# Development of antivirals against norovirus

## linking the bench to the bedside

**Wen Dang**

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The studies presented in this thesis were performed at the Laboratory of Gastroenterology and Hepatology, Erasmus MC-University Medical Center Rotterdam, the Netherlands.

The research was funded by:

- KWF (Dutch Cancer Society) Young Investigator Grant
- Dutch Digestive Foundation (MLDS)
- China Scholarship Council



Cover design: Siluda Advertising Agency, Zhangye, China.
Layout design: the author of this thesis.

Printed by: Ridderprint BV, Ridderkerk, the Netherlands

ISBN: 978-94-6375-096-7

# Development of antivirals against norovirus: linking the bench to the bedside

Ontwikkeling van nieuwe antivirale middelen tegen het norovirus: een verbinding tussen de laboratoriumtafel en het ziektebed

# Thesis

**to obtain the degree of Doctor from the**
**Erasmus University Rotterdam**
**by command of the**
**rector magnificus**

Prof.dr. R.C.M.E. Engels

**and in accordance with the decision of the Doctorate Board**

**The public defense shall be held on**

*Wednesday 12th September 2018 at 9:30*

**by**


## Wen Dang

born in Zhangye, Gansu Province, China


Erasmus University Rotterdam

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**Promoter:**

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**Inner Committee:**

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# Chapter 1

## General Introduction and Outline of This Thesis

Norovirus, a member of the family *Caliciviridae* is a non-enveloped, icosahedral and single-stranded RNA virus with a genome size of approximately 7.5 kilobase (kb). Based on the amino acid sequence comparison of its constituting structural protein virus protein (VP) 1, the genus norovirus is divided into 7 genogroups (G) with GI, II and IV being primarily responsible for infecting humans. During the last two decades, the genogroup II member genotype 4 (GII.4) strain has been responsible for the majority of food-borne outbreaks worldwide [1]. Recently, however, norovirus GII.17 variant has been emerging and even has become predominant in some regions of Asia [2]. In this thesis I aimed to increase our insight into norovirus-provoked pathology.

Currently norovirus is increasingly recognized as the main cause of epidemic nonbacterial gastroenteritis worldwide, especially so since rotavirus vaccines have largely reduced the disease burden associated with infection with the latter virus [3]. Despite being associated with substantial (or even large) economic impact, in addition to considerable mortality, norovirus has been given comparatively less attention when compared to other infectious pathogens. Due to high genetic diversity observed in different noroviruses and the fact that patients that have been sequentially infected with different strains fail to develop cross-protection among distinct genotypes, the development of efficacious norovirus vaccines is still a major challenge [4]. Until now, no licensed antiviral drugs with respect to norovirus are available and clinical management is mainly limited to supportive care with oral or intravenous (IV) rehydration and electrolyte supplementation being the mainstay of treatment [5]. The lack of a robust cell culture model or a convenient small animal model has further hampered the development of strategies aimed at prevention and control of norovirus infection. Limited case reports have demonstrated that ribavirin and nitazoxanide hold promise for combating norovirus-provoked gastroenteritis, yet their therapeutic value and mechanism-of-action need to be further explored.

## *Discovery of norovirus*

In 1929, Zahorsky *et al* first described an epidemic of apparently nonbacterial gastroenteritis and proposed the descriptive name ‘winter vomiting disease’, as this entity was characterized by clinical presentation of acute onset of nausea and vomiting predominantly during winter months [6]. In October 1968, an acute gastroenteritis affected 50% (116 of 232) of the students and teachers in an elementary school in Norwalk, Ohio and

was clinically accompanied by characteristic nausea, vomiting and abdominal pain [7]. Later in 1972, Kapikian *et al* first observed a 27-nm particle with cubic symmetry by immune electro microscopy (IEM) in stool samples derived from the Norwalk outbreak [8]. These particles were then suggested to be the etiological cause of Norwalk gastroenteritis and are now considered the initial discovery of norovirus. But until now, many aspects of norovirus pathobiology remain obscure at best.

## *Clinical features of norovirus infection*

Human norovirus (HuNV) is notorious for being the main cause of gastroenteritis outbreaks worldwide. As a food-borne illness, norovirus infection is predominantly transmitted by the fecal-oral route and through person-to-person contact. Inhalation of infectious aerosols is another possible mode of transmission [9]. Following exposure and after an incubation time of 15 to 36 hours, an acute diarrhea develops which is accompanied by vomiting, abdominal cramps, watery stools and fever. Patients normally resolve the disease within 2 to 3 days, but sometimes patients can persistently and asymptomatically shed virus for up to approximately 8 weeks [10]. Furthermore, chronic and protracted norovirus infection is sometimes also observed especially among young children, the elderly and immunocompromised individuals. Norovirus caused 47 to 96% of acute pediatric gastroenteritis outbreaks and 5 to 36% of sporadic cases [11]. Manish *et al* estimated that each year norovirus resulted in 64,000 hospital admissions and 900,000 polyclinical visits in children from developed countries, and up to 200,000 fatalities of children younger than 5 years old in developing countries [12]. The course of norovirus-mediated disease in older adults is characterized by prolonged duration and fatal outcomes [13]. In my systematic review (**Chapter 2**), I have demonstrated that norovirus infection may persist for weeks to months in hematopoietic stem cell transplant (HSCT) and solid organ transplant (SOT) recipients, and sometimes is accompanied by severe complications that required hospital admission.

## *Features of norovirus biology*

Norovirus demonstrates largely antigenic and genetic diversity. Based on the amino acid sequence of the major capsid protein VP1, norovirus is currently divided into 7 genogroups (G), whereas each genogroup is further subdivided into several genotypes (numbers after G). Genogroup I, II and IV primarily infect humans, and genogroup V is

associated with murine diarrhea [14]. The HuNV genome contains three open reading frames (ORFs), of which ORF1 encodes a nonstructural protein, while ORF2 and ORF3 encode the major and minor capsid protein VP1 and VP2, respectively. The 5' end of norovirus RNA is covalently bound to a virus-encoded protein referred to as VPg, whilst the 3' end is polyadenylated. Gaining understanding of the full life cycle of HuNV had remained largely elusive due to the inability to perform *in vitro* culture of HuNV [15]. Evidence from surrogate models of other animal caliciviruses and murine norovirus (MNV) suggests that a norovirus VPg-bound intact RNA initiates a 'pioneer round' of RNA translation once it enters into a permissive cell line. This leads to the production of the ORF1 polyprotein, which is subsequently co- and post-translationally processed into 6 nonstructural (NS) proteins, namely p48, NTPase, p22, VPg, Pro and Pol. The remaining viral RNAs form a double-stranded replicative form (RF), which further produces positive-sense genomic and subgenomic RNAs in a VPg-dependent manner [16]. The synthesized genomic and subgenomic RNAs are covalently linked to VPg protein at 5' end. The translated structural proteins VP1 and VP2 as well as genomic RNAs are further assembled, encapsulated and released from host cells [15]. However, it is fair to say that further validation and detailing of this proposed life cycle remains necessary.

## *Models to study HuNV*

Although a virus particle was identified as the main cause of gastroenteritis, all attempts to generate and detect the etiological agent using tissue culture techniques and novel organ culture techniques were unsuccessful hitherto [17]. In 2004, Duizer *et al* described the attempt to cultivate HuNV *in vitro* using 16 human carcinoma derived cell lines and 11 non-human hosts derived cell lines. Cell culture systems that mimicked the gastrointestinal tract (GI tract) were also included. However, all such attempts proved to be unsuccessful [18]. In another study, efforts were further made to study the susceptibility of cell cultures of animal origin to HuNV infection. A total of 19 cell cultures from 11 different animal species were inoculated with HuNV-containing fecal samples. Cytopathic effect (CPE) and reverse transcription PCR (RT-PCR) assays were used to detect norovirus replication. The results showed no evidence of any morphological changes or an increase in norovirus RNA [19]. In 2006, Chang *et al* revolutionized the field by pioneering the stable expression of a HuNV RNA replicon in the Huh7 cell line. Even though this replicon model fails to fully

recapitulate the full life cycle of HuNV, it is a superior alternative to the experimental models used earlier for the screening of antivirals for their potential action on HuNV replication [20]. By using this model, a set of substances with anti-norovirus activities was identified and this set included interferons (IFNs), ribavirin, mycophenolic acid (MPA), calcineurin inhibitors and 2'-C-methylcytidine (2CMC) [21-23]. Some further advances were recently made. In 2014, the currently dominant HuNV strain GII.4-Sydney was employed for successful infection of the BJAB human B cell line as evident from a resulting significant increase in viral genomic copy numbers. Importantly, this infection was substantially facilitated by free histo-blood group antigen (HBGA) or by HBGA-expressing enteric bacteria, which probably served as a cofactor for binding and attachment of HuNV to B cells [24]. Later in 2016, the novel human intestinal enteroids (HIEs) derived from intestinal crypts were reported to support HuNV replication. HIEs contained multiple intestinal epithelial cell types and recapitulated most aspects of the human intestinal epithelium, and thus constituted a good culture system to study host-pathogen interactions. In this system, bile was required for strain-dependent HuNV replication, in contrast to the previous report that enteric bacteria was essential for HuNV cultivation in B cells [25]. Hence, further research is needed to fully understand how norovirus can exploit gastrointestinal physiology for successful replication.

Further efforts were also made to develop a robust animal model. In 2006, gnotobiotic (Gn) pigs were evaluated as a potential model to study HuNV pathogenesis and to determine the target cells for HuNV replication. The inoculated Gn pigs developed mild diarrhea and were found to be positive for HuNV genome RNA in rectal swab fluids and intestinal contents. Meanwhile, enterocytes from duodenal and jejunal origin were confirmed to specifically support HuNV replication [26]. A panel of drugs including simvastatin [27, 28] and interferon alpha (IFN$\alpha$) [28] effectively inhibited HuNV replication using this model. Later in 2008, the same group reported that Gn calves developed diarrhea and intestinal lesions upon inoculation with HuNV strain GII.4-HS66. The inoculated calves secreted virus particles in the feces and produced virus-directed antibodies as well as cytokines indicative of ongoing infection and immune reaction [29]. In 2010, chimpanzees i.v. inoculated with HuNV demonstrated no clinical symptoms of gastroenteritis, but shed virus particles in feces. Concurrent with viral shedding in the stools, HuNV RNA became detectable in intestinal and liver biopsies. Analysis of the HuNV-evoked immune response revealed that the chimpanzees developed short-term immunity against HuNV and virus like particles (VLPs),

and displayed protective immunity even after extended exposure [30]. These models have played important roles on fostering our understanding of the pathogenesis of and the immunity against HuNV. Meanwhile, they are useful tools to assess the efficacy of potential interventions aimed at preventing and treating HuNV infection.

## *Murine norovirus (MNV), a model system to study norovirus biology and pathogenesis*

Noroviruses are not restricted to infecting humans. Noroviruses in cattle, swine and mice have also been identified. Among them, MNV is the only norovirus that is permissive for replication in both cell culture and a small animal model. Thus, MNV is useful for studying norovirus biology and associated pathology. The first MNV was described in 2003. It was observed that immunocompromised mice deficient in signal transducer and activator of transcription 1 (STAT1) and recombination-activating gene 2 (RAG2) (RAG/STAT1$^{-/-}$ mice) sporadically succumbed to an infectious nonbacterial agent. Representational difference analysis (RDA) revealed that the pathogen contained an RNA genome homologous to the regions of many calicivirus genomes. It was proposed to name the agent as murine norovirus 1 (MNV1) and place it into a new norovirus genogroup. Further studies demonstrated that MNV1 replicated more efficiently in mice lacking STAT1 than wild type mice, indicating the essential role of innate immunity on combating MNV infections [31].

To further investigate the cellular tropism of MNV, MNV1-infectd STAT1$^{-/-}$ mice underwent immunohistochemistry aimed at confirming the presence of MNV1 protein. MNV1-specific staining was observed in the Kupffer cells residing in the liver, and in the red pulp as well as the marginal zone in the spleen. As expected, bone marrow-derived macrophages (M*φ*) and dendritic cells (DCs) were permissive for MNV1 replication *in vitro*. Further screening in M*φ* cell lines demonstrated that the murine cell line RAW 264.7 supported MNV1 replication yielding visible CPE after infection [32].

Further clonal selection performed both *in vivo* and *in vitro* generated two MNV strains MNV1.CW1 and MNV1.CW3. The former resembled the parental MNV1 with attenuated infectivity in STAT1$^{-/-}$ mice; while the latter demonstrated more significant virulence but a similar growth rate *in vitro* [33]. Clones MNV1.CW1 and MNV1.CW3 only caused sporadic diarrhea and were rapidly cleared by wild-type mice. A new MNV strain MNV1.CR6 can persistently infect wild-type mice and was detected in feces even at day 35

after inoculation. Based on those findings, MNV is thought to be a good model system to study the biology and pathogenesis of norovirus.

## *Host immune response and action of the interferon pathway therein toward norovirus*

The knowledge of how a host responds to norovirus infection has mainly been gained from studies performed in volunteers, due to the lack of a convenient small animal model that sufficiently and effectively supports the natural growth of HuNV. Challenging volunteers with Norwalk agent-containing fecal filtrate provoked antibodies to the Norwalk agent, indicating a potentially important role of acquired immunity on protecting us against the Norwalk gastroenteritis [34]. Consistently, norovirus-associated travelers' diarrhea was associated with higher levels of interleukin (IL)-2 and interferon gamma (IFN*γ*), suggesting that a Th1 immune response was predominant in gut immunity combating norovirus infection [35]. Further studies also explored the therapeutic potential of exogenous IFNs against HuNV replication by using the HuNV replicon and Gn pig model [21, 28]. In the present study, I shall show that HuNV replication was highly sensitive to treatment with type I, II and III IFNs. My mechanistic investigations further identified interferon regulatory factor-1 (IRF-1), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) as the ultimate factors that were responsible for the potency of IFNs against norovirus [36].

Further understanding comes from studies aimed at identifying the predominant factors limiting MNV infection. RNA sequencing of MNV-infected murine macrophages revealed that MNV infection triggered a cellular immune response that involved nuclear factor kappa B (NF-κB), STAT1 and STAT3-based pathways as well as interferon regulatory factor 3 (IRF3) activation [37]. These observations correlated well with the fact that IFNs exerted *in vitro* and *in vivo* efficacy with respect to controlling MNV infection. Type I and II IFNs inhibited the *in vitro* translation of MNV proteins [38]. *In vivo* studies indicated that IFN-*αβ* limited the systemic spreading of the acute strain MNV1.CW3-provoked infection in immunocompetent mice [39], whereas interferon lambda (IFN*λ*) treatment can effectively and completely cure persistent MNV infection irrespective of the presence of adaptive immunity. Two weeks of IFN*λ* treatment reduced virus titers to an undetectable level in the murine mesenteric lymph nodes (MLN) and colon after infection with the persistent MNV

strain MNV1.CR6 [40]. These findings are important. They suggest that exogenous IFNs can be promising antiviral options during norovirus infection and that their effects can become useful for designing drug discovery strategies.

## *Scope of this thesis*

Even though norovirus gastroenteritis (NVGE) is normally self-limiting among immunocompetent individuals, it causes severe complications and fatal outcomes in immunocompromised patients. Hence novel and specific antiviral treatments are urgently needed. In this thesis, I aimed to adequately assess the burden of norovirus infection in hematopoietic stem cell transplant (HSCT) and solid organ transplant (SOT) recipients, and to further explore the potency and mechanism-of-action of several chemicals in this specific setting. I firstly systematically reviewed the prevalence and clinical presentation of NVGE in transplant recipients, and secondly assessed potential treatment strategies for those patients. I next mainly focused on the antiviral potential and the mechanisms of immunosuppressants, exogenous IFNs and nitazoxanide on norovirus biology and related results to clinical experience in NVGE.

## *Outline of this thesis*

As the immune system needs to be highly suppressed in order to prevent rejection, transplant recipients are at high risk of viral infections. Accordingly, diarrhea is a common complication after transplantation. The putative risk factors underlying diarrhea involve regimen-associated toxicity, infections and intestinal graft-versus-host disease (GVHD). Unfortunately norovirus was not routinely detected in those diarrheal patients. This may be due to clinical underestimation of the potential problem as well as insufficiency in studies about norovirus biology and pathogenesis, and the absence of convenient and cheap detection methodology. Presently the situation has improved and the introduction of highly sensitive molecular detection techniques now allows easy identification and characterization of norovirus as an etiological agent for diarrhea in those patients. In **Chapter 2**, I systematically reviewed norovirus infection in HSCT and SOT recipients, and was able to highlight the prevalence, clinical manifestations, diagnosis, risk factors, transmission and evolution, and potential treatments. Immunosuppressants are risk factors for norovirus infection. On the one hand, the weakened immunity thus fails to provide protection against viral invasion. On the other hand, immunosuppressants directly exert multiple effects on

viral replication. Thus, this is a complex discussion. In **Chapter 3**, I provided answers though. I profiled a subset of immunosuppressants and identified MPA as a potent antiviral toward norovirus replication. Using this medication for immunosuppression in transplant recipients should thus provide protection against norovirus infection.

IFNs provide host with the first line of defense against viral invasion. They signal through the Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway and induce hundreds of interferon-stimulated genes (ISGs), which are the ultimate antiviral factors. Given the fact that IFNs have been used in the clinic for decades, in **Chapter 4,** I characterized the antiviral potential of type I, II and III IFNs and found that HuNV possessed high responsiveness to all types of IFNs. I further comprehensively screened a subset of important ISGs by using an overexpression approach and identified IRF-1, RIG-I and MDA5 as potent antiviral effectors.

Nitazoxanide, a thiazolide compound has recently been empirically used in several norovirus cases with unknown mechanism requiring clarification. In **Chapter 5** and **6**, I thus further investigated the antiviral activities and mechanisms of nitazoxanide and its main metabolite tizoxanide against norovirus. The results extended our knowledge about mechanism and application of nitazoxanide in the clinic. An integrating discussion of data generated is also included in this thesis. In conjunction, I feel my studies greatly facilitate the development of novel rational avenues for treating norovirus infection.

# References


1. Esseili MA, Wang Q, Saif LJ. Binding of human GII.4 norovirus virus-like particles to carbohydrates of romaine lettuce leaf cell wall materials. *Appl Environ Microbiol* 2012; **78**: 786-94.
2. Chan MC, Lee N, Hung TN et al. Rapid emergence and predominance of a broadly recognizing and fast-evolving norovirus GII.17 variant in late 2014. *Nat Commun* 2015; **6**: 10061.
3. Kollaritsch H, Kundi M, Giaquinto C et al. Rotavirus vaccines: a story of success. *Clin Microbiol Infect* 2015; **21**: 735-43.
4. Bernstein DI, Atmar RL, Lyon GM et al. Norovirus vaccine against experimental human GII.4 virus illness: a challenge study in healthy adults. *J Infect Dis* 2015; **211**: 870-8.
5. Glass RI, Parashar UD, Estes MK. Norovirus gastroenteritis. *N Engl J Med* 2009; **361**: 1776-85.
6. Zahorsky J. Hyperemesis hiemis or the winter vomiting disease. *Arch Pediat* 1929; **46**: 391-95.
7. Adler JL, Zickl R. Winter vomiting disease. *J Infect Dis* 1969; **119**: 668-73.
8. Kapikian AZ, Wyatt RG, Dolin R et al. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *J Virol* 1972; **10**: 1075-81.
9. Gould D. Management of Norovirus gastroenteritis in the community. *Br J Community Nurs* 2009; **14**: 117, 9-21.
10. Atmar RL, Opekun AR, Gilger MA et al. Norwalk virus shedding after experimental human infection. *Emerg Infect Dis* 2008; **14**: 1553-7.
11. Esposito S, Ascolese B, Senatore L et al. Pediatric norovirus infection. *Eur J Clin Microbiol Infect Dis* 2014; **33**: 285-90.
12. Patel MM, Widdowson MA, Glass RI et al. Systematic literature review of role of noroviruses in sporadic gastroenteritis. *Emerg Infect Dis* 2008; **14**: 1224-31.
13. Cardemil CV, Parashar UD, Hall AJ. Norovirus Infection in Older Adults: Epidemiology, Risk Factors, and Opportunities for Prevention and Control. *Infect Dis Clin North Am* 2017; **31**: 839-70.
14. Karst SM, Wobus CE, Goodfellow IG et al. Advances in norovirus biology. *Cell Host Microbe* 2014; **15**: 668-80.
15. Thorne LG, Goodfellow IG. Norovirus gene expression and replication. *J Gen Virol* 2014; **95**: 278-91.
16. Rohayem J, Robel I, Jager K et al. Protein-primed and de novo initiation of RNA synthesis by norovirus 3Dpol. *J Virol* 2006; **80**: 7060-9.
17. Acute infectious nonbacterial gastroenteritis: etiology and pathogenesis. *Ann Intern Med* 1972; **76**: 993-1008.
18. Duizer E, Schwab KJ, Neill FH et al. Laboratory efforts to cultivate noroviruses. *J Gen Virol* 2004; **85**: 79-87.
19. Malik YS, Maherchandani S, Allwood PB et al. Evaluation of animal origin cell cultures for in vitro cultivation of noroviruses. *J Appl Res Med* 2005; **5**: 312-7.
20. Chang KO, Sosnovtsev SV, Belliot G et al. Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line. *Virology* 2006; **353**: 463-73.
21. Chang KO, George DW. Interferons and ribavirin effectively inhibit Norwalk virus replication in replicon-bearing cells. *J Virol* 2007; **81**: 12111-8.
22. Dang W, Yin Y, Wang Y et al. Inhibition of Calcineurin or IMP Dehydrogenase Exerts Moderate to Potent Antiviral Activity against Norovirus Replication. *Antimicrob Agents Chemother* 2017; **61**.
23. Rocha-Pereira J, Jochmans D, Debing Y et al. The viral polymerase inhibitor 2'-C-methylcytidine inhibits Norwalk virus replication and protects against norovirus-induced diarrhea and mortality in a mouse model. *J Virol* 2013; **87**: 11798-805.

24. Jones MK, Watanabe M, Zhu S et al. Enteric bacteria promote human and mouse norovirus infection of B cells. *Science* 2014; **346**: 755-9.

25. Ettayebi K, Crawford SE, Murakami K et al. Replication of human noroviruses in stem cell-derived human enteroids. *Science* 2016; **353**: 1387-93.

26. Cheetham S, Souza M, Meulia T et al. Pathogenesis of a genogroup II human norovirus in gnotobiotic pigs. *J Virol* 2006; **80**: 10372-81.

27. Bui T, Kocher J, Li Y et al. Median infectious dose of human norovirus GII.4 in gnotobiotic pigs is decreased by simvastatin treatment and increased by age. *J Gen Virol* 2013; **94**: 2005-16.

28. Jung K, Wang Q, Kim Y et al. The effects of simvastatin or interferon-alpha on infectivity of human norovirus using a gnotobiotic pig model for the study of antivirals. *PLoS One* 2012; **7**: e41619.

29. Souza M, Azevedo MS, Jung K et al. Pathogenesis and immune responses in gnotobiotic calves after infection with the genogroup II.4-HS66 strain of human norovirus. *J Virol* 2008; **82**: 1777-86.

30. Bok K, Parra GI, Mitra T et al. Chimpanzees as an animal model for human norovirus infection and vaccine development. *Proc Natl Acad Sci U S A* 2011; **108**: 325-30.

31. Karst SM, Wobus CE, Lay M et al. STAT1-dependent innate immunity to a Norwalk-like virus. *Science* 2003; **299**: 1575-8.

32. Wobus CE, Karst SM, Thackray LB et al. Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. *PLoS Biol* 2004; **2**: e432.

33. Thackray LB, Wobus CE, Chachu KA et al. Murine noroviruses comprising a single genogroup exhibit biological diversity despite limited sequence divergence. *J Virol* 2007; **81**: 10460-73.

34. Parrino TA, Schreiber DS, Trier JS et al. Clinical immunity in acute gastroenteritis caused by Norwalk agent. *N Engl J Med* 1977; **297**: 86-9.

35. Ko G, Jiang ZD, Okhuysen PC et al. Fecal cytokines and markers of intestinal inflammation in international travelers with diarrhea due to Noroviruses. *J Med Virol* 2006; **78**: 825-8.

36. Dang W, Xu L, Yin Y et al. IRF-1, RIG-I and MDA5 display potent antiviral activities against norovirus coordinately induced by different types of interferons. *Antiviral Res* 2018; **155**: 48-59.

37. Levenson EA, Martens C, Kanakabandi K et al. The host response to murine norovirus infection induces significant engagement of IFN and TNF-a immunological programs. *J Immunol* 2017; **198**.

38. Changotra H, Jia Y, Moore TN et al. Type I and type II interferons inhibit the translation of murine norovirus proteins. *J Virol* 2009; **83**: 5683-92.

39. Hwang S, Maloney NS, Bruinsma MW et al. Nondegradative role of Atg5-Atg12/ Atg16L1 autophagy protein complex in antiviral activity of interferon gamma. *Cell Host Microbe* 2012; **11**: 397-409.

40. Nice TJ, Baldridge MT, McCune BT et al. Interferon-lambda cures persistent murine norovirus infection in the absence of adaptive immunity. *Science* 2015; **347**: 269-73.

# Chapter 2

## Norovirus infection in hematopoietic stem cell and solid organ transplant recipients: a systematic review

Wen Dang,[1] Peifa Yu,[1] Mohamad S. Hakim,[1] Wichor M. Bramer,[2] Maikel P. Peppelenbosch,[1] and Qiuwei Pan[1]

[1]Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam, Netherlands

[2]Medical Library, Erasmus MC-University Medical Center, Rotterdam, Netherlands

**Under submission**

## ABSTRACT

Norovirus (NoV) is increasingly reported as an important etiological agent for chronic and severe diarrheal complications in transplant recipients. However, a consensus regarding the exact prevalence, epidemiology, clinical manifestations and potential clinical management of NoV gastroenteritis (NVGE) in those patients is still lacking. We systematically reviewed the burden associated with NoV infection in the transplantation setting. NoV accounted for 2.9 to 60% of severe diarrhea in hematopoietic stem cell transplant recipients and 3.2 to 38.4% in solid organ transplant recipients. NVGE in transplant patients was usually chronic, protracted and sometimes even fatal. Typically, patients required frequent hospital admission and co-infection with other enteric pathogens was also commonly observed. Risk factors contributing to susceptibility, severity and chronicity of NVGE varied markedly between studies. Although several NoV genotypes existed, NoV genotype 2 (GII) was the etiological agent in the majority of cases involved with genotype 1 (GI) being a distant second. Interestingly, chronically infected transplant recipients displayed accelerated NoV evolution and constituted viral reservoirs releasing novel variants. Several studies have explored possible treatment strategies toward NVGE and demonstrated that repurposing nitazoxanide showed promise, even if it failed to clear NVGE in a substantial number of cases. Due to limited size of the studies involved and the absence of clear mechanism-of-action, it cannot yet be considered evidence-based treatment and further evaluation was needed. The burden of NVGE in the transplantation setting is more severe than was expected. Thus, awareness of this problem should be raised.


**Keywords:** norovirus; transplantation; evolution; nitazoxanide.

# INTRODUCTION

Norovirus (NoV) is a member of the family *Caliciviridae*. It is a non-enveloped, positive-sense single-stranded RNA virus with a genome size of approximately 7.5 kilobase (kb). Based on the amino acid sequence of its major capsid protein virus protein (VP) 1, NoV is divided into 7 genogroups (G) with G1, 2 and 4 (GI, II and IV) being primarily responsible for infecting humans [1]. NoV represents the leading etiological agent for human viral gastroenteritis worldwide [2].

NoV gastroenteritis (NVGE) usually manifests as self-limiting diarrheal disease of short duration in immunocompetent individuals. However, chronic and protracted infections have been observed in young children, the elderly and immunocompromised populations [3-5]. In such cases the disease may persist for weeks or even months, and can cause severe weight loss, debilitation and occasionally even death. Recently NoV has been recognized as an important entity for prolonged devastating complications in recipients of hematopoietic stem cell transplants (HSCT) and solid organ transplants (SOT) [6]. With the advent of the widespread use of reverse transcription-polymerase chain reaction (RT-PCR)-based detection of NoV, now an accumulating body of studies have reported the prevalence, epidemiology and pathogenesis of NoV in this specific setting. Unfortunately the development of a NoV vaccine remains challenging partially due to the highly genetic and antigenic diversity of human NoV (HuNoV) as well as the unavailability of small animal models [7]. At present, no licensed treatment for NVGE is available except for fluid replacement and intensive supportive care [8]. However, several clinical studies have explored potential therapies and potentiated the off-label use of FDA-approved nitazoxanide as a promising option for chronically infected patients. Given the fact that detailed knowledge of NoV infection in the transplantation setting is generally segmented and inconclusive, we have now conducted a systematic review to comprehensively evaluate the prevalence, epidemiology, clinical manifestations and potential treatment options of NVGE in HSCT and SOT recipients.

# METHODS

We searched EMBASE, MEDLINE Ovid, Web of science, Scopus, Cochrane Central and Google scholar to identify articles published in English until February 27, 2018, in which NoV

infection was described among HSCT and SOT recipients. The search strategies for each database were designed by an experienced information specialist (WB) and are available in Table S1. After removing duplicates, two independent reviewers (WD and MH) reviewed the title and abstract of all articles to eliminate the irrelevant references and references that did not meet the inclusion criteria. Disagreements were resolved by consensus. To make sure all the relevant publications were captured, we cross-referenced all articles from the bibliographies of the selected publications by WD and MH. After reviewing each article, studies meeting each of the following inclusion criteria were selected: (i) Original research articles or reports describing NVGE in transplant recipients; (ii) Studies using detection methods to clearly confirm the presence of NoV as an etiological agent for diarrhea; (iii) All subjects in the study were HSCT or SOT recipients. Articles were excluded if (i) full-text was not available; (ii) subjects were immunocompromised individuals but not transplant recipients. The detailed algorithm for excluding and including studies is further documented in Fig. 1.

# RESULTS

## *Description of the included studies*

Based on our search criteria and after removal of duplicates, a total of 365 references were found. By reviewing the type, title and abstract of the articles, we excluded 312 references. This left 52 eligible articles and 4 additional articles were identified by manual search from the reference lists. Further assessing the full-text of these 56 articles ultimately resulted in the identification of 47 studies which met the inclusion criteria (Fig. 1).

More in detail, with respect to the subjects we included studies of NoV infection among both HSCT and SOT recipients, and results were taken into account irrespective of gender, age, ethnicity and nationality of the patients involved. In line with the purpose of the current study and based on the contents of selected publications, we categorized topics into 5 aspects, including prevalence and clinical characteristics (*n* = 38), diagnosis (*n* = 5), risk factors (*n* = 4), transmission and evolution (*n* = 12), and treatment (*n* = 10) (Fig. 1). Of note, many publications have addressed several of these topics in the same publication.

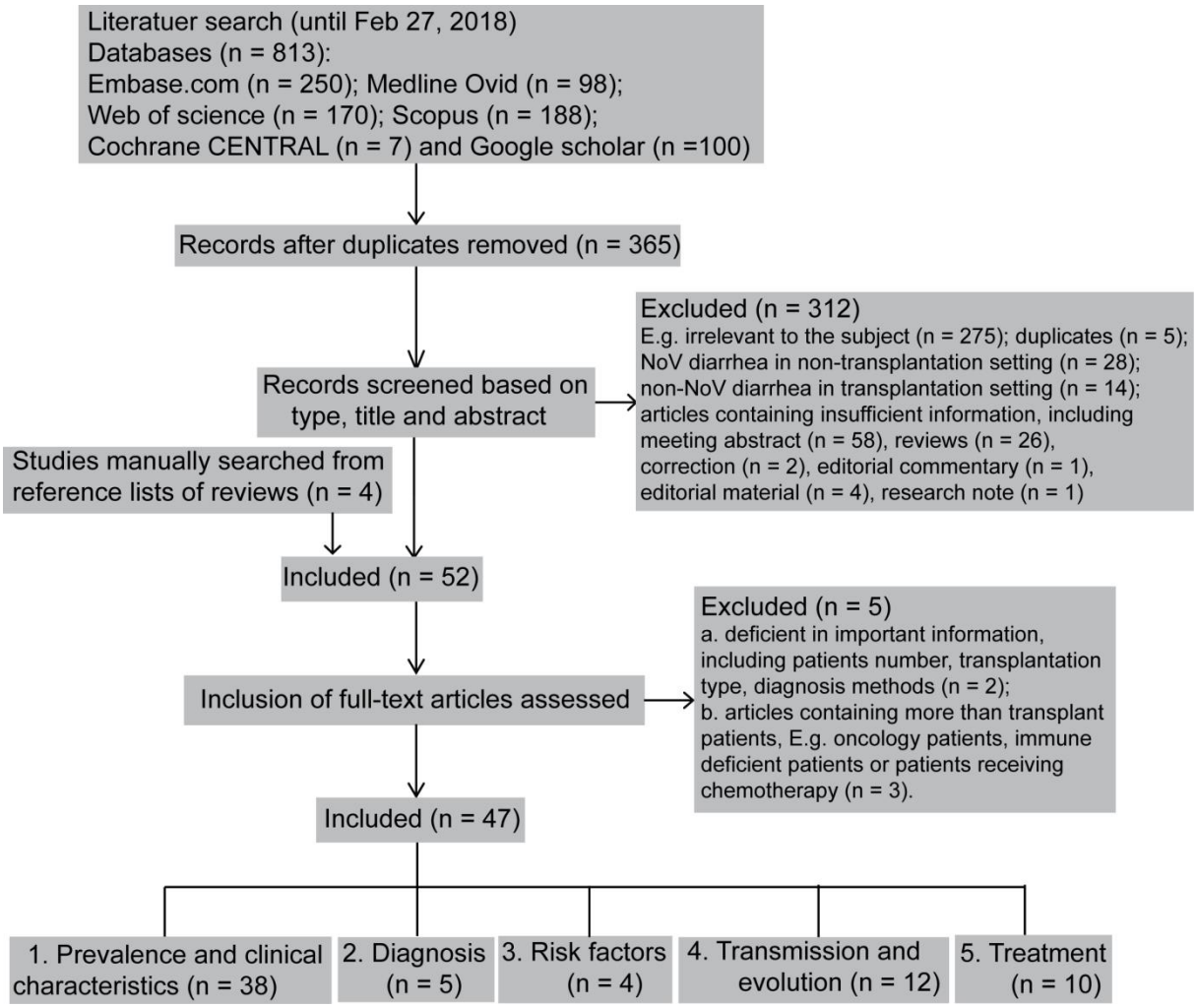



**Figure 1 Flow diagram showing literature search and selection results.**

## *NoV infection in HSCT*

HSCT is a potentially effective treatment for both hematologic malignant and non-malignant disorders. Since hematopoietic stem cells exist not only in the marrow (also called a bone marrow transplantation; BMT) but also in periphery, we collectively referred to HSCT as inclusive HSCT regardless of the origin of the stem cells.

Among HSCT recipients with NVGE, NoV prevalence varied widely within each study, ranging from 2.9 to 26% (Table 1) [9-14]. Highest prevalence of NoV infection (60%) was observed in a study of 10 autologous stem cell transplantation (ASCT) recipients [15]. After an incubation period of 12 to 48 hours following virus exposure, most immunocompetent patients experience a classic set of symptoms including sudden onset of vomiting, abdominal cramps and watery diarrhea [2, 16]. The illness usually resolves within 24 to 72 hours later, although asymptomatic shedding of virus in feces persists for up to 3 weeks [2]. In contrast HSCT recipients form a high-risk group for severe chronic diarrhea following NoV infection.

The duration of symptoms ranged from a median of 8 days to approximately 2 months (Table 1; Table S2) [9-11, 14, 15]. Of 12 NoV-positive ($NoV^+$) HSCT recipients, ten patients presented diarrhea for a median of three months (range 0.5 to 14) and persistently shed high loads of virus for prolonged periods [17] with comparable results observed in two studies that documented viral shedding for a median of 150 days (range 60 to 380) [13] and 145 days (range 13 to 263) [12] among 13 and 8 $NoV^+$ HSCT recipients. Thus, HSCT recipients are clearly compromised in their capacities to mount effective defense against NoV.

NVGE causes severe and devastating complications in HSCT recipients. A comparison of NoV infection (*n* = 12) and clostridium difficile infection (CDI; *n* = 42) in hematopoietic cell transplantation (HCT) recipients demonstrated that NVGE cases provoked more intensive care unit (ICU) admissions (odds ratio, 4.9; 95% confidence interval [CI], 1.1 to 21.6) and resulted in higher mortality (odds ratio, 3.2; 95% IC, 0.74 to 13.5) when compared to CDI [10]. In a retrospective analysis of 63 $NoV^+$ HSCT recipients, 24 (35%) patients required hospitalization with a median length of stay of 5 days (range 2 to 40) [11]. Co-infection with other enteric pathogens was frequently observed. Ten out of 63 $NoV^+$ allogeneic HSCT recipients were co-infected with other gastrointestinal pathogens including adenovirus (AdV; *n* = 3), CDI (*n* = 4), cytomegalovirus (CMV; *n* = 2) and rotavirus (RV; *n* = 1) [11]; while 6 out of 8 $NoV^+$ HSCT patients had co-infections including CDI (*n* = 4) and CMV (*n* = 2) [12]. Moreover norovirus-associated mortality has been observed in several cases [12, 17, 18]. Hence NVGE is clearly a substantial problem in this patient group requiring vigilance during clinical management.

## *Potential role of the reconstructed immune system on NVGE following HSCT*

Hosts exert rapid and broad immune activation when challenged with HuNV and subsequently develop antibody responses, but detailed knowledge of norovirus-host interactions is still largely lacking [19, 20]. HSCT recipients experience a period during which the immune system is rebuilt. Immune response is not well established during this period. This increases the susceptibility of hosts to diverse infections. With respect to NVGE in HSCT recipients, two aspects need especially adequate clarification, one is the time from transplantation to the onset of NVGE, and the other is whether restored T cells contribute to the resolution of NVGE.

**Table 1 Studies reporting the incidence and clinical manifestations of NVGE in HSCT recipients.**

| Author, publication year, [Reference] | Study type | Study period | Country | Total subjects/ Subjects with NVGE/prevalence | Patients group | Duration (days) | Genotype |
|---|---|---|---|---|---|---|---|
| Ueda *et al*, 2015, [9] | Retrospective analysis | Jan 2007-Jun 2011 | Japan | 350/10/2.9% | A | 42 (median, range 3 to 135) | |
| MacAllister *et al*, 2018, [10] | Brief report | Jan 2013-Jul 2016 | USA | 218/12/5.5% | P | 14.5 (IQR:0, 31.5) | GII (10) GI (2) |
| Swartling *et al*, 2018, [11] | Retrospective analysis | 2006-2012 | Sweden | 494/63/12.7% | P + A | 8 (median, range 1 to 328) | |
| Robles *et al*, 2012, [12] | Retrospective study | Jul 2007-Jun 2011 | China | 49/8/16.3% | P | 145 (median, range 13 to 263) | |
| Saif *et al*, 2011, [13] | Case series | | UK | 61/13/21% | P | 150 (median, range 60 to 380) | |
| Doshi *et al*, 2013, [14] | Observational cohort study | Jan 2009-Mar 2009 | USA | 23/6/26% | A | 22.5 (median, range 6 to 33) | |
| Lemes *et al*, 2014, [15] | Prospective study | Oct 2012-Sep 2013 | Brazil | 10/6/60% | A | 61.6 (mean) | GI.3 |

Abbreviation: A, adult; IQR, interquartile range; GI, Genogroup 1; GII, Genogroup 2; P, pediatric.

Evidence suggests that NVGE tends to occur in the first few months after HSCT, which corresponds to the time period at which patients are the most highly immunosuppressed. In a case series the median time from transplantation to diagnosis of NVGE was 25 days (range -80 to 63) among 13 $NoV^+$ HSCT recipients, three of which were diagnosed positive prior to transplantation [13]. This was comparable with two other studies of 8 $NoV^+$ HSCT recipients (a

median of 36.5 days, range 5 to 517) [12] and 10 $NoV^+$ HSCT recipients (a median of 36 days, range 3 to 39) [9]. Another study, however, involving 12 adult allogeneic HSCT recipients with NVGE recorded a much longer time of diarrhea onset (a median of 10.5 months, range 0.25 to 96) following transplantation [17]. Presentation can thus be variable and even long after HSCT, and one should be aware of the possibility of NVGE in such patients.

A few publications highlight the potential role of recovering immunity on the clearance of NoV infection in HSCT recipients. Among 13 pediatric $NoV^+$ HSCT recipients, the median duration of NoV diarrhea (150 days, range 30 to 380) was almost the same as the median time of donor T cell recovery (150 days, range 30 to 390), suggesting a close association of NoV clearance with immune reconstitution [13]. However, T cell recovery was solely defined by the absolute CD3 count instead of the function. Meanwhile the number of patients was rather small requiring further investigation in this respect.

### *NoV infection in SOT recipients*

The prevalence of NoV as a cause of acute and chronic gastroenteritis in SOT recipients ranged from 3.2 to 38.4%, and thus resembled the situation in HSCT recipients (Table 2) [21-32]. Likewise, the former patient groups also displayed protracted NVGE with duration ranging from a median of 12.5 days to 218 days (Table 2) [21, 22, 25-30]. Studies with small sample sizes that were insufficient in evaluating NVGE prevalence and clinical outcomes are listed in Table S3. Common clinical symptoms reported were diarrhea, nausea, vomiting and abdominal pain, whereas severe wasting, profound weight loss and fever were also less commonly reported [25, 30, 33]. Reminiscent from the situation in HSCT recipients, SOT recipients with NVGE required more hospital admissions with longer duration when compared to those with non-NoV diarrhea, showing a specific vulnerability of these patients to NoV. Of 25 $NoV^+$ pediatric HSCT and SOT recipients, 55% (13/25) of patients required hospitalization for diarrhea with 27% being admitted into ICU. The study also performed a matched case-control to compare clinical outcomes of $NoV^+$ diarrhea subjects ($n$ = 22) and non-NoV diarrhea subjects ($n$ = 22). It was found that $NoV^+$ patients required more hospitalization (55% vs 36%, $P$ = 0.23) and ICU admission (27% vs 0%, $P$ = 0.02), meanwhile experienced more weight loss (median 1.6 vs 0.6 kg, $P < 0.01$) [29]. Furthermore, 58% (40/67) of $NoV^+$ SOT patients were hospitalized with duration of on average 9.6 days, which was

**Table 2 Studies reporting the incidence and clinical manifestations of NVGE in SOT and multi-ogran transplant recipients.**

| Author, publication year, [Reference] | Study type | Study period | Country | Total subjects/ Subjects with NVGE/Prevalence | Patients group | Transplant type | Duration (days) | Genotype |
|---|---|---|---|---|---|---|---|---|
| Brakemeier *et al*, 2016, [21] | Retrospective study | Jan 2007-Dec 2011 | Germany | 2010/65/3.2% | A | KTx | 202 (mean, ±62) | |
| Van Beek *et al*, 2016, [22] | Retrospective cohort study | Jan 2006-Dec 2014 | Netherlands | 2182/101/4.% | P + A | SOT and KTx (mainly) | 218 (median, range 32 to 1164) | GII.P4-GII.4 (mainly) |
| Echenique *et al*, 2015, [23] | Retrospective chart review | Mar 2012-Sep 2013 | USA | 534/37/6.8% | A | KTx and LTx (mainly) | | |
| Schvartz *et al*, 2016, [24] | Retrospective case series | | France | 56/7/12.5% | A | KTx | | |
| Avery *et al*, 2017, [25] | Retrospective case series | Jun 2013-Jun 2014 | USA | 193/30/16% | A | KTx (mainly) | 124 (median, range <30 to 600) | |
| Schorn *et al*, 2010, [26] | Case series | Nov 2006-Nov 2008 | Germany | 78/13/16.7% | A | KTx | 150 (median, range 24 to 898) | GII.4 (mainly) |
| Patte *et al*, 2017, [27] | Retrospective case series | 1994-2014 | France | 101/19/18.8% | P + A | ITx | 78 (median, range 20 to 360) | |
| Roos-Weil *et al*, 2011, [28] | Retrospective study | Jul 2008-Nov 2009 | France | 87/16/19.39% | A | RTx | 120 (mean) | |
| Ye *et al*, 2015, [29] | A prospectively enrolled surveillance study | Dec 2012-Sep 2013 | USA | 116/25/22% | P | Combined[a] | 12.5 (median, range 1 to 324) | GII |
| Lee *et al*, 2016, [30] | Retrospective study | Jan 2006-Jul 2013 | USA | 192/67/35% | A | KTx (mainly) | 135 (median, range 1 to 2598) | GII (72%) GI (7%) |
| Coste *et al*, 2013, [31] | Retrospective case series | Sep 2010-Nov 2011 | France | 54/14/36% | A | KTx | | |
| Morotti *et al*, 2004, [32] | Retrospective case series | Jan 2002-Sep 2002 | USA | 13/5/38.4% | P | ITx | | |

a, subjects underwent a combination of HSCT and SOT. Abbreviation: A, adult; GI, Genogroup 1; GII, Genogroup 2; HSCT, hematopoietic stem cell transplantation; ITx, intestinal transplantation; KTx, kidney transplantation; LTx, liver transplantation; NoV, norovirus; P, pediatric; RTx, renal transplantation; SOT, solid organ transplantation.

**Table 3 Co-infections were commonly observed in transplant recipients.**

| Reference | Transplant type | NVGE episodes /Co-infection/Rate | Co-infections |
|---|---|---|---|
| [11] | Allo-HSCT | 63/10/15.8% | AdV (*n* = 3); CDI (*n* = 4); CMV (*n* = 2); RV (*n* = 1) |
| [12] | HSCT | 8/6/75% | CDI (*n* = 4); CMV (*n* = 2) |
| [33] | SOT | 152/28/18% | CDI (*n* = 9); AdV (*n* = 8); CMV (*n* = 5); RV (*n* = 3); CMV and AdV (*n* = 1); Campylobacter spp. (*n* = 1); E. coli (*n* = 1) |
| [34] | SOT (*n* = 20)<br>HSCT (*n* = 3) | 24/8/33.3% | CDI (*n* = 5); RV (*n* = 3) |
| [30] | SOT | 67/10/14.9% | CDI (*n* = 7); CMV (*n* = 3) |
| [29] | SOT (*n* = 9)<br>HSCT (*n* = 25) | 25/5/20% | RV (*n* = 2); CDI (*n* = 1); AdV (*n* = 1); CDI and AdV (*n* = 1) |
| [25] | KTx (*n* = 25) | 31/>9/>28.7% | Pneumonia (*n* = 6); UTI (*n* = 3) |
| [15] | ASCT | 6/5/83% | CMV (*n* = 4); Bacteria (*n* = 1) |
| [27] | Allo-HSCT | 19/4/21% | RV (*n* = 1); AsV (*n* = 1); AdV (*n* = 1); C. jejuni and EnV (*n* = 1) |

AdV, adenovirus; Allo-, allogeneic; AsV, astrovirus; ASCT, allogeneic stem cell transplantation; C. jejuni, Campylobacter jejuni; CDI, Clostridium difficile infection; CMV, cytomegalovirus; E.coli, Escherichia coli; EnV, enterovirus; HSCT, hematopoietic stem cell transplantation; KTx, kidney transplantation; RV, rotavirus; SOT, solid organ transplantation; UTI, urinary tract infection.

much longer than 6.3 days among a matched non-NoV diarrheal group of patients [30]. In accordance, 56% (5/9) $NoV^+$ kidney transplantation (KTx) recipients and 77.4% (24/31) $NoV^+$ SOT recipients required hospitalization [25, 26]; while 80% (121/152) $NoV^+$ SOT recipients required hospitalization with prolonged length of stay of 10 ± 15.2 days [33]. Likewise, 79% (15/19) $NoV^+$ intestinal transplantation (ITx) recipients that were admitted into hospital required a median length of stay of 41 days (range 0 to 119) [27]. Compounding the situation and analysis is that co-infection with other pathogens was commonly observed in SOT recipients including CDI, AdV, RV, CMV, enterovirus (EV), bacterial infections and urinary tract infections (UTIs) (Table 3). Thus, it is necessary to perform multiple microbiological examinations in diarrheal transplant recipients, even if NoV has been detected.

## *Diagnosis*

Various diagnostic assays for establishing NoV status have been employed in the transplantation setting (Table S4). Initially, the Kaplan criteria were used as a tool for detecting possible NVGE outbreaks in healthcare settings, and this approach had an estimated sensitivity of 68% and a specificity of 99% [35, 36]. However, these criteria also applied to non-NoV diarrhea [18]. With the first *bona fide* visualization of NoV particles in 1972, the definitive diagnosis was thus achieved by immune electron microscopy (IEM) [37]. This method is, however, cumbersome, insensitive and only applicable when fecal samples

have a high viral load (> $10^6$ particles per mL of specimen). Subsequently, enzyme-linked immunosorbent assay (ELISA)-based antigen detection assays were developed. Subsequently they were routinely used for detection of NVGE outbreaks. Currently a number of ELISA kits are commercially available including the RIDASCREEN® Norovirus 3rd Generation kit and the RIDA®QUICK Norovirus test [38, 39]. Compared to IEM, these rapid antigen detection tests have a higher sensitivity and specificity. Recently immunochromatography (IC) and histopathological analysis have also been used in several studies [18, 40]. The former shows high specificity and sensitivity, while the latter has only been used for distinguishing NVGE from graft-versus-host disease (GVHD). NVGE is histopathologically characterized by villous blunting and a slight elevation of apoptotic epithelial cells at the tip of the villi; while intestinal GVHD involves a partial loss of surface epithelium and increased numbers of apoptotic crypt epithelial cells [18]. The distinction between NVGE and GVHD is essential for decision-making regarding appropriate treatment options, as the former requires a decrease in or even discontinuation of immunosuppression, whereas the latter requires more harsh immunosuppression. Thus, proper diagnosis is of utmost importance in this respect.

The current 'golden standard' for definitive NoV diagnosis is RT-PCR. This technology shows clear superiority over other diagnostic methods on sensitivity and specificity with respect to NoV detection. Of 54 severe diarrhea events among 49 adult KTx recipients, NoV was undetectable using the classical rapid antigen detection tests, whereas 36% of samples were positive using a Multiplex PCR assays [31]. Among 12 HSCT recipients, NoV was detected only in two patients by electron microscopy (EM), whereas all the patients had a positive signal in RT-PCR assays [17]. Hence PCR-based method is now regarded as the methodology of choice for prompt and accurate diagnosis of NoV infection. Nevertheless, this technique has not yet seen widespread implementation in the transplantation setting, probably because of its high costs and the relatively advanced technical capacity required. Thus, as an alternative in many clinical settings, one resorts to a commercially available IC kit that is marketed in Asia and Europe [2] and provides cheap and easy NoV diagnosis in the transplantation setting, even if superior technology is available.

## *Risk factors*

Many risk factors are associated with a significantly higher risk for NoV infection in transplant recipients. In a retrospective analysis of 350 HSCT recipients, a second or subsequent allogeneic HSCT was associated with increased risk of contracting NVGE [9]. A retrospective study of 55 HSCT recipients showed that recipients who received peripheral blood-derived stem cells or cord blood-derived stem cells were more likely to contract NVGE when compared to those who received bone marrow. Relatively harsh immunosuppression regimens containing fludarabine or alemtuzumab were also associated with a significantly greater risk for subsequent NoV infection [12]. Several risk factors that are linked to more severe and protracted NVGE in transplant recipients are evident as well. In a study of 193 transplant recipients, wasting, MHC incompatible kidney transplant status and plasmapheresis were associated with longer gastroenteritis [25], whereas severe combined immunodeficiency (SCID) was linked to increased chronicity of NoV infection in a study involving 494 allogeneic HSCT recipients [11]. Hence for these patients additional vigilance with respect to NoV infection is called for.

## *NoV evolution*

NoV is divided into 7 distinguished genogroups showing substantial intergenogroup genetic diversity. Most NoV strains in transplant recipients belonged to GII, albeit presenting with highly divergent intragenogroup variants. In addition, GI was also reported. Evidence suggests that chronic NoV infection in transplant recipients accelerates the accumulation of genetic alterations and viral evolution. Viruses that are subject to mutation can potentially evade the immunity of the host, providing a selective advantage of mutated viruses. In transplant recipients this problem may be compounded by a relatively low immune selective pressure that can only partially clear NoV. Thus, immunosuppressed patients may constitute an environment that fosters NoV evolution and serves as potential reservoirs for novel NoV variants [41]. Among 3 HSCT patients with chronic GII.4 Sydney_2012 infection, virus strains accumulated 19, 18, and 8 nucleotide mutations within 110, 113 and 22 days respectively, most of which were non-synonymous [42]. Consistently, gained 46 nucleotide changes in NoV were observed over a period of 683-day virus shedding in a KTx recipient, resulting in 25 amino acid changes with an overall fixation rate of 0.037 amino acids/day. A fixation rate of 0.049 and 0.012 amino acid changes per day was observed in two other patients [26]. Two

GII.7 and one GII.4 NoV strains in 3 chronically infected transplant recipients gained average 5 to 9 mutations within 100 days, most of which were non-synonymous [43]; while in a $NoV^+$ heart transplantation (HTx) recipient, 32 amino acid changes occurred within the ORF2 over a 1-year period, 8 of which located in the hypervariable domain (P2) of the capsid protein [44]. Interestingly, it is recently reported that within immunocompromised hosts NoV has evolved into phylogenetically distinct variants that are genetically different from the circulating strains in the general population [45, 46]. Collectively, the immunocompromised hosts may serve as a reservoir for the emergence of novel infectious NoV variants potentially posing novel threats to the proof of invading herd immunity.

## *Transmission, prevention and control of NVGE*

Although food-borne transmission is the primary mode of transmission, NoV can also be acquired through the fecal-oral route or by inhalation of infectious aerosols [2, 47, 48]. Most NVGE cases in transplant recipients are community-acquired. However, nosocomial transmission is likely to play an important role as well. In a bone marrow transplant (BMT) unit, a nosocomial outbreak happened and involved 5 transplant recipients and 4 healthcare workers [14]. Another reported nosocomial outbreak involved 7 HSCT recipients and 5 staff members following the admission of a $NoV^+$ HSCT index patient. In this case the mode of transmission was potentially through inhalation of infectious aerosols or the fecal-oral route through sharing a lavatory [18]. In a retrospective analysis of 63 $NoV^+$ HSCT recipients, 47% (30/63) patients acquired NoV infection during hospitalization, 6 of which got infected during an outbreak at the transplant ward [11]. Several reports also documented nosocomial outbreaks of NoV infection in transplant recipients, but the potential mode of transmission needed further investigation to allow the development of potentially preventive measures for such patients [15, 49].

Prevention of nosocomial outbreaks is largely dependent on the prompt diagnosis of individual cases and the subsequent elimination of possible transmission routes. It has been proposed that next-generation whole genome sequencing is a good tool to study the direction of NoV spread and potential nosocomial transmission mode [50]. Upon recognition of a NoV outbreak, it is essential to promptly implement preventive measures and exert extra care to contain the situation. Strategies proposed included the transfer of NVGE patients to an isolation ward with in suite toilet facilities, the adherence to strict hygiene

regulations by the medical and nursing staff, the complete disinfection of patient rooms, and evidence for efficacy had been provided [2, 14, 17].

## *Potential treatment strategies toward chronic NVGE*

Chronic NoV infections in transplant recipients often result in serious complications including prolonged diarrhea, allograft failure and mortality. However, no licensed therapies are currently available. Reduction or switch (e.g. to an everolimus-based regimen) of immunosuppression is generally employed in such cases as a first line strategy (Table S5) [17, 51, 52]. Oral and systemic administration of human immunoglobulins (HIGs) as well as oral serum-derived bovine immunoglobulin (OSDBI) have demonstrated efficacies in some studies (Table S5) [34, 53-57]. However, especially the use of nitazoxanide, of which proof of efficacy was found serendipically, appears very promising (Table S5) [58-60].

It is important to state, however, that the effectiveness, mechanism-of-action and extent to which those treatments improve the lot of NVGE patients in the setting of transplantation remain largely unknown. In a series of 31 transplant recipients with NVGE, 6 (19.4%), 2 (6.5%) or 1 (3.2%) patients received monotherapy of nitazoxanide, i.v. immunoglobulin (IVIg) or a reduction in immunosuppression respectively, whereas some patients received a combination of these treatments with nitazoxanide plus reduction of immunosuppression (9; 29%) and nitazoxanide plus IVIg plus reduction of immunosuppression (8; 25.8%) being the most frequently chosen strategy. All the patients ultimately resolved NVGE [25]. A pediatric KTx recipient whose clinical management was complicated by NVGE received oral immunoglobulins (IGs) and medication switching from tacrolimus to sirolimus. This strategy, however, was not effective with respect to the diarrhea. A subsequent fortnight-course of nitazoxanide led to the resolution of diarrhea and complete clearance of the NoV infection as assessed by fecal NoV shedding [59]. Furthermore a $NoV^+$ HSCT recipient was successfully treated with nitazoxanide, whereas other therapeutic options including IVIg and a reduction in immunosuppression were not successful [58]. Thus, these is some evidence that nitazoxanide is a good therapeutic option when confronted with NVGE in the setting of transplantation but in absence of better controlled studies. A stepwise approach to management of NVGE should be considered.

The development of medication useful for combating NoV is now gaining momentum because of the successful cultivation of HuNV in B cells and stem cell-derived human

enteroids [61-63]. Ribavirin, a broad-spectrum antiviral drug has been shown to inhibit *in vitro* NoV replication through depletion of cellular GTP pools [64]. In patients with common variable immunodeficiency (CVID), oral ribavirin resulted in viral clearance in some but not all of the patients [65]. Whether the combination of nitazoxanide with ribavirin will be more effective in transplant recipients deserves further investigation.

## CONCLUSION

This systematic review has comprehensively evaluated the burden inflicted by NVGE in the setting of transplantation. Collectively, the data obtained suggest that NoV infection should be routinely evaluated in transplant recipients with diarrheal complaints, as NVGE results in severe and protracted diarrhea characterized by frequent hospitalization and co-infection, thus requiring special attention. In addition, the highly contagious nature of NoV infection requires special measures to protect other patients as well as hospital staff. Moreover, transplant recipients are prone to develop novel variants of NoV for which there may not be protective immunity in the population. We also explore the candidate therapies toward NVGE, which although still preliminary show promise for combating the virus in the setting of transplantation.

Unfortunately, it appeares not feasible to accurately assess the average prevalence of NVGE in transplant recipients. In some studies, only subjects that experienced diarrhea or were hospitalized for diarrhea were enrolled; while other studies enrolled all the transplant recipients irrespective of whether they had diarrhea or not. Furthermore, different types of transplantation and immunosuppressive medicine may be associated with differential sensitivity to NoV infection. Finally, overall patient number described in the literature was fairly small. Nevertheless, it is evident from our analysis that NVGE in the transplantation setting is a serious concern because of its problematic management and highly contagious nature.

## ABBREVIATION

AdV, adenovirus; ASCT, autologous stem cell transplantation; CDI, clostridium difficile infection; CMV, cytomegalovirus; CVID, common variable immunodeficiency; EM, electron microscopy; G, genogroup; GVHD, graft-versus-host disease; HCT, hematopoietic cell transplantation; HSCT, hematopoietic stem cell transplantation; HTx, heart transplantation; HuNV, human norovirus; IC, immunochromatography; IEM, immune electron microscopy; IGs, immunoglobulins; ITx, intestine transplantation; IVIg, intravenous immunoglobulin; KTx, kidney transplantation; LTx, liver transplantation; NoV, norovirus; NVGE, norovirus gastroenteritis; RT-PCR, reverse transcription-polymerase chain reaction; RV, rotavirus; SOT, solid organ transplantation; SCID, severe combined immunodeficiency; Tx, transplantation.

## ACKNOWLEDGEMENT

This work was supported by the Dutch Digestive Foundation (MLDS) for a career development grant (No. CDG 1304), the Daniel den Hoed Foundation for a Centennial Award fellowship (to Q. Pan), the China Scholarship Council for funding PhD fellowship to W. Dang (201406180072), P. Yu (201708620177) and the Indonesia Endowment Fund for Education (LPDP) for funding PhD fellowship to Mohamad S. Hakim.

## AUTHOR CONTRIBUTIONS

WD contributed to study concept and design, analysis and interpretation of data, and drafting of the manuscript; WB contributed to acquisition of data and critical revision of the manuscript; PY and MH contributed to critical revision of the manuscript for important intellectual content; MP and QP contributed to study concept and design, study supervision and obtaining funding.

