Caspase-mediated cleavage of Murine Norovirus NS1/2 potentiates apoptosis and is required for persistent infection

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Short title: Cleavage of MNV NS1/2 potentiates apoptosis and is required for persistence
Abstract

Human norovirus (HuNoV) is the leading cause of acute gastroenteritis and is spread by fecal shedding that can often persist for weeks to months after infection. Murine norovirus (MNV) is also shed persistently in the feces and provides a tractable model to study molecular mechanisms of enteric persistence. Previous studies have identified non-structural protein 1 (NS1) from the persistent MNV strain CR6 as critical for persistent infection in intestinal epithelial cells (IECs), but its mechanism of action remains unclear. We now find that the function of NS1 in promoting persistence is regulated by apoptosis. Following induction of apoptosis in infected cells, a minority of NS1 is cleaved from the precursor NS1/2 protein, and this cleavage is prevented by mutation of caspase target motifs. MNV strain CR6 with these mutations (CR6Δcasp) is profoundly compromised in infection of IECs and persistence in the intestine. Conversely, replication in tissues outside of the intestine, or in a cultured macrophage cell line, is unchanged, indicating that the requirement of NS1/2 cleavage is intestine-specific. Intriguingly, we also find that cleavage of CR6 NS1/2 potentiates apoptosis, suggesting that regulation of cell death is a novel function of this viral protein. Together, these data indicate that the ability of NS1 to promote MNV persistence in IECs is regulated by host caspases, and suggest that potentiation of apoptosis plays a role in viral tropism in the intestine.
Author Summary

Human Norovirus infection is highly contagious and the most common cause of acute gastroenteritis. Norovirus can persist and be shed for months after infection, leading to continued outbreaks. There are many unanswered questions as to host and viral components of norovirus pathogenesis that can be addressed within the murine norovirus (MNV) model system. We previously identified a critical role for a viral protein, NS1, for intestinal persistence. Herein we describe how the regulation of NS1 is critical for persistent infection in intestinal epithelial cells, but is not required for acute infection of non-epithelial cells, or infection of tissues outside of the gut. Additionally, we demonstrate that NS1 is both regulated by the host cell death machinery, and also reciprocally regulates that machinery to promote cell death during MNV infection, and found that this is specific to persistent strain of MNV. Altogether these data identify a role for how NS1 in a new pathway involved in establishing a persistent norovirus infection in the intestine.
Human norovirus (HuNoV) is the most common cause of epidemic gastroenteritis, and can be particularly dangerous for infants and the elderly [1,2]. Persistent viral shedding often occurs for weeks to months after acute infection, even in asymptomatic individuals [3,4], and is a potential source for continued outbreaks. Despite recent advances in culture systems to study HuNoV [5,6], there is still not an efficient in vitro or small animal system for investigating the molecular components of HuNoV infection. Fortunately, HuNoV and murine norovirus (MNV) share genotypic (ssRNA, positive-sense, ~7.5kb genome) and phenotypic (fecal-oral transmission, persistence in intestinal epithelial cells (IECs), and shedding in the feces) similarities, and utilization of the MNV model system has considerably advanced our molecular understanding of norovirus pathogenesis and persistence [7-9].

All noroviruses encode six non-structural proteins (NS1/2, NS3, NS4, NS5, NS6, and NS7) expressed upon cleavage of the open reading frame 1 (ORF 1) polyprotein by the viral NS6 protease [10] and two structural capsid proteins (VP1 and VP2) from ORFs 2 and 3 [11,12], respectively. MNV also has a fourth ORF that encodes a unique virulence factor not present in HuNoV [13]. Although the non-structural protein NS1/2 is not cleaved by the viral protease, it can be cleaved into two additional proteins (NS1 and NS2) [10,14] by the host protease, caspase 3 [10]. However, definitive data regarding the extent and consequences of such caspase cleavage are lacking.

Prior comparison of an acute and a persistent strain of MNV identified two viral proteins that are key components of MNV pathogenesis and persistence [15-17]: the major capsid protein (VP1) of the acute strain CW3 (VP1\textsuperscript{CW3}) drives viral dissemination outside of the intestine, and NS1 of the persistent strain CR6 (NS1\textsuperscript{CR6}) is critical for viral persistence in the intestine. Additional in vivo analyses demonstrated NS1\textsuperscript{CR6} is absolutely required for infection of a specialized type of IEC, the tuft cell, identified as the cellular reservoir for MNV persistence [18]. Despite the differences in systemic spread and intestinal persistence, both CW3 and CR6 can infect macrophages, dendritic cells, T-cells, and B-cells in vitro and in vivo [6,19,20], and utilize the same cell-surface receptor, CD300lf [21,22]. Thus, VP1\textsuperscript{CW3} and NS1\textsuperscript{CR6} are
functioning via mechanisms other than receptor usage and replicative capacity to promote viral 
dissemination and persistence.

In addition to identified roles of viral proteins in norovirus pathogenesis, critical aspects of the host 
antiviral response have also been identified. Particularly important is the multi-faceted interferon (IFN) 
response [23]. Type I (α/β) and type III (λ) IFNs are both key players in the innate anti-viral response, 
primarily distinguished by the differential expression of their respective receptors [23]. The IFN-α/β 
receptor is widely expressed on most cell types, whereas the IFN-λ receptor is predominantly expressed on 
epithelial cells. In regards to MNV, IFN-α/β signaling is critical for control of MNV in myeloid cells, 
demonstrated by increased titers in macrophages that do not express the IFN-α/β receptor [19], the inability 
to control systemic MNV infection in mice that specifically lack IFN-α/β receptor expression on myeloid 
cells [24,25], and increased dissemination and lethality in mice that do not express the downstream signaling 
molecule, STAT1 [11]. In contrast to systemic spread, intestinal MNV infection is controlled by IFN-
λ signaling in IECs, and persistent infection can be cleared and prevented with IFN-λ treatment [26,27]. 
Interestingly, recent data suggests that NS1^{CR6} may have a role in antagonizing the IFN-λ response during 
intestinal infection of IECs [28], but how NS1^{CR6} may function in this capacity and whether this is its sole 
function during persistent infection is unknown.

