Viruses in Rodent Colonies: Lessons Learned from Murine Noroviruses

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Abstract

Noroviruses (NoVs) are highly prevalent, positive-sense RNA viruses that infect a range of mammals, including humans and mice. Murine noroviruses (MuNoVs) are the most prevalent pathogens in biomedical research colonies, and they have been used extensively as a model system for human noroviruses (HuNoVs). Despite recent successes in culturing HuNoVs in the laboratory and a small animal host, studies of human viruses have inherent limitations. Thus, owing to its versatility, the MuNoV system—with its native host, reverse genetics, and cell culture systems—will continue to provide important insights into NoV and enteric virus biology. In the current review, we summarize recent findings from MuNoVs that increase our understanding of enteric virus pathogenesis and highlight similarities between human and murine NoVs that underscore the value of MuNoVs to inform studies of HuNoV biology. We also discuss the potential of endemic MuNoV infections to impact other disease models.

INTRODUCTION

Commensal bacteria: bacteria living on another host without causing harm or benefit

Virome: the collection of viruses—or their genes—living in a given environment, such as the gastrointestinal tract

Genogroup: related viruses within a genus; can be further subdivided into genotype

The breeding of specific-pathogen-free mice by exclusion of common mouse pathogens has enabled biomedical researchers to increase the consistency of disease models across laboratories and institutions by removing the potentially confounding effects of natural infections. However, the definition of specific pathogen free is unavoidably restricted to previously identified pathogens, most often discovered by their association with specific disease patterns. Murine noroviruses (MuNoVs) cause asymptomatic infections in many mouse strains, including immunocompetent mice and mice lacking adaptive immune responses (1, 2). Thus, their existence went unrecognized until 2003, when we serendipitously discovered the first MuNoV, called MNV-1, in our research facility (2). Since then, diagnostic MuNoV testing has been developed (3), and routine surveillance in mouse facilities has revealed that MuNoVs are the most prevalent type of pathogen in biomedical research facilities worldwide (4–7). Moreover, many MuNoV strains circulate in mouse research colonies and in the wild; over 100 different MuNoV sequences are currently deposited in GenBank.

With the recent advances in high-throughput sequencing technology, the identification of infectious agents no longer depends on their ability to cause overt symptoms. In analogy with commensal bacteria, the virome (viruses quiescently inhabiting a mammalian host), and its likely influences on the health of its host, is receiving increasing attention (8). Thus, the academic research community needs to tackle the difficult questions of which virome constituents are tolerable in mouse colonies and how to control for their inevitable variability. Our analysis of MuNoVs 12 years after their initial discovery—in terms of their utility as a norovirus (NoV) model system and their potential to impact other research models as members of the mouse virome—is meant to inform and stimulate this conversation. Thus, this review will focus on (*a*) how MuNoV research has advanced our understanding of general NoV biology, including cell tropism, promotion of viral pathogenesis by commensal bacteria, persistence, and immunity, and (*b*) the implications of endemic MuNoV infections for other disease models, considering their presence in many barrier facilities.

NOROVIRUSES: THE BASICS

Norovirus Genomic Organization

NoVs are positive-sense, single-stranded RNA viruses that constitute a genus in the Caliciviridae family (9). The genome is an ~7.5-kb polyadenylated RNA that has a viral protein called VPg (viral protein, genome-linked) covalently attached to the 5′ end and is composed of three or four open reading frames (ORFs) (**Figure 1*a***). ORF1 encodes the nonstructural (NS) proteins, which are expressed as a polyprotein that is cleaved by the virus-encoded protease into six mature proteins: NS1/2 (N-terminal protein), NS3 (p22), NS4 (3A-like protein), NS5 (VPg), NS6 (protease), and NS7 [RNA-dependent RNA polymerase (RdRp)] (**Figure 1*a***). ORF2 encodes the major capsid protein VP1, which is divided into shell (S) and protruding (P) domains and forms nonenveloped, icosahedral particles that are 27–40 nm in size (**Figure 1*b***). ORF3 encodes the minor capsid protein VP2, of which a few copies in the interior of the virion are thought to play a role in genome packaging (10, 11). MuNoVs also contain a fourth ORF that is expressed in an alternate reading frame from ORF2 and encodes VF1 (virulence factor 1), an antagonist of the innate immune response (12, 13).

Norovirus Phylogeny

NoVs display a high degree of genetic diversity and are classified according to their VP1 sequences into six established genogroups (GI–VI) (14) and a proposed GVII (15). Human NoVs (HuNoVs)

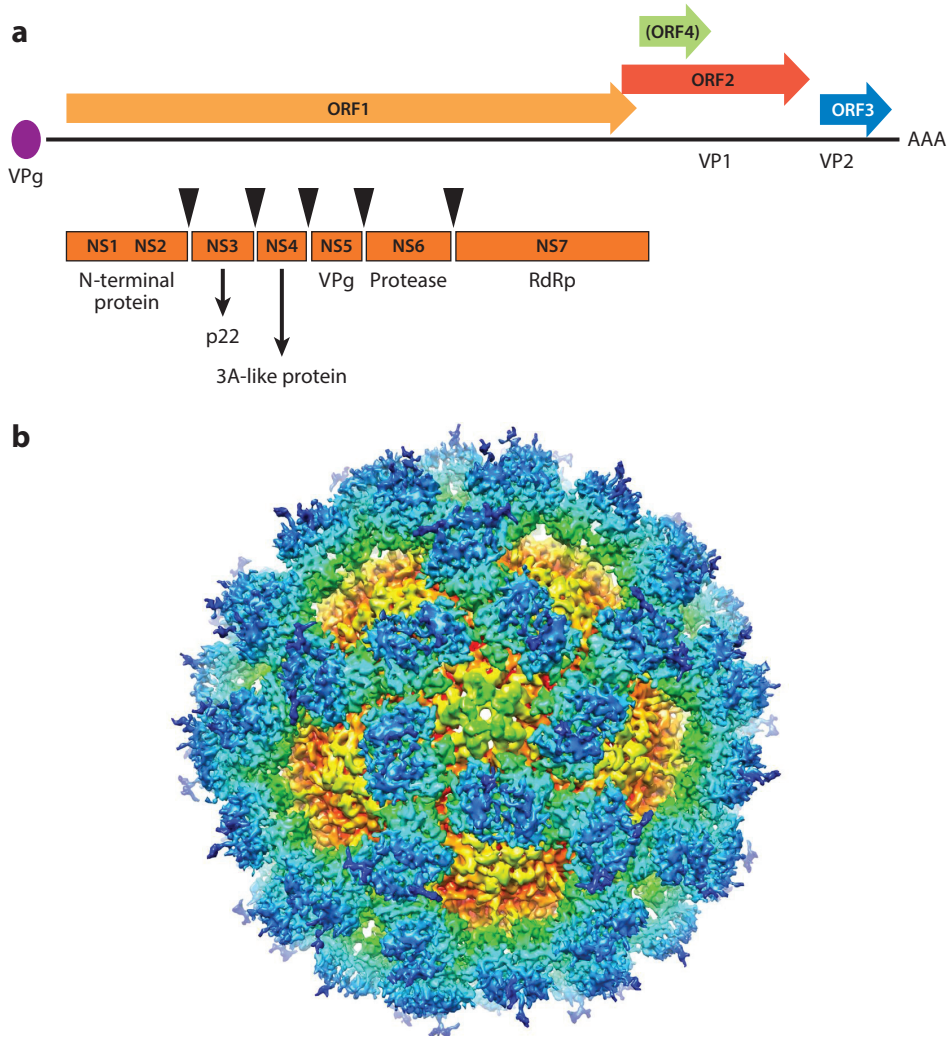


Figure 1

NoV genomic organization and capsid structure. (*a*) NoV genomic organization. ORF1, ORF2, and ORF3 are found in all NoVs, whereas ORF4 is found only in MuNoVs. Triangles indicate cleavage sites in the polyprotein. (*b*) Cryoelectron microscopy image reconstruction of MNV-1. The view is into an icosahedral fivefold axis. Reconstructions are shaded according to radial position. Abbreviations: MuNoV, murine norovirus; NoV, norovirus; NS, nonstructural; ORF, open reading frame; RdRp, RNA-dependent RNA polymerase; VP, viral protein; VPg, viral protein, genome-linked.

