Uncovering and Characterizing the Function and Utility of Human Norovirus NS1

By

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I would also want to take the time to thank the postdocs in lab - Jaewon, Li and Seokoh. I appreciate them for taking me under their wing and being so understanding towards all my questions and concerns I had in lab. This thesis would not have been possible without them. All our conversations we shared will always be close to my heart. To the other lab mates - Lucy, Yao, Christine and Jason - I also extend my gratitude to making life in lab seem pleasant even when experiments were not necessarily going that well.

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Abstract of ***Uncovering and Characterizing the function and utility of Human Norovirus NS1****, by Vasisht Poosapati, ScM, Brown University, May 2026.*

Human norovirus (hNoV) is a leading cause of acute viral gastroenteritis worldwide, yet the molecular mechanisms by which it evades host innate immune responses remain incompletely defined. In this study, we investigated the role of the hNoV non-structural protein NS1 in modulating antiviral signaling, with a focus on MDA5-dependent pathways that drive interferon production. Using a reporter-based system in HEK293T cells, we demonstrate that NS1 proteins derived from both GI and GII genogroups suppress antiviral signaling in a dose-dependent manner. This activity is further conserved in murine norovirus, supporting a broadly conserved mechanism of immune evasion.

Functional assays revealed that NS1 does not inhibit signaling induced by exogenous interferons, indicating that suppression occurs upstream of interferon receptor engagement. These findings support a model in which NS1 targets intracellular antiviral sensing pathways rather than acting through cytokine neutralization. Domain mapping identified the N-terminal region of NS1 as necessary and sufficient for suppressive activity. Notably, a minimal 16-amino acid segment retained robust function, indicating that immune evasion is mediated by a compact and conserved interface. Together, these findings establish NS1 as a conserved antagonist of MDA5-dependent antiviral responses and provide a foundation for future studies aimed at identifying host targets and defining mechanisms of immune evasion.

Introduction

Norovirus is an RNA virus of the *Caliciviridae* family, and is a human pathogen known to cause substantial damage across health care and community settings. They are currently the leading cause of nonbacterial gastroenteritis in the world and is estimated to cause over 200,000 deaths annually[1-3]. The clinical feature of an infection is typically characterized by the sudden onset of nausea, vomiting, diarrhea, and abdominal cramping, which may generally be resolved within the span of one to three days in immunocompetent hosts[4]. However, in elderly individuals, young children, and immunocompromised patients, illness can be substantially more severe and prolonged, carrying a risk of life-threatening dehydration and - in an immunocompromised setting - chronic, progressive gastrointestinal disease[5,6].

Within the United States, norovirus causes an estimated 20 million cases of gastroenteritis each year, resulting in approximately 70,000 hospitalizations and up to 800 deaths annually[7]. Significant disease burden is not the only damaging effect it imposes, as norovirus also has a severe global economic burden. It is estimated that norovirus-associated illness generates approximately $4.2 billion in direct medical expenditures and $60.3 billion in indirect costs from lost workplace productivity annually[8]. In healthcare settings, norovirus is the most frequently identified pathogen implicated in nosocomial gastrointestinal outbreaks and generating considerable institutional costs in terms of infection control and bed days lost[9]. A collection of biological properties render norovirus extremely transmissible. Experimental human challenge studies have demonstrated that as few as 20 viral particles are sufficient to

establish infection in a susceptible host, a remarkably low infectious dose that enables rapid spread even from minimal environmental contamination[10]. The virus is stable and is known to withstand and be broadly resistant to common disinfection strategies including alcohol-based hand rubs[11].

Norovirus presents distinct immunological challenges that complicate prevention efforts. In immunocompromised individuals - including solid organ transplant recipients, hematopoietic stem cell transplant patients, and those with primary immunodeficiencies - norovirus can establish a chronic, persistent infection characterized by continuous viral evolution lasting months to years, and progressive gastrointestinal dysfunction[6,12]. Currently no licensed vaccine or therapy exists for norovirus infection and their ability to evade the host immune system makes it a priority pathogen demanding deeper mechanistic investigation to potentially develop effective therapeutics for the virus [4,13].

Innate Immunity Sensing and Pathway

To work towards developing any form of therapeutic formula against viral pathogens, it is essential to understand the host's immune response and its intricate functioning. The innate immune system acts as the first line of defense against viral intrusion. Intestinal innate immune cells, including IECs, macrophages and DCs, use pathogen-recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs) such as viral RNA from enteric RNA viruses[14]. For positive-sense RNA viruses, like norovirus in this case, the prominent PRR families in charge of initiating an antiviral response include the Toll-like receptors (TLRs), like TLR3 which senses double-stranded RNA

(dsRNA), and TLR7, which senses single-stranded RNA, and the RIG-I-like receptor (RLR) family comprising retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and the regulatory helicase LGP2[15,16].

Within the RLR family, RIG-I and MDA5 are distinguished by their RNA ligand preferences. RIG-I preferentially recognizes short dsRNA species bearing 5′-triphosphate ends whereas MDA5 detects longer dsRNA filaments of at least 1 kilobase pairs that accumulate during later phases of RNA synthesis as the viral replication proceeds[17]. This staggered specificity means the two work in tandem to provide efficient immune coverage across the full cycle of a productive viral infection. RIG-I and MDA5 both have tandem N-terminal caspase recruitment domains (CARDs) with death domain folds, a DExD/H-box helicase (consisting of two RecA-like helicase domains, Hel1 and Hel2, and an insert domain, Hel2i), and a C-terminal domain (CTD)[18]. In the absence of dsRNA, RIG-I has a closed inactive conformation[19]. RNA binding through the helicase and CTD domain releases the CARDs which in turn recruit and activate the signaling adaptor MAVS[20,21,22]. On the other hand, MDA5 does not sequester its CARDs[23] but rather assembles into ATP-sensitive filaments on dsRNA[24]. The CTD in MDA5 is required for this cooperative filament assembly but not for RNA binding[23,25,26]. The MDA5 CARDs instead have been proposed to nucleate the assembly of MAVS into its active polymeric form[23,27].

MAVS is a tail-anchored protein, located on peroxisomes, the mitochondrion-associated membrane, and the outer membrane of mitochondria[28]. Post engagement with either

MDA5 or RIG-I, MAVS becomes activated and functions as a signaling adaptor that recruits downstream signaling proteins[29]. MAVS then recruits the kinases TBK1 and IKKε, which first phosphorylate MAVS itself at a conserved serine-threonine cluster, converting it into a docking platform that recruits IRF3 and IRF7 for their own phosphorylation at their C-terminal activation domains[30,31]. Activated IRF3 and IRF7 form homo- and heterodimers that accumulate in the nuclei where they bind to target sequences along with other complexes to drive IFN-β transcription[30,32]. IFN-β that is produced and secreted as a result of the RLR cascade binds to the IFN receptor in an autocrine or paracrine manner to direct JAK-STAT signaling and the ISGF3-dependent expression of interferon-stimulated genes (ISGs)[30]. In addition to these ISG products, this signaling pathway is also known to induce the expression of the IFN-λ family of IL-10-related cytokines known collectively as type III interferon and various proinflammatory cytokines to control infection[33,34].

Role of Type III Interferons in Mucosal Immunity

While the RIG-I/MDA5-MAVS pathway can lead to production of both type I (IFN-α/β) and type III (IFN-λ) interferons, recent research has revealed that type III interferons play a dominant and non-redundant role in controlling viral infections at mucosal barrier sites, particularly at epithelial surfaces[35,36]. Type III interferons, also designated IFN-λ or lambda interferons, represent a distinct class of antiviral cytokines that, despite sharing only 15-20% amino acid identity with type I interferons, activate similar signaling pathways and transcriptional responses through the JAK-STAT pathway[35,36].