In addition to the IFN response, host cells also initiate apoptosis as a means of eliminating virus-
infected cells. Correspondingly, viruses have evolved ways to hijack apoptotic machinery to their benefit. 
Apoptosis is a non-inflammatory, highly-regulated disassembly of the cell mediated by a family of cysteine 
proteases (caspases), and culminates in the release of the cellular contents within membranous vesicles 
(apoptotic bodies) that are taken up by professional phagocytes, as well as neighboring cells [29]. Because 
induction of apoptosis is an innate response to stress or infection, most viruses have developed ways to 
delay apoptosis in an effort to maximize viral progeny [30], exemplified by a number of large DNA viruses 
that encode viral homologs of the cellular anti-apoptotic protein, Bcl-2 [31]. Conversely, influenza virus 
actively induces apoptosis through manipulation of other Bcl-2 family proteins, which increases viral
replication and enhances pathogenesis [32,33]. In addition to manipulating apoptosis to prevent cell death or increase viral progeny, a number of viruses, including picornaviruses and flaviviruses, utilize ‘apoptotic mimicry’ as an alternate mode of egress and viral dissemination [34], and some viruses rely on apoptotic caspases to cleave viral proteins as an additional level of regulation [35-37]. Thus, apoptosis can be both anti-viral and pro-viral, making its regulation critical for viral pathogenesis.

HuNoV and MNV infection both induce apoptosis, as indicated by association with markers of apoptosis in infected intestinal tissue [38-40]. Thus, these viruses have likely also developed mechanisms to regulate apoptosis to promote infection. Apoptosis in MNV-infected myeloid cells includes downregulation of an inhibitor of apoptosis protein, survivin, and activation of caspases and other cysteine proteases [41,42]. Furthermore, independent expression of the MNV ORF1 polyprotein, which encodes the non-structural proteins (including NS1/2), is also sufficient to induce apoptosis [43], indicating one or more non-structural proteins could be promoting apoptosis during infection. Additional analyses of the non-structural proteins identified two functional caspase motifs in NS1/2, and demonstrated that the NS1/2 protein can be cleaved by recombinant caspase 3 in a cell-free system [10]. Based on these published data, apoptosis during MNV infection may be facilitated by one or more non-structural proteins, and the virus may benefit from apoptotic signaling via utilization of a host caspase to regulate expression of NS1, the critical determinant for intestinal persistence [15].

Herein, we further delineate how NS1\textsuperscript{CR6} functions to promote viral persistence by characterizing NS1/2 cleavage during MNV infection and its role in intestinal persistence. We demonstrate that at late times post-infection, concurrent with induction of apoptosis, a minority of total NS1/2 protein is cleaved in a caspase-dependent manner. Despite the relatively low proportion of total NS1/2 that is cleaved, our data demonstrate that NS1/2\textsuperscript{CR6} cleavage is absolutely required for persistent infection in IECs, and this involves a function separate from IFN antagonism. Moreover, the cleavage of NS1/2\textsuperscript{CR6} potentiates apoptotic cell death \textit{in vitro}, suggesting controlled induction of apoptosis is a novel mechanism required for infection of IECs and intestinal persistence.
Results

Host caspases cleave NS1/2 at late times post-infection. Previous cell-free studies identified two functional caspase motifs (DKAD\textsuperscript{121} and DAMD\textsuperscript{131}) within NS1/2 that are cleaved by host caspase 3 [10] (Fig 1A), however, the necessity and utility of these sites during viral infection was unknown. Therefore, to assess the role of NS1/2 cleavage during viral persistence, we mutated these sites (D\textsubscript{121}G and D\textsubscript{131}G) within the persistent MNV strain, CR6, and named this virus CR6\textsubscript{Δcasp} (Fig 1B). Analyses of viral protein expression during MNV infection of BV2 cells demonstrates cleavage of a minority of total NS1/2 protein at late times post-infection with both an acute (CW3) and persistent (CR6) strain of MNV (Fig 1C). Cleavage of NS1/2 did not occur in CR6\textsubscript{Δcasp}-infected cells, demonstrating that it is dependent on the caspase motifs (Fig 1C), and, importantly, these mutations did not disrupt overall expression of full-length NS1/2 protein. To determine whether host caspases are responsible for NS1/2 cleavage, cells were treated at 10h post-infection (PI) with a pan-caspase inhibitor, ZVAD-fmk, and indeed, the ~28kDa NS2 protein was no longer detected in infected cells at late times post-infection (Fig 1D). The ~18kDa NS1 protein was inconsistently detected in cells via western blot and may be a technical result of the small amounts of overall NS1/2 that get cleaved during infection. Together, these data demonstrate that a small portion of total NS1/2 protein is cleaved by host caspases at relatively late times post-infection with MNV.

Cleavage of NS1/2 is not required for viral replication in vitro. The abundance of full-length NS1/2 following infection with CR6\textsubscript{Δcasp} indicated that viral protein production was not dependent on these cleavage motifs (Fig 1C, D). To further assess whether NS1/2 cleavage is necessary for production of infectious virus, we compared plaque forming units (PFU) produced from cell cultures infected with CR6 vs. CR6\textsubscript{Δcasp} over a 48h time course. Inhibition of NS1/2 cleavage did not significantly impair CR6 replication in BV2 cells (Fig 2A), or within wildtype (WT) bone-marrow derived macrophages (BMDCs) (Fig 2C).
Our in vivo analyses discussed in the following section aimed to assess the role of NS1/2 cleavage on systemic replication as well as intestinal replication of MNV; therefore, we generated CR6 and CR6ACasp viral strains that could spread systemically due to chimeric expression of VP1CW3 (Fig 1B) [15]. We performed growth curves using these capsid chimeras in parallel with growth curves for CR6 and CR6ACasp described above. In vitro growth kinetics in BV2 cells and WT BMDCs displayed minimal differences between CR6-VP1CW3 and CR6ACasp-VP1CW3 (Fig 2B, D). The minor, yet significant, decrease in peak titers of CR6ACasp-VP1CW3 compared to CR6-VP1CW3 in WT BMDCs but not in BV2 cells indicated NS1/2 cleavage may serve a critical function specific to BMDC cultures. Recently published data suggests a role for NS1CR6 in IFN antagonism, therefore, we assessed whether NS1/2 cleavage affected growth in BMDC cultures that are not responsive to IFN (Stat1−/− BMDCs). All the MNV strains we analyzed demonstrated higher peak titers in Stat1−/− BMDCs compared to WT BMDCs (Fig 2C-F), confirming the known inhibitory effect of IFNs on MNV replication [19], and the decreased replicative capacity of CR6ACasp-VP1CW3 was no longer evident (compare Fig 2D to 2F). Altogether, these data indicate that NS1/2 cleavage is not necessary for viral replication but may play a role in counteracting the IFN response.