segregate in GI, GII, and GIV; GII also contains porcine strains. GIII contains bovine and ovine strains; GV contains mouse and rat strains; and GIV, GVI, and the proposed GVII contain canine strains (15). Genogroups are further subdivided into genotypes, with GII genotype 4 (GII.4) strains causing the majority of outbreaks and recurrent epidemics in humans (16, 17). The relationship between the genetic and serologic diversity of NoVs remains largely unknown. One exception is a study of seven MuNoV strains, which segregated into a single genotype and serotype (13). In spite of the genetic and serologic similarity between identified MuNoV strains, they display

significant phenotypic variability, as highlighted in this review. Considering the much greater genetic diversity among HuNoVs, it is likely that they are even more phenotypically variable.

Enteric virus: a virus that infects its host via the gastrointestinal tract

Norovirus Epidemiology

HuNoV infections occur in all age groups and are associated with significant morbidity, mortality, and economic losses. They cause ~20% of all cases of acute gastroenteritis worldwide (18) and an estimated 200,000 deaths in young children in developing countries annually (19). In the United States alone, these viruses cause approximately 21 million infections, 70,000 hospitalizations, and 800 deaths (20). After the introduction of a new rotavirus vaccine, HuNoVs became the most common cause of severe childhood diarrhea (21–23). Food-borne outbreaks due to HuNoVs in the United States cost an estimated 2.2 to 5.8 billion dollars per year (24, 25). The high prevalence of NoVs is attributable to their great stability in the environment and their highly infectious nature. NoV particles remain infectious for weeks to months in the environment (26–28), and the estimated 50% human infectious dose is between 18 and 2,800 particles (29, 30). Other features that make NoVs particularly well adapted to cause large outbreaks are their multiple routes of transmission (e.g., exposure to contaminated food, water, or fomites; person-to-person spread; exposure to aerosolized vomitus particles) and asymptomatic long-term shedding (31–33).

A detailed understanding of the biology of these prevalent pathogens is critical to developing effective prevention and treatment strategies. The identification of MuNoVs allowed the research community to study NoV infection in a tractable and genetically manipulable small animal model system. Moreover, until recently (34), MuNoVs were the only NoVs that could be cultured in the laboratory (35). Together with multiple reverse genetics systems that permit genetic manipulation of the MuNoV genome (36–38), the cell culture and small animal models have greatly advanced our understanding of NoV biology (39, 40). NoV replication strategies were comprehensively reviewed just last year (41), so we focus herein on findings in the MuNoV system and their contribution to our understanding of NoV and enteric virus biology.

NOROVIRUS TROPISM FOR IMMUNE CELLS

The cell tropism of HuNoVs has long eluded the research community, as these viruses appear to infect very few cells in vivo (42), complicating detection of viral antigen in tissue sections from infected hosts. Extensive efforts to culture HuNoVs in established intestinal epithelial cell (IEC) lines have been unsuccessful to date (43–46). Although these studies cannot rule out infection of IECs in vivo, the data outlined below clearly demonstrate that immune cells are MuNoV and HuNoV target cells.

Murine Noroviruses Infect Dendritic Cells and Macrophages

MuNoVs infect macrophages and dendritic cells in culture and in vivo (35, 47). Infection in culture induces apoptosis and cell lysis (13, 48). Although one study found that a HuNoV did not replicate in blood-derived macrophages or dendritic cells in vitro (42), the detection of HuNoV antigen in cells resembling or confirmed to be macrophages or dendritic cells in tissue sections of infected hosts suggests that these cells may be infected in vivo (42, 49–51). To reach their target cells, MuNoVs can cross the intestinal epithelium by transcytosis through microfold (M) cells in the absence of viral replication (52, 53). M cells are specialized IECs that transport particulate antigens from the lumen into the host to initiate immune responses but are also used by numerous enteric

pathogens to overcome host defenses (54, 55). Thus, it is likely that HuNoVs can also use M cells to breach the intestinal epithelial barrier.

Murine Noroviruses Infect B Cells

MuNoVs also infect B cells in culture (34). However, B cell infection is distinct from that of dendritic cells and macrophages. First, MuNoVs display low infectivity in mature B cells, with 5–15% of B cells actively infected, compared with >90% of macrophages (34). Second, MuNoV infection of mature B cells does not result in a loss of cell viability (34). Further studies are required to confirm whether infection is truly noncytopathic or whether the retention of cell viability is due to the low infectivity rate. Finally, mature B cells become persistently infected by MuNoVs despite high levels of progeny virus production and release into the culture supernatant (34). Multiple lines of evidence corroborate that B cells are also in vivo MuNoV targets: Virus titers are reduced in mice lacking functional B cells compared with wild-type mice; viral genomes can be amplified from purified Peyer's patch B cells of infected wild-type mice; and viral NS proteins can be detected in Peyer's patch B cells of infected STAT1$^{-/-}$ and IL-10$^{-/-}$ mice (1, 34, 56).

Peyer's patch: lymphoid follicles present in the lamina propria of the small intestine

Histo–blood group antigens (HBGAs): neutral carbohydrates attached to lipids or proteins presented on some cell types, including intestinal epithelial cells, and in body secretions such as saliva

A Human Norovirus Infects Cultured B Cells

The fact that MuNoVs can infect B cells (34) combined with the finding of HuNoV antigen in intestinal B cells of infected chimpanzees (49) prompted us to test whether HuNoVs similarly infect this cell type. Indeed, a GII.4 HuNoV productively infects the human B cell line BJAB (34). Similar to MuNoV infection, HuNoV infection of B cells in culture appears noncytopathic, and HuNoV can be transcytosed across a polarized epithelium to infect underlying B cells in a coculture system (34). Ongoing studies aim to enhance the robustness of this B cell cultivation system; it currently supports only low levels of viral replication, an apparent contradiction with the high levels of virus shed in the stool of infected people (31). Whether HuNoVs replicate in other cell types either in vitro or in vivo—particularly macrophages, dendritic cells, and IECs—needs to be determined in future studies.

NOROVIRUS INTERACTIONS WITH CARBOHYDRATES

Human and Murine Noroviruses Bind Glycans in a Virus Strain–Specific Manner

In analogy to infection with the related feline calicivirus (FCV) (57, 58), NoV infection of cells starts with viral attachment to cell surface glycans followed by binding to a protein receptor, although the latter has yet to be identified for NoVs. Most HuNoVs bind histo–blood group antigens (HBGAs) in a strain-dependent manner (reviewed, e.g., in 59, 60). Binding to HBGAs also extends to bovine NoVs (61) and canine NoVs (62), but not to MuNoVs (58) or feline NoVs (63). HBGAs, including the ABO and Lewis families of antigens, are neutral carbohydrates that are attached to lipids or proteins presented on multiple cell types, including IECs, and are released into body secretions like saliva (reviewed in 58). Certain HuNoV strains also bind heparan sulfate (64), sialic acid (65), and β-galactosylceramide (66), demonstrating the plasticity of glycan binding. As for HuNoV, MuNoV binding to host cell glycans is virus strain dependent (67). Certain MuNoV strains bind to terminal α2,3-linked sialic acid residues on gangliosides and glycoproteins, whereas others recognize yet-to-be-defined glycans on *N*-linked proteins (67, 68).

