



REVIEW ARTICLE

Norovirus encounters in the gut: multifaceted interactions and disease outcomes

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Noroviruses are major causes of gastroenteritis, with epidemic outbreaks occurring frequently. They are an important global health concern, especially for pediatric and immunocompromised populations, and are challenging pathogens to target immunologically due to their rapid rates of genetic and antigenic evolution and failure to stimulate durable protective immunity. In this Review, we summarize our current understanding of norovirus pathogenesis, noting the prominent role of murine norovirus as a small animal model for norovirus research. We highlight intriguing data supporting the possible involvement of norovirus in sequelae including irritable bowel syndrome and inflammatory bowel diseases, and describe the innate and adaptive immune mechanisms involved in control of both human and murine norovirus infection. Furthermore, we discuss the potential implications of recent discoveries regarding norovirus interactions with the gut microbiota, and briefly detail current understanding of noroviral evolution and its influence on viral pathogenesis. Our mechanistic understanding of norovirus pathogenesis continues to improve with increasing availability of powerful model systems, which will ultimately facilitate development of effective preventive and therapeutic approaches for this pathogen.

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INTRODUCTION

Noroviruses (NoVs) are a major cause of both sporadic cases and epidemic outbreaks of gastroenteritis,¹ and are estimated to be responsible for up to 20% of all acute gastroenteritis cases worldwide.² In the United States alone, human NoVs (HNoVs) are responsible for ~20 million cases of acute gastroenteritis annually, with over 70,000 hospitalizations and nearly 800 deaths each year.³ NoV, in addition to other gastrointestinal pathogens such as rotavirus and enteropathogenic *Escherichia coli*, also remains a major cause of morbidity and mortality in parts of the developing world, especially among children in parts of Africa and southeast Asia.⁴ NoVs can infect a host with a limited number of particles (<20), yet infection results in high levels of viral shedding from infected individuals.^{5,6} This combination of high infectivity and efficient transmission enables HNoVs to cause global epidemics.⁷ NoVs are transmitted between susceptible hosts through the fecal–oral route, and thus contaminated food and water are potential sources of infection, as are aerosol droplets produced during vomiting.^{8–11} Infection with NoV typically causes profuse vomiting and diarrhea, which usually resolves in a period of 1–2 days. Infection of young children, the elderly, and the immunosuppressed, on the other hand, may lead to more severe or protracted illness.^{12–15}

In this article, after introducing the virus and its replication cycle, we will discuss virus-induced pathology and how both innate and adaptive immune mechanisms are involved in NoV control.^{16–23} We will also discuss the interactions with and the influence of the gut microbiota on the course of NoV infection, and finally, we will touch briefly upon evolution of NoVs and its impact on NoV pathogenesis and development of NoV vaccines.

Basic virology of NoVs

NoVs collectively make up one genus of small, positive sense, non-enveloped RNA viruses that belong to the family *Caliciviridae*. Based on sequence similarity, the genus is divided into different genogroups with further subdivisions into genotypes.^{24,25} Genogroups I, II, and IV contain primarily human viruses associated with gastroenteritis,²⁶ while genogroup V contains mouse viruses. NoV has a 7.5 kb genome with three (for human) or four (for mouse) open-reading frames (ORFs). ORF1 encodes a set of at least six non-structural proteins.²⁷ ORF2 encodes the major capsid protein (VP1), whereas ORF3 encodes minor structural protein VP2. In the case of murine NoV (MNoV), a virulence factor (VF1) is encoded from a fourth ORF (ORF4).²⁸ The enclosed genomic RNA is covalently linked on its 5' end with a small virus-encoded protein VPg, and the 3' end is polyadenylated.

With regard to the virion structure, the NoV capsid is composed of 180 monomers of the viral VP1 protein that are ordered into 90 dimers with a $T=3$ icosahedral symmetry.²⁹ In addition to VP1, the VP2 viral protein is present, but only as a few copies in virus particles and is associated with the interior surface of the capsid.³⁰ Structurally, VP1 is composed of two domains, the shell (S) domain and the protruding (P) domain, which are connected by a flexible hinge.³¹ The S domain forms the structural core of the capsid, whereas the P domain, which is further subdivided into subdomains P1 and P2, protrudes from the surface.³² Subdomain P2 is a hypervariable region containing putative receptor-binding sites;^{31,33,34} for MNoV, P2 directly interacts with the proteinaceous viral receptor.^{35,36}

In order to initiate infection, HNoVs bind to histo-blood group antigens (HBGAs), which act as attachment factors on host cells³⁷

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(Fig. 1). HBGAs are glycans that are determinants of the ABO blood group system. They are present in saliva and other bodily secretions, and are expressed on the surface of specific cells including enterocytes.³⁸ HNoVs bind various HBGAs with different specificities among genotypes and genogroups,³⁷ which can further complicate vaccine development approaches aimed at inhibiting virus binding to HBGAs. In mice, MNoVs may also use carbohydrates as attachment receptors, but with varied glycan receptor specificity among virus strains.³⁹ Following attachment to the cell surface, at least three MNoV strains (CW3, CR6, and S7) have been shown to use the CD300 family member CD300lf as a

proteinaceous viral receptor.^{40,41} Deletion of CD300lf from cells renders them resistant to CW3 and CR6 infection, while expression of the molecule converts non-susceptible cells into susceptible cells.^{40,41} Structurally, the CD300 family has a single immunoglobulin variable-like extracellular domain that is involved in the regulation of host immune responses.⁴² For HNoVs, a proteinaceous receptor has not yet been identified. Recently, it has been shown for a feline calicivirus that VP2 forms a portal-like assembly following receptor engagement, which putatively functions as a channel for delivery of the viral genome through the endosomal membrane;⁴³ it remains to be seen if this mechanism applies to NoVs as well.

