Spring 5-1-2019

Exploring Alternative Antiviral Therapeutic Strategies Through Immunomodulation and Learning Novel Innate Immune Responses Against Rotavirus Infection

Zhan Zhang

Follow this and additional works at: https://scholarworks.gsu.edu/biology_diss

Recommended Citation
doi: https://doi.org/10.57709/13419256

This Dissertation is brought to you for free and open access by the Department of Biology at ScholarWorks @ Georgia State University. It has been accepted for inclusion in Biology Dissertations by an authorized administrator of ScholarWorks @ Georgia State University. For more information, please contact scholarworks@gsu.edu.
EXPLORING ALTERNATIVE ANTIVIRAL THERAPEUTIC STRATEGIES THROUGH IMMUNOMODULATION AND LEARNING NOVEL INNATE IMMUNE RESPONSES AGAINST ROTAVIRUS INFECTION

by

ZHAN ZHANG

Under the Direction of Andrew T. Gewirtz, PhD

ABSTRACT

Rotavirus infections are associated with outbreaks of acute gastroenteritis. Non-dividing highly differentiated enterocytes, located at the tips of small intestinal villi, are the primary targets of rotavirus infection. Infants or young children infected with Rotavirus may develop severe diarrhea, become dehydrated and require hospitalization. Despite the broad uses of rotavirus vaccines, rotavirus infections are still one of the leading causes of death among infants and young children nowadays.

Host initiates its innate immune responses through pattern recognition receptors (PPRs) after detects rotavirus invasion. Interferons (IFNs) are considered to be one of the most essential
mediators, and serve as the first frontline defense against viral infections. Our lab activated the innate immunity through a bacterial-derived activator, flagellin, and in turn, the immediate immune responses were able to prevent and eliminate rotavirus infection. Interestingly, flagellin-mediated antiviral protection was neither dependent on interferon signaling nor adaptive immunity but rather elicited by secretion of interleukin (IL)-22 and IL-18, through activation of Toll-like receptor 5 (TLR5) and NOD-like receptor C4 (NLRC4) signaling, respectively. IL-22 promoted overall turnover (proliferation, migration and extrusion) rate of the intestinal epithelial cells (IEC), therefore, the extrusion process expelled the highly differentiated IEC that would preferentially succumb to rotavirus invasion. Meanwhile, IL-18 induced the programmed cell death among cells were infected by rotavirus, thus directly ceased the RV replication cycle. Understanding this novel antiviral mechanism will be essential for the exploration of prophylactic and therapeutic immunomodulatory strategy to substantiate existing medical intervention towards the treatment of viral infectious disease.

INDEX WORDS: Rotavirus, Toll-like receptor 5 (TLR5), NOD-like receptor C4 (NLRC4), Flagellin, Interleukin 22 (IL-22), Interleukin 18 (IL-18).
EXPLORING ALTERNATIVE ANTIVIRAL THERAPEUTIC STRATEGIES THROUGH IMMUNOMODULATION AND LEARNING NOVEL INNATE IMMUNE RESPONSES AGAINST ROTAVIRUS INFECTION

by

ZHAN ZHANG

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the College of Arts and Sciences Georgia State University

2019
EXPLORING ALTERNATIVE ANTIVIRAL THERAPEUTIC STRATEGIES THROUGH IMMUNOMODULATION AND LEARNING NOVEL INNATE IMMUNE RESPONSES AGAINST ROTAVIRUS INFECTION

by

ZHAN ZHANG

Committee Chair: Andrew, Gewirtz

Committee: Richard Plemper
            Timothy Denning
            Sang-Moo Kang
            Jun Zou

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
May 2019
DEDICATION

This dissertation is dedicated to my parents, Lu Zhan and Jiemin Zhang, for their unconditional love and support. Dedication also goes to my family, Pu Zhan, Minwan Wu, Amy Wu, and Sherry Wu, who have supported me to reach my goal.
ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and gratitude to my advisor, Professor Andrew Gewirtz, for his guidance and mentorship. His enthusiasm toward scientific research greatly affected me. Without his support, this dissertation would not be possible.

Many thanks to my committee members Professor Timothy Denning, Professor Sang-Moo Kang, Professor Richard Plemper, and Professor Jun Zou, for their guidance and suggestions over the years.

I would also like to thank all my colleagues and friends I met here in Georgia State University, Lucie Etienne-Mesmin, Hao Tran, Benoit Chassaing, Emilie Viennois, Bo Xiao, Ye Wang, Alexis Bretin, Zhenda, Shi, Benyue Zhang, Mingzhen Zhang, Youri Lee, Yu-Jin Jung, Samantha Spencer, Anuhya Konduru, Yin-Yin Wang, Jin Zhang, Jennifer Miles, Sijia Tang, without their support, and encouragement I wouldn’t make this far.
# TABLE OF CONTENT

ACKNOWLEDGEMENTS .................................................................................................................. V

1 INTRODUCTION ....................................................................................................................... 8

2 EXPERIMENT ........................................................................................................................... 14

2.1 MATERIALS AND METHODS FOR SPECIFIC AIM I ..................................................... 14

2.2 MATERIALS AND METHODS FOR SPECIFIC AIM II .................................................. 22

3 RESULTS OF SPECIFIC AIM I: PREVENTION AND CURE OF ROTAVIRUS INFECTION VIA TLR5/NLRC4–MEDIATED PRODUCTION OF IL-22 AND IL-18 ........................................................................................................... 29

3.1 ABSTRACT ............................................................................................................................. 29

3.2 INTRODUCTION ..................................................................................................................... 29

3.3 RESULTS ................................................................................................................................ 30

3.3.1 FLAGELLIN PROTECTS MICE FROM RV INFECTION AND DIARRHEA 30

3.3.2 FLAGELLIN’S ANTIVIRAL ACTIVITY IS MEDIATED BY TLR5/NLRC4 ON DENDRITIC CELLS ................................................................................................................................. 31

3.3.3 FLAGELLIN MEDIATED PROTECTION AGAINST RV INFECTION REQUIRES BOTH IL-22 AND IL-18 .................................................................................................................. 34

3.3.4 IL-22/IL-18 TREATMENT PREVENTS AND TREATS RV INFECTION ................................................................................................................ 34

3.4 DISCUSSION ............................................................................................................................ 36

4 RESULTS OF SPECIFIC AIM II: IL-22 INDUCED CELL EXTRUSION AND IL-18-INDUCED PYROPTOSIS PREVENT AND CURE ROTAVIRUS INFECTION ......................................................................................................................... 37
1 INTRODUCTION

Rotavirus infections are associated with one of the leading outbreaks of acute gastroenteritis, causing severe diarrhea and dehydration among children <5 years old. In the last decade, the introduction of vaccines (RotaTeq and Rotarix) has reduced the burden of rotavirus disease globally, however, the effectiveness of the vaccines appears to be 30-40% lower in low-income countries than in high-income countries, and still results in >200,000 deaths annually (1). Studies have showed that intestinal microbiota composition (2, 3), oral administration of polio vaccine (4), or maternal antibodies (5, 6) that are possible implications, which are correlated with the diminished efficacy and immunogenicity of rotavirus vaccines. Importantly, all the licensed rotavirus vaccines are live attenuated viruses, which remain their ability to self-replicate inside of the vaccinated children’s bodies. Some studies have discovered that children, who were diagnosed with gastroenteritis, were also infected with virus which contained atypical genome constellation derived from reassortment of typical human rotavirus strain with RotaTeq vaccine virus (7, 8). Therefore, the development of a safer replication-incompetent and a more efficacious rotavirus vaccine with improved immunogenicity are urgent. In order to optimize the effectiveness of rotavirus treatment and vaccines, investigating and understanding of host-enteric virus interactions in terms of basic virology is inevitable.

In the past 40 years, there is an extensive exploration of research focusing on rotavirus structure, replication cycle, and pathogenesis. Studies found that host immune systems aim at virion attachment, transcription, translation, genome synthesis, maturation, and, last but not least, virion release in order to prevent rotavirus multiplication, and cell-to-cell spread. Cytoplasmic viral RNA can be recognized by pattern recognition receptors (PRRs), such as RIG-I (retinoic acid-inducible gene 1) and MDA-5 (melanoma differentiation-associated gene 5). PRRs then
signal through mitochondrial antiviral-signaling protein (MAVS) to activate the interferon (IFN) regulatory factor 3 (IRF3) pathway and nuclear factor-κB (NF-κB) pathway. The transcription factors IRF3 and NF-κB translocate into nucleus, where upregulate IFNs transcription and translation (9). Secreted IFNs can further amplify the antiviral signaling via binding to the IFN receptors, which, in turn, stimulate signal transducer and activator of transcription (STAT)1, STAT2 and IRF9 to induce the downstream antiviral inflammatory responses through activating the IFN-stimulated genes (ISGs) and accumulating IFN transcripts. Nonetheless, rotavirus has also evolved strategies to evade or suppress pro-inflammatory immune responses to maximally curtail the magnitude and persistence of correlated innate immune defense; for example, rotavirus non-structural protein 1 (NSP1) successfully prompts degradation of IRF3, inhibition of NF-κB signaling, and deactivation of STAT1, which leads to poor innate immune responses against rotavirus infection (10-13). Moreover, the established antiviral therapy can only resolute the rotavirus-associated gastrointestinal symptoms. Patients have to rely heavily on their adaptive immune system, including humoral immunity and cell-mediated immunity in order to overcome the rotavirus infection. However, it takes longer time for young babies to build up acquired immune responses to primary rotavirus infection, time during which the invader can multiply and cause severe disease, even death. Hence, rotavirus infection remains a serious clinical problem jeopardizing public health and represents a significant economic burden globally. New approaches involved in modulation of host innate immune responses, also known as immunotherapies, are now being investigated as alternative prophylactic or therapeutic strategies to favor the antimicrobial outcomes (14, 15). The presence of higher activation threshold of innate immunity at gastrointestinal tracts successfully inhibits the excessive inflammation to innocuous microbes. In contrast, successful pathogen invasion will initiate the cellular defense
mechanisms. Lymphocytes such as macrophages and dendritic cells along with intestinal epithelial cells constitutively express Toll-like receptors (TLRs), which can detect the potential harmful components from pathogens, known as pathogen associated molecular patterns (PAMPs), including a number of highly conserved microbial surface structures, such as lipopolysaccharide (LPS), peptidoglycan, and flagellin. Upon the ligation, agonists trigger intracellular signal transduction cascades, and lead to the initiation of innate immune responses. Activation of TLR3, 4, 7, 8 and 9 culminate the secretion of type I (IFN-α/β) and type III (IFN-λ) IFNs (16), and meanwhile, IFNs bind to their cell-surface receptors in a paracrine or autocrine manner, and amplify the antiviral activities via restraining the viral multiplication in infected cells, and establishing an antiviral state in uninfected cells. The interferon-mediated innate immunity serves as the first line defense against virus infection (17). Therefore, prophylactic or therapeutic interventions that target TLRs may benefit the combat against rotavirus infectious disease. Researchers have shown that activation of endosomal TLR3 in either cell compartment (radioresistant cells, also known as non-hematopoietic cells, such as intestinal epithelial cells, or bone marrow-derived cells, also know as hematopoietic cells, such as lamina propria dendritic cells) contribute to the protective roles of innate immune response against rotavirus infection (18). In addition, TLR4, identified as one of the most well studied PRRs, defenses against pathogens, and maintain immune homeostasis in gastrointestinal tract. Upon activation, LPS or viral protein (19) can induce two distinct signaling pathways: MYD88 (myeloid differentiation primary response protein 88)-dependent pathway, which leads to the upregulation of pro-inflammatory cytokines, such as IL-6, TNF-α, IL-1, etc; meanwhile TRIF (Toll-IL-1 receptor domain containing adapter inducing interferon-β)-dependent pathway, which activates the transcription of type I IFN thereby mediate antiviral activity (20). TLR5 also plays an important
role in enteric infection via detection of invasive flagellated bacteria such as *Salmonella typhimurium* (21). A recent publication, Price et al. and his colleagues, defined the spatial compartmentalization of TLR expression on intestinal epithelial cells by using reporter mice, and revealed that except very low levels of TLR5 expression could be detected on Paneth cells, there was no expression of TLR5 in the small intestinal epithelium (22); whereas lamina propria dendritic cells constitutively express TLR5 to a much greater extent compared to IEC (23, 24), therefore, here, we investigated whether activation of TLR5 in the underlying lamina propria could provide the potential to protect the small intestine epithelium from rotavirus invasion.

