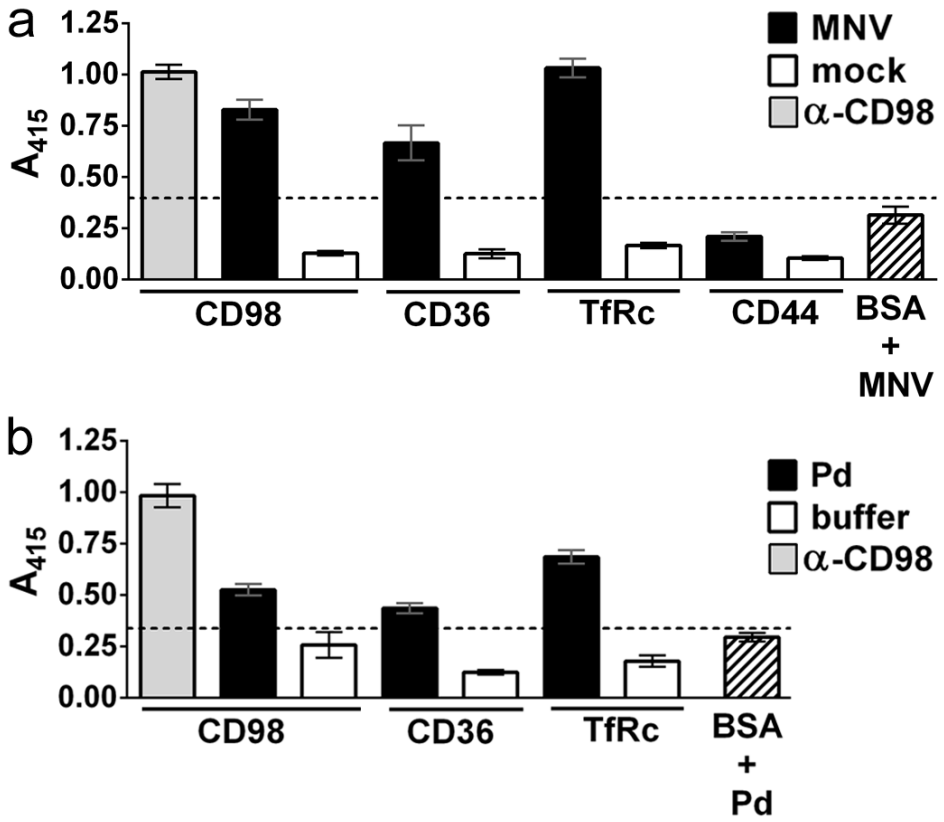

Figure 3.6. MNV-1 interacts with rCD36, rCD98, and rTfRc via its protruding domain (Pd). MNV-1 and MNV-1 Pd binding to the extracellular domain of recombinant proteins was evaluated by ELISA. (a) Recombinant CD36, CD44, CD98, TfRc or an irrelevant protein (BSA) were coated onto 96 well microtiter plates 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 (A415). The dashed line is drawn at the A415 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.

Given that the Pd is the norovirus receptor-binding domain and the outermost 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. 3.6b). 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.

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 examined whether CD98 functions as an entry receptor for MNV-1 in RAW 264.7 cells by investigating 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 cell infection in two ratios; in high molar excess ($2x10^6$-fold excess rCD98:MNV-1 VP1, Fig. 3.7a), and in low molar excess (1,000-fold excess rCD98:MNV-1 VP1, Fig. 3.7b). 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. 3.7a). 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. 3.7b). 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.