The critical distinction between type I and type III interferons lies in their cellular targeting patterns determined by differential receptor expression. While type I IFNs bind and signal through the IFNAR1 and IFNAR2 receptor chains, type III IFNs signal through IFNLR1 and IL10R2 receptor chains, where IL10R2 is also a shared receptor of interleukin (IL)-10, IL-22, and IL-26[35]. In the context of enteric viral infections, the use of murine norovirus has highlighted the dominant role of type III interferons. Mice deficient for Ifnlr1 exhibit drastically elevated intestinal viral replication and enhanced fecal shedding compared to wild-type animals, while mice lacking *Ifnar1* show levels of intestinal viral replication comparable to controls[37]. Additionally, knockout studies highlighted that the expression of IFNLR1 specifically on intestinal epithelial cells is both necessary and sufficient for the antiviral effects of type III interferons, establishing that the intestinal epithelium represents the critical cellular target for IFN-λ-mediated antiviral protection[33].

Interferon-Stimulated Gene Effectors

Antiviral activity led by the interferon signaling is coordinated through interferon-stimulated genes (ISGs) that encode proteins targeting almost every stage of the viral life cycle. In norovirus infections, the most critical ones are the pattern recognition receptors themselves - RIG-I and MDA5 - and their expression is amplified by interferon signaling which creates a positive feedback loop to enhance viral detection sensitivity and promote antiviral responses[38]. IRF1 has been identified as a potent anti-norovirus effector, with overexpression studies demonstrating inhibition of viral replication[38]. Type I and III interferons coordinately induce IRF1, RIG-I, and MDA5 expression, while type II

interferon predominantly induces IRF1 to exhibit anti-norovirus activity[38,39]. In murine norovirus studies, ISG15 has been identified as a critical early-acting antiviral effector that restricts viral entry and uncoating steps of the viral life cycle[40].

Primary Models of Study

The study of norovirus biology and pathogenesis has been fundamentally constrained by the historical inability to cultivate human noroviruses in conventional cell culture systems. This limitation has necessitated and forced the development of alternative experimental approaches like the novel cultivation systems, and animal models.

The murine norovirus (mNoV) is a positive-sense single stranded RNA with a genome of 7.5 kilobases in length[41]. The murine norovirus (mNoV) model is fundamentally the most important model system in place to study the mechanistic features of norovirus infections as it has several advantages working in its favor. First, mNoV is the only norovirus that can be efficiently propagated in standard cell culture, enabling reverse genetics studies, viral mutant construction, and high-throughput antiviral screening[42,43]. Second, the availability of a natural host system allows for in vivo studies of viral pathogenesis, host immune responses, and therapeutic interventions using genetically modified mouse strains[44]. This distinguishes mNoV from hNoV which currently lack a consistently reproducible small host system and a reliable system to maintain long-term culture[45]. Recent work has shown that mNoV exhibits preferential infection of tuft cells, a small and highly specialized group of epithelial cells found in the gut, that are now recognized as key targets in persistent norovirus infections[46,47].

The development of human intestinal enteroid (HIE) culture systems represents a massive shift in the right direction in the field of norovirus research since the discovery of mNoV. They are three-dimensional organoid cultures generated from intestinal stem cells isolated from human jejunal tissue and maintained in enriched media to recapitulate the intestinal microenvironment[48]. These cultures have been shown to reproduce the cellular complexity and specific niche and diversity of the intestinal epithelium including enterocytes, goblet cells and enteroendocrine cells to closely mirror those found in vivo[49]. This gives an added advantage, especially in the context of hNoV as they provide a much more physiologically relevant model to base studies off. Crucially the HIE cultures have been able to support the productive replication of multiple human norovirus genotypes, like GII.1, GII.2, GII.3 and GII.4[50,51].

Despite its seemingly endless bounds, the HIE system faces several technical and practical limitations. Indefinite passaging of human noroviruses in HIEs remains a challenge, even though recent work has begun to overcome this trouble for at least some strains[52]. To add to this, HIE susceptibility also demonstrates donor, segment, and strain-specific variations, with some donors showing complete resistance to specific genotypes[53].

Project Objectives

Noroviruses impose a major global health burden by causing widespread acute gastroenteritis. To tackle them and similar RNA virus pathogens, host immune systems have specially designed immune sensing pathways which include the RLR innate immune sensors like RIG-I and MDA5. This is highlighted in knockout studies, where mice lacking MDA5 displayed a stunted cytokine response in response to mNoV infection[54].

However, despite the presence of MDA5 and its mediated immunity pathway, noroviruses have found a way to bypass this and antagonize the host's ability to sense the viral pathogen and elicit an immune response. Among norovirus proteins, the NS1 protein represents a particularly compelling target for investigation due to its unique characteristics. In a murine norovirus study, NS1 emerged as a potent antagonist of IFN-λ which has been shown to play an integral role in the antiviral charge at mucosal surfaces, including the intestinal epithelium where noroviruses replicate[55]. What leads to the gap in knowledge is that NS1 proteins from different norovirus genotypes exhibit substantial sequence divergence, and this variation is seen even within individual strains like the GII strains[56]. It remains unclear how hNoV NS1 activity interferes with normal immune functioning, viral replication and immune evasion of the virus

The central objective of the thesis is to elucidate the mechanisms by which NS1 helps noroviruses in evading the host immune detection system. With the use of biochemical and virological approaches, this project aims to shine light on the interactions between the NS1 proteins and the MDA5 pathway. Through this project, the aim is to identify the specific domains of the hNoV NS1 strains that are necessary and sufficient for this

suppression. Findings made will improve the knowledge and understanding of norovirus pathogenesis and will provide the basis on which further work can be done to develop therapeutic interventions against noroviruses. By studying these host-viral interactions, this research will significantly contribute to the broader RNA virus field and add to the exemplary work being done in studying the host-pathogen interactions.

Materials and Methods

Bacterial Transformation

Chemically competent *E. coli* BL21(DE3) cells (50 µL) were thawed on ice and transformed with 2 µL of expression plasmid by heat shock. Plasmids designed were derived from pET-BNK and pET-21a+. Cells were incubated on ice for 20 minutes, subjected to heat shock at 42°C for 30 seconds, and immediately returned to ice. Following the addition of 250 µL LB medium, cells were incubated at 37°C for 1 hour to allow antibiotic resistance expression. Transformed cells (100 µL) were plated on LB agar plates containing 50 µg/mL carbenicillin and incubated overnight at 37°C. Single colonies were selected for protein expression.

Protein Expression

A single transformed colony was inoculated into 10 mL LB medium containing 50 µg/mL carbenicillin and grown overnight at 37°C with shaking at 200 rpm. The overnight culture was used to inoculate 1 L of LB medium supplemented with 50 µg/mL carbenicillin. Cultures were grown at 37°C with shaking at 200 rpm until reaching an optical density (OD_{600}) of 0.5-0.8. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. Following induction, cultures were incubated at 16°C with shaking at 150 rpm overnight to promote soluble protein expression.

Cell Lysis

Bacterial cells were harvested by centrifugation at 3,700 rpm for 20 minutes at 4°C. Cell pellets were resuspended in 20 mL of lysis buffer containing 1× Tris-buffered saline (TBS), 0.5% Triton X-100, 1 mM dithiothreitol (DTT), 100 µg/mL lysozyme, 100 units DNase I, and 1× cOmplete protease inhibitor cocktail (Roche). The cell suspension was incubated with gentle rocking at 4°C for 1 hour, followed by sonication using three 30-second bursts at 25% amplitude with 50-second intervals on ice. Cell debris was removed by centrifugation at 20,000 × g for 20 minutes at 4°C, and the clarified supernatant was collected for purification.

Purification and Concentration

His-tagged proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography. Ni-NTA agarose beads (2 mL) were packed into a gravity-flow column and equilibrated with wash buffer (1× TBS containing 30 mM imidazole). Clarified cell lysate was applied to the column and incubated at 4°C with gentle rotation for 1 hour to allow protein binding. The column was washed three times with wash buffer (1 column volume each) to remove unbound proteins and contaminants. Bound proteins were eluted with elution buffer (1× TBS containing 500 mM imidazole) in two sequential applications of 2 mL elution buffer, with 3-minute incubations between applications. Fractions were collected and analyzed by SDS-PAGE.