Flagellin is a subunit component of the bacteria flagellum, which can activate TLR5 on CD103⁺CD11b⁺ lamina propria dendritic cells, and stimulate the secretion of IL-23 (23). Group 3 innate lymphoid cells act downstream of IL-23 by secreting IL-22 (25). IL-22 belongs to IL-10 family, which plays an essential role in defense against bacterial pathogens, such as *Citrobacter rodentium* in GI tract (26). For example, the presence of IL-22 involves support of structure integrity of intestinal epithelium via directly regulating tight junctions between intestinal epithelial cells (27); meanwhile IL-22 also functions as a potent inducer that increase the secretion of antimicrobial peptides (AMPs) from paneth cells (28), which together help prevent bacteria translocation, and contain opportunistic pathogen in the GI tract. Although mice deficient in IL-22 expression showed a significant increase in virus load relative to wild type (29), still there is a lack of evidence indicating that IL-22 can directly elicit antiviral response against enteric viral pathogens. Several groups have reported the antiviral effect of IFN-λ against rotavirus infection. Endogenous secretion or exogenous treatment of IFN-λ successfully restricted rotavirus infection in mice (29-31). Notably, IL-22 is evolutionarily related to type III interferon (IFN-λ) by signaling through the same IL-10Rβ chain, and IL-22Rα chain has the
closest sequence identity compared to IFN-λRα (32-34). Additionally, both receptors are preferentially expressed on intestinal epithelial cells, suggesting a potential role of IL-22 in promoting the virus clearance.

Nucleotide-binding and oligomerization domain (NOD)-like receptors C4 (NLRC4) is an intracellular PRR that can also detect cytosolic flagellin. Researches to date have been focused on the function of NLRC4 in both hematopoietic cell compartment, such as dendritic cells and macrophages, and nonhematopoietic cells, including intestinal epithelial cells (35). For example, *Salmonella typhimurium* is a prevalent mucosal-dwelling pathogen, and upon microbial invasion, NLRC4 together with NAIP (NLR family apoptosis inhibitory proteins) recognize flagellin, recruit adaptor protein ASC (apoptotic speck protein containing a CARD), and form inflammasome complex. Downstream signaling activates protease caspase-1, which mediates the maturation and secretion of proinflammatory cytokines including IL-1β and IL-18, also induces a rapid lytic form of inflammatory cell death, termed as pyroptosis (36). The importance of NLR inflammasome in antiviral responses has also been demonstrated. For example, upon influenza A virus infection, over-exuberant or prolonged stimulation of NLRP3 inflammasome lead detrimental effect to the host, causing tissue damage or dysfunction of the organs (37). Previous studies also suggest that Nlrp9b can recognize double-stranded RNA, and elicit the protection against rotavirus infection in intestinal epithelial cells (38). However, the mechanism by which NLRC4 inflammasome sense and restrict enteric viruses is still largely unknown.

An intact intestinal epithelial monolayer forms a physical segregation between hosts and external environment. Concomitantly, the intestinal epithelium constantly undergoes vigorous self-renewal, which is essential to maintain the structural and functional integrity of the intestine. The main structure of the small intestine contains two compartments: crypt, where the intestinal
stem cells reside, and villi, which contains differentiated cells including enterocytes, goblet cells, enteroendocrine cells, etc. Crypt-resident stem cells manage the continuous renewal of the epithelial cell layers, while differentiated intestinal epithelial cells, except Paneth cells, gradually migrate up the crypt-villus axis to maintain the epithelial barrier functions. After reaching the villus tips, polarized epithelial cells are extruded into the lumen as a result of anoikis during gut homeostatic situation (39). In response to various stimuli, such as microbial infection, mucosal injury, epithelial lining can accelerate the rate of turnover. Recent studies demonstrated that (40, 41).

Therefore, our studies used rotavirus as a model, and aimed at characterizing the synergistic effect of TLR5 and NLRC4 in mediating host innate immunity against enteric virus in GI tract.
2 EXPERIMENT

2.1 Materials and Methods for Specific Aim I

Mice.

WT C57BL/6, Rag1<sup>−/−</sup>, IFNγR1<sup>−/−</sup>, Il1r<sup>−/−</sup>, Il18<sup>−/−</sup>, p40<sup>−/−</sup>, CD11c-DTR, Rag2/Il2rg<sup>−/−</sup> and CD45.1 transgenic mice (on C57BL/6 background) were obtained from Jackson Laboratories (Bar Harbor, ME). IFN I&II R<sup>−/−</sup> and STAT1<sup>−/−</sup> mice, on a C57BL/6 background, were gifts from Dr. Herbert Virgin (Washington University, St. Louis, MO). IFN I R<sup>−/−</sup> mice, which were on a 129 S2 background, were a gift of Dr. Sam Speck (Emory University, Atlanta, GA). Nlrc4<sup>−/−</sup> and Il22<sup>−/−</sup> mice were provided by Genentech, Inc (South San Francisco, CA). Tlr5<sup>−/−</sup> and MyD88<sup>−/−</sup> mice were originally generated by Dr. Shizuo Akira (Osaka University, Japan) and backcrossed to C57BL/6 mice for 10 generations. Generation of Tlr5/Nlrc4<sup>−/−</sup> was previously described<sup>18</sup>. Human IL-18 binding protein (BP) transgenic mice were generated by Dr. Charles Dinarello (University of Colorado, Denver, CO) and maintained as previously described<sup>19</sup>.

Materials.

FliC isoform of flagellin was HPLC-purified, and purity was verified as previously described<sup>(42-45)</sup>. Briefly, flagella were isolated from FljB-deficient S. typhimurium strain SL3201fljB<sup>−</sup> via high-speed centrifugation of bacterial supernatant. Flagella were converted to flagellin monomers by boiling. Flagellin monomers were chromatographically purified using polymixin B agarose, S-sepharose, and Q-separase. This procedure results in a preparation of flagellin that does not activate gene expression (as assessed by RT-PCR and microarray) in mice lacking both known flagellin receptors indicating it does not contain levels of innate immune agonists beyond flagellin. Murine IL-22 was provided Genentech, Inc. Murine IL-18 was purchased from Sino
Biologicals Inc. (Beijing, China). Human IL-22 was purchased from BioLegend (Sand Diego CA). Human IL-18 was purchased from Invivogen (San Diego, CA). Flt3 ligand was a gift of Dr. Robert Mittler (Emory University, Atlanta, GA).

**Rotavirus infection and flagellin and cytokines treatment.**

**Acute models-** Mice were infected with a range of dilutions of a preparation of murine rotavirus EC strain by oral inoculation to determine the amount of virus that resulted in 50% of mice exhibiting a course of detectable fecal RV antigens, referred to as SD50 as per previous studies. Mice were infected with $10^5$ SD50 of murine rotavirus EC strain by oral inoculation after feeding 100 µl of 1.33% sodium bicarbonate to neutralize stomach acid. Except for diarrhea studies and chronic infection studies, we utilized 8-12-week-old mice and gavage volume of 100 µl. Flagellin treatment, unless indicated otherwise, used a dose of 20 µg in 0.2 ml PBS, or 0.2 ml PBS as control, which was given 2 hours prior to inoculation and on days 2, 4, 6 and 8 p.i.

**Diarrhea model-** 7-day-old mice were inoculated with 400 SD50 administered in 50 µl PBS. Flagellin treatment, unless indicated otherwise, used a dose of 10 µg and was given 2 hours prior to inoculation and daily on days 0-9 p.i. Chronic model- Chronic rotavirus infection was performed as previous described (46). Briefly, 3-week-old Rag1−/− mice were fed with 100 µl of 1.33% sodium bicarbonate and then infected with 105 SD50 of murine rotavirus EC strain by oral inoculation. Feces were collected at 3 weeks after infection to confirm the establishment of chronic infection. Where indicated, mice were injected with 0.2 ml of PBS only (control) or 0.2 ml of PBS containing flagellin. Flagellin treatment, unless indicated otherwise, used a dose of 20 µg and was given every other day from days 24-42 p.i. In parallel, the above mice were treated with PBS or PBS containing IL-22, IL-18, or both IL-22 and IL-18 as indicated.
Neutralization of IL-17 and IL-22.

IL-17 and IL-22 were neutralized via i.p. injection of mAb to IL-17 (100 µg) and IL-22 (150 µg, clone 8E11) provided by Genentech, Inc. on days 0, 2, 4, 6 and 8 p.i. as previously described (47, 48).

ELISA to measure fecal RV antigens and antibodies.

A sandwich enzyme-linked immunosorbent assay (ELISA) was performed to detect rotavirus antigen in mouse feces as previously described. Use of multiple dilutions of fecal suspension allowed determination of SD50. Serum anti-RV IgA or IgG and fecal anti-RV IgA were detected as previously described (49).

Immunohistochemistry.

Frozen mouse small intestinal sections were blocked with normal goat serum and incubated with a monoclonal antibody to rotavirus VP6 protein overnight at 4 °C followed by 3 washes in PBS-Tween20. The slides were then incubated with biotinylated secondary antibody to mouse Ig diluted in PBS for 30 minutes at room temperature. After washing again, slides were incubated in HRP-Avidin in PBS for 30 minutes at room temperature. Then, 100 µl freshly made DAB substrate solution was applied to the slides to reveal color, followed by 3 washes in PBS-Tween20. Stained intestinal tissues were then dehydrated through 4 incubations in alcohol (95%, 95%, 100% and 100%). The color change provided by antibody staining was observed using microscopy.

Quantification of RV genomes and replication.
Total RNA was isolated from tissues using TRIzol (Invitrogen, Carlsbad, CA), and quantitative RT-PCR (qRT-PCR) was performed using the Biorad iScriptTM One-Step RT-PCR Kit in a CFX96 apparatus (Bio-Rad, Hercules, CA) with primers targeting the NSP3 region EC.C(+) (5’-GTTGTTGTGCTCATTGC-3’) and EC.C(-) (5’-TCGGAACTCTTGTCGAC-3’). Strand-specific quantitative reverse transcription-PCR (ss-qRT-PCR) assay was used to quantify the ability of rotavirus to spread and replicate, as previously described (50).

Quantification of fecal reoviral RNA shedding using qRT-PCR.

Reovirus T1L was generated as previously described (51). C57BL/6 mice were inoculated orally with 1-4 x 10^8 PFU of reovirus T1L in 0.2 ml PBS. From day 1 to 4 p.i., feces were collected, and total RNA was extracted according to the manufacturer's instructions (MO BIO, Inc., Carlsbad, CA). qRT-PCR was performed to determine viral shedding (forward primer 5’-CGCTTTGAAGGTCGTGTATCA-3’ and reverse primer 5’-CTGGCTGTGCTGATGATTGTTTT-3’ corresponding to the viral S4 gene) (52). Viral shedding level was normalized to fecal weight (53, 54).

Quantification of host cell gene expression.

Total RNA was isolated from colonic tissues using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions, and qRT-PCR was performed using the Biorad iScript™ One-Step RT-PCR Kit in a CFX96 apparatus (Bio-Rad, Hercules, CA) with specific mouse oligonucleotides. The sense and antisense oligonucleotides used include the following:

36B4 5’-TCCAGGCTTTGGGCATCA-3’ and

5’-CTTTATTCCAGCTGCACATCACTCAGA-3’;
KC 5’-TTGTGCAGAAAAGAAGTGCGAG-3’ and
5’-TACAAACACAGCCTCCCAACA-3’;
IFN-λ 5’-AGCTGCAGGCCCTCAAAG-3’ and
5’-TGGGAGTGAATGTGGCTCAG-3’;
p40 5’-GACCATCAGTGCAAAGAGTTTCTAGAT-3’ and
5’-AGGAAAGTCTTTTTTGAATTTTTAA-3’;
IL-22 5’-GTGCTCAACTTCACCCTGGA-3’ and
5’-TGGATGTTCTGCGTCGTCACC-3’.

Results were normalized to the housekeeping gene 36B4.

*In vitro RV infection.*

HT-29 and Caco-2 cells were infected with cell culture-adapted rhesus rotavirus (EC strain cannot infect cultured cells) as previously described (55). 2x10^8 PFU of RRV was activated with 10 µg/ml of trypsin in serum free media (SFM) at 37 °C for 30 minutes. HT-29 cells were grown to confluency in 6 well plates, washed several times with SFM, pre-treated with 100 ng/ml flagellin or left untreated (control). Two hours later, cells were inoculated with virus for 1 hour at 37 °C/5% CO2 to allow for adsorption (in the continued presence of flagellin). Following adsorption, cells were washed again several times with SFM and then incubated with 2 µg/ml trypsin in SFM for 0–48 hours p.i. For Caco-2 cells, Transwell plates (Corning Inc.) were used to allow cytokine treatment in the lower chamber and virus infection in the upper chamber. After IL-22/IL-18 pre-treatment for 1 hour, cells were infected in the presence of IL-22/IL-18 (maintained throughout experiment), and cell lysates prepared at indicated times.
**Generation of bone marrow chimeric mice.**

Bone marrow chimeric mice were generated as previously described (56). Briefly, bone marrow cells were eluted from the femora and tibiae of donor mice with complete RPMI 1640 media. Recipient mice were irradiated at a dose of 11Gy with a 137Cs irradiator before intravenous injection (i.v.) of 2x10^7 donor bone marrow cells. After injection, the mice were maintained in sterile cages and supplied with drinking water containing 2 mg/ml of neomycin (Mediatech). Mice were utilized for experiments 8 weeks later. CD11c-DTR chimeras were used to deplete DC and avoid toxicity that results from DT administration to CD11c- DTR mice (57).