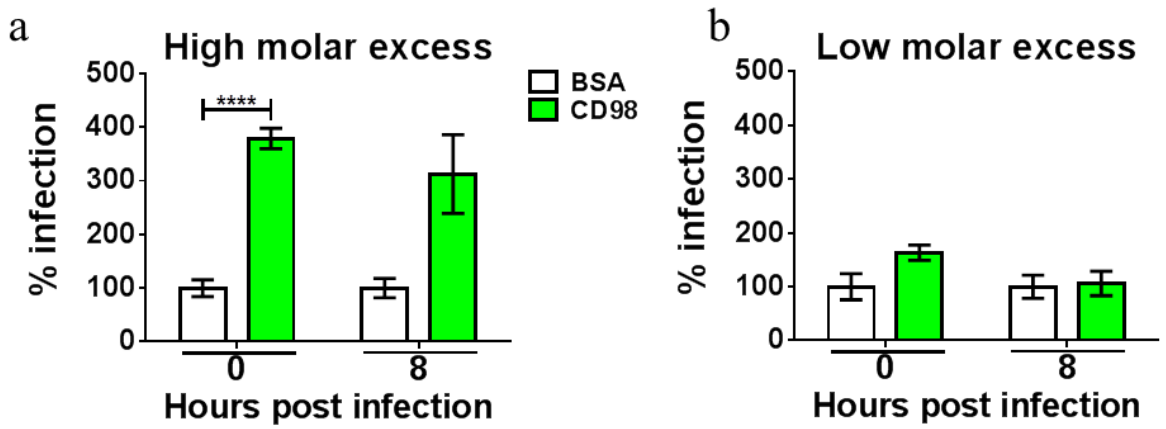

Figure 3.7. Recombinant CD98 enhances MNV-1 infection in a concentration-dependent manner. rCD98 was incubated with MNV-1 prior to infection in 2 x10^6-fold molar excess (a) or 1,000-fold molar excess (b), or with BSA. RAW 264.7 cells were then infected with MNV-1 (MOI of 0.05) and viral genome titers 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.

3.4 Discussion

The early steps of norovirus infection, prior to viral RNA replication, are thought to be key determinants of species-specificity and cell tropism [23]. The carbohydrate moieties that mediate MNV attachment, and the cellular mechanisms involved in MNV internalization have been partially characterized [24,25,29,30]. 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. Their expression is consistent with a role as MNV attachment factors. This expression pattern is not limited to cell types permissive to MNV (e.g. macrophages, dendritic cells and B cells), but is also found in different tissues, such as intestines.

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 [41]. Thus, whether MNV-1 binds to

CD44's stem region, not included in the recombinant protein, 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 binds to glycans present on the ganglioside GD1a and on N- and/or O-linked glycoproteins [24,25]. All recombinant proteins used in our experiments were expressed in mammalian cells and are 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 important 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 [47-49]. 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 [50,51]. 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 [52-55]. 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 [56] and in de-envelopment of herpes simplex 1 virus [57]. Additionally, the light chain xCT of the CD98 amino acid transporter heterodimer is an entry receptor for Kaposi's sarcoma-associated herpesvirus [58]. 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 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 [59]. 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 [60]. 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 [61]. However, whether the enhancement of MNV-1 attachment occurs in a physiological context remains to be determined. Nevertheless, future studies promise to reveal new role(s) for CD98 during MNV-1 infection.

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. Based on our findings and previous work performed in our lab, we propose a working model of MNV-1 infection *in vitro* (Fig. 3.8). MNV-1 uses CD36 and CD44 on the surface of dendritic cells as attachment receptors (Fig. 3.8a). In macrophages, MNV-1 binds to surface expressed CD98, TfRc, and terminal sialic acids on the ganglioside GD1a, and on both N- and O-linked glycoproteins [24,25]. Additionally, CD98 may have post-binding roles during MNV-1 infection of RAW 264.7 cells (Fig. 3.8b).

