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Norovirus mechanisms of immune antagonism

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Noroviruses are a leading cause of gastroenteritis outbreaks globally. Several lines of evidence indicate that noroviruses can antagonize or evade host immune responses, including the absence of long-lasting immunity elicited during a primary norovirus exposure and the ability of noroviruses to establish prolonged infections that are associated with protracted viral shedding. Specific norovirus proteins possessing immune antagonist activity have been described in recent years although mechanistic insight in most cases is limited. In this review, we discuss these emerging strategies used by noroviruses to subvert the immune response, including the actions of two nonstructural proteins (p48 and p22) to impair cellular protein trafficking and secretory pathways; the ability of the VF1 protein to inhibit cytokine induction; and the ability of the minor structural protein VP2 to regulate antigen presentation. We also discuss the current state of the understanding of host and viral factors regulating the establishment of persistent norovirus infections along the gastrointestinal tract. A more detailed understanding of immune antagonism by pathogenic viruses will inform prevention and treatment of disease.

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Introduction

Noroviruses comprise a genus in the *Caliciviridae* family and the human viruses in this genus are notable for their association with numerous and widespread gastrointestinal disease outbreaks. Specifically, human noroviruses are responsible for the majority of severe childhood diarrhoea in regions of the world where a rotavirus vaccine has been implemented and they are the leading global cause of foodborne disease outbreaks [1–4]. Norovirus strains are segregated into genogroups and genotypes/clusters based on the sequence of their major capsid protein VP1 and regions within ORF1, with genogroup I (GI), GII, and GIV containing primarily strains associated with gastroenteritis in humans [5]. Murine noroviruses segregating into GV provide a vital small animal model for norovirus research considering their genetic, molecular, and pathogenic similarities to their human counterparts [6–8].

Several lines of evidence from both human and murine norovirus studies strongly suggest that noroviruses encode mechanisms to circumvent host immune responses: First, early human volunteer studies demonstrated that a subset of people fail to mount lasting protective immunity against a homologous human norovirus challenge [9,10]. Consistent with this, primary murine norovirus infection results in suboptimal immunity that wanes over time [11,12]. Second, although the symptoms of human norovirus infection resolve very quickly in healthy adults, affected individuals can continue to shed low levels of virus for several weeks, reflecting incomplete immune clearance. In fact, it is well-established that even asymptomatically infected individuals can shed human norovirus for extended periods of times, and one recent study reported that shedding duration was similar between symptomatic and asymptomatic subjects [13–15]. This apparent persistent infection is much more pronounced in risk groups including infants, young children, immunocompromised, and transplant patients [16–19]. Likewise, some murine norovirus strains establish persistent infections, with virus remaining detectable primarily in the large intestine for several months post-infection [20–22]. Notably though, highly genetically related intra-cluster murine norovirus strains differ substantially in both induction of adaptive immune responses [12,23] and persistence establishment [20,21], providing highly valuable comparative tools to identify viral determinants of immune antagonism. In this review, we will summarize a growing body of literature describing specific norovirus mechanisms that antagonize host immune responses.

Noroviruses are small, non-enveloped, positive sense RNA viruses with genomes of ~7.5 kb [24]. The 5′ proximal open reading frame ORF1 encodes a polyprotein which is cleaved by the viral protease (Pro) into six nonstructural proteins: (1) NS1/2, also referred to as p48 for human noroviruses; (2) NS3, an NTPase; (3) NS4, also referred to as p22 for human noroviruses; (4) NS5, the VPg protein that is covalently attached to viral RNA molecules; (5) NS6, the viral Pro; and (6) NS7, the viral RNA-dependent RNA polymerase (RdRp). ORF2 and ORF3 are translated from a subgenomic RNA, giving rise to the structural proteins VP1 and VP2, respectively [25,26]. Murine noroviruses express an additional protein

called virulence factor 1 (VF1) from an alternative reading frame within ORF2, designated ORF4 [20,27••]. The p22, p48, VF1, and VP2 proteins have all been described to possess immune-antagonizing activities (VF1 and VP2), or activities with a high likelihood of impeding immune responses (p22 and p48), and will be the focus of this review.