NS1/2 cleavage is critical for persistence in the intestine. To analyze whether NS1/2 cleavage impacts MNV infection of systemic and intestinal tissues in vivo, we perorally challenged WT B6 mice with 1e6 PFU CR6, CR6ACasp, CR6-VP1CW3 or CR6ACasp-VP1CW3, and analyzed tissues at three days post-infection (dpi) for the presence of infectious virus (Fig 3A-D). As expected, CR6 and CR6-VP1CW3 were detected within the mesenteric lymph node (MLN), ileum (devoid of Peyer’s patches), and colon, the eventual site of persistence (Fig 3A, B). Consistent with the high titers in intestinal tissue, we also detected infectious virus shed in the feces of mice infected with CR6 and CR6-VP1CW3 at 3dpi (Fig 3C, D). However, in mice infected with CR6ACasp or CR6ACasp-VP1CW3, we rarely detected infectious virus in intestinal tissues (Fig 3A, B), nor being shed in the feces (Fig 3C, D), indicating that caspase cleavage of NS1/2 is required for promoting early infection of the intestine.
Although production of infectious virus was severely compromised in intestinal tissues of MNV-infected mice in the absence of NS1/2 cleavage, titers in the spleen were only modestly reduced and not significantly different at 3dpi in the absence of NS1/2 cleavage (Fig 3B), indicating that NS1/2 cleavage is not required for systemic spread. Similarly, we were able to detect MNV RNA within the Peyer’s patches of animals infected with CR6Δcasp-VP1CW3 using the ultra-sensitive in situ RNA hybridization platform, RNAscope (Fig 3F), and this was not visibly different from CR6-VP1CW3-infected mice (Fig 3E-G). Together, these data suggest no requirement for NS1/2 cleavage during the initial infection in Peyer’s patches or for subsequent systemic spread (Fig 3B, spleen).

To further verify that NS1/2 cleavage is not required for systemic MNV infection and pathogenesis, we infected Stat1−/− mice with either CR6-VP1CW3 or CR6Δcasp-VP1CW3 (Fig 3H). Previous work demonstrated that VP1CW3 is critical for systemic spread [15,17], and lethality in Stat1−/− mice [16,17,24]. Accordingly, we found that peroral challenge with CR6-VP1CW3 resulted in lethal MNV infection in Stat1−/− mice, and although NS1/2 cleavage is critical for efficient infection in the intestine, CR6Δcasp-VP1CW3 infection also resulted in 100% mortality in Stat1−/− mice (Fig 3H). As a control, we infected Stat1−/− mice with CR6, which persists, but does not result in lethal infection (Fig 3H) [15]. Overall, our characterization of acute infection demonstrates that NS1/2 cleavage is not required for systemic spread of MNV and is not necessary for lethal pathology in Stat1−/− mice. However, NS1/2 cleavage is specifically required for efficient MNV replication in the ileum (outside of Peyer’s patches) and colon, the source of virus shed in the stool and the site of eventual persistence.

Due to limited detection of virus at 3dpi in the intestinal tissues of mice infected with CR6Δcasp and CR6Δcasp-VP1CW3, we predicted that these mutants would also fail to persist. Indeed, whereas CR6 or CR6-VP1CW3 persisted in the ileum and colon at 21dpi (Fig 4A, B), and were shed in the stool (Fig 4C, D), we rarely detected persistent virus in any tissues from mice infected with CR6Δcasp or CR6Δcasp-VP1CW3 (Fig 4). Altogether, these findings support a role for NS1/2 cleavage specifically within the intestinal tissue, and
temporally link the activity of NS1/2 cleavage to the first 3 days post-infection. Thus, NS1/2 cleavage is required for establishing an infection in the intestine and subsequent persistence.

**NS1/2 cleavage functions independently of IFN.** Due to the dominant role IFN-λ plays in protection against persistent MNV infection of the intestine [26], and the potential role of NS1/2 cleavage in counteracting IFN signaling in BMDCs (Fig 2C, D compared to Fig 2E, F) [28], we sought to more thoroughly characterize CR6Δcasp infection in the absence of IFN-λ signaling. To this end, we infected WT or Ifnlr1−/− mice with CR6 or CR6Δcasp and monitored shedding in the stool over time (Fig 5A, B), and persistence in the colon at 21dpi (Fig 5C, D). Additionally, to determine whether an early barrier to MNV infection could be overcome with increased infectious dose, we included an experimental group with 10-fold more CR6Δcasp (1e7 PFU) in the inoculum. MNV was shed in all WT mice infected with CR6 beginning on day 3 and continuing until the experimental endpoint, day 21. Consistent with the protective role of IFN-λ, Ifnlr1−/− mice infected with CR6 shed greater than 10-fold more virus relative to WT mice (Fig 5A, B), and CR6 was detected in colon tissue at levels commensurate with fecal shedding at 21dpi (Fig 5C, D). In contrast, we were unable to detect shedding of CR6Δcasp at any time from WT or Ifnlr1−/− mice (Fig 5A, B). These data indicate that NS1/2CR6 cleavage promotes persistent infection via a novel mechanism separate from IFN antagonism.