Purified proteins were subjected to buffer exchange to remove imidazole and concentrate the sample. Two methods were employed: (1) centrifugal ultrafiltration using Amicon Ultra-15 centrifugal filter units (10 kDa MWCO) at 4,300 × g for 8 minutes

at 4°C, with three sequential washes using 1× TBS; or (2) dialysis using Slide-A-Lyzer dialysis cassettes (10 kDa MWCO) against 2 L of 1× TBS at 4°C overnight with gentle stirring. Following buffer exchange, protein solutions were concentrated to approximately 1 mL using centrifugal filter units.

Cell Line Cultures

Human cell lines utilized in the study include HEK293T (ATCC), HeLa (ATCC, CCl2), HCT116-Dual Reporter (Invivogen, hctd-nfis), A549-Dual Reporter (Invivogen, a549d-nfis). Cell lines were cultured under normal conditions at 37°C in a humidified incubator with 5% CO2 source inlet. Cells were maintained in Dulbeco's Modified Eagle Medium (DMEM; Gibco, 11965092) complemented with 10% fetal bovine serum (FBS; Gibco, 26140079) and 25 mM HEPES buffer solution (Gibco, 15630080). To detach cells during passaging, 0.05% Trypsin-EDTA (Gibco, 25300062) was used, and cells were reseeded at appropriate densities for relevant experiments as described below.

Preparation of Poly (I:C)

Low molecular weight (LMW) polyinosinic-polycytidylic acid (Poly I:C; Invivogen, tlrl-picw) were dissolved into sterile, nuclease-free water (ThermoFisher Scientific, AM9937) to achieve a stock concentration of 10 mg/mL. Aliquots were prepared at adequate volumes and stored at -80 degrees Celsius to prevent multiple freeze-thaw cycles.

Cell Seeding and Transfection

Cells meant to be used for experimental treatments were seeded into 96-well plates 24 hours prior to reach an adequate confluency at the time of transfection on the following day. Primary adherent cell line used was HEK293T, and to reach optimum confluency they were seeded at a concentration of 5 $x10^5$ cells/mL in 100 μL of complete medium in each well.

Transfection was performed using Lipofectamine 3000 (Invitrogen, L3000008) for its efficient nucleic acid delivery across multiple cell types. The transfection complexes were made fresh before each trial, at 24 hours after cell seeding. The process involved making two separate reaction tubes:

1. Poly(I:C) was mixed with P3000 reagent (Invitrogen, L3000008) in Opti-MEM reduced serum medium (Gibco, 31985062) at a 1:1.5 ratio (nucleic acid: P3000) as per the recommendations of the manufacturer.
2. In the second tube, a volume of Lipofectamine 3000 equivalent to the volume of RNA stimuli was diluted in Opti-MEM.

Both mixtures were incubated separately at room temperature for 10 minutes after which they were gently combined, followed by an additional 10-minute incubation at room temperature to allow transformation complex formation. After this incubation period, 10 μL of the RNA-Lipofectamine mixture were add to each well that was being experimentally tested. Proteins being tested were concurrently added.

Reporter Cell Line Assay

A549 and/or HCT116 dual reporter cell lines were seeded into separate white 96-well plates at a density of $4x10^4$ cells per well (4 10^5 cells/mL) in Dulbecco's Modified Eagle Medium (DMEM; Gibco, 1196092) which is supplemented with 10% fetal bovine serum (FBS; Gibco, 26140079) and 25 mM HEPES buffer solution (Gibco, 15630080). Reporter cells were seeded between 20-24 hours before supernatant transfer to ensure sufficient confluency for subsequent detection assays.

At 16 hours post-transfection of the experimental cell lines. Cell supernatants were carefully collected and transferred to corresponding wells of the HCT116 dual reporter plates at primarily two volumes - 5 µL and 2 µL. Reporter cells were incubated for a further 24 hours at 37°C in a 5% CO_2 incubator before luminescence detection.

Luminescence Detection

Following the 24-hour incubation with cell supernatants, IRF was measured using a BioTek Cytation 5 Cell Imaging Multimode Reader (BioTek, CYT5MPW). Luminescence signals were quantified using a 50 ms integration time setting according to the guidelines provided by the manufacturer. Experimental conditions were assayed in biological triplicates to ensure reproducibility and accuracy of results.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism (version 10.4.2; GraphPad Software, San Diego, CA). To assess differences in IRF activity between

control and multiple treatment groups (e.g., LMW-Poly(I:C) stimulated cells transfected with or without viral NS1 proteins at varying concentrations), a one-way analysis of variance (ANOVA) was used. This test was selected to compare the means across three or more independent groups under the assumption of approximate normal distribution and similar variance. No post hoc test was performed, and all conditions were conducted in biological triplicates unless otherwise specified.

Figures

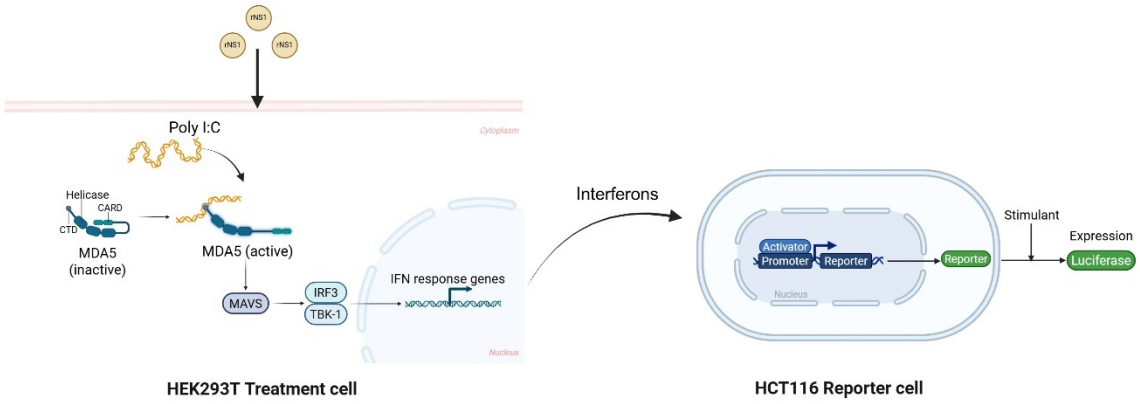

Figure 1: IRF activity assay. Schematic representation of the model assay used to test desired NS1 proteins and check for any IRF activity suppression phenotype. HEK293T cells were used as treatment cell line while HCT116 dual cells were used as the reporter cell line. See *Material and Methods* section for more details.

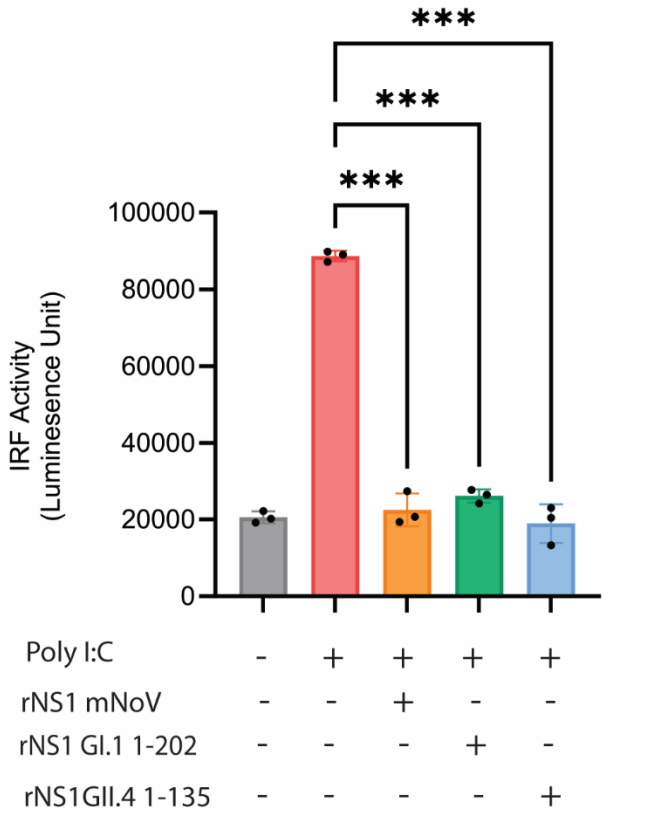

Figure 2: Human Norovirus NS1 from GI.1 and GII.4 strains suppress IRF-mediated responses. IRF activity is suppressed in HEK293T cells transfected with LMW-Poly(I:C) and treated with GI.1 human norovirus full length (FL; aa 1-202), GII.4 human norovirus full length (FL; aa 1-135) or murine NS1 (mNS1; aa 1-121) is used as a positive control. All proteins were tested at 100 µg/mL (+). Data are presented as mean ± SD with individual replicates shown. *** $p < 0.001$.