Conserved Features of Human and Murine Norovirus Glycan Interactions

Although the binding of human and murine NoVs to different glycans likely reflects an adaptation to their respective host species (67, 69), common features of norovirus-glycan interactions emerge. First, the glycan binding sites are located in a similar region of the VP1 P domain (67), topologically (**Figure 2*a–d***) and at the sequence level (**Figure 2*e***). Second, the outermost loops of the MuNoV and HuNoV P domains are highly flexible and are present in two conformations, open and closed (**Figure 2*f***) (70). For MuNoVs, the loop flexibility is important for escape from a neutralizing antibody (71). Future studies are needed to determine whether an anti-HuNoV antibody directed to the corresponding loop (72) is neutralizing and whether conformational changes in the HuNoV capsid can mediate escape.

Germ-free mice: animals born and raised under sterile conditions

Microbiota: the collection of microorganisms (herein used to describe bacteria)—or their genes—living in a given environment, such as the gastrointestinal tract

Carbohydrates May Act as Norovirus Attachment Receptors

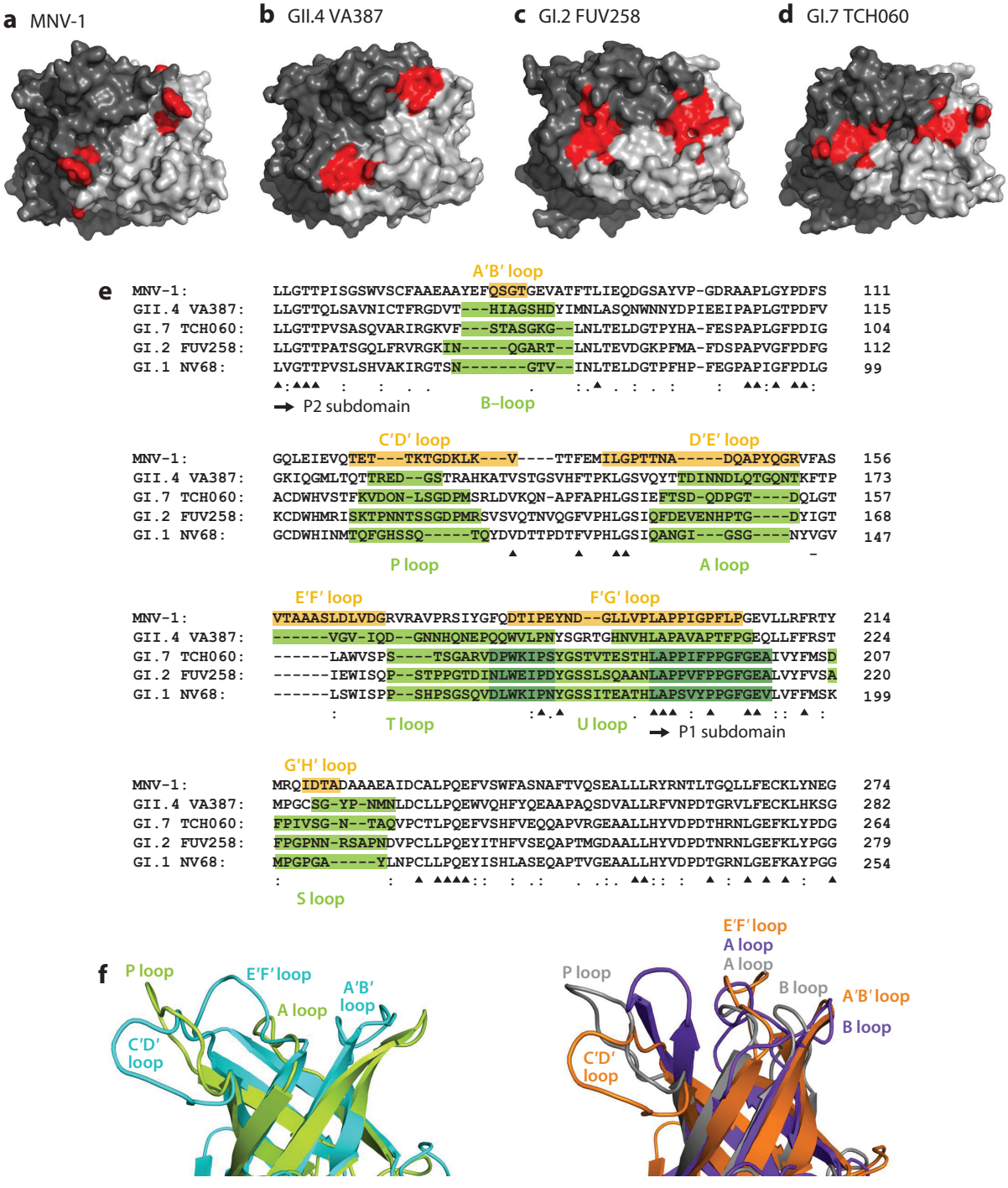
Although the presence of certain HBGAs is linked to susceptibility to specific HuNoV strains (reviewed in 73), the mechanism by which HBGAs facilitate HuNoV infection remains to be determined. HBGAs are not entry receptors: The presence of HBGAs on the cell surface does not permit infection of nonsusceptible cells (74). Instead, HBGAs likely play a role as HuNoV attachment receptors or coreceptors via concentrating viral particles on the cell surface (75). Free HBGAs also increase HuNoV attachment to, and promote infection of, susceptible B cells (34). HuNoV-based clustering of glycosphingolipids causes membrane invaginations (76); thus, binding to glycosphingolipids by murine and human NoVs may represent the first step in NoV entry. Although IECs synthesize HBGAs linked to lipids and/or proteins, immune cells—a NoV target cell population—lack the requisite biosynthetic enzymes and absorb only a low level of HBGA-linked glycolipids from the plasma (77). Thus, additional mechanistic studies are needed to solidify the role(s) of different forms of HBGAs during HuNoV infection.

NOROVIRUSES AND BACTERIA

An emerging theme in the pathogenesis of enteric viruses is their exploitation of commensal bacteria for the purpose of enhancing infection (78, 79). Analysis of viral infections in germ-free mice devoid of an intestinal microbiota and antibiotic (Abx)-treated mice with a significantly

Figure 2

Common features of murine and human NoV glycan binding sites. (*a–e*) The NoV glycan binding site location is conserved. Surface representations of P domain dimers from (*a*) MNV-1 (PDB 3LQ6), (*b*) GII.4 VA387 (PDB 2OBS), (*c*) GI.2 FUV258 (PDB 3ASP), and (*d*) GI.7 TCH060 (PDB 4P12) are shown. The monomeric units are colored in shades of gray and the glycan binding site is shown in red. The functional MNV-1 glycan binding site is based on Reference 67, the VA387 glycan binding site is from Reference 150, and the GI binding sites are from figure 2 of Reference 151. (*e*) A multiple sequence alignment of P domain sequences from representative NoVs was generated with Clustal Omega (152). Numbers represent amino acid residues of the P domain. P domain structures of MNV-1 (PDB 3LQ6), GII.4 VA387 (PDB 2OBS), GI.2 FUV258 (PDB 3ASP), GI.7 TCH060 (PDB 4P12), and Norwalk virus VP1 (PDB 1IHM) were viewed in PyMOL v1.3. Residues forming loops are highlighted in orange for MNV-1 and green for HuNoVs, and corresponding loop names are shown at the top and bottom, respectively. Loop nomenclature is based on References 70 and 153 for MNV-1 and HuNoVs, respectively. For GI HuNoVs, the T and U loops form one large unstructured region in PyMOL (*light and dark green shaded sequences*). (*f*) Antigenic loops of NoVs are highly flexible. Comparison of open and closed loop conformations: The open conformation is shown on the left, with the indicated loops from MNV-1 (A chain; PDB 3LQ6) shown in turquoise and HuNoV GI.7 TCH060 (C chain; PDB 4P12) in light green. The closed conformation is shown on the right, with the indicated loops from MNV-1 (B chain; PDB 3LQ6) shown in orange, HuNoV GII.4 VA387 (PDB 2OBS) in purple, and HuNoV GI.2 FUV258 (A chain; PDB 3ASP) in gray. Abbreviations: HuNoV, human norovirus; MuNoV, murine norovirus; NoV, norovirus; P, protruding.