**Cleavage of NS1/2 is critical for MNV infection of intestinal epithelial cells.** Our preceding data demonstrated that disrupting NS1/2 cleavage leads to an overall reduction of infectious virus produced in intestinal tissues of either WT (Figs 3, 4) or Ifnlr1−/− (Fig 5) mice. Therefore, to attain a more thorough understanding of the role of NS1/2 cleavage on cellular tropism, we infected WT or knockout mice with CR6 or CR6Δcasp and analyzed infection of IECs at 3dpi by flow cytometry and RNAscope (Fig 6). We concurrently quantified MNV infection of Peyer’s patches by qPCR to enable contextualization of the IEC infection data. We were able to detect viral RNA in Peyer’s patches in the majority of CR6-infected mice
and ~50% of CR6\textsubscript{Acasp}-infected mice (Fig 6A). Although CR6\textsubscript{Acasp} infection of Peyer’s patches was less robust than that of CR6, these data confirm that NS1/2 cleavage is not strictly required for initial infection of non-epithelial cells during acute infection (Figs 3E-F, 6A). This detectable but reduced infection by CR6\textsubscript{Acasp} was observed in mice deficient in IFN-\(\lambda\) receptor (Ifnlr1\textsuperscript{-/-}; LRKO), doubly-deficient in IFN-\(\lambda\) and IFN-\(\alpha/\beta\) receptors (Ifnlr1\textsuperscript{-/-}/Ifnar1\textsuperscript{-/-}; DKO), as well as receptor-sufficient controls (WT) (Fig 6A), suggesting NS1/2 cleavage amplifies infection in Peyer’s Patches.

To assess the requirement of NS1/2 cleavage in IEC infection, intestinal tissue devoid of Peyer’s patches was mechanically and enzymatically stripped to obtain a single cell suspension and stained for CD45 and EpCAM as markers of immune cells and epithelial cells, respectively. The presence of MNV infection was detected via dual intracellular staining using two distinct antibodies to NS1/2 in mice infected with CR6 or CR6\textsubscript{Acasp} (Fig 6B, C). We analyzed IEC infection in the same mice as in Fig 6A, including knockouts and littermate controls. Regardless of genotype, we only detected IEC infection when mice were infected with CR6, but not CR6\textsubscript{Acasp} (Fig 6B, C). Additionally, tissue sections were examined via RNAscope, and we reproducibly detected CR6 infection of IECs (Fig 6D, E), as well as non-epithelial cells in the lamina propria (Fig 6E) but were unable to find evidence for CR6\textsubscript{Acasp} infection of IECs at 3dpi (Fig 6F). These data demonstrate the necessity of NS1/2 cleavage for CR6 infection of IECs, the cellular reservoir of MNV persistence. Furthermore, the profound absence of CR6\textsubscript{Acasp} in IECs would translate to a loss of IEC-dependent viral amplification and may explain the reduced titers in Peyer’s patches (Fig 6A) and MLN (Fig 3A, B) during acute infection. Altogether, our data demonstrate that eliminating IFN signaling is not sufficient to rescue CR6\textsubscript{Acasp} infection of IECs (Figs 5 and 6), indicating that cleavage of NS1/2 functions to promote persistent infection of IECs by a mechanism independent of the IFN-\(\lambda\) response.
NS1/2 cleavage promotes MNV-induced apoptosis. We next sought to identify an IFN-λ-independent cellular response altered by NS1/2 cleavage. Because apoptotic-associated caspases have been implicated in cleavage of NS1/2 (Fig 1) [10], and NS1/2 cleavage is critical for developing a persistent MNV infection (Figs. 4, 5), we postulated that apoptosis may be altered by NS1/2 cleavage to promote persistent infection in the gut. Consistent with this hypothesis, we found that CR6 induced significantly more apoptosis in BV2 cells compared to CR6Δcasp (Fig 7). This included an increase in the active form of caspase 3 (Fig 7A, B) and an increase in PARP cleavage (Fig 7A), a known target of activated caspase 3 [44]. The increase in caspase 3 activity at 18hpi (Fig 7B) was not simply due to increased infection in CR6-infected cultures, as there were similar levels of ProPol detected in all infection conditions (Fig 7C). We also measured a significant increase in cells expressing phosphatidylserine on the external plasma membrane via Annexin V staining in CR6-infected cells (Fig 7E-G). Similarly, there was also an increase in these apoptotic markers following infection with CR6-VP1\textsuperscript{CW3} compared to CR6Δcasp-VP1\textsuperscript{CW3} (Fig 7D, E-H). Furthermore, in support of the hypothesis that NS1/2\textsuperscript{CR6} cleavage promotes apoptosis in a manner that is specific for persistence, the acute strain of MNV, CW3, induced less apoptosis in all our analyses (Fig 7), despite demonstrating NS1/2 cleavage (Fig 1C). Interestingly, CR6-VP1\textsuperscript{CW3} infection resulted in increased cell permeability and death relative to CR6 at 17hpi (Fig 7E, H), consistent with our separate findings that VP1\textsuperscript{CW3} drives lytic cell death and inflammatory cytokine production (Jacob VanWinkle, et al, manuscript under review). However, even in the context of increased cell lysis associated with VP1\textsuperscript{CW3}, we still observed a significant decrease in Annexin V-positivity in the absence of NS1/2\textsuperscript{CR6} cleavage (Fig 7F, H), suggesting NS1/2 cleavage is still promoting apoptosis even in the context of lysis and inflammation driven by VP1\textsuperscript{CW3}. Together with the preceding data, these findings suggest that potentiation of apoptotic cell death by caspase-mediated cleavage of NS1/2 is critical for determining IEC tropism and persistence.
Discussion

Herein, we demonstrate that caspase-mediated cleavage of the nonstructural protein, NS1/2, is critical for developing a persistent MNV infection. We engineered and characterized a strain of MNV in which NS1/2 is not cleaved (CR6Δcasp), and our in vitro and in vivo analyses demonstrate that NS1/2 cleavage is not required for viral replication in myeloid cells but is necessary for infection of IECs, the eventual site of persistence. Furthermore, our data indicate that NS1/2 cleavage potentiates apoptosis, suggesting apoptosis is a critical aspect of establishing a persistent MNV infection.