**Cell depletion study.**

For depletion of NK cells, mice were treated with 200 µg of PK136 antibody to NK1.1 (Bio X Cell, Lebanon, NH) by i.p. injection every 2nd day from 2 days before infection to 8 days p.i. For neutrophil depletion, WT C57BL/6 mice were i.p. injected with 200 µg anti-Gr1 (RB6-8C5, Bio X Cell) every 2nd day from 2 days before RV infection to 8 days p.i. Chronically-infected Rag1^−/− mice were injected with 500 µg anti-Gr1 every day from 3 days before flagellin treatment for 5 days. For depletion of macrophages, WT C57BL/6 mice were treated with 0.5 ml and 0.2 ml of clodronate liposomes (50 mg/ml) (ClodronateLiposome.org) by oral gavage and i.p., respectively, every 2nd day from 4 days before infection until day 0 p.i., after which time i.p. injection was continued to maintain depletion. For depletion of DCs, CD11c-DTR chimeric mice were treated with diphtheria toxin (DT, LIST Biological Laboratory, Inc.) at 8 ng/g body weight two and one days before RV infection.

**Preparation of mouse whole blood leukocytes and lamina propria cells.**
Mouse blood was collected with heparinized tubes, centrifuged to removed plasma, resuspended in red blood lysis buffer (BD Biosciences) for 10 minutes, and washed in BD FACS buffer twice. Lamina propria cells were prepared as previously described (58).

**Cell enrichment and adoptive transfer.**

WT C57BL/6 or Tlr5/Nlrc4−/− mice were injected with 30 µg Flt-3 ligand in 0.2 ml PBS every day for 9 days. Mouse splenocytes were stained with fluorochrome-conjugated antibodies to CD45, MHC class II, Ly6G, CD11c, CD11b, NK1.1, F4/80, CD3 and CD19. CD11c+ DCs were FACS-sorted on a BD FACS Aria III cell sorter. DCs with about 99% purity were transferred to recipient mice by i.v. injection. Alternatively, mouse CD11c+ DC were enriched by CD11c microbeads following the manufacturer’s instruction (Miltenyi Biotec, Auburn, CA). The magnetically-enriched CD11c+ DC with purity >95% were injected into Tlr5/Nlrc4−/− mice.

Flow cytometry analysis. Mouse whole blood leukocytes, splenocytes, and intestinal lamina propria cells were prepared and blocked with 10 µg/ml anti-CD16/anti-CD32 (clone 2.4G2 ATCC) for 10 minutes in FACS buffer (PBS supplemented with 0.5% BSA and 0.04% sodium azide). Aliquots of cells (1 × 10^6) were suspended in 0.1 ml FACS buffer on ice for 20 minutes with FITC-, PE-, PerCP-, APC-, PE-Cy7-, Alexa Fluor 700-, and Pacific blue-conjugated mAbs to detect the following surface Ags: CD3, CD11b, CD11c, CD103, CD19, MHC class II, F4/80, NK1.1, CD45.1, CD45.2, Ly-6G (BD Biosciences). Stained cells were analyzed on a BD LSR II flow cytometer. Data analysis was carried out using FlowJo (TreeStar, Ashland, OR).

**Isolation of Intestinal epithelial cells.**
Chronically RV-infected \textit{Rag1}^{−/−} mice were treated as indicated in the figures. At indicated times, mice were sacrificed and small intestines were washed extensively with PBS, cut to 1 cm pieces, and incubated with 2 mM EDTA for 30 minutes with shaking at 4 °C. After vortexing, the supernatant was passed through 100 µm cell strainer. The intestinal epithelial cells (IEC) were centrifuged, resuspended in PBS, and counted under a microscope.

\textit{SDS-PAGE Immunoblotting.}

At indicated time points following in vitro RV infection, cells were washed several times with cold PBS and resuspended in radioimmunoprecipitation assay II buffer (RIPA II) (20mM Tris-HCl, 2.5 mM EDTA, 1% Triton X-100, 10% glycerol, 1% deoxycholate, 0.1% SDS, 50 mM NaF, 10 mM Na$_2$P$_2$O$_7$, 2 mMNaVO$_4$, and protease inhibitor cocktail). Cell lysates were separated on a precasted SDS-PAGE gel (Biorad), transferred to a nitrocellulose membrane, and probed with anti-rotavirus VP6 and human \(\beta\)-actin antibodies. To detect caspase 3, isolated IEC were incubated with 1X SDS lysis buffer (20mM Tris, pH6.8, 1% SDS, 1 mM EDTA) for 30 minutes at room temperature. After full-speed centrifugation, cell lysates were transferred to a fresh tube for analysis using antibodies to cleaved caspase 3 and \(\beta\)-actin antibodies (Cell Signaling Technology)

\textit{RNA sequencing.}

IEC were purified, as described above. RNA was extracted using Qiagen RNeasy kit, according to the manufacturer’s protocol. After purification, RNA concentration and integrity were determined using Epoch Microplate Spectrophotometer (Bio-Tek) and agarose gel electrophoresis, respectively. Total RNA was then prepared for sequencing purposes, using
Illumina TruSeq RNA kit, according to the manufacturer’s protocol. Briefly, mRNA was purified using oligo-dT-coupled beads, fragmented, and converted to cDNA. After end repair and ligation of adapters, mRNA libraries were amplified by PCR and validated using BioAnalyser, according to manufacturer’s recommendations. The purified library was then subjected to sequencing using a HiSeq machine at Cornell University’s core facility. After quality filtering the data, adapter sequences were removed using fastqcliplipper tool (http://hannonlab.cshl.edu/fastx_toolkit/). Tophat2 (http://tophat.cbcb.umd.edu/) was then used to align sequences to the most recently updated mus musculus genome (mm10 version) based on the Bowtie algorithm (59). Alignment summary and normalization were performed through R using EdgeR package including tagwise negative binomial test (60). (http://www.bioconductor.org/packages/release/bioc/html/edgeR.html). The function plotSmear was used to generate plots of the log-fold-changes against log-cpm for each gene of the mus musculus genome. For pathway summarization, Panther classification system was used (61).

2.2 Materials and Methods for Specific Aim II

Mice

All mice used herein were on a C57BL/6 background and bred at Georgia State University (Atlanta, GA). Rotavirus-infected mice were housed in an animal biosafety level 2 facility under institutionally-approved animal use protocols (IACUC # 17047). WT, Rag-1<sup>−/−</sup>, IL-18<sup>−/−</sup>, IL-18-R<sup>−/−</sup>, Stat3<sup>flox</sup>, and Villin-cre were purchased from Jackson Laboratories. NLRC4<sup>−/−</sup>, IL-22<sup>−/−</sup>, and IL-22-R<sup>−/−</sup> mice were provided by Genentech. TLR5<sup>−/−</sup> and TLR5<sup>−/−</sup>/NLRC4<sup>−/−</sup> and WT littersmates were maintained as previously described (62).
Materials

Murine Fc-IL-22 was provided by Genentech, Inc. Murine IL-18 was purchased from Sino Biological Inc (Beijing, China). Procedures for isolation of flagellin, and verification of purity, were described previously (62). Recombinant murine epidermal growth factor (mEGF) was purchased from PEPROTECH.

Rotavirus infection

Acute Models: Age- and sex-matched adult mice (8-12 weeks of age) were orally administrated with 100 µl 1.33% sodium bicarbonate (Sigma), and then inoculated with $10^5$ SD50 of murine rotavirus EC strain. Approach used to determine SD50 has been described previously (62). Chronic model: 5-week-old Rag-1-/- mice were infected with mRV (same infection procedure as described in Acute Models). Feces were collected 3-week post rotavirus inoculation to confirm the establishment of chronic infection. In vitro model: Cell culture-adapted Rhesus RV was trypsin-activated (10 µg/ml trypsin in serum-free RPMI-1640 (Cellgro) at 37 °C for 30 min. The basolateral side of the polarized Caco-2 cells were stimulated with cytokines, 1.5 hours prior to expose to Rhesus RV infection as previously described (62). The upper chamber of the Transwell were infected with Trypsin-pretreated RRV and allowed for adsorption at 37 °C for 40 min before washed with serum-free medium (SFM). The presence of cytokines was maintained constant throughout the experiment.

Fecal Rotavirus Antigen Detection

Fecal pellets were collected daily from individual mouse on days 0-10 post rotavirus inoculation. Samples were suspended in PBS (10% wt./vol.) and, after centrifugation, supernatants of fecal
homogenates were analyzed by enzyme-linked immunosorbent assay (ELISA) after multiple serial dilutions, more detailed descriptions of experimental procedures are previously described (62).

**Generation of Bone Marrow Chimeric mice**

Mice were subjected to X-ray irradiation using 8.5 Gy equivalent followed by injection of $2 \times 10^7$ bone marrow cells administered intravenously as previously described before (62). All mice were afforded an 8-week recovery period before experimental use. For the first 2 weeks post-transfer, mice were maintained in sterile cages, and supplied with drinking water containing 2 mg/ml neomycin (Mediatech/Corning).

**Visual assessment of IEC shed into small intestinal lumen**

Intestinal sections were fixed in methanol-Carnoy’s fixative solution (60% methanol, 30% chloroform, 10% glacial acetic acid) for 48 hours at 4 °C. Fixed tissues were washed two times in dry methanol for 30 min each, followed by two times in absolute ethanol for 20 min each, then incubated in two baths of xylene before proceeded to paraffin embedding. 4-μm-thin sections were sliced from paraffin-embedded tissues, and placed on glass slides after floating on a water bath. The sections were dewaxed by initial incubation at 60 °C for 20 min, and following two bathes in prewarmed xylene substitute solution for 10 min each. Deparaffinized sections were then hydrated in solution with decreasing concentration of ethanol (100, 95, 70, 50, and 30%) every 5 min in each bath. Last, slides were let almost dry completely, and then mounted with Prolong antifade mounting media containing DAPI before analyzed under the fluorescence microscopy.
**Immunohistochemistry for TUNEL staining**

Intestinal sections were fixed in 10% buffered formalin at room temperature for 48 hours, and then embedded in paraffin. Tissues were sectioned at 4 µm thickness and IEC death was detected by TUNEL assay using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche) according to the manufacturer’s instructions.

**Immunoblot Analysis for assay of Cleaved Caspase3 and Phospho-STAT3**

Intestinal epithelial cells lysate (20 µg per lane) were separated by SDS-PAGE through 4%-20% Mini-PROTEAN® TGX™ gel (BIO-RAD), transferred to nitrocellulose membranes, and analyzed by immnoblot, as previously described (62). Briefly, isolated IEC were incubated with RIPA lysis buffer (SANTA CRUZ BIOTECHNOLOGY) for 30 min at room temperature. Subsequently, cell lysates were homogenized with pipette, and then subjected to full-speed centrifugation. The proteins bands were detected for Cleaved Caspase3, phosphor-STAT3 and anti-β-actin (Cell Signaling), and incubated with horseradish peroxidase-conjugated anti-rabbit. Immunoblotted proteins were visualized with Western Blotting Detection Reagents (GE Healthcare), and then imaged using the ChemiDoc XRS⁺ system (Bio-RAD).

**Isolation of intestinal epithelial cells**

The entire small intestine was harvested from different strains of mice according to indicate experimental design, and sliced longitudinally before washed gently in PBS to remove the luminal content. Tissues were processed and maintained in 4 °C at all conditions. Cleaned tissue samples were further minced into 1-2-mm³ pieces, and shaken in 20 ml HBSS containing 2mM EDTA and 10 mM Hepes for 30 min. An additional step of vigorous vortexing in fresh HBSS
(10 mM Hepes) after EDTA incubation would facilitate cell disaggregation. Intestinal epithelial cells (IEC) were then filtered through 70-µm nylon mesh strainer (BD Biosciences), centrifuged, and resuspended in PBS.

**Antibody Staining and Flow Cytometry Analysis**

Bulk leukocytes and intestinal epithelial cells isolated above were incubated with succinimidyl esters (NHS ester)-Alexa Fluor 430, which permitted determination of cell viability. Cells were then blocked by incubation with 10 µg/ml anti-CD16/anti-CD32 (clone 2.4G2 ATCC). 20 min later, cells were stained with fluorescently conjugated antibodies: CD26-PE (clone: H194-112, eBioscience), CD44-PECy7 (clone: IM7, eBioscience), CD45-FITC (clone: 30-F11, eBioscience), CD326-APC (clone: G8.8, eBioscience). Finally, stained cells were followed by fixation with 4% formaldehyde for 10 mins before whole cell population was analyzed on a BD LSR II flow cytometer. Collected data was carried out using FlowJo.

**Quantification of IEC shedding from luminal content**

Host DNA was quantitated from 100 mg of luminal content (100 mg) from small intestine by using QIAamp DNA Stool Mini kit (Qiagen), and subjected to quantitative PCR using QuantiFast SYBR Green PCR kit (Bio-Rad) in a CDX96 apparatus (Bio-Rad) with specific mouse 18S oligonucleotides primers. The sense and antisense oligonucleotides primers used were: 18s-1F: 5’-GTAACCCGTTGAACCCCATT-3’ and 18s-1R: 5’-CCATCCAAATCGGTAGTAGCG-3’. PCR results were expressed as actual numbers of IEC shedding per 100 mg of luminal content, calculated using a standard curve, which was generated using two-fold serial dilutions of mouse colon carcinoma cell line MC26. DNA was extracted
from each vial with known number of MC26 cells after serial dilutions, and then Real-Time quantitative PCR was performed. The cycle quantification (Cq) values (X-axis) are inversely proportional to the amount of target genes (18S) (Y-axis), and this standard-curve plot is applied to estimate the numbers of cell shedding from luminal content based on the quantity of target copies (18S) from each sample.