# REFERENCE


1. van Beek J, de Graaf M, Al-Hello H et al. Molecular surveillance of norovirus, 2005-16: an epidemiological analysis of data collected from the NoroNet network. *Lancet Infect Dis* 2018;**18**(5):545-553.
2. Glass RI, Parashar UD, Estes MK. Norovirus gastroenteritis. *N Engl J Med* 2009;**361**(18):1776-1785.
3. Bok K, Green KY. Norovirus gastroenteritis in immunocompromised patients. *N Engl J Med* 2012;**367**(22):2126-2132.
4. Cardemil CV, Parashar UD, Hall AJ. Norovirus Infection in Older Adults: Epidemiology, Risk Factors, and Opportunities for Prevention and Control. *Infect Dis Clin North Am* 2017;**31**(4):839-870.
5. Esposito S, Ascolese B, Senatore L, Codeca C. Pediatric norovirus infection. *Eur J Clin Microbiol Infect Dis* 2014;**33**(3):285-290.
6. Haidar G, Singh N. Viral infections in solid organ transplant recipients: novel updates and a review of the classics. *Curr Opin Infect Dis* 2017;**30**(6):579-588.
7. Lucero Y, Vidal R, O'Ryan GM. Norovirus vaccines under development. *Vaccine* 2017.
8. Thorne L, Arias A, Goodfellow I. Advances Toward a Norovirus Antiviral: From Classical Inhibitors to Lethal Mutagenesis. *J Infect Dis* 2016;**213** Suppl 1:S27-31.
9. Ueda R, Fuji S, Mori S et al. Characteristics and outcomes of patients diagnosed with norovirus gastroenteritis after allogeneic hematopoietic stem cell transplantation based on immunochromatography. *Int J Hematol* 2015;**102**(1):121-128.
10. MacAllister TJ, Stednick Z, Golob JL, Huang ML, Pergam SA. Underutilization of norovirus testing in hematopoietic cell transplant recipients at a large cancer center. *Am J Infect Control* 2018;**46**(1):100-102.
11. Swartling L, Ljungman P, Remberger M et al. Norovirus causing severe gastrointestinal disease following allogeneic hematopoietic stem cell transplantation: A retrospective analysis. *Transpl Infect Dis* 2018;**20**(2):e12847.
12. Robles JD, Cheuk DK, Ha SY, Chiang AK, Chan GC. Norovirus infection in pediatric hematopoietic stem cell transplantation recipients: incidence, risk factors, and outcome. *Biol Blood Marrow Transplant* 2012;**18**(12):1883-1889.
13. Saif MA, Bonney DK, Bigger B et al. Chronic norovirus infection in pediatric hematopoietic stem cell transplant recipients: a cause of prolonged intestinal failure requiring intensive nutritional support. *Pediatr Transplant* 2011;**15**(5):505-509.
14. Doshi M, Woodwell S, Kelleher K, Mangan K, Axelrod P. An outbreak of norovirus infection in a bone marrow transplant unit. *Am J Infect Control* 2013;**41**(9):820-823.
15. Lemes LG, Correa TS, Fiaccadori FS et al. Prospective study on Norovirus infection among allogeneic stem cell transplant recipients: prolonged viral excretion and viral RNA in the blood. *J Clin Virol* 2014;**61**(3):329-333.
16. Atmar RL, Estes MK. The epidemiologic and clinical importance of norovirus infection. *Gastroenterol Clin North Am* 2006;**35**(2):275-290, viii.
17. Roddie C, Paul JP, Benjamin R et al. Allogeneic hematopoietic stem cell transplantation and norovirus gastroenteritis: a previously unrecognized cause of morbidity. *Clin Infect Dis* 2009;**49**(7):1061-1068.
18. Schwartz S, Vergoulidou M, Schreier E et al. Norovirus gastroenteritis causes severe and lethal complications after chemotherapy and hematopoietic stem cell transplantation. *Blood* 2011;**117**(22):5850-5856.
19. Newman KL, Moe CL, Kirby AE, Flanders WD, Parkos CA, Leon JS. Norovirus in symptomatic and asymptomatic individuals: cytokines and viral shedding. *Clin Exp Immunol* 2016;**184**(3):347-357.

20. Ko G, Jiang ZD, Okhuysen PC, DuPont HL. Fecal cytokines and markers of intestinal inflammation in international travelers with diarrhea due to Noroviruses. *J Med Virol* 2006;**78**(6):825-828.

21. Brakemeier S, Taxeidi SI, Durr M et al. Clinical outcome of norovirus infection in renal transplant patients. *Clin Transplant* 2016;**30**(10):1283-1293.

22. van Beek J, van der Eijk AA, Fraaij PL et al. Chronic norovirus infection among solid organ recipients in a tertiary care hospital, the Netherlands, 2006-2014. *Clin Microbiol Infect* 2017;**23**(4):265 e269-265 e213.

23. Echenique IA, Penugonda S, Stosor V, Ison MG, Angarone MP. Diagnostic yields in solid organ transplant recipients admitted with diarrhea. *Clin Infect Dis* 2015;**60**(5):729-737.

24. Schvartz B, Garcia M, Wolak-Thierry A, De Champs C, Leveque N. Risk factors of asymptomatic shedding of enteric pathogens in renal transplant recipients. *Transpl Infect Dis* 2016;**18**(3):480-482.

25. Avery RK, Lonze BE, Kraus ES, Marr KA, Montgomery RA. Severe chronic norovirus diarrheal disease in transplant recipients: Clinical features of an under-recognized syndrome. *Transpl Infect Dis* 2017;**19**(2).

26. Schorn R, Hohne M, Meerbach A et al. Chronic norovirus infection after kidney transplantation: molecular evidence for immune-driven viral evolution. *Clin Infect Dis* 2010;**51**(3):307-314.

27. Patte M, Canioni D, Fenoel VA et al. Severity and outcome of the norovirus infection in children after intestinal transplantation. *Pediatr Transplant* 2017;**21**(5).

28. Roos-Weil D, Ambert-Balay K, Lanternier F et al. Impact of norovirus/sapovirus-related diarrhea in renal transplant recipients hospitalized for diarrhea. *Transplantation* 2011;**92**(1):61-69.

29. Ye X, Van JN, Munoz FM et al. Noroviruses as a Cause of Diarrhea in Immunocompromised Pediatric Hematopoietic Stem Cell and Solid Organ Transplant Recipients. *Am J Transplant* 2015;**15**(7):1874-1881.

30. Lee LY, Ladner DP, Ison MG. Norovirus infection in solid organ transplant recipients: a single-center retrospective study. *Transpl Infect Dis* 2016;**18**(6):932-938.

31. Coste JF, Vuiblet V, Moustapha B et al. Microbiological diagnosis of severe diarrhea in kidney transplant recipients by use of multiplex PCR assays. *J Clin Microbiol* 2013;**51**(6):1841-1849.

32. Morotti RA, Kaufman SS, Fishbein TM et al. Calicivirus infection in pediatric small intestine transplant recipients: pathological considerations. *Hum Pathol* 2004;**35**(10):1236-1240.

33. Chong PP, van Duin D, Sonderup JL, Grant WJ, Kalil AC, Florescu DF. Predictors of persistent diarrhea in norovirus enteritis after solid organ transplantation. *Clin Transplant* 2016;**30**(11):1488-1493.

34. Florescu DF, Hermsen ED, Kwon JY et al. Is there a role for oral human immunoglobulin in the treatment for norovirus enteritis in immunocompromised patients? *Pediatr Transplant* 2011;**15**(7):718-721.

35. Said MA, Perl TM, Sears CL. Healthcare epidemiology: gastrointestinal flu: norovirus in health care and long-term care facilities. *Clin Infect Dis* 2008;**47**(9):1202-1208.

36. Turcios RM, Widdowson MA, Sulka AC, Mead PS, Glass RI. Reevaluation of epidemiological criteria for identifying outbreaks of acute gastroenteritis due to norovirus: United States, 1998-2000. *Clin Infect Dis* 2006;**42**(7):964-969.

37. Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *J Virol* 1972;**10**(5):1075-1081.

38. Dimitriadis A, Marshall JA. Evaluation of a commercial enzyme immunoassay for detection of norovirus in outbreak specimens. *Eur J Clin Microbiol Infect Dis* 2005;**24**(9):615-618.

39. Morillo SG, Luchs A, Cilli A et al. Norovirus 3rd Generation kit: an improvement for rapid diagnosis of sporadic gastroenteritis cases and valuable for outbreak detection. *J Virol Methods* 2011;**173**(1):13-16.

40. Ueda R, Fuji S, Mori S et al. Characteristics and outcomes of patients diagnosed with norovirus gastroenteritis after allogeneic hematopoietic stem cell transplantation based on immunochromatography. *Int J Hematol* 2015;**102**(1):121-128.

41. Vega E, Donaldson E, Huynh J et al. RNA populations in immunocompromised patients as reservoirs for novel norovirus variants. *J Virol* 2014;**88**(24):14184-14196.

42. Mai H, Gao Y, Cong X et al. GII.4 Sydney_2012 norovirus infection in immunocompromised patients in Beijing and its rapid evolution in vivo. *J Med Virol* 2016;**88**(2):224-233.

43. Hoffmann D, Hutzenthaler M, Seebach J et al. Norovirus GII.4 and GII.7 capsid sequences undergo positive selection in chronically infected patients. *Infect Genet Evol* 2012;**12**(2):461-466.

44. Nilsson M, Hedlund KO, Thorhagen M et al. Evolution of human calicivirus RNA in vivo: accumulation of mutations in the protruding P2 domain of the capsid leads to structural changes and possibly a new phenotype. *J Virol* 2003;**77**(24):13117-13124.

45. Debbink K, Lindesmith LC, Ferris MT et al. Within-host evolution results in antigenically distinct GII.4 noroviruses. *J Virol* 2014;**88**(13):7244-7255.

46. Gallimore CI, Lewis D, Taylor C, Cant A, Gennery A, Gray JJ. Chronic excretion of a norovirus in a child with cartilage hair hypoplasia (CHH). *J Clin Virol* 2004;**30**(2):196-204.

47. Lopman BA, Reacher MH, Vipond IB, Sarangi J, Brown DW. Clinical manifestation of norovirus gastroenteritis in health care settings. *Clin Infect Dis* 2004;**39**(3):318-324.

48. Marks PJ, Vipond IB, Carlisle D, Deakin D, Fey RE, Caul EO. Evidence for airborne transmission of Norwalk-like virus (NLV) in a hotel restaurant. *Epidemiol Infect* 2000;**124**(3):481-487.

49. Kaufman SS, Chatterjee NK, Fuschino ME et al. Characteristics of human calicivirus enteritis in intestinal transplant recipients. *J Pediatr Gastroenterol Nutr* 2005;**40**(3):328-333.

50. Kundu S, Lockwood J, Depledge DP et al. Next-generation whole genome sequencing identifies the direction of norovirus transmission in linked patients. *Clin Infect Dis* 2013;**57**(3):407-414.

51. Engelen MA, Gunia S, Stypmann J. Elimination of norovirus in a chronic carrier under immunosuppression after heart transplantation--effect of everolimus. *Transpl Int* 2011;**24**(11):e102-103.

52. Boillat Blanco N, Kuonen R, Bellini C et al. Chronic norovirus gastroenteritis in a double hematopoietic stem cell and lung transplant recipient. *Transpl Infect Dis* 2011;**13**(2):213-215.

53. Chagla Z, Quirt J, Woodward K, Neary J, Rutherford C. Chronic norovirus infection in a transplant patient successfully treated with enterally administered immune globulin. *J Clin Virol* 2013;**58**(1):306-308.

54. Florescu DF, Hill LA, McCartan MA, Grant W. Two cases of Norwalk virus enteritis following small bowel transplantation treated with oral human serum immunoglobulin. *Pediatr Transplant* 2008;**12**(3):372-375.

55. Gairard-Dory AC, Degot T, Hirschi S et al. Clinical usefulness of oral immunoglobulins in lung transplant recipients with norovirus gastroenteritis: a case series. *Transplant Proc* 2014;**46**(10):3603-3605.

56. Ebdrup L, Bottiger B, Molgaard H, Laursen AL. Devastating diarrhoea in a heart-transplanted patient. *Journal of Clinical Virology* 2011;**50**(4):263-265.

57. Gelfand MS, Cleveland KO. Oral Serum-Derived Bovine Immunoglobulin for Management of Infectious Diarrhea due to Norovirus and Cryptosporidiosis in Solid Organ Transplant Patients. *Infect Dis Clin Prac* 2017;**25**(4):218-220.

58. Siddiq DM, Koo HL, Adachi JA, Viola GM. Norovirus gastroenteritis successfully treated with nitazoxanide. *J Infect* 2011;**63**(5):394-397.

59. Haubrich K, Gantt S, Blydt-Hansen T. Successful treatment of chronic norovirus gastroenteritis with nitazoxanide in a pediatric kidney transplant recipient. *Pediatr Transplant* 2018:e13186.

60. Morris J, Morris C. Nitazoxanide Is Effective Therapy for Norovirus Gastroenteritis after Chemotherapy and Hematopoietic Stem Cell Transplantation (HSCT). *Biol Blood Marrow Tr* 2015;**21**(2):S255-S256.

61. Ettayebi K, Crawford SE, Murakami K et al. Replication of human noroviruses in stem cell-derived human enteroids. *Science* 2016;**353**(6306):1387-1393.

62. Jones MK, Watanabe M, Zhu S et al. Enteric bacteria promote human and mouse norovirus infection of B cells. *Science* 2014;**346**(6210):755-759.

63. Kaufman SS, Green KY, Korba BE. Treatment of norovirus infections: moving antivirals from the bench to the bedside. *Antiviral Res* 2014;**105**:80-91.

64. Dang W, Yin Y, Wang Y et al. Inhibition of Calcineurin or IMP Dehydrogenase Exerts Moderate to Potent Antiviral Activity against Norovirus Replication. *Antimicrob Agents Chemother* 2017;**61**(11).

65. Woodward JM, Gkrania-Klotsas E, Cordero-Ng AY et al. The role of chronic norovirus infection in the enteropathy associated with common variable immunodeficiency. *Am J Gastroenterol* 2015;**110**(2):320-327.

# Supplementary Information

## SUPPLEMENTARY TABLES AND FIGURES

**Table S1 Search terms used in our study.**

| |
|---|
| **Embase.com: 250** |
| ('Norovirus'/exp OR 'norovirus infection'/exp OR (Norovir* OR (norwalk* NEAR/3 virus*)):ab,ti) AND ('transplantation'/exp OR 'graft dysfunction'/exp OR 'recipient'/exp OR (transplant* OR allotransplant* OR autotransplant* OR graft* OR allograft* OR autograft* OR recipient*):ab,ti) |
| **Medline Ovid:** 98 |
| ("Norovirus"/ OR (Norovir* OR (norwalk* ADJ3 virus*)).ab,ti,kf.) AND (exo "transplantation"/ OR transplantation.xs. OR "Primary Graft Dysfunction"/ OR exp Transplants/ OR "Transplant Recipients"/ OR (transplant* OR allotransplant* OR autotransplant* OR graft* OR allograft* OR autograft* OR recipient*).ab,ti,kf.) |
| **Web of science: 170** |
| TS=(((Norovir* OR (norwalk* NEAR/2 virus*))) AND ((transplant* OR allotransplant* OR autotransplant* OR graft* OR allograft* OR autograft* OR recipient*))) |
| **Scopus: 188** |
| TITLE-ABS-KEY(((Norovir* OR (norwalk* W/2 virus*))) AND ((transplant* OR allotransplant* OR autotransplant* OR graft* OR allograft* OR autograft* OR recipient*))) |
| **Cochrane CENTRAL: 7** |
| ((Norovir* OR (norwalk* NEAR/3 virus*)):ab,ti) AND ((transplant* OR allotransplant* OR autotransplant* OR graft* OR allograft* OR autograft* OR recipient*):ab,ti) |
| **Google scholar: 100** |
| Norovirus\|"norwalk virus" transplantion\|allotransplantion\|autotransplantion\|graft\|allograft\|autograft\|recipients |

**Table S2 Studies reporting NVGE in HSCT recipients with limited information.**

| Author, publication year, [Reference] | Study type | Study period | Country | Subjects with NVGE | Patients group | Duration (days) | Genotype |
|---|---|---|---|---|---|---|---|
| Gallimore *et al*, 2004, [1] | Case report | Jan 2000-Jul 2000 | UK | 1 | P | 156 | ARG320/1999/US-like recombinant norovirus (rGII-3) |
| Roddie *et al*, 2009, [2] | Retrospective study | Nov 2004-Aug 2007 | UK | 12 | A | 90 (median, range 15 to 420) | GII-4 (*n* = 9) |

Abbreviation: A, adult; P, pediatric.

**Table S3 Studies reporting NVGE in SOT and multi-organ transplant recipients with limited information.**

| Author, publication year, [Reference] | Study type | Study period | Country | Subjects with NVGE | Patients group | Transplant type | Duration (days) | Genotype |
|---|---|---|---|---|---|---|---|---|
| Westhoff *et al*, 2009, [3] | Exceptional case | | Germany | 2 | A | RTx | >120 | GII |
| Bonani *et al*, 2017, [4] | A prospective parallel cohort pilot study | Jul 2013-Mar 2015 | Switzerland | 7 | A | RTx | 245 (median, range 168 to 322) | GII.4 |
| Chehade *et al*, 2012, [5] | Case report | | Switzerland | 1 | P | KTx | | GII |
| Chagla *et al*, 2013, [6] | case report | | Canada | 1 | A | RTx-PTx | | GII.4 Sydney 2012 |
| Chong *et al*, 2016, [7] | Retrospective chart review | Jan 2010-Apr 2014 | USA | 152 | P + A | KTx (mainly) | 6 (IQR: 2-11) | GII (91%)<br>GI (9%) |
| Echenique *et al*, 2016, [8] | Case report | | USA | 1 | | PTx | | GII.4 |
| Florescu *et al*, 2011, [9] | A matched case-control study | Jul 2006-Nov 2008 | USA | 23 | A + P | SOT | | |
| Florescu *et al*, 2008, [10] | Case report | | USA | 2 | P | SBT | | |
| Gairard-Dory *et al*, 2014, [11] | A retrospective chart review | Jan 2009-May 2013 | France | 12 | | LTx | | |
| Hoffmann et al, 2012, [12] | Research article | | Germany | 3 | | BMT (*n* = 2)<br>SBT (*n* = 1) | >81 | GII.7<br>GII.4 2006b |
| Jurgens *et al*, 2017, [13] | case series | 2016 | USA | 3 | | OHTx | | |
| Karandikar *et al*, 2016, [14] | Research article | Jan 2010-May 2012 | USA | 31 | A + P | HSCT (*n* = 13); SBT (*n* = 18) | | Mainly GII.4 |
| Lee *et al*, 2008, [15] | Case report | | Canada | 1 | P | LTx-PTx-SBTx | 114 | GII Mountain strain |
| Kaufman *et al*, 2003, [16] | Case report | | USA | 1 | P | ITx | 120 | GII Miami Beach |
| Gelfand *et al*, 2017, [17] | Case report | | USA | 1 | A | SOT | 150 | GI/GII |
| Ebdrup *et al*, 2011, [18] | Virology question and answer scheme | | Denmark | 1 | A | HTx | 53 | GII.4 (2006b) |
| Blanco *et al*, 2011, [19] | Letter to the editor | | Switzerland | 1 | A | HSCT-LungTx | 180 | GII.4 |

Abbreviation: A, adult; BMT, bone marrow transplantation; HTx, heart transplantation; HSCT, hematopoietic stem cell transplantation; ITx, intestinal transplantation; IQR, interquartile range; KTx, kidney transplantation; LTx, liver transplantation; LungTx, lung transplantation; OHTx, orthotopic heart transplantation; P, pediatric; PTx, pancrease transplantation; RTx, renal transplantation; SBT, small bowel transplantation; SOT, solid organ transplantation.

**Table S4 Laboratory methods for detection of norovirus.**

| Detection | Reference | Description | Results | Conclusion |
|---|---|---|---|---|
| PCR | Most studies | Mainly used for detection of NoV RNA in stools. | Superior sensitivity and specificity | 1. Gold standard method to diagnose NVGE; 2. No widespread clinical use due to high cost and complicated technique required. |
| EM | [2] | NoV was diagnosed by EM and RT-PCR among 12 HSCT recipients. | EM detected NoV particles in only 2 out of 9 patients tested, whereas RT-PCR diagnosed every instance with NVGE positive. | 1. Insensitive; 2. Expensive and complicated technique required. |
| IC | [20] | 350 diarrheal HSCT recipients were examined by IC using a commercial IC kit. | NoV was detected in 10 out of 350 patients. | 1. Slightly less sensitivity, but significantly cheaper compared to PCR; 2. A promising diagnosis tool in the transplantation setting. |
| ELISA | [11] | NoV was detected by a third-generation commercial RIDASCREEN ELISA kit in 12 LTx recipients. | All the patients were diagnosed positive. | 1. 80% sensitivity and 100% specificity compared to PCR. 2. Patients showing unexplained diarrhea should be repeatedly tested for NVGE due to the limited sensitivity of ELISA diagnostic method. |
| Histological analysis | [3] | Two RTx recipients with persistent NoV excretion for more than 3 months underwent histological analysis. | Deep duodenal biopsies demonstrated a partial villous atrophy and chronic inflammation in the lamina propria. | 1. The pathological analysis showed nonspecific finding toward NVGE; 2. It is a supplemented method to distinguish NVGE from GVHD, as both revealed a distinct histopathologic pattern. |
| | [21] | Duodenal biopsies were available for histological analysis in 3 $NoV^+$ HSCT recipients during GVHD and after onset of NoV infection. | 1. Histopathology findings demonstrated a distinct histopathologic patterns towards GVHD and NVGE. 2. NVGE revealed villous blunting and was characterized by a slight increase in apoptotic epithelial cells pronounced at the luminal surface and a significantly elevated numbers of intraepithelial lymphocytes including CD8+ cells. | |

Abbreviation: EM, electron microscopy; ELISA, enzyme-linked immunosorbent assay; GVHD, graft-versus-host disease; IC, immunochromatography; NoV, norovirus; $NoV^+$, norovirus positive; NVGE, norovirus gastroenteritis; RT-PCR, reverse transcription-polymerase chain reaction.

**Table S5 Studies of potential treatments for NVGE in transplant recipients.**

| Treatment | Reference | Description | Outcome | Conclusion |
|---|---|---|---|---|
| IS reduction | Roddie *et al* [2] | 8 out of 12 $NoV^+$ HSCT recipients required withdrawal or reduction of IS. | 1. A slight decrease of fecal volume and frequency; 2. Some cases presented persistent diarrhea for several months. | IS reduction facilitated the improvement of symptoms. However, standard adjustment was not established. |
| IS change | Engelen *et al* [22] | IS was switched to a mammalian target of rapamycin (mTOR) inhibitor everolimus in a heterotopic HTx recipient with continous and recurrent NVGE. | Diarrhea stopped with negative PCR result for NoV. | Everolimus is a potential option for IS in transplant recipients with chronic NoV infection, but further mechanistic study is needed. |
| | Blanco *et al* [19] | IS was switched from FK506 to sirolimus in a double HSCT-LTx recipient with chronic NVGE. | PCR for NoV was negative in the stool after 3 and half a weeks. | |
| HIG | Florescu *et al* [9] | 1. A matched case control study of 24 $NoV^+$ SOT recipients (12 cases and 12 controls); 2. Each case received 200 mg/kg oral HIG plus standard supportive therapy, whereas each control solely received standard supportive therapy. | HIG favored resolution of diarrhea ($P$ = 0.078, OR = 65.3) and reduction in stool output, but showed no effects on total resolution time, length of hospital stay and cost of hospitalization. | HIG treatment potentially favoured diarrhea resolution and the reduction in stool output, but failed to affect resolution time, length of hospital stay and hospital cost. |
| | Chagla *et al* [6] | An adult $NoV^+$ patient undergoing RTx-PTx received enteral HIG at 45 mg/kg every 6 hours for 2 days. | Diarrhea resolved over the next 24 hours. | |
| | Florescu *et al* [10] | In two cases of NVGE in SBTx recipients, one case recieved oral HIG 25 mg/kg every 6 hours for 2 days; while the other received enteric HIG 25 mg/kg every 6 hours for 2 days. | Diarrhea resolved within 2 days in both cases. | |
| | Gairard-Dory *et al* [11] | Twelve cases of NVGE in LTx recipients received 25 mg/kg of OHIG every 6 hours for 2 days. | 1. Eleven out of 12 patients were successfully treated, whereas 1 case was only mildly improved; 2. No obvious side effects were observed. | |
| | Ebdrup *et al* [18] | A HTx patient with devastating NoV diarrhea underwent HIG 45 mg/kg every 6 hours for 2 days through a duodenal tube. | Over a 3-day course of treatment, stool frequency became normal. | |

To be continued

| Treatment | Reference | Description | Outcome | Conclusion |
|---|---|---|---|---|
| OSDBI | Gelfand *et al* [17] | An adult RTx recipient experiencing a 5-month NoV diarrhea underwent OSDBI at 5 g twice daily. | Over one week of treatment, patient resolved diarrhea and gained weight with fecal negativity of NoV RNA. | OSDBI is safe, well tolerated and relatively cheaper compared to HIG, but controlled clinical studies and *in vitro* research are needed. |
| NTZ | Siddiq *et al* [23] | An adult $NoV^{+}$ HSCT recipient underwent oral administration of NTZ 500 mg twice daily. | A 7-day course of NTZ facilitated the clinical resolution of NVGE, but the patient persistently and asymptomatically shed NoV in the stool. | NTZ is an effective and promising alternative for treating NVGE in transplant recipients, but further detailed research about safety and effectiveness is needed. |
| | Haubrich *et al* [24] | A pediatric KTx recipient experienced long-term and recurrent NVGE and received NTZ 500 mg orally twice daily for 14 days. | 1. A 6-day course for NTZ completeley cleared NoV from the patient; 2. No adverse effects were observed. | |
| | Gorgeis *et al* [25] | Five HSCT patients presenting with NVGE were treated with NTZ 500 mg given orally bid for a course of 3 to 18 days. | 1. Three out of 5 patients had complete resolution of NVGE; 2. One patient remained symptomatic. | |
| | Morris *et al* [26] | Ten patients infected with NoV after HSCT were empirically treated with NTZ. | All patients showed improved clinical symptoms of diarrhea, nausea, and abdominal pain in 2 to 4 days (a median 2 of days). | |

Abbreviation: IS, immunosuppression; NVGE, norovirus gastroenteritis; NTZ, nitazoxanide; NoV, norovirus; HIG, human immunoglobulin; OSDBI, oral serum-derived bovine immunoglobulin.

# REFERENCES


1. Gallimore CI, Lewis D, Taylor C, Cant A, Gennery A, Gray JJ. Chronic excretion of a norovirus in a child with cartilage hair hypoplasia (CHH). *J Clin Virol* 2004;**30**(2):196-204.
2. Roddie C, Paul JP, Benjamin R et al. Allogeneic hematopoietic stem cell transplantation and norovirus gastroenteritis: a previously unrecognized cause of morbidity. *Clin Infect Dis* 2009;**49**(7):1061-1068.
3. Westhoff TH, Vergoulidou M, Loddenkemper C et al. Chronic norovirus infection in renal transplant recipients. *Nephrol Dial Transplant* 2009;**24**(3):1051-1053.
4. Bonani M, Pereira RM, Misselwitz B, Fehr T, Wuthrich RP, Franzen D. Chronic Norovirus Infection as a Risk Factor for Secondary Lactose Maldigestion in Renal Transplant Recipients: A Prospective Parallel Cohort Pilot Study. *Transplantation* 2017;**101**(6):1455-1460.
5. Chehade H, Girardin E, Delich V, Pascual MA, Venetz JP, Cachat F. Acute norovirus-induced agranulocytosis in a pediatric kidney transplant recipient. *Transpl Infect Dis* 2012;**14**(4):E27-29.
6. Chagla Z, Quirt J, Woodward K, Neary J, Rutherford C. Chronic norovirus infection in a transplant patient successfully treated with enterally administered immune globulin. *J Clin Virol* 2013;**58**(1):306-308.
7. Chong PP, van Duin D, Sonderup JL, Grant WJ, Kalil AC, Florescu DF. Predictors of persistent diarrhea in norovirus enteritis after solid organ transplantation. *Clin Transplant* 2016;**30**(11):1488-1493.
8. Echenique IA, Stosor V, Gallon L, Kaufman D, Qi C, Zembower TR. Prolonged norovirus infection after pancreas transplantation: a case report and review of chronic norovirus. *Transpl Infect Dis* 2016;**18**(1):98-104.
9. Florescu DF, Hermsen ED, Kwon JY et al. Is there a role for oral human immunoglobulin in the treatment for norovirus enteritis in immunocompromised patients? *Pediatr Transplant* 2011;**15**(7):718-721.
10. Florescu DF, Hill LA, McCartan MA, Grant W. Two cases of Norwalk virus enteritis following small bowel transplantation treated with oral human serum immunoglobulin. *Pediatr Transplant* 2008;**12**(3):372-375.
11. Gairard-Dory AC, Degot T, Hirschi S et al. Clinical usefulness of oral immunoglobulins in lung transplant recipients with norovirus gastroenteritis: a case series. *Transplant Proc* 2014;**46**(10):3603-3605.
12. Hoffmann D, Hutzenthaler M, Seebach J et al. Norovirus GII.4 and GII.7 capsid sequences undergo positive selection in chronically infected patients. *Infect Genet Evol* 2012;**12**(2):461-466.
13. Jurgens PT, Allen LA, Ambardekar AV, McIlvennan CK. Chronic Norovirus Infections in Cardiac Transplant Patients. *Prog Transplant* 2017;**27**(1):69-72.
14. Karandikar UC, Crawford SE, Ajami NJ et al. Detection of human norovirus in intestinal biopsies from immunocompromised transplant patients. *J Gen Virol* 2016;**97**(9):2291-2300.
15. Lee BE, Pang XL, Robinson JL, Bigam D, Monroe SS, Preiksaitis JK. Chronic norovirus and adenovirus infection in a solid organ transplant recipient. *Pediatr Infect Dis J* 2008;27(4):360-362.
16. Kaufman SS, Chatterjee NK, Fuschino ME et al. Calicivirus enteritis in an intestinal transplant recipient. *Am J Transplant* 2003;**3**(6):764-768.
17. Gelfand MS, Cleveland KO. Oral Serum-Derived Bovine Immunoglobulin for Management of Infectious Diarrhea due to Norovirus and Cryptosporidiosis in Solid Organ Transplant Patients. *Infect Dis Clin Prac* 2017;**25**(4):218-220.
18. Ebdrup L, Bottiger B, Molgaard H, Laursen AL. Devastating diarrhoea in a heart-transplanted patient. *Journal of Clinical Virology* 2011;**50**(4):263-265.
19. Boillat Blanco N, Kuonen R, Bellini C et al. Chronic norovirus gastroenteritis in a double hematopoietic stem cell and lung transplant recipient. *Transpl Infect Dis* 2011;**13**(2):213-215.

20. Ueda R, Fuji S, Mori S et al. Characteristics and outcomes of patients diagnosed with norovirus gastroenteritis after allogeneic hematopoietic stem cell transplantation based on immunochromatography. *Int J Hematol* 2015;**102**(1):121-128.

21. Schwartz S, Vergoulidou M, Schreier E et al. Norovirus gastroenteritis causes severe and lethal complications after chemotherapy and hematopoietic stem cell transplantation. *Blood* 2011;**117**(22):5850-5856.

22. Engelen MA, Gunia S, Stypmann J. Elimination of norovirus in a chronic carrier under immunosuppression after heart transplantation--effect of everolimus. *Transpl Int* 2011;**24**(11):e102-103.

23. Siddiq DM, Koo HL, Adachi JA, Viola GM. Norovirus gastroenteritis successfully treated with nitazoxanide. *J Infect* 2011;**63**(5):394-397.

24. Haubrich K, Gantt S, Blydt-Hansen T. Successful treatment of chronic norovirus gastroenteritis with nitazoxanide in a pediatric kidney transplant recipient. *Pediatr Transplant* 2018:e13186.

25. Turcios RM, Widdowson MA, Sulka AC, Mead PS, Glass RI. Reevaluation of epidemiological criteria for identifying outbreaks of acute gastroenteritis due to norovirus: United States, 1998-2000. *Clin Infect Dis* 2006;**42**(7):964-969.

26. Morris J, Morris C. Nitazoxanide Is Effective Therapy for Norovirus Gastroenteritis after Chemotherapy and Hematopoietic Stem Cell Transplantation (HSCT). *Biol Blood Marrow Tr* 2015;**21**(2):S255-S256.

# Chapter 3

## Inhibition of Calcineurin or IMP Dehydrogenase Exerts Moderate to Potent Antiviral Activity against Norovirus Replication

Wen Dang,[a] Yuebang Yin,[a] Yijin Wang,[a] Wenshi Wang,[a] Junhong Su,[b] Dave Sprengers,[a] Luc J. W. van der Laan,[c] Krzysztof Felczak,[d] Krzysztof W. Pankiewicz,[d] Kyeong-Ok Chang,[e] Marion P. G. Koopmans,[f] Herold J. Metselaar,[a] Maikel P. Peppelenbosch[a] and Qiuwei Pan[a]

[a]*Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam, Netherlands.*

[b]*Medical Faculty, Kunming University of Science and Technology, Kunming, PR China.*

[c]*Department of Surgery, Erasmus MC-University Medical Center, Rotterdam, Netherlands.*

[d]*Center for Drug Design, University of Minnesota, Minneapolis, USA.*

[e]*Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, USA.*

[f]*Department of Viroscience, Erasmus MC-University Medical Center, Rotterdam, Netherlands*

***Antimicrobial Agents and Chemotherapy 2017. 61:e01095-17***

## ABSTRACT

Norovirus is a major cause of acute gastroenteritis worldwide and has emerged as an important issue of chronic infection in transplantation patients. Since no approved antiviral is available, we evaluated the effects of different immunosuppressants and ribavirin on norovirus and explored their mechanisms of action by using a human norovirus (HuNV) replicon-harboring model and a surrogate murine norovirus (MNV) infectious model. The roles of the corresponding drug targets were investigated by gain- or loss-of-function approaches. We found that the calcineurin inhibitors cyclosporin A (CsA) and tacrolimus (FK506) moderately inhibited HuNV replication. Gene silencing of their cellular targets, cyclophilin A, FKBP12, and calcineurin, significantly inhibited HuNV replication. A low concentration, therapeutically-speaking, of mycophenolic acid (MPA), an uncompetitive IMP dehydrogenase (IMPDH) inhibitor, potently and rapidly inhibited norovirus replication and ultimately cleared HuNV replicons without inducible resistance following long-term drug exposure. Knockdown of the MPA cellular targets IMPDH1 and IMPDH2 suppressed HuNV replication. Consistent with the nucleotide synthesizing function of IMPDH, exogenous guanosine counteracted the antinorovirus effects of MPA. Furthermore, the competitive IMPDH inhibitor ribavirin efficiently inhibited norovirus and resulted in an additive effect when combined with immunosuppressants. The results from this study demonstrate that calcineurin phosphatase activity and IMPDH guanine synthase activity are crucial in sustaining norovirus infection; thus, they can be therapeutically targeted. Our results suggest that MPA shall be preferentially considered immunosuppressive medication for transplantation patients at risk of norovirus infection, whereas ribavirin represents as a potential antiviral for both immunocompromised and immunocompetent patients with norovirus gastroenteritis.


**KEYWORDS:** norovirus, mycophenolic acid, calcineurin inhibitors, ribavirin, cell culture model, cell culture, noroviruses.

# INTRODUCTION

Following widespread implementation of rotavirus vaccination, norovirus infection is now becoming the major cause of acute gastroenteritis worldwide [1]. As a single-strand positive-RNA virus belonging to the family *Caliciviridae,* norovirus is classified into 6 distinct genogroups (GI to GVI). Genogroup 2, genotype 4 (GII.4) is the most prevalent, accounting for 96% of all sporadic infections [2]. Although norovirus infection is often self-limiting in adults, every year, it is estimated that noroviruses resulted in up to 200,000 deaths in children below 5 years of age, mainly in developing countries [3]. It is recently estimated that norovirus causes more than 200,000 deaths across all ages per year worldwide, of which more than 90,000 are in children under five [4].

Accumulating evidence indicates that transplant recipients are highly susceptible to norovirus infection, irrespective of their status as a pediatric or adult patient [5, 6]. Norovirus infection has been described in many types of transplant recipients, including those receiving orthotopic transplantation of lung [7], kidney [8-10], liver [11], heart [12, 13], renal [14-16], intestine [17-19], small bowel [20], or hematopoietic stem cells [21-24]. Norovirus infection in such patients often resulted in severe clinical pathology with prolonged illness [21, 25]. Although the exact mechanism of norovirus susceptibility in orthotopic transplant recipients remains unclear, the use of immunosuppressants for preventing rejection is conceivably an important risk factor [5, 26]. It is plausible that an immunosuppressed status weakens host immunity defending virus invasion. Therefore, a cautious reduction and withdrawal of immunosuppressants has been taken into consideration for managing chronic norovirus gastroenteritis in transplant recipients [27]. Intriguingly, immunosuppressants have been reported to directly modulate viral infection [28], but the direct effects on norovirus infection remain to be investigated.

Despite its significant impact on public health, no vaccination or specific antiviral treatment is available. Ribavirin has broad antiviral activity against many viruses *in vitro* and has been approved for treating chronic hepatitis C virus (HCV) patients for decades. A recent study reported that chronic norovirus infection in two patients with common variable immunodeficiency (CVID) was successfully treated by oral ribavirin, indicating the use of ribavirin as a potential therapy against norovirus infection [29], although the definitive effect requires further evaluation.

In this study, we profiled the effects of different immunosuppressants on norovirus in cell culture models. We reveal that cellular calcineurin and IMPDH enzymes are essential factors supporting norovirus replication, which could be targeted by the corresponding immunosuppressants, calcineurin inhibitors and mycophenolic acid (MPA), to exert antinorovirus effects. Furthermore, we demonstrate that ribavirin, a general antiviral drug of a non-immunosuppressive IMPDH inhibitor, has potent antinorovirus effects and exerts an additive effect when combined with calcineurin inhibitors or MPA. These results provide an important reference for managing immunosuppression and antiviral treatment for transplantation patients infected by norovirus or at risk of norovirus infection.

# MATERIALS AND METHODS

## *Reagents*

The immunosuppressants, including dexamethasone (DEX; Chemical Abstract Service [CAS] no. 50-02-2), prednisolone (PRED; CAS no. 50-24-8), rapamycin (RAP; CAS no. 53123-88-9), leflunomide (LEF; CAS no. 75706-12-6), brequinar (BQR) sodium salt hydrate (Method Detection Limit [MDL] no. MFCD21363375), and mycophenolic acid (MPA; CAS no. 24280-93-1) were purchased from Sigma-Aldrich (St Louis, MO). The calcineurin inhibitors (CNIs) cyclosporine (CsA; CAS no. 59865-13-3) and tacrolimus (FK506; CAS no. 104987-11-3) were obtained from Bio-Connect (Huizen, The Netherlands) and Abcam (Cambridge, MA), respectively. Two nonimmunosuppressive CsA derivatives, 431-32 and 440-02, together with a novel calcineurin inhibitor, voclosporin, were provided by the Isotechnika Pharma Incorporation (Edmonto, AB, Canada). All the compounds were dissolved in dimethyl sulfoxide (DMSO) and stored in aliquots at -20℃ before use.

Primary antibodies targeting cyclophilin A (CyPA; polyclonal rabbit, 1:1,000 dilution; Abcam), cyclophilin B (CyPB; polyclonal rabbit, 1:1,000 dilution; Abcam), IMPDH1 (polyclonal rabbit, ab33039, 1:1,000 dilution; Abcam), IMPDH2 (monoclonal rabbit, ab131158, 1:1,000 dilution; Abcam), FKBP12 (polyclonal rabbit, sc-28814, 1:500 dilution; Santa Cruz), and *β*-actin (monoclonal mouse, 1:1,000 dilution; Santa Cruz) were used. Secondary antibodies IRDye® 800CW-conjugated goat anti-rabbit and goat anti-mouse IgGs (1:10,000 dilution; Li-Cor Bioscience, Lincoln, USA) were used, as appropriate.

### *MTT assay*

The cytotoxicities of the compounds on host cells were determined by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. In brief, cells were seeded into 96-well tissue culture plates containing 0.5% DMSO (control) or increasing concentrations of drugs. After the time indicated, 10 mM MTT (Sigma, Zwijndrecht, The Netherlands) was added. With another 3 hour of incubation, the medium was removed and 100 $\mu$L of DMSO was added to each well. The plate was incubated at 37℃ for 50 min. The absorbance at 490 nm was recorded on the microplate absorbance reader (Bio-Rad, CA, USA).

### *Cell cultures and virus propagation*

HG23 (Huh7 cells containing a stable subgenomic HuNV replicon), RAW 264.7, and human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza Verviers, Belgium) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT, USA). A marker cassette containing the neomycin phosphotransferase gene was inserted between nucleotides (nt) 5456 and 6753 of open reading frame 2 (ORF2) of HuNV, conferring HG23 resistance to neomycin. Gentamicin (G418; Gibco) was added to HG23 culture medium at 1.5 mg/mL for selection before experimentation.

The Murine norovirus 1 (MNV-1) [30] was produced by consecutively inoculating MNV-1 (kindly provided by Herbert Virgin, IV, Department of Pathology and Immunology, Washington University School of Medicine) into RAW 264.7 cells. After 4 consecutive passages, the MNV-1 cultures were purified, aliquoted, and stored at -80℃ for all subsequent experiments. The MNV-1 stock was quantified three independent times by the 50% tissue culture infective dose ($TCID_{50}$).

### *TCID50*

MNV-1 was quantified by $TCID_{50}$ assay. Briefly, 10-fold dilutions of MNV-1 were inoculated into RAW cells grown in 96-well tissue culture plate at 1,000 cells/well. The plate was incubated at 37℃ for another 5 days, followed by observing the cytopathic effect (CPE) of each well under a light scope. The $TCID_{50}$ was calculated by using the Reed-Muench method.

### *Quantitative real-time polymerase chain reaction*

Total RNA was isolated with a Macherey NucleoSpin RNA II Kit (Bioke, Leiden, The Netherlands) and measured with a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was reverse transcribed from 500 ng of RNA using a cDNA synthesis kit (TaKaRa Bio, Inc.). The cDNA of a targeted gene transcript was amplified for 50 cycles and quantified with a SYBR Green-based real-time PCR (Applied Biosystems), according to the manufacturer's instructions. All the PCRs were performed in duplicate, and amplification specificity was confirmed by melting-curve analysis. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and murine GAPDH genes were used as reference genes. The relative expression of targeted gene was calculated as $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_{T\text{sample}} - \Delta C_{T\text{control}}$ ($\Delta C_T = C_{T[\text{targeted gene}]} - C_{T[\text{GAPDH}]}$). All primer combinations are listed in Table S1.

### *Antiviral assay with HuNV replicon model*

Before the experiments, HG23 cells were cultured overnight without G418. The next day, HG23 cells were seeded into 48-well tissue culture plates at $5 \times 10^4$ cells per well and treated with 0.5% DMSO (control) or drugs. After 48 hours of treatment, total RNA was extracted from cells, and HuNV replication was determined by qRT-PCR analysis.

### *Antiviral assay with MNV-1*

The antiviral assay with MNV-1 was initiated by inoculating MNV-1 into RAW cells at a multiplicity of infection (MOI) of 0.1. After 1 hour of infection, cells were washed with phosphate-buffered saline (PBS) for 4 times to remove free virus and were subsequently treated with 0.5% DMSO (control) or drugs. After 24 hours of treatment, extracellular RNA and intracellular RNA were extracted from the cell culture supernatant (100 $\mu$L) and cell layer, respectively. The relative intracellular MNV-1 RNA level was normalized to murine GAPDH and calculated with the $2^{-\Delta\Delta CT}$ method. For absolute quantification of MNV-1 RNA in the supernatant, the virus genome copy number was detected using qRT-PCR. In brief, a cDNA-containing target sequence positioned between nt 4972 and 5064 in MNV-1 was amplified, purified, and 10 times serially diluted. The dilutions were quantified by qRT-PCR to generate a standard curve, which was expressed by plotting the log copy numbers against the cycle threshold ($C_T$) value (Fig. S1). The viral genome copy numbers in the MNV-1 culture were calculated by comparing the $C_T$ with that of the standard curve.

MNV-1 could induce cytopathic effect (CPE) in RAW cells [30]. The antiviral activity of drugs on MNV-1 was further verified by using an MTT-based CPE inhibition assay. Briefly, MNV-infected RAW cells were seeded into 96-well tissue culture plates at 10,000 cells/well. After 72 hours of treatment with 0.5% DMSO or drugs, more than 95% CPE was observed in the control well. Then, an MTT assay was initiated, and the absorbance (optical density [OD]) at 490 nm was recorded. CPE inhibition was calculated as $[(OD_{treated})_{MNV} - OD_{VC}]/[OD_{cc} - OD_{vc}]$, where $(OD_{treated})_{MNV}$ represented the OD of virus-infected cells treated with drugs, while $OD_{cc}$ and $OD_{vc}$ represented the OD of the cell control and virus control, respectively.

## *Clearance and rebound assay*

A clearance and rebound assay was performed according to the procedure described by Joana *et al.*, with modifications [31]. In brief, HG23 cells were seeded into 48-well tissue culture plates at $2.5 \times 10^4$ cells per well and treated with increasing concentration of drugs but no G418. After 2 days of treatment, the cells were trypsinized, $2.5 \times 10^4$ cells were sub-cultured into 48-well tissue culture plates containing the same concentration of drugs for another 4 days of treatment and $2 \times 10^4$ cells were collected for qRT-PCR analysis. After 6 days of treatment, the same treatment process was performed for the second time. After 10 days of treatment, $2.5 \times 10^4$ cells were subcultured into a 48-well tissue culture plate with fresh medium containing G418 (1.5 mg/mL). With another 5 days of culture, the cell layers were stained with hematoxylin and eosin, and were visualized with an inverted light microscope. For HuNV RNA level quantification, each passage of drug-treated cells was compared to 0.5% DMSO-treated control cells with the same passage number.

## *In vitro selection of MPA-resistant MNV*

MPA-resistant MNV-1 was selected by culturing MNV-1 for 20 passages under antiviral pressure. Briefly, MNV-infected RAW (MOI, 0.1) cells were treated with 0.5% DMSO or MPA (0.1 or 0.5 $\mu$g/mL). After 24 hours of incubation, the MNV-1 cultures were collected by freezing-thawing process and titrated by qRT-PCR for use in the next passage. After 10 passages, MNV-1 was continuously exposed to the same MPA concentrations for another 10 passages, and MNV-1 was exposed to increased MPA concentrations for another 10 passages. When MNV-1 serial passaging with MPA was done, the inhibitory effect of MPA on 20th-passage MNV-1 was quantified by qRT-PCR.

### *Short-hairpin RNA delivery by lentiviral transduction*

Gene knockdown was performed as described previously [32]. In brief, pLKO.1-based vectors containing the RNA interference sequences were obtained from the Biomics Center at the Erasmus Medical Center. The lentiviral vectors were generated in HEK293T cells and condensed by high-speed centrifugation (if indicated). Transduction was initiated by inoculating short-hairpin RNA (shRNA) vectors into HG23 cells. As the vectors also express a puromycin resistance gene, following 3 days of transduction, cells were selected with 3 $\mu$g/mL puromycin (Sigma-Aldrich). A validated nonsilencing scrambled vector (shCTR) was used as a control. All the shRNA vector sequences are listed in Table S2. For simultaneous knockdown, HG23 cells were transduced with a first lentiviral vector. After selection with puromycin, HG23 cells were subsequently transduced with a second condensed lentiviral vector. The knockdown efficacy and specificity was confirmed by qRT-PCR and Western blot analysis.

### *Western blotting*

Cell samples were lysed and loaded onto a 10 to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis at 120 V for 100 min, the proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (pore size, 0.45 $\mu$M; Invitrogen) for 1.5 h, with an electric current of 250 mA. The membrane was probed with primary antibody plus secondary antibody and detected with Odyssey 3.0 infrared imaging system (Li-Cor Biosciences). Beta-actin served as a standardization for sample loading.

### *Synergy analysis*

To evaluate the interaction of ribavirin and immunosuppressants on norovirus replication, MacSynergy 2 (kindly provided by Mark Prichard) [33], a mathematical model based on the Bliss Independence theory, was employed to analyze the data from 48 hours of combined treatment of ribavirin and immunosuppressants on an HuNV model. In the MacSynergy model, the theoretical additive effect with two compounds could be calculated using the equation Z = X + Y (1 - X), where X and Y represent the inhibition produced by the individual drugs, and Z represents the theoretical effect produced by the combination of two drugs. The theoretical additive surface is subtracted from the actual experimental surface.

When a combination is additive, the data points of the calculated surface lie in the zero plane. A surface that lies >20% above the zero plane indicates a synergistic effect of the combination, and a surface >20% below the zero plane indicates antagonism. The 95% confidence interval was considered to be statistically significant.

### *Statistics*

Data are presented as mean ± standard error of the mean (SEM). Comparisons between groups were performed with Mann-Whitney test using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered significant at a *P* value of <0.05. The 50% cytotoxic concentration ($CC_{50}$) and 50% inhibitory concentration ($IC_{50}$) were determined by fitting the data with a variable-slope dose response-inhibition nonlinear regression equation using GraphPad Prism (Table S3).

## RESULTS

### *Not all immunosuppressants directly affected norovirus replication*

A major challenge in antinorovirus drug development is the lack of an infectious human isolate that recapitulates the entire life cycle of the virus in a cell culture model. Therefore, we used an HuNV subgenomic replicon model that closely mimicked viral replication in the absence of the production of infectious particles [34]. In our study, we observed that glucocorticoids, including prednisone (see Fig. S2A in the supplementary material) and dexamethasone (Fig. S2B), rapamycin (Fig. S2C), brequinar (Fig. S2D), and leflunomide (Fig. S2E) showed no effect on HuNV replication, demonstrating that modulation of norovirus infection is not a general property of immunosuppressants.

### *CsA and its immunosuppressive analogue, voclosporin, inhibited HuNV replication through a CyPA-dependent mechanism*

CsA is a calcineurin inhibitor targeting cellular cyclophilins. In the HuNV model, treatment with CsA (5 $\mu$g/mL) for 48 hours inhibited HuNV replication by 82 ± 3.3% ($P < 0.05$, $n = 6$; Fig. 1A). To further confirm that this inhibitory effect relates to the compound targeting cyclophilins, a series of CsA derivatives were tested. Voclosporin, a novel immunosuppressive cyclophilin inhibitor, diminished cellular HuNV RNA levels by 49 ± 10% ($P < 0.05$, $n = 6$; Fig. 1B) after 48 hours of treatment, even at the low concentration of 1

$\mu$g/mL, whereas the parental compound CsA at this concentration had no effect on HuNV. This observation relates well with the notion that voclosporin is a more potent calcineurin inhibitor than CsA [35]. Two nonimmunosuppressive CsA derivatives, 431-32 and 440-02, which are chemically similar to CsA, if functionally distinct, had no effect on HuNV replication ($P > 0.05$, $n = 4$; Fig. 1C), suggesting that the effects of CsA analogues on norovirus replication are related to calcineurin inhibition.

It is necessary to determine whether long-term exposure to these immunosuppressants completely eliminated HuNV replicons from host cells. For this purpose, a clearance and rebound assay was employed. If the replicons are completely cleared, the cells could not survive and proliferate in the presence of G418. If the cells still carry replicons, they will proliferate. CsA at 5 $\mu$g/mL caused a reduction in HuNV RNA level levels by 79 ± 3.8% ($P < 0.05$, $n = 6$; Fig. 1D) after 10 days of treatment. Consistently, CsA-treated cells survived and proliferated in the presence of the selection marker (Fig. 1E), demonstrating that although CsA inhibited HuNV replication, it did not completely cause elimination of all replicons from cells. Voclosporin at 2.5 $\mu$g/mL provoked potent inhibition of HuNV replication, as evidenced by a reduction of 91 ± 1.5% ($P < 0.05$, $n = 6$; Fig. 1F) in viral RNA. Following subsequent challenge with the selection marker, only a small portion of cells stayed alive (Fig. 1G), showing that voclosporin treatment cleared most HuNV replicons from cultures, but a replicon-containing compartment remained.

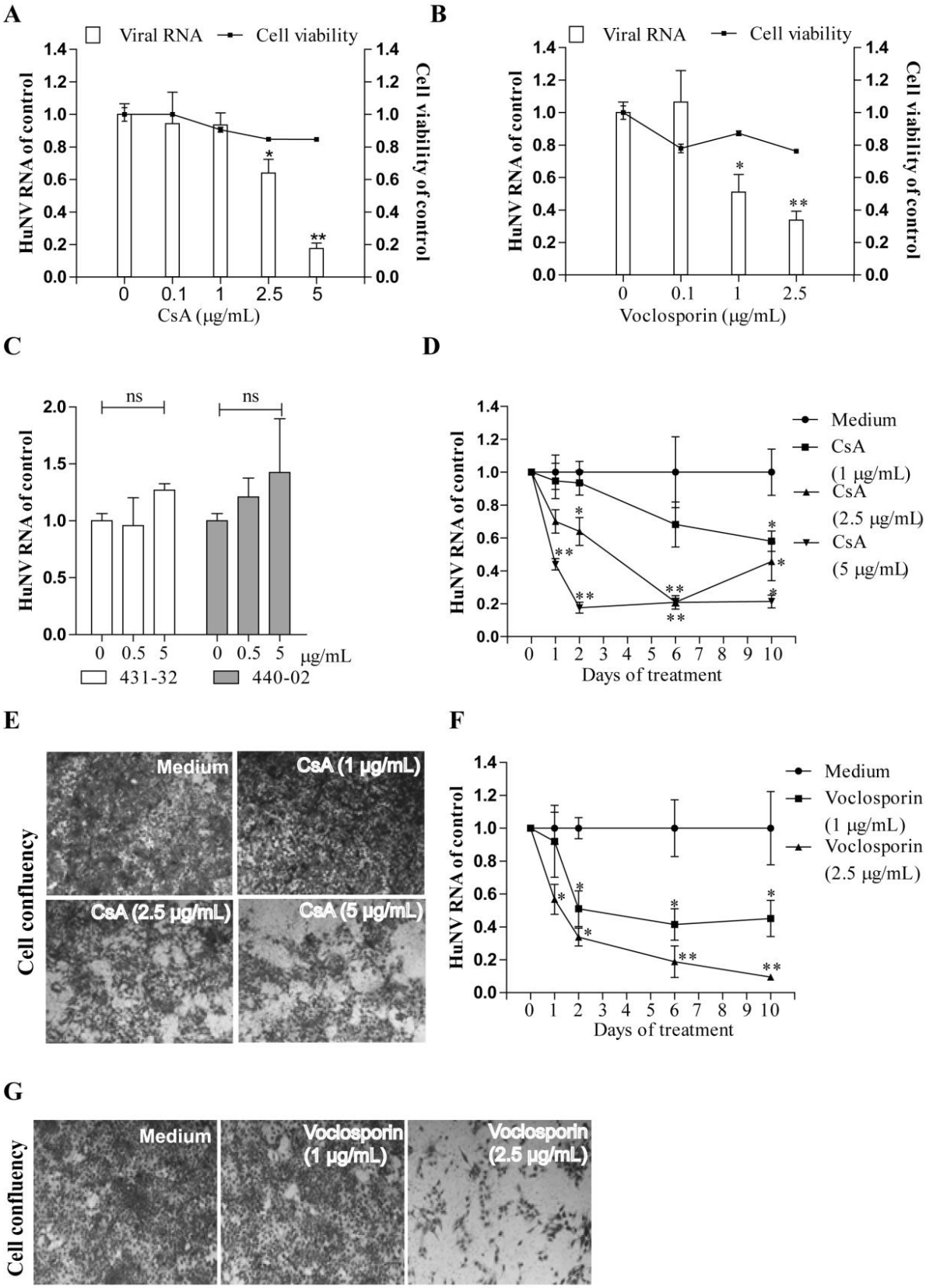



**Figure 1 Antiviral activities of CsA and its derivatives against HuNV replication.** CsA (A) and its immunosuppressive derivative voclosporin (B) dose-dependently inhibited HuNV replication without potent toxicity to host cells after 48 hours of treatment. The level of HuNV RNA was quantified by qRT-PCR and was compared to that in cells treated with 0.5% DMSO (control) (*n* = 3 independent experiments with 2 replicates each). (C) Its nonimmunosuppressive derivatives 431-32

and 440-02 failed to inhibit HuNV replication after 48 hours of treatment, even at the highest concentration tested ($n$ = 2 independent experiments with 2 replicates each). (D and F) Clearance assay with CsA and voclosporin. HG23 cells were treated with CsA or voclosporin for 1, 2, 6 or 10 days. At the end of each treatment period, the level of HuNV RNA was determined by qRT-PCR and normalized to that of the control cells from the same treatment time ($n$ = 3 independent experiments with 2 replicates each). (E and G) Rebound assay with CsA and voclosporin. After 10 days of treatment, drugs were omitted, and HG23 cells (48-well tissue culture plate with $2.5 \times 10^4$ cells per well) were cultured under the selective pressure of G418 (1.5 mg/mL). With another 5 days of culture, the cell layers were stained with hematoxylin and eosin, and visualized by an inverted light microscope. Images are representative of three independent experiments with 2 replicates each. Data are presented as the means ± SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant).

Cellular cyclophilins, in particular the cyclophilin A (CyPA) and cyclophilin B (CyPB) isoforms, are the canonical drug targets of CsA. To study the potential involvement of CyPA and CyPB in CsA-caused inhibition of HuNV replication, an RNA interference (RNAi)-mediated loss-of-function assay was performed. Upon transduction of lentiviral vectors targeting CyPA (shCyPA) or CyPB (shCyPB), specific downregulation of the target genes at the mRNA level was observed at both day 6 and day 10 posttransduction, compared to a scrambled control (shCTR) (Fig. 2A and B). Downregulation at the protein level was observed only at day 10 (Fig. 2C). Consistently, following knockdown, no effect on HuNV was observed at day 6, but a significant reduction in viral replication by 60 ± 5.7% ($P < 0.05$, $n$ = 8; Fig. 2D) was observed by knockdown of CyPA, but not that of CyPB, at day 10 posttransduction. Notably, knockdown of CyPA also resulted in an elevation of CyPB mRNA expression at day 10 posttransduction and *vice versa*, suggesting that a compensatory mechanism may exist between CyPA and CyPB (Fig. 2A and B). Concomitant with CyPA knockdown, CsA ($P < 0.05$, $n$ = 5; Fig. 2E) and voclosporin ($P < 0.05$, $n$ = 5; Fig. 2F) lost their capacity to inhibit HuNV after 48 hours of treatment. Collectively, these results show that CsA and voclosporin inhibited HuNV replication through targeting CyPA, not CyPB.

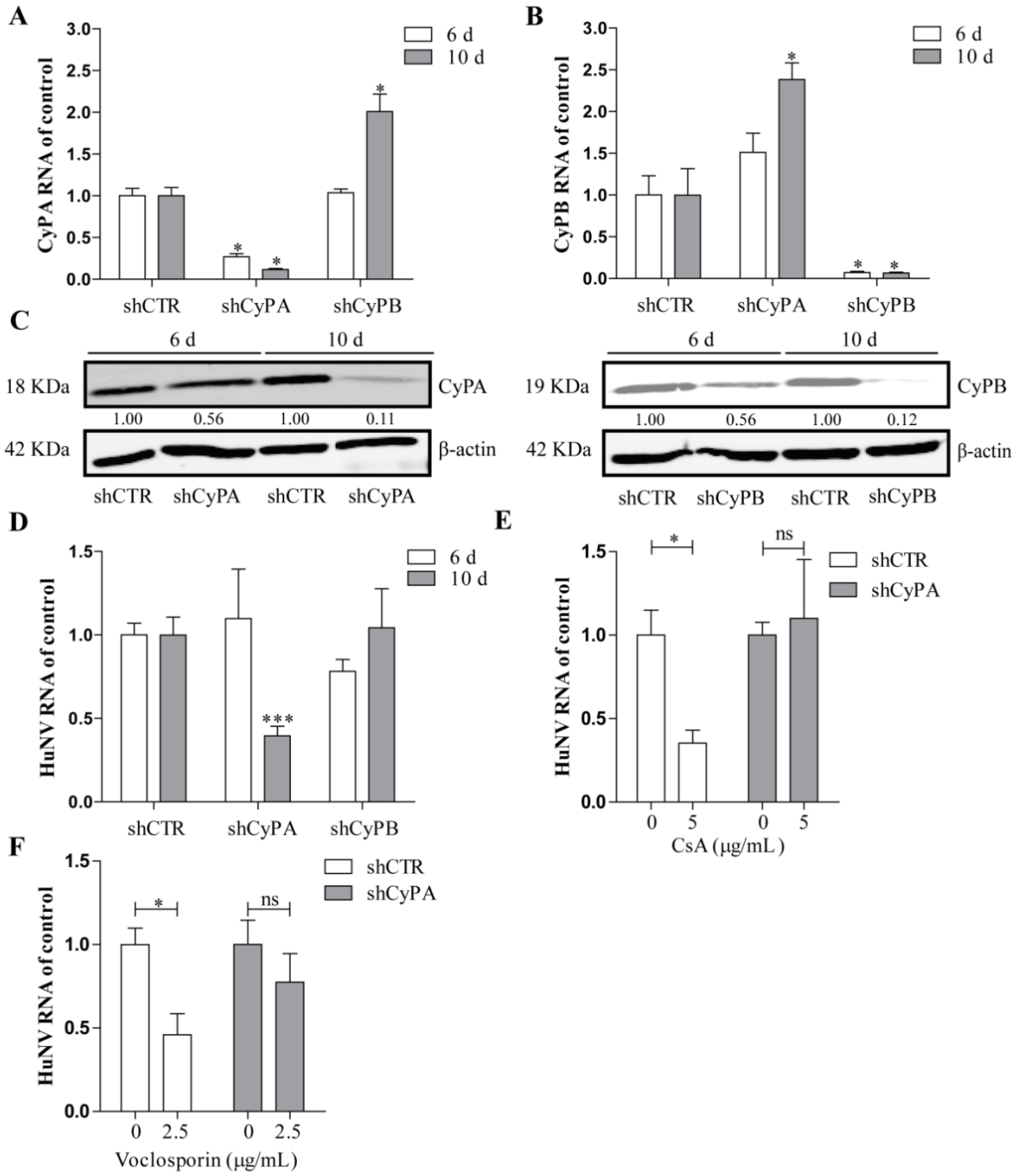



**Figure 2 CyPA, but not CyPB, is involved in CsA-caused inhibition of HuNV replication.** (A and B) Knockdown of CyPA and CyPB through lentiviral shRNA vectors. HG23 cells were transduced with shRNA vectors against CyPA (shCyPA), CyPB (shCyPB), or control (shCTR). At the indicated time points, the level of cyclophilin RNA was measured by qRT-PCR and compared to the control (*n* = 2 independent experiments with 2 replicates each). (C) Western blot analysis of CyPA and CyPB in HG23 cells transduced with shRNAs. Images are representative of three independent experiments. Transduction of CyPA and CyPB shRNAs resulted in a dramatic downregulation of CyPA and CyPB expression at day 10 (10 d) but not at day 6 (6 d) posttransduction. (D) qRT-PCR analysis of HuNV RNA in HG23 cells transduced with shRNAs. Knockdown of CyPA but not CyPB resulted in a significant decrease in viral replication at day 10 but not at day 6 posttransduction (*n* = 3 independent experiments with 2 to 3 replicates each). (E and F) Knockdown of CyPA abrogated the inhibition of HuNV replication by CsA and voclosporin. After successful knockdown of mRNA and protein of CyPA,

the responsiveness of HuNV to CsA and voclosporin treatment was detected after 48 hours of treatment. CsA and voclosporin possessed the anti-norovirus activity against shCTR-treated HG23 cells but failed to suppress HuNV replication in shCyPA HG23 cells. Data are presented as the means ± SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

## *FK506 moderately inhibited HuNV replication via targeting FKBP12*

FK506 (tacrolimus) is another calcineurin inhibitor. FK506 at 2.5 $\mu$g/mL caused a reduction in HuNV replication by 77 ± 5.0% ($P < 0.05$, $n = 6$) and 70 ± 8.3% ($P < 0.05$, $n = 6$) after short-term (2 days, Fig. 3A) and long-term (10 days, Fig. 3B) treatment, respectively. Of note, FK506 at high concentration of 2.5 $\mu$g/mL quickly inhibited HuNV replication being evident from effects even after 1 day of treatment. Similar to CsA analogues, FK506 did not completely clear HuNV replicons from the cultures (Fig. 3C).