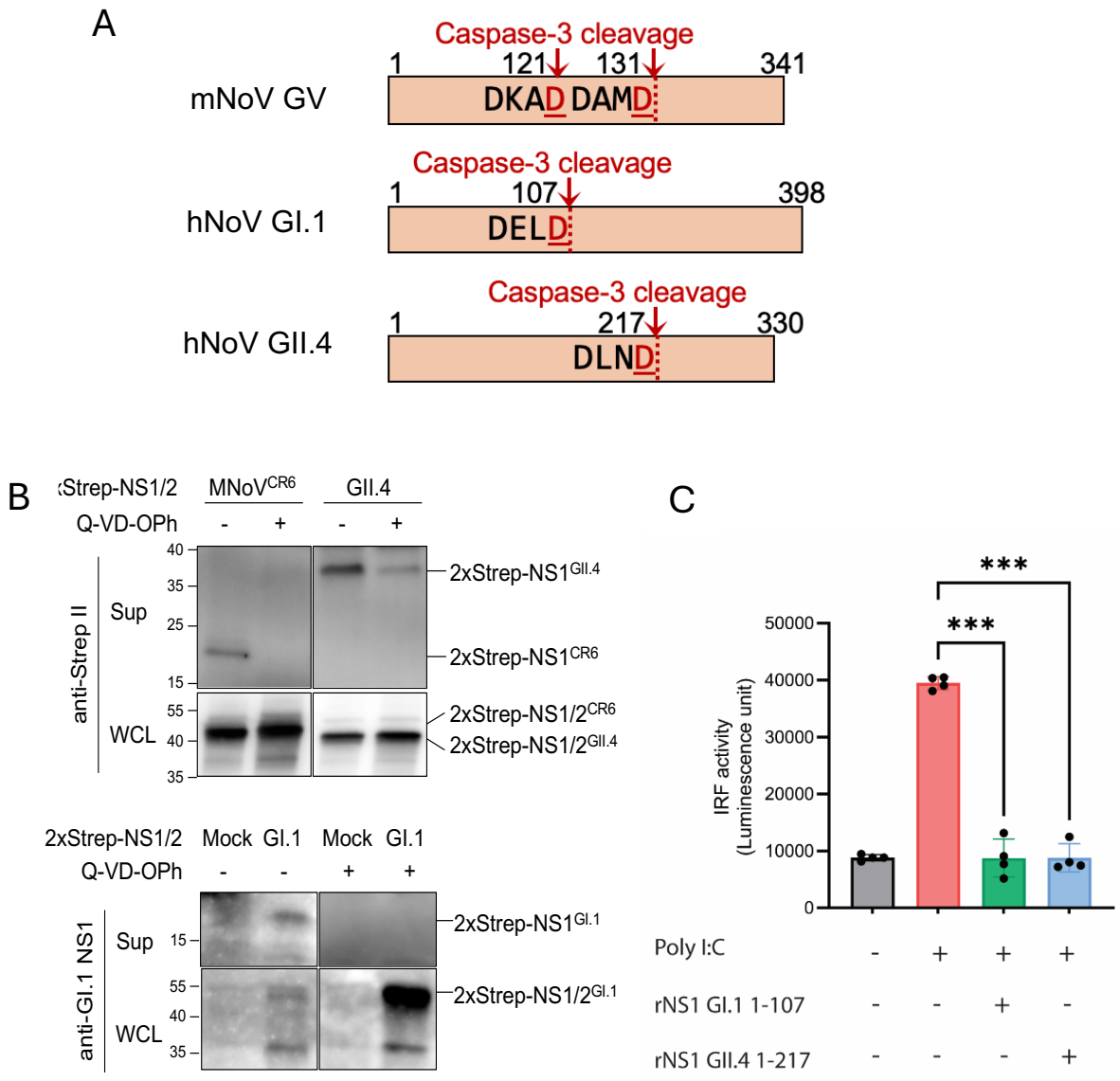

Figure 3: GI.1 and GII.4 NS1 proteins retain IRF suppressive activity following caspase-associated processing. (**A**) Schematic of NS1/2 proteins from MNoV CR6, HNoV GI.1, and HNoV GII.4, indicating predicted caspase-3 cleavage motifs and total protein lengths. (**B**) Immunoblot of 2xStrep-NS1/2-expressing cells treated with or without the pan-caspase inhibitor Q-VD-OPh. Supernatant (Sup) and whole cell lysate (WCL) fractions were probed with anti-Strep II (MNoV CR6 and HNoV GII.4, top) or anti-

GI.1 NS1 (HNoV GI.1, bottom) antibodies. Molecular weights (kDa) and cleavage products are indicated. (**C**) IRF activity was measured in HEK293T cells transfected with LMW-Poly(I:C) and treated with recombinant NS1 constructs. GI.1 NS1 (aa 1–107) and GII.4 NS1 (aa 1–217) at 30 µg/mL (+) alongside mock-infected controls. Data is presented as mean ± SD with individual replicates shown. *** $p < 0.001$.

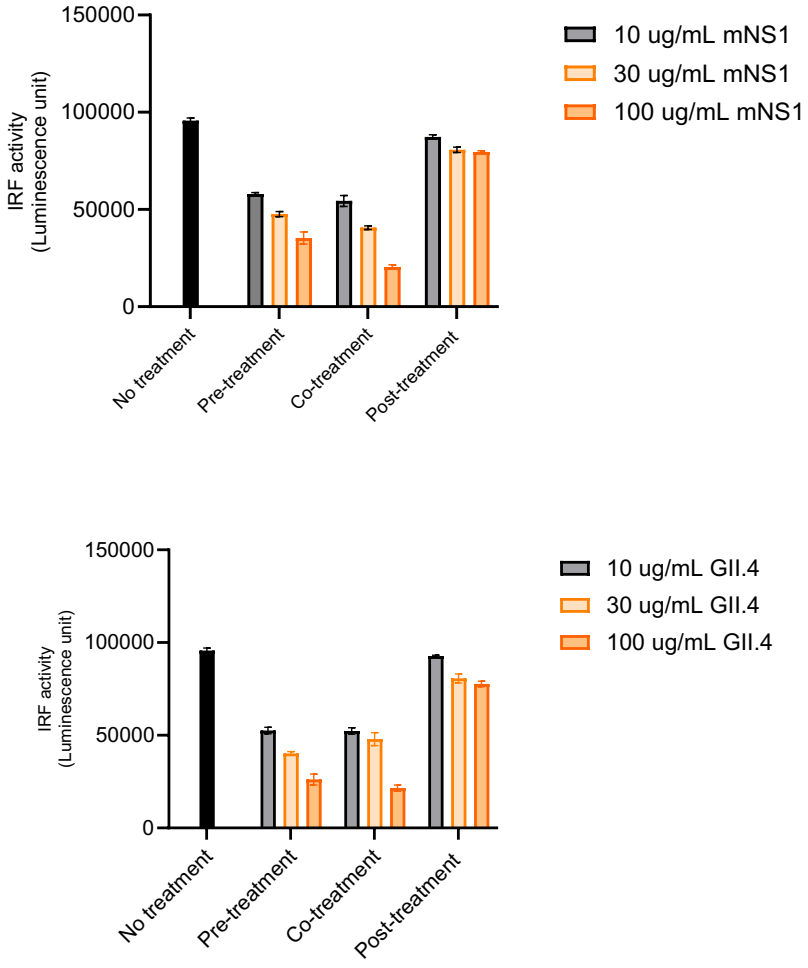

Figure 4: NS1-mediated suppression of IRF signaling is temporally restricted to early pathway activation. IRF activity was quantified in HEK293T cells transfected

with LMW-Poly(I:C) and treated with (top) murine NS1 (aa 1-121) or (bottom) GII.4 NS1 (aa 1-115) at the indicated concentrations under pre-, co-, or post-treatment conditions. at 10, 30, or 100 µg/ml, administered 3 hours before (pre-treatment), simultaneously with (co-treatment), or 3 hours after (post-treatment) LMW poly(I:C) transfection. Data are mean ± SD.