Quantification of RV genomes and replication in IEC and luminal content

To extract RNA, cell pellets were homogenized with TRIzol™ (Invitrogen), and then addition of chloroform to the homogenate allowed separation between RNA (an upper aqueous layer) and DNA plus proteins (a red lower organic layer). Further, isopropanol facilitated the precipitation of RNA, and after centrifugation, the impurities from RNA were removed by washing with 75% ethanol. RNA pellet was resuspended in RNase-free water and preceded to quantitative qRT-PCR. Total RNA from luminal content is purified from RNeasy PowerMicrobiome Kit according to the manufacturer’s instructions. Then primers that target NSP3 region: EC.C (+) (5’-GTTCGTTGTGCTCATTTCG-3’ and EC.C (-) (5’-TCGGAACGTACTTCTGGAC-3’) were applied to quantify the overall viral genomes from IEC and luminal content. RV replication was as quantitated as previously described (50).

BrdU pulse-chase labeling analysis of intestinal enterocyte migration

A pulse-chase experimental strategy by labeling intestinal enterocytes with 5-bromo-2-deoxyuridine (BrdU) was conducted to estimate the IEC migration rate along the crypt-villus axis over a defined period of time. Briefly, 8-week-old mice were intraperitoneally injected with either PBS or cytokine(s) (IL-22 and/or IL-18) 1 hour prior to the BrdU i.p. treatment (50 µg per
mg of mice body weight). After 16 hours, mice were euthanized, and the segment of the jejunum were resected, immediately embedded in OCT and then proceeded to tissue sectioning. 4 µm tissue sections were firstly fixed in 4% formaldehyde for 30 min at room temperature, and then washed 3 times in PBS. DNA denaturation was performed by incubating the sections in prewarmed 1.5 N HCl for 30 min at 37 °C. Then the acid was neutralized by rinsing the sections 3 times in PBS. Before BrdU immunostaining, sections were blocked with rabbit serum (BioGenex, Fremont, CA) for 1 h at room temperature, then incubated with anti-BrdU (Abcam) 2 hours at 37 °C, and counterstained with 4', 6-diamidino-2-phenylindole (DAPI). The BrdU-labeled cells were visualized under the fluorescent microscope.

**Quantification and statistical analysis**

Significance was determined using the one-way analysis of variance (ANOVA), two-way ANOVA, student’s t-test or Chi-Square analysis (GraphPad Prism software, version 6.04). Differences were noted as significant *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
3 RESULTS OF SPECIFIC AIM I: PREVENTION AND CURE OF ROTAVIRUS INFECTION VIA TLR5/NLRC4–MEDIATED PRODUCTION OF IL-22 AND IL-18

3.1 ABSTRACT

Activators of innate immunity may have the potential to combat a broad range of infectious agents. We report that treatment with bacterial flagellin prevented rotavirus (RV) infection in mice and cured chronically RV-infected mice. Protection was independent of adaptive immunity and interferon (IFN, type I and II) and required flagellin receptors Toll-like receptor 5 (TLR5) and NOD-like receptor C4 (NLRC4). Flagellin-induced activation of TLR5 on dendritic cells elicited production of the cytokine interleukin-22 (IL-22), which induced a protective gene expression program in intestinal epithelial cells. Flagellin also induced NLRC4-dependent production of IL-18 and immediate elimination of RV-infected cells. Administration of IL-22 and IL-18 to mice fully recapitulated the capacity of flagellin to prevent or eliminate RV infection and thus holds promise as a broad-spectrum antiviral agent.

3.2 INTRODUCTION

Rotavirus (RV) causes severe dehydrating diarrhea in young children and moderate intestinal distress in adults (63). RV infection of adult mice serves as a well-defined model of intestinal viral infection in which infectivity can be monitored by measuring levels of viral antigen shed in feces (64). RV predominantly infects and replicates in epithelial cells lining the small intestine (63). Bacterial flagellin, the primary component of bacterial flagella, potently activates host defense gene expression in intestinal epithelial cells (IECs) (21) and is viewed as a dominant immune activator in the intestine\textsuperscript{45}. Flagellin-induced gene expression confers both
IECs and mice with resistance to a variety of challenges, including bacteria, chemicals, and radiation (65, 66). Moreover, administration of flagellin reduced susceptibility of mice to a culture-adapted strain of RV (67). Thus, we investigated the potential of flagellin to protect mice against highly contagious pathogenic mouse RV.

3.3 RESULTS

3.3.1 FLAGELLIN PROTECTS MICE FROM RV INFECTION AND DIARRHEA

Oral inoculation of adult C57BL/6 mice with mouse RV (EC strain), using a dose 100,000 times that required to infect 50% of mice, resulted in uniform RV shedding that peaked 3 to 4 days after inoculation and resolved several days later, indicating viral clearance. Administration of a single dose of flagellin via intraperitoneal injection shortly before infection temporarily prevented infection (fig. S1A). Repeated administration of flagellin further delayed (fig. S1B) or completely prevented infection (Fig. 1A). In contrast, repeated administration of bacterial lipopolysaccharide (LPS) only transiently protected against RV infection (fig. S2).

RV infection induces an adaptive immune response, which is normally required to clear infection (68). However, we saw very little elevation in antibodies to RV in flagellin-treated mice (fig. S3), suggesting that flagellin’s protection against RV was independent of adaptive immunity. Confirming this notion, the protective effect of flagellin was fully maintained in mice lacking recombination activating gene 1 (Rag1−/−), making them deficient in mature B and T lymphocytes and susceptible to chronic RV infection (Fig. 1B) (46).

Analogous to young children, infection of neonatal mice with RV causes watery diarrhea (69). The incidence, duration, and active days of such RV-induced diarrhea and associated viral
loads were reduced via flagellin treatment (Fig. 1, C and D). Flagellin treatment also diminished reovirus load after oral inoculation, suggesting broad antiviral activity (fig. S4).

We next investigated whether flagellin could treat established chronic RV infection in immune-compromised mice. Although Rag1−/− mice of all ages develop chronic RV infection, high viral loads are attained by infecting 3-week-old Rag1−/− mice (46). Flagellin treatment eliminated detectable RV shedding by 2 days after treatment. Moreover, 10 doses of flagellin treatment over a 20-day period abolished RV shedding for at least 150 days, whereas untreated mice shed virus over their lifetime (Fig. 1E). Accordingly, RV antigens, which were detectable by means of immunostaining in villus epithelial cells in untreated Rag1−/− mice, were absent in mice treated with flagellin by 24 hours (fig. S5A). Moreover, treatment with flagellin resulted in reduced levels of RV RNA within hours of administration, and RV RNA was undetectable 48 hours after treatment. Reduced levels of RV replication preceded a reduction in levels of RV genomic RNA (fig. S5B). In Rag1−/− mice, a substantial degree of replication occurs extra-intestinally, especially in the liver, resulting in hepatitis (70). Levels of RV RNA in the liver were greatly reduced within 24 hours of flagellin treatment and undetectable within 48 hours (fig. S5C). Similarly, flagellin treatment also eliminated the more modest levels of RV RNA in the spleen (fig. S5D). These results indicate that flagellin treatment cures chronic RV infection in immune-compromised mice.

3.3.2 FLAGELLIN’S ANTIVIRAL ACTIVITY IS MEDIATED BY TLR5/NLRC4 ON DENDRITIC CELLS

Flagellin-induced remodeling of intestinal gene expression requires Toll-like receptor 5 (TLR5) (71). Flagellin can also be recognized intracellularly by the NOD-like receptor C4
(NLRC4) inflammasome, resulting in caspase 1–mediated production of the cytokines interleukin-1β (IL-1β) and IL-18 (72, 73). We expected that only TLR5 would be required for flagellin protection against RV infection. However, loss of either pathway of flagellin recognition reduced, but did not completely eliminate, the capacity of flagellin to prevent RV infection (Fig. 2, A and B). In contrast, absence of both TLR5 and NLRC4 or myeloid differentiation primary response gene 88 (MyD88), which is required for signaling by TLR5 and inflammasome-associated cytokines, eliminated flagellin’s protection against RV infection (Fig. 2, C and D).

Next, we generated bone marrow chimeric mice using wild-type (WT) and Tlr5/Nlrc4−/− mice to determine the extent to which flagellin-mediated protection against RV infection requires recognition of flagellin by hemopoietic or nonhemopoietic cells. In contrast to our expectation that IEC recognition of flagellin would be essential, transplant of WT bone marrow to irradiated Tlr5/Nlrc4−/− mice completely restored the capacity of flagellin to prevent RV infection, whereas administration of Tlr5/Nlrc4−/− bone marrow to WT mice eliminated flagellin’s anti-RV activity (fig. S6, A and B). Subsequent studies demonstrated that TLR5-dependent protection was restricted to hemopoietic cells, whereas NLRC4 expression by either cell compartment was sufficient for flagellin-mediated protection (fig. S6, C to F). This is consistent with reports of NLRC4 expression in macrophages and IECs (35, 74). Furthermore, hemopoietic and nonhemopoietic cells produce IL-18 in response to flagellin (fig. S7).

We next examined the role of hemopoietic cell types in mediating flagellin’s antiviral effect. Although flagellin induced neutrophil recruitment to the intestine (fig. S8), neutrophils were not required for flagellin’s antiviral effect (fig. S9). Nor were natural killer (NK) cells or intestinal macrophages required (figs. S10 and S11). Dendritic cells (DCs) mediate intestinal
production of antibacterial peptides in response to flagellin (23). To investigate whether these cells play a role in flagellin’s antiviral effect, we generated chimeric mice in which bone marrow–derived cells were engineered to express the diphtheria toxin receptor (DTR) under control of the CD11c promoter so that CD11c-expressing cells, primarily DCs, could be depleted by the administration of diphtheria toxin (DT) (75). Both DT-treated WT mice and untreated chimeric mice were protected against RV infection by flagellin treatment (Fig. 2E and fig. S12). However, flagellin was unable to protect DT-treated chimeric mice against RV infection (Fig. 2E). Next, we investigated whether DCs expressing TLR5 and NLRC4 are sufficient for flagellin protection against RV infection. Tlr5/Nlrc4−/− mice were intravenously administered purified DCs [CD19−/major histocompatibility complex (MHC) class II+/CD11c+/F4/80−] isolated from WT or Tlr5/Nlrc4−/− mice. Only the WT DC were capable of partial restoration of flagellin-mediated protection against RV infection, which was dependent on the number of DCs transferred (Fig. 2, F to H). Together, these results indicate that activation of TLR5/NLRC4 on DCs is necessary and sufficient for flagellin’s antiviral effect.

Interferons (IFNs) play a central role in antiviral immunity. However, flagellin’s antiviral effect was fully maintained in mice lacking type I and/or II IFN receptors or in mice lacking signal transducer and activator of transcription 1 (STAT1), which mediates IFN signaling (fig. S13, A to D). Nor did flagellin induce expression of type III IFN (fig. S13E), which is induced by RV (76), or IFN-associated gene expression (77). Thus, flagellin prevents and clears RV infection by a previously unrecognized antiviral pathway.
3.3.3 FLAGELLIN MEDIATED PROTECTION AGAINST RV INFECTION REQUIRES BOTH IL-22 AND IL-18

DC TLR5 activates the IL-12/IL-23 axis, resulting in innate lymphoid cells’ (ILCs) production of IL-17 and IL-22 (23, 78). Hence, we investigated whether ILC and this axis was involved in flagellin’s antiviral action. Mice deficient in p40, which is a component of both IL-12 and IL-23, and Rag2/Il2rg−/− mice, which lack ILCs (and mature B and T lymphocytes), were not effectively protected by flagellin treatment (Fig. 3, A and B). Flagellin protection against RV infection was not affected by neutralization of IL-17 (Fig. 3C) but was almost completely abolished by genetic or antibody-mediated blockade of IL-22 (Fig. 3, D and E). These results suggest a central role for ILC-mediated IL-22 production in mediating flagellin’s antiviral action. Accordingly, LPS does not elicit robust IL-22 production (78). Conversely, the requirement of NLRC4 suggested possible roles for inflammasome cytokines IL-1β and IL-18. Ablation of IL-1 receptor signaling modestly impaired flagellin’s protection against RV infection, whereas two different means of IL-18 ablation markedly reduced flagellin’s antiviral effect (Fig. 3, F to H). In contrast to flagellin-induced IL-22 production, which requires ILCs, and DC expression of TLR5 (fig. S7) (79), flagellin-induced IL-18 production was unimpaired in Rag2/Il2rg−/− or DC-ablated mice (fig. S7), indicating that distinct signaling events drive IL-22 and IL-18 production in distinct cell types.