a MNV-1
b GII.4 VA387
c GI.2 FUV258
d GI.7 TCH060
e
A'B' loop
MNV-1: LLGTTPISGSWVSCFAAEAAYEFQSGTGEVATFTLIEQDGSAYVP-GDRAAPLGYPDFS 111
GII.4 VA387: LLGTTQLSAVNICTFRGDVT---HIAGSHDYIMNLASQNWNNYDPIEEIPAPLGTPDFV 115
GI.7 TCH060: LLGTTPVSASQVARIRGKVF---STASGKG--LNLTELDGTPYHA-FESPAPLGFPDIG 104
GI.2 FUV258: LLGTTPATSGQLFRVRGKIN-----QGART--LNLTEVDGKPFMA-FDSPAPVGFPDFG 112
GI.1 NV68: LVGTTPVSLSHVAKIRGTSN-------GTV--INLTELDGTPFHP-FEGPAPIGFPDLG 99
P2 subdomain
B–loop
C'D' loop
D'E' loop
MNV-1: GQLEIEVQTET---TKTGDKLK---V----TTFEMILGPTTNA-----DQAPYQGRVFAS 156
GII.4 VA387: GKIQGMLTQTTRED--GSTRAHKATVSTGSVHFTPKLGSVQYTTDINNDLQTGQNTKFTP 173
GI.7 TCH060: ACDWHVSTFKVDQN-LSGDPMSRLDVKQN-APFAPHLGSIEFTSD-QDPGT----DQLGT 157
GI.2 FUV258: KCDWHMRISKTPNNTSSGDPMRSVSVQTNVQGFVPHLGSIQFDEVENHPTG----DYIGT 168
GI.1 NV68: GCDWHINMTQFGHSSQ-----TQYDVDTTPDTFVPHLGSIQANGI---GSG----NYVGV 147
P loop
A loop
E'F' loop
F'G' loop
MNV-1: VTAAASLDLVDGRVRAVPRSIYGFQDTIPEYND--GLLVPLAPPIGPFLPGEVLLRFRTY 214
GII.4 VA387: ------VGV-IQD--GNNHQNEPQQWVLPNYSGRTGHNVHLAPAVAPTFPGEQLLFFRST 224
GI.7 TCH060: ------LAWVSPS----TSGARVDPWKIPSYGSTVTESTHLAPPIFPPGFGEAIVYFMSD 207
GI.2 FUV258: ------IEWISQP--STPPGTDINLWEIPDYGSSLSQAANLAPPVFPPGFGEALVYFVSA 220
GI.1 NV68: ------LSWISPP--SHPSGSQVDLWKIPNYGSSITEATHLAPSVYPPGFGEVLVFFMSK 199
T loop
U loop
P1 subdomain
G'H' loop
MNV-1: MRQIDTADAAAEAIDCALPQEFVSWFASNAFTVQSEALLLRYRNTLTGQLLFECKLYNEG 274
GII.4 VA387: MPGCSG-YP-NMNLDCLLPQEWVQHFYQEAAPAQSDVALLRFVNPDTGRVLFECKLHKSG 282
GI.7 TCH060: FPIVSG-N--TAQVPCTLPQEFVSHFVEQQAPVRGEAALLHYVDPDTHRNLGEFKLYPDG 264
GI.2 FUV258: FPGPNN-RSAPNDVPCLLPQEYITHFVSEQAPTMGDAALLHYVDPDTNRNLGEFKLYPGG 279
GI.1 NV68: MPGPGA-----YLNPCLLPQEYISHLASEQAPTVGEAALLHYVDPDTGRNLGEFKAYPGG 254
S loop
f
P loop
E'F' loop
A'B' loop
A loop
C'D' loop
E'F' loop
A loop
A loop
P loop
B loop
A'B' loop
B loop
C'D' loop

reduced intestinal microbial load demonstrated that infection with, and in some cases transmission of, different types of enteric viruses—poliovirus, reovirus, mouse mammary tumor virus (MMTV), and rotavirus—is enhanced by the microbiota (80–83).

Commensal Bacteria Promote Murine Norovirus Infection of Mice

Mirroring results for these other enteric viruses, commensal bacteria also promote MuNoV infections (**Figure 3*a***). Commensal bacteria enhance acute MNV-1 and MNV-3 infections, as virus titers in mucosal tissues were reduced in Abx-treated mice (34). Commensal bacteria also facilitate the establishment of persistent MNV-CR6 infection (84): Persistent intestinal infection was not observed in Abx-treated mice, but fecal transplantation from microbially colonized (but not Abx-treated) mice restored the ability of MNV-CR6 to establish persistence. A third study demonstrated reduced MNV-CR6 infection in germ-free mice, indicating that bacteria enhance, but are not absolutely required for, MuNoV infection (85). This is consistent with findings that MuNoV infection of cultured cells (34, 35), and its transcytosis across the epithelial barrier in vitro (52), does not require bacteria.

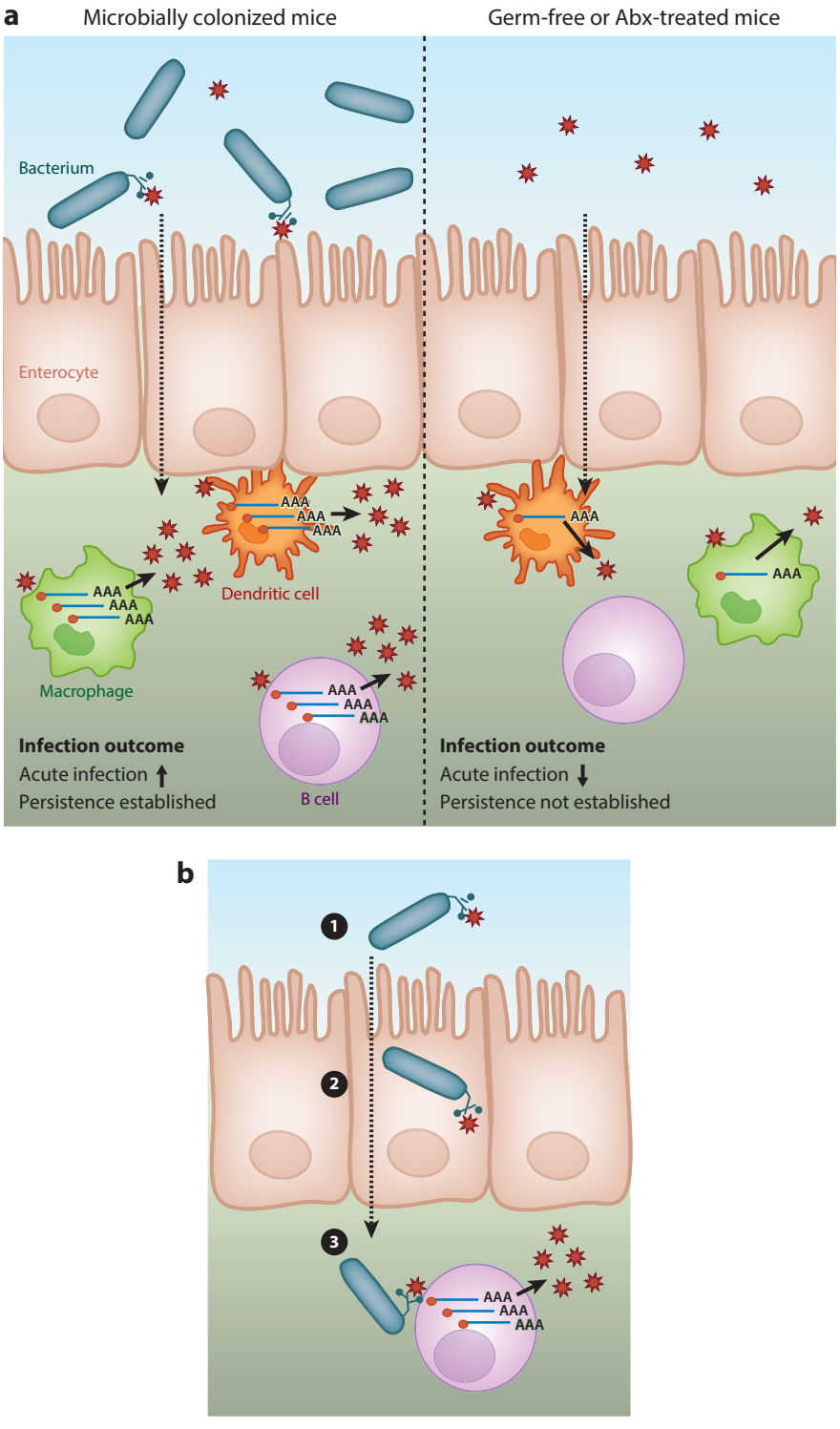
Commensal Bacteria Stimulate Human Norovirus Infection of B Cells