FK506 suppresses the immune system by binding to FKBP12, resulting in inhibition of the phosphatase activity of calcineurin. To understand the role of FKBP12 in FK506-mediated inhibition of HuNV, we knocked down the expression of FKBP12. It is demonstrated that knockdown of FKBP12 (Western blot analysis in Fig. 3D and reverse transcription-quantitative PCR [qRT-PCR] analysis in Fig. S3), but not FKBP8 (a control; qRT-PCR analysis in Fig. S3) resulted in reduced HuNV replication (Fig. 3E). At the same time, FKBP12 knockdown partially abrogated FK506-mediated inhibition of HuNV ($P < 0.05$, $n = 8$; Fig. 3F), suggesting that FK506 inhibited HuNV replication through a pathway involving FKBP12. Thus, the calcineurin pathway appears to be essential for norovirus propagation, and experiments were initiated to confirm this notion.

## *Calcineurin activation is indispensable for efficient replication of norovirus*

Calcineurin inhibitors are effective against norovirus replication, whereas nonimmunosuppressive CsA derivatives are ineffective, suggesting that calcineurin activity is required for CsA and FK506-mediated inhibition of norovirus. To confirm this hypothesis, we used RNAi to knock down protein phosphatase 3, catalytic subunit, alpha isozyme (PPP3CA) to inhibit calcineurin phosphatase activity. After successful knockdown of PPP3CA (Fig. 3G), it is shown that HuNV replication was inhibited (Fig. 3H). Thus, calcineurin activation is vital for norovirus replication, supporting the idea that CsA and FK506 exerted anti-norovirus activity through the inhibition of calcineurin.

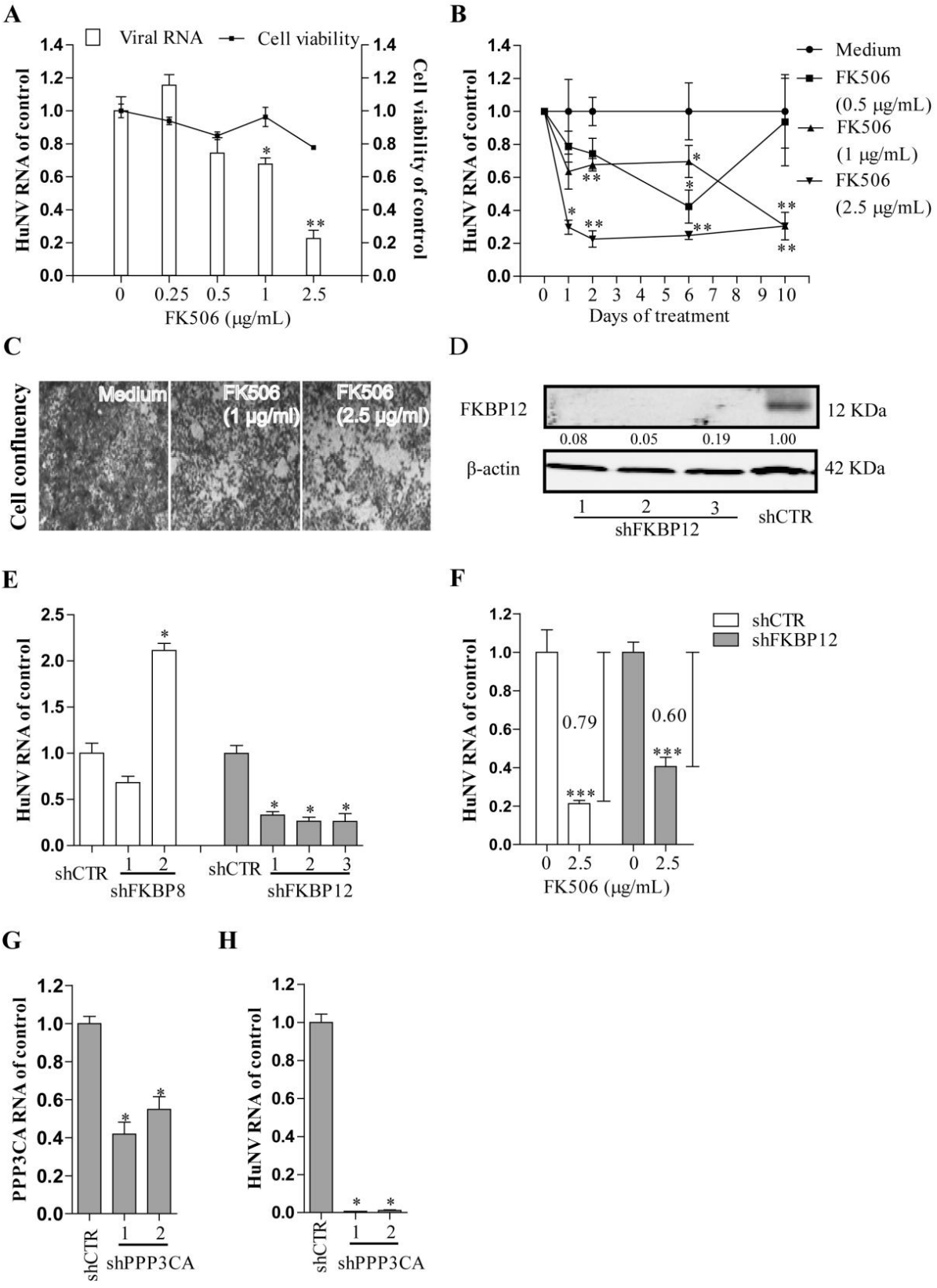



**Figure 3 FK506 moderately inhibited HuNV replication through FKBP12 and calcineurin.** (A) Concentration-dependent antiviral effects of FK506 on HuNV replication after 48 hours of treatment (*n* = 3 independent experiments with 2 replicates each). (B) Clearance assay with FK506. Long-term exposure to FK506 resulted in a moderate reduction of HuNV replication (*n* = 3 independent experiments with 2 replicates each). (C) Rebound assay with FK506. After long-term clearance phase, HG23 cells were cultured in the presence of G418 for another 5 days. Reduced cell confluency was

observed, indicating that FK506 partially cleared the HuNV replicons from host cells. (D) Western blot analysis of FKBP12 knockdown by lentiviral shRNA vectors. Images are representative of three independent experiments. (E) qRT-PCR analysis of HuNV RNA level in shCTR, shFKBP8, and shFKBP12 cells after transduction of lentiviral shRNA vectors for 10 days. FKBP12 but not FKBP8 knockdown inhibited HuNV replication ($n$ = 2 independent experiments with 2 replicates each). (F) FKBP12 knockdown partially blocked FK506-induced inhibition of HuNV after 48 hours of treatment ($n$ = 3 independent experiments with 2 to 3 replicates each). (G) qRT-PCR analysis of calcineurin knockdown at the RNA level. The level of calcineurin subunit PPP3CA RNA was presented as the relative value to the shRNA control ($n$ = 2 independent experiments with 2 replicates each). (H) PPP3CA knockdown inhibited HuNV replication, as determined by qRT-PCR ($n$ = 2 independent experiments with 2 replicates each). Data are presented as the means ± SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

## *MPA potently inhibited norovirus replication and completely cleared HuNV replicons from host cells*

Examining the effects of MPA on HuNV replication, we observed that treatment with only 0.25 $\mu$g/mL of MPA, which was even lower than the blood concentration (approximately 1 to 10 $\mu$g/mL) in transplantation patients [36], potently inhibited HuNV replication by 79 ± 2.7% ($P < 0.05$, $n$ = 6; Fig. 4A) after 48 hours of treatment. Likewise, following long-term treatment with 0.5 $\mu$g/mL MPA (10 days; Fig. 4B), HuNV replication was reduced by 94 ± 1.7% ($P < 0.05$, $n$ = 6). Correspondingly, in the rebound experiment (Fig. 4C), MPA-treated cells completely lost the ability to proliferate and succumbed to the selection marker, suggesting that MPA treatment totally cleared replicons from the host cells. In apparent support, when we tested other 4 IMPDH inhibitors with differential inhibitory efficacies on IMPDH enzymatic activity (Ki values toward IMPDH1 and IMPDH2 are shown in the Table S4), all of them exerted significant anti-norovirus activities at a 1 $\mu$M concentration ($P < 0.05$, $n$ = 6; Fig. 4D).

In the face of a lack of a cell culture system for HuNV, we considered MNV a suitable surrogate for studying HuNV biology and pathogenesis. Consistent with the results in the replicon model, MPA at 0.1 $\mu$g/mL reduced MNV-1 cellular viral RNA by 83 ± 8% ($P < 0.05$, $n$ = 6 to 8; Fig. 4E) and virus production in the supernatant ($P < 0.05$, $n$ = 6; Fig. 4F) after 24 hours of treatment. The inhibitory effect was also confirmed in a cytopathic effect (CPE) inhibition assay, demonstrating that MPA at 0.1 $\mu$g/mL protected RAW cells from MNV-1 induced CPE formation by 57 ± 14% ($P < 0.05$, $n$ = 8; Fig. 4E). Collectively, our results demonstrated that MPA potently inhibited norovirus replication and could even completely clear the virus.

## *High barrier to resistance development*

In the HuNV replicon model, MPA completely cleared the viral replicons, which obviously prevented the emergence of resistance and thus leaves the question unanswered about whether norovirus develops resistance to MPA treatment. To address this issue, MNV-infected RAW cells were exposed to MPA in different setups for 20 passages. As shown in Fig. 4G, MNV-1 retained its sensitivity to MPA treatment, even at passage 20, with inhibitory efficacy varying from 98.5 ± 0.07% to 99.6 ± 0.07% following treatment with 0.5 $\mu$g/mL MPA, which was comparable to that in the unchallenged control (99.6 ± 0.05% inhibition). Thus, norovirus is not prone to developing resistance to MPA.

## *MPA inhibited norovirus replication by simultaneously targeting IMPDH1 and IMPDH2*

MPA acts through inhibition of IMPDH. There are two isoforms of IMPDH, IMPDH1 and IMPDH2, in mammals. MPA has a 5-fold more potent inhibitory effect on IMPDH2 than on IMPDH1 [33]. In view of the low concentrations of MPA required to counteract norovirus, IMPDH2 appears to be the more likely drug target with respect to its effects on the norovirus life cycle. However, knockdown of IMPDH2 had no significant effect on HuNV replication (Fig. 5C), even though such knockdown profoundly decreased IMPDH2 mRNA and protein levels, as assessed by qRT-PCR (Fig. S4) and Western blot (Fig. 5A). Similarly, knockdown of IMPDH1 decreased IMPDH1 expression at the RNA level (Fig. S4) and protein level (Fig. 5B) but caused no significant change in HuNV replication (Fig. 5C). Surprisingly, simultaneous knockdown of IMPDH1 and IMPDH2 (qRT-PCR analysis in Fig. S5 and Western blot analysis in Fig. 5D) suppressed HuNV replication by 64 ± 5.3% ($P < 0.05$, $n = 4$; Fig. 5E), showing the interchangeable roles of IMPDH1 and IMPDH2 on norovirus life cycle.

## *Guanosine restored norovirus replication in MPA-treated and IMPDH1/2 knockdown cells*

MPA inhibits *de novo* guanosine nucleotide biosynthesis by inhibiting IMPDH. To examine whether MPA exerts an inhibitory effect on norovirus via guanosine depletion, we challenged norovirus cultures either with only MPA or with a combination of MPA and guanosine (with guanosine at 1, 10 or 100 $\mu$g/mL concentrations). After 24 hours of incubation, norovirus replication was determined by qRT-PCR analysis. We observed that the

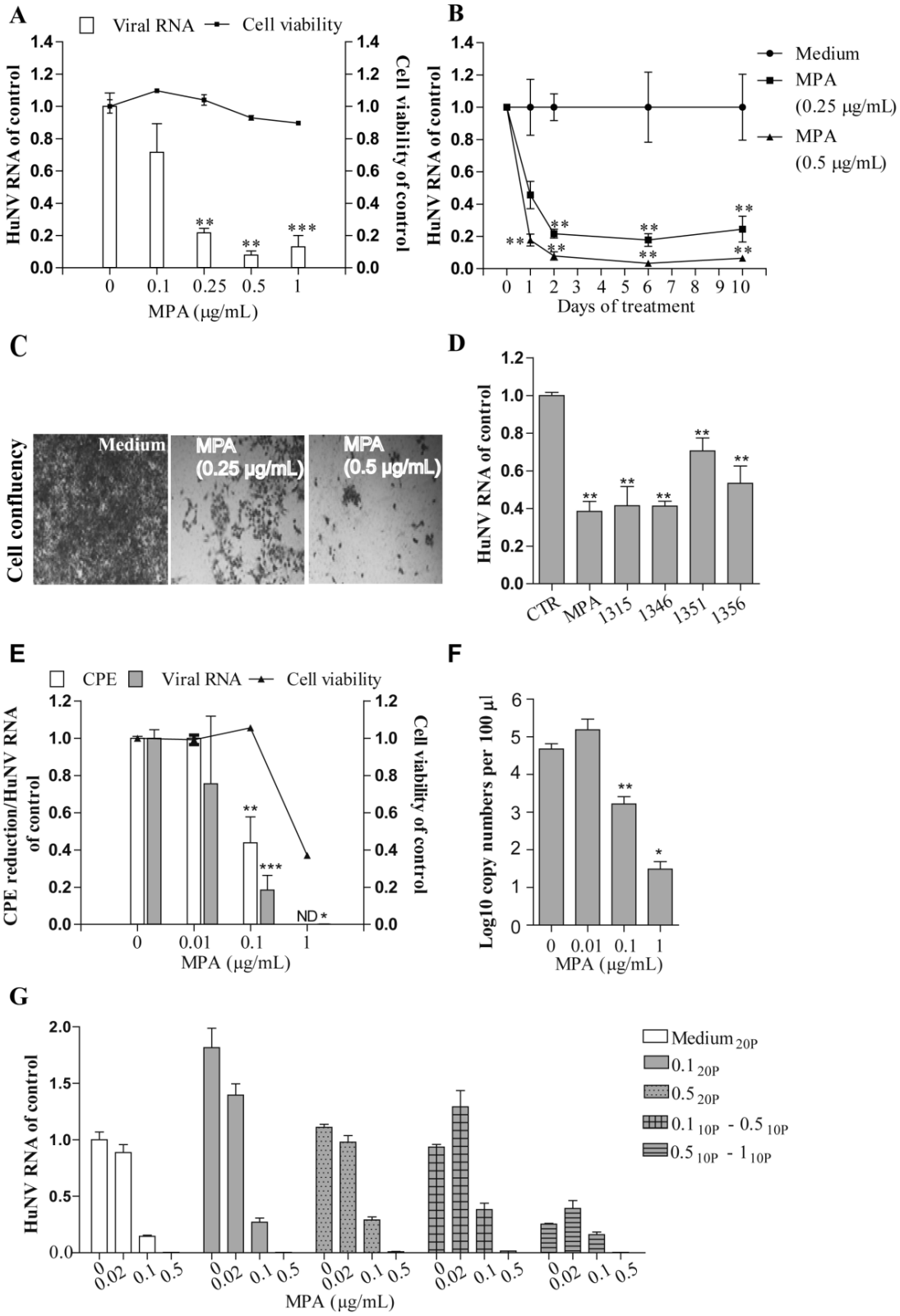



**Figure 4 MPA potently inhibited norovirus replication and completely eliminated HuNV replicons from host cells without concomitant drug resistance.** (A) Treatment with MPA for 48 hours potently inhibited HuNV replication, as determined by qRT-PCR (*n* = 3 independent experiments with 2 replicates). (B) Clearance assay with MPA (*n* = 3 independent experiments with 2 replicates). (C) Rebound assay with MPA. Long-term treatment with 0.5 $\mu$g/mL MPA completely cleared HuNV replicons from host cells, and no colony formation was observed. (D) Comparison of

the antinorovirus effects of MPA and 4 IMPDH inhibitors on HuNV. In the HG23 cells, treatment with 4 IMPDH inhibitors (the same concentration of 1 $\mu$M as MPA) resulted in a reduction in HuNV replication ($n$ = 3 independent experiments with 2 replicates). (E) The antinorovirus effects of MPA were validated on MNV-1 as quantified by means of an MTT-based CPE reduction assay and qRT-PCR ($n$ = 2 independent experiments with 2 to 3 replicates). ND, not detected. (F) Same as panel E for detecting the cellular MNV-1 RNA level, the viral RNA copy numbers in the supernatant (secreted viruses) were also detected after 24 hours of treatment with MPA. MPA potently inhibited MNV-1 virus particle production ($n$ = 2 independent experiments with 2 or 3 replicates). (G) The inhibitory efficacy of MPA against MNV-1 following 20 passages (20P) exposed to MPA or vehicle control. In the selection process, MNV-1 was either directly cultured in the presence of a fixed MPA concentration (0.1 or 0.5 $\mu$g/mL) or in a lengthy stepwise selection concentration from 0.1 to 0.5 $\mu$g/mL or from 0.5 to 1 $\mu$g/mL. After selection, the antinorovirus effects of MPA on the 20th passage of MNV-1 were determined by qRT-PCR. Data are presented as the means ± SEM (*, $P$ < 0.05; **, $P$ < 0.01; ***, $P$ < 0.001). 10P, 10 passages.

inhibitory effect of MPA on norovirus replication was sensitive to supplementation with guanosine. An 100 $\mu$g/mL guanosine concentration completely negated MPA effects in both the MNV-1 model ($P$ < 0.05, $n$ = 6 to 8; Fig. 5F) and HuNV replicon model ($P$ < 0.05, $n$ = 5; Fig. 5G). In apparent agreement, exogenous supplementation of guanosine reversed the inhibitory effects of IMPDH1/2 knockdown on HuNV replication ($P$ < 0.05, $n$ = 5; Fig. 5H). Thus, norovirus biology requires substantial intracellular guanosine levels, and the inhibitory effects of MPA and IMPDH knockdown are mediated by the depletion of this guanosine pool.

## *Ribavirin efficiently inhibited norovirus partially through nucleotide depletion*

Ribavirin at 2.5 $\mu$g/mL concentration resulted in a reduction of HuNV replication by 64 ± 6.1% ($P$ < 0.05, $n$ = 6; Fig. 6A) and 61 ± 8.5% ($P$ < 0.05, $n$ = 6; Fig. 6B) after short-term and long-term treatment, respectively. Additionally, ribavirin at higher concentrations (5 and 10 $\mu$g/mL) was capable of clearing cells of most replicons following 10 days of consecutive culture (Fig. 6C). The antiviral activity of ribavirin was confirmed in the MNV-1 model. Ribavirin at concentration of 5 $\mu$g/mL effectively decreased MNV-1 cellular RNA ($P$ < 0.05, $n$ = 6; Fig. 6D) and extracellular viral particles in the supernatant ($P$ < 0.05, $n$ = 6; Fig. 6E). In the CPE inhibition experiment, treatment with ribavirin for 3 days dose-dependently increased cell viability and decreased CPE formation by inhibiting MNV-1 replication ($P$ < 0.05, $n$ = 8; Fig. 6D), further validating the antiviral activity of ribavirin on MNV-1. To further explore the mechanism of ribavirin, we investigated whether the addition of exogenous guanosine would revert the antiviral effect of ribavirin. As shown in Fig. S6, a high concentration of guanosine partially reverted the antiviral activity of ribavirin for both HuNV and MNV, indicating that guanosine depletion contributed to the antiviral activity of ribavirin.

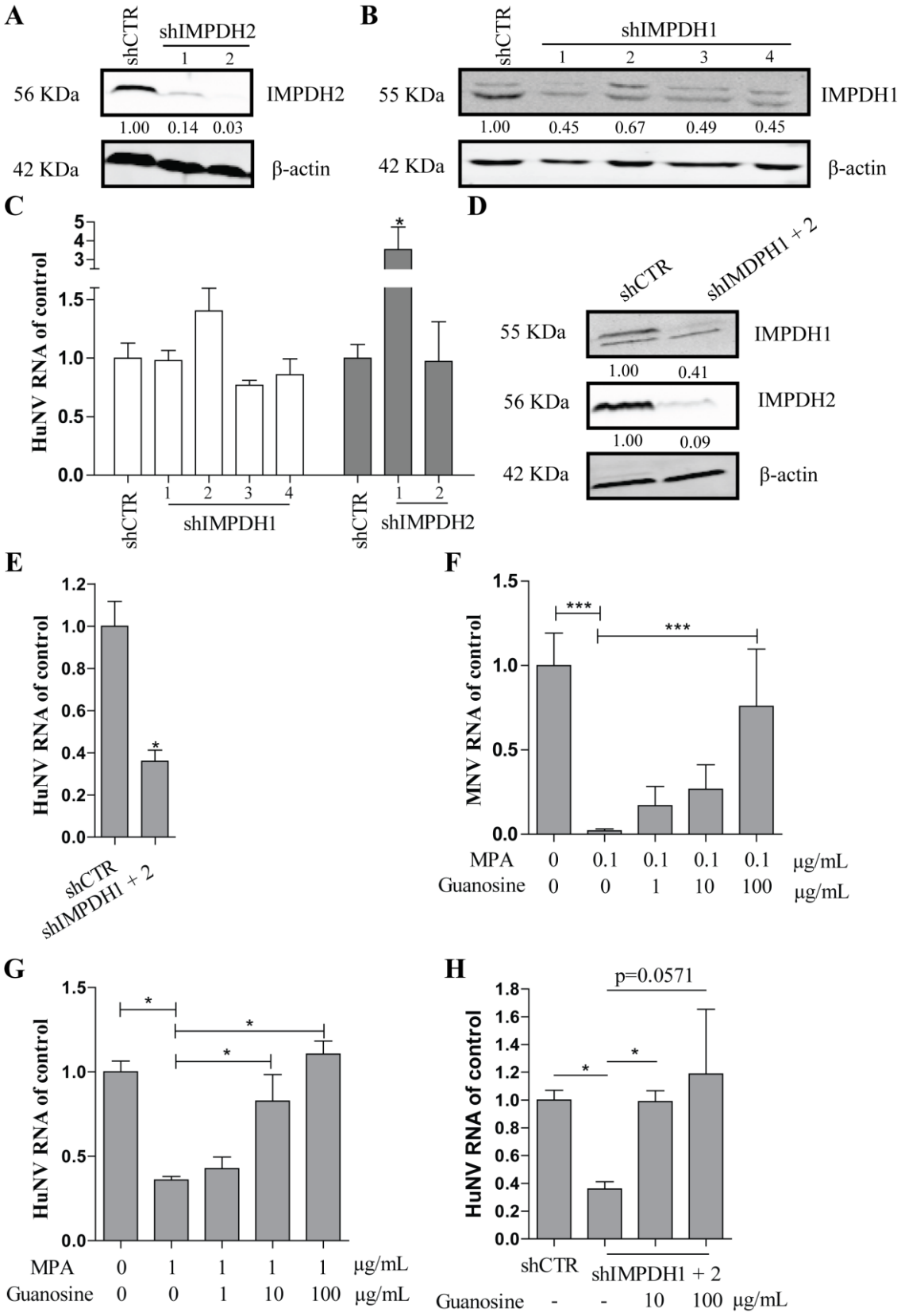



**Figure 5 Simultaneous knockdown of IMPDH1/2 reduced HuNV replication, which was reversed by exogenous guanosine.** (A and B) Validation of IMPDH downregulation in shCTR cells and shIMPDH cells by Western blotting. Images are representative of three independent experiments. (C) qRT-PCR analysis of HuNV RNA level in IMPDH knockdown cells. Compared to control cells,

knockdown of IMPDH1 or IMPDH2 alone had no significant effect on HuNV replication (*n* = 2 independent experiments with 2 replicates each). (D) Validation of IMPDH downregulation after simultaneous knockdown of IMPDH1/2 by Western blotting. Images are representative of three independent experiments. (E) Simultaneous silence of IMPDH1/2 decreased HuNV replication (*n* = 2 independent experiments with 2 replicates). (F and G) Guanosine restored norovirus replication in MPA-treated cells. MNV-1 and the HuNV replicon were treated with MPA alone or combined with guanosine (1, 10 or 100 *μ*g/mL). After 24 hours of incubation, norovirus replication was quantified by qRT-PCR. (H) IMPDH1/2 knockdown cells were cultured with medium or increasing concentrations of guanosine. After 24 hours of incubation, the HuNV RNA level was analyzed by qRT-PCR and was compared to the shCTR cells (*n* = 2 independent experiments with 2 replicates). Data are presented as the means ± SEM (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).

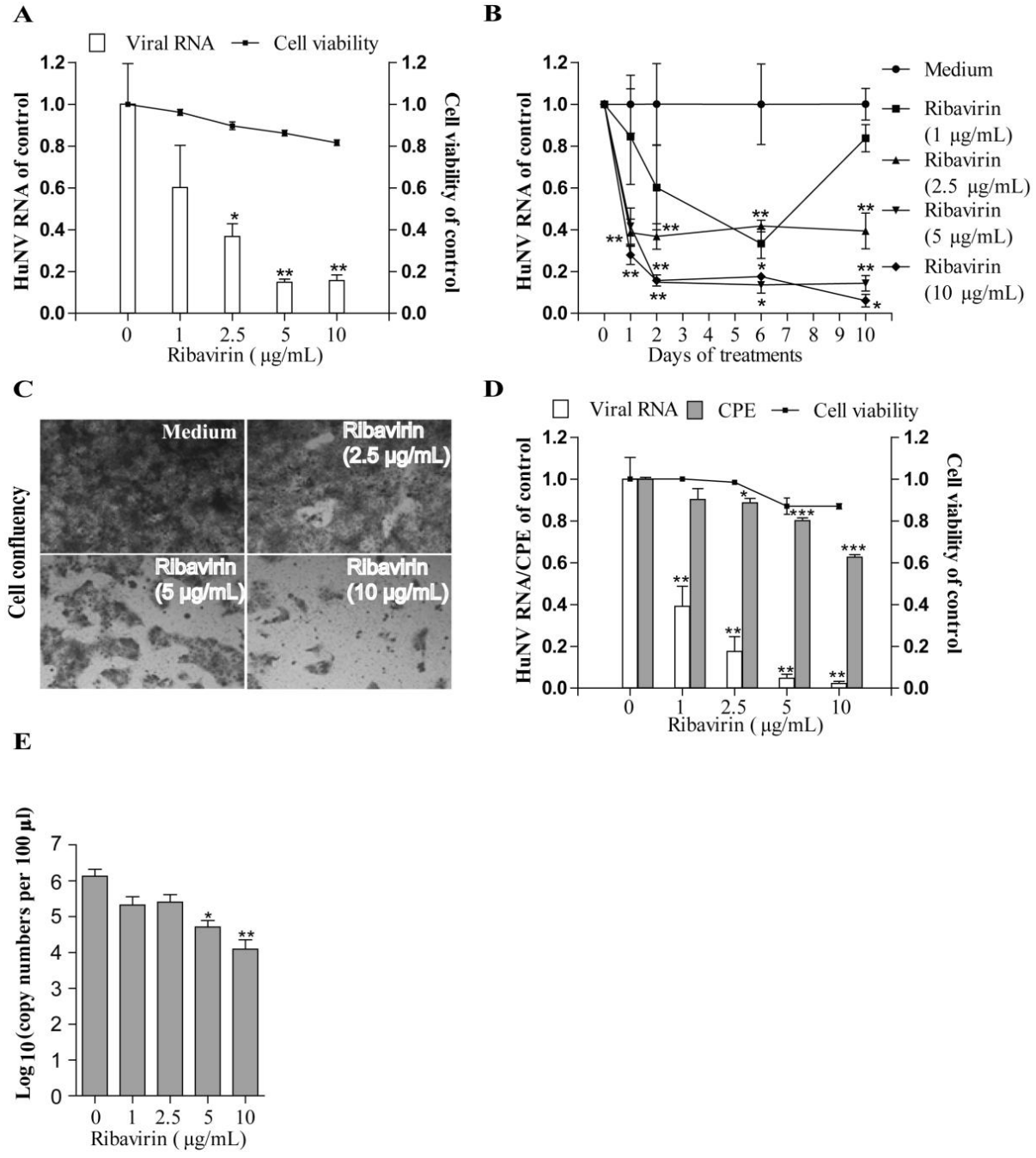



**Figure 6 Ribavirin effectively inhibited norovirus replication.** (A) Treatment with ribavirin for 48 hours dose-dependently decreased HuNV replication, as determined by qRT-PCR (*n* = 3

independent experiments with 2 replicates each). (B) Clearance assay with ribavirin (*n* = 3 independent experiments with 2 replicates each ). (C) Rebound assay with ribavirin. (D) The antinorovirus activity of ribavirin was also confirmed on MNV-1. Ribavirin treatment decreased MNV-1-induced CPE and viral RNA replication in RAW 264.7 cells (*n* = 3 independent experiments with 2 to 3 replicates). (E) Ribavirin treatment also decreased MNV-1 virus particle production in supernatant as quantified by viral RNA copy numbers (*n* = 3 independent experiments with 2 replicates). Data are presented as the means ± SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

## *Combinatory effects of ribavirin and immunosuppressants on norovirus replication*

Immunosuppressants are often used in combination in the clinic. First, we evaluated the interaction of immunosuppressants on HuNV using MacSynergy 2 combination analysis. As shown in Fig. 7A, the combined calcineurin inhibitors, FK506 and CsA, exerted a moderately synergistic antiviral effect, with a log volumes of 5.55. The maximal degree of combined inhibition between FK506 and CsA was achieved at 1 $\mu$g/mL FK506 and 0.4 $\mu$g/mL CsA, with a synergy volume of 35.06 $\mu M^2$% above the expected value. The combination of MPA with FK506 (Fig. 7B) or CsA (Fig. 7C) achieved a synergy volume of -7.79 $\mu M^2$% (log volume of -0.93) or -14.58 $\mu M^2$% (log volume of -1.32), respectively, indicating an additive effect. These results highlight the dependency of norovirus biology on both calcineurin phosphatase activity and the presence of an adequate intracellular guanosine pool.

Next, we assessed the combinatory antiviral effects of ribavirin and immunosuppressants. The combined effects of ribavirin and immunosuppressants, including CsA (Fig. 7D), FK506 (Fig. 7E), and MPA (Fig. 7F) were additive, with synergy volumes of 0 $\mu M^2$% (log volume of 0), 0 $\mu M^2$% (log volume of 0) and -4.04 $\mu M^2$% (log volume of -0.37), respectively.

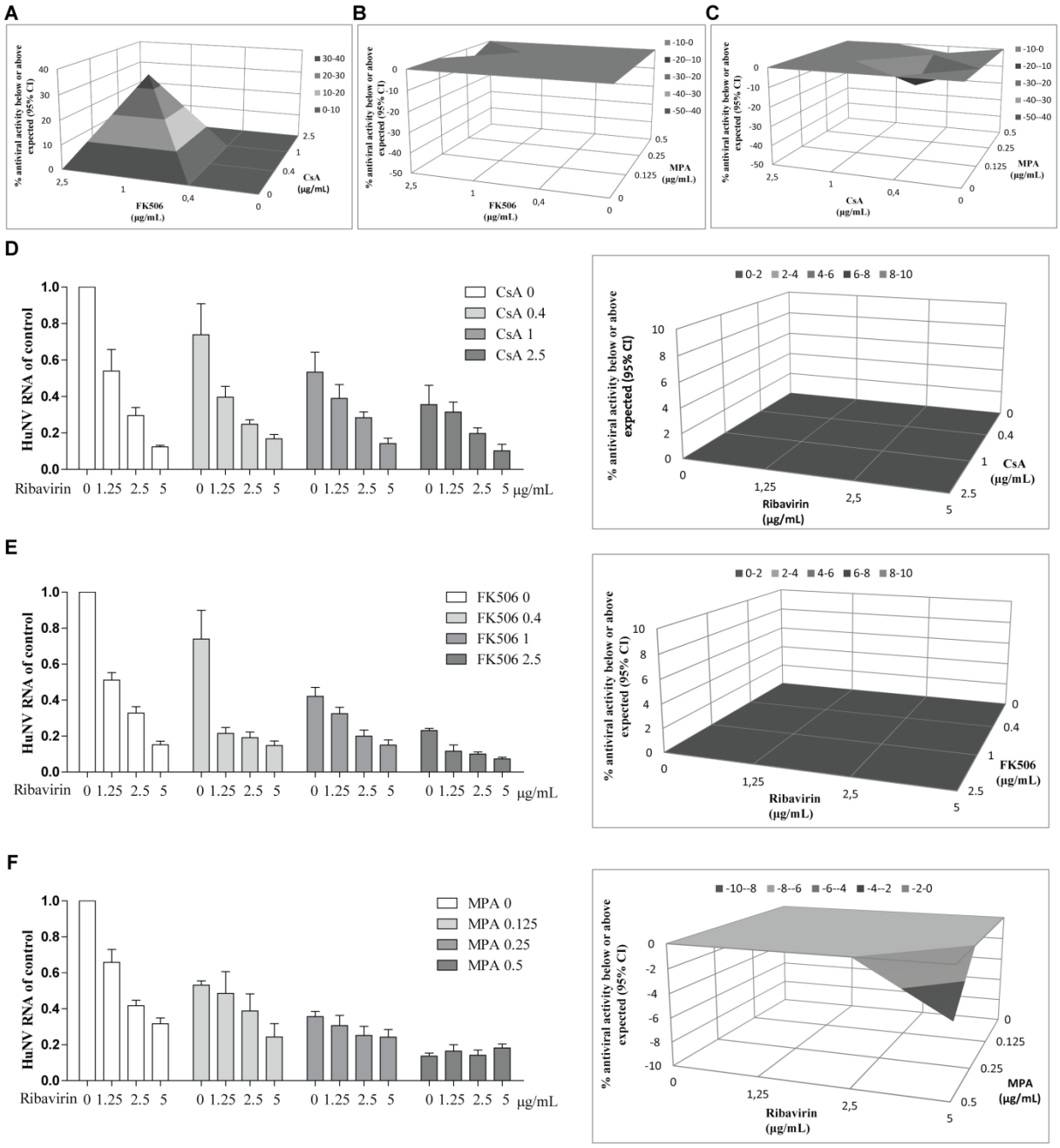



**Figure 7 The combinatory effects of ribavirin and immunosuppressants on norovirus replication.** The combinatory effects of two drugs in the 48-hour antiviral assay with HuNV were analyzed using the mathematical model MacSynergy. The three-dimensional surface plot represents the differences (within 95% confidence interval [95% CI]) between actual experimental effects and theoretical additive effects of the combination at various concentrations of the two compounds (*n* = 5). The antiviral effects of FK506 in combination with CsA (A) or MPA (B) as well as CsA in combination with MPA (C) was analyzed by MacSynergy model. Combinations of ribavirin with CsA (D), FK506 (E) or MPA (F) were analyzed by the relative HuNV RNA level compared to the control and MacSynergy model.

# DISCUSSION

Immunosuppressants are universally used to deliberately induce immunosuppression after organ transplantation to prevent allograft rejection. However, this results in increased susceptibility to opportunistic infections, and especially norovirus infection has emerged as a major concern in this respect. A recent study reported that among 116 immunocompromised pediatric hematopoietic stem cell and solid organ transplant recipients, norovirus infection was associated with prolonged diarrhea and frequent intensive care unit (ICU) admission [5]. Although new classes of immunosuppressive medications have been introduced to the transplant community during the last decades, T-lymphocytes remain the key targets of these agents, as this cell type is a major effector in graft rejection. T-lymphocytes, however, also play an important role in the adaptive immune response following viral infection; thus, reduced resistance to viral infection is an expected side effect of graft tolerance-inducing medications. Intriguingly, immunosuppressive medications may also directly interfere with the viral life cycle. For various viruses, it is now clear that the choice of immunosuppressive medications relates to patient sensitivity to infection [38]. Norovirus biology, however, is relatively poorly understood, and its interaction with different immunosuppressive regimens remains obscure at best. Here, we have profiled the commonly used immunosuppressants on norovirus infection and related the effects to their cellular effectors. Thus, our data provide guidance as to the host biochemical mechanisms essential for norovirus replication and may guide clinical management of transplantation patients at risk for norovirus infection. With respect to the latter, the remarkable sensitivity of norovirus to MPA is especially important, particularly in view of the absence of norovirus-directed vaccines or antiviral medications.

Interestingly, our study reveals a hitherto unexpected role for calcineurin phosphatase activity in the norovirus life cycle. Calcineurin inhibitors include FK506 and CsA. FK506 is currently a widely used immunosuppressive agent for managing transplantation patients. CsA binds to cyclophilins, and FK506 binds to FK binding proteins (FKBPs). Both events result in a profound inhibition of the phosphatase activity of calcineurin, which in turn suppresses T cell proliferation. In general, FK506 is thought to have no effect on viral infections [28]. In contrast, CsA has been demonstrated to interact with the biology of a broad range of viruses, mainly through inhibiting cyclophilins. Of note, CsA inhibited HCV

replication both *in vitro* and *in vivo* in an apparently specific manner. HCV RNA replication requires the non-structural HCV NS5A protein that serves as a direct ligand for CyPA. CsA could disrupt the CyPA-NS5A interaction, further reducing HCV RNA replication [39]. In our study, FK506 and CsA exerted a moderate inhibitory effect on norovirus replication through FKBP12 and CyPA, respectively. Furthermore, nonimmunosuppressive CsA analogues, which do not inhibit calcineurin, failed to suppress norovirus replication, supporting the hypothesis that calcineurin activation is required for the norovirus life cycle.

Calcineurin is a heterodimeric $Ca^{2+}$- and calmodulin-dependent serine/threonine protein phosphatase composed of a 60-kDa catalytic subunit (PPP3CA) and a 19-kDa calcium binding regulatory subunit (PPP3R1) [40]. Our results showed that knockdown of PPP3CA resulted in a dramatic reduction of HuNV replication, demonstrating that calcineurin is required for HuNV replication. Although the exact mechanism as to how calcineurin facilitates norovirus replication is unknown, it may resemble its role in the life cycle of the polyomavirus BK [41]. The observation that CsA, but not NIM811 (a nonimmunosuppressive CsA derivative), inhibited BK virus (BKV) replication by inhibiting CyPA and calcineurin activation is highly reminiscent of the results obtained in the present study, but concluding that the effects are mechanistically identical requires further validation, especially with respect to the role of the nuclear factor of activated T cells (NFAT).

Mycophenolate mofetil (MMF) has been widely used in kidney, pancreas, liver and heart transplantation [42]. MPA, the active form of MMF *in vivo*, is a potent inhibitor of IMPDH, a rate-limiting enzyme in the *de novo* synthesis of guanosine nucleotides. By inhibiting IMPDH, MPA results in the depletion of the intracellular GTP and dGTP pools and exerts potent immunosuppressive effects. MPA has been demonstrated to inhibit a variety of viruses, ranging from DNA virus, including hepatitis B virus (HBV), to RNA viruses, including HCV, dengue virus, yellow fever virus, and Chikungunya virus [28]. Here, we demonstrated that MPA potently inhibited both MNV and HuNV replication through simultaneously targeting IMPDH1 and IMPDH2. Furthermore, MPA at levels below the serum concentrations in patients completely eliminated HuNV replicons from cells after long-term treatment. In apparent agreement, 4 IMPDH inhibitors also inhibited HuNV replication in our experiments. Apparently, MPA has a high barrier against developing drug resistance with respect to its action on norovirus biology. Of note, characterization of the potential mutagenesis of the viral genome in response to MPA exposure is interesting for

future research. In conjunction, our data provide compelling evidence that MPA should be the immunosuppressant of choice in transplantation patients at risk for norovirus infection.

The optimization of immunosuppression might help the clearance of norovirus infection in a proportion of infected organ recipients. However, for the transplant or other types of patients who fail to clear the virus, effective antiviral therapy is urgently needed, whereas no proven antiviral medications are available. Ribavirin, a general antiviral, has been used in the clinic for decades to treat various types of viral infections. A recent study reported that ribavirin cleared norovirus and resulted in complete symptomatic and histological recovery in two CVID patients, which raises the possibility of ribavirin as a potential antiviral therapy for norovirus infection. Several studies have attempted to understand the antiviral activity and the mechanism of ribavirin on norovirus. It has been reported that addition of guanosine to the ribavirin treatment could moderately reversed ribavirin inhibition on HuNV [43], which is consistent with our results. Another two studies demonstrated that ribavirin inhibited MNV through increased mutagenesis and quasispecies diversity [44, 45]. In our study, ribavirin at concentrations comparable to serum concentrations in patients [46] inhibited norovirus replication. Ribavirin, combined with MPA or calcineurin inhibitors, additively inhibited norovirus replication, which supports the potential of ribavirin as a therapy for norovirus infection in transplant patients. However, it remains to be further investigated whether the optimal combination with ribavirin is MPA, CsA or FK506.

In summary, we profiled the effects of clinically relevant immunosuppressants on norovirus replication and observed that norovirus was sensitively dependent on IMPDH guanine synthesizing activity but also required calcineurin phosphatase activity. MPA represents a potent inhibitor of norovirus replication, with a high barrier toward the development of drug resistance. Ribavirin is a promising candidate in treating norovirus infection, athough further evaluation, particularly in patients, is needed.

## ACKNOWLEDGEMENTS

We gratefully acknowledge Professor Herbert W. Virgin (Washington University, St Louis, MO, USA ) for providing us the MNV-1 and Dr Kyeong-Ok Chang (Kansas State University, USA) for providing the HuNV replicon. We also thank the general support of the Center for Drug Design (University of Minnesota, USA) for developing the IMPDH inhibitors.

# REFERENCE


1. McAtee CL, Webman R, Gilman RH, Mejia C, Bern C, Apaza S, Espetia S, Pajuelo M, Saito M, Challappa R, Soria R, Ribera JP, Lozano D, Torrico F. 2016. Burden of Norovirus and Rotavirus in Children After Rotavirus Vaccine Introduction, Cochabamba, Bolivia. Am J Trop Med Hyg **94**:212-217.
2. Hoa Tran TN, Trainor E, Nakagomi T, Cunliffe NA, Nakagomi O. 2013. Molecular epidemiology of noroviruses associated with acute sporadic gastroenteritis in children: global distribution of genogroups, genotypes and GII.4 variants. J Clin Virol **56**:185-193.
3. Patel MM, Widdowson MA, Glass RI, Akazawa K, Vinje J, Parashar UD. 2008. Systematic literature review of role of noroviruses in sporadic gastroenteritis. Emerg Infect Dis **14**:1224-1231.
4. Bartsch SM, Lopman BA, Ozawa S, Hall AJ, Lee BY. 2016. Global Economic Burden of Norovirus Gastroenteritis. PLoS One **11**:e0151219.
5. Ye X, Van JN, Munoz FM, Revell PA, Kozinetz CA, Krance RA, Atmar RL, Estes MK, Koo HL. 2015. Noroviruses as a Cause of Diarrhea in Immunocompromised Pediatric Hematopoietic Stem Cell and Solid Organ Transplant Recipients. Am J Transplant **15**:1874-1881.
6. van Beek J, van der Eijk AA, Fraaij PL, Caliskan K, Cransberg K, Dalinghaus M, Hoek RA, Metselaar HJ, Roodnat J, Vennema H, Koopmans MP. 2017. Chronic norovirus infection among solid organ recipients in a tertiary care hospital, the Netherlands, 2006-2014. Clin Microbiol Infect **23**:265 e269-265 e213.
7. Boillat Blanco N, Kuonen R, Bellini C, Manuel O, Estrade C, Mazza-Stalder J, Aubert JD, Sahli R, Meylan P. 2011. Chronic norovirus gastroenteritis in a double hematopoietic stem cell and lung transplant recipient. Transpl Infect Dis **13**:213-215.
8. Coste JF, Vuiblet V, Moustapha B, Bouin A, Lavaud S, Toupance O, de Rougemont A, Benejat L, Megraud F, Wolak-Thierry A, Villena I, Chemla C, Le Magrex E, de Champs C, Andreoletti L, Rieu P, Leveque N. 2013. Microbiological diagnosis of severe diarrhea in kidney transplant recipients by use of multiplex PCR assays. J Clin Microbiol **51**:1841-1849.
9. Florescu DF, Hermsen ED, Kwon JY, Gumeel D, Grant WJ, Mercer DF, Kalil AC. 2011. Is there a role for oral human immunoglobulin in the treatment for norovirus enteritis in immunocompromised patients? Pediatr Transplant **15**:718-721.
10. Schorn R, Hohne M, Meerbach A, Bossart W, Wuthrich RP, Schreier E, Muller NJ, Fehr T. 2010. Chronic norovirus infection after kidney transplantation: molecular evidence for immune-driven viral evolution. Clin Infect Dis **51**:307-314.
11. Lee BE, Pang XL, Robinson JL, Bigam D, Monroe SS, Preiksaitis JK. 2008. Chronic norovirus and adenovirus infection in a solid organ transplant recipient. Pediatr Infect Dis J **27**:360-362.
12. Engelen MA, Gunia S, Stypmann J. 2011. Elimination of norovirus in a chronic carrier under immunosuppression after heart transplantation--effect of everolimus. Transpl Int **24**:e102-103.
13. Ebdrup L, Bottiger B, Molgaard H, Laursen AL. 2011. Devastating diarrhoea in a heart-transplanted patient. J Clin Virol **50**:263-265.
14. Chagla Z, Quirt J, Woodward K, Neary J, Rutherford C. 2013. Chronic norovirus infection in a transplant patient successfully treated with enterally administered immune globulin. J Clin Virol **58**:306-308.
15. Westhoff TH, Vergoulidou M, Loddenkemper C, Schwartz S, Hofmann J, Schneider T, Zidek W, van der Giet M. 2009. Chronic norovirus infection in renal transplant recipients. Nephrol Dial Transplant **24**:1051-1053.
16. Roos-Weil D, Ambert-Balay K, Lanternier F, Mamzer-Bruneel MF, Nochy D, Pothier P, Avettand-Fenoel V, Anglicheau D, Snanoudj R, Bererhi L, Thervet E, Lecuit M, Legendre C, Lortholary O, Zuber J. 2011. Impact of norovirus/sapovirus-related diarrhea in renal transplant recipients hospitalized for diarrhea. Transplantation **92**:61-69.

17. Kaufman SS, Chatterjee NK, Fuschino ME, Magid MS, Gordon RE, Morse DL, Herold BC, LeLeiko NS, Tschernia A, Florman SS, Gondolesi GE, Fishbein TM. 2003. Calicivirus enteritis in an intestinal transplant recipient. Am J Transplant **3**:764-768.

18. Kaufman SS, Chatterjee NK, Fuschino ME, Morse DL, Morotti RA, Magid MS, Gondolesi GE, Florman SS, Fishbein TM. 2005. Characteristics of human calicivirus enteritis in intestinal transplant recipients. J Pediatr Gastroenterol Nutr **40**:328-333.

19. Morotti RA, Kaufman SS, Fishbein TM, Chatterjee NK, Fuschino ME, Morse DL, Magid MS. 2004. Calicivirus infection in pediatric small intestine transplant recipients: pathological considerations. Hum Pathol **35**:1236-1240.

20. Florescu DF, Hill LA, McCartan MA, Grant W. 2008. Two cases of Norwalk virus enteritis following small bowel transplantation treated with oral human serum immunoglobulin. Pediatr Transplant **12**:372-375.

21. Robles JD, Cheuk DK, Ha SY, Chiang AK, Chan GC. 2012. Norovirus infection in pediatric hematopoietic stem cell transplantation recipients: incidence, risk factors, and outcome. Biol Blood Marrow Transplant **18**:1883-1889.

22. Saif MA, Bonney DK, Bigger B, Forsythe L, Williams N, Page J, Babiker ZO, Guiver M, Turner AJ, Hughes S, Wynn RF. 2011. Chronic norovirus infection in pediatric hematopoietic stem cell transplant recipients: a cause of prolonged intestinal failure requiring intensive nutritional support. Pediatr Transplant **15**:505-509.

23. Lemes LG, Correa TS, Fiaccadori FS, Cardoso D, Arantes Ade M, Souza KM, Souza M. 2014. Prospective study on Norovirus infection among allogeneic stem cell transplant recipients: prolonged viral excretion and viral RNA in the blood. J Clin Virol **61**:329-333.

24. Roddie C, Paul JP, Benjamin R, Gallimore CI, Xerry J, Gray JJ, Peggs KS, Morris EC, Thomson KJ, Ward KN. 2009. Allogeneic hematopoietic stem cell transplantation and norovirus gastroenteritis: a previously unrecognized cause of morbidity. Clin Infect Dis **49**:1061-1068.

25. Echenique IA, Stosor V, Gallon L, Kaufman D, Qi C, Zembower TR. 2016. Prolonged norovirus infection after pancreas transplantation: a case report and review of chronic norovirus. Transpl Infect Dis **18**:98-104.

26. Karst SM, Baric RS. 2015. What is the reservoir of emergent human norovirus strains? J Virol **89**:5756-5759.

27. Atmar J, Mullen E. 2013. Norovirus in immunocompromised patients. Oncol Nurs Forum **40**:434-436.

28. Pan Q, Tilanus HW, Metselaar HJ, Janssen HL, van der Laan LJ. 2012. Virus-drug interactions--molecular insight into immunosuppression and HCV. Nat Rev Gastroenterol Hepatol **9**:355-362.

29. Woodward JM, Gkrania-Klotsas E, Cordero-Ng AY, Aravinthan A, Bandoh BN, Liu H, Davies S, Zhang H, Stevenson P, Curran MD, Kumararatne D. 2015. The role of chronic norovirus infection in the enteropathy associated with common variable immunodeficiency. Am J Gastroenterol **110**:320-327.

30. Wobus CE, Karst SM, Thackray LB, Chang KO, Sosnovtsev SV, Belliot G, Krug A, Mackenzie JM, Green KY, Virgin HW. 2004. Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. PLoS Biol **2**:e432.

31. Rocha-Pereira J, Jochmans D, Debing Y, Verbeken E, Nascimento MS, Neyts J. 2013. The viral polymerase inhibitor 2'-C-methylcytidine inhibits Norwalk virus replication and protects against norovirus-induced diarrhea and mortality in a mouse model. J Virol **87**:11798-11805.

32. Pan Q, de Ruiter PE, von Eije KJ, Smits R, Kwekkeboom J, Tilanus HW, Berkhout B, Janssen HL, van der Laan LJ. 2011. Disturbance of the microRNA pathway by commonly used lentiviral shRNA libraries limits the application for screening host factors involved in hepatitis C virus infection. FEBS Lett **585**:1025-1030.

33. Prichard MN, Shipman C, Jr. 1990. A three-dimensional model to analyze drug-drug interactions. Antiviral Res **14**:181-205.

34. Chang KO, Sosnovtsev SV, Belliot G, King AD, Green KY. 2006. Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line. Virology **353**:463-473.

35. Stalder M, Birsan T, Hubble RW, Paniagua RT, Morris RE. 2003. In vivo evaluation of the novel calcineurin inhibitor ISATX247 in non-human primates. J Heart Lung Transplant **22**:1343-1352.

36. Patel CG, Akhlaghi F. 2006. High-performance liquid chromatography method for the determination of mycophenolic acid and its acyl and phenol glucuronide metabolites in human plasma. Ther Drug Monit **28**:116-122.

37. Carr SF, Papp E, Wu JC, Natsumeda Y. 1993. Characterization of human type I and type II IMP dehydrogenases. J Biol Chem **268**:27286-27290.

38. Brennan DC, Aguado JM, Potena L, Jardine AG, Legendre C, Saemann MD, Mueller NJ, Merville P, Emery V, Nashan B. 2013. Effect of maintenance immunosuppressive drugs on virus pathobiology: evidence and potential mechanisms. Rev Med Virol **23**:97-125.

39. Gallay PA. 2012. Cyclophilin inhibitors: a novel class of promising host-targeting anti-HCV agents. Immunol Res **52**:200-210.

40. Rusnak F, Mertz P. 2000. Calcineurin: form and function. Physiol Rev **80**:1483-1521.

41. Li YJ, Wu HH, Weng CH, Chen YC, Hung CC, Yang CW, Wang RY, Sakamoto N, Tian YC. 2012. Cyclophilin A and nuclear factor of activated T cells are essential in cyclosporine-mediated suppression of polyomavirus BK replication. Am J Transplant **12**:2348-2362.

42. Shipkova M, Armstrong VW, Oellerich M, Wieland E. 2005. Mycophenolate mofetil in organ transplantation: focus on metabolism, safety and tolerability. Expert Opin Drug Metab Toxicol **1**:505-526.

43. Chang KO, George DW. 2007. Interferons and ribavirin effectively inhibit Norwalk virus replication in replicon-bearing cells. J Virol **81**:12111-12118.

44. Arias A, Thorne L, Goodfellow I. 2014. Favipiravir elicits antiviral mutagenesis during virus replication in vivo. Elife **3**:e03679.

45. Julian TR, Baugher JD, Rippinger CM, Pinekenstein R, Kolawole AO, Mehoke TS, Wobus CE, Feldman AB, Pineda FJ, Schwab KJ. 2016. Murine norovirus (MNV-1) exposure in vitro to the purine nucleoside analog Ribavirin increases quasispecies diversity. Virus Res **211**:165-173.

46. Nicot F, Legrand-Abravanel F, Lafont T, Dubois M, Saune K, Pasquier C, Chatelut E, Izopet J. 2008. Serum concentrations of ribavirin and pegylated interferon and viral responses in patients infected with HIV and HCV. J Med Virol **80**:1523-1529.

# Supplementary Information

## SUPPLEMENTARY TABLES AND FIGURES

**Table S1 PCR primer sequences were listed.**

| Name | Sequences |
|---|---|
| Neomycin phosphotransferase | F: 5′-CCGGCTACCTGCCCATTC-3′<br>R: 5′-CCAGATCATCCTGATCGACAA G-3′ |
| MNV-1[a] | F: 5′-CACGCCACCGATCTGTTCTG-3′<br>R: 5′-GCGCTGCGCCATCACTC-3′ |
| Human GAPDH | F: 5′-GTCTCCTCT GACTTCAACAGCG-3′<br>R: 5′-ACCACCCTGTTGCTGTAGTAGCCAA-3′ |
| Murine GAPDH | F: 5′-TTCCAGTATGACTCCACTCACGG-3′<br>R: 5′-TGAAGACACCAGTAGACTCCACGAC-3′ |
| CyPA | F: 5′-TAAAGCATACGGGTCCTGG-3′<br>R: 5′-TCGAGTTGTCCACAGTCAG-3′ |
| CyPB | F: 5′-ATGTAGGCCGGGTGATCTTT-3′<br>R: 5′-TTTATCCCGGCTGTCTGTCT-3′ |
| FKBP12 | F: 5′- TGCTAGGCAAGCAGGAGGTGAT-3′<br>R: 5′- GTGGCACCATAGGCATAATCTGG-3′ |
| FKBP8 | F: 5′- ACTCCTACGACCTCGCCATCAA-3′<br>R: 5′- GGTAGTGGTCGAGCTTCAGCTG-3′ |
| PPP3CA | F: 5′-GCCCTGATGAACCAACAGTTCC-3′<br>R: 5′-GCAGGTGGTTCTTTGAATCGGTC-3′ |
| IMPDH1 | F: 5′-GCACACTGTGGGCGAT-3′<br>R: 5′-GAGCCACCACCAGTTCA-3′ |
| IMPDH2 | F: 5′-TCTTCAACTGCGGAGAC-3′<br>R: 5′-CTGTAAGCGCCATTGCT-3′ |

a accession# NC008311

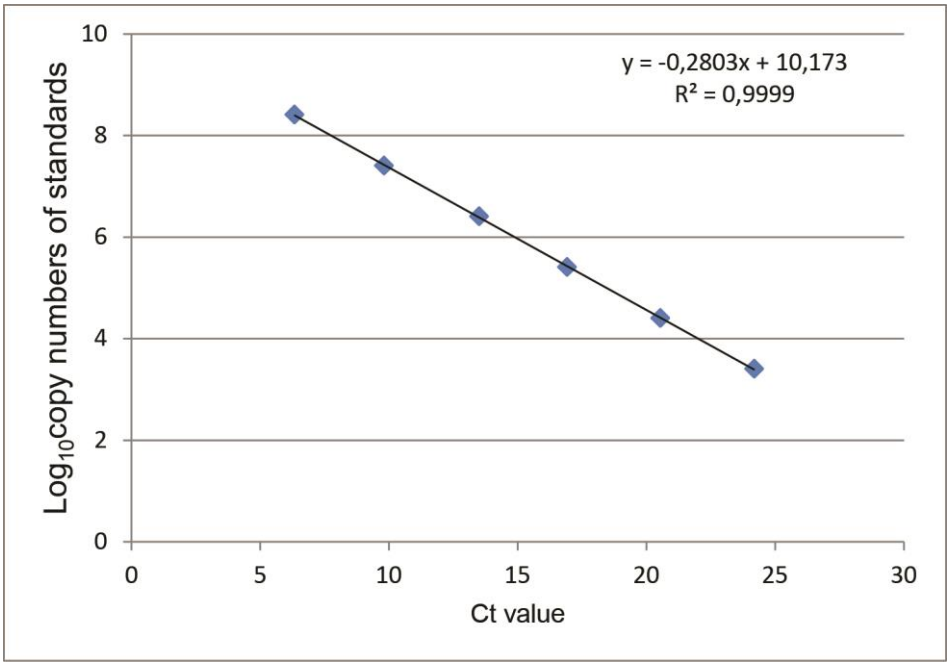



**Figure S1 Standard curve for quantifying MNV-1 genome copy numbers.**

**Table S2 Sequences of shRNA-mediated vectors were listed.**

| Name | sequences |
|---|---|
| Scrambled vector | 5′-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCAT CTTGTTGTTTTT-3′ |
| CyPA | 5′-CCGGTGGTGACTTCACACGCCATAACTCGAGTTATGGCGTGTGAA GTCACCATTTTTG-3′ |
| CyPB | 5′-CCGGGCCTTAGCTACAGGAGAGAAACTCGAGTTTCTCTCCTGTAG CTAAGGCTTTTTG-3′ |
| shFKBP8-1 | 5′-CCGGACGTCGCTGGAGAATGGCACACTCGAGTGTGCCATTCTC CAGCGACGTTTTTT-3′ |
| shFKBP8-2 | 5′-CCGGAGTGGACATGACGTTCGAGGACTCGAGTCCTCGAACGTC ATGTCCACTTTTTT-3′ |
| shFKBP12-1 | 5′- CCGGAGAGAGCCAAACTGACTATATCTCGAGATATAGTCAGTTTG GCTCTCTTTTTT-3′ |
| shFKBP12-2 | 5′-CCGGGCCAAACTGACTATATCTCCACTCGAGTGGAGATATAGTCA GTTTGGCTTTTT-3′ |
| shFKBP12-3 | 5′-CCGGGAGAGCCAAACTGACTATATCCTCGAGGATATAGTCAGTTTG GCTCTCTTTTT-3′ |
| shPPP3CA-1 | 5′-CCGGCACCACAACATAAGATCACTACTCGAGTAGTGATCTTATGTT GTGGTGTTTTT-3′ |
| shPPP3CA-2 | 5′-CCGGGAATAATAACAGAGGGTGCATCTCGAGATGCACCCTCTGTTA TTATTCTTTTT-3′ |
| shIMPDH1-1 | 5′-CCGGCCAGGATTCATAGACTTCATACTCGAGTATGAAGTCTATGAA TCCTGGTTTTT-3′ |
| shIMPDH1-2 | 5′-CCGGCGGAAGGTCAAGAAGTTTGAACTCGAGTTCAAACTTCTTG ACCTTCCGTTTTT-3′ |
| shIMPDH1-3 | 5′-CCGGGTGACGTTGAAAGAGGCAAATCTCGAGATTTGCCTCTTTCA ACGTCACTTTTT-3′ |
| shIMPDH1-4 | 5′-CCGGCCTGAAGAAGAACCGAGACTACTCGAGTAGTCTCGGTTCTT CTTCAGGTTTTT-3′ |
| shIMPDH2-1 | 5′-CCGGCACCTACAATGACTTTCTCATCTCGAGATGAGAAAGTCATTG TAGGTGTTTTT-3′ |
| shIMPDH2-2 | 5′-CCGGGACTGTTTCTTGGAAGAGATACTCGAGTATCTCTTCCAAGAA ACAGTCTTTTT-3′ |

**Table S3 *In vitro* anti-norovirus and cytotoxic activity of compounds.**

| | **Compounds** | **Inhibition of viral RNA replication $EC_{50}$, $\mu$g/mL** | **Inhibition of cell proliferation $CC_{50}$, $\mu$g/mL** | **Selectivity index** |
|---|---|---|---|---|
| Human norovirus model | Cyclosporin A (CsA) | 3.90 | 14.34 | 3.67 |
| | Voclosporin (VCS) | 0.94 | 4.74 | 5.04 |
| | Tacrolimus (FK506) | 2.92 | 3.72 | 1.27 |
| | Mycophenolic acid (MPA) | 0.13 | 4.11 | 31.61 |
| | Ribavirin | 1.25 | 14.94 | 11.95 |
| Mouse norovirus model | Mycophenolic acid (MPA) | 0.05 | 0.80 | 40 |
| | Ribavirin | 0.74 | 30.87 | 41 |

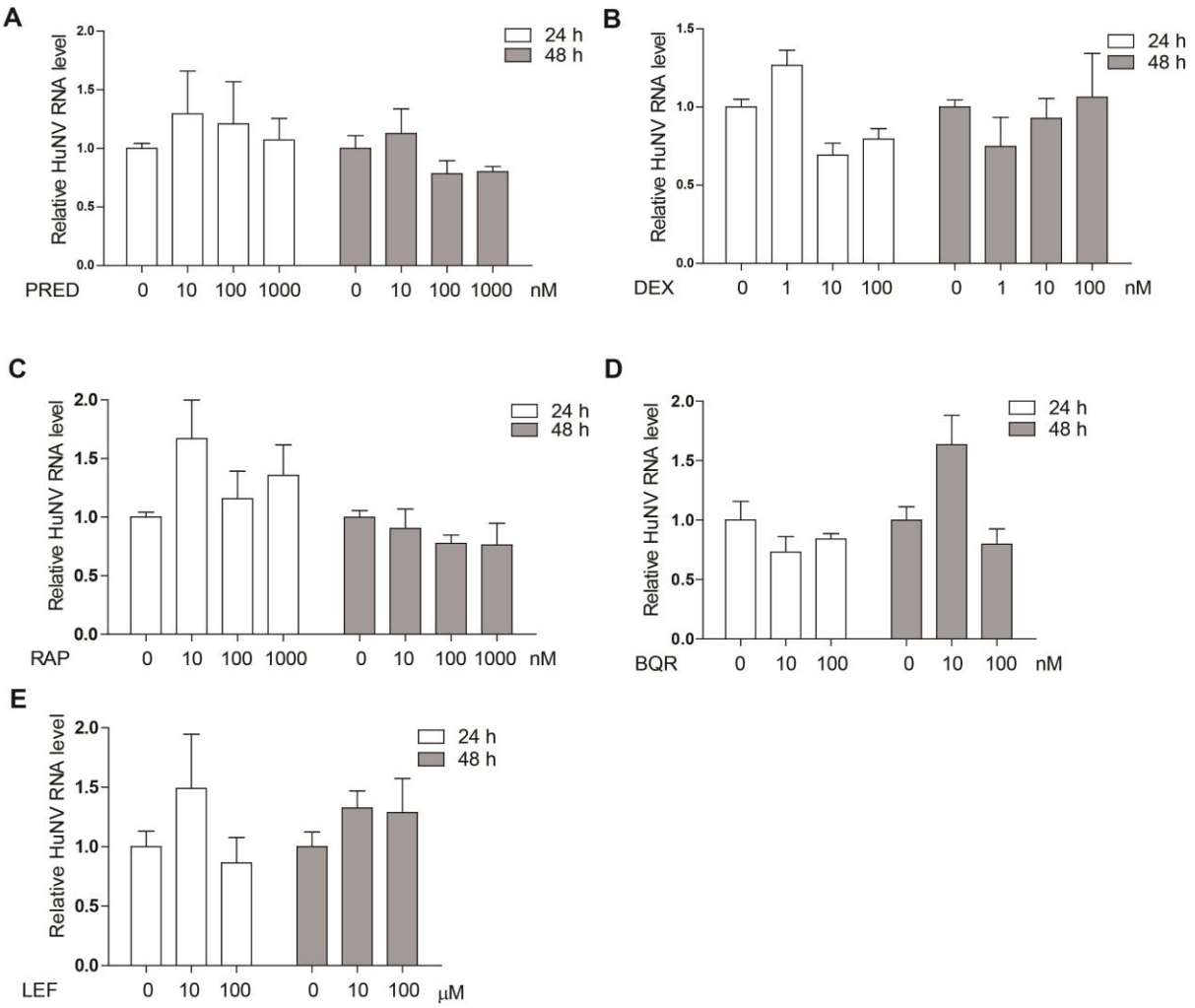



**Figure S2 Not all immunosuppressants directly affect norovirus replication.** Glucocorticoids, including PRED (A) and DEX (B) showed no effects on HuNV replication. Likewise, rapamycin (C), an mTOR inhibitor produced no significant changes in HuNV replication. Similarly, BQR (D) and LFM (E), which are in clinical development for use in transplantation medicine, showed no significant effects on HuNV replication. Six replicates were performed (three independent experiments, each with two replicates) and data are presented as the means ± SEM.