3.3.4 IL-22/IL-18 TREATMENT PREVENTS AND TREATS RV INFECTION

We next investigated the extent to which recombinant IL-22 and IL-18 recapitulate flagellin’s antiviral action. When administered prophylactically, IL-18 or IL-22 had only partial protective efficacy, but together conferred complete protection against a broad range of RV
inoculation (Fig. 4A and fig. S14). Moreover, co-administration of IL-18 and IL-22 eliminated viral shedding in chronically RV-infected Rag1\(^{-/-}\) mice (Fig. 4B). These results did not reflect a strong dependence on one of these cytokines for driving the expression of the other in that flagellin-induced IL-18 expression is independent of TLR5 (45), whereas flagellin-induced IL-22 is largely independent of NLRC4 (fig. S15). Rather, our results suggest that parallel signaling pathways activated by IL-22 and IL-18 protect against RV infection and promote clearance of this virus, respectively. Consequently, combined treatment with IL-22 and IL-18 recapitulated the capacity of flagellin to cure RV infection in mice lacking mature T and B lymphocytes. Such combined IL-22/IL-18 treatment eliminated RV from Rag1\(^{-/-}\) mice within 24 hours (versus 48 hours for flagellin) and, in contrast to flagellin, was effective in mice lacking ILCs (fig. S16). Like flagellin, prophylactic IL-22/IL-18 administration also afforded protection against the severe diarrhea seen in neonates (Fig. 4C). Furthermore, treating neonate mice with IL-22/IL-18 after diarrhea manifested moderately shortened its course (fig. S17). Thus, recapitulating flagellin’s antiviral effect with IL-22/IL-18 might offer broad antiviral therapeutic potential even in immune-compromised hosts.

Neither flagellin nor IL-22/IL-18 altered RV infection in cultured IECs (fig. S18). Hence, to investigate mechanisms by which IL-22 and IL-18 treatment cleared RV infection, we examined signaling events in gut epithelial cells isolated from chronically RV-infected mice treated with IL-22, IL-18, or both cytokines. RNA sequencing revealed that IL-22 treatment induced rapid reprogramming of epithelial cell gene expression, implicating genes involved in a broad array of cellular processes (Fig. 4D and figs. S19 and S20). A more modest effect was observed in response to IL-18, whereas the combination of IL-22 and IL-18 induced a number of changes in gene expression not seen with either cytokine alone. In contrast, administration of IL-
18 but not IL-22 to RV-infected mice resulted in rapid activation of caspase 3, suggesting involvement of apoptosis (fig. S21). Thus, IL-22 and IL-18 induced changes in gene expression and caspase 3 activation that correlated with rapid blockade of RV replication and elimination of RV genomes within 24 hours of cytokine treatment (Fig. 4, E and F). Together, these data suggest that IL-18 induces signaling events that lead to rapid reduction in RV levels, whereas IL-22 reprograms epithelial cell gene expression, resulting in resistance to RV infection.

3.4 DISCUSSION

We demonstrate that innate immunity can be harnessed to prevent and treat viral infection. Given the health burden caused by RV, including 600,000 annual deaths in children (80) and chronic infections in immune-compromised hosts, this strategy presents a therapeutic opportunity, differences between human and mouse RV strains (71) notwithstanding. Moreover, the complex antiviral action of flagellin was fully recapitulated by IL-22 and IL-18, whose use could circumvent differences in NLRC4 function between mice and humans (66). The action of these cytokines would likely be synergistic with therapies that directly target viruses and/or those that promote adaptive immunity. Thus, we propose activation of innate immunity with flagellin, or IL-22 and IL-18, as a potential strategy to combat emerging and recalcitrant viral pathogens.
4 RESULTS OF SPECIFIC AIM II: IL-22 INDUCED CELL EXTRUSION AND IL-18-INDUCED PYROPTOSIS PREVENT AND CURE ROTAVIRUS INFECTION

4.1 ABSTRACT

Administration of bacterial flagellin elicits production of TLR5-mediated IL-22 and NLRC4-mediated IL-18 that act in concert to cure and prevent rotavirus (RV) infection. This study investigated the mechanism by which these cytokines act to impede this virus. Although IL-18 and IL-22 induce each other’s expression, we found that IL-18 and IL-22 both impeded RV independently of each other and did so by distinct mechanisms, in both cases via activation of their cognate receptors in intestinal epithelial cells (IEC). IL-22 drove IEC proliferation and migration toward villus tips, which resulted in increased extrusion of highly differentiated IEC that serve as the site of RV replication. In contrast, IL-18 induced pyroptotic death of RV-infected IEC thus directly interrupting the RV replication cycle, resulting in spewing of incompetent virus into the intestinal lumen and causing a rapid drop in levels of RV-infected IEC. Together, these actions resulted in rapid and complete expulsion of RV, even in hosts with severely compromised immune systems. These results suggest that IL-18/22 might be a means of treating viral infections that preferentially target short-lived epithelial cells.

4.2 INTRODUCTION

Rotavirus (RV) remains a scourge to humanity, causing severe distress to many and thousands of childhood deaths annually, particularly in developing countries wherein RV vaccines have only moderate efficacy (1). RV is a double-stranded RNA virus that primarily infects intestinal epithelial cells (IEC) that line the villus tips of the ileum, resulting in severe
life-threatening diarrhea in young children and moderate gastrointestinal distress in adults (29, 81, 82). Such tropism and pathogenesis is faithfully recapitulated in RV-infected mice making the mouse model of RV useful for studying basic aspects of RV immunity and disease pathophysiology. Further, the RV mouse model may prove a useful platform for discovery of novel means to treat and prevent RV infection, especially in scenarios when adaptive immunity, which normally plays an essential role in clearing RV, is not functioning adequately. Toward this end, we previously reported that administration of bacterial flagellin rapidly cured, and/or protected against, RV infection. Such protection was independent of interferon and adaptive immunity and dependent upon generation of both toll-like receptor 5 (TLR5)-mediated IL-22 and NOD-like receptor C4 (NLRC4)-mediated IL-18, which together, resulted in prevention and/or cure of RV infection, and its associated diarrhea (62). However, the mechanisms by which these cytokines impede RV infection remained unknown and hence was the focus of this study. Herein, we report that IL-22 acts upon IEC to drive proliferation, migration, and ultimately extrusion of infected IEC into the intestinal lumen while IL-18 drives rapid necrotic/pyroptotic death of RV-infected IEC. Together, such actions of IL-22 and IL-18 eliminate RV from the intestine independent of adaptive immunity.

4.3 RESULTS

4.3.1 IL-22 AND IL-18 ACTIVATE THEIR RECEPTORS ON EPITHELIAL CELLS TO PROTECT AGAINST ROTAVIRUS

We previously reported that systemic administration of bacterial flagellin elicits TLR5-mediated production of IL-22 and NLRC4-mediated generation of IL-18 that can act in concert to prevent or treat rotavirus (RV) and some other enteric viral infections (62). Specifically, as
shown in Figure 5A and our previous work, the chronic RV infections that developed in RV-inoculated immune-deficient C57BL/6 Rag-1\(^{-}\) mice were cured by combined systemic treatment with IL-18 and IL-22 while injection of either cytokine alone reduced RV loads but did not clear the virus, regardless of cytokine dose and duration of administration. While in these particular experiments, RV infection was assayed by measuring fecal RV antigens by ELISA; assay of RV genomes in the intestine yields very similar results (62). In WT mice, while sufficiently high doses of recombinant IL-22 can, by itself, fully prevent RV infection, at lower doses exogenously administered IL-22 and IL-18 reduced the extent of RV infection, the combination of these cytokines eliminated evidence of infection (Figure 5B). The central goal of this study was to elucidate mechanisms by which these cytokines act in concert to treat and prevent RV infection.

In the context of parasitic infection, both IL-18 and IL-22 promote expression of each other and loss of either impairs immunity to Toxoplasma gondii (26). Hence, we hypothesized that administration of IL-18 might impede RV primarily as a result of its previously reported ability to induce IL-22 expression. This hypothesis predicted that ability of IL-18 to protect against RV infection would be largely absent in IL-22\(^{-}\) mice. However, that administration of IL-18 upon RV inoculation clearly reduced the extent of RV infection in IL-22\(^{-}\) mice argued strongly against this hypothesis (Figure 5C). Next, we considered the converse hypothesis, namely that IL-22 might impede RV infection by elicitation of IL-18 but, analogously, observed that recombinant IL-22 markedly prevented RV infection in IL-18\(^{-}\) mice (Figure 5D). Thus, while IL-18 and IL-22 may well play important roles in inducing each other’s expression, our results indicate that they also each activate distinct signaling pathways that cooperate to impede RV infection.
Next, we examined the extent to which IL-18 and IL-22 acted upon the hematopoietic or non-hematopoietic compartment to impede RV infection. We used WT, IL-18-R\(^{-/-}\), and IL-22-R\(^{-/-}\) mice to generate irradiation bone marrow chimeric mice that expressed the receptors for IL-22 or IL-18 in only bone marrow-derived or radioresistant cells. Such mice were inoculated with RV, treated with recombinant IL-22 or IL-18, and RV infection monitored via measuring fecal RV antigens by ELISA. Mice that expressed the IL-22 receptor only in bone marrow-derived cells were not protected from RV infection by IL-22 (Figure 6A), whereas mice with IL-22 receptor only in radioresistant cells were strongly protected by this cytokine (Figure 6B). These results suggest that IL-22 protects mice from RV infection by acting on IEC, which is known to be populated from radioresistant stem cells and responsive to IL-22 (83). In accord with this notion, we observed that multiple IEC cell lines are responsive to IL-22 in vitro via STAT3 phosphorylation although IL-22, like flagellin and IL-18, did not impact RV infection in vitro (Figure S22). Studies with IL-18-R chimeric mice similarly revealed that expression of this receptor in only bone marrow-derived cells conferred a modest reduction in the extent of RV infection upon IL-18 administration (Figure 6C) although the impact of IL-18 on RV infection was clearly more evident in mice that expressed IL-18-R in only radioresistant cells (Figure 6D), likely IEC. Together, these results that agonizing IL-18 and IL-22 receptors on IEC result in generation of signals that impede RV in vivo but not in vitro.

### 4.3.2 IL-22 AND IL-18 PROMOTES IEC PROLIFERATION/MIGRATION

In cell culture and organoid models, IL-22 promotes IEC proliferation, migration, and stem cell regeneration (84-86), which together are thought to contribute to ability of IL-22 to promote healing in response to an array of insults including exposure to radiation and dextran
sodium sulfate (DSS) in vivo (25, 87-89). In contrast to such severe injuries, RV infection is generally characterized by a lack of overt intestinal inflammation (90, 91). Nonetheless, we envisaged that promoting IEC proliferation and/or migration, IL-22 might reduce extent of RV infection by increasing the rate of turnover of IEC, especially cells near villus tips, which is the predominant site of RV infection (29, 81, 82). We further reasoned that, perhaps IL-18 might share such actions and thus would further increase IEC proliferation and turnover. To begin to examine these possibilities, mice were administered BrdU and treated with IL-22 and/or IL-18. Sixteen hours later, mice were euthanized and intestines subjected to fluorescence microscopy to measure rates at which IEC migrated toward villus tips, from where they are extruded into the lumen (92). In accord with our hypothesis, administration of IL-22 approximately doubled the rate at which IEC migrated toward villus tips (Figure 7, A and B). IL-18 administration also increased rate of IEC migration albeit to a lesser extent. Yet, the combination of these cytokines did not result in a faster rate of IEC migration relative to IL-22 alone. Epidermal growth factor (EGF) is known to promote IEC proliferation and migration (93, 94). Hence, we next tested whether this cytokine might protect against RV infection. We observed EGF indeed had ability to reduce extent of RV infection (Figure 7C). Together these results support the hypothesis that promoting IEC replication and migration contributes to ability of IL-22 and IL-18 to protect against RV infection but was not able to explain the ability of these cytokines to work cooperatively toward this end.