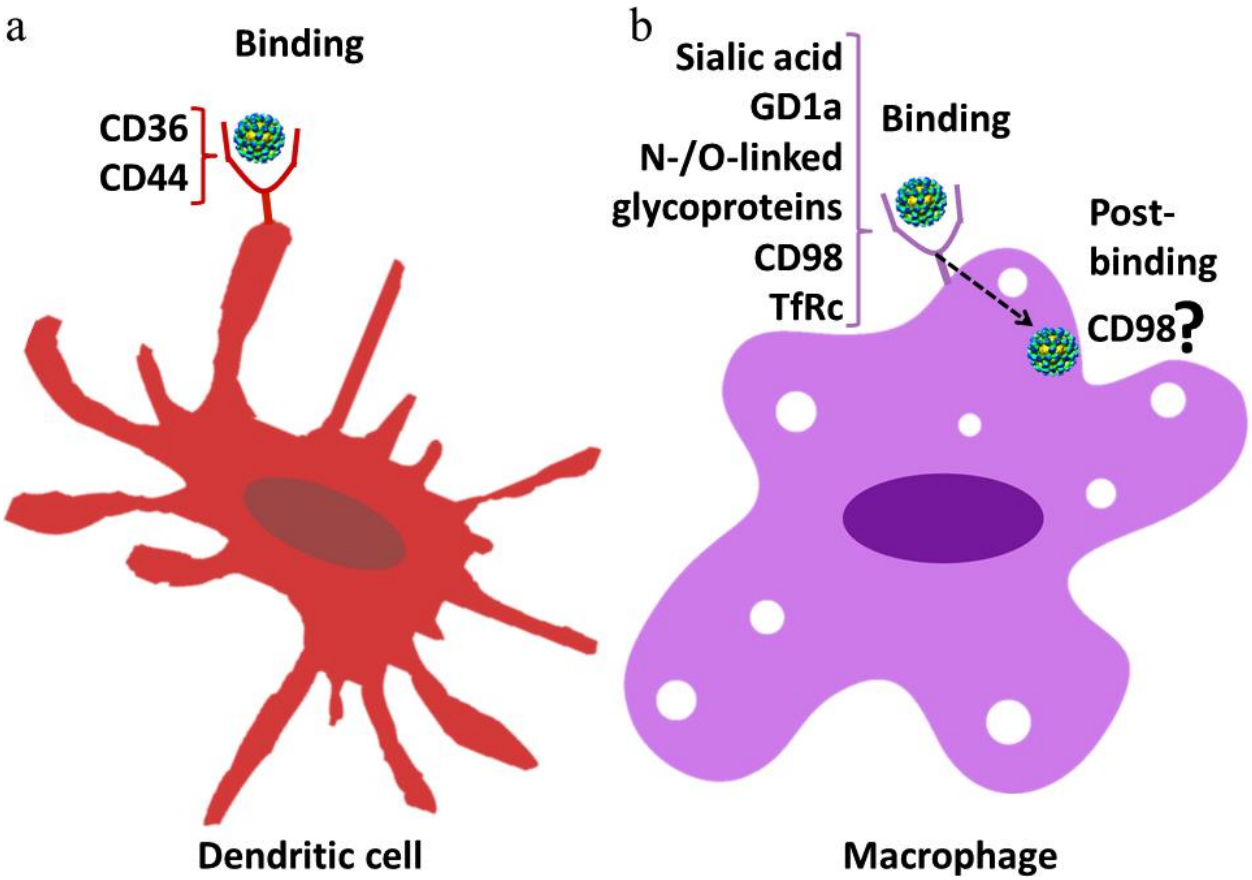

Figure 3.8. Working model of MNV-1 infection *in vitro*. (a) In dendritic cells, MNV-1 uses CD36 and CD44 as attachment receptors to bind to cells and initiate infection. (b) In macrophages, CD98, TfRc, and terminal sialic acids on the ganglioside GD1a, and on N- and O-linked glycoproteins are attachment receptors used by MNV-1 to bind to cells and initiate infection. CD98 may have post-binding roles during MNV-1 infectious cycle.

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.

3.5 References

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Chapter 4. Discussion and future directions

In this dissertation, two fundamental aspects of murine norovirus infection were addressed: i) what mechanisms are used by these enterically transmitted viruses to cross the intestinal barrier to gain access to its target cells in the intestinal lamina propria; and ii) what proteins mediate MNV-1 binding and entry to target cells. The specific aims of this dissertation were to verify the role M cells played in MNV-1 and MNV.CR3 intestinal infection *in vivo* by using a conditional knockout mouse model of M cell deficiency [1], and to identify protein receptors involved in binding and entry of MNV-1 in cell culture.

4.1 Summary of results

Data presented in chapter two of this dissertation show that two highly genetically related MNV strains, MNV-1 and MNV.CR3, have biological differences during infection *in vivo*. Some of the differences observed were previously unknown. i) MNV-1 and MNV.CR3 had different infection kinetics in C57BL/6 mice. Specifically, the MNV-1 infectious cycle required approximately 9 hours to generate infectious progeny viruses detectable by plaque assay, while MNV.CR3 required approximately 12 hours. ii) MNV-1 initiated infection in the small intestine (jejunum and ileum), while MNV.CR3 initiated infection in the large intestine (cecum) of C57BL/6 mice. iii) Trafficking of MNV-1 to the MLN occurred early in infection (at 9 hpi), and virus titers increased throughout time. In contrast, MNV.CR3 trafficking to the MLN was delayed with virus first detectable in the MLN by 24 hpi and reduced compared to MNV-1. Additionally, MNV-1 trafficking to the MLN of M-less mice was reduced compared to that of the littermate controls at 24 hpi. One explanation for these findings, particularly the difference in trafficking to the MLN amongst the two MNV strains, is consistent with the hypothesis that MNV-1 and MNV.CR3 have different cellular tropism early in infection *in vivo*. Future studies using highly sensitive RNA-based detection systems such as PrimeFlow RNA assay (eBioscience) or RNAscope (ACD) using virus strain-specific probes (*in situ* hybridization-based technique) associated to flow cytometric-based or immunohistochemistry-based