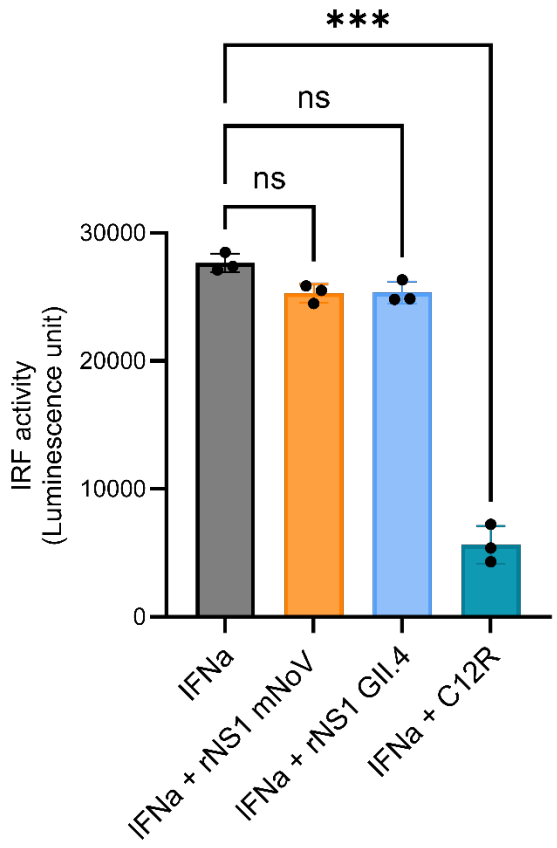

Figure 5: NS1 does not suppress IRF activation downstream of IFN-α signaling. IRF activity in HEK293T cells treated with exogenous IFN-α in the presence of the indicated murine NS1 nor GII.4 NS1 (100 µg/mL).The viral IFN-α decoy receptor C12R was included as a positive inhibitory control. Data are presented as mean ± SD with individual replicates shown. *** $p < 0.001$; ns, not significant.

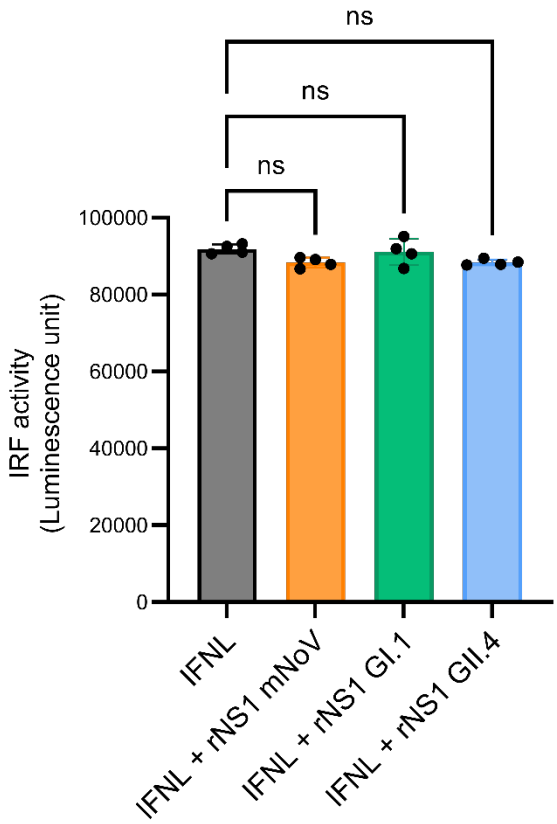

Figure 6: NS1 does not inhibit IRF activation downstream of IFN-λ signaling. IRF activity in HEK293T cells stimulated with exogenous IFN-λ and treated with murine NS1, GI.1 NS1, or GII.4 NS1 at 100 μg/ml. Data are mean ± s.d. with individual replicates shown. ns, not significant.

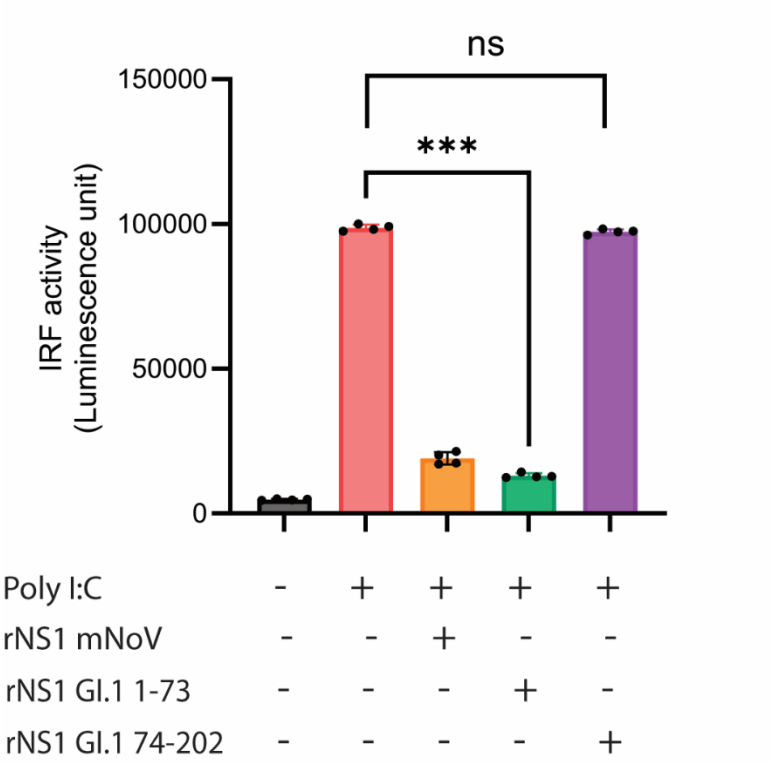

Figure 7: GI.1 NS1 N-terminal domain is necessary and sufficient for IRF suppression. IRF activity in HEK293T cells transfected with LMW poly(I:C) and treated with GI.1 NS1 N-terminal (aa 1-73) or C-terminal (aa 74-202) truncation constructs at 100 µg/ml (+). Murine NS1 (aa 1-121) was included as a positive control. Data are mean ± s.d. with individual replicates shown. Statistical significance compared to Poly(I:C) control: *** $p < 0.001$; ns, not significant.