Impairment of protein trafficking pathways by human norovirus p48 and p22 nonstructural proteins

Many gastrointestinal pathogens disrupt the secretory pathway in intestinal epithelial cells, altering the balance of ions and fluids between the epithelial barrier and the intestinal lumen and thus contributing to diarrheal disease symptoms [28]. The main purpose of encoding proteins that interfere with cellular secretion pathways is likely to facilitate microbial replication but an unavoidable consequence of this activity is the impairment of vital host cell functions including those necessary for mounting immune responses at the cellular level. For example, cytokine secretion and surface expression of MHC and costimulatory molecules are both dependent on a functional protein trafficking pathway.

Two human norovirus proteins have been demonstrated to possess anti-secretory activity when overexpressed in cultured cells. First, the p48 nonstructural protein colocalizes with markers of the Golgi apparatus and induces Golgi rearrangement into discrete aggregates indicative of Golgi disassembly [29]. An independent study reported a vesicular staining pattern of fluorescently tagged p48 protein consistent with ER/Golgi localization [30••]. Localization of p48 to the Golgi or vesicles did not require a predicted transmembrane domain at the carboxyl terminus of the protein, although this domain was sufficient to induce Golgi localization of a reporter protein [29,30••]. Finally, p48 was revealed to bind vesicle-associated membrane protein-associated protein A (VAP-A) that functions in SNARE-mediated vesicular transport and to block the transport of the vesicular stomatitis virus (VSV) G glycoprotein to the cell surface at a post-Golgi trafficking step [30••]. Collective evidence thus strongly argues that the human norovirus p48 protein interferes with intracellular protein trafficking when overexpressed in cultured cells.

Second, the p22 nonstructural protein also contributes to Golgi disassembly and inhibition of the cellular secretory pathway [31••]: Transient expression of fluorescently tagged p22 resulted in disassembly of the Golgi and inhibition of protein secretion as measured by a secreted alkaline phosphatase reporter assay. The p22 protein specifically blocked trafficking of COPII-coated vesicles from the ER to the Golgi. Notably, p22 contains a motif which closely resembles a well-defined ER export signal that is conserved across many human norovirus strains. Mutations in this motif ablated the ability of p22 to induce Golgi disassembly and inhibit protein secretion; moreover this motif could substitute for an established ER export signal. The murine norovirus p22 protein homologue called p18 does not contain this motif; although p18 did cause Golgi disassembly when expressed in 293T cells, it was less efficient at blocking the secretory pathway than human norovirus p22 [32]. Available evidence thus supports a model whereby p22 localizes to COPII-coated vesicles and prevents their fusion with the Golgi, ultimately leading to Golgi disassembly and impaired protein trafficking within the cell. Although the precise mechanism by which p22 alters normal trafficking of COPII-coated vesicles has not been elucidated, it requires a specific motif that has been coined a mimic of an endoplasmic reticulum export signal (MERES) [31••,32].

Although the above-described studies examined the activity of p48 or p22 in overexpression systems, there is evidence supporting the notion that inhibition of host secretory pathways also occurs during norovirus infections: Transfection of Huh7 cells with human norovirus genomic RNA, and infection of permissive RAW 264.7 cells with a murine norovirus, both induce Golgi disassembly [31••,33]. Murine norovirus replication complexes initially assemble on membranes derived from the ER, Golgi, and endosomes ultimately leading to accumulation of numerous cytoplasmic vesicles where intracellular replication occurs [33,34], supporting the idea that noroviruses hijack the secretory pathway for the purpose of assembling replication factories on cellular membranes. While it has yet to be determined whether the murine norovirus p48 and p22 homologues (referred to as NS1/2 and NS4, respectively) possess anti-secretory activity, they both localize to organelles involved in the secretory pathway so it is tempting to speculate that they promote replication complex formation on host membranes [35]. Moreover, a recent study demonstrated that NS1/2 regulates the ability of a murine norovirus to efficiently infect the colon and establish a persistent infection at this site [36]. By consolidating *in vitro* observations of human norovirus p48 and *in vivo* observations of its murine norovirus homologue NS1/2, it is reasonable to speculate that norovirus persistence establishment is directly related to the anti-secretory (and possibly the related immune antagonist) activity of this nonstructural protein.

Overall data thus support a general model whereby specific norovirus nonstructural proteins localize to organelles of the host secretory pathway and encode mechanisms to impede normal trafficking within this pathway so that host membranes can be used as scaffolds for viral replication complex assembly. An unavoidable consequence of inhibiting vesicular transport within the secretory pathway is disassembly of the Golgi apparatus,