NS1/2 was not previously known to play a role in cell death during MNV infection, but is required for viral replication beginning at the earliest stages of replication complex assembly. Norovirus replication occurs in the cytoplasm on cellular membranes with a variety of organelle markers [45], and occurs near membranous vesicles [19,45]. MNV NS1/2 targets to the endoplasmic reticulum (ER) [46,47] and promotes replication via membrane re-organization and through an interaction with the vesicle-associated membrane protein, VAPA [48,49]. Similarly, HuNoV NS1/2 also interacts with VAPA, targets to vesicles, and disrupts vesicle trafficking when ectopically expressed [49]. Our data indicate that the functions of NS1/2 during viral replication remain intact when the caspase cleavage sites are mutated, and viral replication occurs normally (Fig 2). NS1/2 cleavage only occurs after the bulk of viral replication has completed indicating that its role in promoting apoptosis, IEC infection, and persistence involves relatively late events in the viral lifecycle.

The last step of the viral replication cycle is egress. Therefore, NS1/2 cleavage and associated apoptotic death may be important for regulating release of viral progeny. How noroviruses are released from infected cells is not well understood, but infection of macrophages and DCs does result in cell lysis [19], and non-enveloped viruses generally rely on cell lysis to release newly assembled virions. Death by apoptosis may alter this process by reducing lytic virion release and favoring retention of virions within cellular membranes. Indeed, a number of non-enveloped viruses utilize vesicle-associated egress as a means of evasion from immune detection, and vesicles with exposed phosphatidylserine allow for
phagocytic uptake of novel target cells that may not express the viral receptor [50,51]. This type of vesicle
egress, known as ‘apoptotic mimicry’, is also utilized by enveloped viruses which can incorporate
phosphatidylserine directly into their viral envelope [34]. Viruses, or virus-filled vesicles, decorated with
phosphatidylserine can inhibit IFN responses via activation of Tyro3/Axl/Mer (TAM) receptor tyrosine
kinases when phagocytosed by dendritic cells (DCs) [52], which would allow more efficient viral
replication in those target cells. Thus, vesicular egress has the potential to dramatically change the outcome
of infection.

Previous work identified CD300lf as the MNV receptor [21], and accordingly, it is expressed on
MNV target cells, including myeloid cells and a specialized IEC subtype, tuft cells. Tuft cells are a rare
cell type in the intestinal epithelium and are dispersed among non-tuft IECs [53]. Interestingly, our analyses
often found intestinal villi with multiple, adjacent epithelial cells infected (Fig 6E) even though MNV
infection has not been shown to alter numbers of tuft cells in the intestine [18]. These findings raise the
possibility of receptor-independent transfer of MNV to neighboring IECs, which could be mediated by
apoptosis or vesicular egress regulated by NS1/2 cleavage. Future work will be necessary to determine
whether vesicle-associated egress occurs during norovirus infection and whether it is required for viral
persistence.

Regular maintenance of the highly proliferative intestinal epithelium means differentiated epithelial
cells have a short lifespan, and undergo a specialized form of apoptotic cell death (anoikis) triggered as the
cell detaches from the basal membrane and is shed into the lumen [54]. Specific Bcl-2 family proteins are
critical players in anoikis [55,56], and display increased expression as IECs transit up the villus [56]. It is
possible cleavage of NS1/2 (and perhaps NS1 directly) interferes with Bcl-2 family proteins as a means of
altering the kinetics of the highly-regulated pathways that control apoptosis within differentiated IECs.
Interestingly, similar to NS1/2 cleavage promoting apoptosis during infection, some Bcl-2 proteins (e.g.
Bid) are also regulated via cleavage, in which the truncated version is the active, pro-apoptotic form of the
It will be interesting to examine whether NS1/2 interactions with the apoptotic machinery plays a role in maintaining IEC infection.

Death by apoptosis could also function to avoid immune detection and to limit inflammation associated with cell lysis. Virus production that results from cell lysis would promote inflammatory signals to alert the host of an infection. Thus, promotion of an apoptotic cell death in myeloid cells, mediated by NS1/2<sup>Cr6</sup> cleavage (Fig 7), would limit inflammation and benefit persistence. In addition to professional phagocytes, phagocytosis by non-professional phagocytes (e.g. epithelial cells) is critical for efficient clearance of apoptotic cells, and for limiting inflammation in tissues with high cellular turnover [58]. Specifically, in a mouse model of colonic injury, phagocytic uptake of apoptotic cells by IECs was sufficient for limiting inflammation in the colon [59], and phagocytosis mediated by epithelial cells is similarly critical for reducing inflammation in lung tissue [60] and mammary glands [61]. Thus, intestinal infection that promotes apoptotic death and the phagocytic uptake by IECs would limit host detection and provide a non-inflammatory environment that would allow more efficient infection of IECs and benefit MNV persistence. Regardless of whether apoptotic death limits inflammation or functions by another mechanism, our studies here have suggested potentiation of apoptosis following cleavage of NS1/2 as a new pathway critical for sustained infection of IECs and expanded our understanding of the requirements for viral persistence in the intestine.
Materials and Methods