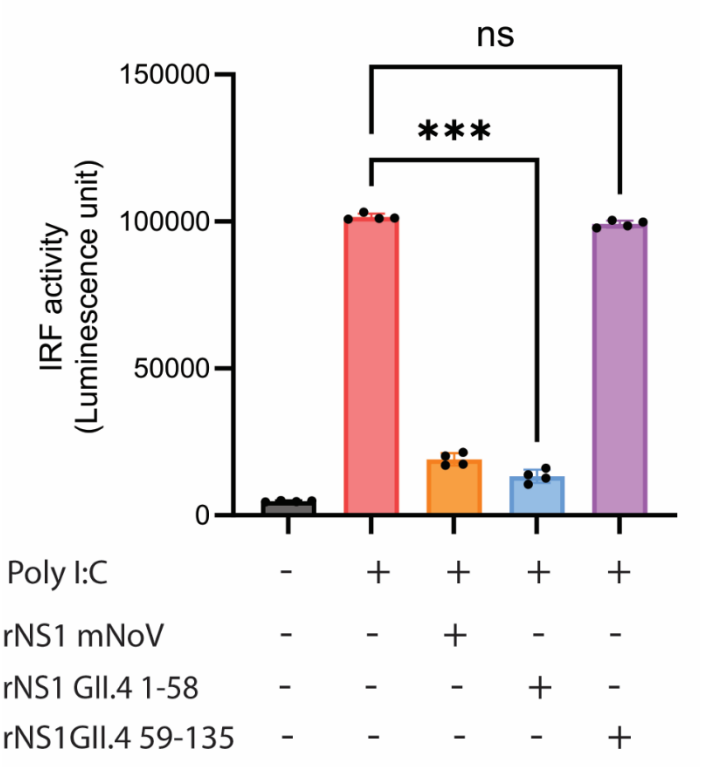

Figure 8: GII.4 NS1 N-terminal domain is necessary and sufficient for IRF suppression IRF activity in HEK293T cells transfected with LMW poly(I:C) and treated with GII.4 NS1 N-terminal (aa 1-58), C-terminal (aa 59-135) truncation constructs or Murine NS1 (aa 1-121) at 100 µg/ml (+). Data are mean ± s.d. with individual replicates shown. Statistical significance compared to Poly(I:C) control: *** $p < 0.001$; ns, not significant.

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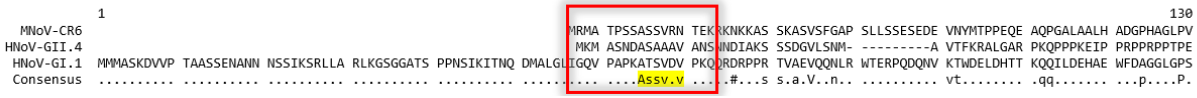

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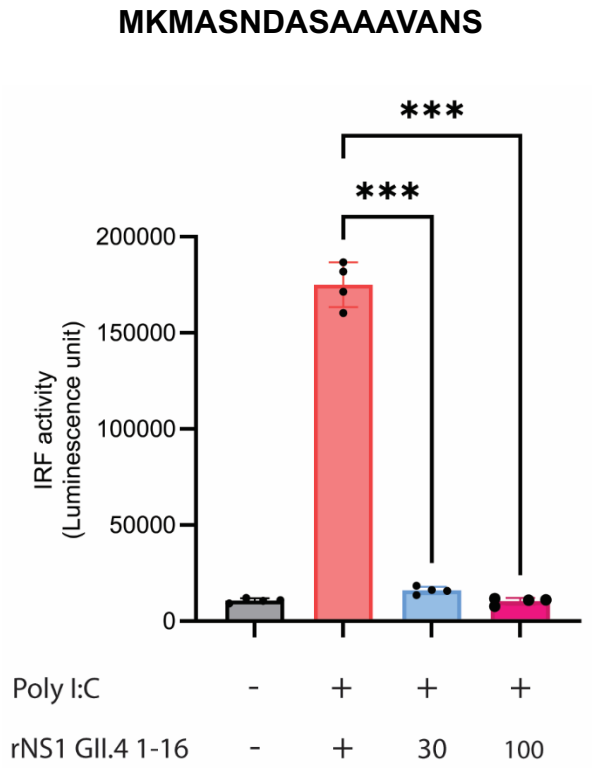

Figure 9: GII.4 NS1 minimal domain (aa 1-16) is sufficient for IRF suppression.

(**A**) Visual representation of the sequence lengths and alignment of NS1 proteins for GV, GII.4 and GI.1; consensus sequence is also included. **(B, top)** Exact 1-16 aa sequence of GII.4 NS1 used to make a construct with SUMO tag attached. **(B, bottom)** IRF activity was measured in HEK293T cells transfected with LMW-Poly(I:C) and

treated with GII.4 NS1 (aa 1-16)-SUMO at 30 μg/mL and 100 μg/mL. Data are presented as mean ± SD with individual replicates shown. *** $p < 0.001$.

Results

Full length hNoV NS1 display suppression of IRF activity

To investigate whether IRF suppression is conserved across human norovirus genotypes when compared to murine GV strain, we examined NS1 proteins from GI.1 and GII.4 strains using our model assay **(Figure 1).** The boundaries for these human NS1 constructs were defined based on prior functional mapping of murine NS1 (mNS1) and aimed to mimic the sequence length. To enable this direct comparison between the murine and human norovirus mechanisms, we used GI.1 (1-202) and GII.4 (1-135) constructs for our experimental study and our referred to internally as “full length”. HEK293T cells transfected with LMW Poly (I:C) show robust IRF activation in the absence of NS1. Treatment with recombinant His-tagged murine NS1 (residues 1-121) at 30 μg/mL and 100 μg/mL resulted in dose-dependent suppression of IRF activity relative to the mock transfection. This suppression phenotype is conserved across both human norovirus (hNoV) NS1 proteins tested: GI.1 (1-182) and GII.4 (1-135). With GI.1, there was approximately a 2-fold reduction at 100 μg/mL, and we observe nearly a 4-fold reduction when treated with GII.4 at the same concentration **(Figure 2)**. These findings indicate that hNoV NS1 proteins suppress the IRF activity like the murine NS1 protein (mNS1, 1-121) which was previously shown to exhibit such a phenotype.

Caspase-3 processing preserves NS1 suppressive function

Bioinformatic analysis predicted hNoV NS1 proteins to have Caspase-3 cleaved sites that are broadly conserved within each GI and GII genogroup **(Figure 3A).** To determine whether Caspase-3-mediated processing occurs in the context of NS1 secretion, we expressed 2xStrep-tagged NS1/2 proteins with or without pan-caspase inhibitor Q-VD-Oph and analyzed supernatants and whole cell lysates by immunoblot. Cleavage products were detected in the supernatants and abolished upon inhibitor treatment **(Figure 3B).** We tested Caspase-cleaved constructs, GI.1 (1-107) and GII.4 (1-217), post LMW Poly (I:C) transfection. Supernatant amount used to measure IRF activity in reporter cell lines was 2 µL. Both caspase-processed proteins retained IRF suppressive activity, with GI.1 showing approximately 3-fold reduction and GII.4 demonstrating 2.5-fold reduction in reporter activity compared to mock-treated cells (Figure 3C). These findings helped narrow down the hNoV NS1 proteins integral in suppressing the IRF activity.

Temporal requirements of NS1-mediated immune suppression:

To determine temporal and dose requirements for NS1-mediated IRF suppression, murine NS1 (10, 30, or 100 µg/mL) was administered 3 hours before, simultaneously with, or 3 hours after Poly(I:C) transfection. Both pre-treatment and co-treatment demonstrated robust dose-dependent suppression, with 100 µg/mL reducing IRF activity by 2 to 3-fold **(Figure 4, top).** Intermediate suppression occurred at 30 µg/mL, while 10 µg/mL showed minimal effect. Post-treatment failed to achieve suppression at any concentration, with activity remaining at control levels. We also aimed to explore this with the human NS1 strain. Human GII.4 NS1 displayed identical temporal kinetics,

with pre-treatment and co-treatment achieving comparable dose-dependent suppression; 100 µg/mL reduced IRF activity by approximately 4-fold, while post-treatment remained ineffective across all concentrations **(Figure 4, bottom).** These results demonstrate that NS1 has optimum performance during or prior to pathway initiation to achieve dose-dependent suppression, indicating potential targeting of early, saturable steps in IRF activation and the conservation of this across both murine and human NS1 strains indicates a potential fundamental mechanism of norovirus immune evasion. This indicates that NS1 seems to act before the proficient production of interferons.

NS1 proteins do not function as interferon decoy receptors

We wanted to next investigate whether NS1 proteins suppress cytokine sequestration or rather directly target the signaling pathway, we tested their ability to neutralize exogenous IFN- α. HEK293T cells were treated with recombinant IFN-α displayed robust reporter activation that remained unchanged in the presence of either murine NS1 or human GII.4 NS1 (100 µg/mL) **(Figure 5).** All IFN-α treatment conditions produced comparable activities, indicating NS1 proteins do not sequester extracellular interferon. In contrast when tested with the C12R protein, a well-characterized IFN-α decoy receptor[57], we see dramatically reduced IFN-α-stimulated activity, confirming assay sensitivity for cytokine neutralization. Similar results were observed with IFN-λ stimulation, where murine NS1, GI.1 NS1, and GII.4 NS1 failed to reduce IFN-λ-induced reporter activity **(Figure 6).** The inability of NS1 proteins to neutralize either type I or type III interferons confirms that norovirus immune suppression via the NS1 proteins

potentially occurs through direct intracellular pathway targeting rather than extracellular cytokine sequestration.