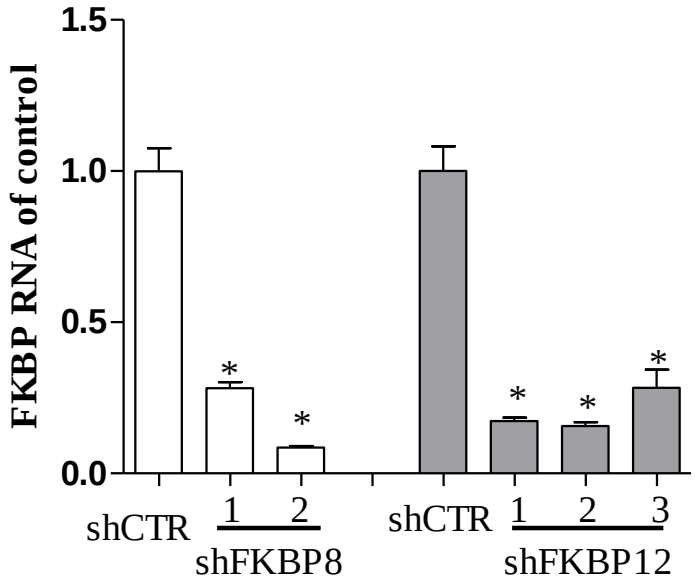



**Figure S3 qRT-PCR analysis of FKBP8 and FKBP12 RNA levels after shRNA-based knockdown.**

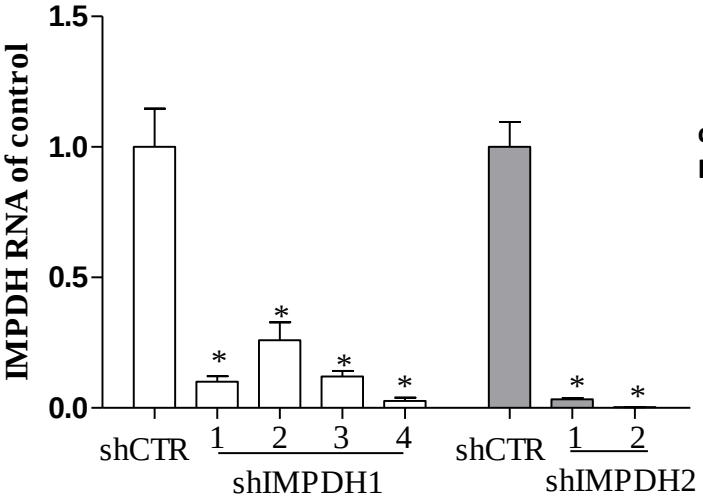



**Figure S4 qRT-PCR analysis of IMPDH RNA levels in IMPDH1 or IMPDH2 knockdown cells.**

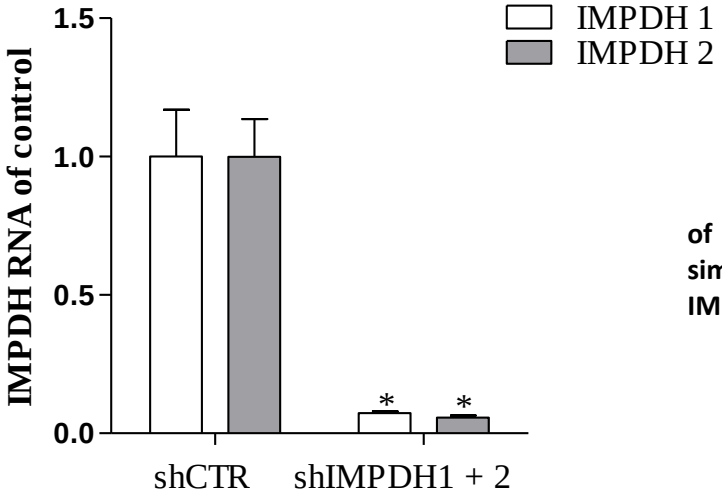



**Figure S5 qRT-PCR analysis of IMPDH RNA levels after simultaneous knockdown of IMPDH1 and IMPDH2.**

**Table S4 The Ki values of 4 IMPHD inhibitors toward IMPDH1 and IMPDH2.** Because of confidentiality, the structures are not shown.

| # | IMPDH I Ki in nM | IMPDH II Ki in nM | MW |
|---|---|---|---|
| 1315 | 0.60 | 13.90 | 744.53 |
| 1346 | 859.1 | 243.9 | 546.54 |
| 1351 | 618.40 | 185.90 | 549.59 |
| 1356 | 70.12 | 65.69 | 588.67 |

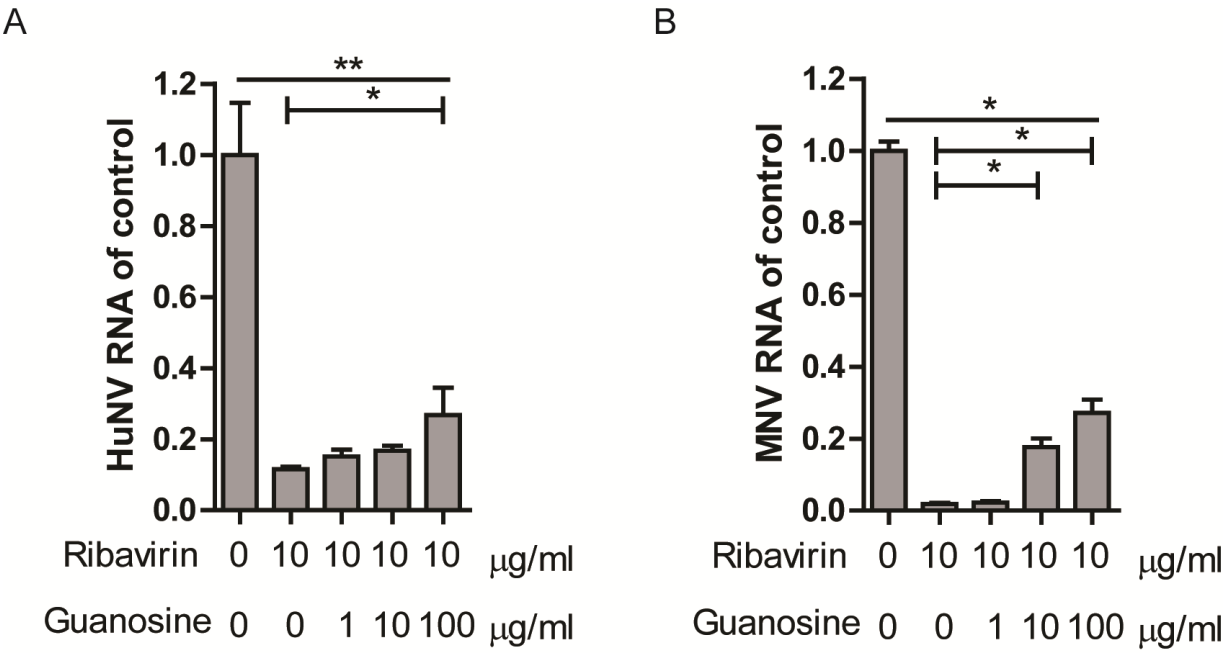



**Figure S6 The effects of exogenous guanosine on the antiviral effect of ribavirin toward HuNV (A) and MNV (B) model.**

## SUPPLEMENTARY DISCUSSION

Glucocorticosteroids were widely used during the early years of organ transplantation. Nowadays clinicians generally attempt to avoid prescribing steroids in case of active infection because of their potential of facilitating the infectious process and the concern about long-term complications associated with such treatment regimens [1]. For instance, steroids boluses used to treat acute rejection in hepatitis C virus (HCV) infection-related liver transplantation were often associated with an increase in viral load and the severity of HCV recurrence [1, 2]. In cell culture, steroids had no effect on HCV replication [3], but can specifically facilitate viral entry by enhancing the expression of the HCV co-receptors [4]. In the present study, however, we observed no direct effects of steroids on norovirus replication *in vitro*, although their impact on norovirus infection in patients remained to be further addressed. Rapamycin when complexed to FKBP12 to form the FKBP-rapamycin complex that directly binded to mTOR complex 1 and thus inhibited the mTOR pathway, appeared likewise safe but was associated with anti-norovirus activity *per se.* This contrasted the situation with respective to HCV infection. Rapamycin potently inhibited HCV RNA replication, but did not influence the early replication cycle steps associated with its biology such as cell entry and viral RNA translation [5]. Diametrically opposing the situation with HCV, another study showed that rapamycin facilitated hepatitis E virus (HEV) replication through inhibition of PI3K-PKB-mTOR pathway. The latter pathway limited HEV infection and acted as a gate-keeper with respect to HEV in human HEV target cells. In our study, rapamycin treatment resulted in no change on norovirus replication in cell culture models. Thus, effects of mTOR inhibitors on viral life cycle appear highly virus specific, hampering design of rational immunosuppressive therapies using this medication. Brequinar and leflunomide, two best-known inhibitors of dihydroorotate dehydrogenase (DHOD), interfere with cell proliferation by inhibiting pyrimidine nucleotide biosynthesis [6]. Leflunomide has been extensively studied and is approved for the treatment of psoriatic arthritis and rheumatoid arthritis [7]. Leflunomide has been shown to inhibit HIV-1 replication mainly through pyrimidine nucleotide pool depletion [8]. In our study, both leflunomide and brequinar showed no effects on HuNV replication and appeared thus not specifically useful for the management of patients at risk for norovirus infection.

# SUPPLEMENTARY REFERENCES

1. McGee S, Hirschmann J. 2008. Use of corticosteroids in treating infectious diseases. Arch Intern Med **168:**1034-1046.

2. Fong TL, Valinluck B, Govindarajan S, Charboneau F, Adkins RH, Redeker AG. 1994. Short-term prednisone therapy affects aminotransferase activity and hepatitis C virus RNA levels in chronic hepatitis C. Gastroenterology **107:**196-199.

3. Henry SD, Metselaar HJ, Van Dijck J, Tilanus HW, Van Der Laan LJ. 2007. Impact of steroids on hepatitis C virus replication in vivo and in vitro. Ann N Y Acad Sci **1110:**439-447.

4. Ciesek S, Steinmann E, Iken M, Ott M, Helfritz FA, Wappler I, Manns MP, Wedemeyer H, Pietschmann T. 2010. Glucocorticosteroids increase cell entry by hepatitis C virus. Gastroenterology **138:**1875-1884.

5. Stohr S, Costa R, Sandmann L, Westhaus S, Pfaender S, Anggakusuma, Dazert E, Meuleman P, Vondran FW, Manns MP, Steinmann E, von Hahn T, Ciesek S. 2015. Host cell mTORC1 is required for HCV RNA replication. Gut doi:gutjnl-2014-308971 [pii]
10.1136/gutjnl-2014-308971.

6. Hey-Mogensen M, Goncalves RL, Orr AL, Brand MD. 2014. Production of superoxide/H2O2 by dihydroorotate dehydrogenase in rat skeletal muscle mitochondria. Free Radic Biol Med **72:**149-155.

7. Munier-Lehmann H, Vidalain PO, Tangy F, Janin YL. 2013. On dihydroorotate dehydrogenases and their inhibitors and uses. J Med Chem **56:**3148-3167.

8. Schlapfer E, Fischer M, Ott P, Speck RF. 2003. Anti-HIV-1 activity of leflunomide: a comparison with mycophenolic acid and hydroxyurea. AIDS **17:**1613-1620.

# Chapter 4

## IRF-1, RIG-I and MDA5 display potent antiviral activities against norovirus coordinately induced by different types of interferons

Wen Dang[a], Lei Xu[a], Yuebang Yin[a], Sunrui Chen[a], Wenshi Wang[a], Mohamad S. Hakim[a], Kyeong-Ok Chang[b], Maikel P. Peppelenbosch[a], Qiuwei Pan[a, *]

[a]Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam, Netherlands.

[b]Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, USA

**Antiviral Res 2018. 155: 48-59.**

## ABSTRACT

Norovirus represents the main cause of acute nonbacterial gastroenteritis worldwide. In immunocompromised patients, it bears high risk of causing chronic infection with significant morbidity and mortality. The lack of specific treatment prompts the development of anti-norovirus agents. In this study, we have investigated the role of interferon (IFN) response and evaluated antiviral activities of different IFNs against human norovirus (HuNoV) replication using a HuNoV replicon model. We found that HuNoV RNA replication was sensitive to all types of IFNs, including IFN*α* (type I), IFN*γ* (type II), IFN*λ*1 and 3 (type III). IFNs canonically induce interferon-stimulated genes (ISGs) to exert their antiviral activities. By profiling a subset of important human ISGs using an overexpression approach, we have identified RTP4 and HPSE as moderate anti-norovirus ISGs, whereas IRF-1, RIG-I (also known as *DDX58*) and MDA5 (also known as *IFIH1*) were identified as potent anti-norovirus effectors. Interestingly, type I and III IFNs coordinately induced IRF-1, RIG-I and MDA5, whereas type II IFN predominantly induced IRF-1 to exhibit their anti-norovirus activities. Combination of different IFNs revealed that IFN*γ* worked cooperatively with type I or type III IFNs to induce ISGs and subsequently inhibit HuNoV replication. Of note, replication of HuNoV did not interfere with antiviral IFN response. In summary, we showed the potent anti-norovirus activities of different types of IFNs and identified the key anti-norovirus effectors. These findings are important for understanding norovirus-host interactions and developing antiviral therapies.


**KEYWORDS:** Norovirus; Interferon; IRF-1; RIG-I; MDA5

# INTRODUCTION

Norovirus represents the main cause of infectious viral gastroenteritis worldwide with significant morbidity and mortality. It is estimated that norovirus infection causes over 200,000 deaths annually of which more than 90,000 deaths occur in children under five years [1]. Although norovirus gastroenteritis is usually self-limiting, it has been recently recognized as a risk factor for chronic gastroenteritis in specific populations, particularly transplant patients [2, 3]. In transplant settings, norovirus gastroenteritis is characterized by severe complications, including protracted illness with malnutrition, organ failure and chronic viral shedding [4].

As an important component of the innate immune system, interferons (IFNs) constitute the first line of defense against invading pathogens. Currently, IFNs are classified into three major groups including type I, II and III IFNs. Upon viral infections, both type I and III IFNs are induced by the stimulation of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I) like helicases. Type I IFNs consisting of 13 subtypes are expressed by most cell types and secreted into the extracellular milieu. Secreted type I IFNs bind to the IFNAR receptors in an auto- or paracrine fashion and activate the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway, leading to the expression of a broad range of IFN-stimulated genes (ISGs), which are ultimate antiviral effectors [5]. Type III IFNs consist of three members including IFN$\lambda$1, $\lambda$2 and $\lambda$3 (IL29, IL28A and IL28B, respectively) and also induce a type I IFN-like response. Unlike type I IFNs which target most of the cell types, type III IFNs primarily target epithelial cells [6]. They play an important role in the epithelial antiviral host defense and consequently constitute a major barrier for enteric virus infections. Notably, type II IFN has solo member (IFN$\gamma$) and is involved in resisting a broad range of intracellular microorganisms [7].

First discovered in 1957, IFN$\alpha$ was clinically applied for treating hepatitis C virus (HCV) infection in 1986 [8]. It has been the backbone regimen for chronic HCV therapy for decades. In addition, PEG-IFN$\alpha$ has also been clinically used for treating chronic hepatitis B virus (HBV) infection [9]. Type II IFN$\gamma$-1b branded under the name Actimmune was approved in 2000 by the Food and Drug Administration (FDA) for the treatment of chronic granulomatous disease and severe malignant osteopetrosis [10]. However, IFN$\gamma$ failed to reduce viral load in human

immuno-deficiency virus (HIV) [11] or HCV [12] patients. Type III IFNs displayed comparable antiviral efficacy but reduced side effects when compared with IFN$\alpha$ for treating chronic HCV in phase III trial [9]. The success of IFNs as antiviral treatment in the clinic has promoted us to foresee their anti-norovirus potential, since no specific medication for norovirus gastroenteritis is currently available.

In this study, we have characterized the antiviral activities of type I, II and III IFNs against norovirus using a human norovirus (HuNoV) replicon model [13]. Furthermore, we have identified the key anti-norovirus effectors that are coordinately activated by different types of IFNs. These results have revealed new insights into norovirus-host interactions and provided a basis for future therapeutic applications of IFNs against norovirus gastroenteritis.

# METHODS

## *Cell lines and Reagents*

Human Huh7 hepatocellular carcinoma cells expressing a genotype 1 HuNoV replicon (HG23) and human embryonic kidney 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza Verviers, Belgium) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT, USA), 100 $\mu$g/mL of streptomycin, and 100 IU/mL of penicillin. A neomycin resistant gene was engineered into ORF2, thereby conferring resistance of HG23 cells to gentamicin (G418; Gibco) treatment. So G418 was added to HG23 culture medium at 1.5 mg/mL for selection before experimentation.

Human IFN alpha 2a recombinant protein (IFN$\alpha$) (Thermo-Fisher Scientific, Cat # 111001) and human IFN beta 1a recombinant protein (IFN$\beta$) (Sigma-Aldrich, Cat # 14151) were stocked in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) at $1 \times 10^5$ units/mL based on international units. Recombinant human IFN gamma (IFN$\gamma$) (BioLegend, Cat # 570202), human recombinant IL29 protein (IFN$\lambda$1) (Abnova, Cat # P3635), IL28A (IFN$\lambda$2) (Abnova, Cat # P5985) and IL28B (IFN$\lambda$3) (Abcam, Cat # ab201872) were stocked in PBS containing 0.1 % BSA at 100 $\mu$g/mL. To avoid repeated freeze-thaws, the compounds were aliquoted and stored at -80℃. During experimentation the compounds were stepwise diluted to desired concentration. CP-690550 (CAS 477600-75-2; Santa Cruz Biotechnology) was dissolved in DMSO at 1 mg/mL. The cytotoxicity of IFNs against HG23 cells was determined by MTT assay.

### *HuNoV antiviral assay*

HG23 cells were seeded into 48-well cell culture plates ($2.5 \times 10^4$ cells per well), treated with IFNs alone or in combination and assayed for HuNoV RNA levels after 2 days of treatment. To further evaluate the long-term effects of IFNs on HuNoV, a clearance and rebound assay was performed (Fig. S1) [14]. During the clearance phase, HG23 cells were treated with IFNs for 2, 6 or 10 days. At the end of each treatment period, the levels of HuNoV RNA were determined. After clearance phase, IFNs were omitted. HG23 cells were plated into 48-well cell culture plate ($2.5 \times 10^4$ per well) and cultured under the selective pressure of G418 (1 mg/mL). With another 5 days of culture, the cell layers were stained with hematoxylin and eosin, and visualized by an inverted Zeiss Axiovert 200 microscope equipped with Zeiss AxioCam MRm camera (LLC, Thornwood, NY, USA).

### *Generation of ISG-expressing lentiviral pseudoparticles and lentiviral transduction*

HEK293T cells were used to generate ISG-expressing lentivirus vectors. Twenty-four hours before transfection, $2 \times 10^6$ cells were plated into T75 flask. Two hours before transfection, the cell monolayers were washed with PBS for 3 times and refreshed with FCS-free DMEM. The plasmids expressing the pTRIP.CMV.IVSb.ISG.ires.TagRFP, HIV-1 gag-pol and VSV-G were combined in Opti-MEM at a ratio of 1:0.8:0.2, respectively. For each transfection, 150 $\mu$g PEI was added to 30 $\mu$g of total DNA to a final volume of 1 mL Opti-MEM. After 20 min of incubation at room temperature, the mixture was added dropwise to the cells. Six hours later the cells were gently washed once with PBS and refreshed with growth medium. After 48 hours and 72 hours of transfection conditioned medium were pooled and filtered through a 0.45-$\mu$m pore size filter. Two hours of ultracentrifugation (22,000 rpm) was used to concentrate lentiviral particles. The pellet was subsequently re-suspended and stored at -80℃ with the dilution of $1 \times 10^7$ viral RNA copies per mL.

For transduction assay, HG23 cells were inoculated into 48-well cell culture plate ($5 \times 10^4$ cells per well) and transduced with lentiviral pseudoparticles encoding ISGs. After 2 days of culture the cells were collected for detection of transduction efficiency by using a FACSAria™ flow cytometer (BD Bioscience) equipped with a 561-nm laser.

### *Statistics*

Data are presented as the mean ± SEM. Comparisons between groups were performed with Mann-Whitney test using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered significant at a *P* value less than 0.05.

## RESULTS

### *Type I, II and III IFNs universally and effectively inhibited HuNoV replication*

To determine the antiviral potential of different types of IFNs against HuNoV, we use a HuNoV replicon model to monitor HuNoV RNA replication after IFNs treatment. HG23 cells were treated with individual IFNs and viral replication was measured after 2 days of treatment by qRT-PCR. As shown in Fig. 1A and Table S1, IFNs reduced the levels of HuNoV RNA in a dose-dependent manner (EC50 and EC90): IFN$\alpha$ (59.88 and 330.7 IU/mL), IFN$\beta$ (156.20 and 935.0 IU/mL), IFN$\gamma$ (8.26 and 11.53 ng/mL), IFN$\lambda$1 (0.79 and 1.34 ng/mL), IFN$\lambda$2 (2.69 and 8.64 ng/mL) and IFN$\lambda$3 (1.43 and 10.97 ng/mL). The effects of IFNs on host cells were monitored by MTT assay. Even at the highest concentration, IFNs showed no major effects on the viability of HG23 cells with the exception of IFN$\lambda$1. Highest concentration of IFN$\lambda$1 (100 ng/mL) inhibited HG23 cell proliferation by 21 ± 3.1%. We next examined the kinetics of antiviral activities of IFNs over a time course ranging from 2 to 10 days. As shown in Fig. 1B, 10 days of treatment with IFN$\alpha$ (1,000 IU/mL), IFN$\beta$ (1,000 IU/mL), IFN$\gamma$ (10 ng/mL), IFN$\lambda$1 (10 ng/mL), IFN$\lambda$2 (10 ng/mL) or IFN$\lambda$3 (10 ng/mL) decreased HuNoV RNA levels by 99.9 ± 0.05%, 87 ± 5.3%, 80 ± 10%, 99.98 ± 0.01%, 47 ± 16% and 99.6 ± 0.01%, respectively.

To further study whether IFNs could completely clear HuNoV replicons from host cells after long-term treatment, gel-based RT-PCR assay was performed using RNA extracted from 10-day treated HG23 cells. Two primer sets, including neomycin primers (Neo) and HuNoV GI-specific diagnostic primers (NVp36/35) were used (Table S2, Fig. S2). We observed that the intensity of Neo and NVp36/35 products in HG23 cells was almost undetectable after treatment with 1,000 IU/mL of IFN$\alpha$, 100 ng/mL of IFN$\gamma$, 10 ng/mL of IFN$\lambda$1 or IFN$\lambda$3, whereas there was a moderate decrease in HG23 treated with 100 IU/mL of IFN$\alpha$, 1,000

IU/mL of IFN*β* or 100 ng/mL of IFN*λ*2 (Fig. 2A). Consistently, a rebound assay was designed to confirm the complete clearance of HuNoV replicons from HG23 cells after IFN treatment.

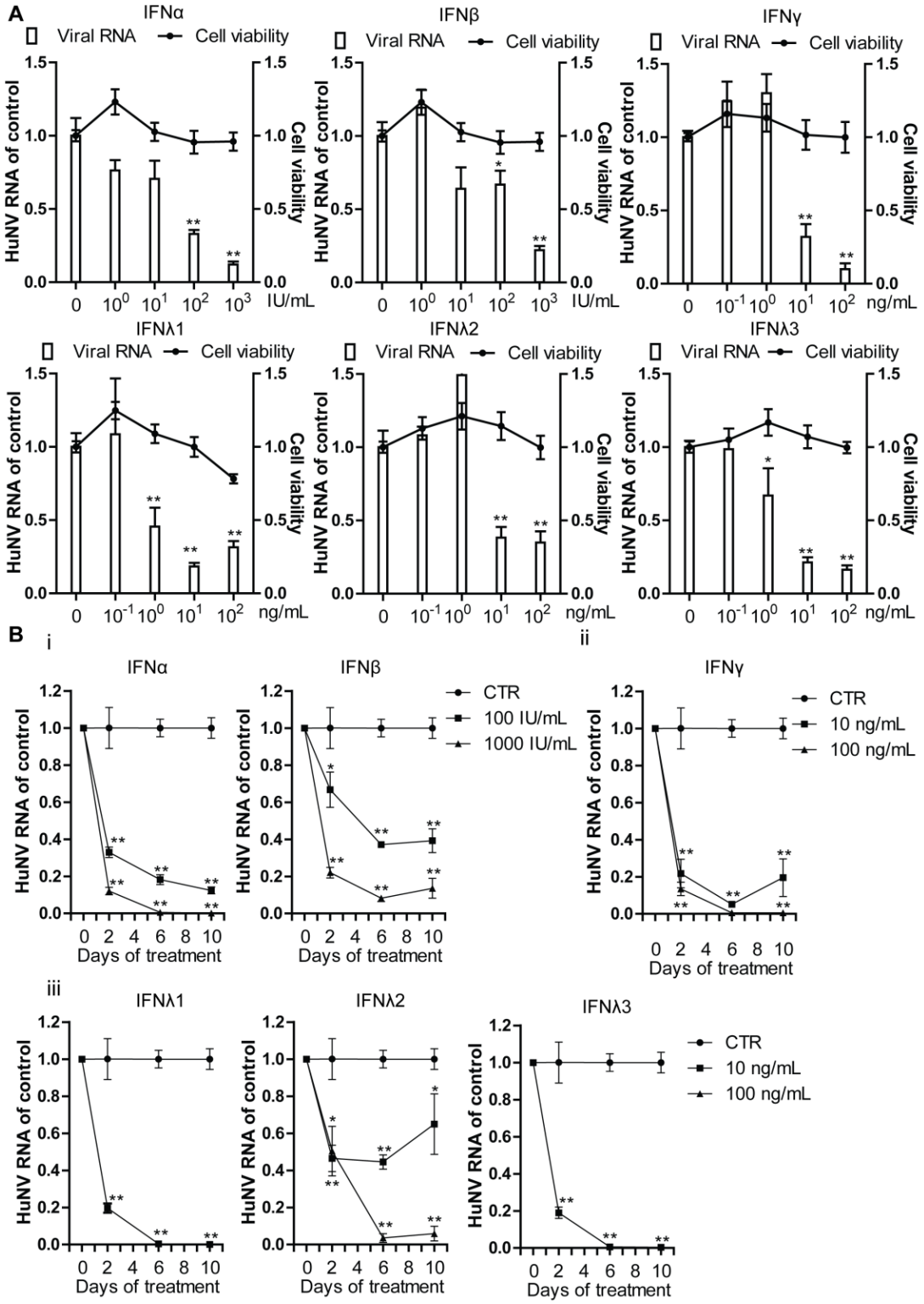



**Figure 1 IFNs robustly inhibited HuNoV replication at concentrations without significant cytotoxicity.** (A) HG23 cells were treated with different concentrations of IFNs as indicated. After 2 days of treatment the antiviral activities and cytotoxicity were measured by qRT-PCR and MTT assay, respectively. The concentration of type I IFNs is given as international units (IU/mL); while that of type II and III IFNs is given as weight per volume (ng/mL). The left *y* axis displayed the relative HuNoV

RNA levels compared with those of the untreated control; while the right *y* axis showed cell viability compared with medium group. (B) In prolonged treatment, HG23 cells were treated with different concentrations of IFNs for 2, 6 or 10 days. At the end of each treatment, HuNoV RNA was determined by qRT-PCR and normalized to human GAPDH gene. The level of remaining HuNoV RNA in IFN-treated cells was compared to that in untreated cells at the same time point. Shown are the means ± SEM from three independent experiments with duplicates each (Mann-Whitney test; *, $P < 0.05$; **, $P < 0.01$).

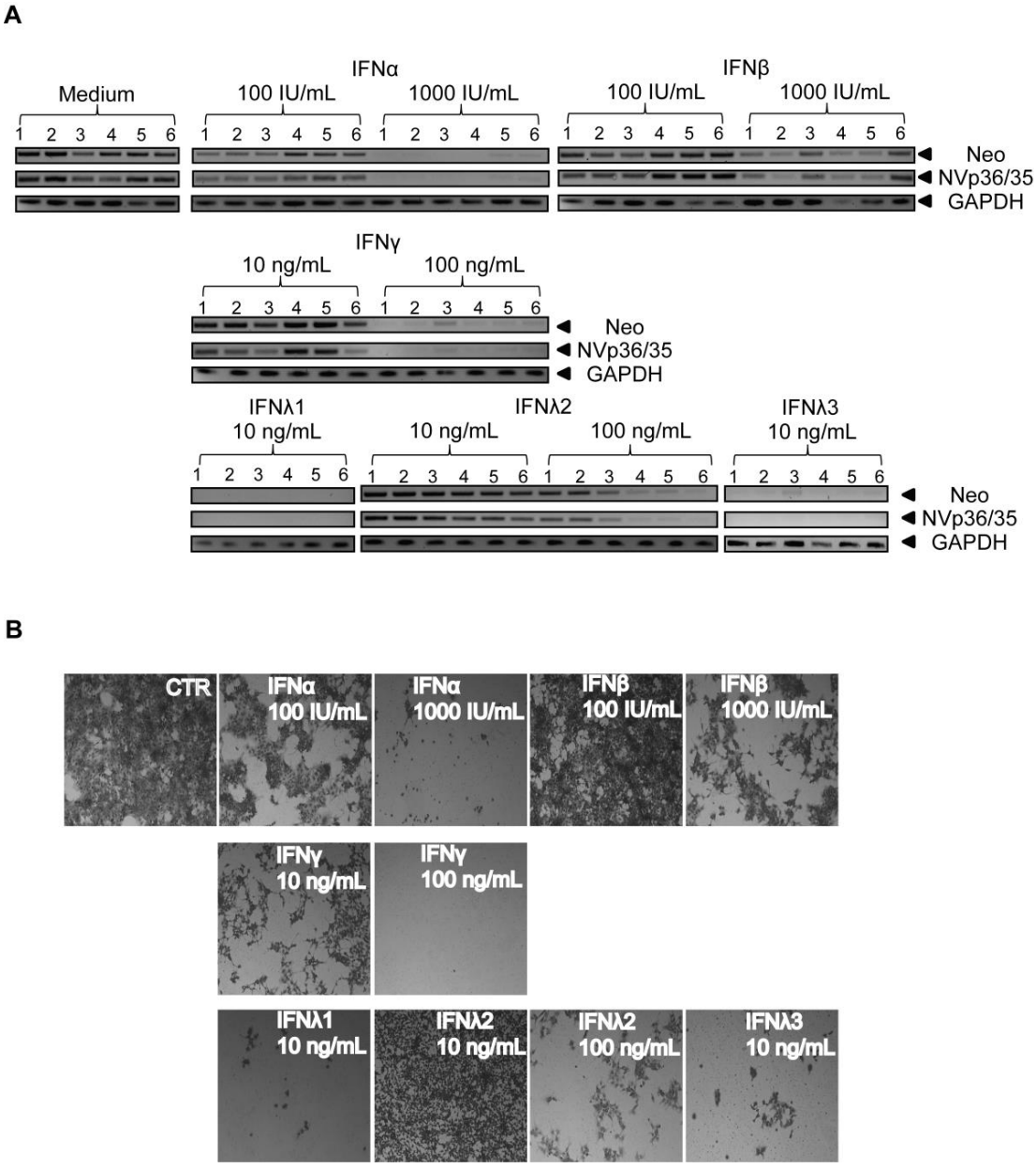



**Figure 2 High concentrations of IFNs completely cleared HuNoV replicons.** (A) Gel-based RT-PCR analysis was performed to determine HuNoV replicon levels in HG23 cells after long-term treatment with IFNs. Equal amounts of total cellular RNAs isolated from HG23 samples were first reverse transcribed into cDNA and then subjected to RT-PCR analysis by using neomycin primer pair (804 bp) and HuNoV GI-specific primer pair (470 bp). Human GAPDH primer set was used as a reference gene. A total of 6 samples were detected and indicated by numbers. (B) Rebound assay. IFNs-treated HG23 cells were cultured with G418 (1 mg/mL). After 5 days of culture the plate was stained and visualized under light microscope. Images are representative of three independent experiments.

Ten-day IFN-treated HG23 cells were grown in the presence of G418 (1 mg/mL). If HG23 cells contained HuNoV replicons, they could survive in G418 selection and even proliferate. Treatment with IFN*β* (1,000 IU/mL), IFN*γ* (10 ng/mL) or IFN*λ*2 (10 ng/mL) resulted in a relatively moderate reduction of HuNoV replicon (Fig. 2A). Therefore HG23 cells proliferated and formed colonies (Fig. 2B). In contrast, treatment with IFN*α* (1,000 IU/mL), IFN*λ*1 (10 ng/mL) or IFN*λ*3 (10 ng/mL) resulted in a more pronounced drop in HuNoV RNA levels during the long-term treatment. Therefore HG23 cells lost the ability to survive (Fig. 2B), indicating that these IFNs were able to completely clear HuNoV replicons from the host cells.

## *Profiling important ISGs identifys RTP4 and HPSE as mild antiviral ISGs against HuNoV*

ISGs are the ultimate antiviral effectors induced by IFNs. After IFN treatment hundreds of ISGs are induced. The induction of a subset of ISGs by different types of IFNs in HG23 cells was shown in Fig. S3. To identify the key ISGs that are potentially responsible for the anti-norovirus effects of IFNs, we have profiled well-known antiviral ISGs, including IRF-1, C6orf150 (also known as *MB21D1*), HPSE, RIG-I (also known as *DDX58*), MDA5 (also known as *IFIH1*), IFITM3, DDX60, IFI44L, IFI6, IFITM2, MAP3K14, MOV10, NAMPT (also known as *PBEF1*), OASL, RTP4, TREX1, ADAR, FAM46C, MCOLN2, IRF-2, UNC84B (also known as *SUN2*), IFITM1, LY6E and IRF-7 on HG23 cells. A bicistronic lentiviral vector co-expressing an ISG and a red fluorescent protein TagRFP was used to overexpress the ISG (Fig. 3A) [15]. With a pilot experiment, we have found that a 48-hour transduction was an optimal time point for transgene expression (Fig. S4A). Next, all ISGs were tested for their anti-norovirus activities using HG23. After 2 days of transduction expression levels of different ISGs and their relative anti-norovirus effectiveness were determined (Table S3, Fig. 3B). Three of these lentiviral vectors including IFI44L, IFITM2 and MOV10 failed to mediate high-level transduction in host cells, perhaps resulting from ISG-mediated toxicity and poor packaging of the vector genome. Most of the successfully expressed ISGs did not have major effects on HuNoV replication; whereas RTP4 and HPSE showed moderate antiviral activities and inhibited HuNoV replication by 50.5 ± 6.6 % and 42.2 ± 4.3 %, respectively (Table S3, Fig. 3B). The kinetics of HPSE and RTP4 expression were further determined after 24, 48 and 72 hours of transduction by detecting the ISGs mRNA (Fig. 3C and E) and RFP protein (Fig. S4B and C).

After verifying the successful overexpression of HPSE and RTP4, we found that HPSE and RTP4 exerted the most potent inhibition of HuNoV replication at 48 hours (Fig. 3D and F).

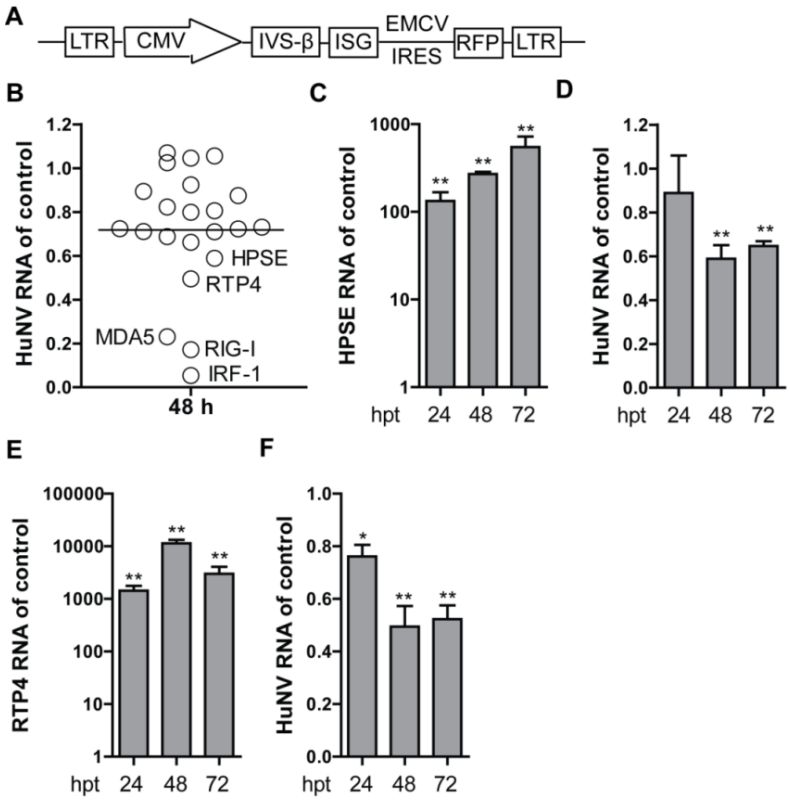



**Figure 3 Profiling ISGs identifies HPSE and RTP4 as mild anti-norovirus effectors.** (A) Schematic of the Gateway-compatible, bicistronic lentiviral vector. (B) Dot plots of HuNoV replication levels in the presence of overexpressed ISGs at 48 hours (*n* = 4 independent experiments with duplicates each). The *y* axis displayed the relative HuNoV RNA levels normalized to the Fluc control. The black line reflected the population mean. (C) HG23 cells were transduced with HPSE overexpression lentiviral vector or Fluc vector. After 24, 48 and 72 hours of transduction, HPSE mRNA levels were confirmed by qRT-PCR (*n* = 4 independent experiments with duplicates each). HPSE mRNA expression was increased as early as 24 hours and persisted to 72 hours. (D) HuNoV replication was determined by qRT-PCR and compared to that in Fluc control samples at the same points (*n* = 4 independent experiments with duplicates each). (E and F) HG23 cells were transduced with RTP4 overexpression lentiviral vector. The overexpression efficiency and antiviral effectiveness were determined by qRT-PCR (*n* = 4 independent experiments with duplicates each). The abbreviation used was: hpt, hours post-transduction. Data are presented as the mean ± SEM (*, $P < 0.05$; **, $P < 0.01$).

## *IRF-1, RIG-I and MDA5 were potent anti-norovirus effectors*

Among these tested ISGs, IRF-1, RIG-I and MDA5 exerted strong anti-norovirus activities (Fig. 3B). We also profiled the antiviral dynamics of these three potent effectors after 12, 24 and 48 hours of transduction. The transduction efficiency was monitored by RFP expression with flow cytometry analysis (Fig. S5). IRF-1, RIG-I and MDA5 expression was determined by qRT-PCR and Western blotting (Fig. 4A, C and E). After 2 days of transduction, IRF-1, RIG-I and MDA5 inhibited HuNoV RNA replication by 95 ± 2%, 83 ± 3% and 77 ± 6%, respectively (Table S3, Fig. 4B, D and F).

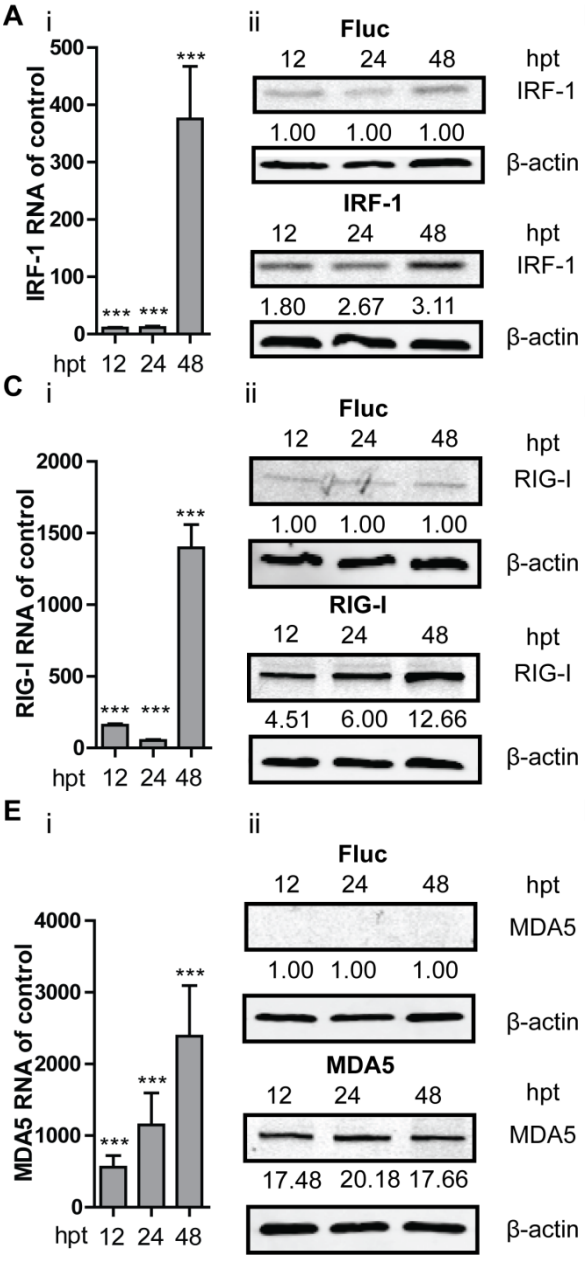



**Figure 4 Identification of IRF-1, RIG-I and MDA5 that potently inhibited HuNV replication.** (A, C and E) HG23 cells were transduced with ISG lentiviral vectors. After 12, 24 and 48 hours of transduction, cells were collected for determination of ISG mRNA and protein expression. (i) qRT-PCR was employed to quantify the expression of ISGs. The results were normalized to GAPDH and compared to Fluc control (*n* = 4 independent experiments with duplicates each). (ii) Western blotting was used for analysis of ISG proteins. IRF-1, RIG-I and MDA5 mRNA levels were significantly induced as early as 12 hours and were sustained for 48 hours. (B, D and F) To quantify HuNoV replication, qRT-PCR analysis was performed and the results were compared to Fluc control (*n* = 4 independent experiments with duplicates each). The abbreviation used was: hpt, hours post-transduction. Data are presented as the mean ± SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

## *RTP4, HPSE, IRF-1, RIG-I and MDA-5 were differentially induced by different types of IFNs*

Although all three types of IFNs exerted anti-norovirus effects, their potency varied considerably (Fig. 1A and B). Therefore, we have investigated whether it was due to the different induction levels and patterns of these anti-norovirus effectors stimulated by different types of IFNs. We found that type I and III IFNs coordinately and strongly induced RTP4, HPSE, IRF-1, RIG-I and MDA5. On the contrary, IFN*γ* slightly stimulated RIG-I, MDA5 and RTP4, but robustly and predominantly induced IRF-1 (Fig. 5A, Table S4). These results

were further confirmed by Western blot (Fig. 5B). These results suggested that different types of IFNs exerted variable anti-norovirus effects by inducing distinct subsets of ISGs.

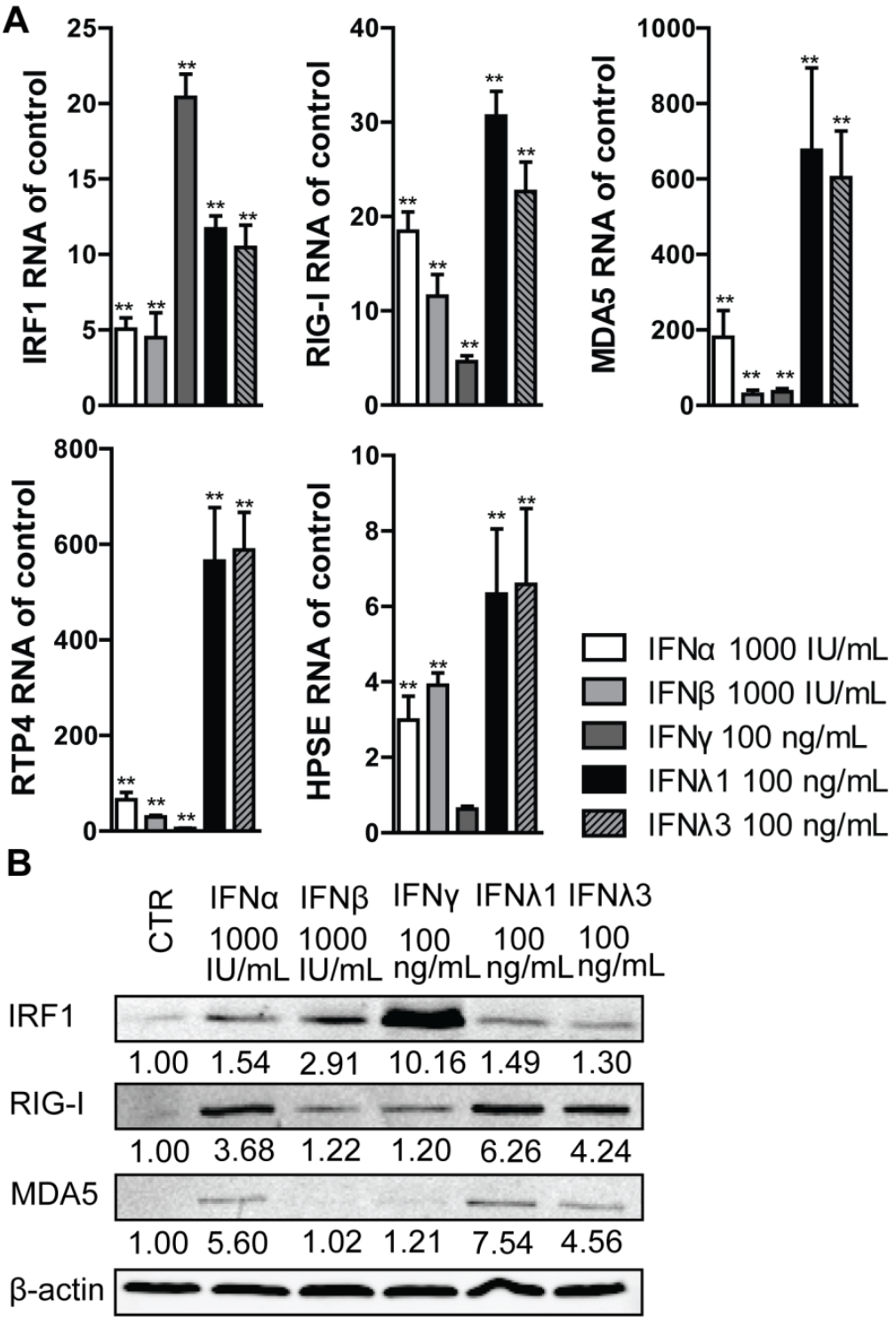



**Figure 5 Type I and III IFNs coordinately induced IRF-1, RIG-I and MDA5; whereas type II IFN predominantly induced IRF-1 to exert their antiviral activities against HuNoV.** HG23 cells were treated with high concentrations of IFNs. After 2 days of treatment the expression of ISG mRNA and protein was detected by qRT-PCR (A) (*n* = 3 independent experiments with duplicates each) and Western blotting (B), respectively. Data are presented as the mean ± SEM (*, $P < 0.05$; **, $P < 0.01$).

## *IFNγ potentiated the anti-norovirus effects of type I and III IFNs by cooperative induction of IRF-1, RIG-I and MDA5*

Different types of IFNs bind to their unique receptors and activate a distinct pattern of antiviral effectors. We therefore explored the combination of different IFNs to inhibit HuNoV replication. As shown in Fig. 6A, the combination of IFN*γ* with IFN*λ*1 resulted in a pronounced decline of HuNoV RNA replication (83 ± 1.8%) after 2 days of treatment when compared with IFN*γ* (10 ng/mL; 70 ± 4.2%) or IFN*λ*1 (10 ng/mL; 54 ± 12%) alone. IFN*γ* combined with IFN*α* were more effective and decreased HuNoV RNA level by 98.2 ± 0.2% when compared with IFN*γ* (10 ng/mL; 74 ± 4%) or IFN*α* (100 IU/mL; 85 ± 4%) alone. However this was not observed in the combination of IFN*α* with IFN*λ*1.

Consistently, the combination of IFNs significantly enhanced the magnitude of IRF-1, RIG-I and MDA5 expression when compared with individual IFNs alone (Fig. 6B and C). These results suggested that cooperative induction of essential anti-norovirus ISGs by the combination of different IFNs augmented their antiviral activities.

## *Pharmacological inhibition of JAK completely abolished the anti-norovirus effects of type I and III IFNs, but partially attenuated the effects of type II IFN*

The JAK-STAT pathway mediates the induction of ISGs and antiviral effects of IFNs. CP-690550 (tofacitinib), a pharmacological inhibitor of JAK has been approved by FDA for the treatment of rheumatoid arthritis [16]. Thus, we have investigated the role of CP-690550 on IFNs-induced inhibition of HuNoV replication. Of note, a high concentration (1,000 ng/mL) of CP-690550 alone had a mild inhibition of HuNoV replication by 30 ± 9% after 2 days of treatment (Fig. 7A). Combination of CP-690550 with type I or III IFNs could completely restore the HuNoV RNA replication. However, it partially restored HuNoV replication when combined with IFN*γ*. Consistently, CP-690550 effectively blocked ISG expression induced by IFNs (Fig. 7B, Fig. S6), demonstrating that CP-690550 counteracted the inhibition of HuNoV replication by IFNs through blocking ISG induction.

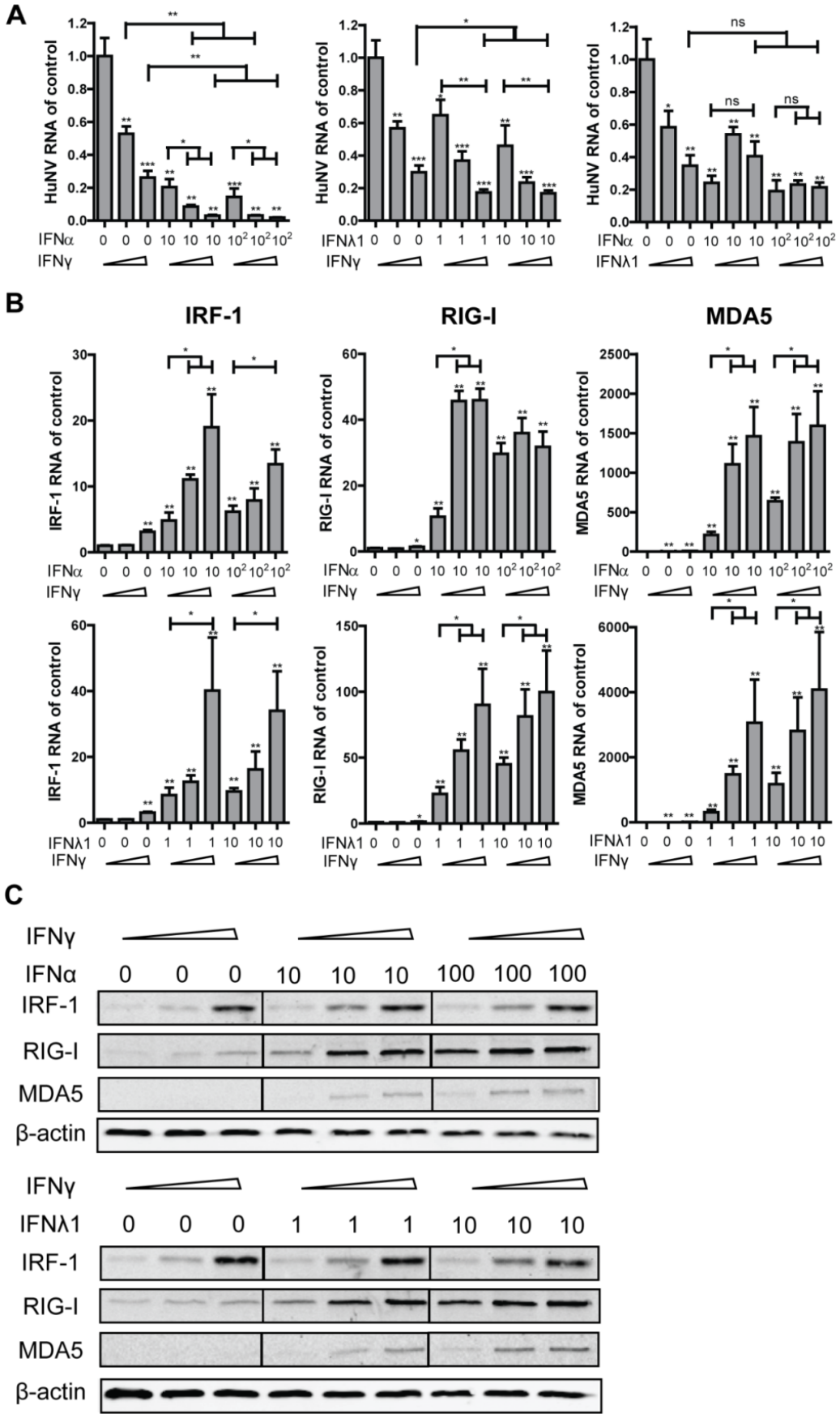



**Figure 6 Combinatory effects of different IFNs on HuNoV replication and ISG induction.** (A) HG23 cells were treated with IFN*α* (0, 10 and 100 IU/mL), IFN*γ* (0, 1 and 10 ng/mL), IFN*λ*1 (0, 1 and 10

ng/mL) or in combination. After 2 days of treatment, the inhibitory effects of different IFN combinations on HuNoV replication were compared to those of the IFN-only treated samples (*n* = 3 independent experiments with duplicates each). The expression of anti-norovirus ISGs at mRNA and protein levels were detected by qRT-PCR (B) and Western blotting (C), respectively. The qRT-PCR results were normalized to GAPDH and displayed as fold change relative to untreated control (*n* = 3 independent experiments with duplicates each). The Western blot images were representative of three independent experiments. Data are presented as the mean ± SEM (*, *P* < 0.05; **, *P* < 0.01).