techniques (PrimeFlow and RNAscope, respectively) provide an approach to investigate this hypothesis. These systems enable us to determine whether MNV-1 and MNV.CR3 have different initial cellular tropism *in vivo*, by detecting viral RNA in cells and performing phenotypic characterization of these infected cells in the intestine.

Regarding my investigation into the role of M cells, my studies uncovered a more complex role of these cells in MNV infection than was previously appreciated. My findings combined with published work indicated that their role may be mouse- and/or virus-strain dependent. As discussed in more detail in chapter two, this hypothesis is supported by the observation that MNV-1 and MNV.CR3 intestinal infection was not affected by the absence of M cells in the conditional knockout mouse model of M cell deficiency [1]. Nonetheless, in the absence of M cells, MNV-1 trafficking to the MLN was impaired in this model.

In chapter three of this dissertation, the identification and role of four transmembrane glycoproteins during MNV-1 infection *in vitro* are described. Our data show that MNV-1 used distinct transmembrane glycoproteins in different cell types during infection. CD36 and CD44 were required for efficient MNV-1 binding to BMDC, while MNV-1 binding to RAW 264.7 cells was reduced in CD98- and TfRc-depleted cells. Furthermore, CD98 depletion reduced MNV-1 infection. Conversely, MNV-1 infection of SRDC cells was not affected by CD98 depletion, but it was enhanced upon TfRc depletion. Additionally, MNV-1 binding to the four proteins was assessed by ELISA. MNV-1 bound directly to the extracellular domain of rCD36, rCD98, and rTfRc via its VP1 P domain. Intriguingly, incubation of rCD98 with MNV-1 prior to infection enhanced virus infectivity in RAW 264.7 cells in a concentration dependent manner. Therefore, we concluded that CD36, CD44, CD98 and TfRc modulated MNV-1 infection in a cell type-specific manner and that CD98 may be an entry coreceptor for MNV-1 in RAW 264.7 cells. However, further studies are needed to confirm this hypothesis and determine the cell-type specific functions of CD36, CD44 and TfRc during MNV infection in macrophages and dendritic cells.

In summary, we uncovered a potentially more complex role for M cells during MNV infection *in vivo*, and putative MNV-1 attachment protein receptors for dendritic cells and macrophages. Additionally, one of these proteins (CD98) may have post-binding role(s) in MNV-1 infection of RAW 264.7 cells, such that of a coreceptor.

4.2 Discussion and future directions

4.2.1 MNV-1 and MNV.CR3 have distinct biological features *in vivo*

MNV-1 and MNV.CR3 had different initial tissue tropism in C57BL/6 mice. The initial site of MNV-1 infection was the small intestine, while MNV.CR3 initially infected the large intestine. A possible explanation for the different initial tissue tropism presented by these two strains is their differing glycan-binding requirements. MNV-1 binding to macrophages relies on terminal sialic acid residues on the ganglioside GD1a, N- and O-linked glycoproteins; while MNV.CR3 uses N-linked glycoproteins to infect murine macrophages [2,3]. Although the different glycan-binding phenotypes described were observed during MNV infection of macrophages *in vitro* [2,3], it is likely that the strain-dependent glycan-binding pattern is retained during *in vivo* infections. If this hypothesis is correct, these attachment receptors may also play a role in MNV binding to the intestinal mucosa. Indeed, the intestinal epithelium is differentially glycosylated [4-6]. For example, sialylated and sulfated glycans are the predominant glycans in the small intestine, whereas in the large intestine fucosylated glycans are the main glycans [4]. Additionally, differences in immune cells in the lamina propria and intestinal microbiota contribute to distinct glycosylation patterns in the intestine [6].