N-terminal domain of NS1 is sufficient for immune suppression

Previous work done in the lab was able to identify the critical region of the mNoV NS1 required to result in IRF activity suppression (data not shown). Now, the aim was to follow a similar plan. To map functional domains responsible for IRF suppression, we generated truncation constructs for both genotypes. GI.1 NS1 was divided into a N-terminal domain (1-73) and a C-terminal domain (74-202). At 100 µg/mL, the N-terminal domain recapitulated the suppression phenotype observed with full-length protein as well as the mNS1, displaying nearly 5-fold lower IRF activity compared to C-terminal domain treatment, which showed close to no suppression **(Figure 7).** Similarly, GII.4 NS1 N-terminal (aa 1-58) and C-terminal (aa 59-135) domains were tested. The N-terminal domain produced approximately 5-fold decrease in IRF activity relative to mock-treated cells, while the C-terminal domain showed no suppressive effect **(Figure 8).** This helps us further narrow down the required domain that helps the virus suppress immune responses.

Minimal amino acid region of NS1 is found sufficient for immune suppression

While sequence homology is poorly maintained across the strains, it is important to note that the murine GV strain more closely resembles the GII strain than the GI strain does to either of them. While the GII and GV strain have similar amino acid start positions at the N-terminal (GV has one extra base in the front), GI has an overhanging sequence over the latter two **(Figure 9A).** Analysis of amino acid sequence conservation between GII.4 and murine norovirus identified the 1-16 amino acid region as having the most

significant overlap with the murine counterpart. A construct with a SUMO-tag attached was generated and tested for functional activity. Cells transfected with LMW Poly(I:C) showed a near 10-fold reduction in IRF activity when treated at 100 and 30 μg/mL as compared to the mock infected cells **(Figure 9B).** These findings indicated that this small domain of the GII.4 NS1 strain was sufficient for complete IRF suppression.

Discussion

Limitations of Study

While the work presented here provides new insight into the role of human norovirus NS1 in suppressing IRF-mediated signaling, several limitations should be acknowledged. First, our experimental system relied on the delivery and overexpression of recombinant NS1 proteins rather than the use of a live viral infection model. Although this approach enabled precise control over protein dosage and facilitated mechanistic interrogation, it does not fully recapitulate the temporal dynamics, protein stoichiometry, or post-translational modifications that occur during authentic norovirus infection. In addition, variability in protein purification yields introduced challenges in maintaining strictly equivalent treatment conditions across experiments.

Second, the use of low molecular weight poly(I:C) as a surrogate for viral RNA, while effective in activating RIG-I-like receptor signaling, does not fully capture the complexity of norovirus-derived RNA species or the spatial and temporal context of viral replication intermediates. As such, the signaling responses observed here may not entirely reflect those elicited during natural infection.

Finally, all experiments were performed in vitro, limiting our ability to assess the physiological relevance of NS1-mediated immune suppression in the context of an intact organism. How NS1 activity influences viral replication, immune evasion, and disease progression in vivo, where additional factors such as tissue architecture, immune cell interactions, and cytokine gradients play critical roles should be explored in detail. Together, these limitations define a clear path forward, emphasizing the necessity of validating NS1 function in physiologically relevant infection models to establish its role in shaping norovirus pathogenesis.

Significance and Implications

Over the past few years, RNA viruses and host innate immunity have intertwined and co-evolved to get the better of each other. This has resulted in sophisticated viral countermeasures that target critical nodes of antiviral signaling cascades. Our investigation of human norovirus (hNoV) NS1-mediated immune evasion reveals that immune suppressive activity is preserved across phylogenetically divergent genotypes despite substantial sequence variation. These findings illuminate fundamental principles governing virus-host coevolution and establish NS1 as an exemplar of precision-engineered viral antagonism of pattern recognition receptor signaling.

Using a reductionist reporter-based system we observed the phenotype of NS1-mediated IRF suppression across taxonomically distinct norovirus genogroups, representing a compelling case study in functional, constraint-driven molecular evolution. High mutation rates and quasispecies dynamics are defining features of RNA

virus evolution and contribute to sequence variation during adaptation[58,59]. Against this background, the preservation of IRF antagonistic activity across GI.1, GII.4, and murine genotypes in our study suggests strong functional constraint on this immunosuppressive property. This conservation pattern parallels observations in other RNA virus families, including the NS1 proteins of influenza A viruses and the V proteins of measles virus, where antiviral suppression capabilities are maintained despite antigenic diversification[60,61]. Our results identify and strengthen the notion that hNoV NS1 is a dedicated immune antagonist and employs a role analogous but mechanistically different to antagonistic factors in other RNA viruses like influenza A and its NS1 proteins. It is important to reiterate that NS1's suppressive effect seen in all our assays was highly pathway specific. Previous work in lab showed that NS1 blunted interferon signaling initiated using a MDA5 specific agonist like HMW Poly (I:C) whereas exclusive stimulation of the RIG-I pathway remained largely unaffected (data not shown).

Our optimization experiments further highlight the temporal constraints governing NS1-mediated immune suppression and reveal mechanistic features that distinguish this strategy from post-transcriptional or post-translational regulation. What seemingly appears to be necessary, the requirement for NS1 presence during pathway initiation, coupled with the complete inefficacy of post-stimulus treatment, strongly implicates targeting of proximal signaling events within the RLR cascade. Similar mechanistic strategies have been described for viral antagonists that target early steps in antiviral signaling, including hepatitis C virus NS3/4A-mediated cleavage of MAVS and foot-and-mouth disease virus Lpro-mediated inhibition of interferon regulatory factors, supporting

convergent evolution toward disruption of proximal nodes in antiviral signal transduction[62,63]. This shared strategy across several viral species solidifies the targeting of upstream RLR pathway elements to be an optimal strategy to increase viral amplification potential. The requirement for NS1 presence prior to or during pathway activation suggests disruption of early, rate-limiting steps in antiviral signaling. The rapid kinetics of norovirus replication, with detectable viral proteins appearing within 4-6 hours post-infection[64], further supports the idea that NS1 is expressed within a critical temporal window that precedes full activation of host antiviral responses. From a therapeutic standpoint, this gives us further motivation to develop small-molecule inhibitors or biologics that block NS1's function or find a way to enhance MDA5 and other upstream elements of the signaling pathway, and control norovirus infections.

A key observation from our assays is that the NS1 proteins do not seem to directly abrogate or neutralize the interferons themselves. We observed that when treated along with exogenous IFN-α and IFN-λ, NS1 proteins from both murine and human strains were unable to neutralize the exogenously treated interferons. This provides further evidence for the hypothesis that the intracellular pathway is being directly targeted. This would distinguish norovirus immune evasion from the soluble cytokine-binding proteins employed by poxviruses which function as interferon decoy receptors to neutralize host interferons[65,66]. Instead, NS1 proteins from the norovirus strains we tested align more closely with viral proteins that modulate intracellular signaling pathways, including the NS1 and NS2 proteins of respiratory syncytial virus, which suppress IRF3 and NF-κB activation[67]. The selectivity of NS1 for IRF pathway suppression further distinguishes it

from broader viral immunomodulators such as vaccinia virus A46R, which targets multiple Toll-like receptor adaptor proteins to suppress diverse innate immune pathways[68], suggesting that norovirus NS1 has evolved a highly targeted mechanism of immune evasion optimized for disrupting antiviral signaling while preserving cellular functions required for viral replication. This would indicate that NS1 seems to play a role upstream of interferon and ISG expression and potentially targets the MDA5 oligomers or the MAVS complex.