The importance of commensal bacteria for enteric MuNoV infection led Jones et al. (34) to test whether HuNoV infection of cultured cells was enhanced by the presence of bacteria. Indeed, removal of commensal bacteria from virus-positive stool via filtration drastically reduced viral infectivity of human B cells, although viral titers in the inoculum were unaffected, and a specific species of commensal bacteria—*Enterobacter cloacae*—was able to restore infectivity of the filtered virus inoculum (34).

In contrast to poliovirus and MMTV (80, 81, 83), NoVs do not appear to bind bacterial lipopolysaccharide: Free lipopolysaccharide was unable to stimulate HuNoV infection of B cells (34), and the lipopolysaccharide sensor TLR4 and its downstream signaling molecules were dispensable for establishment of persistent MuNoV infections (84). Instead, HBGAs expressed on certain commensal bacteria (86) promoted HuNoV infection of B cells, whereas a non-HBGA-expressing bacterial strain did not (34). Importantly, synthetic HBGA restored infectivity of filtered virus inoculum comparably to HBGA-expressing bacteria (34). A model emerges whereby a HuNoV binds an HBGA-expressing commensal bacterium in the gut lumen, the virus-bacteria

Figure 3

Commensal bacteria promote NoV infections. (*a*) MuNoV infection of Abx-treated or germ-free mice (*right*) is significantly reduced compared with infection of microbially colonized mice (*left*), demonstrating that commensal bacteria stimulate NoV infections in vivo by an unknown mechanism. Because infection of germ-free mice is not completely ablated, bacteria-independent mechanisms exist to allow the virus to breach the intestinal epithelium and access target immune cells. This is represented by free virus interacting with target cells. (*b*) A model for bacterial stimulation of HuNoV B cell infection, based on the work of Jones et al. (34). In this model, (❶) a HuNoV binds commensal bacteria expressing the appropriate histo–blood group antigen in the gut lumen, (❷) the virus-bacteria complex is cotranscytosed across the polarized epithelium, (❸) and the bacterial glycan stimulates viral attachment to permissive B cells. This model is based on data showing that filtration-based removal of bacteria from a virus-positive stool inoculum significantly reduced HuNoV infection of B cells both in direct B cell infection and in a coculture system where the viral inoculum (placed into the apical supernatant) and target B cells (cultured in the basolateral chamber) were separated by a layer of polarized intestinal epithelial cells. Abbreviations: Abx, antibiotic; HuNoV, human norovirus; MuNoV, murine norovirus; NoV, norovirus.


a
Microbially colonized mice
Germ-free or Abx-treated mice
Bacterium
Enterocyte
AAA
Dendritic cell
Macrophage
B cell
Infection outcome
Acute infection
Persistence established
Infection outcome
Acute infection
Persistence not established
b
1
2
3

complex is transcytosed across the intestinal epithelium, and the bacterial glycan stimulates viral attachment to underlying intestinal B cells (**Figure 3*b***) (87). Overall, identification of commensal bacteria as a stimulatory factor for in vivo MuNoV infection led directly to the development of the first HuNoV culture system, underscoring the power of animal models in advancing our understanding of human pathogens.

Many open questions remain regarding the physiological significance of NoV interactions with commensal bacteria. For example, what are the immune implications of virus binding to commensal bacteria? Corecognition of bacterial and viral ligands may result in a tolerogenic environment that suppresses robust antiviral immunity, similar to findings with MMTV (81). Consistent with this idea, MuNoV infection of wild-type mice is only weakly inflammatory, whereas infection of IL-$10^{-/-}$ mice results in intestinal inflammation but only in the presence of commensal bacteria (**Figure 4*a***) (56). This suggests that the anti-inflammatory cytokine IL-10 suppresses immune responses to NoVs in a bacteria-dependent manner.

NOROVIRUS PERSISTENCE AND IMMUNITY

Two immune mechanisms that control (*a*) acute NoV infections and (*b*) protective immunity determinants were reviewed recently (40). In brief, type I interferon (IFN) and components of the adaptive immune system coordinate control and clearance of primary NoV infections, and antibody and $CD4^+$ T cells are critical for mediating protective immunity to a secondary NoV challenge, although there are virus strain–specific distinctions in the magnitude of memory immune responses and the overall duration of these responses is fairly short lived. Below we focus on the ability of NoVs to establish prolonged, or persistent, infections and on more recent studies pertaining to NoV immunity that illuminate (*a*) a critical role for type III IFN in controlling persistent enteric infection and (*b*) the influences of nutritional status and coinfection on anti-NoV immune responses.