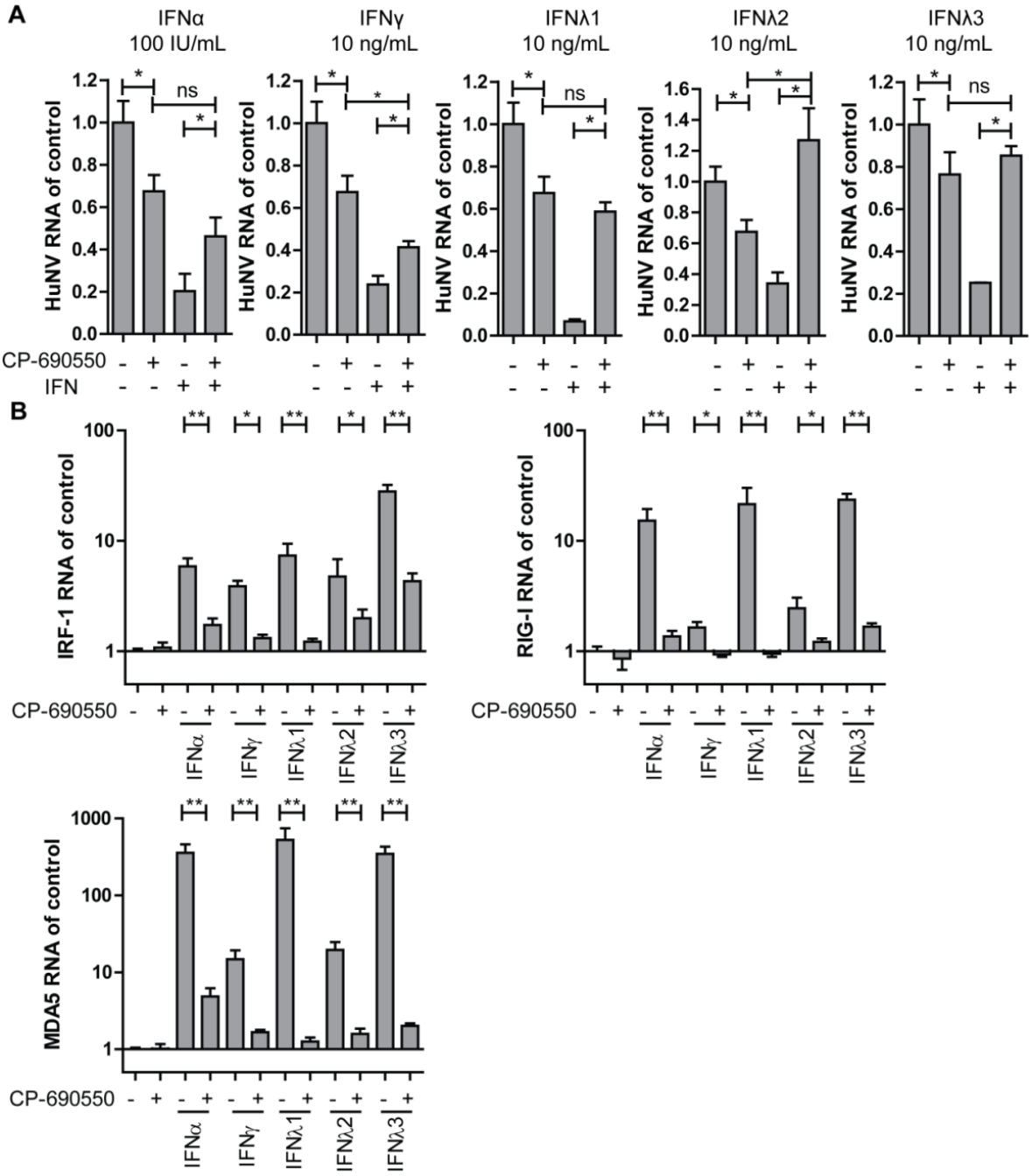



**Figure 7 Inhibition of JAK negated the anti-norovirus activities and the induction of ISGs by IFNs.** (A) HG23 cells were treated with IFNs, JAK inhibitor CP-690550 (1,000 ng/mL) or in combination. After 2 days of treatment HuNoV replication was quantified by qRT-PCR and compared to medium control (*n* = 3 independent experiments with duplicates each). ISG expression was quantified by qRT-PCR and compared to medium control (B; *n* = 3 independent experiments with duplicates each). Data are presented as the mean ± SEM (*, *P* < 0.05; **, *P* < 0.01).

## DISCUSSION

Although the burden of norovirus gastroenteritis has been well-recognized [1, 4], no specific anti-norovirus treatment is currently available except for supportive care and supplementation of fluids. Ribavirin is effective in inhibiting both HuNoV and murine norovirus (MNV) RNA replication *in vitro* [17]. However, *in vivo* study has revealed that only a subset of patients responded to ribavirin treatment [18]. Nitazoxanide, a broad-spectrum antimicrobial agent, has been reported to be a safe therapeutic option in managing norovirus gastroenteritis in immunocompetent adults [19] and transplantation patients [20, 21]. However, a case report has described that nitazoxanide was ineffective for treatment of chronic norovirus gastroenteritis in patient with X-Linked Agammaglobulinemia [22]. Thus, the investigation of anti-norovirus effects of IFNs in this study bears important clinical implications.

The lack of suitable tissue culture or a convenient small animal model is a great barrier for understanding the true nature of HuNoV pathogenesis and developing specific antivirals. Cultivation of HuNoV has been attempted in 27 cell lines, including eight human gastrointestinal epithelia cells, but was unsuccessful [23]. HuNoV was recently found to infect B cells *in vitro* requiring the presence of histo-blood group antigen (HBGA)-expression enteric bacteria [24]. In addition, multiple HuNoV strains have been successfully cultivated in stem cell-derived, nontransformed human intestinal enteroids [25]. However, both B cells and intestinal enteroids have not been widely used yet, probably due to technical challenges and limitations. In our study, we employed a widely used HuNoV replicon model and revealed that IFN$\alpha$ and type III IFNs (IFN$\lambda$1 and IFN$\lambda$3) exerted rapid and potent antiviral activities against HuNoV replication. Moreover, IFN$\gamma$ also robustly inhibited HuNoV RNA replication. As type III IFNs are expressed in a tissue-dependent fashion and primarily act on epithelial cells *in vivo* [26], IFN$\lambda$ holds considerable therapeutic potential in restricting norovirus gastroenteritis.

Though highly sensitive to IFN treatment, HuNoV is still less sensitive to IFNs when compared with HCV. Treatment with 1 IU/mL of IFN$\alpha$ resulted in robust inhibition of HCV replication in the Huh7-based subgenomic replicon containing a luciferase reporter and almost completely suppressed HCV replication after 72 h of treatment. Consistently, in the full-length HCV model, IFN$\alpha$ (10-1,000 IU/mL) dose-dependently inhibited viral replication

[27], which was almost 10 times more sensitive than that in our HuNoV study. The same phenomenon was observed in respect to type II and III IFNs on HCV and HuNoV. However, our study demonstrated that IFNs at clinically achievable concentrations exerted a strong inhibition of HuNoV replication. Meanwhile combination of IFN$\alpha$ with ribavirin showed additive effects on the inhibition of HuNoV replication [17], indicating that IFN monotherapy or combination therapy with ribavirin had the potential for treating norovirus gastroenteritis.

It has been previously reported that IFN$\alpha/\beta$ and IFN$\gamma$ potently inhibited HuNoV replication in the replicon-bearing cells *in vitro* [13, 17]. By transfecting stool-derived HuNoV RNA into mammalian cells, HuNoV RNA replication and virus particle production were sensitive to type I and III IFNs treatment [28]. Consistently, both type I and II IFNs inhibited MNV replication in permissive myeloid cells by restricting the translation of MNV proteins [29]. Moreover, IFN$\alpha$ and IFN$\beta$ were found to prevent systematic spread of MNV, only IFN$\lambda$ was capable of curing persistent MNV infection, suggesting its great therapeutic potential of treating norovirus infection [30]. However, the exact mechanism of how IFNs inhibited HuNoV RNA replication is unclear. Upon IFN treatment, hundreds of ISGs are induced. However, only a subset of them have broad or targeted antiviral effects. Interestingly, a few ISGs are even pro-viral for particular viruses, highlighting the complexity of the highly pleiotropic IFN system [15]. By profiling the well-known ISGs, we found that IRF-1, RIG-I and MDA5 were potent anti-norovirus ISGs. Further studies have showed that type I and III IFNs robustly induced a large set of ISGs, especially IRF-1, RIG-I and MDA5 to inhibit HuNoV replication. In contrast, IFN$\gamma$ strongly and predominantly induced IRF-1, which contributed to its anti-norovirus effects.

The exact mechanism of how IRF-1, RIG-I and MDA5 counteract HuNoV replication requires future research. IRF-1 has been reported to induce the expression of ISGs, similar to type I IFNs. IRF-1 specifically binded to the upstream regulatory region of the IFN$\beta$ and activated IFN$\beta$ gene expression in virus-infected fibroblasts [31]. IRF-1 also effectively counteracted hepatitis E virus (HEV) replication through the activation of the JAK-STAT pathway and the subsequent induction of antiviral ISGs, but independent of IFN production [32]. Similarly, it was classically recognized that RIG-I exerted its antiviral activity by inducing IFN production upon sensing invading virus infection. However, in HEV study RIG-I stimulated the cellular innate immunity against HEV partially through the JAK-STAT pathway, regardless of IFN production [33]. Like RIG-I, MDA5 functioned as a sensor to detect many

viral infections and was critical for type I IFN induction during virus infections [34]. Notably, MDA5 recognized MNV strain MNV-1 infection *in vivo* and stimulated the production of type I IFNs and other cytokines [35]. To further address this issue in our model, we have evaluated the effects of these three ISGs on the expression of other ISGs including two known anti-norovirus ISGs PKR [29] and ISG15 [36]. Overexpression of any of the three ISGs stimulated the expression of other ISGs in our HuNoV model (Fig. S7). Nevertheless, the exact antiviral mechanism of these ISGs remains to be further investigated.

Because different types of IFNs activate distinct patterns of ISGs, we hypothesized that they may function cooperatively to inhibit HuNV replication. Indeed, we found that IFN*γ* exerted cooperative anti-norovirus activity when combined with type I or III IFNs. Previous study has shown that combination of IL29 with IFN*α* or IFN*γ* synergistically inhibited HCV replication. The combination treatment mechanistically resulted in increased activation of the ISRE promoter elements to further enhance ISG expression [37]. Similarly, we have demonstrated that the combination of IFN*γ* with IFN*α* or IFN*λ*1 cooperatively induced the expression of IRF-1, RIG-I and MDA5, contributing to their augmented anti-norovirus activity. These results indicate a cooperation of different types of IFNs in combating norovirus replication, but also provide rationale for potential combination therapy.

High responsiveness of HuNoV to IFN treatment potentiated the application of exogenous IFNs as immunotherapy against norovirus gastroenteritis. Conversely, IFN antagonism has been employed by some viruses, including zika virus [38] and HEV [39] to escape the immune control. By further investigating the JAK-STAT pathway and mRNA levels of different ISGs in the presence or absence of HuNoV after IFN*α* treatment, we found that HuNoV showed no major effect on counteracting IFN response (Supplementary Materials and Results), which further highlighted the vital role of IFNs on combating norovirus infection.

## CONCLUSION

In summary, we have demonstrated the anti-norovirus effects of different types of IFNs and identified the key antiviral effectors including IRF-1, RIG-I and MDA5. Furthermore, HuNoV displayed no major effect on IFN signaling pathway. Given the fact that both type I and II IFNs have been widely used in the clinic and type III IFNs are at the stage of clinical

development, our results bear important implications for the future application of IFNs for treating norovirus gastroenteritis.

## ACKNOWLEDGEMENT

We gratefully acknowledge Mary K. Estes (Baylor College of Medicine, Houston) for providing the pHuNoV$_{U201F}$ and pHuNoV$_{U201F}$-based constructs. We also thank Charles M. Rice (The Rockefeller University, New York) for providing ISG lentiviral vectors. This work was supported by Dutch Digestive Foundation (MLDS) for a career development grant (No. CDG 1304), the Daniel den Hoed Foundation for a Centennial Award fellowship (to Q. Pan), the China Scholarship Council for funding PhD fellowship to W. Dang (201406180072), L. Xu (201306300027), Y. Yin (201307720045), W. Wang (201303250056) and S. Chen (201606760056), and Indonesia Endowment Fund for Education (LPDP) for funding PhD fellowship to Mohamad S. Hakim.

## AUTHOR CONTRIBUTIONS

WD, LX, YY, SC and WW performed the experiments; MH and KO contributed to the scientific discussion and facilities; WD, MP and QP conceived the project and wrote the manuscript.

# REFERENCE


1. Bartsch SM, Lopman BA, Ozawa S, Hall AJ, Lee BY. 2016. Global Economic Burden of Norovirus Gastroenteritis. PLoS One **11:**e0151219.
2. Ye X, Van JN, Munoz FM, Revell PA, Kozinetz CA, Krance RA, Atmar RL, Estes MK, Koo HL. 2015. Noroviruses as a Cause of Diarrhea in Immunocompromised Pediatric Hematopoietic Stem Cell and Solid Organ Transplant Recipients. Am J Transplant **15:**1874-1881.
3. Bok K, Green KY. 2012. Norovirus gastroenteritis in immunocompromised patients. N Engl J Med **367:**2126-2132.
4. Angarone MP, Sheahan A, Kamboj M. 2016. Norovirus in Transplantation. Curr Infect Dis Rep **18:**17.
5. Sen GC. 2001. Viruses and interferons. Annu Rev Microbiol **55:**255-281.
6. Ank N, Iversen MB, Bartholdy C, Staeheli P, Hartmann R, Jensen UB, Dagnaes-Hansen F, Thomsen AR, Chen Z, Haugen H, Klucher K, Paludan SR. 2008. An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. J Immunol **180:**2474-2485.
7. de Weerd NA, Nguyen T. 2012. The interferons and their receptors--distribution and regulation. Immunol Cell Biol **90:**483-491.
8. Hoofnagle JH, Mullen KD, Jones DB, Rustgi V, Di Bisceglie A, Peters M, Waggoner JG, Park Y, Jones EA. 1986. Treatment of chronic non-A,non-B hepatitis with recombinant human alpha interferon. A preliminary report. N Engl J Med **315:**1575-1578.
9. Lin FC, Young HA. 2014. Interferons: Success in anti-viral immunotherapy. Cytokine Growth Factor Rev **25:**369-376.
10. Razaghi A, Owens L, Heimann K. 2016. Review of the recombinant human interferon gamma as an immunotherapeutic: Impacts of production platforms and glycosylation. J Biotechnol **240:**48-60.
11. Roff SR, Noon-Song EN, Yamamoto JK. 2014. The Significance of Interferon-gamma in HIV-1 Pathogenesis, Therapy, and Prophylaxis. Front Immunol **4:**498.
12. Couzigou P, Perusat S, Bourliere M, Trimoulet P, Poynard T, Leroy V, Marcellin P, Foucher J, Bronowicki JP, Chene G, Group AHGT. 2013. Interferon-gamma with peginterferon alpha-2a and ribavirin in nonresponder patients with chronic hepatitis C (ANRS HC16 GAMMATRI). J Gastroenterol Hepatol **28:**329-334.
13. Chang KO, Sosnovtsev SV, Belliot G, King AD, Green KY. 2006. Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line. Virology **353:**463-473.
14. Dang W, Yin Y, Peppelenbosch MP, Pan Q. 2017. Opposing Effects of Nitazoxanide on Murine and Human Norovirus. J Infect Dis **216:**780-782.
15. Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P, Rice CM. 2011. A diverse range of gene products are effectors of the type I interferon antiviral response. Nature **472:**481-485.
16. Boor PPC, de Ruiter PE, Asmawidjaja PS, Lubberts E, van der Laan LJW, Kwekkeboom J. 2016. JAK-inhibitor tofacitinib suppresses interferon alfa production by plasmacytoid dendritic cells and inhibits arthrogenic and antiviral effects of interferon alfa. Translational Research.
17. Chang KO, George DW. 2007. Interferons and ribavirin effectively inhibit Norwalk virus replication in replicon-bearing cells. J Virol **81:**12111-12118.
18. Woodward JM, Gkrania-Klotsas E, Cordero-Ng AY, Aravinthan A, Bandoh BN, Liu H, Davies S, Zhang H, Stevenson P, Curran MD, Kumararatne D. 2015. The role of chronic norovirus infection in the enteropathy associated with common variable immunodeficiency. Am J Gastroenterol **110:**320-327.
19. Rossignol JF, El-Gohary YM. 2006. Nitazoxanide in the treatment of viral gastroenteritis: a randomized double-blind placebo-controlled clinical trial. Aliment Pharmacol Ther **24:**1423-1430.

20. Siddiq DM, Koo HL, Adachi JA, Viola GM. 2011. Norovirus gastroenteritis successfully treated with nitazoxanide. J Infect **63:**394-397.

21. Rossignol JF. 2014. Nitazoxanide: a first-in-class broad-spectrum antiviral agent. Antiviral Res **110:**94-103.

22. Kempf B, Edgar JD, Mc Caughey C, Devlin LA. 2017. Nitazoxanide Is an Ineffective Treatment of Chronic Norovirus in Patients With X-Linked Agammaglobulinemia and May Yield False-Negative Polymerase Chain Reaction Findings in Stool Specimens. J Infect Dis **215:**486-487.

23. Duizer E, Schwab KJ, Neill FH, Atmar RL, Koopmans MP, Estes MK. 2004. Laboratory efforts to cultivate noroviruses. J Gen Virol **85:**79-87.

24. Jones MK, Watanabe M, Zhu S, Graves CL, Keyes LR, Grau KR, Gonzalez-Hernandez MB, Iovine NM, Wobus CE, Vinje J, Tibbetts SA, Wallet SM, Karst SM. 2014. Enteric bacteria promote human and mouse norovirus infection of B cells. Science **346:**755-759.

25. Ettayebi K, Crawford SE, Murakami K, Broughman JR, Karandikar U, Tenge VR, Neill FH, Blutt SE, Zeng XL, Qu L, Kou B, Opekun AR, Burrin D, Graham DY, Ramani S, Atmar RL, Estes MK. 2016. Replication of human noroviruses in stem cell-derived human enteroids. Science **353:**1387-1393.

26. Sommereyns C, Paul S, Staeheli P, Michiels T. 2008. IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. PLoS Pathog **4:**e1000017.

27. Zhou X, Xu L, Wang W, Watashi K, Wang Y, Sprengers D, de Ruiter PE, van der Laan LJ, Metselaar HJ, Kamar N, Peppelenbosch MP, Pan Q. 2016. Disparity of basal and therapeutically activated interferon signalling in constraining hepatitis E virus infection. J Viral Hepat **23:**294-304.

28. Qu L, Murakami K, Broughman JR, Lay MK, Guix S, Tenge VR, Atmar RL, Estes MK. 2016. Replication of Human Norovirus RNA in Mammalian Cells Reveals Lack of Interferon Response. J Virol **90:**8906-8923.

29. Changotra H, Jia Y, Moore TN, Liu G, Kahan SM, Sosnovtsev SV, Karst SM. 2009. Type I and type II interferons inhibit the translation of murine norovirus proteins. J Virol **83:**5683-5692.

30. Nice TJ, Baldridge MT, McCune BT, Norman JM, Lazear HM, Artyomov M, Diamond MS, Virgin HW. 2015. Interferon-lambda cures persistent murine norovirus infection in the absence of adaptive immunity. Science **347:**269-273.

31. Miyamoto M, Fujita T, Kimura Y, Maruyama M, Harada H, Sudo Y, Miyata T, Taniguchi T. 1988. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. Cell **54:**903-913.

32. Xu L, Zhou X, Wang W, Wang Y, Yin Y, Laan LJ, Sprengers D, Metselaar HJ, Peppelenbosch MP, Pan Q. 2016. IFN regulatory factor 1 restricts hepatitis E virus replication by activating STAT1 to induce antiviral IFN-stimulated genes. FASEB J **30:**3352-3367.

33. Xu L, Wang W, Li Y, Zhou X, Yin Y, Wang Y, de Man RA, van der Laan LJW, Huang F, Kamar N, Peppelenbosch MP, Pan Q. 2017. RIG-I is a key antiviral interferon-stimulated gene against hepatitis E virus regardless of interferon production. Hepatology **65:**1823-1839.

34. Siren J, Imaizumi T, Sarkar D, Pietila T, Noah DL, Lin R, Hiscott J, Krug RM, Fisher PB, Julkunen I, Matikainen S. 2006. Retinoic acid inducible gene-I and mda-5 are involved in influenza A virus-induced expression of antiviral cytokines. Microbes Infect **8:**2013-2020.

35. McCartney SA, Thackray LB, Gitlin L, Gilfillan S, Virgin HW, Colonna M. 2008. MDA-5 recognition of a murine norovirus. PLoS Pathog **4:**e1000108.

36. Rodriguez MR, Monte K, Thackray LB, Lenschow DJ. 2014. ISG15 functions as an interferon-mediated antiviral effector early in the murine norovirus life cycle. J Virol **88:**9277-9286.

37. Pagliaccetti NE, Eduardo R, Kleinstein SH, Mu XJ, Bandi P, Robek MD. 2008. Interleukin-29 functions cooperatively with interferon to induce antiviral gene expression and inhibit hepatitis C virus replication. J Biol Chem **283:**30079-30089.

38. Grant A, Ponia SS, Tripathi S, Balasubramaniam V, Miorin L, Sourisseau M, Schwarz MC, Sanchez-Seco MP, Evans MJ, Best SM, Garcia-Sastre A. 2016. Zika Virus Targets Human STAT2 to Inhibit Type I Interferon Signaling. Cell Host Microbe **19:**882-890.

39. Todt D, Francois C, Anggakusuma, Behrendt P, Engelmann M, Knegendorf L, Vieyres G, Wedemeyer H, Hartmann R, Pietschmann T, Duverlie G, Steinmann E. 2016. Antiviral Activities of Different Interferon Types and Subtypes against Hepatitis E Virus Replication. Antimicrob Agents Chemother **60:**2132-2139.

# Supplementary Information

## SUPPLEMENTARY METHODS

### *Antibody*

STAT1 (Rabbit mAb, #9172), phosphor-STAT1(Tyr$^{701}$) (58D6, Rabbit mAb, #9167), IRF-1 (D5E4, Rabbit mAb, #8478), MDA5 (D74E4, Rabbit mAb, #5321) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). RIG-I (H-300, Rabbit polyclonal, #sc-98911), STAT2 (C-20, Rabbit polyclonal, sc-476) and *β*-actin (C-4, Mouse mAb, #sc-47778) were purchased from Santa Biotechnology (Santa Cruz, CA, USA). Secondary antibodies IRDye® 800CW-conjugated goat anti-rabbit and goat anti-mouse IgGs (1:10,000 dilution; Li-Cor Bioscience, Lincoln, USA) were used, as appropriate.

### *Clearance of HuNoV replicons from HG23 cells*

HG23 cells were treated with 0.5 $\mu$g/mL of mycophenolic acid (MPA) and harvested after 2 days of treatment. Cells were subsequently re-challenged with same concentrations of MPA for 4 days. Another round of 4-day treatment was applied with the same concentration. After 10 days of treatment the resulting cells were designated $HG23_{DEPL}$ and cultured in the absence of G418.

### *Colony formation assay*

To detect whether HuNoV replicons were completely cleared from $HG23_{DEPL}$ cells, $HG23_{DEPL}$ or HG23 cells were plated into 6-well cell culture plate (1,000 cells per plate) and cultured in the medium containing G418 (1 mg/mL). On day 3 and 6, the wells were replaced by fresh medium containing the same concentration of G418. After 9 days of treatment, colonies were stained with hematoxylin and eosin and visualized.

### *Transfection of HuNoV plasmids*

To examine the effects of HuNoV on IFN signaling, a plasmid construct containing the full-length HuNoV genome ($HuNoV_{U201F}$; GII.3 U201 strain) and a construct harboring the same genome coupled with a GFP reporter gene were used [1]. Briefly, 293T cells were seeded into 6-well cell culture plates at a density of $2 \times 10^5$ cells per well. After overnight culture, 200 ng of pEGFP-C1 or 2000 ng of $HuNoV_{U201F\text{-}ORF1\text{-}IRES\text{-}GFP}$ or a mixture of $HuNoV_{U201F}$ (2000ng) and pEGFP-C1 (200ng) plasmids (a ratio of 10:1) were transfected with FuGene®

HD transfection reagent in a total volume of 1 mL Opti-MEM per well. After 6 hours of transfection, fresh DMEM medium containing 10% FCS was added into each well. Transfected cells were harvested after 2 days of culture and processed for cell sorting using FACSAria™ flow cytometer (BD Bioscience) equipped with a 640-nm laser. GFP-positive cells were treated with 1,000 IU/mL of IFN$\alpha$ and analyzed for type I IFN signaling after 24 hours of treatment.

### *Western blot*

Cell samples were lysed and loaded onto a 10 to 15% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). After electrophoresis at 120 V for 100 mins, the proteins were electrotransferred to polyvinylidene difluoride (PVDF) membrane (Invitrogen) for 1.5 h with an electric current of 250 mA. The membrane was probed with primary antibody plus secondary antibody and detected with Odyssey 3.0 Infrared Imaging System (LI-COR Biosciences). $\beta$-actin was served as standardization of sample loading.

### *Reverse transcription PCR (RT-PCR)*

Two primer sets were designed to detect HuNoV replicons in HG23 cells. The primer pair designated Neo-F and Neo-R is located in the neomycin resistant gene [2] and is predicted to produce a product of 804 bases. Primers NVp36/35 are located in a highly conserved RNA polymerase region of HuNoV GI genome and are predicted to give a product of 470 bases [3]. For HuNoV reverse genetics system, a primer pair designated NV-4661 [4] and NVp110 [5] were used for specific detection of HuNoV GII genome. For RT, cDNA was reverse transcribed from extracted cellular RNA by using TakaRa PrimeScript™ RT reagent kit, according to the manufacturer's instructions. Briefly, 8 $\mu$L of total cellular RNA (500 ng) was mixed with 2 $\mu$L of 5× PrimeScript Buffer on ice. The mixture was incubated at 37℃ for 15 mins; this was followed by heat inactivation at 85℃ for 5 s. The mixture was 20 times diluted and stored at -20℃ for further use. PCR was performed using Q5® High-Fidelity DNA polymerase kit (New England Biolabs Inc.) in a 25 $\mu$L reaction mixture containing 5 $\mu$L of 5× Q5 Reaction Buffer, 0.5 $\mu$L of a 10 mM dNTPs, 1.25 $\mu$L of 10 $\mu$M Forward Primer, 1.25 $\mu$L of 10 $\mu$M Reverse Primer, 5 $\mu$L of template cDNA and 0.25 $\mu$L of Q5 High-Fidelity DNA Polymerase. Amplification was carried out with initial denaturation at 98℃ [30 s]; 30 cycles of 98℃ [10 s], 50-70℃ [30 s], and 72 ℃ [30 s]; and an additional extension step of 72℃ for 2 min. The GAPDH primer set [6] was used as a reference gene and was amplified in 25 cycles.

The products were loaded onto 1 to 2% agarose gel containing SERVA DNA Stain G, and DNA electrophoresis at 100 V for 45 min was performed. An 100 base pair (bp) DNA ladder (Promega) was also electrophoresed on each gel. Bands were visualized and recorded with the Gel.Doc 2000 system (Bio-Rad). All primer combinations were listed in Table S2.

### *Quantitative real-time PCR (qRT-PCR)*

Total RNA was isolated with a Machery-NucleoSpin RNA II Kit (Bioke, Leiden, The Netherlands) and measured with a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was reverse transcribed from 500 ng RNA using a cDNA Synthesis Kit (TAKARA BIO INC). The cDNA of a targeted gene transcript was amplified for 50 cycles and quantified with a SYBRGreen-based real-time PCR (Applied Biosystems), according to the manufacturer's instructions. All the PCR reactions were performed in duplicates and amplification specificity was confirmed by melting-curve analysis. Human GAPDH was used as a reference gene. The relative expression of targeted gene was calculated as $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_{T\text{sample}} - \Delta C_{T\text{control}}$ ($\Delta C_T = C_{T\,[\text{targeted gene}]} - C_{T[\text{GAPDH}]}$). All primer combinations were listed in Table S5.

### *MTT assay*

The cytotoxicity of IFNs on host cells was determined by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. In brief, cells were seeded into 96-well cell culture plates containing 0.5% DMSO (control) or increasing concentrations of IFNs. After 2 days of treatment, 10 mM MTT (Sigma, Zwijndrecht, The Netherlands) was added. With another 3 hours of incubation, the medium was removed and 100 $\mu$L of DMSO was added to each well. The plate was incubated at 37℃ for 50 min. The absorbance at 490 nm was recorded on the microplate absorbance reader (Bio-Rad, CA, USA).

## SUPPLEMENTARY RESULTS

### *HuNV replication did not interfere with IFNα signaling*

These *in vitro* data support the potential therapeutic use of IFNs against norovirus infection. Conversely, it is important to evaluate whether norovirus interferes with the antiviral IFNs response. Previously, we have demonstrated that mycophenolic acid (MPA) exerted potent anti-norovirus activity and completely cleared HuNoV replicons from HG23 cells [7]. Thus, we depleted HuNoV replicons from HG23 cells by MPA and compared the IFNs response in HG23 and replicon-depleting HG23 cells ($HG23_{DEPL}$) after IFN$\alpha$ treatment. We

first confirmed the complete depletion of HuNoV replicons from HG23 cells by using RT-PCR and colony formation assay. RT-PCR yielded strong bands using neomycin primers and NVp36/35 primers in HG23 cells. In the contrast, no bands were observed in $HG23_{DEPL}$ cells (Fig. S8A). After 9 days of culture with G418, HG23 cells formed colonies, whereas $HG23_{DEPL}$ cells failed to grow any colonies (Fig. S8B), consistently demonstrating the complete clearance of HuNoV replicons from HG23 cells. To address the question about the effects of HuNoV on IFNs signaling, HG23 and $HG23_{DEPL}$ cells were treated with 1,000 IU/mL of IFN$\alpha$. At the indicated time points induction of JAK-STAT signaling pathway was detected by Western blot. As shown in Fig. S8C, phosphorylation of STAT1 was quickly induced, even after 30 mins of IFN$\alpha$ treatment in both cells. IFN$\alpha$ stimulated STAT1 phosphorylation in a time-dependent manner. There was no significant difference in both cells, indicating that HuNoV protein expression and RNA replication had no major effect on JAK-STAT pathway. Furthermore, there was no major effect on levels of expression for IFN$\alpha$-triggered ISGs (Fig. S8D; Fig. S9).

A plasmid-based HuNoV reverse genetics system ($pHuNoV_{U201F}$) has been recently developed (Fig. S10A). It expressed complete genome of the HuNoV GII.3 U201 strain and produced viral particles when transfected into cells [1]. We transfected 293T cells with $pHuNoV_{U201F}$. To monitor transfection efficiency, a plasmid with a GFP expression vector pGFP-C1 was co-transfected with $pHuNoV_{U201F}$ to act as a reporter. Cell sorting was performed after 2 days of transfection and GFP-positive cells were collected (Fig. S10B). As the amount of pGFP-C1 was much higher than $pHuNoV_{U201F}$ (a ratio of 10:1), this indicated high transfection efficiency of $pHuNoV_{U201F}$ in GFP-positive cells. In addition, $pHuNoV_{U201F\text{-}ORF1\text{-}IRES\text{-}GFP}$ was used as a polymerase KO construct, because it contained a stop codon after the GFP. Vector pGFP-C1 alone was used as control. After cell sorting, the expression of plasmids was detected. Because of the lack of HuNoV commercial antibodies, we detected HuNoV RNA expression using qRT-PCR (Fig. S11A) and RT-PCR (Fig. S11B). qRT-PCR data showed the abundant expression of HuNoV RNA in $pHuNoV_{U201F}$ and $pHuNoV_{U201F\text{-}ORF1\text{-}IRES\text{-}GFP}$ transfected cells by using self-designed HuNoV GII.3 U201-specific primers and GII-specific diagnostic primers. In RT-PCR experiment, we used a GII-specific diagnostic primer set. The specificity of the primer set was illustrated in Fig. S12. RT-PCR results showed the same results, indicating the successful expression of HuNoV reverse genetics system in 293T cells. The cells were subsequently treated with IFN$\alpha$ (1,000 IU/mL) for another 24 hours. HuNoV proteins and RNA expression in 293T cells had no major effects on the total levels of STAT1

and STAT2 proteins as well as phosphorylated STAT1 protein (Fig. S11C), and did not inhibit the induction of ISGs (Fig. S11D). In conclusion, HuNoV proteins and RNA expression did not interfere with the IFN signaling pathway.

## SUPPLEMENTARY TABLES AND FIGURES

**Table S1 *In vitro* anti-norovirus activities of IFNs after 2 days of treatment.**

| | $EC_{50}$ | $EC_{90}$ |
|---|---|---|
| IFN*α* [IU/mL] | 59.88 | 330.70 |
| IFN*β* [IU/mL] | 156.20 | 935.00 |
| IFN*γ* [ng/mL] | 8.26 | 11.53 |
| IFN*λ*1 [ng/mL] | 0.79 | 1.34 |
| IFN*λ*2 [ng/mL] | 2.69 | 8.64 |
| IFN*λ*3 [ng/mL] | 1.43 | 10.97 |

**Table S2 Primers used by RT-PCR.**

| Target | Primer | | Sense | Sequence (5'-3')[a] | Location | Annealing |
|---|---|---|---|---|---|---|
| HuNoV | HuNoVGI-specific | NVp36 | + | ATAAAAGTTGGCATGAACA | 4487-4505[b] | 57℃ |
| | | NVp35 | - | CTTGTTGGTTTGAGGCCATAT | 4936-4956[b] | |
| | HuNoV GII-specific | NV-4611 | + | CWGCAGCMCTDGAAATCATGG | 4611-4631[c] | 57℃ |
| | | NVp110 | - | ACDATYTCATCATCACCATA | 4865-4884[b] | |
| Neomycin phosphotransferase | Neo-F | | + | ATGGGATCGGCCATTGAAC | | 59℃ |
| | Neo-R | | - | TCAGAAGAACTCGTCAAG | | |
| GAPDH | GAPDH-F | | + | TCGTGGAAGGACTCATGACC | | 68℃ |
| | GAPDH-R | | - | TCCACCACCCTGTTGCT | | |

[a]Y = C + T; M = A + C; D = A+T+G; W = A+T
[b]Nucleotide positions are the positions in Hu/NLV/Norwalk/68/US (accession no. M87661).
[c]Nucleotide positions are the positions in Hu/NLV/Lordsdale/93/UK (accession no. X86557).

**Table S3 Levels of expression for transfected ISGs and their relative anti-norovirus effectiveness.** Data are presented as the means ± SEM (*n* = 8 replicates). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

| Gene | Gene expression | *P* value | HuNV replication (relative) | *P* value |
|---|---|---|---|---|
| IRF-1 | 375,60 ± 91,63 | ** | 0,05 ± 0.01 | *** |
| C6orf150 | 128224,36 ± 12444.49 | ** | 0,68 ± 0.09 | *** |
| HPSE | 269,30 ± 16,49 | ** | 0,58 ± 0.06 | *** |
| RIG-I | 1389.00 ± 160.90 | ** | 0,17 ± 0.02 | *** |
| MDA5 | 2389.00 ± 705,00 | ** | 0,23 ± 0.06 | *** |
| IFITM3 | 508,44 ± 5,57 | ** | 1,04 ± 0.19 | NS |
| DDX60 | 83,52 ± 15,91 | ** | 0,92 ± 0.16 | * |
| IRF-2 | 504,50 ± 110,60 | ** | 1.05 ± 0.15 | NS |
| RTP4 | 11382.00 ± 1903.00 | ** | 0,49 ± 0.07 | *** |
| UNC84B (SUN2) | 8,51 ± 1,10 | ** | 0,60 ± 0.05 | *** |
| FAM46C | 12,12 ± 1,73 | ** | 1,04 ± 0.17 | NS |
| MCOLN2 | 45,88 ± 8.72 | ** | 0,82 ± 0.11 | NS |
| IFI44L | 1,10 ± 0,11 | NS | 0,66 ± 0.06 | ** |
| IFI6 | 506,33 ± 104.88 | *** | 0,87 ± 0.08 | NS |
| IFITM2 | 1,29 ± 0,10 | NS | 0,79 ± 0.16 | NS |
| MAP3K14 | 588,44 ± 45.66 | *** | 0,70 ± 0.13 | NS |
| MOV10 | 1,05 ± 0,41 | NS | 1.02 ± 0.38 | NS |
| NAMPT (PBEF1) | 6,30 ± 1.22 | *** | 0,73 ± 0.10 | * |
| OASL | 94,27 ± 17,45 | *** | 1,07 ± 0.12 | NS |
| IFITM1 | 118,59 ± 18.11 | *** | 0,68 ± 0.06 | ** |
| TREX1 | 44,38 ± 9,26 | *** | 0,89 ± 0.23 | NS |
| ADAR | 9,20 ± 2.43 | *** | 0,80 ± 0.15 | * |
| LY6E | 11306.00 ± 1001.00 | *** | 0,72 ± 0.04 | *** |
| IRF-7 | 1,72 ± 0,20 | * | 0,72 ± 0.09 | ** |

**Table S4 Levels of expression for IRF-1, RIG-I and MDA5 following IFN treatment.** HG23 cells were treated with high concentration of IFNs. After 2 days of treatment, the ISG mRNA expression was detected by qRT-PCR (*n* = 3 independent experiments with duplicates each). Data are presented as the means ± SEM.

| Gene | IFN*α* 1,000 IU/mL | IFN*β* 1,000 IU/mL | IFN*γ* 100 ng/mL | IFN*λ*1 100 ng/mL | IFN*λ*3 100 ng/mL |
|---|---|---|---|---|---|
| IRF-1 | 5.06 ± 0.75 | 4.48 ± 1.65 | 20.40 ± 1.54 | 11.70 ± 0.85 | 10.47 ± 1.47 |
| RIG-I | 18.45 ± 2.04 | 11.54 ± 2.32 | 4.62 ± 0.63 | 30.67 ± 2.63 | 22.64 ± 3.15 |
| MDA5 | 180.70 ± 70.50 | 29.38 ± 10.97 | 36.14 ± 8.10 | 676.00 ± 218.40 | 603.50 ± 123.30 |
| RTP4 | 65.56 ± 15.23 | 29.50 ± 3.24 | 5.84 ± 0.40 | 564.80 ± 112.10 | 588.70 ± 78.09 |
| HPSE | 2.98 ± 0.63 | 3.91 ± 0.32 | 1.62 ± 0.07 | 6.33 ± 1.72 | 6.58 ± 2.01 |

**Table S5 Primers used by qRT-PCR.**

| Name | Sequences[a] |
|---|---|
| HuNoV GI-specific primer | COG1F (+): 5'- CGYTGGATGCGNTTYCATGA-3'<br>COG1R (-): 5'- CTTAGACGCATCATCATTYAC-3' |
| HuNoV GII-specific primer | QNIF2d (+):5'-ATGTTCAGRTGGATGAGRTTCTCWGA-3'<br>COG2R (-):5'-TCGACGCCATCTTCATTCACA-3' |
| HuNoV GII.3 U201-specific primer | F: 5'- ACCACCTGCTGCAATAAGTC-3'<br>R: 5'- GTCAAAGGCTGTGTATGGGA -3' |
| Neomycin phosphotransferase | F: 5'-CCGGCTACCTGCCCATTC-3'<br>R: 5'-CCAGATCATCCTGATCGACAA G-3' |
| CXCL10 | F: 5'- GGTGAGAAGAGATGTCTGAATCC-3'<br>R: 5'- GTCCATCCTTGGAAGCACTGCA-3' |
| DDX60 | F: 5'-GGTGTTTTCACCAGGGAGTATCG-3'<br>R: 5'-CCAGTTTTGGCGATGAGGAGCA-3' |
| GAPDH | F: 5'-TGTCCCCACCCCCAATGTATC-3'<br>R: 5'-CTCCGATGCCTGCTTCACTACCTT-3' |
| HPSE | F: 5'-GAATGGACGGACTGCTACCAAG-3'<br>R: 5'-CTCCTAACCAGACCTTCTTGCC-3' |
| IFIT1 | F: 5'-GCCTTGCTGAAGTGTGGAGGAA-3'<br>R: 5'-ATCCAGGCGATAGGCAGAGATC-3' |
| IFIT2 | F: 5'-GGAGCAGATTCTGAGGCTTTGC-3'<br>R: 5'-GGATGAGGCTTCCAGACTCCAA-3' |
| IFIT3 | F: 5'- CCTGGAATGCTTACGGCAAGCT-3'<br>R: 5'- GAGCATCTGAGAGTCTGCCCAA-3' |
| IFITM1 | F: 5'-GGCTTCATAGCATTCGCCTACTC-3'<br>R: 5'-AGATGTTCAGGCACTTGGCGGT-3' |
| IFITM3 | F: 5'-CTGGGCTTCATAGCATTCGCCT-3'<br>R: 5'-AGATGTTCAGGCACTTGGCGGT-3' |
| ISG15 | F: 5'-CTCTGAGCATCCTGGTGAGGAA-3'<br>R: 5'-AAGGTCAGCCAGAACAGGTCGT-3' |
| IRF-1 | F: 5'-GAGGAGGTGAAAGACCAGAGCA-3'<br>R: 5'-TAGCATCTCGGCTGGACTTCGA-3' |
| IFI44L | F: 5'-TGCACTGAGGCAGATGCTGCG-3'<br>R: 5'-TCATTGCGGCACACCAGTACAG-3' |
| IFI6 | F: 5'-TGATGAGCTGGTCTGCGATCCT-3'<br>R: 5'-GTAGCCCATCAGGGCACCAATA-3' |
| MDA5 (IFIH1) | F: 5'-GCTGAAGTAGGAGTCAAAGCCC-3'<br>R: 5'-CCACTGTGGTAGCGATAAGCAG-3' |
| MX1 | F: 5'- GGCTGTTTACCAGACTCCGACA-3'<br>R: 5'- CACAAAGCCTGGCAGCTCTCTA-3' |
| OAS3 | F: 5'-CCTGATTCTGCTGGTGAAGCAC-3'<br>R: 5'-TCCCAGGCAAAGATGGTGAGGA-3' |
| PKR | F: 5'-GAAGTGGACCTCTACGCTTTGG-3'<br>R: 5'-TGATGCCATCCCGTAGGTCTGT-3' |
| RIG-I | F: 5'-CACCTCAGTTGCTGATGAAGGC-3'<br>R: 5'-GTCAGAAGGAAGCACTTGCTACC-3' |
| RTP4 | F: 5'-GACGCTGAAGTTGGATGGCAAC-3'<br>R: 5'-GTGGCACAGAATCTGCACTTGG-3' |

[a]Y = C + T; R = A + G; W = A+T; N = any

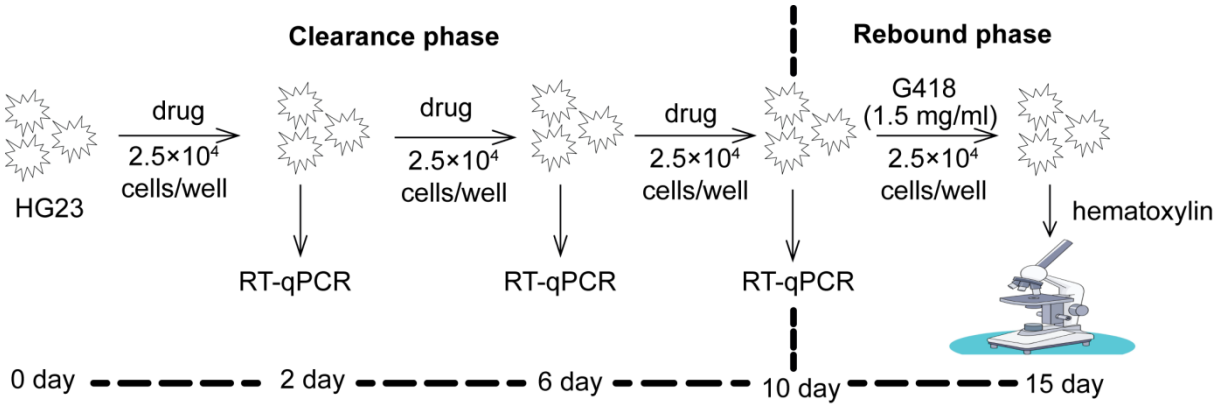



**Figure S1 The design of long duration experiment.** HG23 cells were seeded into 48-well cell culture plates ($2.5 \times 10^4$ cells per well) and treated with IFNs. After 2 days of treatment, cells were trypsinized, $2.5 \times 10^4$ cells were subcultured into 48-well cell culture plates containing the same concentration of IFNs for another 4 days, and $2.5 \times 10^4$ cells were collected for qRT-PCR analysis. After 6 days of treatment, the same treatment process was performed for the second time. After 10 days of treatment, $2.5 \times 10^4$ cells were subcultured into a 48-well cell culture plate with fresh medium containing G418 (1 mg/mL). With another 5 days of culture, the cell layers were stained with hematoxylin and eosin and visualized with an inverted light microscopy.

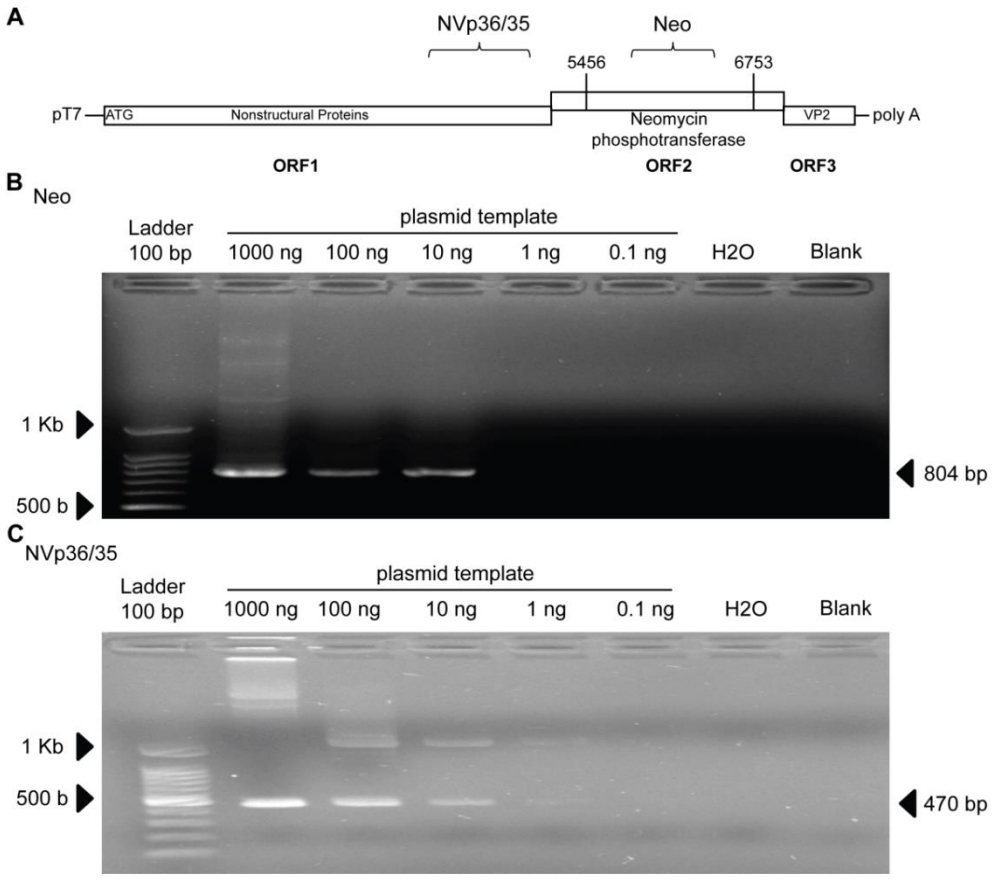



**Figure S2 Specificity of primer sets used to detect the HuNoV genome in HG23 cells.** (A) Schematic diagram of the HuNoV subgenomic replicon construct designated pNV-neo. A full-length cDNA clone of a HuNoV GI genome was previously engineered into the pSPORT1 plasmid. Meanwhile, the neomycin resistance gene was engineered into ORF2 as a selection marker. RNA transcripts were synthesized *in vitro* and were transfected into human Huh7 cells to generate a stably expressed

HuNoV replicon model, designated HG23. The location of the two primer sets including HuNoV GI-specific primers (NVp35 and NVp36) and neomycin primers (Neo-F and Neo-R) were indicated. (B) The specificity of primers Neo-F and Neo-R was verified by using purified plasmid pNV-neo as initial template. After 30 cycles of amplification a single band at 804 bp was indicative of the specificity of the primer pair. (C) Primer pair NVp35 and NVp36 produced a specific and dominant band of 470 bp and showed robust specificity.

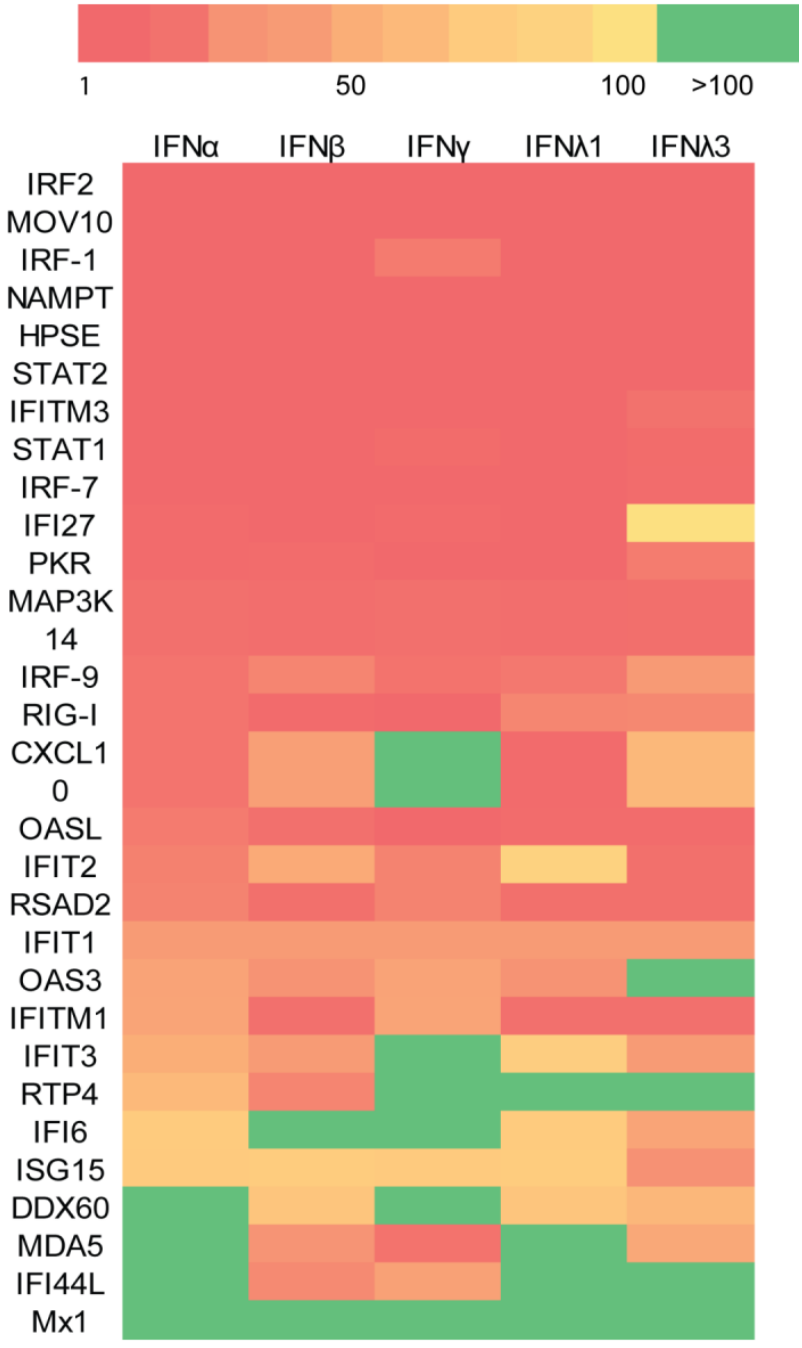



**Figure S3 The mRNA expression of 29 ISG genes was assessed by qRT-PCR, normalized to GAPDH and shown as fold-change expression compared to the control sample.** HG23 cells were treated with high concentration of IFNs (1,000 IU/mL IFN*α*, 1,000 IU/mL IFN*β*, 100 ng/mL IFN*γ*, 100 ng/mL IFN*λ*1 and 100 ng/mL IFN*λ*3) or medium. After 2 days of treatment, the gene expression (nfold) was shown as a color scale.

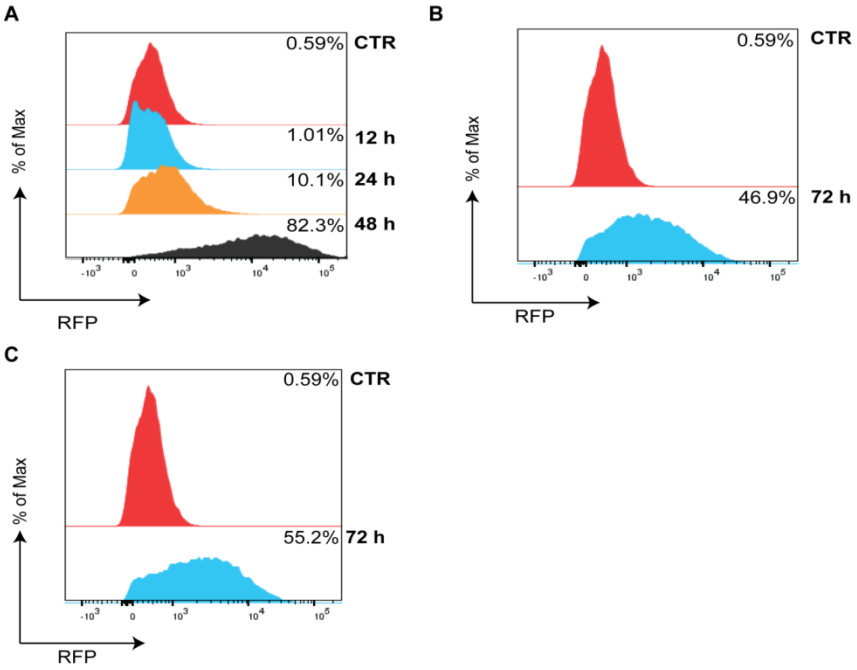



**Figure S4 Analysis of RFP expression by flow cytometry showed the successful deliver and expression of ISG genes in HG23 cells.** (A) HG23 cells were transduced with a control vector that expressed a Photinus pyralis luciferase (Fluc) gene. At indicated time points of 12, 24 and 48 hours, flow cytometry was performed to detect the expression of RFP. The *x* axis represented fluorescence intensity of RFP, while *y* axis indicated the percentage of the total cell population. (B) HG23 cells were transduced with HPSE overexpression lentiviral vector. After 72 hours of transduction, the expression level of HPSE was confirmed by detecting RFP. (C) Flow cytometry data demonstrated the successful expression of RTP4 after 72 hours of transduction.

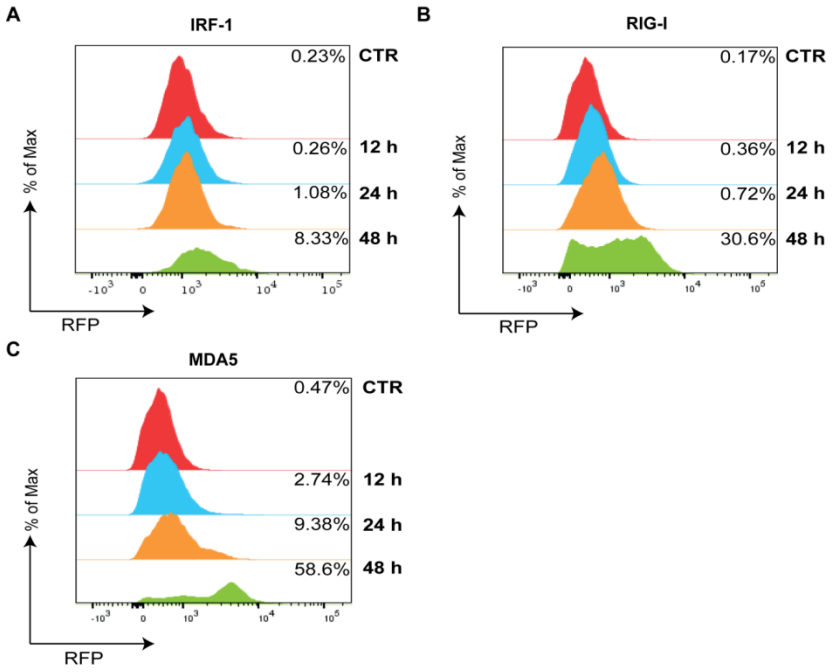



**Figure S5 Flow cytometry data demonstrated the successful overexpression of IRF-1, RIG-I and MDA5 in HG23 cells.** HG23 cells were transduced with lentiviral vectors IRF-1 (A), RIG-I (B) or MDA5 (C). After 12, 24 and 48 hours of transduction, the percentage of RFP-positive cells was detected by flow cytometry.

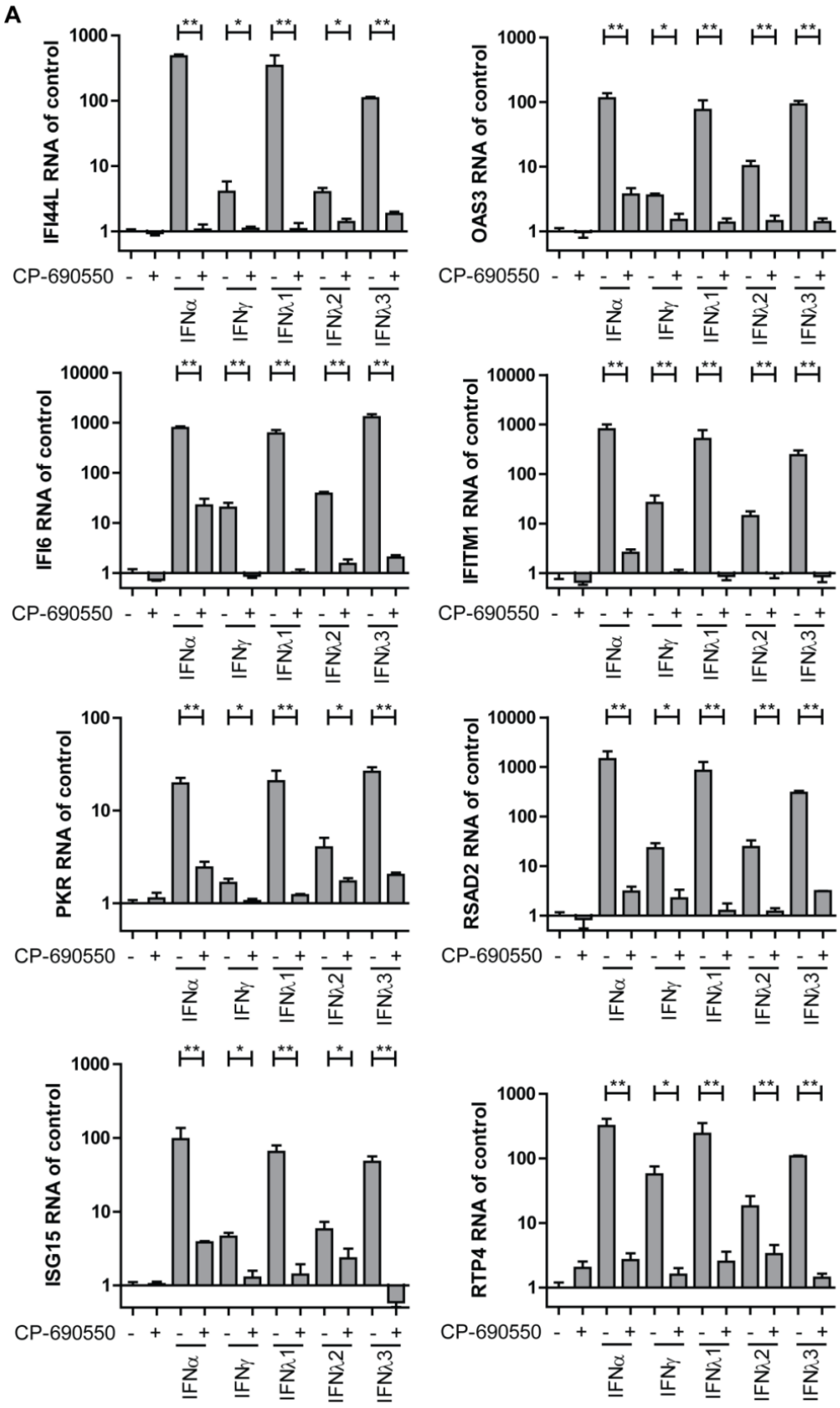



**Figure S6 Besides IRF-1, RIG-I and MDA5, CP-690550 blocked the induction of other ISGs by IFNs.** (A) HG23 cells were treated with IFNs, JAK inhibitor CP-690550 (1,000 ng/mL) or in combination. After 2 days of treatment, the expression levels for ISGs were quantified by qRT-PCR and compared to medium control (*n* = 3 independent experiments with duplicates each). Data are presented as the mean ± SEM (*, $P < 0.05$; **, $P < 0.01$).

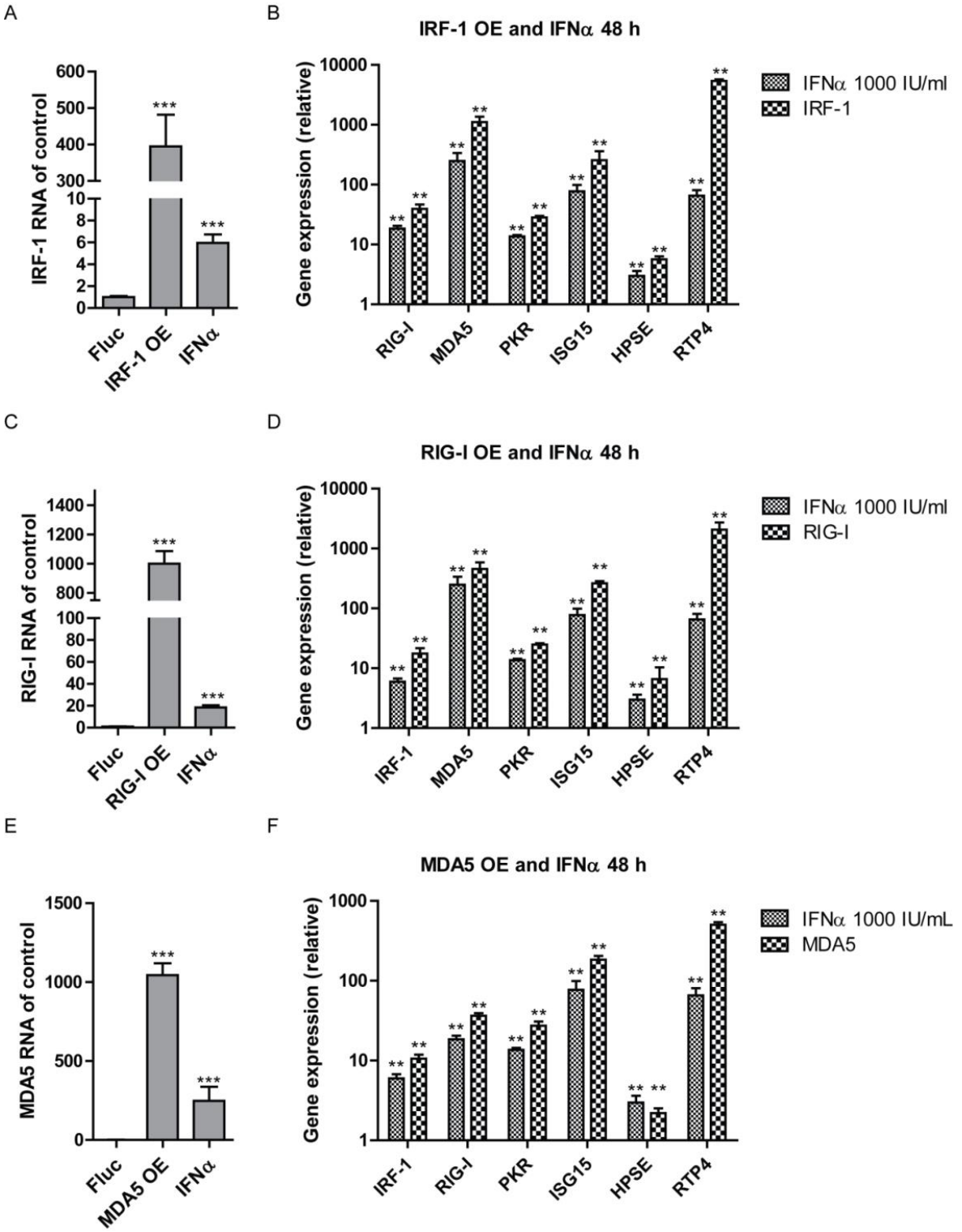



**Figure S7 Overexpression of IRF-1, RIG-I and MDA5 activated the expression of other ISGs.** (A, C and E) qRT-PCR analysis of IRF-1, RIG-I and MDA5 expression in HG23 cells transduced with ISG OE vector or Fluc vector or treated with IFN*α* (1,000 IU/mL) for 48 hours (*n* = 3 independent experiments with duplicates each). (B, D and F) The qRT-PCR analysis of ISG RNA levels in HG23 cells transduced with ISG OE vector or Fluc vector or treated with IFN*α* (1,000 IU/mL) for 48 hours (*n* = 3 independent experiments with duplicates each). Data are presented as the mean ± SEM. **P* < 0.05, ***P* < 0.05, ****P* < 0.05.