Upon successful virus attachment, entry and viral particle uncoating occur, and NoV positive-sense RNA (+RNA) is then exposed in the cytoplasm where viral RNA translation takes place. The VPg protein attached at the 5' end of the genome is responsible for recruiting necessary host translation factors.⁴⁴ After successful translation, the large polyprotein encoded by ORF1 is then post-translationally cleaved by the virus-encoded protease (Pro, also known as NS6) into individual proteins: p48 (also known as NS1/2 or N term), NTPase (also known as NS3), p22 (also known as NS4), VPg, Pro, and RNA-dependent RNA polymerase. It has been shown that NS1/2 is further cleaved by host caspases to produce NS1 and NS2 proteins.^{45,46} Typical for RNA viruses, during genome replication +RNA is first converted into negative-sense RNA (-RNA), which is then used as a template for the synthesis of new genomic and subgenomic +RNAs. Capsid proteins VP1 and VP2 are produced from subgenomic +RNAs that contain only ORF2 and ORF3. Newly forming capsids assemble around genomic, and possibly subgenomic, +RNAs and eventually virus particles are released from the infected cell through mechanisms that are not fully understood.

In humans, as shown in intestinal biopsies from immunocompromised patients, VP1 can be detected in enterocytes, macrophages, T cells, and dendritic cells (DCs), indicating possible active infection of these cell types.⁴⁷ In vitro, HNoVs have been shown only relatively recently to infect differentiated human intestinal organoid cultures enriched in mature enterocytes, which are absorptive columnar intestinal epithelial cells (IECs), and a subset of established human B cell lines.⁴⁸⁻⁵⁰ In mice, MNoVs cause either acute or prolonged persistent infections.³⁹ Strains MNV-1 and CW3 cause acute infection with virus being cleared within 1 week.⁵¹ During the early phase of infection by acute strains, macrophages, DCs, B cells, and T cells in gut-associated lymphoid tissues are infected followed by virus shedding only in the first several days post infection.⁵² An infection model for MNoV has been proposed, whereby the virus uses microfold (M) cells to overcome the epithelial barrier in order to infect lymphocytes, macrophages, and DCs in the intestine (Fig. 2a), before being trafficked to local lymph nodes and distal sites by DCs.⁵³⁻⁵⁵ Alternately, persistent strains such as CR6 cause a prolonged infection with infectious virus shedding in the stool for potentially the lifetime of the animal.⁵¹ CR6 persistently infects a specialized IEC type,⁵⁶ tuft cells, which are chemosensory cells upregulated by parasite infection.⁵⁷ It remains possible that in addition to this persistent tuft cell reservoir, immune cells in the gastrointestinal lymphoid tissues may also be targets during early infection with persistent strains.³⁹ The distinct infection phenotypes observed with different MNoV strains suggest differential immune regulation mechanisms employed depending on the strain of the virus and the mode of infection.

NoV immune regulation

An intact immune system is critical to effective control of NoV infection, and data from responses to both human and mouse NoV infection support important roles for both the innate and adaptive arms of immunity to keep NoVs in check.