Alternatively, the different initial tissue tropism observed for MNV-1 and MNV.CR3 could be explained by different cellular tropism early in infection. The population of MNV target cells in the intestinal lamina propria differs greatly between the small and large intestines, and even within different segments of the small or large intestines (e.g. duodenum vs. ileum and cecum vs. colon, respectively) [7]. Thus, it is possible that the initial site of MNV-1 replication is the small intestine because this would be the segment where the virus encounters more of its preferential target cells. Conversely, MNV.CR3 would encounter more of its preferential target cells in the large intestine. Distinct preferential target cells population of MNV-1 and MNV.CR3 early in infection would provide an explanation for the initially high titers in the small and large intestine, respectively. Consistent with that hypothesis is also my observation that the kinetics of virus detection in the MLN varied between MNV-1 and MNV.CR3 presented. At 9 hpi, MNV-1 was present in the MLN of infected C57BL/6 mice, and viral titers increased as the infection progressed. On the other hand, MNV.CR3 trafficking to the MLN was modest and delayed (24

hpi) compared to MNV-1. Later in infection, when MNV spreads to other intestinal segments, the virus would then be postulated to infect additional target cell types as well.

Migratory DCs (CD103+ CX3CR1-) and non-migratory gut resident DCs (CD103- CX3CR1+) are predominant subsets of lamina propria DCs found in the intestine. Antigen trafficking from the lamina propria to the MLN is performed by migratory DCs, but not by gut resident DCs [8,9]. Based on the differences observed for MNV-1 and MNV.CR3 trafficking to the MLN in C57BL/6 mice and on MNV-1's dependence on M cells to traffic to the MLN (discussed in section 4.3), we hypothesize that MNV-1 preferentially infects CD103+ CX3CR1- migratory DCs, while MNV.CR3 preferentially infects CD103- CX3CR1+ non-migratory gut-resident DCs early in infection. MNV-1-infected migratory DCs in the lamina propria would then quickly migrate to the MLN and contribute to infection in that organ. This mechanism is likely dependent on M cell transcytosis of MNV-1 from the intestinal lumen into the lamina propria, since M-less mice had decreased MNV-1 yield in the MLN at 24 hpi compared to littermate controls, and migratory DCs use M cells-transcytosed antigens to sample the intestinal lumen [8-10]. Previous work performed by our group showed that migratory DCs are not the preferential cells targeted by MNV.CR3 during intestinal infection, but they are essential for the virus to reach the MLN [11], which further supports this hypothesis. Additionally, early in infection, MNV.CR3 would be taken up by gut resident DCs during their active sampling of antigens from the intestinal lumen (by their extended dendrites through the intestinal epithelium into the intestinal lumen).

Further work characterizing the cellular population infected by MNV-1 and MNV.CR3 in the lamina propria of C57BL/6 mice, BALB/c mice, M-less mice and littermate controls will be essential to test these hypotheses. To determine whether MNV-1 and MNV.CR3 have different initial cellular tropism *in vivo*, phenotypic characterization of target cells in the intestinal lamina propria will be performed by PrimeFlow RNA assay (eBioscience). This will allow us to assess whether these two strains have distinct preferential target cells early in infection (9 hpi and 12 hpi for MNV-1 and MNV.CR3, respectively).

4.2.2 MNV-1 trafficking to the MLN is dependent on M cells

One unexpected finding of my work was that MNV-1 trafficking to the MLN was reduced in M-less mice compared to littermate controls at 24 hpi. This finding suggests that M

cells are required for optimal MNV-1 dissemination from the lamina propria to the MLN. A possible explanation for the observed reduction in MNV-1 trafficking to the MLN is that M-less mice could have different cell populations (absolute and relative number of specific cell types) in the intestinal lamina propria and/or MLN compared to littermate controls. Accordingly, a decrease in the number of MNV-1 target cells might explain the phenotype observed. This hypothesis can be easily tested by isolating intestinal lamina propria and MLN leukocytes of M-less mice and littermates and performing phenotypic characterization and quantification of infected target cells by PrimeFlow RNA assay (eBioscience). This experiment would allow us to verify whether there is a decrease in the number of infected target cells (macrophages, dendritic cells and B cells) that could explain the MLN phenotype observed.