Cells, virus, and plaque assays

BV2 cells (gift from Herbert Virgin IV, Washington University, St. Louis, MO), a mouse microglial cell line [62], were maintained in DMEM (Life Technologies, ThermoFisher, Carlsbad, CA) with 5% fetal bovine serum (FBS) (VWR Seradigm, Radnor, PA), 1X Penicillin/Streptomycin/Glutamine (P/S/G) solution, and 10mM HEPES. For bone-marrow derived dendritic cells (BMDCs), bone marrow was isolated from the long bones of each hind leg of either a WT or Stat1−/− mouse and suspended in RPMI (Life Technologies). Red blood cells were lysed using red blood cell lysis solution (Sigma, St Louis, MO) and remaining bone marrow cells were cultured in RPMI with 10% FBS, 1X P/S/G solution, 10mM HEPES, 1X Sodium Pyruvate, 1X non-essential amino acids, and 20ng/mL GM-CSF obtained from supernatants of GM-CSF-secreting cells (J558L, [63]). After seven days of culture (37C, 5% CO2), non-adherent cells were collected, infected with indicated MNV strains (MOI = 1), and maintained without GM-CSF. For indicated experiments, ZVAD-fmk (Cell Signaling Technology [CST], Danvers, MA) was re-suspended in DMSO and added to cells at 70µM. As a positive control for apoptosis assays, cells were treated with 1uM staurosporine (SS) (CST) for 3.5h, at 37C.

CR6Δcasp MNV was generated using site directed mutagenesis as previously described [15] with the following primers: CR6 D121G, 5’-GCCTAAGGAAGATAAAGGCCGTTGCGCCCTCCCATGCG and CR6 D131G, 5’-TGCGGAGGACGCCATGGGTGCAAGGGAGCCCCATAATTGG. The targeted residue is underlined with the mutation in bold. Correct mutations were verified via sanger sequence analysis.

MNV stocks were generated from plasmids, as described [17]. Briefly, plasmids encoding viral genomes of parental CW3 (GenBank accession no. EF014462.1), parental CR6 (GenBank accession no. JQ237823), CR6-VP1 CW3 [15,17] and the two NS1/2Δcasp viral genomes generated herein, were individually transfected into 293T cells (ATCC #CRL-3216). Forty-eight hours post-transfection, cells were frozen, thawed, vortexed, and spun at 3,000xg to remove large debris. Supernatants were expanded
by two passages at MOI <0.05 in BV2 cells, and p2 virus supernatant was filtered (0.45µm), concentrated
by ultra-centrifugation through a 30% sucrose cushion, and titered via plaque assay.

Plaque assays were performed in BV2 cells, similar to previously described methods [15]. Briefly, BV2 cells were grown in 6well plates, infected with serial dilutions of each sample (500µl per well, 1h, RT, on a rocking platform), after which the inoculum was removed and cells were overlaid with 1%
methylcellulose in complete DMEM. At 2-3d post-overlay, cells were fixed and stained with 20% EtOH / 0.1% crystal violet.

**Mice, infections, and tissue collection**

Stat1\(^{-/-}\) (Stat1\(^{tm1Dlv}\)) mice were originally obtained from the Jackson Laboratories (stock #012606). Ifnlr1\(^{-/-}\) (generated from Ifnlr1\(^{tm1a(EUCOMM)Wtsi}\)) and Ifnar1\(^{-/-}\) (Ifnar1\(^{tm1Agt}\)) were originally obtained from Washington University in St. Louis. Ifnlr1\(^{-/-}\) and Ifnar1\(^{-/-}\) were bred to generate Ifnlr1\(^{-/-}\)/Ifnar1\(^{-/-}\) mice (DKO) and Ifnlr1\(^{+/-}\)/Ifnar1\(^{+/-}\) (Dhet). Dhet and DKO crossbreeding yielded littermate mice deficient in neither (Dhet), both (DKO), or a single (Ifnlr1\(^{+/-}\)/Ifnar1\(^{-/-}\) or Ifnlr1\(^{-/-}\)/Ifnar1\(^{+/-}\)) IFN receptor gene(s); these littermates were used for experiments to control for variability between litters.

All mice (7-9weeks old) were perorally infected with 1e6 PFU in a 25µL volume administered by pipet, with the exception of experiments in Figure 6 where 1e6 or 1e7 PFU (as indicated in figure) was administered by oral gavage in a 100µL volume. An equal proportion of males and females were maintained among experimental groups to mitigate results due to any unidentified sex-dependent variables. Same-sex mice, infected with the same MNV strain, were often co-housed for acute infection analyses (less than three days), but singly-housed for extended experiments to prevent continued transmission between animals.

Tissue samples, or a single fecal pellet, were collected in 2ml cryovials with 1mm zirconia/silica beads (Biospec, Bartlesville, OK), frozen in a dry ice/EtOH bath and stored at -80C. For plaque assay or
RT-qPCR analyses, either 1ml DMEM or 1ml RiboZol (VWR) was added, respectively, and tissues were homogenized via bead-beating (1min, RT) prior to continued analysis.

Ethics Statement

All mice were bred on C57BL/6 background and maintained in specific-pathogen-free barrier facilities at Oregon Health & Science University under animal protocols approved by the institutional animal care and use committee at Oregon Health & Science University (protocol # IP00000228) according to standards set forth in the Animal Welfare Act.

RNA, cDNA, and RT-qPCR

RNA from tissues was extracted via RiboZol (VWR) protocols, and RNA from cells was extracted with theZR Viral RNA kit (ZymoResearch, Irvine, CA). DNA contamination was removed with Turbo DNase (ThermoFisher), and 1ug of RNA was used to generate cDNA with the ImpromII reverse transcriptase (Promega, Madison, WI). Quantitative PCR was performed using PerfeCTa qPCR FastMix II (QuantaBio, Beverly, MA), and the following oligo/probes were used for detection: MNV genomic RNA - Forward primer CACGCCACCGATCTGTTCTG, Reverse primer GCGCTGCGCCATCCTC, and Probe TGCGCTTTGGAACAATGG; RPS29 - Forward primer GCAAATACGGGCTGAACATG, Reverse primer GTCCAACTTAATG AAGCCTATGTC, and Probe CCTTCGCGTACTGCCGGAAGC.