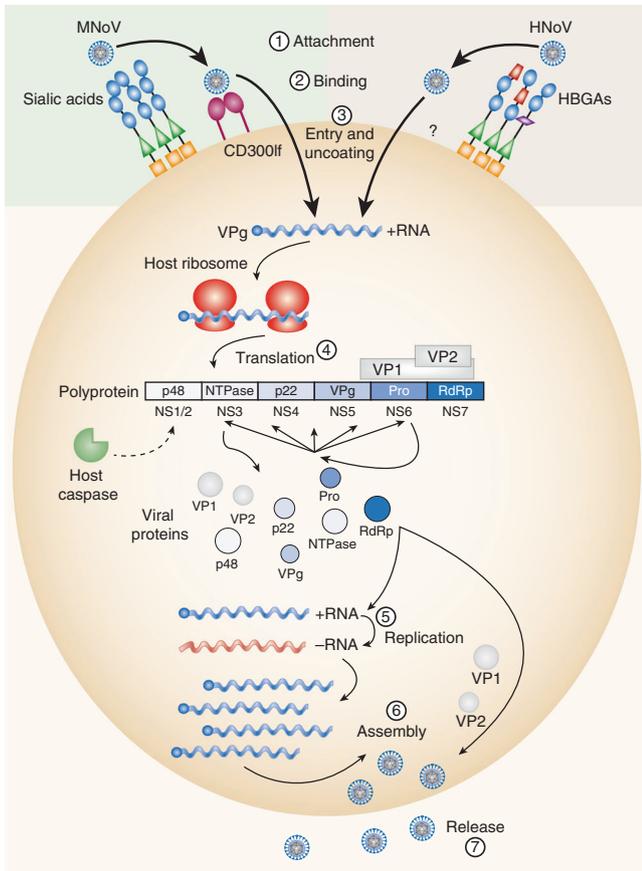


Fig. 1 The replication cycle of noroviruses. The replication cycle of NoV begins with attachment (1) of the virus to carbohydrates on the cell surface, where human norovirus (HNoV) binds histo-blood group antigens (HBGAs) and murine norovirus (MNoV) binds other carbohydrates including sialic acids. The proteinaceous receptor is currently only known for MNoVs, which utilize the CD300lf molecule (2), enabling virus entry and uncoating (3) into the host cell. The positive sense RNA genome is then exposed in the cytoplasm, bound at its 5' end to viral protein VPg. VPg recruits and engages host translation factors, leading to translation (4) of a large polyprotein of at least six non-structural (NS) viral proteins in addition to structural proteins VP1 and VP2, and in the case of MNoV, a virus immune evasion factor VF1 that is produced from an additional open-reading frame (not shown). NS6 (protease) cleaves the viral polyprotein into distinct viral proteins, and host caspases further cleave NS1/2 into NS1 and NS2. The viral RNA-dependent RNA polymerase then engages viral +RNA to start transcription and replication of the virus genome (5). Typical for RNA viruses, replication ensues through a -RNA replication intermediate that serves as a template to produce new viral +RNA genomes. Viral structural proteins then combine with nascent viral +RNA molecules for assembly (6) of new virus particles that exit the cell (7) through yet-to-be-discovered mechanisms

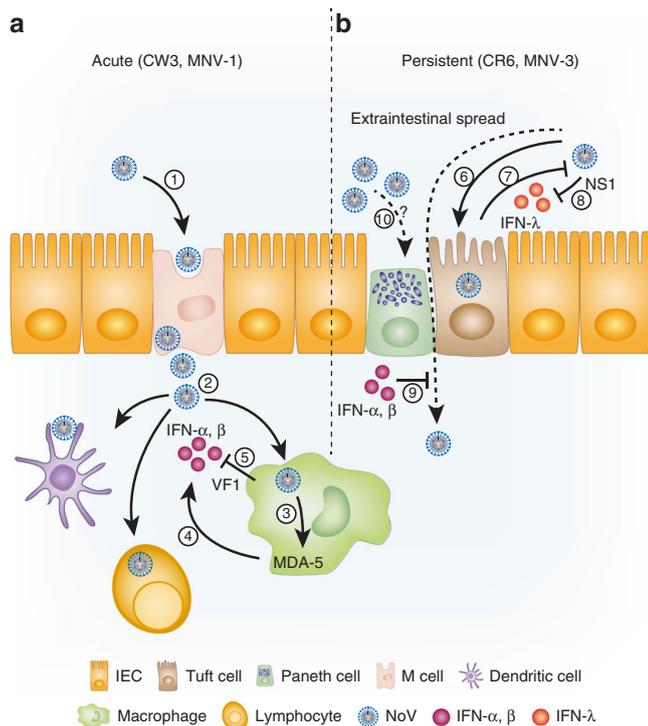


Fig. 2 Innate immune control of noroviruses. **a** For acute murine norovirus (MNoV) strains, virus is thought to cross the epithelial barrier through microfold (M) cells (1) infecting immune cell types including macrophages, dendritic cells, and lymphocytes (2). The virus is recognized by intracellular immune receptor MDA-5 (3), leading to an interferon (IFN) response (4) that is countered by the viral immune evasion factor VF1 (5). **b** Persistent MNoV strains infect tuft cells (6). IFN- λ plays a critical role in regulating persistent virus in vivo (7), which is counteracted by viral protein NS1 (8). Type I IFNs have been shown to prevent extraintestinal spread of persistent strains (9). It has also been shown that in the context of underlying genetic susceptibility, persistent MNoV can drive Paneth cell abnormalities linked to inflammatory bowel disease (10)

Innate control

In humans, pro- and anti-inflammatory cytokine levels peak during the symptomatic period of infection, which typically resolves within 1–2 days, indicating activation of the host immune system upon NoV infection.^{58,59} Experimentally infected symptomatic individuals showed greater immune system activation as measured by serum cytokines compared to asymptomatic subjects,⁶⁰ suggesting that symptomatology may be immune mediated in NoV infection. Further, symptoms did not directly correlate with greater viral burden, measured by either titer or duration of virus shedding, again highlighting the role of immune activation in symptom development in NoV infection. Although studies in human subjects are critical to understand the specific interaction of HNoV with the different arms of the immune system, much of our understanding comes from studies in wild-type and immunodeficient mice.