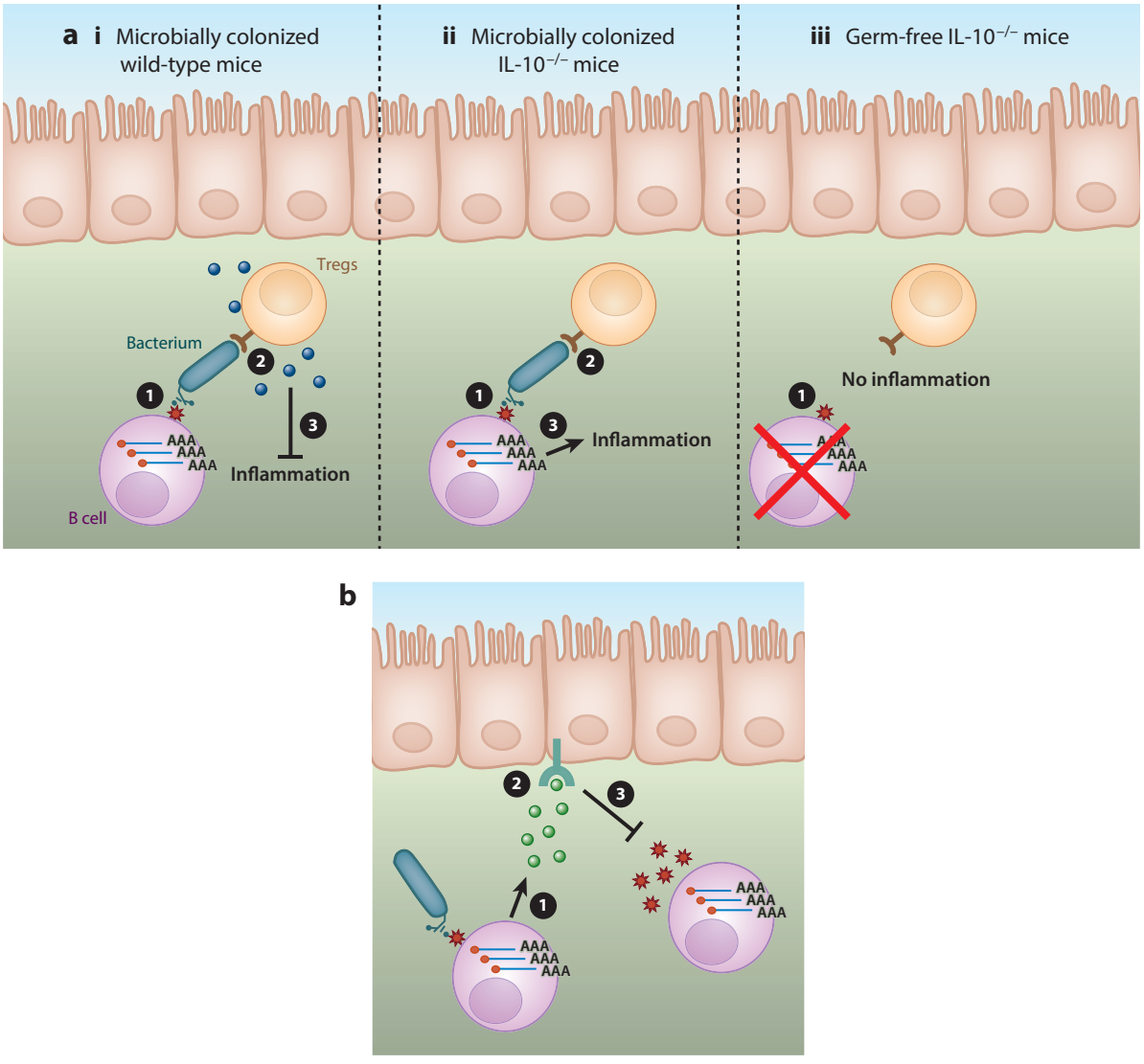
Murine Noroviruses Can Persist in the Colon, Contributing to Prolonged Fecal Shedding

The classical symptoms of HuNoV infection (e.g., vomiting, diarrhea, and nausea) develop and resolve within a few days, but viral shedding following symptomatic and asymptomatic infections

Figure 4

Commensal bacterial interactions may drive the immune response during NoV infections. (*a*) Commensal bacteria stimulate a tolerogenic IL-10-dependent microenvironment that suppresses inflammation in response to MuNoV infection. Basic et al. (56) demonstrated that MuNoV infection is inflammatory in IL-$10^{-/-}$ but not wild-type mice and that inflammation in the absence of IL-10 was dependent on commensal bacteria, as it did not occur in germ-free IL-$10^{-/-}$ mice. Based on these results, together with the observations of Jones et al. (34), Baldridge et al. (84), and Kernbauer et al. (85) revealing that commensal bacteria stimulate MuNoV infection, we propose the following model. (*i*) In wild-type mice with a normal intestinal microbiota, commensal bacteria stimulate MuNoV infection of immune cells (❶); commensal bacteria–specific Tregs recognize bacterial antigens (❷) and secrete IL-10 (*blue spheres*), which prevents virus-induced inflammation (❸). There is currently no evidence that Tregs are producers of IL-10 in this model, and future studies will be required to determine the cellular source of this immunosuppressive cytokine. (*ii*) In microbially colonized mice deficient in IL-10 expression, viral infection proceeds normally because stimulatory commensal bacteria are present (❶). However, because Tregs cannot secrete IL-10 in response to bacterial antigen recognition (❷), immune responses to the virus are not suppressed and instead induce an inflammatory response (❸). (*iii*) In germ-free IL-$10^{-/-}$mice, viral infection is significantly reduced due to the absence of stimulatory commensal bacteria (❶); thus, no inflammation is observed. (*b*) Based on findings by Nice et al. (96), we propose a model whereby acute NoV strains, such as MNV-1, induce IFNλ (*green spheres*) (❶); IFNλ signals to intestinal epithelial cells or other nonimmune cells (❷); and this signaling indirectly reduces viral replication and prevents persistence (❸). Persistent MuNoV strains such as MNV-CR6 fail to induce significant IFNλ, likely a key strategy for persistence establishment. Abbreviations: IFN, interferon; MuNoV, murine norovirus; NoV, norovirus; Tregs, regulatory T cells.

lasts for weeks to months (31, 88, 89). Moreover, immunocompromised patients can be chronically infected (90), generating highly diverse viral populations that may serve as reservoirs of phenotypically distinct HuNoV strains (91, 92). Similar to HuNoVs, most MuNoVs, with the exception of MNV-1, are shed in the feces for extended periods of time (13). Long-term shedding of MuNoVs has been associated with ongoing low-level persistent infection of the colon (93). A single amino acid difference in the NS1/2 protein between MNV-1 (which causes acute infection) and MNV-CR6 (which causes persistent infection) regulates colonic tropism and establishment of persistence (94). In addition, antiviral $CD8^+$ T cell induction is reduced during persistent MNV-CR6 infection compared with T cell responses during acute MNV-1 infection (95), suggesting that immune evasion contributes to the ability of a NoV to establish persistence. The cell type acting as a persistent reservoir of NoVs is undefined, but we speculate that it is B cells, considering they are persistently infected by MuNoVs in culture (34).



Type III Interferon Controls Persistent Murine Norovirus Infection of the Colon

Although type I IFN plays a critical role in preventing severe MuNoV infections and limits replication in peripheral tissues (1, 2, 96), the type I IFN receptor (encoded by *Ifnar1*) is dispensable for controlling persistent colonic infection and fecal shedding of MNV-CR6 (96). Instead, the type III IFN, or IFNλ, receptor (encoded by *Ifnlr1*) controls intestinal infection independent of type I IFN signaling or adaptive immune cells. IFNλ treatment one day after MNV-CR6 infection prevented persistent infection, and IFNλ treatment after establishment of persistent infection resulted in viral clearance from tissues and feces. This suggests type III IFN inhibition of intestinal infection occurs indirectly (**Figure 4*b***), a conclusion further supported by the following findings. First, IFNλ (but not IFNβ) treatment of permissive dendritic cells in culture failed to inhibit MNV-CR6 replication. Second, bone marrow chimera experiments showed increased MNV-CR6 shedding into the feces when *Ifnlr1*$^{-/-}$ recipient mice received wild-type donor (immune) cells but not when wild-type recipient mice received *Ifnlr1*$^{-/-}$ donor cells. Finally, the ability of a MuNoV strain to establish persistence correlated with a lack of induction of IFNλ (but not IFNβ) in infected tissues.

Overall, findings from MuNoV indicate that NoVs evade both innate (e.g., IFN induction) and adaptive (e.g., robust CD8$^+$ T cell response) immunity to establish persistence (12, 95). The influence of commensal bacteria on persistence is complex, as underscored by the observation that MNV-CR6 persistence in *Ifnlr1*$^{-/-}$ mice not only is enhanced compared with its persistence in wild-type control mice but also occurs in a bacteria-independent manner, whereas persistence establishment in wild-type mice is dependent on bacteria (84). Thus, commensal bacteria may normally suppress IFNλ and thereby facilitate enteric viral infections.