4.2.3 M cells are not required for intestinal infection of MNV-1 or MNV.CR3 in a conditional knockout mouse model of M cell deficiency

In the conditional knockout mouse model of M cell deficiency, MNV infection was independent of M cells. This is in contrast to previous studies by our lab, which show decreased intestinal virus titers in MNV-1- and MNV.CR3-infected animals, either BALB/c mice depleted of M cells [12] or transgenic mice on a BALB/c background that lack gut-associated lymphoid tissue [13]. In the current study, the transgenic mouse we used was on a C57BL/6 background. Thus, it will be important to determine whether the mouse strain background contributes to the mechanism MNV uses to breach the intestinal barrier. This is especially the case when we consider that in both phenotypes of transgenic C57BL/6 mice (M-less and littermates), the MNV-1 initial site of infection was the large intestine as opposed to the small intestine seen in non-transgenic C57BL/6 mice. Additionally, the MNV infectious dose needed to achieve consistent infection had to be increased in these transgenic mice compared to the parental strain C57BL/6. These findings suggest that these transgenic mice have intrinsic differences from the non-transgenic parental strain, which could account for the M cell-independent MNV intestinal infection phenotype. In summary, my data suggest that the use of M cells by MNV may be mouse strain-specific.

Another alternative is that the M cell antibody depletion protocol used previously by our lab may affect intestinal homeostasis in an unprecedented way. In this protocol, a neutralizing anti-RANKL antibody was administered to mice every other day for a total of four doses. Then,

animals were infected with MNV [12]. The RANK:RANKL pathway is involved in diverse cellular signaling pathways, including osteogenesis, immunity, and cancer [14]. Therefore, due to its wide involvement in different organic systems, RANKL neutralization may have had effects beyond M cells, for example in intestinal homeostasis in general and in extra-intestinal sites. We hypothesize that one such "off-target" effect would have caused decreased MNV infection in mice treated with anti-RANKL antibody, but not in the isotype-treated control group. Additionally, RANKL neutralization may have caused shifts in the intestinal microbiota of mice. Since commensal bacteria enhance MNV infection *in vivo* [15], mice treated with anti-RANKL may have had decreased MNV titers due to changes in their intestinal microbiota. Depletion of M cells by using the neutralizing anti-RANKL antibody in C57BL/6 mice, BALB/c mice, M-less mice and littermate controls will be essential to elucidate several questions that arouse from our study: i) whether M cell-independent MNV intestinal infection is a mouse strain-specific finding; ii) if the anti-RANKL antibody treatment causes "off-target" effects beyond M cell depletion; and iii) if the antibody treatment alters the intestinal microbiota.

To address these questions, we will perform RANKL antibody depletion of M cells in BALB/c mice, C57BL/6 mice, M-less mice, and littermate controls followed by MNV infection to determine whether MNV dependency on M cells is mouse strain-specific. To address whether the antibody treatment has "off target" effects that could have contributed to the M cell-dependent infection phenotype observed in previous studies by our lab, we will perform histological analysis (e.g. H&E staining) of intestinal sections to determine whether there are changes between the control and anti-RANKL treated groups. If no changes are observed, we will isolate intestinal epithelial and lamina propria cells of control and anti-RANKL treated groups and perform phenotypic characterization of the intestinal cell population by flow cytometry. Lastly, to determine whether the antibody treatment has "off target" effects that alter the intestinal microbiota of mice, we will perform 16S rRNA sequencing and compare the operational taxonomic units (OTUs) present in the different conditions. Assessing the role other antigen transport mechanisms (such as goblet cell-associated passage) [16] play in MNV infection *in vivo* will be important to expand our current understanding on how MNV breaches the intestinal barrier.

4.2.4 Identification of putative MNV-1 protein receptors

One goal of this dissertation was to identify cellular proteins that participate in the early steps of MNV-1 infection *in vitro.* We took an unbiased proteomic approach (VOPBA followed by mass spectrometry analysis) to identify putative MNV-1 protein receptors. To increase the likelihood of identifying conserved receptors between different target cell types, we created inclusion criteria to help us narrow down candidates that would then have their role assessed during MNV-1 infection. All of the following criteria needed to be met for a hit to be considered a putative MNV-1 protein receptor: 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).