Generally upon virus entry into cells, viral components are sensed by multiple host cell factors.^{61,62} On the plasma membrane as well as in endosomal compartments, Toll-like receptors are important components of the sensing machinery of the cell.⁶³ RIG-like helicases like Rig-I and MDA-5 are located in the cytoplasm and recognize foreign RNA.^{64–66} Sensing of foreign virus components in the cell eventually leads to induction of a robust innate immune response. For MNoV, it has been shown that MDA-5 is required to control infection through induction of interferon (IFN)⁶⁷ (Fig. 2a). MDA-5 activation requires heme-oxidized IRP2 ubiquitin ligase 1, or HOIL1, which is part of the

linear ubiquitin chain assembly complex that is a crucial regulator of multiple immune signaling pathways, although the mechanism of regulation is still unclear.⁶⁸ Transcription factors IFN regulatory factor 3 (IRF-3) and IRF-7 are induced upon MNoV infection, which eventually stimulate type I IFN (IFN- α and IFN- β) production.¹⁸

IFN responses in the mouse limit MNoV viral replication, as shown for mice lacking type I IFN receptor (*Ifnar1*^{-/-}), which die after acute MNoV challenge.^{16,18–20,22,23} Moreover, lethality is exacerbated if the type II IFN (IFN- γ) receptor (*Ifngr1*) is also mutated.^{22,69} These findings suggest combinatorial antiviral effects of type I and II IFNs, although these factors seem to interfere with MNoV replication in distinct ways. Although both IFN types inhibit translation of viral proteins, IFN- γ acts in a double-stranded RNA-activated protein kinase-dependent manner⁷⁰ while IFN- α is independent. IFN- γ has additionally been shown to disrupt cytoplasmic MNoV replication complexes via modulation of autophagy proteins and induction of IFN-inducible GTPases.⁷¹ Type III IFN (IFN lambda, IFN- λ) has been also shown to be significantly involved in MNoV control in vivo (Fig. 2b). Intraperitoneal administration of IFN- λ clears persistent MNoV infection, even in the absence of adaptive immunity.⁷² In the gut, expression of IFN- λ receptor (*Ifnlr1*) on IECs in the small intestine and colon is required for this antiviral activity.⁷³ Furthermore, IFN- λ protects naive mice against transmission of MNoV when co-housed with mice shedding high loads of virus.⁷⁴ IFN- λ signaling in gut epithelial cells, induced by chronic astrovirus infection, has also been shown to protect immunodeficient mice from MNoV and murine rotavirus infections.⁷⁵ Interestingly, IFN- λ has also been implicated in counteracting the proviral effects of the microbiota in persistent MNoV infection,⁷⁶ discussed in further detail below.

Although effective in inhibiting and regulating virus replication, endogenous IFN responses are counteracted by a number of viral evasion mechanisms (Fig. 2a, b). Immune evasion molecule VF1 is encoded by an extra ORF (ORF4) that is present in the MNoV genome but absent in HNoVs.²⁸ VF1 expression delays upregulation of immune effector molecules such as CXCL10, ISG54, and IFN- β , hence encouraging viral replication. Another viral component involved in MNoV immune evasion is the viral non-structural protein NS1, which facilitates CR6 persistence in vivo by blocking IFN- λ responses.^{46,56,77} Collectively, as with other pathogens, an active virus–host interaction has evolved with the host reacting antivirally to infection and the virus avoiding these pathways through multiple mechanisms.

Adaptive control

Adaptive immune responses in humans are important for immunologic memory to help prevent HNoV reinfection. Early studies of infection in humans suggested that protective autologous immunity against HNoVs is short term, in the range of 8–14 weeks post infection.^{78,79} More recent reports, however, have indicated that protective immunity might be longer lasting than initially thought, with estimates ranging from 1 to 4 years.^{80,81}

Human subjects in HNoV experimental trials show signs of induction of adaptive immune responses.^{17,21} While limited explorations of cellular immune responses to HNoV have been conducted,⁸² humoral immune responses have been studied more broadly. Several candidate vaccines target the major HNoV capsid protein VP1.^{83,84} VP1 spontaneously self-assembles into virus-like particles (VLPs) when expressed in eukaryotic cells. These VLPs are morphologically and antigenically similar to the complete viral particle.^{85,86} Some HNoV vaccine candidates that are based on VLPs of the GI.1 genotype or a combination of GI.1 and GI.4 genotypes have already completed phase I and phase II clinical trials.⁸⁷ In one trial, human subjects received two doses of bivalent genogroup I genotype I (GI.I) VLPs made from recombinant VP1, and then were subsequently challenged with

an oral live GII.4 strain. Participants showed serum antibody responses against both GI.1 and GII.4 antigens.²¹ In another study, HNoV VLPs of a prototypic GI.1 strain were orally administered to 36 healthy adult volunteers at different doses, and all vaccines developed significantly increased anti-VLP IgA antibody-secreting cells detectable in blood.¹⁷ Additionally, a randomized, double-blind, placebo-controlled, multicenter trial to assess the safety, immunogenicity, and efficacy of an experimental, intranasally delivered HNoV genotype GI.1 VLP vaccine showed that 70% of vaccine recipients developed significantly increased virus-specific IgA. Moreover, vaccination significantly reduced the frequencies of both NoV infection and gastroenteritis weeks after vaccine administration.⁸⁸ GII.4-specific monoclonal antibodies have been successfully isolated from infected patients.⁸⁹ Interestingly, an antibody recognizing a neutralizing epitope conserved across three decades of pandemic strains was recently identified in vaccinated individuals, signifying that vaccination has the potential to elicit broadly effective neutralizing antibody responses.⁹⁰