We next considered how promoting IEC proliferation might impede RV. One seemingly likely consequence of increased IEC proliferation/migration might be increased extrusion of IEC into the lumen, which is thought to occur such that cells remain alive until extrusion is completed thus allowing the gut barrier to not be compromised (95). Hence, we hypothesized that increased
proliferation/migration induced by IL-22 and/or IL-18 treatments might result in increased extrusion of villus tip cells, which are the site of RV infection. We first investigated this hypothesis by an approach used by others (96), namely examining cross sections of H&E stained pieces of ileum for visual evidence of cell shedding, but found it difficult to distinguish IEC from other luminal contents (data not shown). Therefore, we sought to visualize such cells via a DNA stain, DAPI. While this approach suggested greater presence of IEC in the lumen of mice treated with cytokines, particularly IL-22 (Figure 8A), it was difficult to quantitate such a difference via cell counting. Hence, analogous to approaches used to quantitate gut bacteria via their 16s DNA, we sought to evaluate levels of host cells via qPCR of 18s DNA in the ileum. While the highly degradative environment of the intestine would likely degrade IEC shed into the lumen, we reasoned that since such cells are extruded in a relatively intact state, their DNA might survive long enough to enable quantitation by qPCR. Hence, as detailed in Methods, small intestinal contents were extracted and 18s DNA quantitated and expressed as number of cells per 100 mg of luminal content using known numbers of mouse epithelial cells to generate a standard curve. This approach indicated that, indeed, IL-22 treatment markedly increased the level of IEC present in the lumen (Figure 8B) indicating increased IEC shedding. IL-18 induced only a modest level of IEC shedding that appeared to be additive to the shedding induced by IL-22. A generally similar pattern was observed in the cecum (Figure 8D). In contrast, these cytokines did not impact levels of 18s DNA present in the lumen of the colon (Figure 8E), perhaps reflecting that the impact of these cytokines on IEC shedding is specific to the ileum/cecum and/or that the DNA of shed IEC is quickly degraded in the bacterial-dense colon. An even greater amount of shedding of IEC into the ileum was induced by treating mice with flagellin although 2 treatments of IL-18/22 could match this level suggesting that production of these cytokines might be
sufficient to recapitulate the IEC shedding (Figure 8C) induced by flagellin. Moreover, use of IL-22<sup>-/-</sup> and IL-18<sup>-/-</sup> mice revealed that these cytokines, both of which are necessary for flagellin’s anti-RV action [5], were both absolutely necessary for flagellin-induced cell shedding (Figure 8F). Collectively, these results support the notion that increased extrusion of IEC, particularly in response to IL-22 might be central to this cytokine’s ability to impede RV infection but did not offer much insight into how IL-22 and IL-18 cooperate to offer stronger protection against this virus.

### 4.3.3 IL-18 INDUCES DEATH OF RV-INFECTED IEC.

Next, we examined how IL-22 and IL-18 might impact IEC in the absence and presence of an active RV infection. Initially, we sought to use the chronic RV infection model but the very high variance of RV levels within such animals made this approach hard to interpret (data not shown). Hence, we utilized WT mice that had been infected with RV on day 3 post-inoculation, a time approaching peak levels of RV shedding (Figure 5B). Such RV infected mice (or uninfected) mice were administered IL-22 and/or IL-18, euthanized 6 h later, and small intestinal content isolated. Like IL-18/22 administration, RV infection, by itself, upregulated IEC extrusion with a marked further increase in IEC extrusion being observed by administration of IL-18/22 to RV-infected mice (Figure 9A). This suggests that increased IEC extrusion may normally contribute to innate defense against RV [2] and that exogenously administered IL-18/22 (or flagellin) enhance this protective mechanism. Yet, like the case in uninfected mice, the promotion of IEC extrusion seemed driven by IL-22 and not IL-18 (Figure 9B).

Next, we sought to investigate events in IEC that remained part of the small intestine at the time of increased IEC extrusion. Specifically, we sought to examine if IL-18 and/or IL-22
might impact signals associated with necrotic/pyroptotic, cell death. First, we assayed levels of cleaved caspase-3, which is known to drive such cell death pathways by SDS-PAGE immunoblot. We observed that IL-18/22 and RV, by themselves, induced modest and variable induction of Cleaved Caspase3 while these cytokines induced marked induction of Cleaved Caspase3 when administered to RV-infected mice (Figure 9C). Such induction of Caspase3 was observed in response to IL-18 but not IL-22 (Figure 9D). Quantitation of cell death by TUNEL staining also indicated that both IL-18/22 and RV by themselves resulted in an increase in TUNEL-positive cells with an approximate 2-fold further elevation being observed when IL-18/22 was administered to RV-infected mice. Yet, the localization of the TUNEL-positivity was quite striking. Specifically, IL-18/22 and RV by themselves resulted in sporadic TUNEL-positive cells throughout the villus whereas administration of IL-18/22 resulted in striking TUNEL-positivity at the villus tips, which is the primary site of RV infection (Figure 9, E to G). Analogous to the Cleaved Caspase3 activity, striking induction of TUNEL-positivity at the villus tips in RV-infected mice was seen in response to IL-18 but not IL-22. These results suggest that IL-18 might impede RV infection by causing death of RV-infected cells.

4.3.4 IL-18 INTERRUPTS VIRAL REPLICATION

Lastly, we examined the extent to which IL-22-induced IEC extrusion and IL-18-induced IEC death associated with RV reduction in the ileum at 6h and 24h following administration of these cytokines. Specifically, we measured, in both the lumen and IEC, levels of RV genomes and the ratio of +/− RV strands, which reflects levels of active replication since most + strands serve in generation of RV proteins and do not get incorporated into RV virions (97). In accord with our previous work, we observed that in the epithelium, both IL-22 and IL-18 led to a clear
reduction in both the level of RV genomes and RV replication by 6h (Figure 10, A and B). In contrast, in the small intestinal lumen, there was a marked, albeit variable, increase in the level of RV genomes and a stark increase in RV +/− strand ratios 6h following administration of IL-18, the combination of IL-18 and IL-22, but not IL-22 by itself (Figure 10, C and D). By 24h, levels of RV in the lumen had dropped dramatically while the miniscule levels of remaining virus appeared to not be actively replicating (Figure 6, E and F). Collectively, these results support a model wherein IL-18-induced cell death interrupts active RV replication, spewing incompletely replicated virus into the lumen while IL-22 induces IEC migration and subsequent extrusion of the mature IEC that RV targets, thus together working in concert to resolve RV infection.

4.4 DISCUSSION

The central focus of this study was to determine the mechanism by which IL-18 and IL-22, which are elicited via bacterial flagellin, cures, and/or prevents, rotavirus infection. We initially considered the possibility that the ability of IL-18 and IL-22 to promote each other’s expression allowed them to cooperate to promote RV clearance by a common mechanism. However, we found that, irrespective of such mutual promotion, IL-18 and IL-22 both impeded RV independent of each other and did so by distinct mechanisms. Specifically, IL-22 drove intestinal epithelial cells (IEC) proliferation and migration toward villus tips, which resulted in increased extrusion of highly differentiated IEC that serve as the site of RV replication. In contrast, IL-18 induced pyroptotic death of RV-infected IEC thus directly interrupting the RV replication cycle and causing a rapid drop in levels of RV-infected IEC. We conclude that, together, these actions result rapid and complete expulsion of RV, even in hosts with severely compromised immune systems.
RV does not induce detectable increases in IL-22 expression nor does genetic deletion of IL-22 appear to markedly augment RV infection (62), thus arguing that IL-22 does not normally play a major role in clearance of this pathogen. Nonetheless, the downstream action of IL-22, particularly its promotion of IEC turnover may be shared by endogenous anti-RV host defense mechanisms. While the role of adaptive immune-independent host defense against RV is most easily appreciated in immune compromised mice wherein RV loads decline markedly from their peak levels, it may also play a role in protecting against RV even in immune competent mice. While innate host defense against RV is likely multifactorial, and may involve type III interferon (29), our observation that RV infection increases IEC extrusion, combined with previous observation that RV infection activates intestinal stem cell proliferation suggests a role for increased IEC turnover in limiting RV infection (81). Hence, we presume that IL-22 is but one means, albeit a potent one, of activating a very basic primitive mechanism of host defense against a variety of challenges.

IEC are highly rapidly proliferating cells with average lifetimes of about 3 days (97). Hence, the intestine must continuously eliminate vast numbers of cells. The overwhelming majority of IEC are eliminated via cell extrusion at villus tips via a process termed anoikis. A central tenet of anoikis is that cells remain alive at the time of extrusion followed by the lack of attachment to other cells resulting in induction of a programmed death process (39). A key aspect of this process is that it permits elimination of cells without comprising gut barrier function and thus avoiding infectious and inflammation that might otherwise result therefrom. Accordingly, administration of IL-22 is associated with few adverse effects and in a variety of scenarios shows clear ability to resolve inflammation (98). It is possible that increasing anoikis via IL-22 results in extrusion of RV-containing cells in a manner that prevents viral escape and, consequently,
infection of other IEC. However, IL-22’s lack of induction of a detectable increase in luminal RV argues against this possibility. Rather, we envisage that the cell death process that follows IEC extrusion might result in destruction of RV in those cells. Additionally, and/or alternatively, we hypothesize that the accelerated IEC turnover induced by IL-22 results in villus IEC being less differentiated and thus less susceptible to RV infection. In accord with this possibility, we’ve observed that flagellin administration resulted in an IL-22-dependent increase in CD44+26-IEC (Figure. S23), which are known to be RV-resistant (99). While it is difficult to discern the relative importance of IL-22’s induction of IEC extrusion versus its impact on differentiation state of villus IEC, that IL-22-induced reduction in RV levels in chronically infected Rag-1−/− mice occurs over a course of several days supports a role for the latter mechanism. Use of IL-22 receptor bone marrow chimera mice demonstrated that this cytokine’s impact on RV is mediated by its direct impact on IEC (83). IL-22 induced signaling is generally thought to be mediated by STAT3 (62, 89). Accordingly, IL-22 induced phosphorylation of STAT3 in IECs in vivo. However, generation of IECs-specific STAT3-KO mice, in which we verified that STAT3 was faithfully depleted, could still be strongly protected against RV by IL-22 suggesting this aspect of IL-22’s action need not proceed by a characterized signaling mechanism (Figure. S24). Thus, how the IL-22 receptor signals to impact IECs phenotype remains incompletely understood.

In contrast to IL-22, recent work indicates induction of IL-18 plays a role in endogenous innate immunity against RV. Specifically, Zhu et al. demonstrated a role for RV-induced increases in IL-18, mediated by activation of the NLR9pb inflammasome, in mediating clearance of this virus in immune competent mice. Such IL-18 induction correlated with, and was necessary for, gasdermin-dependent pyroptosis, the absence of which resulted in delayed clearance of RV (38, 100). Based on this work, we hypothesize that exogenously administered
IL-18 might enhance RV-induced death of RV-infected cells and/or more generally increase IEC turnover analogous to IL-22. In accord with the latter, administration of IL-18 in the absence of RV elicited a modest increase in the number of TUNEL positive cells as well as a modest increase in IEC proliferation/migration that was not accompanied by increased IEC extrusion suggesting the increased proliferation compensated for cell death. However, such TUNEL positive cells were scattered along the villus rather than being concentrated toward the tips where RV would be located. In contrast, in RV infected mice, IL-18 led to TUNEL positive cells at the villus tips in a manner that strongly implicated pyroptosis in mediating IL-18’s anti-RV effect. These results suggest that induction of IL-18 receptor-mediated signaling by itself is not sufficient to induce cell death in villus tip epithelial cells but rather triggers death only in cells primed as a result of RV infection. The nature of such priming is not understood but could conceivably involve IEC signaling pathways, including NLR9pb, TLR3, and PKR, that are capable of recognizing RV components and/or responding to intracellular stress in general (38, 101, 102). In this context, the ability of IL-22 to enhance IL-18-induced TUNEL positivity in RV-infected might possibly reflect an intersection of IL-22-R and IL-18-R signaling or be a manifestation of these cytokines to promote each other’s expression.

The improved understanding of the mechanism by which IL-18/22 treats RV infection reported herein should inform use of these cytokines to treat viral infection in humans. While chronic RV infections, which occur in immune compromised humans, are one potential use of IL-18/22, there are a number of chronic viral infections in need of additional therapeutic options. Our results suggest that this cytokine treatment would likely be effective for viruses that preferentially infect villus epithelial cells and perhaps epithelial cells with high turnover rates in general. In contrast, this combination of cytokines seems unlikely to impact viruses that inhabit
more long-lived cells, including hematopoietic cells that are generally not responsive to IL-22. In accord with this reasoning, we’ve observed that flagellin and IL-18/22 has some efficacy against reovirus, particularly early in infection when it infects gut epithelial cells, as well as some efficacy against influenza, which initially infects lung epithelial cells, but did not show any impact on hepatitis C virus as assayed in mice engrafted with human hepatocytes (data not shown), which are thought to be long lived cells. Nor did IL-18/22 protect mice against norovirus infection, wherein the virus infects B-cells and tuft cells (103, 104). In contrast, human norovirus is thought to primarily infect epithelial cells, particularly in immunocompromised persons who develop chronic norovirus infections (105). Hence, we envision that chronic rotavirus and/or norovirus infections in person with immune dysfunction might be reasonable targets of IL-18/22-based therapy.

5 CONCLUSION

Rotavirus infections are associated with outbreaks of acute gastroenteritis among young children that result in over 200,000 deaths globally every year (1). Pioneering work achieved by our lab has discovered that flagellin induce innate immune responses in gastrointestinal tract, which successfully prevented and treated rotavirus infections in mice. Such antiviral protection was neither dependent on interferon signaling nor adaptive immunity but rather elicited via activation of Pattern Recognition Receptors (PRRs)-Toll-like receptor 5 (TLR5) and NOD-like receptor C4 (NLRC4). Flagellin-mediated antiviral protection enlightened us to explore novel therapeutic opportunities whereby harness of innate immunity via immunomodulation would combat a broad spectrum of infectious diseases. However, owing to the intricacies of agonist molecules-stimulated innate immune responses, excessive activation or inappropriate
propagation of the immune responses can lead to detrimental inflammation, cause tissue damages and even organs failure. Therefore, my dissertation was focused on gaining an insightful overview of the mechanism by which treatment of flagellin clear the rotavirus infection, and aimed at proposing an efficient therapeutic strategy to fight against rotavirus invasion.