Malnutrition and Helminth Coinfections Suppress Antinorovirus Immunity

People in developing countries are susceptible to more severe enteric infections than people in industrialized nations are, and they fail to respond as robustly to oral vaccines (97–100). The basis for these phenomena is multifactorial, and studying these processes in tractable animal models is critical to the development of strategies to enhance protection in this particularly vulnerable population. Studies using the MuNoV model have made important contributions. First, malnourished mice developed more severe and prolonged MNV-1 infections, and they failed to develop protective immunity to a secondary challenge, which correlated with a severely muted antiviral mucosal IgA response (101). They also supported enhanced viral evolution, raising the possibility that malnourished individuals could represent reservoirs of novel viral variants. Second, the antiviral CD8$^+$ T cell response to MNV-1 or MNV-CR6 was reduced in mice coinfected with the helminth *Trichinella spiralis* (102). This reduction was associated with potent activation of alternatively activated macrophages, suggesting an immunomodulatory role for these innate effector cells. Because MuNoV infections are regulated by the intestinal microbiota, by nutrition, and by coinfections with other common enteric pathogens, they represent an excellent model with which to study the complex relationships that ultimately dictate disease outcome.

DEVELOPMENT OF NOROVIRUS ANTIVIRALS

Although the rapid nature of typical NoV infections may limit the utility of therapeutic antiviral treatment strategies in immunocompetent individuals, NoV drug discovery efforts are needed to treat the high number of immunocompromised patients chronically infected with HuNoVs (90). Moreover, prophylactic drugs that limit viral transmission can greatly reduce the economic costs

associated with NoV infections (25). Due to the difficulties in developing a robust HuNoV cell culture system (43–46), the inability to orally infect mice with HuNoVs (51), and the high cost of HuNoV large animal studies, many investigators have used the MuNoV model for initial drug discovery efforts, often complemented by studies in HuNoV replicon–bearing cells (103). Several recent reviews on the topic of NoV antiviral drug development have been published (e.g., 104, 105). Thus, we focus on examples that have used the MuNoV model for drug development and efficacy testing both in vitro and in vivo.

Antivirals Targeting Viral Proteins

Although all NoV proteins represent putative targets for antiviral development, most work to date has focused on the viral protease and polymerase (105). The conserved nature of these two NoV proteins (106, 107) increases the likelihood that findings from MuNoVs will translate to HuNoVs in future clinical trials. Furthermore, the NoV RdRp is similar to other RdRps from positive-sense RNA viruses (107), allowing the use of previously developed RdRp inhibitors such as the nucleoside analogs 2′-*C*-methylcytidine (2CMC) and favipiravir. 2CMC and the closely related analog 2′-F′-2CMC displayed antiviral activity against MuNoV in vitro in the low-micromolar range, and drug-resistant escape mutants were not detected through 30 passages (108, 109). Importantly, mice lacking type I and II IFN receptors treated with 2CMC prior to MNV-1 infection were protected from developing diarrhea, and they developed protective immunity to a subsequent lethal challenge (110). 2CMC also has potential as a prophylactic in preventing NoV transmission (111) and was effective in clearing a HuNoV from replicon-bearing cells (110). However, the likelihood of widespread use in humans is low given the previously observed side effects of the 2CMC derivative valopicitabine (also called NM283) in clinical trials of HCV-infected patients (112, 113).

Favipiravir (T-705) is in late-stage clinical development for influenza virus treatment and exhibits antiviral activity against multiple other RNA viruses in animal models (e.g., 114–117). Favipiravir prevented MuNoV replication in culture early during infection (118). More importantly, it reduced MuNoV titers in vivo, and virus was eliminated from the feces in the majority of persistently infected animals (119). Favipiravir is a guanosine and adenosine analog (120) that induced mutations in the MuNoV genome in vivo (119), providing in vivo proof that lethal mutagenesis (i.e., increasing mutations in the viral genome to cause extinction of the viral population) can be used therapeutically. Considering the broad-spectrum antiviral activity of favipiravir (121), this drug will likely exhibit antiviral activity against diverse HuNoV strains.

Antivirals Targeting Host Proteins

Numerous host proteins facilitate NoV infections, and those representing candidate drug targets were recently reviewed (104). However, in vivo studies of host-targeted antivirals that reduce NoV infections without significant toxicity remain scarce. One example is WP1130, a small molecule that inhibits a subset of cellular deubiquitinases (DUBs) (122). Ubiquitin-modifying enzymes, including DUBs, have critical regulatory functions in many cellular processes and can influence viruses at all stages of the viral life cycle. WP1130 exhibits antiviral activity in cell culture against several RNA viruses, including MuNoV in murine macrophages and Norwalk virus in replicon-containing cells by induction of the unfolded protein response through cellular DUB inhibition (123). Oral administration of WP1130 inhibited MuNoV infection in the proximal small intestine of mice, although it failed to block infection in more distal regions of the intestine because of the poor solubility and/or bioavailability of the compound. These studies suggest that cellular

DUBs represent promising targets for host-targeted broad-spectrum antivirals, but future efforts are needed to identify more effective derivatives.

Although anti-NoV drug development efforts are still at an early stage, studies with MuNoVs have identified many putative targets. The recent development of a HuNoV culture model (34) now provides an important tool to test the translatability of MuNoV findings to HuNoVs, thus significantly enhancing future efforts.

INFLUENCE OF MURINE NOROVIRUSES ON BIOMEDICAL RESEARCH MODELS

MuNoVs are highly prevalent in mouse colonies at academic research institutions and in pet stores (4, 124). Most strains of mice naturally infected with a MuNoV do not display noticeable signs of illness; thus, their presence easily goes unrecognized. Eradicating MuNoVs from mouse colonies is time-consuming and expensive (125); thus, many academic research institutions tolerate the presence of natural MuNoVs. This can significantly compromise experimental interpretability. For example, in a large microarray analysis of germ-free versus conventionally housed mice, certain conventional animals were contaminated with a MuNoV, causing upregulation of antiviral genes (126). Considering the tropism of MuNoVs for innate and adaptive immune cells (34, 35) and their ability to establish persistent infections (2, 13), multiple studies have directly tested the influence of MuNoV infection on various models of inflammatory diseases and other infections. As summarized below, these studies demonstrate that MuNoV infections can have effects on intestinal inflammatory conditions, dependent on host genetic background and MuNoV strain, but most MuNoV infections minimally impact other disease models.

Effects of Murine Norovirus Infections on Extra-Intestinal Inflammatory and Infectious Conditions

With the exception of the inflammatory bowel disease (IBD) models discussed below, studies examining the effect of MuNoV infection on inflammatory conditions have observed no or modest conditional phenotypes. MNV-4 infection resulted in a modest increase in the size of atherosclerotic lesions in *Ldlr*$^{-/-}$ mice fed a high-fat, high-cholesterol diet, but not in those fed a high-fat, high-sucrose diet (127). MNV-4 infection did not affect the development or severity of obesity or insulin resistance in wild-type C57BL/6 mice fed a high-fat diet or in *Ldlr*$^{-/-}$ mice (127, 128), nor did it affect *Helicobacter*-induced colon cancer in *Smad3*$^{-/-}$ mice (129).