Remarkably, however, even after induction of specific adaptive responses and after resolution of symptoms, virus shedding from infected symptomatic as well as asymptomatic individuals can still continue up to 60 days post infection.⁹¹ Thus, while adaptive immune responses are important for HNoV regulation, there may be mechanisms of immune evasion or intrahost evolution that permit HNoV to be maintained even in healthy individuals (Fig. 3b). Further, although NoV gastroenteritis in healthy humans is generally self-limiting, patients with compromised immune responses and consequently partial or complete inability to control the virus may shed HNoV for months to years, with gastrointestinal disease that, over time, can become increasingly debilitating and life-threatening.^{92–94} Underlying medical conditions like inherited immune disorders, kidney and hematopoietic stem cell transplantation (HSCT), and cancer or cancer treatment could lead to prolonged shedding of the virus.⁹⁴ These chronic infections have been speculated to lead to nosocomial outbreaks.⁹⁵

MNoV studies have been critical for assessing the significance of adaptive immune responses in clearing NoV infection,⁹⁶ and especially useful for exploration of cellular immune responses. An effective adaptive immune response against acute MNoV requires both B and T cells. *Rag1*^{-/-} and *Rag2*^{-/-} mice, which lack both cell types, develop chronic infections of normally acute strains with high viral loads²³ (Fig. 3a). Evidence indicates that antibody production is required for clearance of acute MNoV strains,⁹⁷ as *Rag1*^{-/-} mice that receive B cells incapable of producing MNoV antibodies fail to clear infection. Both CD4 and CD8 T cells have also been shown to be required for efficient clearance of primary acute MNoV infection from the intestine and intestinal lymph nodes.⁹⁸ Adoptive transfer of either polyclonal anti-MNoV serum or neutralizing anti-MNoV monoclonal antibodies is sufficient to reduce MNoV infection both systemically and in the intestine. Similarly, *Rag1*^{-/-} mice that receive a transfer of activated MNoV-specific CD8⁺ T cells have significantly reduced viral loads.⁹⁹ As anticipated, the magnitude of innate immune response affects adaptive responses to NoVs in vivo. Acute MNoV strain CW3 stimulates greater type I and III IFN responses compared to persistent CR6, which may contribute to the development of more effective adaptive immune responses and eventually viral clearance.⁷² Further, CW3 can persist systemically in vivo if CD11c-positive DCs are defective in type I IFN responses,¹⁶ again highlighting the connection between an initial effective induction of innate immune response and adaptive immune responses essential for viral clearance.

Persistent strains of MNoV are associated with lower levels of activated MNoV-specific CD8⁺ T cells, which was initially speculated to be a mechanism by which the virus can be maintained without being cleared.⁹⁹ Persistent MNoV infection

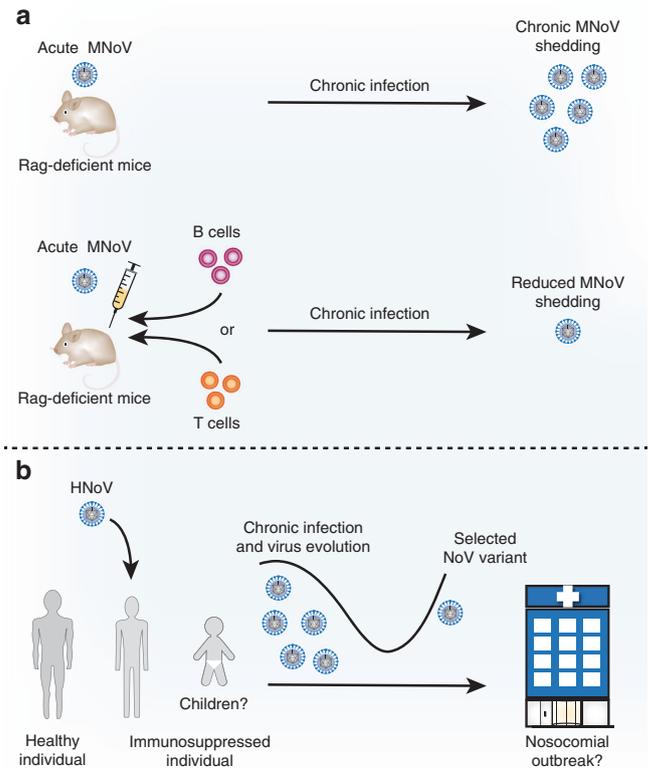


Fig. 3 Adaptive immune control in norovirus infection. **a** Both B and T cells have been implicated in murine norovirus (MNoV) immune control as *Rag*-deficient mice lacking both cell types exhibit protracted infection by acute MNoV strains. *Rag1*^{-/-} mice that receive either specific B or activated T cells against MNoV show reduced virus loads. **b** In humans, human norovirus (HNoV) can be shed for prolonged times from immunosuppressed individuals, children, and remarkably even from asymptomatic healthy humans. Chronic infections in these individuals lead to emergence of new virus variants that might lead to nosocomial outbreaks in health care facilities