By using genetically modified mice, we found that flagellin performed an agonistic function that interacted with TLR5 in dendritic cells, triggered the secretion of interleukin 23, and Group 3 innate lymphoid cells (ILCs) act downstream of IL-23 by secreting IL-22; meanwhile, flagellin also stimulated NLRC4-mediated inflammasome cytokine IL-18 maturation. Co-administration of IL-22 and IL-18 recapitulated flagellin’s antiviral responses, as treatment of cytokines conferred a complete protection against rotavirus inoculation and cleared established infection within a short period of time. Notably, the immunomodulation that is elicited by the synergistic effects of cytokines could be even more beneficial, since this therapeutic paradigm circumvent the distinct expression and function patterns of NLRC4 between mice and humans (106), therefore cytokine treatment can be introduced as a more competent therapeutic strategy to fight against rotavirus infection.

Intriguing findings presented by Professor Estes and her team showed upon rotavirus infection, ISCs underwent rapid expansion; while differentiated intestinal epithelial cells (IECs) (except Paneth cells) migrated upward along the crypt-villus axis to maintain the epithelial barrier functions, and replaced the infected and damaged enterocytes (81), which suggested an intrinsic linkage between the enhancement of basal cell turnover rate and modulation of host defenses against pathogenic invasion. IL-22, a member of the IL-10 family, is preferentially produced by lymphoid cells, such as T cells, ILCs. ILC-derived IL-22 contributes to the
homeostasis of gastrointestinal tract during pathogenic invasion. Irrespective of undetectable perturbation is displayed in IL-22-ablated naïve mice compared to wild-type counterparts (107); IL-22\(^{-/-}\) mice showed increased susceptibility during enteric pathogenic invasion (26), due to incapability in preventing bacterial translocation, and releasing antimicrobial peptides. IL-22 has also been extensively studied for its critical roles in promoting intestinal stem cell (ISC) proliferation, tissue regeneration, and wound healing (86); herein, my dissertation further demonstrated that IL-22 was required for flagellin-upregulated proliferation of IECs. In addition, exogenous treatment of IL-22 could promote IEC migration frequency, and markedly induce the extrusion of IEC at villus tips. Hence, we presumed that treatment of IL-22 substantiated the host innate defense mechanism against rotavirus invasion in a manner that cleared the infected IEC without disrupting the homeostasis of mucosal barrier. Signal transducer and activator of transcription (STAT)3 is an essential transcriptional factor activated by a variety of cytokines, including IL-22. IL-22-linked STAT3 activation, which believes as the predominating signaling cascade in the gastrointestinal tract, assists epithelial cell proliferation, migration, and mucosal wound healing (86, 108, 109). Notably, IL-22 receptor is preferentially expressed on the mucosal epithelial cells (83, 110); however, mice with depletion of STAT3 in all IECs still elicited full protection against rotavirus inoculation after treatment of IL-22. The data has been documented for the first time that IECs have the capacity to sensitize IL-22, and limit rotavirus multiplication through a STAT3-independent signaling pathway. Future project will delve deeper into the mechanistic understanding of how signaling acts downstream of IL-22. Nevertheless, the approach to study gastrointestinal tract is stymied, due to the diverse biological functions of cellular communication and signal transduction in GI tract, and complex pathology paradigm of rotavirus in vivo (111). Herein, we will adopt an novel technique, which propagate three-
dimensional “mini-intestines”, named enteroids, from isolated intestinal stem cells, and allow us to reconstitute intestinal functions ex vivo to a large extent, and, meanwhile, study enteric virus-host interaction at cellular level.

Interleukin 18, which belongs to the member of the IL-1 family of cytokines, plays a dichotomous role in intestinal homeostasis and inflammation (112-116). Over the past decade, irrespective of the great strides have been made in understanding of IL-18, the mechanism of IL-18 is yet fully elucidated. Zhu et al. showed that mice genetically deficient in Caspase-1 and gasdermin d (Gsdmd) were more susceptible to rotavirus infection (100). The assembly of Caspase-1-associated inflammasome complex promotes the maturation of IL-1β, and IL-18, and cleavage of gasdermin D. Activated gasdermin D causes membrane rupture, extravasation of pro-inflammatory cytokines, and induces pyroptotic cell death. All together, it suggests that inflammasome-regulated IEC pyroptosis restrict rotavirus infection in vivo. Exogenous treatment of pro-inflammatory cytokine, IL-18, to acute rotavirus infected mice model also triggered cell death in IEC monolayer where cellular compartment are RV-preferentially targeted. Interestingly, the invading viral pathogen is a prerequisite for IL-18-mediated cell death, as naïve mice that were subjected to the IL-18 treatment, did not show increased cell death within the same time point compared with infected mice model. It is still unclear how IL-18 manages to drive distinct cellular responses upon either homeostatic or viral-induced immunopathological conditions, but conceivable that IL-18 may directly impede RV-evolved strategies that inhibit innate immune responses. Gac et al. noted a potential evasive mechanism of RV in early stage of viral invasion, as rotavirus upregulated mitochondrial superoxide dismutase expression, while inhibited reactive oxygen species (ROS) accumulation inside the infected cells. Elevated intracellular levels of ROS promoted the death of infected cells, whereas superoxide dismutase
successfully prolonged the progression of the cell death by alleviating the rapid production of ROS inside infected cells (117). IL-18-mediated synthesis of ROS has been extensively studied in many diseases models (118, 119); Meanwhile, pretreatment of IL-18BP, a natural antagonist of IL-18, drastically reduced oxidative stress (120), indicating that IL-18 may elicit the protection against rotavirus infection through ROS-induced pyroptotic cell death. Therefore, mechanistic clarification as to the IL-18-mediated pyroptosis will be further examined in future studies.

In summary, my dissertation mainly focused on the functional synergy in respective of mechanistic pathways elicited via IL-22/18 during protection against rotavirus infection. Our current data also suggests that treatment of IL-22/18 has potential in combating a broad rang of virus that also preferentially target on epithelial monolayer with high turnover rate, such as reovirus and influenza virus. On the contrary, the combination of cytokines seems unlikely to prevent virus, such as Hepatitis C virus, which multiply within long-lived hepatocytes. Future studies we will investigate the therapeutic opportunity in immune-compromised hosts.
APPENDICES: LIST OF FIGURES

Appendix A: Figures of Specific Aim I

Appendix A.1: Major Figures of Specific Aim I
Figure 1. Flagellin protects mice from RV infection and diarrhea.

(A and B) Eight-week-old female (A) C57BL/6 mice or (B) Rag1⁻/⁻ mice were orally inoculated with mouse RV, EC strain. Mice were administered 0.2 ml of phosphate-buffered saline (PBS) (vehicle) ± 20 µg of flagellin by means of intraperitoneal injection, and then every other day from 0 to 18 days after inoculation. Feces were collected daily and assayed for RV antigens by means of enzyme-linked immunosorbent assay (ELISA). Results are shown as mean ± SEM [two-way analysis of variance (ANOVA), n = 4 mice, P < 0.001) for (A) and (B). p.i., post-inoculation. (C) Seven-day-old C57BL/6 mice were orally inoculated with RV (supplementary materials). Mice were treated with PBS or flagellin (10 µg) every day from 0 to 9 days after inoculation and monitored for incidence of diarrhea daily (χ² test, n = 11 mice, *P < 0.01). Flagellin-treated mice exhibited significantly reduced duration of diarrhea and days of active diarrhea (Student’s t test, n = 11 mice, *P < 0.01). (D) Seven-day-old mice, treated as in (C), were euthanized 3 days after inoculation. Total RNA of small intestines from those mice were prepared and analyzed for RV NSP3 RNA level (Student’s t test, n = 6 mice, *P < 0.05). (E) Three-week-old Rag1⁻/⁻ mice were inoculated with mouse RV. Three weeks after inoculation, at which point a chronic infection had been established, mice were treated with PBS ± flagellin (20 µg) every 2nd day from 22 to 40 days after inoculation as indicated with red arrows. Feces were collected at indicated days and assayed for RV antigens by means of ELISA. Asterisk indicates that the difference between mice given PBS and flagellin was statistically significant. Results are shown as mean ± SEM (two-way ANOVA, n = 5 mice, P < 0.0001).
Figure 2. Flagellin’s antiviral activity is mediated by TLR5/NLRC4 on dendritic cells.

(A to D) Eight-week-old (A) Tlr5−/−, (B) Nlr4−/−, (C) Tlr5/Nlrc4−/−, and (D) MyD88−/− mice were orally inoculated with mouse RV and administered PBS ± flagellin (20 µg) every other day from 0 to 8 days after inoculation. Feces were assayed for RV antigens by means of ELISA. Results in (A) to (D) are shown as mean ± SEM [Student’s t test, n = 5 mice, P < 0.05 on day 3 in (A) and day 5 in (B)]. Differences between PBS and flagellin groups in (C) and (D) were not significant [two-way ANOVA, n = 4 to 5 mice, P = 0.6361 for (C) and P = 0.3871 for (D)]. (E) CD11c-DTR reconstituted bone marrow chimeras were either untreated or injected with DT at 8 ng/gram body weight once a day for 2 days. Flow cytometry plots show the extent of depletion of DCs (CD45+CD19−MHC class II+CD11c+ splenocytes). Mice were then studied as in (A) to (D). The difference between the PBS and flagellin groups was statistically significant in absence of
DT (two-way ANOVA, n = 4 mice, P < 0.001) and nonsignificant in DT-treated group (two-way ANOVA, n = 6 to 7 mice, P = 0.3821). (F and G) Tlr5/Nlrc4−/− mice were adoptively transferred with 4 × 106 fluorescence-activated cell–sorted DCs (purity > 98.5%) from Tlr5/Nlrc4−/− (F) or WT C57BL/6 (G) mice. Twelve hours later, the mice were studied as in (A) to (D). The difference between the PBS and flagellin groups was statistically significant in (G) (Student’s t test on days 2 to 4, n = 4 mice, P < 0.05) but not (F) (Student’s t test, n = 4 mice, P > 0.05 on all days). (H) Tlr5/Nlrc4−/− mice were adoptively transferred with 20 million magnetic bead–sorted DCs (purity > 95.0%) from WT C57BL/6 mice, and 12 hours later, the mice were studied as in (A) to (D) (Student’s t test on days 2 to 5, n = 4 mice, P < 0.05).
Figure 3. Flagellin-mediated protection against RV infection requires both IL-22 and IL-18.

(A to H) Indicated strains of genetically modified 8-week-old mice were orally inoculated with mouse RV, EC strain. Mice were treated with PBS ± flagellin (20 µg), via intraperitoneal injection, every other day from 0 to 8 days after inoculation. Feces were collected daily and assayed for RV antigens by means of ELISA. The following strain was used in each panel: (A) p40−/−, (B) Rag2/Il2rg−/−, (C) WT C57BL/6 mice treated with IL-17–neutralizing mAb, (D) Il22−/−, (E) WT C57BL/6 mice treated with IL-22–neutralizing mAb, (F) Il1r−/−, (G) IL-18BP TG, and (H) Il18−/− mice. Results in (A) to (H) are shown as mean ± SEM (n = 4 to 6 mice). The difference between mice given PBS and flagellin was statistically significant for (C) and (F) (two-way ANOVA, P < 0.0001) and significant at individual days of (A), (B), (D), (E), (G), and (H) [Student’s t test, P < 0.05 on day 3 in (A), days 7 and 9 of (B), day 5 of (D), day 2 of (E), days 3 and 4 in (G), and days 3 and 4 in (H)].
Figure 4. IL-22/IL-18 treatment prevents and treats RV infection.
(A) Eight-week-old C57/BL6 mice were treated with 0.2 ml PBS (vehicle), 2 µg IL-22, 1 µg IL-18, or 2 µg IL-22 plus 1 µg IL-18 by means of intraperitoneal injection and subsequently inoculated with RV. Such cytokines were administered every other day from 0 to 8 days after inoculation. Feces were assayed for RV antigens by means of ELISA. Results are shown as mean ± SEM. Difference between PBS and IL-22/IL-18 groups were statistically significant (two-way ANOVA, n = 4 mice, P < 0.0001). (B) Rag1−/− mice, chronically infected with RV, were treated with PBS, 10 µg IL-22, 1 µg IL-18, or both on days 24 and 26 after inoculation (indicated with red arrows). Difference between PBS and IL-22/IL-18 groups was statistically significant (two-way ANOVA, n = 4 mice, P < 0.0001). (C) Seven-day-old C57BL/6 mice were orally inoculated with RV. Mice were administered 50 µl PBS (vehicle) or 1 µg IL-22 plus 0.2 µg IL-18 immediately before inoculation, and 1 to 9 days after inoculation, and monitored for incidence of diarrhea daily (χ² test, n = 5 to 6 mice, *P < 0.05), duration and active days of diarrhea (Student’s t test, n = 5 to 6 mice, *P < 0.001). (D to F) Chronically RV-infected Rag1−/− mice were treated with one injection of PBS, PBS containing 10 µg IL-22, 1 µg IL-18, or 10 µg IL-22 plus 1 µg IL-18. (D) Venn diagram representation of significant changes in intestinal epithelial gene expression 3 hours after cytokine treatment. (E) Intestinal levels of RV genomes and replication rates as reflected by NSP3 RNA levels and +/- RV strand ratios at 3 hours (Student’s t test, n = 4 mice, *P < 0.001 for RV genome, *P < 0.05 for RV RNA +/- strand ratio). (F) RV genomes levels at indicated time (Student’s t test, n = 4 mice, *P < 0.0001).
Appendix A.2: Supplementary Figures of Specific Aim I

Figs. S1 to S21

Supplementary Figure 1. Flagellin delays/protects mice from RV infection.
(A and B) Eight-week-old female C57BL/6 mice were orally inoculated with murine RV, EC strain. Two hours prior to infection, mice were given an i.p. injection of 0.2 ml PBS (vehicle) or 0.2 ml of PBS containing 20 µg of flagellin. Feces were collected daily and assayed for RV antigens by ELISA. (A) Mice were administered PBS or flagellin only prior to inoculation with RV. (B) Mice were administered PBS or flagellin on day 0, 2, 4, 6 and 8 days p.i. Levels of fecal RV antigens shown as mean +/- S.E.M. The protection effect of flagellin versus PBS was statistically significant in (A) (Student’s t-test, N=6, P<0.0001, on days 2-4) and (B) (2-way ANOVA, N=6, P<0.001).
Supplementary Figure 2. LPS provides temporary protection against RV infection.