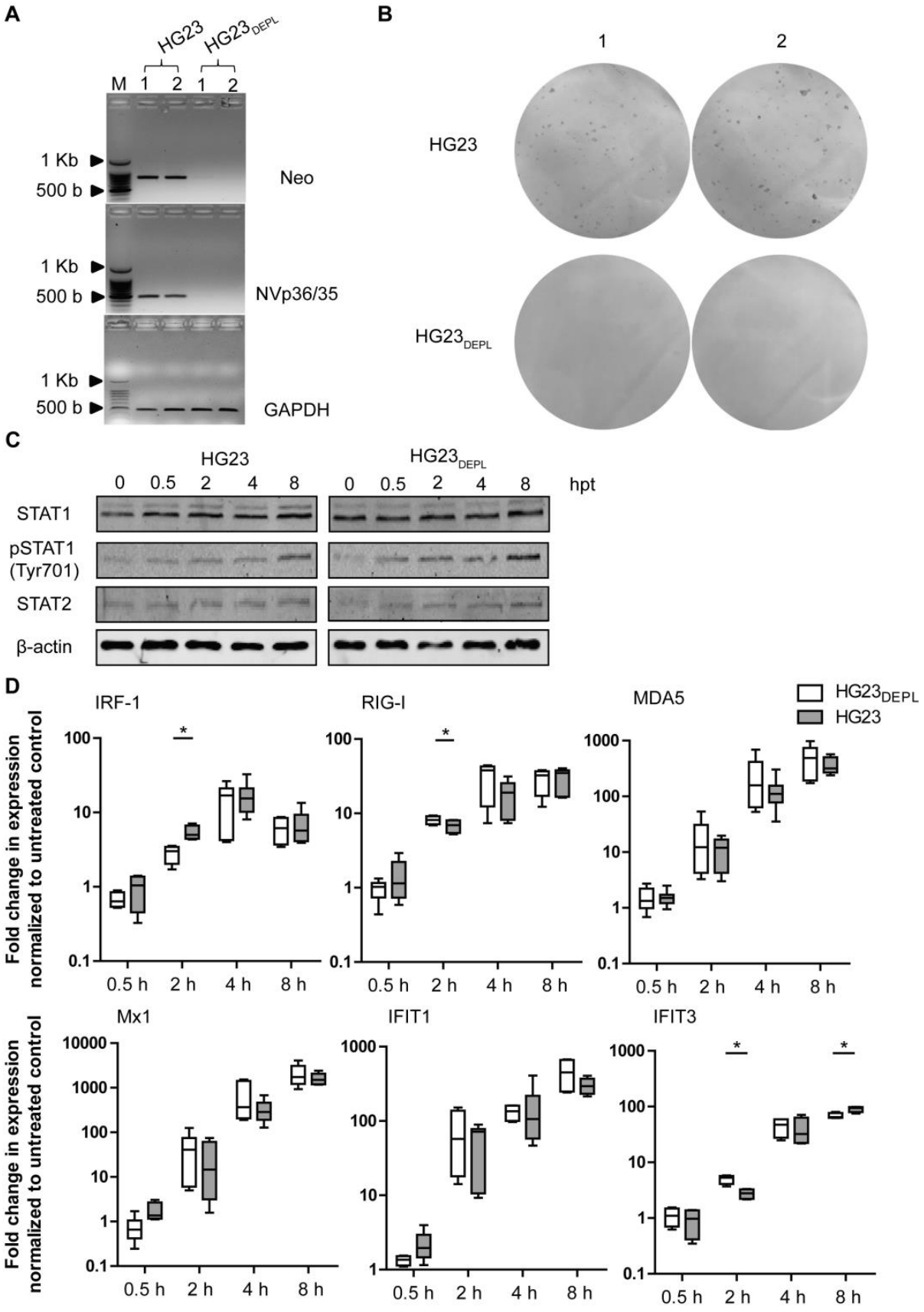



**Figure S8 HuNoV RNA replication did not interfere with IFN signaling.** After consecutively treating HG23 cells with 0.5 $\mu$g/mL MPA for 10 days, the resulting cells were designated $HG23_{DEPL}$. Complete clearance of HuNoV replicons in $HG23_{DEPL}$ was verified by RT-PCR (A) and colony formation

assay (B). In the RT-PCR, lane 1 and 2 represented cells from two different passages. M indicated the 100 bp DNA ladder. In the colony formation assay, 1,000 cells of HG23 or $HG23_{DEPL}$ were plated into 6-well cell culture plate and maintained in the presence of 1 mg/mL of G418. After 10 days of culture, the plates were washed, fixed and stained. Representative data are from independent experiments. To compare the JAK/STAT pathway signaling, HG23 or HG23DEPL cells were treated with 1,000 IU/mL of IFN*α*. At indicated time points, the IFN*α* response was detected. (C) Western blotting of STAT1, p-STAT1 and STAT2. *β*-actin was used as a reference. (D) Levels of expression for ISGs in HG23 and $HG23_{DEPL}$ cells were determined after the addition of IFNs via qRT-PCR. The *y* axis represented the fold changes in the expression levels normalized to a respective control without IFN treatment (*n* = 3 independent experiments with duplicates each). Data are presented as the mean ± SEM (Mann-Whitney test; *, $P < 0.05$; **, $P < 0.01$).

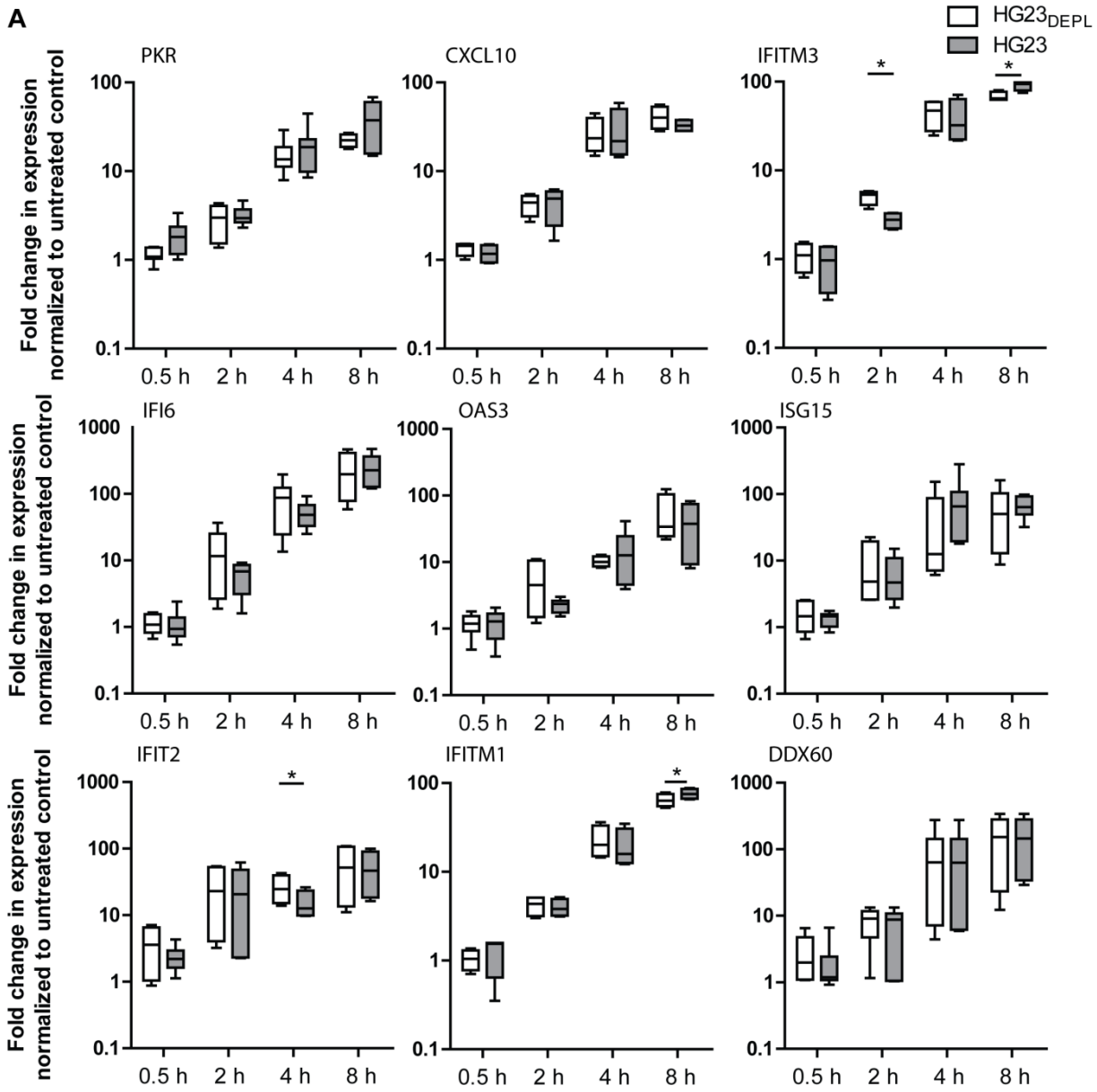



**Figure S9 HuNoV RNA replication had no effect on ISG induction.** (A) qRT-PCR analysis of ISG expression in HG23 cells and $HG23_{DEPL}$ cells after IFN*α* treatment. Data was normalized to GAPDH and compared to a respective control without IFN treatment (*n* = 3 independent experiments with duplicates each). Data are presented as the mean ± SEM (*, $P < 0.05$).

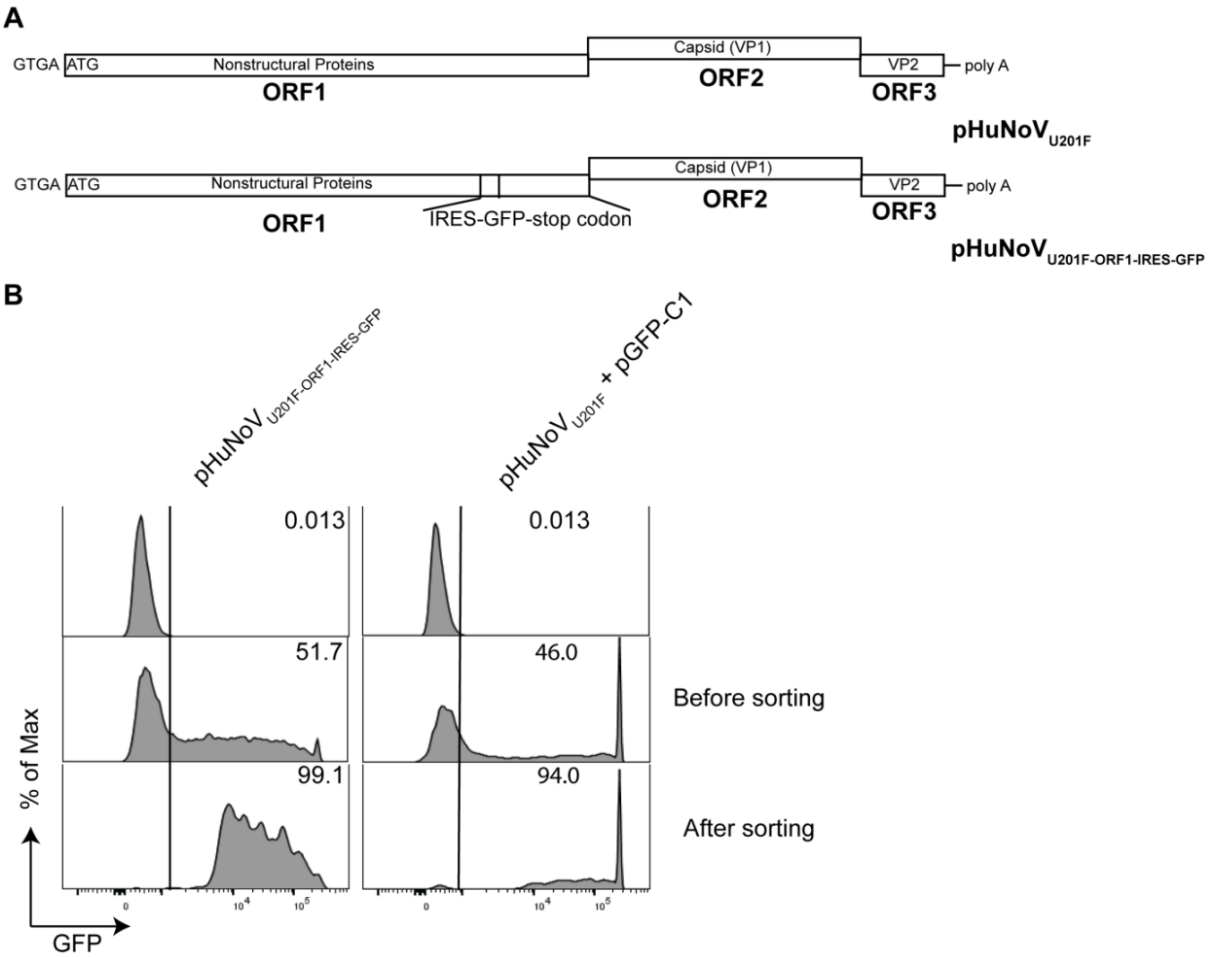



**Figure S10 Cell sorting of GFP-positive cells after transfection of HuNoV reverse genetics in 293T cells.** (A) Schematic diagrams of pHuNoV$_{U201F}$- and pHuNoV$_{U201F}$-based constructs. (Upper) The HuNoV GII.3 U201 genome was represented. ORF1 of the HuNoV genome encoded a polyprotein, while ORF2 and ORF3 encoded the capsid protein VP1 and VP2, respectively. The whole genome was inserted into an expression cassette under the control of the promoter regulating the human EF-1$\alpha$. (Lower) Construct pHuNoV$_{U201F\text{-}ORF1\text{-}IRES\text{-}GFP}$ had an encephalomyocarditis virus, internal ribosomal entry site and GFP (EMCV-IRES-GFP) gene with a stop codon that was inserted into the ORF1 region. (B) In order to improve the transfection efficiency, we used pGFP-C1, a plasmid expressing green fluorescent protein (GFP) as a transfection marker. HEK293T cells were transiently transfected with the plasmid pGFP-C1 alone (control) or pHuNoV$_{U201F\text{-}ORF1\text{-}IRES\text{-}GFP}$ alone or pHuNoV$_{U201F}$ combined with pGFP-C1. After 48 h of transfection FACS was performed to sort GFP-positive cells. The ration of pGFP-C1: pHuNoV$_{U201F}$ was 10:1, so it was convincing that GFP positive cells were successfully transfected with pHuNoV$_{U201F}$.

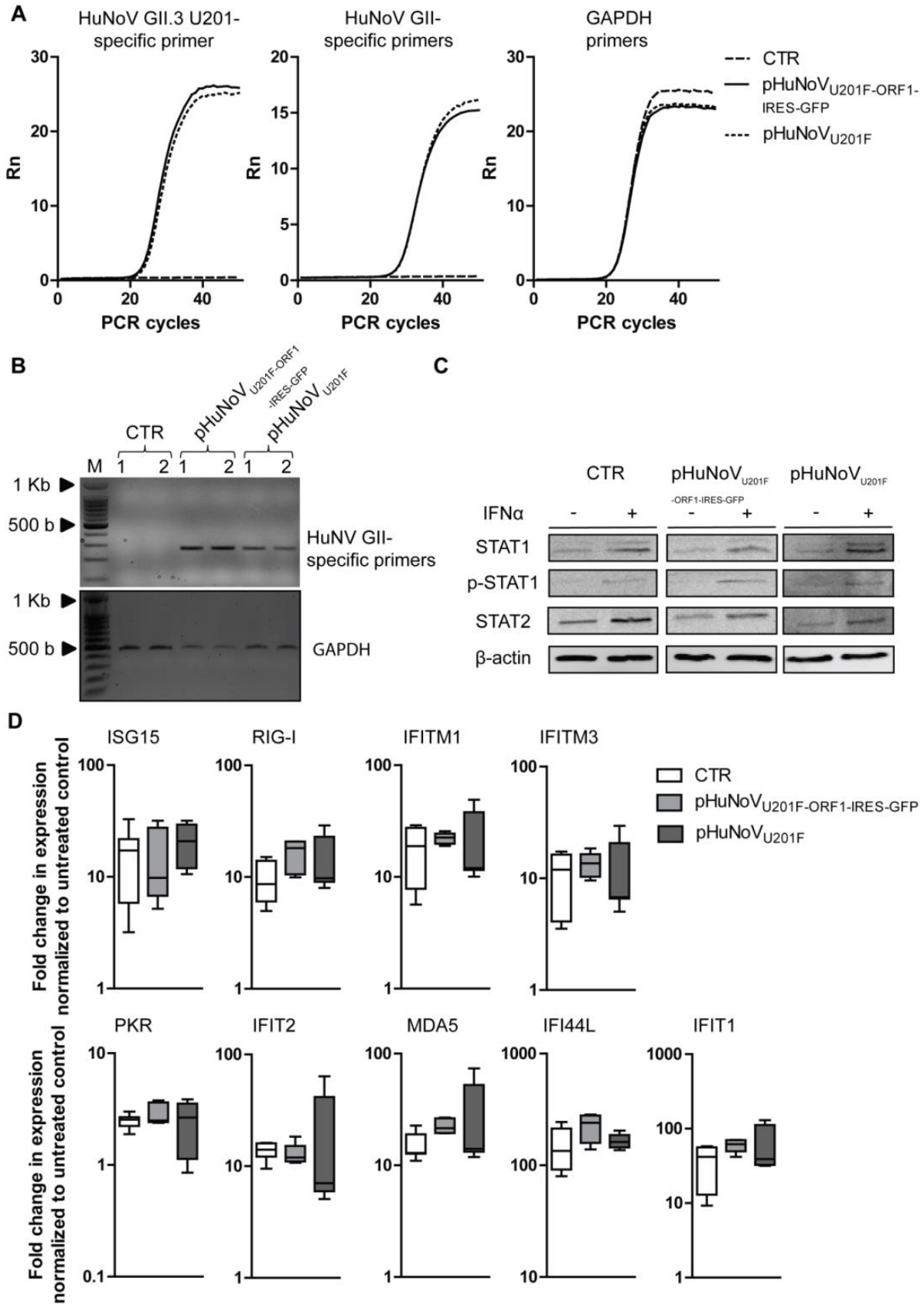



**Figure S11 HuNoV protein and RNA expression had no effect on IFN signaling.** After transfection of HuNoV reverse genetics into 293T cells, the successful expression of HuNoV RNA was verified by qRT-PCR and RT-PCR. (A) In the qRT-PCR, two primer sets were used. One was self-designed HuNoV GII.3 U201-specific primer pair and the other was GII-specific diagnostic primer pair.

Human GAPDH was used as a reference gene. (B) Gel-based RT-PCR analysis of HuNoV RNA expression by using HuNoV GII-specific primer set. Human GAPDH was used as a reference gene. Lane 1 and 2 represented cells from two independent experiments. M indicated the 100 bp DNA ladder. HEK293T cells expressing HuNoV reverse genetics were treated with IFN*α* (1,000 IU/mL) for 24 hours and used to assess the JAK-STAT pathway. The results were compared to those in pGFP-C1-transfected control cells. (C) Western blot of STAT1, p-STAT1 and STAT2. *β*-actin was used as a reference. (D) Levels of expression for ISGs in 293T cells with and without transfected HuNoV constructs were determined at 24 hours after the addition of IFN*α* via qRT-PCR. The *y* axis represents the fold changes in the expression levels normalized to a respective control without IFN treatment (*n* = 3 independent experiments with duplicates each). Data presented as mean ± SEM (Mann-Whitney test; *, $P < 0.05$; **, $P < 0.01$).

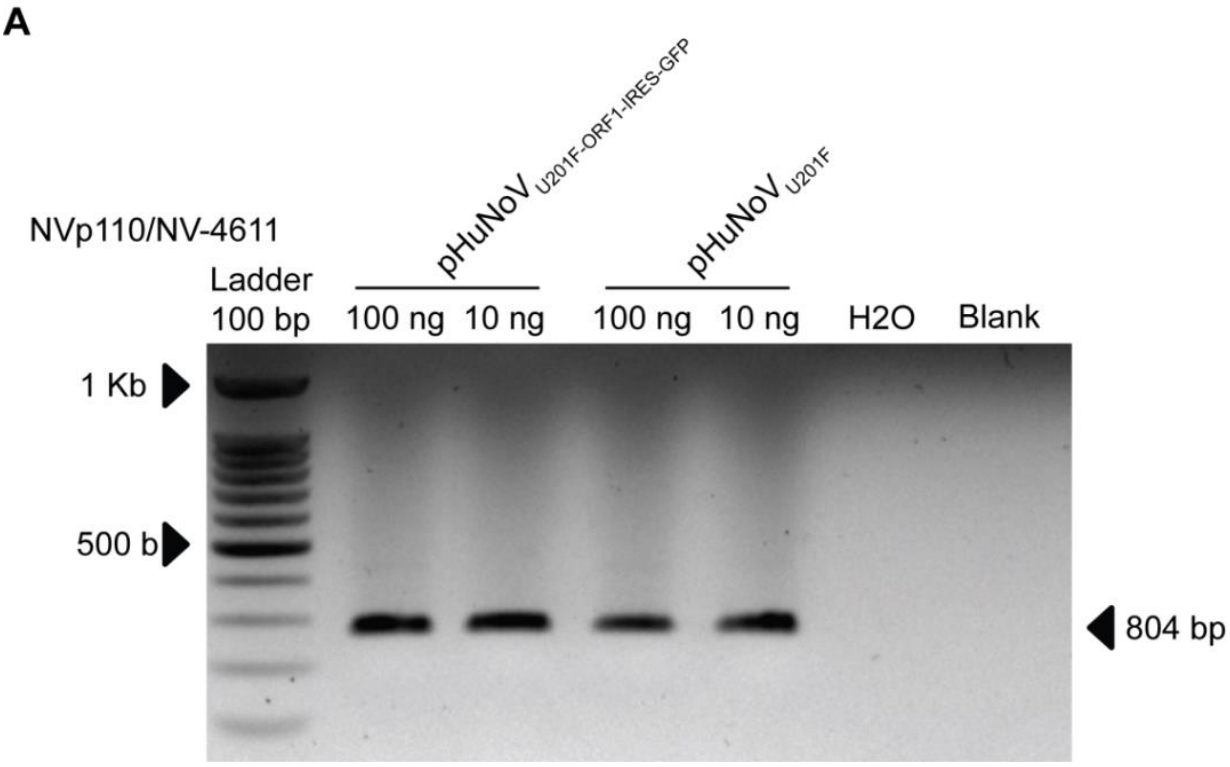



**Figure S12 Specificity of primer sets used to detect the HuNoV reverse genetics.** (A) The specificity of primers NVp110 and NV-4611 were verified by using purified plasmid $pHuNoV_{U201F}$ and $pHuNoV_{U201F\text{-}ORF1\text{-}IRES\text{-}GFP}$ as initial template. After 30 cycles of amplification, a single band at 234 bp was indicative of the specificity of the primer pair.

# SUPPLEMENTARY REFERENCES


1. Katayama K, Murakami K, Sharp TM, Guix S, Oka T, Takai-Todaka R, Nakanishi A, Crawford SE, Atmar RL, Estes MK. 2014. Plasmid-based human norovirus reverse genetics system produces reporter-tagged progeny virus containing infectious genomic RNA. Proc Natl Acad Sci U S A **111:**E4043-4052.
2. Chang KO, Sosnovtsev SV, Belliot G, King AD, Green KY. 2006. Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line. Virology **353:**463-473.
3. Wang J, Jiang X, Madore HP, Gray J, Desselberger U, Ando T, Seto Y, Oishi I, Lew JF, Green KY, et al. 1994. Sequence diversity of small, round-structured viruses in the Norwalk virus group. J Virol **68:**5982-5990.
4. Yuen LK, Catton MG, Cox BJ, Wright PJ, Marshall JA. 2001. Heminested multiplex reverse transcription-PCR for detection and differentiation of Norwalk-like virus genogroups 1 and 2 in fecal samples. J Clin Microbiol **39:**2690-2694.
5. Le Guyader F, Estes MK, Hardy ME, Neill FH, Green J, Brown DW, Atmar RL. 1996. Evaluation of a degenerate primer for the PCR detection of human caliciviruses. Arch Virol **141:**2225-2235.
6. Li Y, Pong RC, Bergelson JM, Hall MC, Sagalowsky AI, Tseng CP, Wang Z, Hsieh JT. 1999. Loss of adenoviral receptor expression in human bladder cancer cells: a potential impact on the efficacy of gene therapy. Cancer Res **59:**325-330.
7. Dang W, Yin Y, Wang Y, Wang W, Su J, Sprengers D, van der Laan LJW, Felczak K, Pankiewicz KW, Chang KO, Koopmans MPG, Metselaar HJ, Peppelenbosch MP, Pan Q. 2017. Inhibition of Calcineurin or IMP Dehydrogenase Exerts Moderate to Potent Antiviral Activity against Norovirus Replication. Antimicrob Agents Chemother **61:**e01095-17.

# Chapter 5

## Opposing Effects of Nitazoxanide on Murine and Human Norovirus

Wen Dang[1], Yuebang Yin[1], Maikel P. Peppelenbosch[1] and Qiuwei Pan[*1]

[1]Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam, Netherlands

**J Infect Dis. 2017; 216:780-2**

# TO THE EDITOR

Norovirus is a major cause of acute gastroenteritis worldwide, and chronic infections frequently occur in immunocompromised patients. However, no US Food and Drug Administration-approved specific medication is available for treating norovirus infection. Interestingly, substantial clinical evidence has suggested that nitazoxanide, originally developed as an antiprotozoal agent, is a potential antiviral therapy for norovirus infection [1]. Both clinical trials and case studies have reported its effects on reduction of symptom duration in immunocompetent patients [2] or clearance of the infection in immunocompromised patients [3, 4]. On the contrary, a recent study in the *Journal of Infectious Diseases* by Kempf et al reported that nitazoxanide was not effective for treating chronic norovirus gastroenteritis in a patient with X-Linked Agammaglobulinemia [5]. These results have sparked concern regarding whether this drug holds promise in treating norovirus infection, indicating the need for further assessment of the efficacy and working mechanism of nitazoxanide [6].

Although the lack of robust cell culture models is a key bottleneck in the norovirus research field, there are a few surrogate models currently available, including a human subgenomic norovirus replicon (HuNV) [7] and the murine norovirus (MNoV) [8] infectious model. Therefore, we have assessed the direct effects of nitazoxanide on norovirus in these models to better clarify the current debate.

We first investigated the effects in the HG23 cell culture model, a Huh-7 cell line-based HuNV replicon. This subgenomic replicon, derived from a GI genogroup strain, encodes the neomycin phosphotransferase gene by replacing part of the viral ORF2 sequence, thereby conferring resistance of HG23 cells to G418 treatment (**Figure 1A**). In this model, we found that nitazoxanide at 5 $\mu$g/mL, a clinically achievable concentration, significantly inhibited viral replication by 90% ± 6.4 after 48 hours of treatment without toxicity to host cells ($P < .001$) (**Figure 1B**). To further evaluate the long-term effects, a clearance and rebound assay was performed in this model (**Figure 1C**). During the clearance phase, HG23 cells were constantly treated with nitazoxanide for 10 days, after which the cells were cultured in medium containing G418 (1.5 mg/mL) for another 5 days. As shown in **Figure 1D**, nitazoxanide at 5 $\mu$g/mL dramatically inhibited HuNV replication and cleared most

of the HuNV from host cells, as evidenced by the low confluency of replicon cells after G418 selection (**Figure 1E**).

On the contrary, we found that nitazoxanide significantly increased the RNA replication (by 6-fold; $P < .01$) (**Figure 1F**) and virus particle production (**Figure 1G**) of a mouse norovirus strain (MNoV-1; GV). We further evaluated this effect on another two MNoV strains with distinct biological behaviors, the acutely cleared strain $MNoV^{CW3}$ and the persistent strain $MNoV^{CR6}$. Notably, nitazoxanide had no effect on $MNoV^{CW3}$ RNA replication and virus production but slightly facilitated the infection of $MNoV^{CR6}$.

Our results indicate the complexity of evaluating the effects of nitazoxanide on norovirus infection. This is in line with previous findings that nitazoxanide inhibits hepatitis B virus, hepatitis C virus, rotavirus, or influenza virus infection through multiple mechanisms [9]. Given the opposing effects of nitazoxanide on human and murine norovirus, we postulate that the virus genotype and strain are critically important for responsiveness. Currently, there are six well-classified norovirus genogroups, with GI, GII and GIV affecting humans. Among these genogroups, GII.4 is responsible for most of the pandemics, and GII.17 is an emerging pathogenic genotype. Furthermore, chronically infected immunocompromised individuals and transplant patients are reservoirs for the emergence of new strains of norovirus [10]. Because the diversity of norovirus genotypes circulating in immunocompromised patients is much greater than that in healthy populations, larger and well-designed clinical studies may help us to understand the distinct responsiveness to nitazoxanide among different populations and possibly move us toward more personalized treatment. Encouragingly, our results demonstrating a specific antiviral effect in the human norovirus replicon system highlight the great potential of nitazoxanide for treating norovirus infection, although further research is required to confirm and expand these results.

## NOTES

**Acknowledgement.** We thank Dr Kyeong-Ok Chang (Kansas State University) for providing us the human norovirus replicon and Dr Herbert W. Virgin IV (Washington University School of Medicine) for providing the mouse norovirus strains.

**Financial support.** This work was supported by the Dutch Digestive Foundation for a career development grant (no. CDG 1304) and the China Scholarship Council for funding a PhD fellowship to W. D. (201406180072).

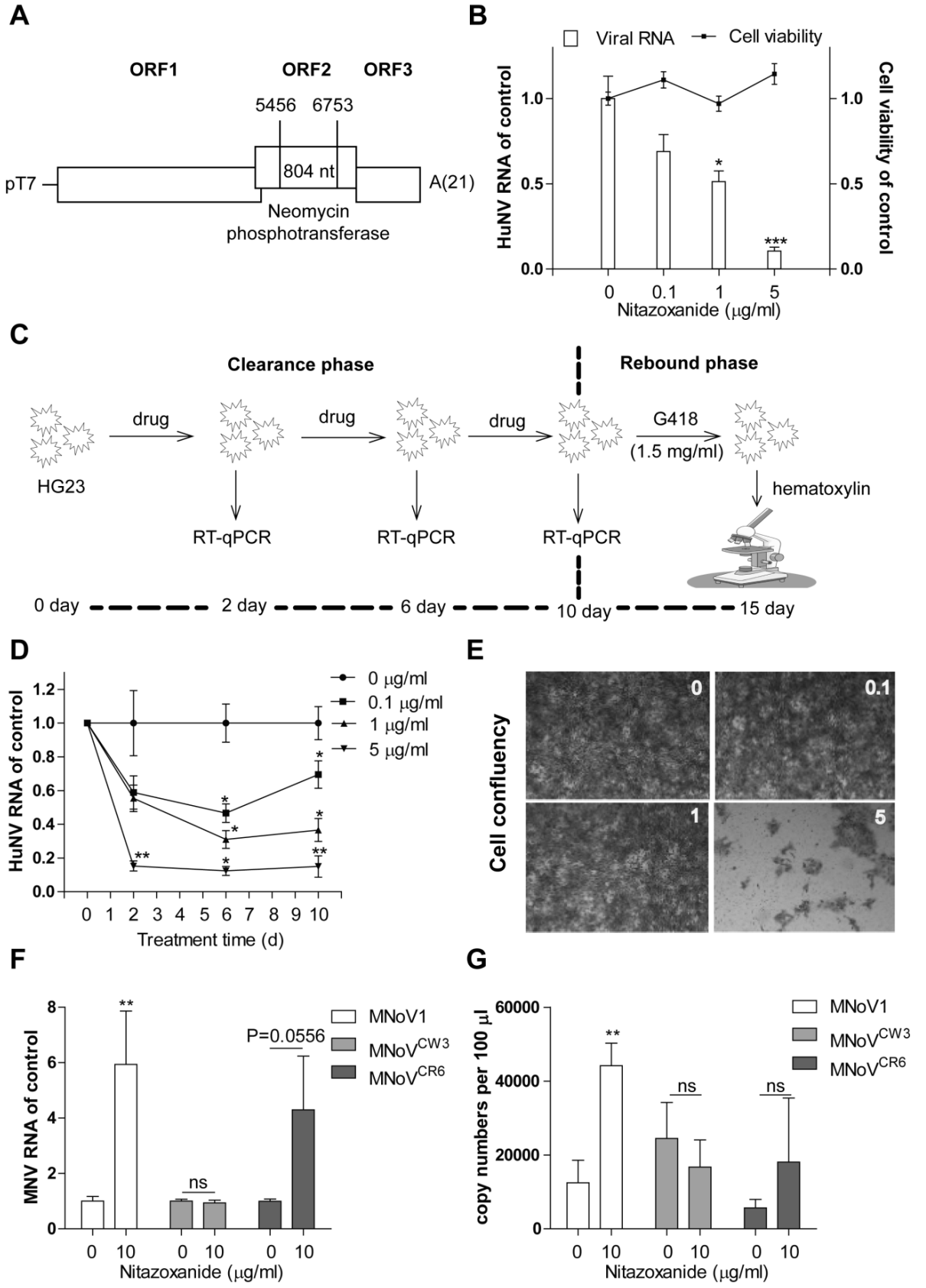



**Figure 1.** *A*, Schematic diagram of the human subgenomic norovirus replicon (HuNV) construct. RNA transcripts were synthesized *in vitro* and were transfected into the human Huh-7 cells to generate the HG23 model system. After the transfection, the cells were cultured in medium complemented with G418 (1.5 mg/mL). *B*, Nitazoxanide dose-dependently inhibited replication of

the HG23 HuNV subgenomic replicon after 48 hours of treatment. The levels of HuNV RNA were quantified by quantitative real-time polymerase chain reaction (RT-qPCR) and were compared with cells treated with 0.5% dimethyl sulfoxide (DMSO) (control) (*n* = 3 independent experiments with 2 replicates each). *C*, HuNV replicon clearance-rebound assay. During the clearance phase, HG23 replicon cells were cultured in the presence of nitazoxanide without G418. After 10 days, nitazoxanide was removed, and cells were incubated in the presence of G418 (1.5 mg/mL) for 5 days. *D*, Clearance assay with nitazoxanide. Cells were treated with nitazoxanide for 2, 6, or 10 days. At the end of each treatment period, the levels of HuNV RNA were determined by RT-qPCR and compared with the control cells of the same treatment time (*n* = 3 independent experiments with 2 replicates each). *E*, Rebound assay with nitazoxanide. After 10 days of treatment, nitazoxanide was omitted, and HG23 cells ($2.5 \times 10^4$ per 48-well) were cultured under the selective pressure of G418 (1.5 mg/mL). With another 5 days of culture, the cell layers were stained with hematoxylin and eosin. Units: $\mu$g/mL. *F* and *G*, Nitazoxanide exerts differential effects on murine norovirus replication. RAW 264.7 cells were infected with 3 murine norovirus strains (MNoV-1, $MNoV^{CW3}$ or $MNoV^{CR6}$) at a multiplicity of infection of 0.1 and were subsequently treated with different concentrations of nitazoxanide. After 24 hours of incubation, the relative levels of cellular murine norovirus (MNV) RNA and extracellular viral RNA copy numbers were determined by RT-qPCR (*n* = 3 independent experiments with 2 replicates each). Data presented as means ± SEM. *, $P < .05$; **, $P < .01$; ***, $P < .001$; ns, not significant; Mann-Whitney test using GraphPad Prism.

# REFERENCES

1. Thorne L, Arias A, Goodfellow I. Advances toward a norovirus anti-viral: from classical inhibitors to lethal mutagenesis. J Infect Dis 2016; **213**(suppl 1):S27–31.

2. Rossignol JF, El-Gohary YM. Nitazoxanide in the treatment of viral gastroenteritis: a randomized dou-ble-blind placebo-controlled clinical trial. Aliment Pharmacol Ther 2006; **24**:1423–30.

3. Siddiq DM, Koo HL, Adachi JA, Viola GM. Norovirus gastroenteritis successfully treated with nitazoxanide. J Infect 2011; **63**:394–7.

4. Rossignol JF, Lopez-Chegne N, Julcamoro LM, Carrion ME, Bardin MC. Nitazoxanide for the empiric treatment of pediatric infectious diarrhea. Trans R Soc Trop Med Hyg 2012; **106**:167–73.

5. Kempf B, Edgar JD, Mc Caughey C, Devlin LA. Nitazoxanide is an ineffective treatment of chronic norovirus in patients with X-linked agammaglobulinemia and may yield false-negative polymerase chain reaction findings in stool specimens. J Infect Dis 2017; **215**:486–7.

6. Thorne L, Goodfellow I. Reply to Kempf et al. J Infect Dis 2017; **215**:487–8.

7. Chang KO, Sosnovtsev SV, Belliot G, King AD, Green KY. Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line. Virology 2006; **353**:463–73.

8. Wobus CE, Karst SM, Thackray LB, et al. Replication of norovirus in cell culture reveals a tropism for dendritic cells and macrophages. PLoS Biol 2004; **2**:e432.

9. Rossignol JF, La Frazia S, Chiappa L, Ciucci A, Santoro MG. Thiazolides, a new class of anti-influenza molecules targeting viral hemagglutinin at the post-translational level. J Biol Chem 2009; **284**:29798–808.

10. Karst SM, Baric RS. What is the reservoir of emergent human norovirus strains? J Virol 2015; **89**:5756–9.

# Chapter 6

## Nitazoxanide inhibits Human Norovirus Replication and Synergizes with Ribavirin by Activation of Cellular Antiviral Response

Wen Dang,[a] Lei Xu,[a] Buyun Ma,[a] Sunrui Chen,[a] Yuebang Yin,[a] Kyeong-Ok Chang,[b] Maikel P. Peppelenbosch,[a] Qiuwei Pan[a]*

[a]Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam, Netherlands

[b]Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, USA

***Antimicrobial Agents and Chemotherapy 2018 (in press).***

## ABSTRACT

Norovirus is the main cause of viral gastroenteritis worldwide. Although norovirus gastroenteritis is self-limiting in immunocompetent individuals, chronic infections with debilitating and life-threatening complications occur in immunocompromised patients. Nitazoxanide (NTZ) has been empirically used in the clinic and demonstrated effectiveness against norovirus gastroenteritis. In this study we aimed at uncovering the antiviral potential and mechanisms of NTZ and its active metabolite, tizoxanide (TIZ) using a human norovirus (HuNV) replicon. NTZ and TIZ, collectively referred to as thiazolides (TZD) potently inhibited the replication of HuNV and a norovirus surrogate feline calicivirus. Mechanistic studies revealed that TZD activated cellular antiviral response and stimulated the expression of a subset of interferon-stimulated genes (ISGs), particularly IRF-1 not only in Huh7-based HuNV replicon but also in naïve Huh7, Caco-2 and novel human intestinal organoids. Overexpression of exogenous IRF-1 inhibited HuNV replication; whereas knockdown of IRF-1 largely attenuated the anti-norovirus potency of TZD, suggesting that IRF-1 mediated TZD-induced inhibition of HuNV. By using a JAK inhibitor CP-690550 and STAT1 knockout approach, we found that TZD induced antiviral response independent of the classical Janus Kinase/signal transducers and activators of transcription (JAK/STAT) pathway. Furthermore, TZD and ribavirin synergized to inhibit HuNV replication and completely depleted the replicons from host cells after long-term treatment. In summary, our results demonstrated that TZD combated HuNV replication through activation of cellular antiviral response, in particular inducing a prominent antiviral effector IRF-1. NTZ monotherapy or combination with ribavirin represented promising options for treating norovirus gastroenteritis, especially in immunocompromised patients.

**Keywords:** norovirus, nitazoxanide, cell culture model, IRF-1, ribavirin, synergy

# INTRODUCTION

Norovirus is one of the main causative agents for acute viral gastroenteritis in all age groups. It is estimated to cause a median number of 699 million illness and 219,000 deaths every year, of which more than 90,000 deaths occur in children under five years old [1]. Nowadays, norovirus gastroenteritis is also recognized as an emerging burden in immunocompromised populations, particularly transplant recipients [2]. Suppression of immune system facilitates norovirus infection, resulting in chronic diarrhea and other complications [3, 4]. Furthermore, norovirus gastroenteritis has been reported to cause graft-versus-host disease (GVHD) and sepsis, due to the breakdown of gastrointestinal mucosa in those patients [5]. Besides careful fluid replacement and intensive supportive care, no specific antivirals are currently available for treating norovirus gastroenteritis.

Nitazoxanide (NTZ) was developed in the early 1970s and originally commercialized as an anti-parasitic drug. Following oral administration, NTZ is absorbed from the gastrointestinal (GI) tract and rapidly hydrolyzed by plasma esterase to form its active circulating metabolite tizoxanide (TIZ). The concentration of TIZ in serum maximally reaches 10 $\mu$g/mL [6]. Both drugs belong to a class of agents known as thiazolides (TZD). NTZ is currently licensed in the United States (Alinia®, Romark Laboratories) for treating diarrhea caused by Cryptosporidium parvum and Giardia intestinalis in adults and children above 12 months age [7]. Interestingly, NTZ has also been reported to exert potent and broad-spectrum antiviral activities against many viruses including influenza virus, hepatitis B virus (HBV), hepatitis C virus (HCV), human immune-deficiency virus (HIV) and rotavirus [7, 8]. Mechanistically, it has been demonstrated that NTZ selectively blocks the maturation of the viral hemagglutinin of influenza viruses at post-translational level, thus inhibiting the proper assembly and release of the virus from the host cells [9]. With regard to HCV, NTZ is involved in activation of protein kinase R (PKR), a key kinase that regulates the host innate anti-HCV response [10]. For rotavirus, NTZ reduces the size and alters the architecture of rotavirus viroplasm, thus decreasing the viral dsRNA formation [8]. NTZ has been recently reported to elicit anti-viral innate immunity to combat HIV and other virus infections [11]. However, a general consensus regarding the antiviral mechanism-of-action of NTZ is not well established, and seemingly depends on the virus itself and host cells.

Several clinical studies have recently demonstrated the off-label use of NTZ in treating norovirus gastroenteritis. In a randomized double-blind placebo-controlled clinical trial, 50 adults and adolescents presenting diarrhea with stool positive for norovirus, rotavirus or adenovirus were enrolled. The median resolution time for all subjects as well as the ones infected with norovirus was significantly reduced in NTZ-treated group when compared to placebo group [12]. Later in a retrospective study comprising 12 patients with norovirus gastroenteritis after chemotherapy and hematopoietic stem cell transplantation (HSCT), 11 patients clinically responded with improvement in symptoms following NTZ administration [5]. Similar results were observed in another study comprising 5 patients with norovirus gastroenteritis after HSCT. Oral administration of NTZ resulted in the resolution of gastroenteritis and complete viral clearance [13]. These clinical studies have indicated the potential of repurposing NTZ as a viable therapeutic option for norovirus gastroenteritis. However, further experimental research is required to characterize its *bone fide* anti-norovirus activity and mechanistic insight. In a review article based on personal communication, It has been mentioned that the 50% inhibitory concentration (IC50) and 90% inhibitory concentration (IC90) for TIZ were 0.5 and 1.2 $\mu$g/mL respectively using a human norovirus (HuNV) replicon [7]. In our previous study, we also found that NTZ displayed potent anti-norovirus activity using the same replicon model [14].However, the exact antiviral mechanisms of NTZ against norovirus have not been revealed. Thus, we have systematically explored the anti-norovirus potential of TZD and studied its potential mechanism-of-action. Our results have revealed that TZD triggered cellular innate immune response to combat norovirus replication and synergized with ribavirin, a broad-spectrum antiviral drug that showed effectiveness against norovirus gastroenteritis *in vivo* [15].

# MATERIALS AND METHODS

## *Cell lines and virus propagation*

Human Huh7 hepatocellular carcinoma cells expressing HuNV genotype 1 replicons (HG23) [16] and human embryonic kidney 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza Verviers, Belgium) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT, USA). Caco-2 cells (Human Caucasian colon adenocarcinoma; ECACC) were cultivated in DMEM supplemented with 20% (vol/vol) FCS. Crandell Rees feline kidney cells (CRFK) were maintained in Minimum Essential Medium

with Earle's Salts without glutamine (EME), 1% L-glutamine, 0.05% (w/v) sodium bicarbonate, 0.02 M Hepes buffer, 0.2% nystatin solution and 10% (vol/vol) fetal bovine serum (FBS). A neomycin resistant gene was engineered into open reading frame 2 (ORF2), thereby conferring HG23 resistance to gentamicin (G418; Gibco) [16]. G418 was added to HG23 culture medium at 1.5 mg/mL for selection before experimentation.

Feline calicivirus (FeCV; kindly gifted from Dr. Erwin Duizer, National Institute for Public Health and the Environment, the Netherlands) strain F9 was propagated in monolayers of CRFK cells as previously described [17]. Briefly, FeCV was inoculated into CRFK monolayers at a multiplicity of infection (MOI) of 0.1. After 3 days of incubation, the FeCV cultures were collected by repeated freezing and thawing, and clarified by centrifugation at 4,000 rpm for 10 min. The supernatant was filtered through a 0.45-$\mu$m filter (Waters Millipore), titrated three independent times by the 50% tissue culture infectious doses (TCID50) and stocked at -80℃ for all subsequent experiments.

## *Reagents*

Nitazoxanide (NTZ; Chemical Abstract Service [CAS] no. 55981-09-4; Sigma-Aldrich), tizoxanide (TIZ; CAS no. 173903-47-4; Cayman Chemical) and CP-690550 (CAS no. 477600-75-2; Santa Cruz Biotechnology) were dissolved in dimethyl sulfoxide (DMSO) at 20 mg/mL, 5 mg/mL and 1 mg/mL, respectively. Ribavirin (CAS no. 36791-04-5; Sigma-Aldrich) was stocked in water at 10 mg/mL. Human interferon alpha 2a recombinant protein (IFN$\alpha$) (Catalog no. 111001; Thermo-Fisher Scientific) was stocked in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) at 100,000 units/mL based on international units. Matched concentration of DMSO (0.05%) were used as vehicle control. To avoid repeated freeze-thaws, the compounds were aliquoted and stored at -80℃. During experimentation the compounds were stepwise diluted to desired concentration.

Interferon regulatory factor 1 (IRF-1) (D5E4, Rabbit mAb, #8478) antibody was obtained from Cell Signaling Technology and $\beta$-actin (C-4, Mouse mAb, #sc-47778) was purchased from Santa Biotechnology (Santa Cruz, CA, USA). Secondary antibodies IRDye® 800CW-conjugated goat anti-rabbit and goat anti-mouse IgGs (1:10,000 dilution; Li-Cor Bioscience, Lincoln, USA) were used, as appropriate.

## *Culturing and passaging human primary intestinal organoids*

Culture of human primary intestinal organoids was performed as described before [18]. Intestinal biopsies were dissected, cut longitudinally and completely washed with pre-cold PBS. After removing villus and fat, small intestine was minced into small pieces (~ 1 $mm^3$) in a 10 cm culture dish using a scalpel and washed with complete chelating solution (CCS) (1.0 g/L $Na_2HPO_4.2H_2O$, 1.08 g/L $KH_2PO_4$, 5.6 g/L NaCl, 0.12 g/L KCl, 15 g/L sucrose, 10 g/L D-Sorbitol and 80 $\mu$g/L DL-dithiothreitol dissolved in MilliQ $H_2O$) for 3 times by pipetting up and down 15 times, followed by further incubation with 8 mM EDTA on a shaking platform for 15 min at 4℃. Supernatant was discarded and biopsies were gently rinsed with PBS to completely get rid of EDTA. It was thoroughly suspended in CCS solution by pipetting up and down 10 times with a 10-mL pipet to loosen crypts, and 2 mL of FCS was added. The supernatant contained crypts and was collected in a 50 mL tube; whereas the remaining biopsies were further digested with EDTA for 2 to 3 times and supernatant with crypts was pooled. Crypt suspension was first centrifuged at 300 g for 5 min. Supernatant was gently removed and the pellet was resuspended in 2 mL complete medium growth factor (GF)-(CMGF-, advanced DMEM/F12 supplemented with 1% [vol/vol] of GlutaMAX™ Supplement [Gibco, Grand island, USA], 10 mM of Hepes and 100 U/mL of Penicillin-Streptomycin]. Second round of centrifugation at 130g for 5 min was performed to harvest crypt. The crypt pellet was finally suspended in ice-cold growth factor reduced phenol-red free Matrigel (Corning, Bedford, USA) and dropped into the center of a pre-warmed (37℃) 24-well tissue culture plate at 100 to 500 crypts per well. The plate was incubated at 37℃, 15 min for Matrigel solidification. Crypts were finally cultured in culture medium (Table S1) and passaged every 6 days.

After approximately 6 days, organoids were passaged. Briefly, organoids were collected to a 15 mL falcon tube and dissociated mechanically by pipetting up and down 15 to 20 times by passing through a 5-mL pipet inserting a 200 $\mu$L tip. The resulting suspension was centrifuged at 150 g for 5 min at 4℃ and pellet was cultured in fresh Matrigel with a 1:3 split ratio.

### *HuNV antiviral assay*

Twenty-four hours before experimentation, HG23 cells were cultured in the medium without G418. HG23 cells were seeded into 48-well tissue culture plates ($2.5 \times 10^4$ cells per well), treated with compounds alone or in combination, and quantified for HuNV RNA levels

after 2 days of treatment by qRT-PCR using a primer pair targeting the RNA-dependent RNA polymerase (RdRP) of HuNV. To further evaluate the long-term effects of compounds on HuNV replication, long-term treatment and rebound assay was designed, as described previously [14, 19, 20]. During long-term treatment HG23 cells were treated with compounds for 2, 6 or 10 days. Cells were passaged to avoid overgrowth and drugs were replenished at times in between. At the end of each treatment period, HuNV RNA levels were determined by qRT-PCR. After 10 days of treatment, compounds were omitted. HG23 cells were plated into 48-well tissue culture plate ($2.5 \times 10^4$ per well) and cultured under the selective pressure of G418 (1 mg/mL). Following 5 days of culture, cell colonies were stained with hematoxylin and eosin, and visualized by an inverted Zeiss Axiovert 200 microscope equipped with Zeiss AxioCam MRm camera (LLC, Thornwood, NY, USA).

### *FeCV TCID50 and antiviral assay*

FeCV stock was quantified by TCID50 assay. In briefly, 50 $\mu$L of 10-fold dilutions of FeCV were inoculated into 100 $\mu$L of CRFK cells in 96-well tissue culture plates at 1,000 cells per well. After 5 days of incubation at 37℃, each well was scored under a light scope for the cytopathic effect (CPE). A TCID50 was calculated from 6 replicates by using the Reed-Muench method.

The antiviral activities of TZD against FeCV were detected by qRT-PCR and CPE reduction assay. The antiviral assay with FeCV was initiated by inoculated virus into CRFK monolayers at an MOI of 0.1 ($2.5 \times 10^4$ cells per well of 48-well tissue culture plate). Following 90 min incubation at 37℃, cells were washed with PBS for 3 times to remove free virus and increasing concentrations of TZD were added. After 24 hours of treatment, extracellular RNA and intracellular RNA were extracted from the supernatant (virus particles, 100 $\mu$L) and cell monolayers respectively. The relative intracellular FeCV RNA levels were normalized to feline glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and calculated with the $2^{-\Delta\Delta C}{}_T$ method. To further quantify the absolute numbers of virus genomes in the supernatant, FeCV genome copy number was detected using qRT-PCR using a primer pair of forward primer (5'-GAACTACCCGCCAATCAACAT-3'; corresponding to nucleotide [nt] 2420-2440) and reverse primer (5'-CGGCTCTGATGGCTTGAAACTG-3'; corresponding to nt 2507-2528). Briefly, a cDNA-containing target sequence in FeCV was amplified using Q5® High-Fidelity DNA polymerase kit (New England Biolabs Inc.) in a 25 $\mu$L reaction mixture

containing 5 $\mu$L of 5× Q5 Reaction Buffer, 0.5 $\mu$L of a 10 mM dNTPs, 1.25 $\mu$L of 10 $\mu$M forward primer (5'-GAACTACCCGCCAATCAACAT-3'; corresponding to nt 2420-2440), 1.25 $\mu$L of 10 $\mu$M reverse primer (5'-CGGCTCTGATGGCTTGAAACTG-3'; corresponding to nt 2507-2528), 5 $\mu$L of template cDNA and 0.25 µL of Q5 High-Fidelity DNA Polymerase. Amplification was carried out with initial denaturation at 98℃ [30 s]; 35 cycles of 98℃ [10 s], 66℃ [30 s], and 72 ℃ [30 s]; and an additional extension step of 72℃ for 2 min. The product was subjected to agarose gel electrophoresis, purified using the ChargeSwitch-Pro PCR clean-up kit (Invitrogen) and 10-time serially diluted. The dilutions were quantified by qRT-PCR to generate a standard curve, which was expressed by plotting the log copy numbers against the cycle threshold ($C_T$) values (Fig. S1). The FeCV genome copy numbers in the medium were calculated by comparing the $C_T$ with that of the standard curve.

FeCV could induce significant CPE in CRFK cell line [21]. The antiviral activities of TZD against FeCV were further confirmed by using an MTT-based CPE induction assay. Briefly, 10 $\mu$L of FeCV was inoculated into 80 $\mu$L of CRFK cells ($1 \times 10^4$ cells per well of 96-well tissue culture plate) at an MOI of 0.5, followed by addition of 10 $\mu$L of increasing concentrations of TZD. After 2 days of incubation, a clear CPE was observed characterized by complete destruction of the cell monolayers in the control well. Then, an MTT assay was performed and the absorbance (optical density [OD]) at 490 nm was recorded. CPE reduction was calculated as $[(OD_{treated})_{FeCV}\text{-}OD_{VC}]/[OD_{CC}\text{-}OD_{VC}]$, where $(OD_{treated})_{FeCV}$ represented the OD of virus-infected cells treated with drugs, while $OD_{CC}$ and $OD_{VC}$ represented the OD of the cell control and virus control, respectively. The protective effect of TZD on FeCV-infected CRFK cells could also be directly visualized by observing the survival of cells. In brief, each well was stained with hematoxylin and eosin, and visualized with an inverted light microscope.

### *Drug treatment on Caco-2 and organoids*

Caco-2 cells were plated into 48-well tissue culture plate ($2 \times 10^4$ cells per well) and incubated with 10 $\mu$g/mL TZD. After 2 days of treatment, the medium was discarded and cell monolayers were washed 3 times with PBS. Cells were subsequently lysed for RNA extraction.

The organoid experiment was performed on monolayer cultures of organoids, as described [22]. A 24-well tissue culture plate was pre-covered with 50% Collagen R and incubated at 37℃ for 30 mins. Organoids were harvested, transferred to 24-well tissue

culture plate (100 to 200 organoids per well) and centrifuged at 1500 rpm for 10 min. The drug was added and the cultures were further incubated for 48 hours. After treatment the supernatant was aspirated and the monolayers were washed with PBS for 3 times and lysed for qRT-PCR.

### *Generation of IRF-1-expressing lentiviral pseudoparticles and lentiviral transduction*

HEK293T cells were used to generate IRF-1-expressing lentivirus vectors. Briefly, 293T cells were co-transfected with IRF-1-expressing plasmid pTRIP.CMV.IVSb.IRF-1.ires.TagRFP, HIV-1 gag-pol and VSV-G (a ratio of 1:0.8:0.2) in Opti-MEM using polyethyleneimine (PEI). After 6 hours of transfection, cells were gently washed once with PBS and refreshed with growth medium. After 48 and 72 hours of transfection, lentivirus-containing supernatants were pooled and filtered through a 0.45-$\mu$m pore size filter. Two hours of ultracentrifugation (22,000 rpm) was used to concentrate lentiviral particles. The pellet was subsequently re-suspended and stored at -80℃ with the dilution of 7 log viral RNA copies per mL.

For transduction assay, HG23 cells were seeded into 48-well tissue culture plate (5 × $10^4$ cells per well) and transduced with lentiviral pseudoparticles encoding IRF-1. After 2 days of culture cells were collected for detecting IRF-1 by qRT-PCR and Western blot.

### *STAT1 knockout in Huh7 using the CRISPR/Cas9 system*

STAT1 knockout (KO) Huh7 clone was established by Lenti-CRISPR/Cas9 system (STAT1 sgRNA: TCCCATTACAGGCTCAGTCG). In brief, HEK293T cells were co-transfected with lentiCRISPRv2-STAT1, pVSVg and psPAX2 (a ratio of 1:0.5:0.75) using FUGEN HD Transfection Reagent (Qiagen), according to the manufacturer's instructions. After 24 hours of incubation, medium was replaced with fresh medium supplemented with 1% FCS. Lentivirus-containing supernatants were pooled at 24 and 48 hours, and subsequently filtered through a 0.45-$\mu$m pore size filter. Huh7 cells were infected with lentiviral supernatant for 2 days and further incubated with selection medium containing 4 $\mu$g/mL puromycin for 7 days. To obtain knockout clones, single cells were sorted into 96-well tissue culture plates and cultured with puromycin (4 $\mu$g/mL). Medium were refreshed every 4 days during selection. Cell colonies were collected and identified by Western blot and genome sequence.

### *Western blot*

Cell samples were lysed and loaded onto a 10 to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis at 120 V for 100 mins, the proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (pore size, 0.45 $\mu$m; Invitrogen) for 1.5 h, with an electric current of 250 mA. The membrane was probed with primary antibody plus secondary antibody and detected with Odyssey 3.0 infrared imaging system (LI-COR Biosciences). $\beta$-actin was served as standardization of sample loading.

### *Quantitative real-time polymerase chain reaction (qRT-PCR)*

Total RNA was isolated with a Machery NucleoSpin RNA II kit (Bioke, Leiden, The Netherlands) and measured with a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was reverse transcribed from 500 ng of RNA using a cDNA synthesis kit (TaKaRa Bio, Inc.). The cDNA of a targeted gene transcript was amplified for 50 cycles and quantified with a SYBR Green-based real-time PCR (Applied Biosystems), according to the manufacturer's instructions. All the PCRs were performed in duplicates and amplification specificity was confirmed by melting-curve analysis. Human and feline GAPDH were used as reference genes. The relative expression of targeted gene was calculated as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_{T\text{sample}} - \Delta C_{T\text{control}}$ ($\Delta C_T = C_{T[\text{targeted gene}]} - C_{T[\text{GAPDH}]}$). All primer combinations were listed in Table S2.

### *Statistics*

Data are presented as mean ± standard error of the mean (SEM). Comparisons between groups were performed with Mann-Whitney test using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered significant at a *P* value less than 0.05.

## RESULTS

### *TZD exhibited potent antiviral activities toward HuNV and FeCV replication without significant cytotoxicity*

A Huh7-based HuNV replicon model (HG23) representing one of very few options in modeling HuNV replication in cell culture has been widely used for studying anti-norovirus agents. After 2 days of treatment, NTZ and TIZ dose-dependently inhibited HuNV replication (Fig. 1A). The toxicities of TZD toward HG23 cells were determined by a 3-(4,5-dimethyl-2-

thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (supplementary material). TZD showed no major toxicities toward HG23 cells at the clinically relevant concentration of 10 $\mu$g/mL (Fig. 1A). These results have demonstrated the potent anti-norovirus effects of TZD without triggering major cytotoxicities.