drives MNoV-specific tissue-resident memory CD8⁺ T cells to a differentiation state similar to that involved in responses against latent cytomegalovirus. Interestingly, more recent work suggests that tuft cell infection may provide persistent strains with an immune-privileged enteric niche,^{57,100} as even enhancement of CD8⁺ T cell responses to persistent MNoV is insufficient to drive clearance. Further study will be needed to determine whether immune-privileged niches play a role in longer-term shedding in humans.

NoV pathology

HNoV infection is mostly acute with symptoms manifested after an incubation period of 12–48 h. Symptoms typically include vomiting, abdominal cramps, fever, the presence of mucus in stool, watery diarrhea, headache, chills, and myalgia. In healthy adults, gastroenteritis is self-limiting, and clinical symptoms tend to last for 1–2 days.^{5,101} However, in more susceptible groups like the elderly and young children or those who have impaired immune functions, clinical symptoms can be much more severe and last longer.^{14,102,103} Protracted HNoV infections have been reported in individuals with inherited immunodeficiencies, in transplant patients receiving immunosuppressive therapy, and in patients undergoing cancer chemotherapy, as well as in individuals who are infected with HIV.^{14,94} On the other hand, NoV infections might often be asymptomatic in early childhood,

possibly due to the effect of breastfeeding and transferred maternal antibodies.¹⁰⁴

In addition to the acute symptomatic manifestations of NoV infection, there has been some evidence linking NoV infections to sequelae including post-infectious irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD).^{105–107} In a 2-year prospective cohort study analyzing an outbreak of severe gastroenteritis attributed to a foodborne HNoV,¹⁰⁷ the prevalence of IBS was significantly higher in subjects who had experienced acute gastroenteritis than in control subjects. Another study following individuals from a massive outbreak of viral gastroenteritis also suggests that HNoV gastroenteritis can lead to the development of post-infectious IBS in a substantial proportion of patients.¹⁰⁸

An interesting connection between MNoV and IBD has also been identified. Persistent MNoV infection in mice with a mutation in human IBD susceptibility gene *Atg16l1* was found to cause enhanced intestinal pathology in the context of dextran sodium sulfate treatment.¹⁰⁵ Paneth cells, epithelial cells at the base of ileal crypts, are dedicated secretory cells secreting a wide spectrum of anti-microbial compounds and proteins, such as α -defensins and tumor necrosis factor- α (TNF- α), that collectively act as key mediators of host–microbe interactions.^{109–112} Histological changes in Paneth cells are observed in human IBD patients homozygous for the mutant *ATG16L1* allele,¹¹³ and similarly, mice hypomorphic for *Atg16l1* expression (*Atg16l1*^{HM}) show dramatic changes in both the morphology and transcriptional profile of Paneth cells with increased expression of pro-inflammatory cytokines and lipid metabolism genes.¹¹⁴ Infection of *Atg16l1*^{HM} mice with persistent MNoV strain CR6 was shown to drive this altered transcriptome, unique Paneth cell morphology, and abnormal granule packaging.¹⁰⁵ Consistent with the recent discovery that tuft cells are the persistent reservoir of MNoV,⁵⁷ Paneth cells themselves were shown not to be infected, and *Atg16l1*^{HM} mice exhibited similar viral loads as controls. In a related study, deletion of *Atg16l1* specifically in intestinal epithelial cells led to increased lethality and clinical disease upon MNoV infection and DSS treatment of mice.¹¹⁵ Thus, both the host mutation, specifically in the intestinal epithelium, and viral infection are required for the Crohn's disease-like phenotype, raising the possibility that enteric infections could contribute to IBD in humans. Intriguingly, the enhanced intestinal pathology observed in infected mice could be reduced using blocking antibodies against TNF α or IFN- γ , or upon treatment with broad-spectrum antibiotics.¹⁰⁵ The antibiotic responsiveness of this model served as an early indication that the microbiota may be important for effects of MNoV in the intestine.

A similar finding was observed in interleukin-10 (IL-10)-deficient mice, which have been used extensively as a model for IBD.^{116,117} In this study, mucosal inflammation was noted in *Il10*^{-/-} mice 2–4 weeks after infection compared to wild-type MNoV-infected mice that remained asymptomatic.¹¹⁸ Remarkably, the inflammatory lesions were absent in germ-free *Il10*^{-/-} mice, again indicating a dependence on the presence of the enteric microbiota for the observed virus-related IBD effects. A recent study builds upon this observation to suggest that the specific composition of intestinal microbiota is important for MNoV-triggered pathology, as segmented filamentous bacteria may protect against pathology, but only in some microbial contexts.¹¹⁹ In sum, these studies have suggested that NoVs may serve as viral triggers for IBS or IBD in some environmental and genetic contexts, and also indicated that the microbiota may be important for NoV pathogenesis. Further recent work has in turn revealed numerous interesting interactions between NoVs and bacteria in the gut.