Cell Lysates and Western blot

BV2 cells were grown and infected in 12well plates (2 e5 cells/well). Cells were lifted with trypsin/0.25% EDTA (Gibco, ThermoFisher), washed with 1X cold PBS, resuspended in 100ul NP40 lysis buffer (Alfa Aesar, Havervill, MA) with protease inhibitor cocktail (Sigma, St Louis, MO), incubated on ice (15min with intermittent mixing), cleared via centrifugation (1min, 4C, full-speed), and diluted 1:1 with 2x SDS loading buffer (0.125 M Tris [pH 6.8], 4% SDS, 20% glycerol, 0.004% bromophenol blue, 10% β-
mercaptoethanol). Lysates were run on Novex Tris-Glycine gels (ThermoFisher), transferred to 0.2µm PVDF (BioRad, Hercules, CA), blocked with 5% nonfat dry milk, and probed with the following antibodies (Ab): caspase 3 pAb (CST), PARP pAb (CST), β-Actin pAb (Invitrogen), MNV NS1/2 pAb (a gift from Dr. Vernon Ward, U. of Otago, Australia), or MNV ProPol pAb (a gift from Dr. Kim Green, NIH). Signal was detected using goat, α-rabbit IgG-HRP (Jackson ImmunoResearch), developed with ECL substrate (Pierce, ThermoFisher), and imaged with an ImageQuant LAS4000 (GE Healthcare, Little Chalfont, UK).

Intestinal epithelial cell collection

Epithelial fractions were prepared by non-enzymatic striping as previously described [24]. Mice were euthanized and distal small intestine, cecum, and colon (regions of highest MNV replication) were isolated. Peyer’s patches were removed from small intestine and intestinal tissues were incubated in stripping buffer (10% bovine calf serum, 15 mM HEPES, 5 mM EDTA, 5 mM dithiothreitol [DTT] in PBS) with shaking for 20 min at 37°C. The dissociated cells were collected as the epithelial fraction, consisting predominantly of IECs and directly used for flow cytometry.

Surface and intracellular staining and flow cytometry

BV2 cells were trypsinized and washed once with 1X PBS, stained with the amine-reactive viability dye, Zombie Aqua (Biolegend, San Diego, CA) (20min, on ice), washed once with cold 2% FBS/PBS, washed once with cold Annexin V binding buffer (CST), and stained with Annexin V-FITC (CST) (15min, RT). Cells were immediately analyzed via flow cytometry on an LSR Fortessa (Becton Dickinson [BD], Franklin Lakes, NJ) with FACSDiva software (BD), and data was analyzed using FlowJo software (FlowJo LLC, Ashland, OR).

IECs isolated as described above were stained with the Zombie Aqua viability dye (Biolegend), Fc receptors were blocked with purified CD16/CD32 pAb (clone93; Biolegend), and cells were probed for
surface markers, EpCAM (clone G8.8; Biolegend) and CD45 (clone 30-F11; Biolegend). Cells were subsequently fixed with 2% paraformaldehyde/PBS (10 min, RT), and blocked/permeabilized in 0.2% Triton X/PBS with 2% normal goat serum (20 min, RT). Fixed and permeabilized cells were probed for intracellular viral antigen with 2 distinct antibodies: Rabbit polyclonal anti-NS1/2 (a gift from Dr. Vernon Ward, U. of Otago, Australia) and mouse monoclonal anti-NS1 (clone CM79, a gift from Dr. Ian Clark, U. of Southampton, UK). These unconjugated antibodies were detected with anti-Rabbit Alexa Fluor 647 (Molecular Probes, Eugene, OR), and anti-mouse Dylight 405 (Jackson ImmunoResearch), respectively. Cells were analyzed via flow cytometry, and viable cells that were EpCAM+, CD45-, NS1/2 pAb+, and NS1 mAb+ were labeled as infected epithelial cells.

**RNA in situ hybridization via RNAscope®**

Sections of approximately two cm from the ileum that contained a Peyer’s patch (PP) were selected, cut open lengthwise, pinned apical side up in wax trays, and fixed in 10% neutral-buffered formalin (Sigma) (18-24 h, RT), then moved to 70% EtOH. Tissues were embedded in 2% agar (ThermoFisher), the PP was vertically transected, flipped 90 degrees, and re-embedded in 2% agar. Agar blocks were maintained in 70% EtOH prior to being paraffin-embedded. Tissue sections (5 μm) were cut and maintained at RT with desiccant until processed. RNA in situ hybridization was performed using the RNAscope Multiplex Fluorescent v2 kit (Advanced Cell Diagnostics [ACDBio], Newark, CA) per protocol guidelines. Probes were designed by ACDBio for detection of CW3 genomic RNA (471891-C1), CR6 genomic RNA (502631-C1), and EpCAM mRNA (418151-C2), and signals were amplified and detected via ACDBio protocols using TSA® plus technology (Perkin Elmer, Waltham, MA). Slides were mounted with ProLong Gold antifade reagent (ThermoFisher), and imaged using a Zeiss ApoTome2 on an Axio Imager, with a Zeiss AxioCam 506 (Zeiss, Jena, Germany).

**Statistical Analyses**
Data were analyzed with Prism 7 software (GraphPad Prism Software, La Jolla, CA), with specified tests and significance noted in the figure legend.
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Figure Legends

Fig 1. MNV NS1/2 is cleaved by host caspases at late times-post-infection. (A) Schematic of MNV genomic organization, illustrating initial NS1/2 expression within the ORF1 polyprotein. Cleavage sites in ORF1 are marked accordingly - sites cleaved by the viral protease (v) and sites cleaved by host caspases (*), the latter at aspartic acid residues 121 and 131 within the 2 functional caspase motifs, DKAD\textsuperscript{121} and DAMD\textsuperscript{131} [10]. (B) Schematic representation of the five MNV clones used in these studies. Clones with D\textsubscript{121}G and D\textsubscript{131}G mutations are referred to as Δcasp, and indicated by the X. (C) BV2 cells were infected with indicated strains of MNV (MOI=1), and whole cell extracts (WCE) were analyzed via western blot (WB) at indicated times post-infection. Image is representative of 4 independent experiments. Arrows are pointing to the full-length NS1/2 at ~45kD, and NS2 at ~28kD. * Denotes a non-specific band at ~30kD. (D) BV2 cells were infected as in C, but subsequently treated with 50μM ZVAD-fmk at 10hpi, and WCE were analyzed via WB. Image is representative of 3 independent experiments. SS, staurosporine.