Based on these criteria, five proteins were identified: CD36, CD44, CD98, HSP90 and TfRc. HSP90 aids in VP1 stability and plays a role later in MNV-1 infection [17]. Thus, we excluded it from our study. Four putative MNV-1 protein receptors, which were previously unknown to play a role in MNV-1 infection, were validated and had their role in MNV-1 infection assessed. CD36 and CD44 are two transmembrane glycoproteins that facilitated MNV-1 binding to BMDC. In RAW 264.7 cells, CD98 and TfRc promoted MNV-1 binding. Additionally, CD98 likely has post-binding roles in MNV-1 infection of RAW 264.7 cells, since in CD98-depleted cells MNV-1 binding and infection were reduced and rCD98 enhanced MNV-1 infectivity in a concentration-dependent manner. Additionally, we demonstrated that three of these proteins (CD36, CD98 and TfRc) directly bind to MNV-1 via its VP1 P domain. The recombinant proteins used to test MNV-1 binding by ELISA were all expressed in mammalian cells and are glycosylated. Since MNV-1 is known to interact with glycosylated proteins [2], future studies are needed to determine whether the MNV-1 Pd interaction with the extracellular domain of these proteins is via glycan moieties or the protein backbone. This can be easily tested by performing ELISA with recombinant proteins that are expressed in bacteria (lacking glycans) and in mammalian cells (having glycans). If MNV-1 and its VP1 P domain bind equally to the bacterially expressed proteins (compared to the proteins expressed in mammalian cells), this would indicate a protein-protein interaction. On the other hand, if binding to bacterially expressed proteins is decreased, this would indicate a carbohydrate-protein interaction. Additionally, we would like to verify binding of MNV-1 and P domain to CD44 by ELISA using the full-length extracellular domain of CD44 (standard and variant isoforms). Lastly, we would

like to test whether the extracellular domain of rCD36, rCD44, and rTfRc is able to competitively inhibit MNV-1 binding to target cells.

4.2.5 CD98 may be an entry coreceptor for MNV-1 infection of RAW 264.7 cells

One interesting finding of my work was that optimal MNV-1 infection of RAW 264.7 cells (at 0 and 8 hpi) was dependent on the presence of CD98. Consequently, this protein may play further roles in addition to mediating MNV-1 binding to cells. Moreover, the observation that the extracellular domain of rCD98 enhanced MNV-1 infectivity of RAW 264.7 cells suggests that this molecule may be a coreceptor for MNV-1.

Based on data presented in Chapter 3, we propose a working model that illustrates the potential mechanisms by which CD98mediates MNV-1 infection of RAW 264.7 cells (Fig. 4.1). In CD98-depleted cells, we observed decreased MNV-1 binding and infection. However, contrary to what we predicted, when soluble rCD98 was incubated with MNV-1 in high molar excess prior to infection, binding to and possibly infection of (although not statistically significant) RAW 264.7 cells were increased. Therefore, we hypothesize that CD98 acts as a binding receptor and coreceptor facilitating internalization of MNV-1 into RAW 264.7 cells.

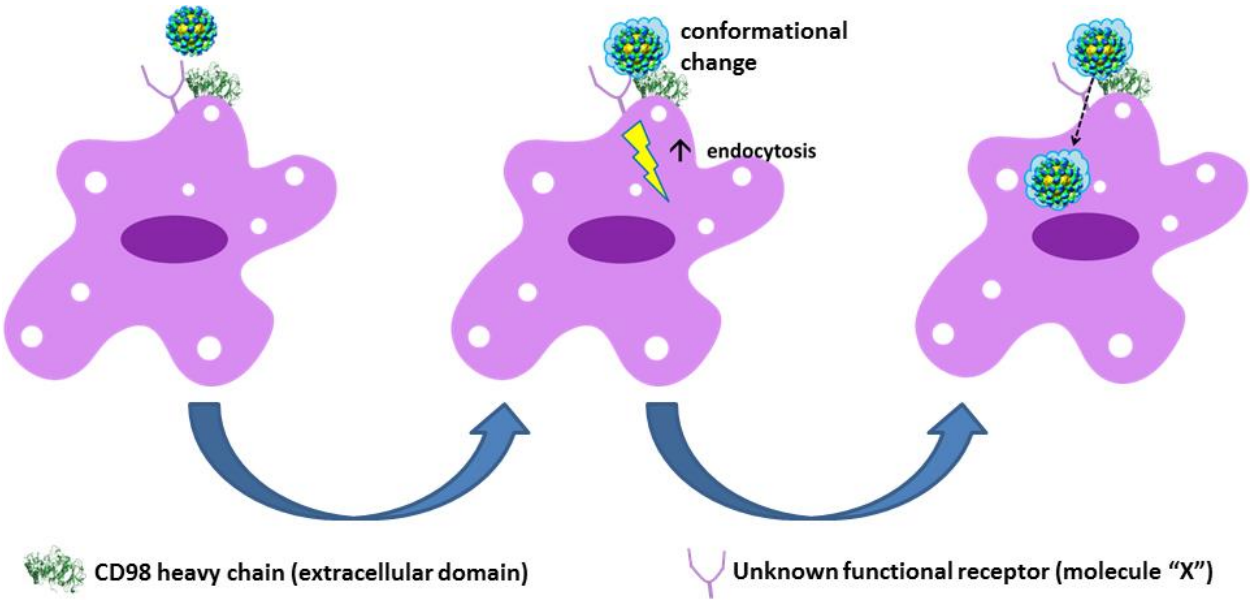

Figure 4.1. Working model of CD98-mediated MNV-1 infection *in vitro*. See text for detailed description of the model.