Norovirus interactions with the microbiota

The entire collection of microorganisms inhabiting a specific niche, such as the gut, is referred to as the microbiota. The

microbiota plays a fundamental role in the induction, training, and function of the host immune system,¹²⁰ and the gut microbiota has an important role in maintaining intestinal homeostasis by preventing colonization by exogenous pathogens and potentially harmful indigenous microorganisms.¹²¹ The microbiota also promotes mucosal barrier functions, and is required for maintenance of the mucus that serves as an efficient barrier against both pathogens and commensal bacteria.^{122–124} Invading NoVs encounter members of the host microbiota and are influenced by their presence, recently reviewed in ref. ¹²⁵ Direct interactions of HNoV capsids with members of the microbiota have been shown for commensal as well as pathogenic bacterial species,^{126,127} interactions that are mediated via HBGA-like carbohydrates expressed on the surfaces of these bacteria. Similar to HNoVs, MNoVs bind carbohydrate moieties on the cell surface including sialic acid residues¹²⁸ (Fig. 1), which are widely expressed on bacteria.¹²⁹ Thus, MNoVs are also likely to directly interact with members of the microbiota, although experimental evidence is still pending.

Experimental alteration of the microbiota dramatically alters MNoV infection, as pretreatment of mice with an antibiotic cocktail prevents infection of the intestine by both acute strain MNV-1 and by persistent MNoV strains MNV-3 and CR6.^{50,76} Importantly, at least in the case of CR6, viral infection can be restored with a fecal transplant from untreated to antibiotic-treated animals.⁷⁶ No single antibiotic was found to be solely responsible for resistance, suggesting that there may be a variety of microbes able to promote MNoV infection. Further, antibiotic-mediated resistance to CR6 infection required intact innate immune responses, specifically IFN- λ , signal transducer and activator of transcription 1, and IRF-3, suggesting that the proviral effects of commensal bacteria may serve to counteract innate host immune responses against NoV infection.⁷⁶ It was also recently found that antibiotic treatment decreases the number of tuft cells in the colon, and that administration of IL-4 to antibiotic-treated mice can rescue persistent MNoV infection by promoting tuft cell proliferation.⁵⁷ Thus, regulation of permissive cell numbers appears to be an important mechanism by which the microbiota can affect NoV infection.

Another mechanism by which the microbiota promotes NoV infection involves secretory Igs. Secretory IgA (sIgA) molecules are secreted from epithelial cells via the polymeric Ig receptor (pIgR) into the intestinal lumen, where they bind antigens and serve as a first line of mucosal defense against enteric pathogens. The gut microbiota regulates IgA production, and the number of IgA-producing cells in the intestine is markedly decreased in germ-free mice.¹³⁰ Surprisingly, acute MNoV infection was significantly reduced in *Pigr*^{-/-} mice compared to controls, and *Pigr*^{-/-} mice had increased levels of the antiviral molecules IFN- γ and inducible nitric oxide synthase in the ileum. Germ-free *Pigr*^{-/-} mice, however, were equivalent to wild-type germ-free mice and exhibited normalized cytokine levels, indicating that the gut microbiota and sIgA promote infection through alterations in microbial immune responses.¹³¹

In addition to regulation of immune-related pathways, the gut microbiota may also influence the course of NoV infection via modification of cofactors required for NoV replication. Bile acids are heavily modified by the gut microbial community,¹³² including alteration of total amounts of bile acids within the gut.¹³³ HNoV capsids bind directly to bile acids at a partially conserved pocket on the HNoV P domain,¹³⁴ and addition of specifically the non-proteinaceous component of human bile to organoids greatly increases HNoV replication in a dose-dependent manner.⁴⁸ Furthermore, bile acids enhance HBGA binding to GII.10 VLPs, suggesting that bile acids may facilitate interactions of HNoVs with host cells.¹³⁴ Bile acids have also been found to be essential for efficient replication of a porcine enteric calicivirus in vitro,¹³⁵ and serve as cofactors for MNoV cell binding



and infectivity.³⁵ The MNoV VP1 has two bile acid binding sites at the P domain dimer interface, distant from receptor-binding sites.³⁵ In a recent study, bile acids were shown to induce conformational changes in the MNoV P domain, allowing for a higher degree of saturation of receptor onto the virus.¹³⁶ Beyond direct interactions with NoVs, bile acids also interact with host receptors, such as TGR5 and FXR, to regulate intestinal inflammation and integrity,^{137,138} and thus may play a role in regulating NoV infection indirectly as well.

There is some evidence that NoV infection itself may also have effects on the composition of the gut microbiome.¹³⁹ A subset of individuals infected by HNoVs exhibit alterations of the microbiota, with changes observed similar to those linked to liver fibrosis and obesity,^{140,141} with an enhanced bacterial firmicutes-to-bacteroidetes ratio.¹³⁹ MNV-1 has been also shown to enhance the firmicutes-to-bacteroidetes ratio in C57BL/6 mice,¹⁴² although another study did not detect changes in the microbiota following acute or persistent MNoV infection,¹⁴³ suggesting the possibility of facility-dependent effects.