Consistently, TZD dose-dependently reduced a norovirus surrogate FeCV cellular viral RNA and viral genomes in the supernatant after 24 hours of treatment without significant cytotoxicities (Fig. 1B and C). The inhibitory effect was also confirmed in a CPE reduction assay, demonstrating that TZD at 5 and 10 $\mu$g/mL protected CRFK cells from FeCV-induced CPE formation (Fig. 1D). Meanwhile, TZD at 5 and 10 $\mu$g/mL could significantly increase the survival of FeCV-infected CRFK cells (Fig. 1E). Collectively, our results demonstrated that TZD potently inhibited HuNV and its surrogate FeCV replication.

## *TZD activated the expression of interferon-stimulated genes (ISGs), especially IRF-1*

TZD have been recently reported to stimulate innate antiviral immunity in peripheral blood mononuclear cells *in vitro* potentially accounting for its anti-HIV activity [11]. IFN signaling is a vital component of the innate immunity against viral pathogens. It signals through the Janus Kinase/signal transducers and activators of transcription (JAK-STAT) pathway and transcriptionally induces hundreds of ISGs as ultimate antiviral effectors [23, 24]. We thus investigated the antiviral state in HG23 cells after TZD treatment. Treatment with 10 $\mu$g/mL NTZ or TIZ for 2 days induced the expression of a panel of important ISGs, including IRF-1, interferon regulatory factor (IRF) 9, retinoic acid-inducible gene I (RIG-I), IFI27 (also known as ISG12 or p27), RNA-dependent protein kinase PKR, ISG15, Mx1 and melanoma differentiation-associated protein (MDA) 5 (Fig. 2A). Some of those ISGs exerted broad activities against many virus [25]. Interestingly, compared with exogenous IFN$\alpha$ (1,000 IU/mL) serving as a positive control, TZD were more potent at inducing IRF-1 mRNA expression (NTZ [11.26 ± 1.94] vs TIZ [8.26 ± 1.70] vs IFN$\alpha$ [4.89 ± 0.75]; Fig. 2A). Western blot analysis confirmed the potent induction of IRF-1 in response to TZD treatment at protein level (Fig. 2B; Fig. S2). We next excluded the implication of HuNV replicon in TZD-mediated stimulation of ISGs, as TZD treatment also induced ISGs in naïve Huh7 cells. Consistent with HG23, IRF-1 mRNA was stimulated by TZD more effectively than 1,000 IU/mL of IFN$\alpha$ in Huh7 cells (NTZ [17.31 ± 2.94] vs TIZ [14.86 ± 3.49] vs IFN [2.57 ± 0.17]; Fig. 2C).

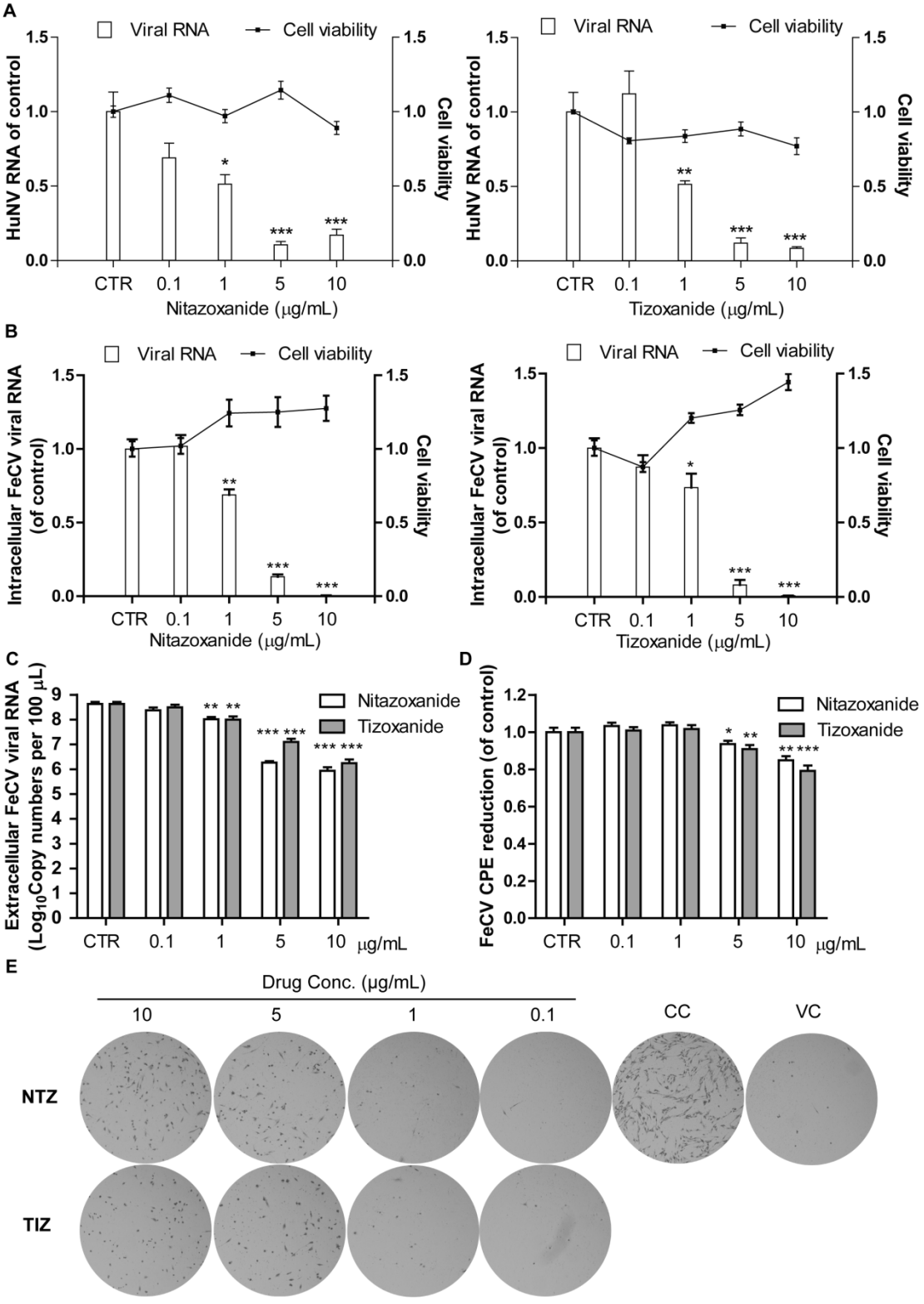



**Figure 1 TZD potently inhibited HuNV and its surrogate FeCV replication without significant cytotoxicities.** (A) Nitazoxanide and its active metabolite tizoxanide dose-dependently inhibited HuNV replication without clear toxicities to host cells after 2 days of treatment. The level of HuNV replicon RNA was quantified using qRT-PCR and compared to vehicle control (0.05% DMSO, set as 1) (*n* = 3 independent experiments with duplicates each). (B) TZD elicited potent antiviral potential against FeCV. CRFK cells were first infected with FeCV (an MOI of 0.1) and incubated with vehicle control or increasing concentrations of TZD. After 24 hours of treatment, cellular FeCV RNA level was

quantified by qRT-PCR, normalized to feline GAPDH and compared to vehicle control (set as 1). (C) Same as (B) for detecting the cellular FeCV RNA, viral RNA copy numbers in the supernatant (secreted viruses) were also detected after 24 hours of treatment with vehicle control or TZD. TZD significantly reduced extracellular FeCV viral genomes (*n* = 3 independent experiments with duplicates each). (D and E) The anti-FeCV activities of TZD were further validated by an MTT-based and hematoxylin and eosin staining-based CPE reduction assay. CRFK cells were first infected with FeCV (an MOI of 0.5) and incubated with vehicle control or TZD. After 2 days of treatment, CPE was quantified by the MTT assay, and residual cells were observed after fixation and staining with hematoxylin and eosin. In Figure E, CC and VC represented cell control and virus control respectively. Images are representative of three independent experiments. Data are presented as the means ± SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

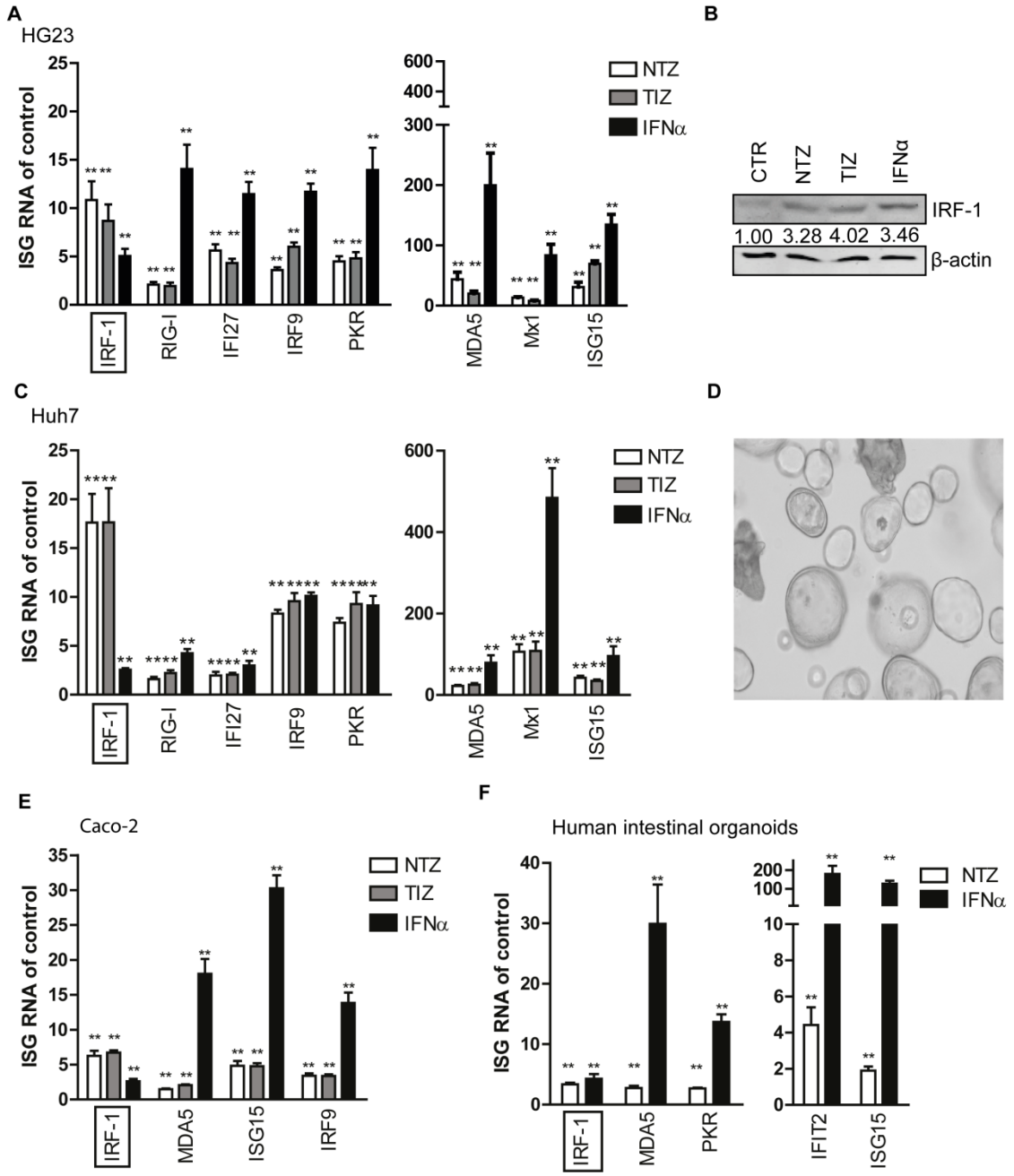



**Figure 2 TZD robustly stimulated the expression of IRF-1 and several other ISGs in HuNV replicon model and intestinal models of Caco-2 and primary organoids.** (A) Fold changes of ISGs induced by NTZ (10 $\mu$g/mL), TIZ (10 $\mu$g/mL) or IFN$\alpha$ (1,000 IU/mL) in HG23 cells. After 2 days of

treatment, levels of ISG mRNA were quantified by qRT-PCR and normalized to human GAPDH. Results were expressed as fold changes compared to the vehicle control (DMSO; *n* = 3 independent experiments with duplicates each). (B) Western blot analysis of IRF-1 protein expression in response to NTZ (10 $\mu$g/mL), TIZ (10 $\mu$g/mL) or IFN$\alpha$ (1,000 IU/mL). Data are representative of three independent experiments. (C) Naïve Huh7 cells were treated with NTZ (10 $\mu$g/mL), TIZ (10 $\mu$g/mL), IFN$\alpha$ (1,000 IU/mL) or vehicle control. After 2 days of treatment, relative levels of ISG mRNA were quantified by qRT-PCR (*n* = 3 independent experiments with duplicates each). (D) Morphology of 3-D human primary intestinal organoids in Matrigel. (E) Relative levels of ISG RNA were quantified by qRT-PCR after 2 days of treatment with NTZ (10 $\mu$g/mL) or IFN$\alpha$ (1,000 IU/mL) in Caco-2 cells (*n* = 3 independent experiments with duplicates each). (C) Relative levels of ISG RNA in human intestinal organoids after treatment with NTZ (10 $\mu$g/mL) or IFN$\alpha$ (1,000 IU/mL) for 2 days (*n* = 3 independent experiments with duplicates each). Data are presented as the means ± SEM (*, $P < 0.05$; **, $P < 0.01$).

## *TZD robustly induced ISGs in human intestinal cell line and primary organoids*

Though the cellular tropism of HuNV is not completely understood, gastrointestinal (GI) tract is believed to be the reservoir for HuNV replication. We further extended TZD treatment to human intestinal epithelial cells (Caco-2) and primary human intestinal organoids that recapitulated the nature of intestinal epithelium (Fig. 2D). Consistently, TZD triggered antiviral response in those two models, even though to a lesser extent when compared to HG23 cells and Huh7 cells (Fig. 2E and F). TZD stimulated higher or comparable levels of IRF-1 mRNA compared to 1,000 IU/mL IFN$\alpha$ in two models.

Although the expression levels and patterns of ISGs differed among different cell models after TZD treatment, IRF-1 was consistently highly induced. IRF-1 has been reported to be one of the most important ISGs exerting antiviral activities against 14 different viruses within 7 families [26]. Thus, we further specifically investigated the role of IRF-1 on TZD-mediated inhibition of HuNV replication.

## *IRF-1 inhibited HuNV replication and contributed to TZD-mediated anti-norovirus effects*

To dissect the role of IRF-1 on HuNV replication, we first overexpressed IRF-1 in HG23 cells. A bicistronic lentiviral vector co-expressing an IRF-1 and the red fluorescent protein TagRFP as well as a control virus containing a luciferase gene from *Photinus pyralis* (Fluc) were used in the study [25]. IRF-1 mRNA and protein levels were significantly increased in overexpressing cells after 2 days of transduction (Fig. 3A). Exogenous IRF-1 overexpression resulted in a robust inhibition of HuNV RNA replication by 94.4 ± 2.9% (Fig. 3B). To further explore the role of basal IRF-1, a loss-of-function assay was performed to silence IRF-1 by transducing a lentiviral-based hairpin RNA (shRNA) construct targeting IRF-1. A nontargeting

scrambled vector was used as a control. Successful knockdown of endogenous IRF-1 was confirmed by qRT-PCR (by 52.4 ± 9.1%) and Western blot analysis (by 72%) (Fig. 3C). In the absence of TZD, IRF-1 knockdown had minor effect on HuNV RNA replication (Fig. 3D). However, anti-norovirus effects of TZD were largely attenuated in IRF-1 knockdown cells when compared to control cells. This attenuated effect in TZD treatment was greater than that in TIZ treatment (Fig. 3E). These results indicated that IRF-1 exerted antiviral activity and contributed to TZD-mediated inhibition of HuNV replication.

## *Triggering antiviral response by TZD was independent of JAK-STAT pathway*

To elucidate the mechanisms by which TZD stimulated cellular antiviral immunity, we first examined whether TZD induced the production and secretion of interferons (IFNs). IFN genes including IFN$\alpha$, IFN$\beta$ and IL28A/B were detected in HG23 and intestinal organoids following NTZ treatment. NTZ unexpectedly showed minor effects on IFN mRNA expression (Fig. S3).

The JAK/STAT pathway is the principal cascade for IFN signaling. Upon binding to the two IFN receptor subunits (IFNAR1 and IFNAR2), type I IFNs activate the Janus kinases (Jak1 and Tyk2) and signal transducers of transcription (STAT1 and STAT2), resulting in the elevation of hundreds of ISGs. To examine whether TZD stimulated ISGs through the JAK-STAT pathway, TZD were combined with CP-690550 (Tofacitinib), a potent and selective JAK inhibitor. An 100 IU/mL IFN$\alpha$ was used as a control. The antiviral activity of IFN$\alpha$ was partially abolished and HuNV RNA levels were restored from 4.5 ± 1.7% to 28.9 ± 14.6% when combined with CP-690550 (Fig. 4A). In contrast, CP-690550 did not interfere with the anti-norovirus effects of TZD. Correspondingly, CP-690550 significantly suppressed IFN$\alpha$ but not TZD-mediated induction of ISGs including IRF-1 (Fig. 4B), PKR, ISG15 and MDA5 (Fig. 4C).

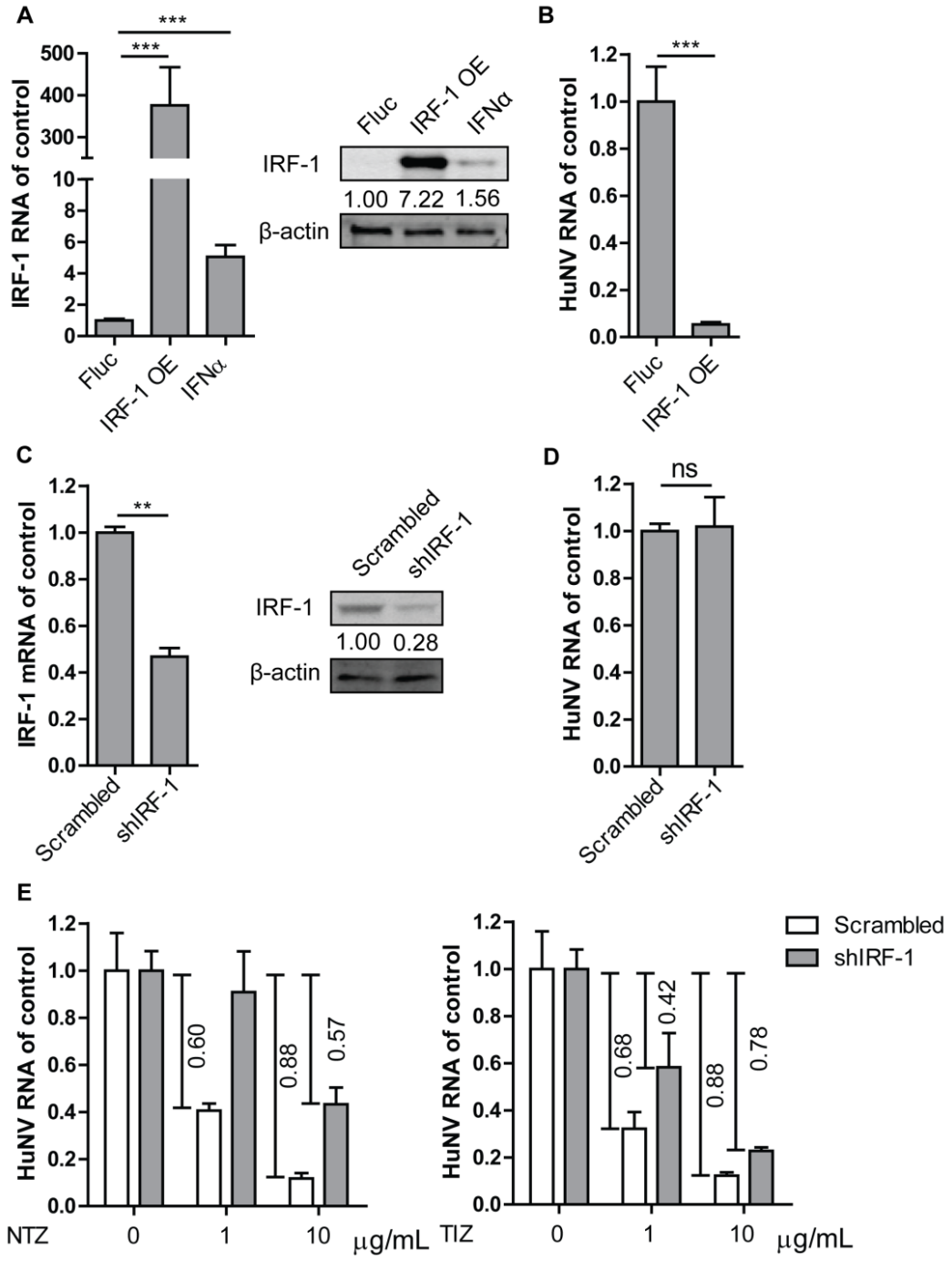



**Figure 3 IRF-1 mediated TZD-triggered inhibition of HuNV replication.** (A) To overexpress IRF-1, HG23 cells were transduced with lentiviral IRF-1-expressing vector or Fluc-expressing vector (control). After 2 days of transduction, the level of IRF-1 expression was detected by qRT-PCR (*n* = 3 independent experiments with duplicates each) and Western blot (*n* = 3 independent experiments). INF*α* (1,000 IU/mL) was used as a positive control. (B) Overexpression of IRF-1 potently inhibited HuNV replication (*n* = 3 independent experiments with duplicates each). (C) To further evaluate the role of basal IRF-1 on HuNV replication, HG23 cells were transduced with lentiviral shRNA vector targeting IRF-1. A scrambled vector was used as a nontargeting control. Successful knockdown of IRF-1 was confirmed by qRT-PCR (*n* = 3 independent experiments with duplicates each) and Western blot (*n* = 3 independent experiments). (D) Knockdown of IRF-1 had minor effects on HuNV replication (*n* = 3 independent experiments with duplicates each). (E) IRF-1 shRNA and nontargeting control cells were mock-treated or treated with indicated concentration of TZD. After 2 days of treatment, the

levels of HuNV RNA were quantified by qRT-PCR and compared to a respective control ($n$ = 3 independent experiments with duplicates each). Data are presented as the means ± SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

STAT1 is a key component of the JAK-STAT pathway. CRISPR/Cas9 mediated approach was used to efficiently knockout STAT1 in Huh7 cells. The successful knockout was confirmed by Western blot. Compared to Huh7 control cells, STAT1 protein was undetectable in Huh7 KO cells (Fig. 4D). To further confirm the complete knockout of STAT1, Huh7 control and KO cells were treated with IFN$\alpha$. As expected, IFN$\alpha$ treatment (1,000 IU/mL) significantly induced STAT1 expression in Huh7 control cells but not in KO cells, while no major difference was observed in STAT2 mRNA in both cells (Fig. 4E). These data indicated the successful knockout of STAT1. Next, Huh7 control and KO cells were treated with TZD or 1,000 IU/mL IFN$\alpha$ for 2 days and the expression of ISGs was measured by qRT-PCR. We first observed that STAT1 KO slightly decreased the basal levels of a subset of ISGs including MDA5, Mx1 and DDX60. This was probably due to the fact that STAT1 was critical to maintain cellular innate immune homeostasis. As expected, the induction levels of ISGs in KO cells were significantly diminished in response to IFN$\alpha$ treatment when compared to control cells. However, TZD induced similar levels of ISGs in both control cells and KO cells including IRF-1 (Fig. 4F), MDA5, Mx1, DDX60, ISG15 and IFIT1 (Fig. 4G), suggesting that TZD induced antiviral response and ISG expression independent of the JAK-STAT pathway.

Nuclear factor-kappa B (NFκB) signaling participates in modulating the activation of various proinflammatory cytokine genes and ISGs [27], contributing to a vital role on combating pathogen invasion. By using Huh7- and Caco-2-based NFκB luciferase reporter cells, we further excluded the activation of the NFκB pathway by TZD (Fig. S4). Thus, a novel mechanism is likely involved in TZD-induced cellular antiviral response.

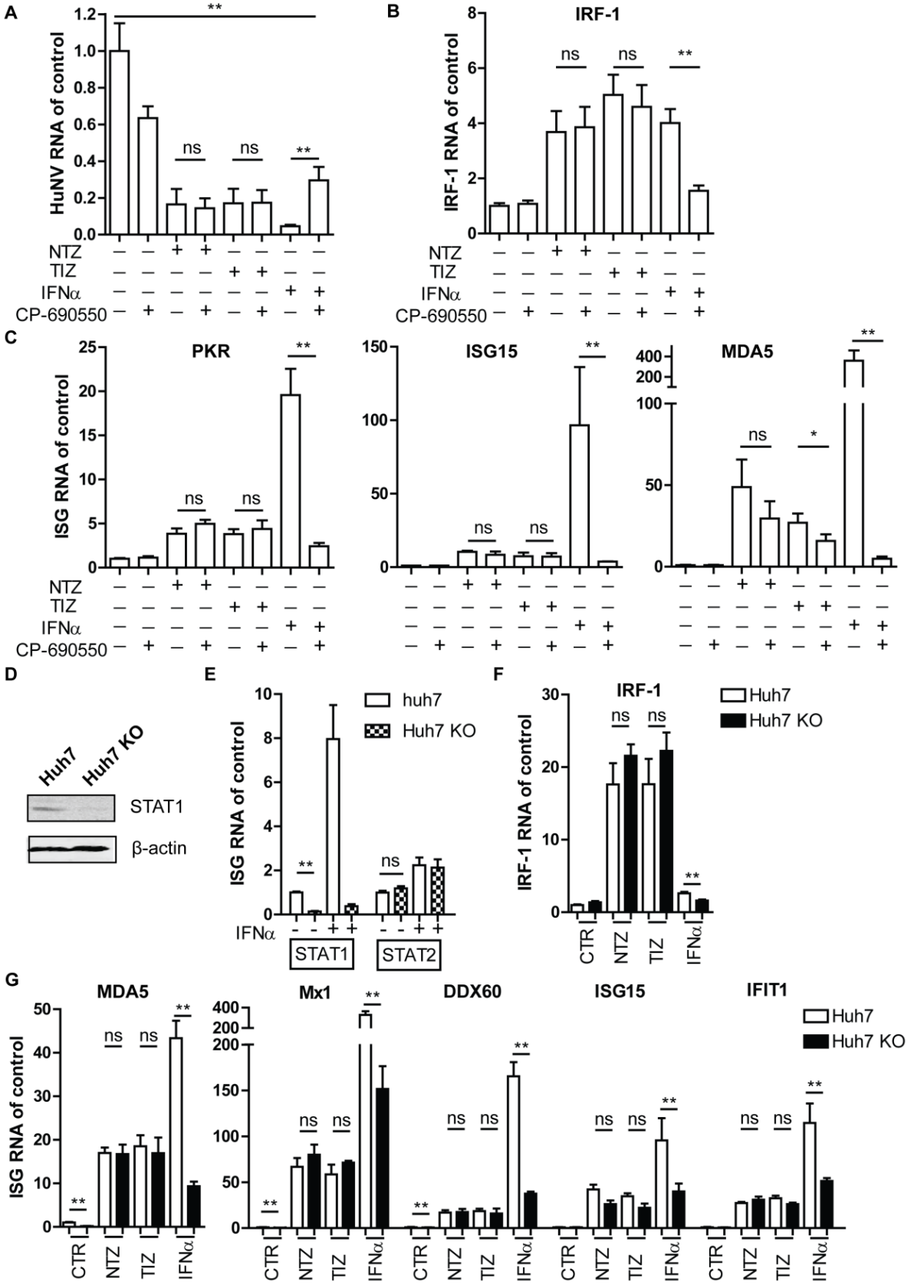



**Figure 4 Stimulation of antiviral response by TZD was independent of JAK-STAT pathway.** (A) HG23 cells were treated with vehicle only (DMSO; control), NTZ (10 $\mu$g/mL), TIZ (10 $\mu$g/mL), IFN$\alpha$ (100 IU/mL) alone or in combination with CP-690550 (1,000 ng/mL). After 2 days of treatment, the HuNV replication was quantified by qRT-PCR ($n$ = 3 independent experiments with duplicates each).

(B and C) The expression of IRF-1 and other ISGs including PKR, ISG15 and MDA5 was quantified by qRT-PCR (*n* = 3 independent experiments with duplicates each). (D) STAT1 knockout (KO) clone was established from Huh7 cells expressing STAT1 sgRNAs. Successful KO of STAT1 was confirmed by Western blot. (E) To further confirm successful KO of STAT1, Huh7 control and STAT1 KO cells were treated with IFN$\alpha$ (1,000 IU/mL) for 2 days. Levels of STAT1 and STAT2 RNA were evaluated by qRT-PCR (*n* = 3 independent experiments with duplicates each). Knockout of STAT1 abolished the induction of STAT1 but not STAT2 after IFN$\alpha$ treatment. (F and G) Huh7 control and STAT1 KO cells were mock-treated (DMSO; control) or treated with NTZ (10 $\mu$g/mL), TIZ (10 $\mu$g/mL), or IFN$\alpha$ (1,000 IU/mL). After 2 days of treatment, the expression levels of IRF-1, MDA5, Mx1, DDX60, ISG15 and IFIT1 were detected by qRT-PCR. The results were first normalized to human GAPDH and then compared to control cells (*n* = 3 independent experiments with duplicates each). Data are presented as the means ± SEM (*, $P < 0.05$; **, $P < 0.01$).

## *Synergistic anti-norovirus activity and augmented ISG induction by TZD and ribavirin combination*

To achieve better antiviral efficacy, we have evaluated the combination effect of TZD and ribavirin against HuNV replication. In short-term (2 days) treatment, combination of NTZ and ribavirin did not achieve significantly enhanced inhibition of HuNV replication and showed additive effect as calculated by the Mac Synergy II software (Fig. 5A). Similar results were observed in TIZ and ribavirin combination (Fig. 5B).

We next prolonged treatment time in a long-term assay (10-day), and HuNV RNA was quantified after 2, 6 and 10 days of treatment. Importantly, NTZ combining with ribavirin resulted in an enhanced inhibition of HuNV replication after prolonged treatment (Fig. 6A). Antiviral data at 10 day suggested potent synergistic effect in combination as shown in the 3-D MacSynergy II plot (Fig. 6B). To further detect the residual HuNV replicons in host cells after prolonged combination treatment, a rebound assay was designed by culturing HG23 cells with selection marker G418. If HuNV replicons were completely deleted from host cells, HG23 could not proliferate to form colonies. As shown In Fig. 6C, no colonies were found in the combination of 2.5 $\mu$g/mL NTZ and 5 $\mu$g/mL ribavirin, indicating the complete clearance of HuNV replicons from host cells. To gain more evidence to support this finding, gel-based RT-PCR assay was performed to specifically detect HuNV replicons in the cell culture RNA. No bands were detectable after high concentration of combination treatment, confirming the depletion of HuNV replicons from host cells (Fig. S5). Similar results were obtained with TIZ and ribavirin combination (Fig. 7; Fig. S5). Concurrently, we have observed synergistic induction of ISGs in combination treatment including IRF-1, MDA5, PKR and Mx1 (Fig. 8). Of note, this synergistic induction of ISGs was modest on day 2, but more robust on day 6 and

10 (Fig. 8). This may support the combined anti-norovirus effects of TZD and ribavirin that were additive in short-term treatment but synergistic after long-term treatment.

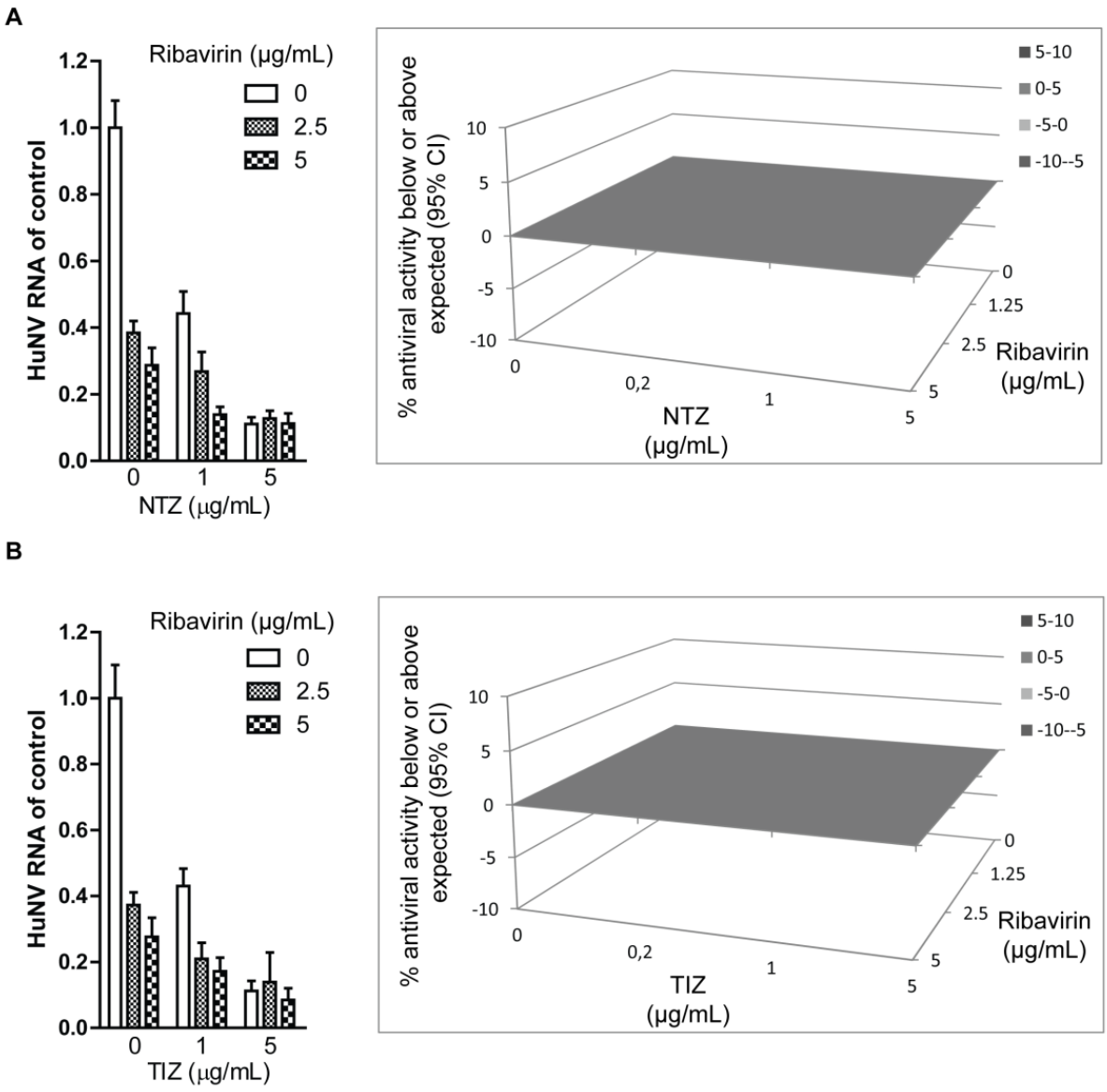



**Figure 5 TZD worked addtively with ribavirin to inhibit HuNV replication after short-term treatment.** (A) HG23 cells were treated with various concentrations of NTZ alone, ribavirin alone or the two in combination for 2 days. Antiviral activities were determined by qRT-PCR (*n* = 3 independent experiments with duplicates each; Left). To further explore the drug-drug interaction, antiviral results were analyzed with a mathematical model (Right). The 3-D surface plot shown represented the difference (within 95% confidence interval [CI]) between the actual experimental effects and theoretical additive effect of the combination at various concentrations of the two compounds. (B) The combination of TIZ with ribavirin.

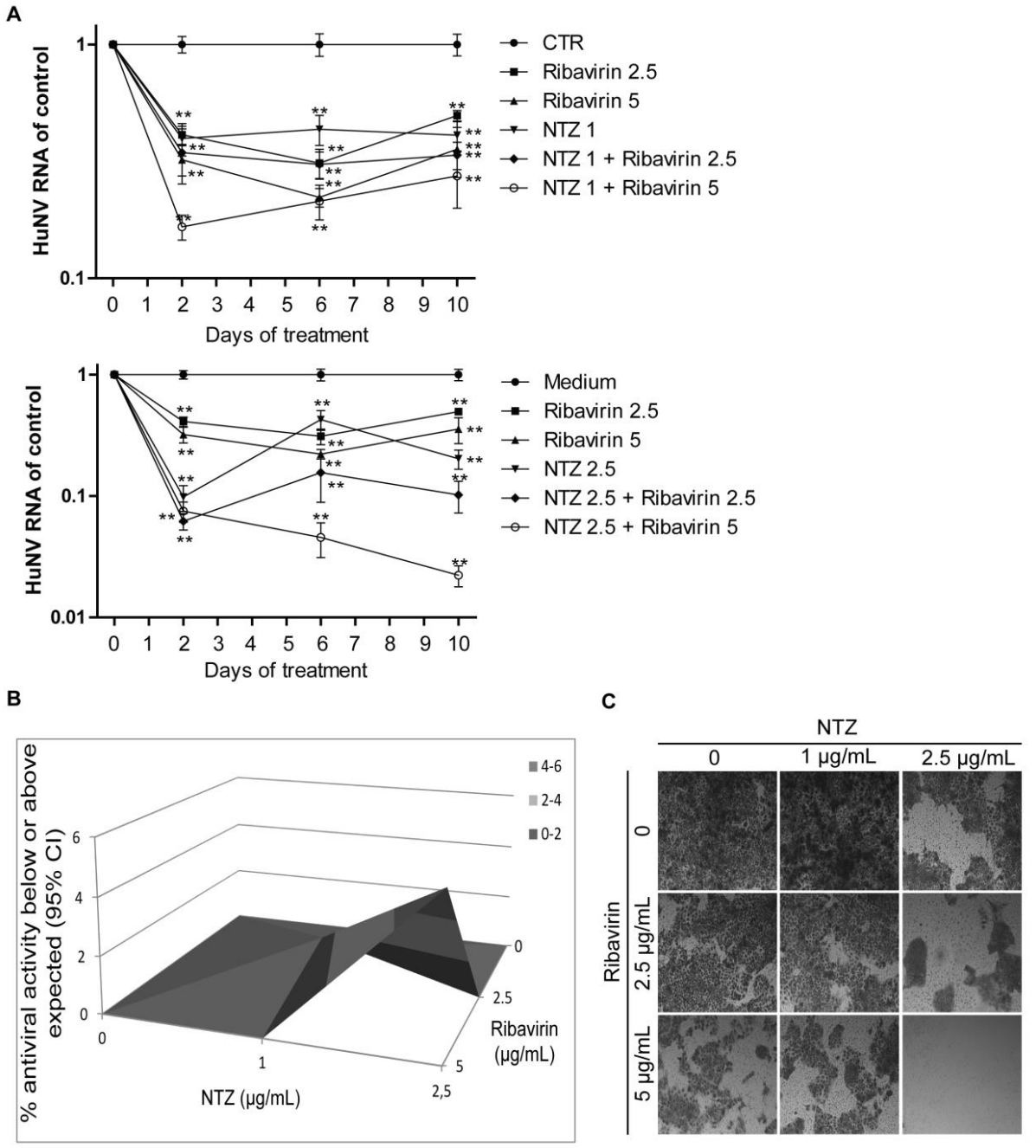



**Figure 6 Long-term combination of NTZ with ribavirin synergistically inhibited HuNV replication and completely depleted HuNV replicons from host cells.** (A) HG23 cells were incubated with NTZ alone, ribavirin alone or two drugs in combination. After 2 days of culture, cells were passaged to fresh drug-containing medium for another 4 days of incubation. After 6 days, cells were passaged with another round of 4 days of treatment. At the end of each treatment, HG23 cells were harvested and analyzed for the levels of HuNV RNA ($n$ = 3 independent experiments with duplicates each). (B) Synergy analysis. The antiviral results after 10 days of treatment were analyzed with MacSynergy. (C) Rebound assay. After 10 days of treatment, HG23 cells were plated into 48-well tissue culture plates ($2 \times 10^4$ cells per well) containing 250 $\mu$L of medium with 1 mg/mL G418. After 5 days of culture, cell colonies were stained and visualized using an inverted light microscope. Data presented are presentative of 3 independent experiments. Data are presented as the means ± SEM (*, $P < 0.05$; **, $P < 0.01$).

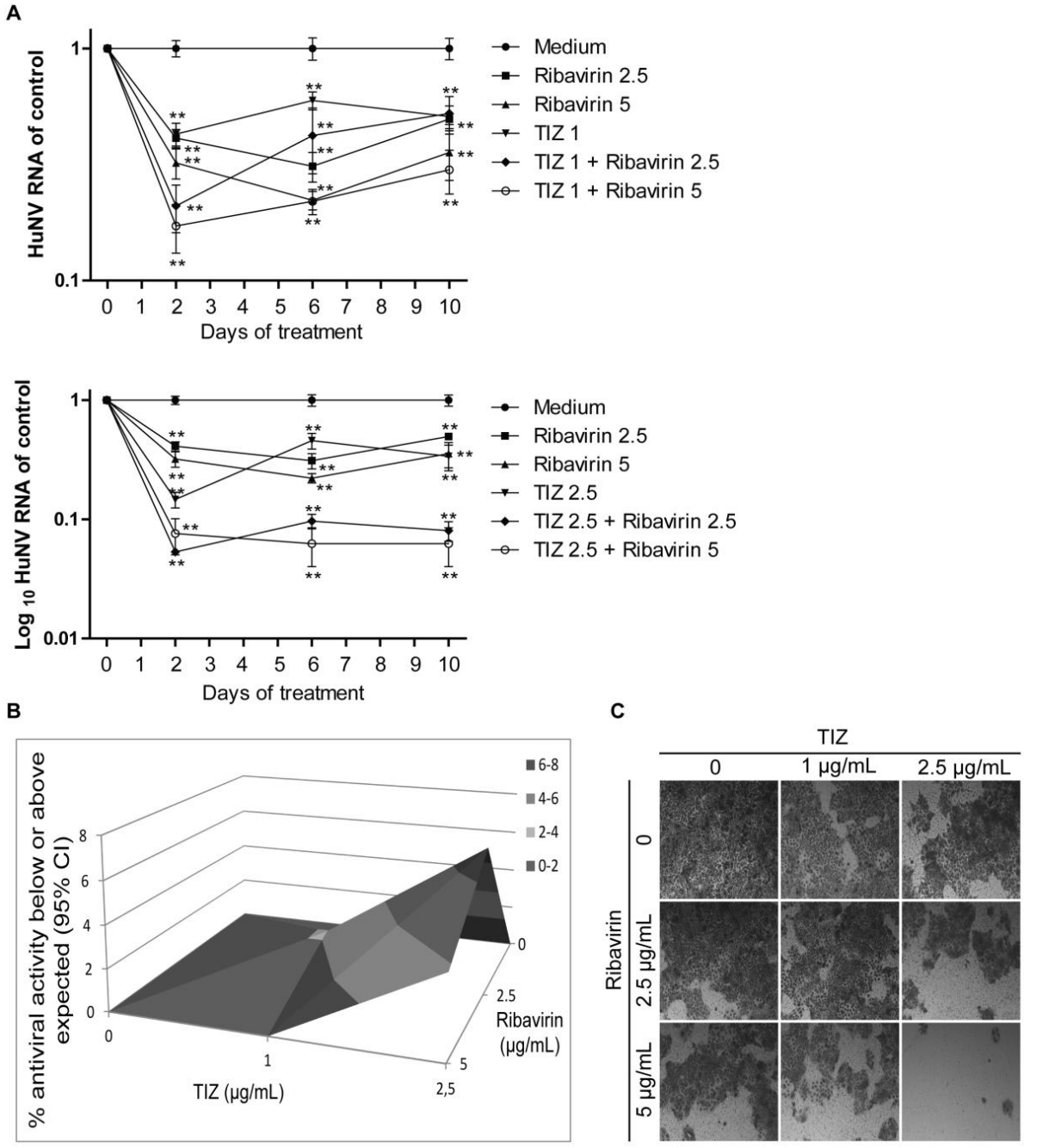



**Figure 7 Combining TIZ with ribavirin synergistically inhibited HuNV replication.** (A) Combination of TIZ with ribavirin showed greater efficacy against HuNV replication compared to individual drug (*n* = 3 independent experiments with duplicates each). (B) Synergy analysis. (C) Rebound assay after long-term treatment. Data are presented as the means ± SEM (*, $P < 0.05$; **, $P < 0.01$).

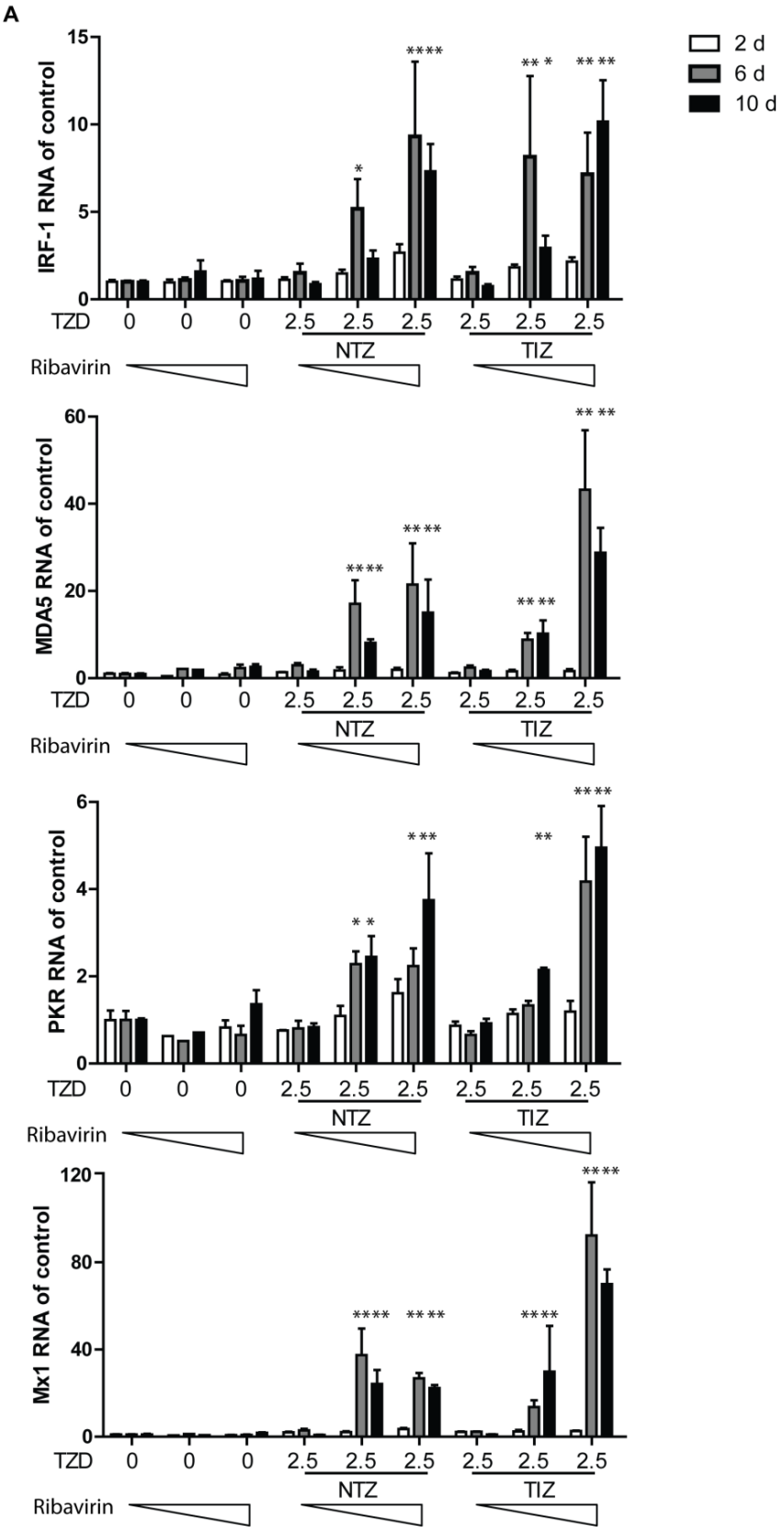



**Figure 8 Augmented induction of ISGs after combination treatment of TZD and ribavirin.** The qRT-PCR analysis of the expression levels of IRF-1, MDA5 and PKR after treatment with TZD (2.5 $\mu$g/mL NTZ and 2.5 $\mu$g/mL TIZ) and ribavirin (0, 2.5 and 5 $\mu$g/mL) in HG23 cells for 2, 6 and 10 days ($n$ = 3 independent experiments with duplicates each). Data are presented as the means ± SEM (*, $P$ < 0.05; **, $P$ < 0.01).

# DISCUSSION

With the accessibility of rotavirus vaccine [28], norovirus contributes substantially to the main cause of acute viral gastroenteritis across all settings and all age groups. In healthy general populations, norovirus normally causes self-limiting gastroenteritis. However, it often results in prolonged symptoms and virus shedding in immunocompromised patients. In a retrospective study comprising 2183 sold organ transplant (SOT) recipients, 4.6% recipients were diagnosed norovirus-positive and 22.8% of these patients developed chronic norovirus gastroenteritis [29]. Meanwhile, norovirus gastroenteritis is a great global economic burden due to the resulting huge health system costs and productivity losses [1]. Some clinical studies have reported the successful use of human immunoglobulin (HIG) [30] and ribavirin [31] for empirical treatment. Due to inadequate clinical data and experimental studies, both drugs require further evaluation as potential therapies. NTZ was recently demonstrated to be effective therapy for norovirus gastroenteritis in immunocompromised patients [7]. It showed high tolerance and minor adverse effects even after several weeks of administration in those patients [5]. Herein we documented that TZD exerted potent anti-norovirus efficacy through inducing innate antiviral response, in particular IRF-1 in a HuNV replicon (HG23). It exhibited synergistic anti-norovirus activity with ribavirin, which supported the potential of the combination treatment.

The HuNV replicon represents one of the few options for anti-HuNV drug evaluation. However, it dose not fully recapitulate the HuNV life cycle with deficiency of viral attachment, entry, assembly and release. Meanwhile, the relatively low replication level (ranging from 1,000 to 3,000 copies per cell) [16] may not fully resemble the fact that the peak amount of virus shedding reached $95 \times 10^9$ genomic copies/g feces in patients upon HuNV infection [32]. The shortage of HuNV infectious model prompted us to exploit HuNV surrogates to further confirm the anti-norovirus properties of TZD. Murine norovirus (MNV) is a good model system to study norovirus biology and pathogenesis as well as antiviral therapies [33]. Surprisingly, NTZ exerted opposing effects on human and murine norovirus replication. High concentration of NTZ (10 $\mu$g/mL) increased MNV strain MNV-1 replication and elicited no effects on the replication of another two strains $MNV^{CW3}$ and $MNV^{CR6}$ [14]. FeCV belongs to the family *Caliciviridea* and possesses many biochemical characteristics, similar genomic organization and primary RNA sequence compared to norovirus. Thus, FeCV

has been used as a surrogate model to study norovirus, especially in the aspects of antiviral development, inactivation methods and effective intervention in transmission [34-36]. Moreover, FeCV could be persistently cultivated in CRFK cells. We have demonstrated similar anti-calicivirus effects elicited by TZD against FeCV. Those results collectively suggest that TZD possessed potent antiviral potential against HuNV.

NTZ was originally developed as an antiprotozoal agent and was later reported to be a broad-spectrum antiviral drug toward many virus including influenza, rotavirus, HBV, HCV, HIV, yellow fever virus and Japanese encephalitis viruses [7]. It has been repurposed for the treatment of influenza in a phase 2b/3 clinical trial [7]. However, the exact mechanism-of-action remained largely elusive. NTZ was previously reported to stimulate anti-viral innate immunity, which contributed to its anti-HIV activity [11]. In our study we have demonstrated that TZD inhibited HuNV replication by inducing a panel of ISGs. Amongst the induced ISGs with generic antiviral effects, some were previously noted to have anti-norovirus activity. ISG15 mitigated murine norovirus 1 (MNV1) replication *in vitro* and *in vivo* by targeting an early step in the viral life cycle [37]. MDA5, a cellular sensor recognized MNV-1 and further triggered host immune response to MNV-1 [38]. PKR mediated type II IFN inhibition of MNV-1 translation [39].

Intriguingly, IRF-1, a broad antiviral ISG was potently induced by TZD at comparable or even higher levels when compared to 1,000 IU/mL IFN$\alpha$ treatment. This was observed in multiple cell types including HG23, naïve Huh7, Caco-2 and primary intestinal organoids. Importantly, we demonstrated that IRF-1 possessed potent anti-norovirus effect and mediated the antiviral effects of TZD. Interesting, IRF-1 by itself was able to induce the expression of many ISGs, resulting in an ampler magnitude of antiviral immunity by inducing IFN production [40, 41] or by directing activating the JAK-STAT pathway [42]. Classically, transcription of ISGs is via the JAK-STAT pathway. By pharmacological and genetic approaches, we found that the induction of ISGs by TZD was independent of this cascade. Further studies also excluded the activation of NFκB pathway and the production of IFNs in the action of TZD. One possible mechanism-of-action in the up-regulation of ISGs by TZD may be due to the inhibition of a transcriptional repressor. Accumulating evidence suggests that many DNA-binding proteins act as a transcriptional repressor for the induction of ISGs [43]. NTZ is involved in multiple biological roles including interfering with crucial metabolic and pro-death signaling [44]. Thus, NTZ may inhibit those transcriptional repressors, resulting

in the induction of ISG expression. Further research is called to reveal the exact mechanism-of-action.

Although NTZ has been empirically used in several cases for treating norovirus gastroenteritis [45-47], the efficacy remained controversial. NTZ was reported to be ineffective in a patient with X-Linked Agammaglobulinemia [48]. Ribavirin, a broad-spectrum antiviral agent has been reported to inhibit norovirus *in vitro* [19]. Clinical studies demonstrated that ribavirin treatment resulted in complete viral clearance in a subset of patients, but failed in others [31]. In this study, the combination of these two regimens resulted in synergistic anti-norovirus effects and could completely clear the virus replicons from host cells, even below or at clinically achievable concentrations. One potential antiviral mechanism of ribavirin was through modulating the cellular immunity and inducing ISGs [49]. Consistently, we observed augmented induction of ISGs by combination of TZD and ribavirin.

In summary, we have demonstrated the *bone fide* anti-norovirus effects of TZD through induction of cellular antiviral response. They further synergized with ribavirin in antiviral activities and ISG induction. These knowledge bear important implications of repurposing NTZ or in combination with ribavirin for treating norovirus gastroenteritis.

## ACKNOWLEDGEMENT

We gratefully acknowledge Dr. Erwin Duizer (National Institute for Public Health and the Environment, Bilthoven, the Netherlands) for providing us the FeCV and CRFK cells. We also thank Dr. Charles M. Rice (Rockefeller University, New York, NY, USA) for generously providing the overexpressing lentiviral vector.

## FUNDING

This work was supported by the Dutch Digestive Foundation (MLDS) for a career development grant (No. CDG 1304), the KWF (Dutch Cancer Society) Young Investigator grant (No. 10140) (to Q. Pan) and the China Scholarship Council for funding PhD fellowship to W. Dang (201406180072), L. Xu (201306300027), B. Ma (201508330291), Y. Yin (201307720045) and S. Chen (201606760056).

## TRANSPARENCY DECLARATIONS

None.

# REFERENCE


1. Bartsch SM, Lopman BA, Ozawa S, Hall AJ, Lee BY. 2016. Global Economic Burden of Norovirus Gastroenteritis. PLoS One **11:**e0151219.
2. Ye X, Van JN, Munoz FM, Revell PA, Kozinetz CA, Krance RA, Atmar RL, Estes MK, Koo HL. 2015. Noroviruses as a Cause of Diarrhea in Immunocompromised Pediatric Hematopoietic Stem Cell and Solid Organ Transplant Recipients. Am J Transplant **15:**1874-1881.
3. Kaufman SS, Chatterjee NK, Fuschino ME, Morse DL, Morotti RA, Magid MS, Gondolesi GE, Florman SS, Fishbein TM. 2005. Characteristics of human calicivirus enteritis in intestinal transplant recipients. J Pediatr Gastroenterol Nutr **40:**328-333.
4. Schwartz S, Vergoulidou M, Schreier E, Loddenkemper C, Reinwald M, Schmidt-Hieber M, Flegel WA, Thiel E, Schneider T. 2011. Norovirus gastroenteritis causes severe and lethal complications after chemotherapy and hematopoietic stem cell transplantation. Blood **117:**5850-5856.
5. Morris J, Morris C. 2015. Nitazoxanide Is Effective Therapy for Norovirus Gastroenteritis after Chemotherapy and Hematopoietic Stem Cell Transplantation (HSCT). Biology of Blood and Marrow Transplantation **21:**S255-S256.
6. Stockis A, De Bruyn S, Gengler C, Rosillon D. 2002. Nitazoxanide pharmacokinetics and tolerability in man during 7 days dosing with 0.5 g and 1 g b.i.d. Int J Clin Pharmacol Ther **40:**221-227.
7. Rossignol JF. 2014. Nitazoxanide: a first-in-class broad-spectrum antiviral agent. Antiviral Res **110:**94-103.
8. La Frazia S, Ciucci A, Arnoldi F, Coira M, Gianferretti P, Angelini M, Belardo G, Burrone OR, Rossignol JF, Santoro MG. 2013. Thiazolides, a new class of antiviral agents effective against rotavirus infection, target viral morphogenesis, inhibiting viroplasm formation. J Virol **87:**11096-11106.
9. Rossignol JF, La Frazia S, Chiappa L, Ciucci A, Santoro MG. 2009. Thiazolides, a new class of anti-influenza molecules targeting viral hemagglutinin at the post-translational level. J Biol Chem **284:**29798-29808.
10. Elazar M, Liu M, McKenna SA, Liu P, Gehrig EA, Puglisi JD, Rossignol JF, Glenn JS. 2009. The anti-hepatitis C agent nitazoxanide induces phosphorylation of eukaryotic initiation factor 2alpha via protein kinase activated by double-stranded RNA activation. Gastroenterology **137:**1827-1835.
11. Trabattoni D, Gnudi F, Ibba SV, Saulle I, Agostini S, Masetti M, Biasin M, Rossignol JF, Clerici M. 2016. Thiazolides Elicit Anti-Viral Innate Immunity and Reduce HIV Replication. Sci Rep **6:**27148.
12. Rossignol JF, Abu-Zekry M, Hussein A, Santoro MG. 2006. Effect of nitazoxanide for treatment of severe rotavirus diarrhoea: randomised double-blind placebo-controlled trial. Lancet **368:**124-129.
13. Gorgeis J, Sizemore C, Bashey A, Holland HK, Solomon SR, Morris LE, Solh M. 2017. Nitazoxanide is effective therapy for norovirus gastroenteritis after hematopoietic stem cell transplantation. Biol Blood Marrow Transplant **23:**S197-S198.
14. Dang W, Yin Y, Peppelenbosch MP, Pan Q. 2017. Opposing Effects of Nitazoxanide on Murine and Human Norovirus. J Infect Dis **216:**780-782.
15. Woodward J, Gkrania-Klotsas E, Kumararatne D. 2017. Chronic norovirus infection and common variable immunodeficiency. Clin Exp Immunol **188:**363-370.
16. Chang KO, Sosnovtsev SV, Belliot G, King AD, Green KY. 2006. Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line. Virology **353:**463-473.
17. Duizer E, Bijkerk P, Rockx B, De Groot A, Twisk F, Koopmans M. 2004. Inactivation of caliciviruses. Appl Environ Microbiol **70:**4538-4543.
18. Yin Y, Bijvelds M, Dang W, Xu L, van der Eijk AA, Knipping K, Tuysuz N, Dekkers JF, Wang Y, de Jonge J, Sprengers D, van der Laan LJ, Beekman JM, Ten Berge D, Metselaar HJ, de Jonge

H, Koopmans MP, Peppelenbosch MP, Pan Q. 2015. Modeling rotavirus infection and antiviral therapy using primary intestinal organoids. Antiviral Res **123:**120-131.

19. Dang W, Yin Y, Wang Y, Wang W, Su J, Sprengers D, van der Laan LJW, Felczak K, Pankiewicz KW, Chang KO, Koopmans MPG, Metselaar HJ, Peppelenbosch MP, Pan Q. 2017. Inhibition of Calcineurin or IMP Dehydrogenase Exerts Moderate to Potent Antiviral Activity against Norovirus Replication. Antimicrob Agents Chemother **61**.

20. Dang W, Xu L, Yin Y, Chen S, Wang W, Hakim MS, Chang KO, Peppelenbosch MP, Pan Q. 2018. IRF-1, RIG-I and MDA5 display potent antiviral activities against norovirus coordinately induced by different types of interferons. Antiviral Res **155:**48-59.

21. Bidawid S, Malik N, Adegbunrin O, Sattar SA, Farber JM. 2003. A feline kidney cell line-based plaque assay for feline calicivirus, a surrogate for Norwalk virus. J Virol Methods **107:**163-167.

22. Ettayebi K, Crawford SE, Murakami K, Broughman JR, Karandikar U, Tenge VR, Neill FH, Blutt SE, Zeng XL, Qu L, Kou B, Opekun AR, Burrin D, Graham DY, Ramani S, Atmar RL, Estes MK. 2016. Replication of human noroviruses in stem cell-derived human enteroids. Science **353:**1387-1393.

23. Wang W, Xu L, Su J, Peppelenbosch MP, Pan Q. 2017. Transcriptional Regulation of Antiviral Interferon-Stimulated Genes. Trends Microbiol **25:**573-584.

24. Xu L, Wang W, Peppelenbosch MP, Pan Q. 2017. Noncanonical Antiviral Mechanisms of ISGs: Dispensability of Inducible Interferons. Trends Immunol **38:**1-2.

25. Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P, Rice CM. 2011. A diverse range of gene products are effectors of the type I interferon antiviral response. Nature **472:**481-485.

26. Schoggins JW, MacDuff DA, Imanaka N, Gainey MD, Shrestha B, Eitson JL, Mar KB, Richardson RB, Ratushny AV, Litvak V, Dabelic R, Manicassamy B, Aitchison JD, Aderem A, Elliott RM, Garcia-Sastre A, Racaniello V, Snijder EJ, Yokoyama WM, Diamond MS, Virgin HW, Rice CM. 2014. Pan-viral specificity of IFN-induced genes reveals new roles for cGAS in innate immunity. Nature **505:**691-695.

27. Wang W, Xu L, Brandsma JH, Wang Y, Hakim MS, Zhou X, Yin Y, Fuhler GM, van der Laan LJ, van der Woude CJ, Sprengers D, Metselaar HJ, Smits R, Poot RA, Peppelenbosch MP, Pan Q. 2016. Convergent Transcription of Interferon-stimulated Genes by TNF-alpha and IFN-alpha Augments Antiviral Activity against HCV and HEV. Sci Rep **6:**25482.

28. Bines JE, Danchin M, Jackson P, Handley A, Watts E, Lee KJ, West A, Cowley D, Chen MY, Barnes GL, Justice F, Buttery JP, Carlin JB, Bishop RF, Taylor B, Kirkwood CD, Program RVRV. 2015. Safety and immunogenicity of RV3-BB human neonatal rotavirus vaccine administered at birth or in infancy: a randomised, double-blind, placebo-controlled trial. Lancet Infect Dis **15:**1389-1397.

29. van Beek J, van der Eijk AA, Fraaij PL, Caliskan K, Cransberg K, Dalinghaus M, Hoek RA, Metselaar HJ, Roodnat J, Vennema H, Koopmans MP. 2017. Chronic norovirus infection among solid organ recipients in a tertiary care hospital, the Netherlands, 2006-2014. Clin Microbiol Infect **23:**265 e269-265 e213.

30. Florescu DF, Hermsen ED, Kwon JY, Gumeel D, Grant WJ, Mercer DF, Kalil AC. 2011. Is there a role for oral human immunoglobulin in the treatment for norovirus enteritis in immunocompromised patients? Pediatr Transplant **15:**718-721.

31. Woodward JM, Gkrania-Klotsas E, Cordero-Ng AY, Aravinthan A, Bandoh BN, Liu H, Davies S, Zhang H, Stevenson P, Curran MD, Kumararatne D. 2015. The role of chronic norovirus infection in the enteropathy associated with common variable immunodeficiency. Am J Gastroenterol **110:**320-327.

32. Atmar RL, Opekun AR, Gilger MA, Estes MK, Crawford SE, Neill FH, Graham DY. 2008. Norwalk virus shedding after experimental human infection. Emerg Infect Dis **14:**1553-1557.

33. Wobus CE, Thackray LB, Virgin HWt. 2006. Murine norovirus: a model system to study norovirus biology and pathogenesis. J Virol **80:**5104-5112.

34. Nims R, Plavsic M. 2013. Inactivation of caliciviruses. Pharmaceuticals (Basel) **6:**358-392.

35. McCann KB, Lee A, Wan J, Roginski H, Coventry MJ. 2003. The effect of bovine lactoferrin and lactoferricin B on the ability of feline calicivirus (a norovirus surrogate) and poliovirus to infect cell cultures. J Appl Microbiol **95:**1026-1033.

36. Slomka MJ, Appleton H. 1998. Feline calicivirus as a model system for heat inactivation studies of small round structured viruses in shellfish. Epidemiol Infect **121:**401-407.

37. Rodriguez MR, Monte K, Thackray LB, Lenschow DJ. 2014. ISG15 functions as an interferon-mediated antiviral effector early in the murine norovirus life cycle. J Virol **88:**9277-9286.

38. McCartney SA, Thackray LB, Gitlin L, Gilfillan S, Virgin HW, Colonna M. 2008. MDA-5 recognition of a murine norovirus. PLoS Pathog **4:**e1000108.

39. Changotra H, Jia Y, Moore TN, Liu G, Kahan SM, Sosnovtsev SV, Karst SM. 2009. Type I and type II interferons inhibit the translation of murine norovirus proteins. J Virol **83:**5683-5692.

40. Miyamoto M, Fujita T, Kimura Y, Maruyama M, Harada H, Sudo Y, Miyata T, Taniguchi T. 1988. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. Cell **54:**903-913.

41. Fujita T, Reis LF, Watanabe N, Kimura Y, Taniguchi T, Vilcek J. 1989. Induction of the transcription factor IRF-1 and interferon-beta mRNAs by cytokines and activators of second-messenger pathways. Proc Natl Acad Sci U S A **86:**9936-9940.

42. Xu L, Zhou X, Wang W, Wang Y, Yin Y, Laan LJ, Sprengers D, Metselaar HJ, Peppelenbosch MP, Pan Q. 2016. IFN regulatory factor 1 restricts hepatitis E virus replication by activating STAT1 to induce antiviral IFN-stimulated genes. FASEB J **30:**3352-3367.

43. Hu S, Xie Z, Onishi A, Yu X, Jiang L, Lin J, Rho HS, Woodard C, Wang H, Jeong JS, Long S, He X, Wade H, Blackshaw S, Qian J, Zhu H. 2009. Profiling the human protein-DNA interactome reveals ERK2 as a transcriptional repressor of interferon signaling. Cell **139:**610-622.

44. Di Santo N, Ehrisman J. 2014. A functional perspective of nitazoxanide as a potential anticancer drug. Mutat Res **768:**16-21.

45. Rossignol JF, El-Gohary YM. 2006. Nitazoxanide in the treatment of viral gastroenteritis: a randomized double-blind placebo-controlled clinical trial. Aliment Pharmacol Ther **24:**1423-1430.

46. Rossignol JF, Lopez-Chegne N, Julcamoro LM, Carrion ME, Bardin MC. 2012. Nitazoxanide for the empiric treatment of pediatric infectious diarrhea. Trans R Soc Trop Med Hyg **106:**167-173.

47. Siddiq DM, Koo HL, Adachi JA, Viola GM. 2011. Norovirus gastroenteritis successfully treated with nitazoxanide. J Infect **63:**394-397.

48. Kempf B, Edgar JD, Mc Caughey C, Devlin LA. 2017. Nitazoxanide Is an Ineffective Treatment of Chronic Norovirus in Patients With X-Linked Agammaglobulinemia and May Yield False-Negative Polymerase Chain Reaction Findings in Stool Specimens. J Infect Dis **215:**486-487.

49. Thomas E, Feld JJ, Li Q, Hu Z, Fried MW, Liang TJ. 2011. Ribavirin potentiates interferon action by augmenting interferon-stimulated gene induction in hepatitis C virus cell culture models. Hepatology **53:**32-41.

# Supplementary Information

## SUPPLEMENTARY METHODS

### *Reverse transcription PCR (RT-PCR)*

Two primer sets were designed to detect HuNV replicons in HG23 cells. Primers Neo-F/Neo-R are located in the neomycin resistant gene [1] and predicted to produce a 804 bp product. Primers NVp36/35 are located in a highly conserved RNA polymerase region of HuNV GI genome and predicted to give a 470 bp product [2]. For RT, cDNA was reverse transcribed from extracted cellular RNA by using TakaRa PrimeScript™ RT reagent kit. Briefly, 8 $\mu$L of total cellular RNA (500 ng) was mixed with 2 $\mu$L of 5× PrimeScript Buffer on ice. The mixture was incubated at 37℃ for 15 mins; this was followed by heat inactivation at 85℃ for 5 s. The mixture was 20 times diluted and stored at -20℃ for further use. PCR was performed using Q5® High-Fidelity DNA polymerase kit (New England Biolabs Inc.) in a 25 $\mu$L reaction mixture containing 5 $\mu$L of 5× Q5 Reaction Buffer, 0.5 $\mu$L of a 10 mM dNTPs, 1.25 $\mu$L of 10 $\mu$M Forward Primer, 1.25 $\mu$L of 10 $\mu$M Reverse Primer, 5 $\mu$L of template cDNA and 0.25 $\mu$L of Q5 High-Fidelity DNA Polymerase. Amplification was carried out with initial denaturation at 98℃ [30 s]; 30 cycles of 98℃ [10 s], 50-70℃ [30 s] and 72 ℃ [30 s], and an additional extension step of 72℃ for 2 min. The GAPDH primer set was used as a reference gene and was amplified in 25 cycles [3]. The products were loaded onto 1 to 2% agarose gel containing SERVA DNA Stain G. DNA electrophoresis at 100 V for 45 min was performed. An 100 bp DNA ladder (Promega) was also electrophoresed on each gel. Bands were visualized and recorded with the Gel.Doc 2000 system (Bio-Rad). All primer combinations were listed in Table S2.

### *MTT assay*

Cytotoxicities of the compounds against host cells were determined by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. In brief, cells were seeded into 96-well tissue culture plates containing 0.05% DMSO (control) or increasing concentrations of drugs. After the time indicated, 10 mM MTT (Sigma, Zwijndrecht, Netherlands) was added. With another 3 hours of incubation, the medium was removed and 100 $\mu$L of DMSO was added to each well. The plate was incubated at 37℃ for 50 min. The absorbance at 490 nm was recorded on the microplate absorbance reader (Bio-Rad, CA, USA).

# SUPPLEMENTARY TABLES AND FIGURES

**Table S1 Overview of culture medium components for human intestinal organoids.**

| Components | Storage (℃) | Stocks | Final | Company |
|---|---|---|---|---|
| Wnt 3a | 4 | Conditioned medium | 50% | Self-produced |
| R-Spondin 1 | -20 | Conditioned medium | 20% | Self-produced |
| Noggin | -20 | Conditioned medium | 10% | Self-produced |
| B27 | -20 | 50× | 1 | Gibco |
| N2 | -20 | 100× | 1 | Gibco |
| N-Acetylcysteine (mol/L) | -20 | 0.5 | $1 \times 10^{-3}$ | Invitrogen |
| Nicotinamide (mol/L) | -20 | 1 | $1 \times 10^{-2}$ | Sigma-Aldrich |
| A83-01(TGF-beta inhibitor) (mol/L) | -20 | $5 \times 10^{-4}$ | $5 \times 10^{-7}$ | Tocris |
| SB202190 (p38 inhibitor) (mol/L) | -20 | $3 \times 10^{-2}$ | $3 \times 10^{-6}$ | Sigma-Aldrich |
| Human EGF (g/L) | -20 | $5 \times 10^{-2}$ | $5 \times 10^{-5}$ | Sigma-Aldrich |
| Prostaglandin E2 (mol/L) | -20 | $1 \times 10^{-4}$ | $1 \times 10^{-8}$ | PeproTech |
| Gastrin (mol/L) | -20 | $1 \times 10^{-4}$ | $1 \times 10^{-8}$ | PeproTech |
| Y-27632 (Rho kinase inhibitor) (mol/L) | -20 | $1 \times 10^{-2}$ | $1 \times 10^{-5}$ | Bio-Connect BV |

**Table S2 Primers used by qRT-PCR.**

| Name | Sequences[a] | Location |
|---|---|---|
| HuNoV GI-specific primer | COG1F (+): 5'- CGYTGGATGCGNTTYCATGA-3'[b]<br>COG1R (-): 5'- CTTAGACGCATCATCATTYAC-3'[b] | 5291[b]<br>5375[b] |
| FeCV | F: 5'- GAACTACCCGCCAATCAACAT -3'<br>R: 5'- CGGCTCTGATGGCTTGAAACTG-3' | 2420-2440<br>2507-2528 |
| DDX60 | F: 5'-GGTGTTTTCACCAGGGAGTATCG-3'<br>R: 5'-CCAGTTTTGGCGATGAGGAGCA-3' | |
| Human GAPDH | F: 5'-TGTCCCCACCCCCAATGTATC-3'<br>R: 5'-CTCCGATGCCTGCTTCACTACCTT-3' | |
| Feline GAPDH | GAPDH.57f: 5'- GCCGTGGAATTTGCCGT-3'<br>GAPDH.138r: 5'- GCCATCAATGACCCCTTCAT-3' | |
| IFIT1 | F: 5'-GCCTTGCTGAAGTGTGGAGGAA-3'<br>R: 5'-ATCCAGGCGATAGGCAGAGATC-3' | |
| IRF9 | F: 5'- CCACCGAAGTTCCAGGTAACAC-3'<br>R: 5'- AGTCTGCTCCAGCAAGTATCGG-3' | |
| IFI27 | F: 5'- CGTCCTCCATAGCAGCCAAGAT-3'<br>R: 5'- ACCCAATGGAGCCCAGGATGAA-3' | |
| IFITM3 | F: 5'-CTGGGCTTCATAGCATTCGCCT-3'<br>R: 5'-AGATGTTCAGGCACTTGGCGGT-3' | |
| ISG15 | F: 5'-CTCTGAGCATCCTGGTGAGGAA-3'<br>R: 5'-AAGGTCAGCCAGAACAGGTCGT-3' | |
| IRF-1 | F: 5'-GAGGAGGTGAAAGACCAGAGCA-3'<br>R: 5'-TAGCATCTCGGCTGGACTTCGA-3' | |
| IFI6 | F: 5'-TGATGAGCTGGTCTGCGATCCT-3'<br>R: 5'-GTAGCCCATCAGGGCACCAATA-3' | |
| MDA5 (IFIH1) | F: 5'-GCTGAAGTAGGAGTCAAAGCCC-3'<br>R: 5'-CCACTGTGGTAGCGATAAGCAG-3' | |
| Mx1 | F: 5'- GGCTGTTTACCAGACTCCGACA-3'<br>R: 5'- CACAAAGCCTGGCAGCTCTCTA-3' | |
| OAS3 | F: 5'-CCTGATTCTGCTGGTGAAGCAC-3'<br>R: 5'-TCCCAGGCAAAGATGGTGAGGA-3' | |
| OASL | F: 5'-GTGCCTGAAACAGGACTGTTGC-3'<br>R: 5'-CCTCTGCTCCACTGTCAAGTGG-3' | |

| | |
|---|---|
| PKR | F: 5’-GAAGTGGACCTCTACGCTTTGG-3’<br>R: 5’-TGATGCCATCCCGTAGGTCTGT-3’ |
| RIG-I | F: 5’-CACCTCAGTTGCTGATGAAGGC-3’<br>R: 5’-GTCAGAAGGAAGCACTTGCTACC-3’ |
| RSDA2 | F: 5’-CCAGTGCAACTACAAATGCGGC-3’<br>R: 5’-CGGTCTTGAAGAAATGGCTCTCC-3’ |

[a]Y = C + T; R = A + G; W = A+T; N = any
[b]Corresponding nucleotide position of Norwalk/68 virus (accession no. M87661) of the 5’end.

**Table S3 Primers used by RT-PCR.**

| Target | Primer | | Sense | Sequence (5’-3’)[a] | Location | Annealing |
|---|---|---|---|---|---|---|
| HuNV | HuNV GI-specific | NVp36 | + | ATAAAAGTTGGCATGAACA | 4487-4505[b] | 70℃ |
| | | NVp35 | - | CTTGTTGGTTTGAGGCCATAT | 4936-4956[b] | |
| Neomycin phosphotransferase | Neo-F | | + | ATGGGATCGGCCATTGAAC | | 59℃ |
| | Neo-R | | - | TCAGAAGAACTCGTCAAG | | |
| GAPDH | GAPDH-F | | + | TCGTGGAAGGACTCATGACC | | 68℃ |
| | GAPDH-R | | - | TCCACCACCCTGTTGCT | | |

[a]Y = C + T; M = A + C; D = A+T+G; W = A+T
[b]Nucleotide positions are the positions in Hu/NLV/Norwalk/68/US (accession no. M87661).

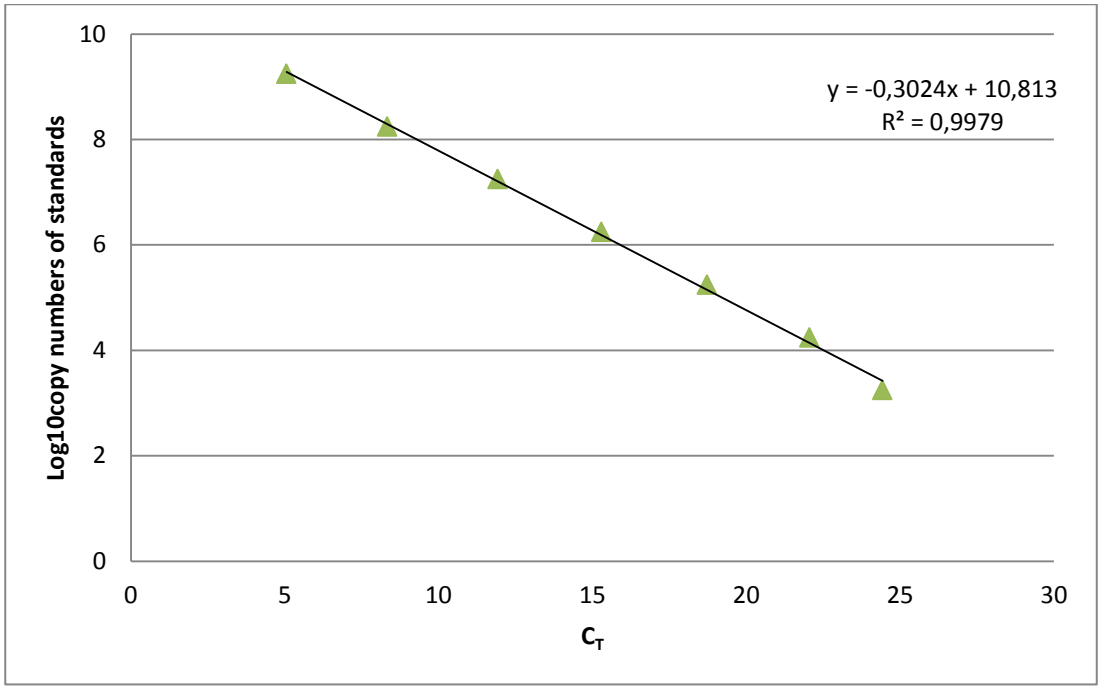



**Fig. S1 Standard curve for quantifying FeCV genome copy numbers.**

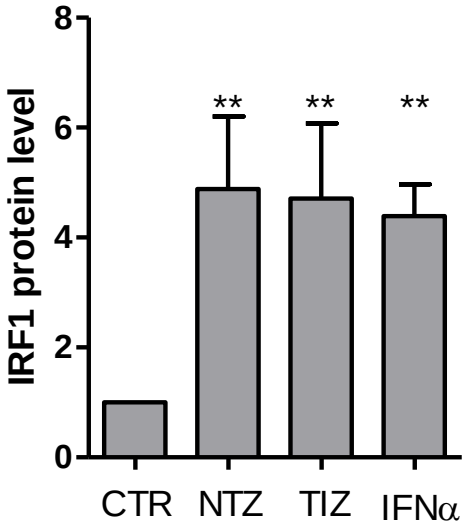



**Fig. S2 Densitometric analysis of Western blot of IRF-1 protein expression after treatment with NTZ (10 μg/mL), TIZ (10 μg/mL), IFNα (1,000 IU/mL) or matched concentration of DMSO as vehicle control (CTR).** The data are plotted as the means ± SEM and statistical comparisons were analyzed by t test ($n$ = 5). $^{**}P < 0.01$.

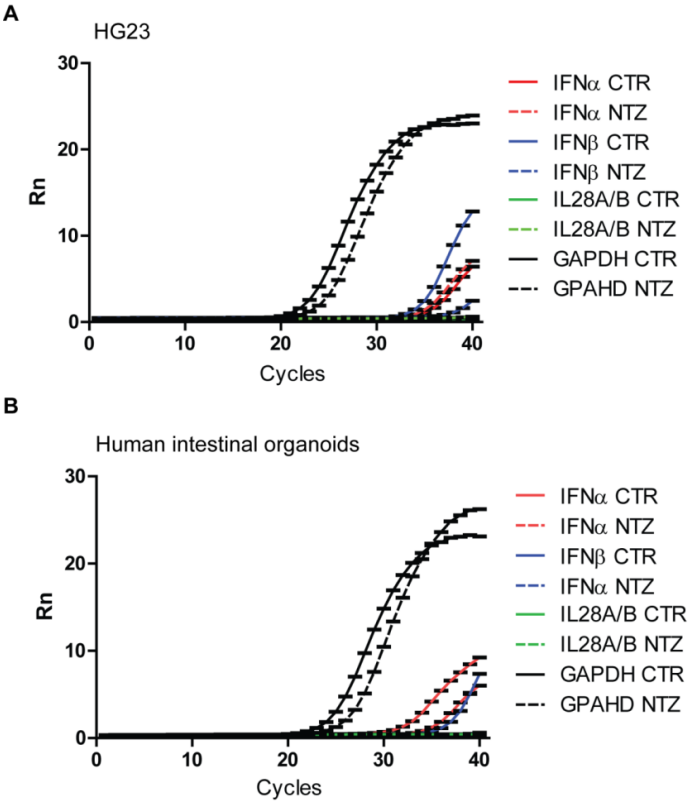



**Fig. S3 NTZ had no major effects on the induction of IFN genes.** The qRT-PCR analysis of IFN genes including IFNα, IFNβ and IL28A/B after NTZ treatment (10 μg/mL for 2 days) in HG23 (A) and human intestinal organoids (B). Data are representative of 3 independent experiments.

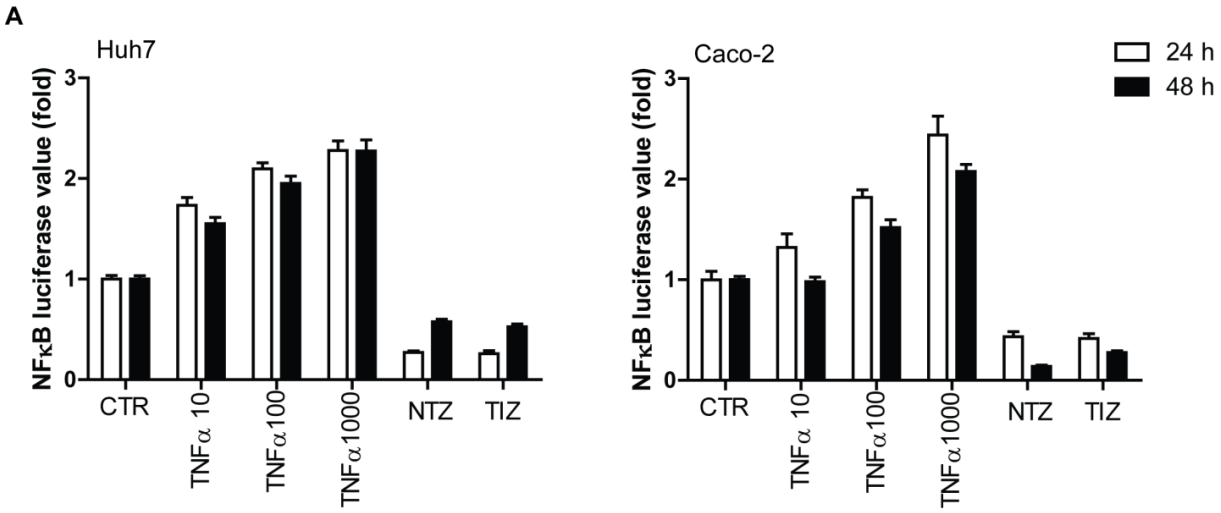



**Fig. S4 Stimulation of ISGs by TZD was independent of NFκB pathway.** Huh7 and Caco-2-based NFκB luciferase reporter cells were mock-treated (DMSO) or stimulated for 24 and 48 hours with tumor necrosis factor-alpha (TNF*α)* (10, 100 and 1,000 ng/mL), NTZ (1 $\mu$g/mL) or TIZ (1 $\mu$g/mL). Luciferase activities were determined and normalized to the DMSO control (*n* = 3 independent experiments with duplicates each). TZD treatment failed to induce NFκB-dependent reporter gene activity.

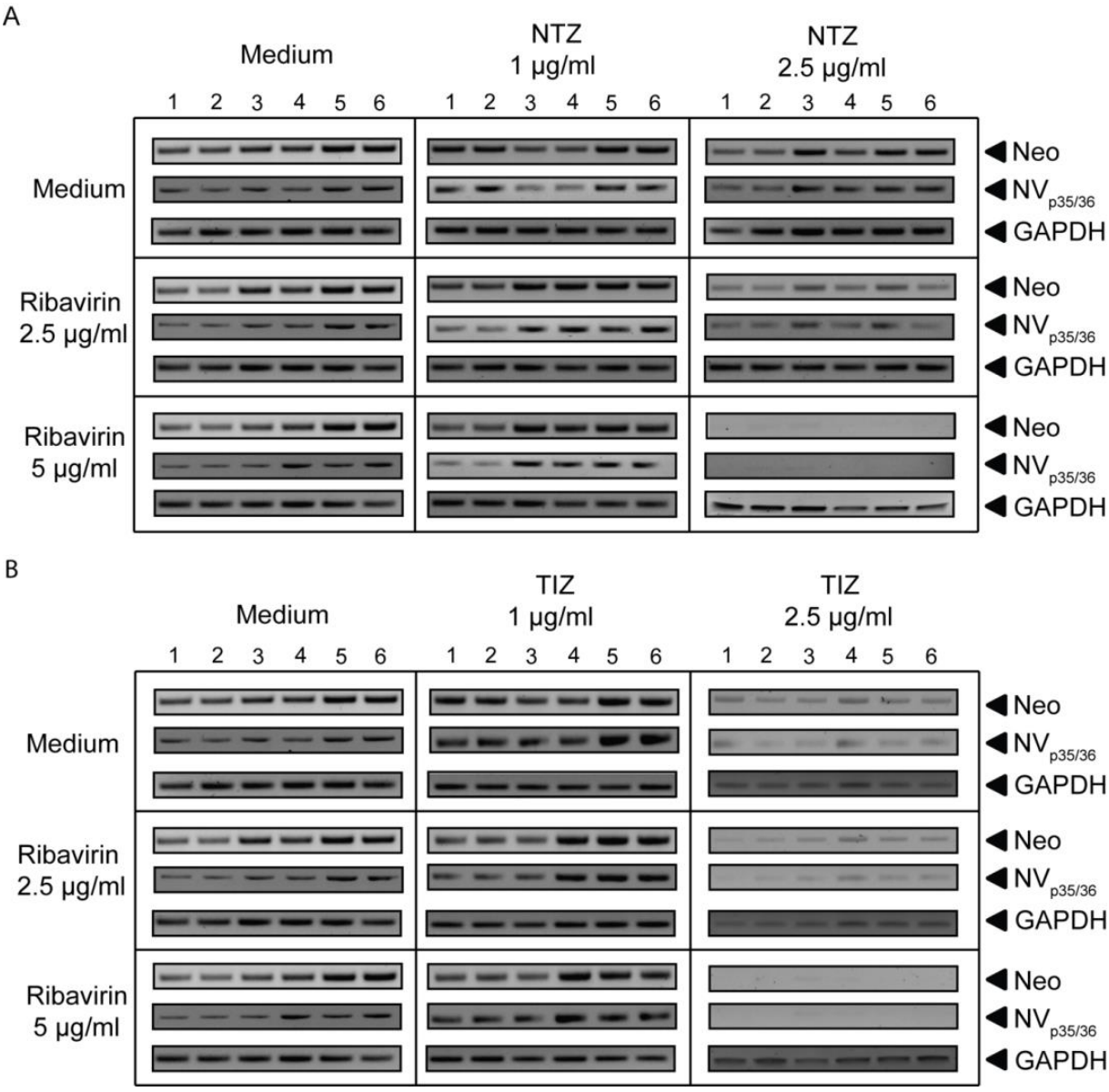



**Fig. S5 Combination of TZD with ribavirin completely depleted HuNV replicons from HG23 cells after prolonged treatment.** After 10 days of treatment, total cellular RNA was isolated from HG23 cells and gel-based reverse transcription polymerase chain reaction (RT-PCR) was performed to detect HuNV replicons with two primer pairs. The Neo-F/R pair was designed to amplify the full length neomycin phosphotransferase (804 base pair [bp]); while the $NV_{p35/36}$ pair specifically detected genogroup I (GI) norovirus and yielded a 470 bp DNA amplicons. Human GAPDH primer set was used as a reference gene. A total of 6 samples were detected and indicated by numbers.

# SUPPLEMENTARY REFERENCES

1. Chang KO, Sosnovtsev SV, Belliot G, King AD, Green KY. 2006. Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line. Virology **353:**463-473.

2. Wang J, Jiang X, Madore HP, Gray J, Desselberger U, Ando T, Seto Y, Oishi I, Lew JF, Green KY, et al. 1994. Sequence diversity of small, round-structured viruses in the Norwalk virus group. J Virol **68:**5982-5990.

3. Li Y, Pong RC, Bergelson JM, Hall MC, Sagalowsky AI, Tseng CP, Wang Z, Hsieh JT. 1999. Loss of adenoviral receptor expression in human bladder cancer cells: a potential impact on the efficacy of gene therapy. Cancer Res **59:**325-330.

# Chapter 7

## Summary and Discussion

Despite being the responsible etiological agent for approximately one fifth of all cases of acute diarrhea, norovirus receives less attention when compared to other infectious pathogens [1]. The inability to cultivate human norovirus (HuNV) largely impedes the growth of our understanding and further study of the biology and pathogenesis of norovirus and the associated diseases. Moreover, a lack of accurate and cheap detection methodology further thwarts diagnosis, leading to lag behind in raising the awareness of the importance and significance of norovirus. The introduction of PCR technology has significantly improved and simplified the detection of norovirus RNA, and this technology is currently considered the 'golden-standard' for norovirus diagnosis [2]. Accumulating evidence has confirmed norovirus as the etiological agent for chronic and severe gastroenteritis in immunocompromised populations. However, regarding the clinical manifestations, epidemiology and potential treatment of norovirus infection in hematopoietic stem cell transplant (HSCT) and solid organ transplant (SOT) recipients, no generally accepted consensus has been reached. In order to address this issue, in **Chapter 2**, I systematically reviewed the burden provoked by and potential therapy for norovirus infection in the transplantation setting. I focused on five aspects, including prevalence and clinical characteristics, diagnosis, risk factors, transmission and evolution, and potential therapies. I hope my review will foster reaching a consensus and raising the awareness of the problem in the field. Furthermore, I hope it shall help expanding our understanding of the burden of norovirus diarrhea in immunocompromised populations and provide some clues for the development of novel antiviral strategies. Finally, it sets the stage for the remainder of this thesis.

The immune system is highly suppressed in transplant recipients due to the intake of immunosuppressants. Thus, these individuals are susceptible to a variety of infections. Recent studies have demonstrated that immunosuppressants can directly affect viral replication [3]. The calcineurin inhibitors cyclosporine A (CSA) and tacrolimus (FK506) stimulated hepatitis E virus (HEV) replication [4], whereas rapamycin and everolimus unexpectedly facilitated HEV replication [5]. With respect to rotavirus, CsA and FK506 did not affect its replication [6]. In contrast, mycophenolic acid (MPA) potently inhibited a variety of virus replication including HEV [4], rotavirus [6], dengue virus [7], hepatitis C virus (HCV) [8] and hepatitis B virus (HBV) [9]. In **Chapter 3**, I screened a subset of immunosuppressants for their potential as anti-noroviral agents and found that MPA exerted strong inhibition of norovirus replication, whereas the calcineurin inhibitors CsA and FK506 moderately decreased HuNV

replication. I further established that ribavirin, a broad-spectrum antiviral agent inhibited norovirus replication and resulted in an additive effect when combined with these norovirus counteracting immunosuppressants. These observations bear important implications for the optimization and management of immunosuppressants in transplant recipients. Meanwhile, ribavirin holds promise for combating norovirus infection, though further *in vivo* mechanistic studies are needed.

Treatment of norovirus infection is still a challenge. Immunocompromised patients with norovirus gastroenteritis are in greatest need of an effective and specific treatment. Several chemicals have been reported to display *in vitro* activities against norovirus replication [10], however the potential drug efficacies have not been clearly defined *in vivo*. Thus, I tried to develop new tricks for old drugs and reincarnate them as anti-norovirus drugs. In **Chapter 4**, I profiled interferons (IFNs) for their anti-norovirus potential. The results demonstrated that HuNV RNA replication was largely inhibited by the application of all types of IFNs, including IFN-*αβ* (type I), IFN*γ* (type II), IL-29 and IL28B (type III), but to a lesser extent when compared to the effects these cytokines have on HCV replication. Interferon-stimulated genes (ISGs) are the ultimate antiviral effectors of the IFN-induced signal transduction cascade. By using an overexpression approach, I profiled a subset of important human ISGs for their action and function as anti-norovirus proteins. Among all the ISGs I investigated, interferon regulatory factor 1 (IRF-1), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) were identified as being the most potent anti-norovirus effectors of the IFN signal transduction cascade. Interestingly, type I and III IFNs coordinately and cooperatively induced these three ISGs, whereas type II IFN predominantly induced IRF-1 to accomplish their antiviral activities. Those findings have great clinical importance, as it provides a rational foundation for developing IFN-based therapies to combat norovirus-associated diseases. Furthermore, exploiting the actions of IRF-1, RIG-I and MDA5 on HuNV may allow future development of ISG-mediated therapeutic strategies that potentially have less side effects as compared to IFN-based therapy. Acitretin, a retinoic acid derivative approved by the United States Food and Drug Administration (FDA) was shown to enhance RIG-I signaling *ex vivo* of HIV-infected cells, contributing to controlling HIV infection [11]. The natural ligand of RIG-I, 5'-triphosphate RNA (5'-pppRNA) was reported to activate RIG-I to stimulate an antiviral response that inhibited HEV replication [12]. Further insight into the biochemical mechanisms of these ISGs may provide a

platform for the development of antivirals acting against norovirus –associated gastroenteritis.

Nitazoxanide is recently discovered to be potentially useful against norovirus diarrhea and has been used in several empirical studies that demonstrates effectiveness toward norovirus gastroenteritis [13, 14]. However, a recent Letter to the Editor described the failure of nitazoxanide treatment in clearing norovirus chronic infection in a patient with X-linked agammaglobulinemia (XLA) [15]. To uncover the reason behind this apparent inconsistency, I explored the *in vitro* effects of nitazoxanide on HuNV and MNV replication (I did this in **Chapter 5**) and I was the first to discover the opposing effects of nitazoxanide on these viruses. I attributed this inconsistency to the genetic differences in the norovirus strains, as HuNV belongs to genogroup II and MNV belongs to genogroup V. Meanwhile, different host cell cultures may alternatively respond to nitazoxanide treatment, which potentially contributed to the opposing activity of nitazoxanide observed. In **Chapter 6**, I continued to investigate the antiviral mechanism of nitazoxanide. I established that nitazoxanide profoundly and robustly triggered a host immune response and induced the production of a subset of ISGs, particularly IRF-1. I further established that nitazoxanide acted in synergy with ribavirin, which thus potentiated nitazoxanide monotherapy as well as combination therapy with ribavirin as promising strategies for combating norovirus infections in immunocompromised patients as well as in the setting of acute outbreaks. Thus, in conjunction I hope my studies open new rational avenues for combating norovirus-associated disease.

# REFERENCES


1. Bartsch SM, Lopman BA, Ozawa S et al. Global Economic Burden of Norovirus Gastroenteritis. *PLoS One* 2016; **11**: e0151219.
2. Scipioni A, Bourgot I, Mauroy A et al. Detection and quantification of human and bovine noroviruses by a TaqMan RT-PCR assay with a control for inhibition. *Mol Cell Probes* 2008; **22**: 215-22.
3. Pan Q, Tilanus HW, Metselaar HJ et al. Virus-drug interactions--molecular insight into immunosuppression and HCV. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 355-62.
4. Wang Y, Zhou X, Debing Y et al. Calcineurin inhibitors stimulate and mycophenolic acid inhibits replication of hepatitis E virus. *Gastroenterology* 2014; **146**: 1775-83.
5. Zhou X, Wang Y, Metselaar HJ et al. Rapamycin and everolimus facilitate hepatitis E virus replication: revealing a basal defense mechanism of PI3K-PKB-mTOR pathway. *J Hepatol* 2014; **61**: 746-54.
6. Yin Y, Wang Y, Dang W et al. Mycophenolic acid potently inhibits rotavirus infection with a high barrier to resistance development. *Antiviral Res* 2016; **133**: 41-9.
7. Takhampunya R, Ubol S, Houng HS et al. Inhibition of dengue virus replication by mycophenolic acid and ribavirin. *J Gen Virol* 2006; **87**: 1947-52.
8. Henry SD, Metselaar HJ, Lonsdale RC et al. Mycophenolic acid inhibits hepatitis C virus replication and acts in synergy with cyclosporin A and interferon-alpha. *Gastroenterology* 2006; **131**: 1452-62.
9. Gong ZJ, De Meyer S, Clarysse C et al. Mycophenolic acid, an immunosuppressive agent, inhibits HBV replication in vitro. *J Viral Hepat* 1999; **6**: 229-36.
10. Kaufman SS, Green KY, Korba BE. Treatment of norovirus infections: moving antivirals from the bench to the bedside. *Antiviral Res* 2014; **105**: 80-91.
11. Li P, Kaiser P, Lampiris HW et al. Stimulating the RIG-I pathway to kill cells in the latent HIV reservoir following viral reactivation. *Nat Med* 2016; **22**: 807-11.
12. Xu L, Wang W, Li Y et al. RIG-I is a key antiviral interferon-stimulated gene against hepatitis E virus regardless of interferon production. *Hepatology* 2017; **65**: 1823-39.
13. Rossignol JF, El-Gohary YM. Nitazoxanide in the treatment of viral gastroenteritis: a randomized double-blind placebo-controlled clinical trial. *Aliment Pharmacol Ther* 2006; **24**: 1423-30.
14. Siddiq DM, Koo HL, Adachi JA et al. Norovirus gastroenteritis successfully treated with nitazoxanide. *J Infect* 2011; **63**: 394-7.
15. Kempf B, Edgar JD, Mc Caughey C et al. Nitazoxanide Is an Ineffective Treatment of Chronic Norovirus in Patients With X-Linked Agammaglobulinemia and May Yield False-Negative Polymerase Chain Reaction Findings in Stool Specimens. *J Infect Dis* 2017; **215**: 486-7.

# Chapter 8

## Nederlandse Samenvatting

## Dutch Summary

Norovirus is de verzamelnaam van een groep van virussen welke een belangrijke oorzaak vormen van diarree. Er wordt aangenomen dat mondiaal de helft van alle acute buikgriepepisoden (acute gastro-enteritis) door norovirussen worden veroorzaakt. Het virus veroorzaakt regelmatig epidemieën van een relatief kleine schaal voor. Het norovirus is uiterst besmettelijk en kan mensen treffen van eigenlijk iedere leeftijd. Norovirus wordt overgedragen via water en voedsel dat met ontlasting besmet is, maar ook via een direct contact met besmette personen. Tenslotte verspreidt het virus zich ook door aerosolvorming waarna het virus verschillende oppervlakten kan besmetten (bijvoorbeeld de deurkruk of het lichtknopje van het toilet zijn berucht in dit opzicht). In dit proefschrift wil ik een bijdrage leveren aan de strijd van de mensheid tegen het norovirus.

Probleem bij onderzoek naar norovirus is dat het niet gekweekt kan worden in cellijnen en dat er eigenlijk geen goede/doelmatige proefdiermodellen zijn. Recentelijk is de situatie wat verbetert door het beschikbaar komen van zogenaamde repliconmodellen die in ieder geval een gedeelte van de levenscyclus van norovirus kunnen ondersteunen alsook door de beschikbaar komen van organoïden. Deze nieuwe modellen openen een venster op beter onderzoek waarvan ik in dit proefschrift gebruik heb gemaakt. In **hoofdstuk 1** geef in een uitgebreide rechtvaardiging van mijn onderzoek en leg ik aan de lezer uit waarom ik voor de uitgevoerde strategie met betrekking tot deze dissertatie gekozen heb.

Maar in eerste instantie begon ik met een onderzoek om uit te zoeken hoe groot het probleem van norovirus infectie is en dan met name in de transplantatiegeneeeskunde. Dit doe ik in **hoofdstuk 2**. Hierin verzamel ik op systematische wijze alle primaire studies die in de literatuur verschenen zijn en ik stel vast dat transplantatiepatiënten uiterst gevoelig zijn voor norovirus, dat als zij besmet zijn ernstig ziek worden en ook heel lang ziek zijn omdat ze het virus niet klaren. Ook zijn ze dat zeer besmettelijk en zowel medepatiënten alsook de medische staf kunnen gemakkelijk besmet worden. Er zijn dan ook zeer uitgebreide hygiënische voorzorgsmaatregelen nodig. Ook stel ik vast dat er nog geen goede medicatie is om deze patiënten te behandelen. Verminderen van de immunosuppressie (riskant vanwege orgaanafstoting) en intraveneus immunoglobuline kunnen wat helpen. Interessant is dat nitazoxadine (een antiparasitair middel) lijkt helpen. Echter data is beperkt en in de afwezigheid van een rationele verklaring hoe dit middel zou kunnen helpen bij norovirus-geïnfecteerde patiënten moet men voorzichtig blijven.

Het zou echter ook kunnen dat de juiste keuze van immunosuppressie al veel kan betekenen met betrekking tot preventie van norovirusinfectie. Patiënten met orgaantransplantatie moeten sowieso middelen krijgen die afstoting van het getransplanteerde orgaan verkomen. Dergelijke middelen grijpen vaak diep in op de cellulaire fysiologie. Omdat de virale levenscyclus van het norovirus complex is (zie ook **hoofdstuk 1**) is het wel mogelijk dat sommige afstotingvoorkomende medicatie ook interfereert met de levenscyclus van het norovirus. Ik onderzoek deze mogelijkheid in **hoofdstuk 3**. In dit hoofdstuk onderzoek de invloed van een aantal immunosuppressiva die gebruikt worden om afstoting te voorkomen in een repliconmodel. Inderdaad bleken sommige medicijnen interactie te vertonen met de levenscyclus van het norovirus. Het meest interessant was wellicht Mycofenolzuur, de werkzame stof van de geneesmiddelen Mycofenolaatmofetil (MMF, CellCept van Roche) en Mycofenolzuur natrium (EC-MPS, entric coated mycophenolic acid sodium, Myfortic van Novartis. Deze stof remt het enzym IMPDH, waardoor het afweersysteem verzwakt en in de combinatie met andere immunosuppresiva is het profylactisch met betrekking tot de afstoting van transplantie-organen. Gebruik van dit middel lijkt dan ook aangewezen voor patiënten die een hoog risico lopen op besmetting met norovirus of reeds een infectie hebben opgelopen. Ik heb deze resultaten gepubliceerd: Dang W, Yin Y, Wang Y, Wang W, Su J, Sprengers D, van der Laan LJW, Felczak K, Pankiewicz KW, Chang KO, Koopmans MPG, Metselaar HJ, Peppelenbosch MP, Pan Q. Inhibition of Calcineurin or IMP Dehydrogenase Exerts Moderate to Potent Antiviral Activity against Norovirus Replication. *Antimicrob Agents Chemother*. 2017 Oct 24;61(11). pii: e01095-17. doi: 10.1128/AAC.01095-17. *Antimicrobial Agents and Chemotherapy* is een vooraanstaand tijdschrift uitgegeven door de *American Society for Microbiology* en ik heb dus goede hoop dat ik met deze publicatie de relevante doelgroep kan bereiken.

In een volgend **hoofdstuk 4** onderzoek ik de interactie van norovirus met interferonen. Interferonen zijn glycoproteïnen die de cellen van het immuunsysteem afscheiden na contact met virussen. De naam interferon komt van de observatie dat interferonen interfereren met de virale replicatie. Interferonen zijn onder andere werkzaam bij chronische hepatitis B en hepatitis C, maar de werking van interferonen op de afweer tegen norovirus was nog niet onderzocht. Ik stel vast in dit hoofdstuk dat alle geteste interferonen norovirusreplicatie kunnen onderdrukken en ik karakteriseer de betrokken moleculaire mechanismen in enig detail. Ik heb deze resultaten gepubliceerd: Dang W, Xu L,

Yin Y, Chen S, Wang W, Hakim MS, Chang KO, Peppelenbosch MP, Pan Q. IRF-1, RIG-I and MDA5 display potent antiviral activities against norovirus coordinately induced by different types of interferons. *Antiviral Res*. 2018 May 10;155:48-59. doi: 10.1016/j.antiviral.2018.05.004. *Antiviral Research* is een vooraanstaand tijdschrift en het officiële tijdschrift van de *International Society for Antiviral Research* en ik heb dus goede hoop dat ik met deze publicatie de relevante doelgroep kan bereiken.

Zoals gezegd zijn er aanwijzingen dat nitazoxadine een goede optie is bij de behandeling van norovirus infectie, maar is het bewijs anekdotisch van aard. In **hoofdstuk 5** probeer ik het gebruik van deze medicatie voor dit doel te rationaliseren. Ik laat zien dat het middel direct aangrijpt op de levenscyclus van het humaan norovirus maar niet op het muis norovirus, waar het zelfs virusreplicatie lijkt te stimuleren (het muis norovirus is vroeger veel gebruikt voor preklinisch onderzoek in proefdieren, maar mijn resultaten laten zien dat dit wellicht een ongelukkige keuze is geweest). Deze bevindingen dragen bij aan het groeiende momentum dat gebruik van nitazoxadine voor de behandeling van norovirusinfectie nu ondergaat. Ik heb deze resultaten gepubliceerd: Dang W, Yin Y, Peppelenbosch MP, Pan Q. Opposing Effects of Nitazoxanide on Murine and Human Norovirus. *J Infect Dis*. 2017 Sep 15;216(6):780-782. *The Journal of Infectious Diseases* is een vooraanstaand tijdschrift en het officiële tijdschrift van *de Infectious Diseases Society of America* en ik heb dus goede hoop dat ik met deze publicatie de relevante doelgroep kan bereiken.

In **Hoofdstuk 6** bouw ik deze bevindingen verder uit en karakteriseer ik het mechanisme van werking van nitazoxadine in detail, waarmee ik de geschiktheid van deze medicatie voor de behandeling van transplantatiepatiënten die lijden aan een nororvirusinfectie verder objectiveer. Een belangrijk punt is dat de potentie van dit middel nog beter wordt als het gecombineerd wordt met ribavirine. Ribavirine is een antiviraal geneesmiddel dat de werking van alpha-interferon versterkt en al gebruikt wordt voor de behandeling van hepatitis C. Ik laat nu zien dat het middel ook effectief is tegen norovirus en met de nu ontdekte synergie met nitazoxadine lijkt een geheel nieuw venster of effectieve behandeling van norovirusinfectie bij transplantatiepatiënten te zijn geopend. Deze resultaten zijn buiten het bestek van dit proefschrift nog niet gepubliceerd maar wel goed ontvangen door *Antimicrobial Agents and Chemotherapy* waar ik snel een versie naar toe wil sturen die acceptabel voor dit vooraanstaande tijdschrift zou moeten zijn.

In **hoofdstuk 7**, tenslotte vat ik de resultaten uit dit proefschrift nog eens samen en bediscussieer ik hun samenhang, ook in de contekst van de contemporaine biomedische literatuur. In haar totaliteit hoop ik met dit proefschrift een wezenlijke bijdrage te hebben geleverd in het gevecht der mensheid met de gesel die het norovirus op de mensheid doet nederdalen.

# Appendix

**Acknowledgements**

**Publications**

**PhD Portfolio**

**Curriculum Vitae**


## Acknowledgement

## 致谢

To my dear colleagues. I am highly appreciative of your companion and assistance.
It is time to say goodbye, but I hope our friendship never dies.
I love you more than I did before.

To Prof. dr. Maikel P. Peppelenbosch. You are knowledgeable and open-minded.
Thank you for your support and supervision.
I sincerely invite you and your families to visit China.

To Dr Pan. Thank you for everything you've done for me.

To Prof. dr. H.J. Metselaar, Prof. dr. C.A.B. Boucher and Prof. dr. E. Claassen.
Thank you very much for your critical revision of my thesis.
Your constructive comments largely improved my work.

To Prof. Haixue Zheng and Prof. Hong Tian.
Thank you for serving me with delicious hotpot and chinese wine.
You are the leading scientists in the field of FMDV.
I really enjoyed the scientific discussion with you and all your lab members.

I would like also give my great thanks to the financial support from the China Scholarship Council.

## To Prof. Rui Wang and Prof. Kairong Wang, Lanzhou University
## 致兰州大学王锐院士和王凯荣教授

### 卜算子•咏梅
*Ode to the Plum Blossom*

——毛泽东
*Mao Zedong*

| | |
|---|---|
| 风雨送春归 | Wind and rain escorted Spring's departure, |
| 飞雪迎春到 | Flying snow welcomes Spring's return. |
| 已是悬崖百丈冰 | On the ice-clad rock rising high and sheer |
| 犹有花枝俏 | A flower blooms sweet and fair. |
| 俏也不争春 | Sweet and fair, she craves not Spring for herself alone, |
| 只把春来报 | To be the harbinger of Spring she is content. |
| 待到山花烂漫时 | When the mountain flowers are in full bloom |
| 她在丛中笑 | She will smile mingling in the midst. |

# To My Friends in Holland
# 致我在荷兰的朋友

## 送杜少府之任蜀州
## Bidding Deputy Magistrate Du Farewell

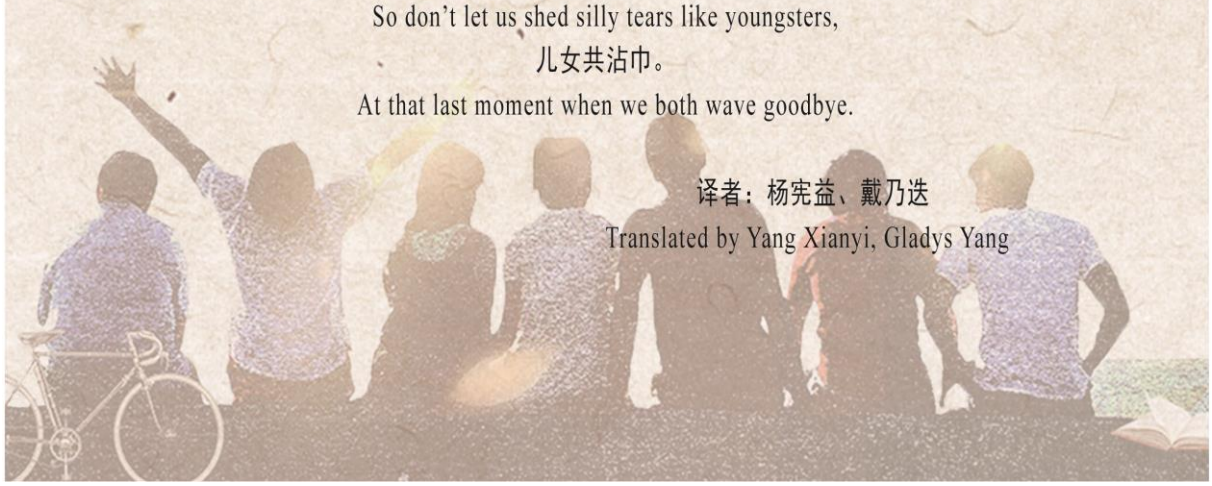
——王勃

Wang Bo

城阙辅三秦，
The capital and place are guarded by the land of three Qin kingdoms,
风烟望五津。
In the distance the Five Ferries are screened by wind and mist.
与君离别意，
Now comes the time for us to bid farewell to each other,
同是宦游人。
And we will both be officials away from home on duty.
海内存知己，
So long as we remain bosom friends in our heart of hearts,
天涯若比邻。
We'll still feel like neighbors despite the distance apart.
无为在歧路，
So don't let us shed silly tears like youngsters,
儿女共沾巾。
At that last moment when we both wave goodbye.

译者：杨宪益、戴乃迭

Translated by Yang Xianyi, Gladys Yang



## To Meng and Sunrui, My Dear Paranimf
## 致我亲爱的伴郎伴娘

### 爱莲说
### The Language of Flowers

——周敦颐
Zhou Dunyi

出淤泥而不染，　　How stainless it rises from its slimy bed,
濯清涟而不妖。　　How modestly it responds on the clear pool.

Translated by Herbert A. Giles

### 红楼梦
### Dream of the Red Chamber

——曹雪芹
Cao Xueqin

气质美如兰，　　By nature fair as an orchid,
才华馥必仙。　　With talents to match an immortal.

## To My Love
## 致我的最爱

Hey, you
We've been playing hide and seek for almost 30 years
Now game is over and I finally have everything of you
What a lucky guy I am

I've got the words to pour my praise on you
But I am bitting my tongue and stuck in silence
You see everything through my eyes
No language describes how I feel when you let me in
It is warm like sunlight and pure like snow
Your love shines my way into future
So I offer my life as a sacrifice to you

Can't wait going back to Lanzhou
Every tik tok is a torture
Future is full of challenge
But who cares if love is always there

# To My Beloved Father
# 致我敬爱的父亲

谢谢爹爹

从我开始记事起， 我们一直在相互对立
高中的时候，我的叛逆达到了极点
争吵，冷战，相互看不顺眼
那是一段艰难的日子，也是我成长最快的岁月

现在的我们开始慢慢和解
可能因为您老了，对我更加宽容
也可能因为我慢慢长大了，对您更加理解
每次回家，父子俩喝一场酒是必不可少的家庭仪式
只有喝醉的时候，我们才会向对方倾诉各自的易与不易
您说不用在意那些父慈子孝的虚名
但是我能感受到您为了我而做的改变

我很佩服您的为人之道
大度，平和，一切的如意和不如意都能看得淡
我在努力继承您的这些大智慧
祝福爹爹健康长寿
下一个三十年，下下个三十年
儿子陪您一醉方休

# To My Great mother
# 致我最伟大的母亲

谢谢妈妈

三十载春秋，很幸运有您一直陪我左右，庇我风雨
女子本弱，为母则强
您是一位伟大，坚强，不服输和有骨气的母亲
这些品质一直影响着我

四年的异国生活，我能感受到您对我时时刻刻的牵挂和思念
每次打电话，您都是兴高采烈的
但从不跟我诉说家里艰难的时刻
我很感激这份母子亲情
在我处于低谷时能从您那里找到慰藉和动力
而当我有所成就时您也不会吝啬对我的肯定和嘉奖

祝福妈妈健康长寿
下一个三十年，下下个三十年
妈妈在，家就在
有您的支持，我不惧去闯荡任何的不可能
因为在您面前
我永远是有妈妈爱的孩子

## To My Future Child
## 致我未来的孩子

My dear baby, how are you?
I am sorry for delaying your presence in the world
Life is not easy, I need more time to fully prepare myself before embracing you
So just take it easy and wait with patience

Life is a circle
I will hear your first cry and you will witness my last breath
In between, we are connected by blood and bound by love
You will never imagine the countless happiness and hope you bring to me
This is the best gift

I hope you are born as a rule-breaker with a brave heart
You can defy all the rules as long as you are the rule-maker in your world
Pave your own path and dominate your own universe
Never let the stupid and mediocre consensus shape you as an obedient puppy
Be a tiger and show your teeth

Now I am ready for welcoming you
I call you
I feel you
I dream of you
I wait for you
Are you ready for introducing yourself to me?

# Publication list

1. **Dang W**, Yin Y, Peppelenbosch MP et al. Opposing Effects of Nitazoxanide on Murine and Human Norovirus. ***J Infect Dis*** 2017; **216**: 780-2.

2. **Dang W**, Yin Y, Wang Y et al. Inhibition of Calcineurin or IMP Dehydrogenase Exerts Moderate to Potent Antiviral Activity against Norovirus Replication. ***Antimicrob Agents Chemother*** 2017; **61**.

3. **Dang W**, Xu L, Ma B et al. Nitazoxanide Inhibits Human Norovirus Replication and Synergizes with Ribavirin by Activation of Cellular Antiviral Response. ***Antimicrob Agents Chemother*** (***in press***)**.**

4. **Dang W**, Xu L, Yin Y et al. IRF-1, RIG-I and MDA5 display potent antiviral activities against norovirus coordinately induced by different types of interferons. ***Antiviral Res*** 2018; **155**: 48-59.

5. **Dang W**, Yu P, Mohamad H et al. Norovirus Infection in Hematopoietic Stem Cell and Solid Organ Transplant Recipients: a Systematic Review. **Under submission.**

6. Yin Y, **Dang W**, Zhou X et al. PI3K-Akt-mTOR axis sustains rotavirus infection via the 4E-BP1 mediated autophagy pathway and represents an antiviral target. ***Virulence*** 2018; **9**: 83-98.

7. Yin Y, Bijvelds M, **Dang W** et al. Modeling rotavirus infection and antiviral therapy using primary intestinal organoids. ***Antiviral Res*** 2015; **123**: 120-31.

8. Yin Y, Wang Y, **Dang W** et al. Mycophenolic acid potently inhibits rotavirus infection with a high barrier to resistance development. ***Antiviral Res*** 2016; **133**: 41-9.

9. Wang K, **Dang W**, Yan J et al. Membrane perturbation action mode and structure-activity relationships of Protonectin, a novel antimicrobial peptide from the venom of the neotropical social wasp Agelaia pallipes pallipes. ***Antimicrob Agents Chemother*** 2013; **57**: 4632-9.

10. Yan J, Wang K, **Dang W** et al. Two hits are better than one: membrane-active and DNA binding-related double-action mechanism of NK-18, a novel antimicrobial peptide derived from mammalian NK-lysin. ***Antimicrob Agents Chemother*** 2013; **57**: 220-8.

11. Wang K, Yan J, **Dang W** et al. Dual antifungal properties of cationic antimicrobial peptides polybia-MPI: membrane integrity disruption and inhibition of biofilm formation. ***Peptides*** 2014; **56**: 22-9.

12. Wang K, Yan J, **Dang W** et al. Membrane active antimicrobial activity and molecular dynamics study of a novel cationic antimicrobial peptide polybia-MPI, from the venom of Polybia paulista. ***Peptides*** 2013; **39**: 80-8.

13. Wang K, **Dang W**, Xie J et al. Antimicrobial peptide protonectin disturbs the membrane integrity and induces ROS production in yeast cells. ***Biochim Biophys Acta*** 2015; **1848**: 2365-73.

14. Wang K, Jia F, **Dang W** et al. Antifungal effect and action mechanism of antimicrobial peptide polybia-CP. ***J Pept Sci*** 2016; **22**: 28-35.

# PhD Portfolio

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|---|---|
| **Name of PhD student** | Wen Dang |
| **Department** | Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam |
| **PhD Period** | September 2014 – September 2018 |
| **Promotor** | Prof. dr. Maikel P. Peppelenbosch |
| **Copromotor** | Dr. Qiuwei Pan |

## PhD training

### Seminars

- 2014-2018, Weekly MDL seminar program in experimental gastroenterology and hepatology (attending); (42 weeks/year; @1.5 h) (ECTS, 9.0).
- 2014-2018, Weekly MDL seminar program in experimental gastroenterology and hepatology (presenting); (preparation time 16 h; 2 times/year) (ECTS, 4.6).
- 2014-2018, Biweekly research group education (attending); (20 times/year; @1.5 h) (ECTS, 4.3).
- 2014-2018, Biweekly research group education (presenting); (preparation time 8 h; 4 times/year) (ECTS, 4.6).

### General Courses and Workshops

- 2015, Course in Virology 2015 (ECTS, 1.8).
- 2018, Biomedical English Writing Course for MSc and PhD-students (ECTS, 2.0).
- 2018, The annual Course on Molecular Medicine (ECTS, 0.6).
- 2018, the Workshop on Microsoft Excel 2010: Basic/Advanced (ECTS, 0.6).
- 2018, The Erasmus MC Cancer Institute Research Day (ECTS, 0.3).
- 2018, The Course Scientific Integrity.

## National and International Conferences

- 2016, Annual Day of the Molecular Medicine, Postgraduate School Molecular Medicine, Rotterdam, the Netherlands (Poster presentation).
- 2016, 9th Dutch Experimental Gastroenterology and Hepatology (DEGH) meeting, Veldhoven, The Netherlands (Poster presentation).
- 2018, Virus 2018-Breakthrough in Viral Replication, Barcelona, Spain (Poster presentation).
- 2018, 31st International Conference on Antiviral Research (ISAR, International Society For Antiviral Research), Porto, Portugal (Poster presentation).

## Academic Awards

- 2018, 31st International Conference on Antiviral Research (ISAR, International Society For Antiviral Research), Porto, Portugal ($ 400,00).
- 2014, China Scholarship Council (CSC) Scholarship (File No. 201406180072)
- 2014, National Scholarship for Graduate Students (¥ 20,000).

# Curriculum Vitae

Wen Dang was born on February 8th 1989 in Shandan, Zhangye, Gansu province, China. He was raised by his beloved parents Zhenguo Dang and Meiying Sun and finished his primary, secondary and high school in his hometown.

In 2007, he started his undergraduate study of life sciences and technology at the School of Life Sciences in Lanzhou University, Lanzhou, China (4 years). In 2011, he initiated his master research in the same university at the School of Basic Medical Sciences under the supervision of Prof. Rui Wang. His main focus was to discovery and synthesize novel antimicrobial peptides (AMPs) as novel agents to combat emerging resistant bacterial and fungal infections.

In 2014, with the support of China Scholarship Council (CSC) scholarship, he started his PhD research at the Department of Gastroenterology and Hepatology, Erasmus University Medical Center, Rotterdam, the Netherlands. Under the supervision of Prof. Maikel P. Peppelenbosch and Dr. Qiuwei Pan, his main research was to study norovirus-host interactions and to develop antivirals against norovirus infection.