The remarkable compaction of suppressive activity within a 16-amino acid GII.4 domain represents an extreme example of functional miniaturization in viral proteins. This peptide length approaches the lower limits required for stable protein-protein interactions and suggests that NS1 may function through mechanisms analogous to intrinsically disordered protein regions that adopt structured conformations upon target binding[69,70]. It will be interesting to see if the GII.4 NS1 has more than one such compact domain that can suppress activity, and ongoing efforts in lab aim to further explore this avenue and identify further functional domains or claim exclusivity of the 1-16 aa domain.

Our findings contribute to the growing understanding of type III interferon's dominant role in mucosal antiviral defense and highlight why noroviruses have evolved specific mechanisms to counteract this pathway. The preferential targeting of IRF signaling by NS1 is consistent with the critical role of type III interferons in controlling norovirus infections at intestinal epithelial surfaces. Discovering a modular function of specific hNoV NS1 domain opens new avenues to explore the development of novel antiviral

interventions. This brings about a change in the way noroviruses were seen so far; they were always thought to have minimum ability in manipulating and disarming the host's interferon response unlike the likes of flaviviruses and paramyxoviruses. This discovery will undoubtedly increase urgency in the pursuit of therapeutic discoveries to tackle viral infections. The clinical urgency is particularly acute for immunocompromised populations, where chronic norovirus infections represent a growing healthcare challenge with limited therapeutic options[6,12]. Recent clinical studies highlight the limited and inconsistent efficacy of current therapeutic approaches, including ribavirin and immunoglobulin, in the management of chronic norovirus infection in immunocompromised patients[71]. The identification of functional domains in our study can be translated into viable targets for drug design. Small molecules or peptides that mimic region and can overcome solubility issues would be helpful as they could be used to dampen excessive interferon responses in autoimmune conditions or on the contrary block the NS1-MDA5 interaction during norovirus infection and restore normal antiviral functioning. This work expands the current understanding of viral immune evasion mechanisms by identifying a potentially novel viral evasion mechanism and makes significant contributions to the field of immunology and virology. Studying the interplay between NS1 and host mechanisms will not only help in the advancement of norovirus study but also aid in the broader fields of host-pathogen interactions and signaling pathway studies.

Future Directions

Our discovery of NS1 immune evasive ability opens the door to several exciting and important avenues to explore and further understand the underlying mechanisms and relevance of this phenotype. A central priority for future investigations will be to identify and define the host factors that mediate NS1-dependent suppression of antiviral signaling and to determine whether these interactions differ or conserved between the full length NS1 proteins we used and the further minimal suppressive domains identified in this study. Our work hinted at NS1 activity being temporally specific, wherein suppression seems to be observed only when the protein is present during or prior to pathway activation. This would implicate that the components of proximal signaling events within the MDA5-MAVS axis might be the viable targets for NS1. However, as our results were only part of an initial optimization run, it is still plausible that NS1 interacts with a broader set of host factors that collectively regulate immune signaling, intracellular localization, and protein trafficking.

To address this, we suggest a systematic mapping of NS1-host interactions. A comparative approach in which our full-length NS1, suppressive truncation constructs, and the minimal functional domain are independently used as baits in affinity purification-mass spectrometry (AP-MS) or proximity-labeling experiments. This would allow direct assessment of whether a shared or distinct set of host proteins underlies the observed suppressive phenotype. This would help in identifying whether all our proteins and truncation mutants interact and share a common interaction partner and would suggest a core inhibitory interface that mediates suppression. While this is more likely, there is also the chance of the existence of different binding profiles that would

suggest that NS1 functions through domain-specific interactions, where each region could play several key antagonizing roles.

Based on the findings from our search for candidate host factors, target validation would be a viable next step. We suggest doing so by using co-immunoprecipitation assays using tagged NS1. If it is found that NS1 directly interacts with the components of the RLR signaling cascade, its effects can be mapped onto discrete and key pathway activation steps, like MDA5 oligomerization and filament assembly, MAVS activation, recruitment of TBK1 or IRF3 phosphorylation. There is an alternate possibility that NS1 associates with membrane-associated or trafficking proteins, which would suggest a model in which immune suppression is achieved indirectly through the disruption of signaling complex organization or localization rather than direct inhibition of a single signaling adaptor. Live-cell imaging of signaling complex assembly could be one technique to see if this theory is more applicable in the case of NS1.

A key next step will be to determine whether full-length NS1 and the minimal suppressive domain engage the same host target. Shared interactions would indicate that the minimal domain constitutes the core inhibitory interface, with the remaining regions contributing to stability or localization. In contrast, divergence in interaction profiles despite preserved suppressive activity would suggest that NS1 functions through multiple, modular domains. If additional functional regions are identified, domain-resolved interaction mapping will be required to assign specific targets and

mechanisms to each region. Conversely, if suppression is fully captured by a single minimal domain, future work can focus on defining the inhibitory interface at residue-level resolution through systematic mutagenesis. To keep exploring using our in vitro assay, scrambling the key domain, specifically the 16-amino acid region would be interesting as it would give us a better insight into the specificity of this suppression phenotype.

Recent findings show that in murine norovirus infection, NS1 secretion depends on caspase-3 processing and NINJ1-dependent pathway, supporting the idea that released NS1 could act beyond the cell[72]. Although our data presented for hNoV NS1 favor a predominantly intracellular mechanism, it remains plausible that extracellular NS1 engages a surface binding partner on neighboring epithelial cells to modulate antiviral signaling in a paracrine manner. This could be tested first by measuring binding of tagged recombinant NS1 to intact cells by flow cytometry and microscopy and then by determining whether bound NS1 alters facets like IRF3 phosphorylation or ISG induction in recipient cells. Candidate surface receptors could be identified by crosslinking-based proteomics or by a surfaceome CRISPR activation screen in which activation of plasma membrane protein genes in poorly responsive cells is coupled to sorting for gained NS1 binding or signaling responsiveness. This will help further explore the path and mechanism NS1 follows to exert its immunosuppressive effects within infected cells and tissues.

In parallel with mechanistic studies, an important gap in the field that warrants further investigation is the discovery of host receptors governing human norovirus strains entry.

Whereas murine norovirus uses the proteinaceous receptor CD300lf, the corresponding receptor or receptor complex for human norovirus remains incompletely defined. Future studies should combine unbiased CRISPR-based loss-of-function screening, gain-of-function receptor expression approaches, and staged attachment-versus-entry assays in permissive human intestinal models to distinguish true entry receptors from attachment factors. Integration of these approaches with VLP-based pulldown proteomics and validation in human intestinal enteroids should provide a tractable path toward defining the host determinants of human norovirus tropism and entry competence. Integration of receptor biology with NS1 function may provide additional insight into how early stages of viral entry and subsequent immune evasion are coordinated.

Finally, it will be essential to validate these mechanisms in physiologically relevant systems. Human intestinal enteroids provide a model that more closely recapitulates the cellular composition and immune environment of the intestinal epithelium, where noroviruses establish infection. Although technically challenging, the practice of enteroid culturing can preserve the cellular diversity, polarity, and differentiation state of the intestinal epithelium, including enterocytes, goblet cells, and other specialized cell types makes them the most relevant in vitro model currently available. Infection assays in these systems will be necessary to determine whether the host factors identified in reductionist systems are indeed responsible for NS1-mediated immune suppression in vivo. Recombinant GI.1 and GII.4 NS1 proteins, as well as their suppressive truncation constructs, can be applied to differentiated enteroid monolayers before or during stimulation with Poly (I:C) or interferon-inducing agonists to assess their effects on IRF

activation, IFN-λ production, and ISG induction. These systems also make it possible to examine whether NS1-mediated suppression varies across epithelial cell states or differentiated populations, including tuft-cell-enriched cultures, thereby addressing whether the activity of NS1 is linked to the specialized cellular niches that support norovirus infection. Together, such experiments will bridge mechanistic insight with physiological relevance and determine whether the immune evasion phenotypes observed with human norovirus NS1 proteins in simplified reporter systems are preserved in the epithelial environment in which infection is established.

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