Interestingly, in the absence of the gut microbiota, NoV alone may have the capacity to contribute to gut homeostasis.¹⁴⁴ Germ-free mice exhibit aberrant intestinal morphology and deficiencies in the lymphocyte compartment,¹⁴⁵ but persistent MNoV infection restores intestinal morphology and lymphocyte function in a type I IFN-dependent fashion, without inducing overt inflammation and disease.¹⁴⁴ These data highlight the possibility that not only bacteria but also eukaryotic viruses might contribute to homeostasis and mucosal immunity.

Finally, in addition to complex interactions with the bacterial component of the microbiota, other microorganisms in the gut can also influence NoV infection. Intestinal helminths increase small intestinal tuft cell numbers through multiple mechanisms including via succinate signaling,^{146–148} and helminth coinfection results in higher levels of fecal shedding of persistent MNoV.¹⁴⁹ As briefly mentioned earlier, virome member murine astrovirus can mediate antiviral effects against MNoV.⁷⁵ Beyond these intriguing observations, there certainly may be a number of additional as-yet-undiscovered microbial regulators for NoV infections, especially in the context of human infection where the microbiota has substantially greater interindividual variation.

NoV evolution is critical to pathogenesis

NoVs are a diverse but related group of viruses that have the ability to infect different hosts including humans, rodents, felines, canines, and pigs.²⁴ Genogroups share only 51–56% sequence similarity with one another when whole-genome sequences are considered, whereas strains in a genotype share 69–97% nucleotide sequence similarity.¹⁵⁰ With regard to sequences for VP1, HNoVs show a very high calculated mutation rate (1.21×10^{-2} to 1.41×10^{-2} substitutions per site per year).¹⁵¹

One of the major genotypes responsible for human outbreaks is genotype 4 of genogroup II (GII.4). GII NoVs are responsible for more than 90% of NoV disease in the United States, with GII.4 NoVs causing 50–80% of disease from year to year.¹⁵² GII.4 strains are characterized by high rates of evolution.¹⁵³ Comparison of over 2000 ORF2 sequences of HNoVs of the GI and GII genotypes shows a limited number (≤ 5) of distinct intra-genotypic variants within each genotype, except for the GII.4 genotype that contains the largest number of variants (>10).¹⁵⁴ Interestingly, analysis of full-length sequences of HNoV in healthy individuals infected with GII.4, GII.6, or GII.17 viruses indicates that GII.4 viruses accumulate mutations rapidly within and between hosts, while the GII.6 and GII.17 viruses remained relatively stable. The viral capsid is seemingly under evolutionary pressure and is responsible for the emergence of the new antigenically distinct variants.^{37,155} Emergence of these new GII.4 strains coincides with increased HNoV outbreak activity.¹⁵⁶ The broad genetic and antigenic diversity of

circulating HNoV strains poses a challenge for the development of broadly protective vaccines.¹⁵⁷ Immunity elicited due to natural infection or immunization may be specific only to the genogroup of that HNoV.^{158,159}

The MNoV system has been important for highlighting how even minor genetic changes can be associated with dramatic phenotypic changes. For example, a single amino acid substitution in the MNoV capsid protein is sufficient to confer lethality in *Stat1*^{-/-} mice.^{19,160} Another important example is the finding that a single amino acid change in NS1/2 will permit an acute viral strain to persist, possibly via modifications in the protein's tertiary conformation.^{161,162} Because minor changes in the NoV genome can dramatically alter pathogenesis, development of effective vaccination and antiviral treatments to limit HNoV persistence and transmission are urgently needed to prevent emergence of novel pathogenic variants.

CONCLUSIONS AND FUTURE DIRECTIONS

Our basic understanding of NoV infection and associated pathogenesis has steadily expanded over time. Exciting recently developed systems for culturing HNoV offer promise for additional mechanistic insight into infection as well as exploration of therapeutic approaches. The use of MNoV as a small animal model system has also contributed critical knowledge regarding host and microbial control of NoVs, highlighting interesting aspects of virus replication, tropism, immune evasion, and induced pathogenesis.

As more pieces of the NoV puzzle come together, however, there are still many interesting open questions that need to be addressed in order to fully solve the puzzle. With no currently licensed vaccine or antiviral against HNoVs, important avenues for investigation include (1) the role of interindividual microbiota variation in HNoV disease outcomes and effects on vaccine responses; (2) alternate therapeutic approaches to cure individuals chronically infected with HNoV; and (3) improved understanding of regulators of and phenotypic outcomes from NoV mutation and evolution. Limitation of the detrimental effects of this epidemic-causing viral pathogen will require continued exploration and development of creative approaches to target the virus, the microbiota, and the host.

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AUTHOR CONTRIBUTIONS

E.H. and M.T.B. wrote and edited the manuscript. E.H. generated the figures.

ADDITIONAL INFORMATION

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