My working model postulates that MNV-1 first binds to CD98 on the cell surface of RAW 264.7 cells, forming a complex MNV-1+CD98. CD98 in the complex, when in proximity

to a still unknown functional receptor (molecule "X"), binds to it and another complex is formed: MNV-1+CD98+X. This complex formation may trigger conformational changes in the virus capsid and/or enhance endocytic activity of the host cell. In any case, MNV-1 internalization takes place upon formation of the MNV-1+CD98+X complex. It is important to emphasize that in our model, the crucial step that leads to virus internalization is the formation of the MNV-1+CD98+X complex on the cell surface. Only in this context MNV-1 would become internalized. Alternatively, MNV-1+CD98 could become internalized, but only when the complex MNV-1+CD98+X is formed that MNV-1 is able to cross the limiting membrane and reach the cytosol.

Our proposed model (Fig. 4.1) explains the finding of decreased MNV-1 binding and infection in CD98-depleted cells. In this situation, there is less CD98 for MNV-1 to bind to, and there is less CD98 to form a complex with molecule "X". Consequently, there is less MNV internalization as well. Our proposed model also provides a potential explanation to the soluble rCD98 enhancement of infection (observed only in high molar excess). When soluble rCD98 is mixed with MNV-1 in high molar excess, likely all MNV-1 particles are covered by rCD98. This way, the binding step of MNV-1 to CD98 on the cell plasma membrane is bypassed. Since there is a high amount of MNV-1+CD98, there is a greater chance that the complex will encounter molecule "X" (greater than under normal conditions, where the only CD98 source is the plasma membrane). Therefore, there would be more formation of MNV-1+CD98 and MNV-1+CD98+X complexes, and this would result in the increased binding and infection observed. Regardless of whether MNV-1 capsids undergo conformational changes or cells have enhanced endocytic activity (diffused or localized), it is the complex MNV-1+CD98+X formation that triggers MNV-1 internalization.

A first step to test whether soluble rCD98 could mediate interaction between MNV-1 and the plasma membrane is to assess the ability of the recombinant protein to bind to the plasma membrane by performing flow cytometric analysis (using anti-CD98) of RAW 264.7 cells incubated with rCD98 or BSA control. If rCD98 binds to the cell plasma membrane, we would observe increased fluorescence intensity in these cells compared to BSA-incubated cells. Additionally, defining the molecular mechanism(s) involved in CD98 facilitation of MNV-1 infection will be crucial to our better understanding of the MNV-1 infectious cycle *in vitro*. We would like to determine which steps of the infectious cycle and by which signaling pathway

CD98 exerts its role during MNV-1 infection of RAW 264.7 cells. Future studies will involve loss of function assays (e.g. siRNA knockdown) of pathways CD98 participates and proteinase K resistance assay [18] combined with light-sensitive MNV-1 infection of RAW 264.7 cells. These studies will help us further characterize the mechanism(s) by which CD98 facilitates MNV-1 infection *in vitro*.

4.3 Conclusion

My PhD dissertation has provided new information on the different biological features of two highly genetically similar MNV strains during infection *in vivo*. Additionally, this dissertation challenges the broadly applicable nature of the MNV M cell-dependent infection paradigm. The findings suggested that other non-M cell host factors determine the mechanisms through which MNV breaches the intestinal barrier. This work has also identified four transmembrane glycoproteins that are responsible for optimal MNV-1 binding to target cells *in vitro*. One of them (CD98) may have additional, post-binding role(s) during MNV-1 infection, but future experiments will be needed to test its putative role as a coreceptor.

As in most scientific research, the current study generated many more questions than answers. The field of norovirus-host interaction remains largely unexplored and full of interesting and challenging areas that await to be addressed. Ideally, this work will inspire and/or provoke curiosity in others to join the Wobus Laboratory and carry on some of the work I initiated.

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