Similarly, MuNoVs have no to modest effects on other infections. Persistent MNV-G infection enhanced a subsequent mouse parvovirus infection and extended the duration of mouse parvovirus shedding (130). However, neither acute MNV-1 nor persistent MNV-CR6 infection altered the severity of, or immunity to, influenza A virus or vaccinia virus infection (131). MNV-1 also failed to affect murine cytomegalovirus infection establishment or reactivation, although MNV-1 infection modestly reduced the T cell response to murine cytomegalovirus (132). Furthermore, MNV-CR6 did not alter the immune response to Friend retrovirus (133).

Murine Norovirus Effects on Inflammatory Bowel Disease Models

Crohn's disease, a common form of IBD in people, is associated with genetic polymorphisms in numerous loci, including the autophagy gene *Atg16L1* (134, 135). Mice with an *Atg16L1* mutation are susceptible to intestinal abnormalities displaying features of Crohn's disease (136–138). This phenotype is linked to persistent MuNoV infection, an association that was revealed when

researchers rederived mice into a barrier facility free of endemic MuNoV and observed a loss of the intestinal abnormalities, particularly in Paneth cells, that were previously detected in the *Atg16L1*-deficient mice (137). Aberrant Paneth cell morphology (e.g., in granule packaging) was fully restored upon experimental MNV-CR6, but not MNV-1, infection (138). Moreover, intestinal abnormalities in MNV-CR6-infected *Atg16L1*-deficient mice were dependent on commensal bacteria and the inflammatory cytokines TNFα and IFNγ. A model emerges whereby *Atg16L1* deficiency causes a reduction in the amount of antimicrobial peptides produced in, or packaged into granules of, Paneth cells while increasing inflammatory cytokine production in response to a viral infection (139). This is a perfect example of how endemic infections in colonies of research animals can confound data interpretation and introduce lab-to-lab variability within the same model system. It is also, however, an elegant and powerful example of how animal models can facilitate the dissection of complex multifactorial disease processes that are responsible for many inflammatory and autoimmune disorders common in the human population. Future studies are needed to investigate how the newly recognized interdependence between the microbiota, MuNoV infection, and the mucosal immune system (34, 84, 85) contributes to these pathologies.

Other models of IBD showed less dramatic effects, underscoring the conditional nature of MuNoV effects on inflammatory disease models. *Helicobacter* infection induces IBD in certain knockout strains of mice, and MNV-4 infection exacerbated this condition in *Mdr1a*$^{-/-}$ mice (140). However, MNV-4 failed to exacerbate disease in *Helicobacter*-infected *Smad3*$^{-/-}$ or *Il10*$^{-/-}$ mice (129, 141). *Salmonella typhimurium* infection of wild-type mice treated with streptomycin is used as a model of intestinal fibrosis, a complication in IBD, but acute MNV-1 or persistent MNV-4 infection did not alter major features of this model (142).

Potential for Murine Norovirus Infection to Shape the Intestinal Microbiota

Endemic viral infections may influence the composition of the intestinal microbiota, which strongly shapes host mucosal immunity (143–145). Conflicting reports exist as to whether MuNoV infection alters the composition of the microbiota in immunocompetent mice. No major changes were observed in the intestinal microbiota of inbred C57BL/6 or outbred Swiss Webster mice infected with MNV-1, MNV-4, or MNV-CR6 (146). In another study, a significant reduction in the Bacteroidetes/Firmicutes ratio was observed in MNV-1-infected C57BL/6 mice compared with mock-inoculated controls (101). The reason for these discordant results is unclear, but institutional variability may play a role. Reduced Bacteroidetes and increased Proteobacteria were observed in a minority of HuNoV-infected subjects (147), although very limited health data and inability to perform repeated sampling confound the observation. These limited data suggest that NoV infections may alter the microbiota under certain conditions.

Murine Noroviruses Provide Beneficial Effects of Commensal Bacteria in Germ-Free Mice

The beneficial effect of a "healthy" microbiota is widely recognized (e.g., 148, 149). Remarkably, one study recently demonstrated that a single MuNoV could restore in germ-free mice the beneficial functions typically attributed to commensal bacteria despite reduced viral loads (85). Germ-free mice infected with different MuNoV strains (MNV-CR6, MNV-1, and MNV-SKI) resembled bacterially colonized mice in terms of villus width, lymphocyte number and function, antibody levels, and basal suppression of innate lymphoid cell expansion. These phenotypes were dependent on type I IFN signaling, because MNV-CR6 was not able to restore intestinal health in *Ifnar1*$^{-/-}$ germ-free mice. MuNoV infection was also protective against infectious and chemical

forms of intestinal injury in Abx-treated mice. These results suggest that under certain conditions MuNoVs may be considered commensals in nature, shifting the perception that viral infections are strictly pathogenic. However, as this applied only to a bacteria-free environment, the translatability to other NoV infections is unclear.

CONCLUSIONS

The discovery of MNV-1 and the subsequent development of cell culture and reverse genetics systems have provided important tools to the NoV field to study molecular aspects of NoV biology. Combined with advances in HuNoV research, these studies have yielded important insights into unifying principles underlying NoV replication mechanisms. Further comparative studies between NoVs from different host species will likely reveal additional shared mechanisms that deepen our understanding of these viruses. In addition, the study of MuNoVs as a model for enteric virus infections has yielded exciting new findings regarding the role of commensal bacteria during acute and persistent viral infections, effects on host physiology, and innate immunity in the intestine. Because these studies are in their infancy, much remains to be discovered regarding the host-microbiota-virus interplay.

Complicating but also enriching these studies is the phenotypic diversity of MuNoV strains. Although the strains are genetically highly related and segregate into a single cluster within genogroup V, there are striking differences in the duration of infections, virulence, cytopathogenicity in cultured cells, immune responses, and effects on other inflammatory conditions. Even for the strains preferentially studied in the MuNoV field (i.e., MNV-1 as the prototype acute MuNoV strain and MNV-3, MNV-4, MNV-CR3, and MNV-CR6 as representative persistent strains), the viral determinants regulating the varied outcomes of infection with these viruses are only beginning to be defined. HuNoVs exhibit much greater genetic diversity between different clusters and genogroups. Thus, one implication for HuNoV research is the existence of a variety of unrecognized differential infection outcomes and immune responses. Dissecting and defining these differences and developing the tools to do so remain a major challenge for the future. Knowledge from studies of MuNoVs may help guide such efforts. It may be in this capacity that a common contaminant to mouse research facilities makes its greatest contribution to improving human health.

SUMMARY POINTS

1. The MuNoV system has made significant contributions to a greater understanding of NoV biology.
2. MuNoV studies have driven the identification of B cells as NoV targets and commensal bacteria as cofactors for NoV infection.
3. Although the specific glycan structures used during NoV infection display a wide degree of variability across virus strains, carbohydrate usage by NoVs is highly conserved.
4. NoVs have evolved specific features to successfully infect and spread in an intestinal ecosystem dominated by bacteria.
5. Most MuNoV infections minimally impact other disease models, but effects on intestinal inflammatory conditions need to be evaluated on a case-by-case basis.

FUTURE ISSUES

1. MuNoV and HuNoV share many similarities, but additional comparative studies are needed to better characterize the extent of similarities and differences and identify the unifying principles in NoV biology.
2. Studies investigating the mechanisms of NoV disease pathogenesis are needed.
3. The field has a need for improved animal models of HuNoV disease that incorporate the microbiota and a full complement of immune functions.
4. Future research should aim to gain a mechanistic understanding regarding the role(s) of carbohydrates and enteric bacteria during NoV infection.
5. Studies are needed to identify HuNoV target cells in vivo and to develop new or more robust cell culture models.

DISCLOSURE STATEMENT

S.M.K. is coinventor on a patent application pertinent to this work (US patent application no. 61/992,040, "Methods and Compositions for Caliciviridae"). The authors are not aware of any other affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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