Fig 2. Cleavage of NS1/2 is not required for MNV replication in vitro. (A, B) BV2 cells were infected with the indicated strain of MNV (MOI=1) and infectious virus was analyzed at indicated times post-infection via plaque assay. Data are an average of 3 independent experiments. WT (C, D) or Stat1\textsuperscript{-/-} (E, F) BMDCs were generated as described in methods, and infected and analyzed as in A-B. Data are an average of 2 independent experiments. Dotted lines indicate limit of detection. Statistical significance was determined via two-way ANOVA with Bonferroni’s multiple comparisons test. *, p ≤ 0.05. BMDCs, bone marrow-derived dendritic cells.

Fig 3. Cleavage of NS1/2 is specifically required for early replication in intestinal tissues. WT B6 mice were perorally infected with indicated viruses (1e6 PFU). At 3 days post-infection (dpi), tissues (A, B) and stool (C, D) were harvested and analyzed via plaque assay. Data are from 8-9 individual mice within 3 separate experiments, and mean values are indicated. (E-H) Peyer’s patches within the ileum were...
analyzed via RNA *in situ* hybridization, using RNAScope commercial protocols and probes. MNV genomic RNA was probed within ORF2, which encodes the major capsid (VP1), and is labeled in magenta, epithelial cells were probed via EpCAM, and labeled in green, and DAPI is labeling nuclei in blue. Images are representative of 2-3 mice for each group. (I) Mice (5-6 per group) were infected with indicated virus and survival was plotted over the course of 35 days. Statistical significance was determined via Mann-Whitney test for A-D. *, p ≤ 0.05; **, p ≤ 0.01; ns, not significant; MLN, mesenteric lymph node; yellow scale bar, 50 µm; white scale bar, 20 µm.

**Fig 4.** Cleavage of NS1/2 is critical for persistent infection in the intestine. (A, B) WT B6 mice were perorally infected with indicated viruses, sacrificed at 21 days post-infection (dpi), and indicated tissues were analyzed via RT-qPCR for MNV genomic RNA. Data are combined from 2 separate experiments with data points representing individual mice, and mean values are indicated. (C, D) Fecal pellets were also collected at 21 dpi, and similarly analyzed. Statistical significance was determined via Mann-Whitney test. *, p ≤ 0.05; **, p ≤ 0.01; ns, not significant; MLN, mesenteric lymph node.

**Fig 5.** Caspase cleavage of NS1/2 is required independent of the IFN-λ response. WT (A, C) or *Ifnlr*-/- (B, D) mice were infected by oral gavage with either 1e6 PFU or 1e7 PFU, as indicated. Fecal samples (A, B) were collected at 3, 7, 14, and 21 days post-infection (dpi), and colon tissue (C, D) was harvested at 21dpi. All samples were analyzed via RT-qPCR, and the mean of each group (N=4) is indicated. Dotted lines indicate limit of detection.

**Fig 6.** NS1/2 cleavage is necessary for infection of intestinal epithelial cells. (A-C) Littermate mice deficient for the type III IFN receptor (*Ifnrl1-/-* or *Ifnrl1-/-/Ifnar-/-*, “LRKO”), deficient for both type I and III IFN receptors (*Ifnrl1-/-/Ifnar-/-*, “DKO”), and matched receptor-sufficient controls (*Iflr1+/+ / Ifnar+/+ or *Iflr1+/+/*Ifnar+/+, “WT”) were infected with indicated viruses (9-13 mice per group combined from 3 separate
experiments), and analyzed at 3 dpi. (A) Peyer’s patches were collected and analyzed via RT-qPCR for 
MNV genomic RNA and normalized to RPS29. (B, C) Intestinal epithelial cells (CD45-, EpCAM+) were 
collected and doubly stained for NS1/2 as described in methods. Dotted line indicates limit of detection. 
(C) Flow plots representing data from 5 mice in B (each mouse is a separate color). (D-F) Ileum tissue 
from CR6-infected or CR6Δcasp-infected WT mice was analyzed via RNAscope at 3 dpi, as in Figure 3. 
MNV RNA is in magenta, EpCAM is in green, and nuclei are stained with DAPI in blue. Images are 
representative of 5 mice infected with each virus. white scale bar, 20µm. Legend refers to data in A and 
B. Statistical significance was determined by Mann-Whitney test. *, p £ 0.05.

Fig 7. Cleavage of NS1/2 potentiates apoptotic cell death. BV2 cells were infected (MOI=1) with 
indicated MNV strains, and (A-D) whole cell extracts were collected at indicated times and analyzed via 
WB for caspase 3, PARP, actin, and MNV ProPol. (A, D) Arrows denote cleaved fragments of caspase 3 
and PARP, indicating activation. Images are representative of 3 independent experiments. Cleaved caspase 
3 (B), and ProPol (normalized to actin) (C) were analyzed via densitometry, with average and SD shown. 
(E-H) Cells were harvested at 14h and 17hpi, stained with Annexin V and zombie LIVE/DEAD viability 
stain, and analyzed via flow cytometry. (E) Representative flow plots are shown from one of six 
independent experiments. (F) Total Annexin V+ cells were averaged at 17hpi, and individual and mean 
values are indicated, and (G) live, Annexin V+ (bottom right quadrant of flow plots in E), and (H) dead, 
Annexin V+ (top right quadrant of flow plots in E) populations were also analyzed separately. Statistical 
significance was determined by repeated measures one-way ANOVA, with Bonferroni’s multiple 
comparison test. *, p £ 0.05; **, p £ 0.01; ns, not significant. SS, staurosporine.