Eight-week-old female C57BL/6 mice were orally inoculated with murine RV, EC strain. Mice were treated with PBS, LPS (10 µg), or flagellin (20 µg), via i.p. injection, every 2nd from day 0-8 p.i. Feces were collected daily and assayed for RV antigens by ELISA. Results are shown as mean +/- S.E.M. The difference was statistically significant between mice given PBS and flagellin (2-way ANOVA, N=5, P<0.001) and not significant between PBS and LPS (2-way ANOVA, N=5, P=0.2147).
Supplementary Figure 3. Flagellin mediates prevention of RV infection without activating RV-specific adaptive immunity.

(A to C) Sera and fecal samples from C57BL/6 mice, inoculated with RV and treated with PBS or flagellin as described in Fig. 1A, were isolated 14 days p.i. and assayed for anti-RV IgG (A) and IgA (B) by ELISA. Fecal samples on day 0, 2, 4, 6, 8, 10 and 14 p.i. were assayed for fecal
IgA (C). The difference between mice given PBS and flagellin was statistically significantly from each other. Statistical analysis are shown as [Student’s t-test, N=6, P<0.001, (A)], [Student’s t-test, N=4, P<0.05, (B)] and [2-way ANOVA, N=5, P<0.001, (C)].
Supplementary Figure 4. Flagellin decreases fecal reovirus shedding in WT C57BL/6 mice. C57BL/6 mice were inoculated per orally with 1-4 X 10^8 PFU of reovirus strain Type 1 Lang (T1L) in 0.2 ml PBS. Mice were i.p injected with PBS or flagellin (20 µg) 2 hours before reovirus inoculation and every second day thereafter. The number of viral genome copies in feces on days 1, 2, 3, and 4 was determined using qRT-PCR using primers specific to the reovirus S4 gene. Results are expressed as the mean +/- S.E.M. Asterisk indicates significant difference between untreated and flagellin-treated mice. (Student’s t-test, N=4, *P<0.05).
Supplementary Figure 5. Flagellin decreases RV antigen in chronically infected \textit{Rag1}^\text{-/-} mice.

(A) Chronically RV-infected \textit{Rag1}^\text{-/-} mice were administered 1 dose of flagellin, euthanized day 1 after flagellin treatment and small intestine immunostained for RV antigens. Representative images are shown. (B to D) Chronically RV-infected \textit{Rag1}^\text{-/-} mice were administered 1 dose of flagellin, euthanized at 3, 6, 12, 24 and 48 hours. (B) Total RNA of small intestines were analyzed for NSP3 RNA level and ratio of positive strand to negative strand of NSP3 by strand-specific qRT-PCR which indicates viral replication rate. (C) Total RNA of liver was analyzed for RV NSP3 RNA level. (D) Total RNA of spleen was also analyzed for RV NSP3 RNA level. Asterisk indicates significant difference between untreated and flagellin-treated mice. [Student t-test, N=3, *P<0.05, for (B), (C) and (D)].
Supplementary Figure 6. Flagellin's antiviral activity requires TLR5 on hematopoietic cells and NLRC4 in either compartment.

(A to F) Indicated bone marrow chimeric mice were inoculated with RV and treated with PBS or flagellin from day 0-8 p.i. Feces were collected daily and assayed for RV antigens by ELISA. Measure of RV antigens in feces, are shown as mean +/- S.E.M. The difference between mice
given PBS and flagellin was statistically significant in (A) (2-way ANOVA, N=6-7, P<0.001) and [(C) to (F)] (2-way ANOVA, N=4-8, P<0.001), and non-significant in (B) (2-way ANOVA, N=5, P=0.4183).
Supplementary Figure 7. Cellular source of flagellin-induced IL-18 and IL-22.

(A) Bone marrow chimeric mice generated from WT and Il18⁻/⁻ mice were treated with flagellin. Sera were collected at 3 hours post treatment and assayed for IL-18 by ELISA (Student’s t-test, N=4-5, *P<0.01). (B and C) CD11c-DTR mice were injected with DT. After 24 hours, the
CD11c-DTR and Rag2/Il2rg−/− mice were treated with flagellin, and, 3 hours post flagellin treatment, sera were assayed for IL-22 (B) and IL-18 (C) by ELISA (Student’s t-test, N=3-4, *P<0.05). Asterisk indicates significant difference between untreated and flagellin-treated mice.
Supplementary Figure 8. Flagellin-treated RV-infected mice exhibit massive neutrophil infiltration in the gut.

(A and B) Rag1−/− mice were orally inoculated with murine RV, EC strain. Mice were treated with PBS or flagellin (20 µg), via i.p. injection, in day 0 and 2 p.i. On day 3 p.i., lamina propria of small intestines and colons were analyzed for neutrophil infiltration by flow cytometry. (A) Scatter plots using CD45, Ly-6G, and CD11b to quantitate relative numbers of neutrophils in each condition/tissue. (B) Mean quantitation of neutrophils ± S.E.M. Induction of small intestinal and colonic neutrophils by flagellin was significant (Student’s t-test, N=4, *P<0.01) for both tissues.
Supplementary Figure 9. Flagellin cures chronically RV-infected, neutrophil-depleted Rag1\(^{-/-}\) mice and prevents RV infection in neutrophil-depleted WT C57BL/6 mice. (A and B) (A) Rag1\(^{-/-}\) mice, which had been chronically infected with RV for 168 days were subjected to antibody-mediated neutrophil depletion beginning day 168 p.i. and then treated with PBS or 20 \(\mu\)g flagellin on day 171 and 173 p.i. Feces were collected on indicated day and assayed for RV antigens by ELISA. Mice were euthanized on day 174 at which time intestinal
tissue was assayed for relative numbers of neutrophils as in Fig. S8. Relative numbers of neutrophils in each condition/tissue and levels of fecal RV antigens shown as mean +/- S.E.M were shown. Differences between PBS and flagellin groups were significant (2-way ANOVA, N=3, P<0.01) beginning 2 days post-treatment. (B) Eight-week-old C57BL/6 mice were treated with neutrophil-depleting antibody 24 hours before RV infection and then once every 2nd day from day 0-8 p.i. The mice were given PBS or 20 µg flagellin 2 hours before RV inoculation. Feces were assayed for RV antigens by ELISA. Differences between PBS and flagellin groups were significant (2-way ANOVA, N=4, P<0.0001).
Supplementary Figure 10. Flagellin-induced antiviral effect is maintained in mice depleted of NK cells.

Fig. S10. Flagellin-induced antiviral effect is maintained in mice depleted of NK cells.

(A and B) C57BL/6 mice were subjected to antibody-mediated NK cell depletion. Two days later, mice were orally inoculated with RV and treated via i.p. injection with PBS or flagellin (20 µg) every 2nd day from day 0-8 p.i. Feces were collected daily and assayed for RV antigens by ELISA. (A) Scatter plots using CD45, NK1.1 and CD11b to quantitate relative numbers of NK cells in each condition. (B) Levels of fecal RV antigens shown as mean +/- S.E.M. The difference between mice given PBS and flagellin was statistically significant (2-way ANOVA, N=4, P<0.001).
Supplementary Figure 11. Flagellin-induced antiviral effects are maintained in mice depleted of macrophages.
(A to C) C57BL/6 mice were subjected to clodronate liposome-mediated macrophage depletion from 4 days before infection. Clodronate liposome-treated mice were orally inoculated with RV and treated via i.p. injection with PBS or flagellin (20 µg) every 2nd day from day 0-8 p.i. Feces were collected daily and assayed for RV antigens by ELISA. (A) Scatter plots using CD45, MHC class II, CD11b and F4/80 to quantitate relative numbers of macrophages in each condition and (B) percentage of decrease of macrophages after depletion was calculated. (C) Levels of fecal RV antigens shown as mean +/- S.E.M. The difference between mice given PBS and flagellin was statistically significant (2-way ANOVA, N=4, P<0.001).
Supplementary Figure 12. Diphtheria toxin does not affect flagellin-mediated protection against RV in WT C57BL/6 mice from RV.

(A and B) Eight-week-old female C57BL/6 mice were treated DT on 2 consecutive days. Twenty-four hours after the second DT treatment, the mice were orally inoculated with murine RV, EC strain. Two hours prior to infection and every 2nd day thereafter from day 0-8 p.i., mice were given an i.p. injection of 0.2 ml PBS (vehicle) or 0.2 ml of PBS containing 20 µg of flagellin. Feces were collected daily and assayed for RV antigens by ELISA. Levels of fecal RV antigens shown as mean +/- S.E.M. The difference between flagellin and PBS was statistically significant (2-way ANOVA, N=5, P<0.001).
Supplementary Figure 13. Flagellin induces intestinal expression of IL-12/IL-23 and IL-22 but not type III IFN.

(A to D) Indicated strains of genetically-modified 8-week-old mice were orally inoculated with murine RV, EC strain. Mice were treated with PBS or flagellin (20 µg), via injection, every other day from 0-8 days p.i. Feces were collected daily and assayed for RV antigens by ELISA. (A)
IFN I R<sup>−/−</sup>, (B) IFNγR1<sup>−/−</sup>, (C) IFN I & II R<sup>−/−</sup>, (D) STAT1<sup>−/−</sup>. Levels of fecal RV antigens shown as mean +/- S.E.M. The difference between mice given PBS and flagellin was statistically significant for (A) to (D) (2-way ANOVA, N=5-6, P<0.0001). (E) Rag1<sup>−/−</sup> mice were administered 20 µg of flagellin and euthanized at indicated time point. Small intestinal mRNA level of indicated genes was measured by qRT-PCR. Data are shown as means +/- S.E.M. Asterisk indicates significant difference between untreated and flagellin-treated mice (Student’s t-test, N=3-6, *P<0.05).
Supplementary Figure 14. IL-22/IL-18 protects WT C57BL/6 mice against high and low levels of RV inocula.

(A to C) Eight-week-old female C57BL/6 mice were orally inoculated with (A) 10¹, (B) 10³ and (C) 10⁵ SD50 of murine RV. Two hours prior to infection and every 2nd day thereafter from day 0-8 p.i., mice were given an i.p. injection of 0.2 ml PBS (vehicle) or 0.2 ml of PBS containing 2 µg of IL-22 and 1 µg of IL-18. Feces were collected daily and assayed for RV antigens by ELISA. Levels of fecal RV antigens shown as mean +/- S.E.M. The protection effect of IL-22/IL-18 versus PBS was statistically significant in all three groups (2-way ANOVA, N=5, P<0.001).
Supplementary Figure 15. Flagellin-induced activation of IL-22 is independent of NLRC4 signaling.

(A and B) WT C57BL/6, TLR5<sup>−/−</sup>, NLRC4<sup>−/−</sup>, and TLR5/NLRC4<sup>−/−</sup> mice were administered 20 µg of flagellin and euthanized at 3 hours. Small intestinal mRNA level of IL-22 (A) and p40 (B) was measured by qRT-PCR. Data are the means ± S.E.M. Asterisk indicates significant difference between PBS and flagellin-treated mice (Student’s t-test, N=4, *P<0.05).
Supplementary Figure 16. IL-22/IL-18 treatment cures chronically RV-infected Rag2/Il2rg<sup>-/-</sup> mice.

Three-week-old Rag2/Il2rg<sup>-/-</sup> mice were inoculated with murine RV. Three weeks following inoculation, mice were treated with PBS, 10 µg IL-22 plus 1 µg IL-18 by i.p. injection on day 24 and 26 p.i. as indicated by red arrows. Feces were collected daily until day 34 p.i. and assayed for RV antigens by ELISA. Results are shown as the mean +/- S.E.M (2-way ANOVA, N=3, P<0.0001).
Supplementary Figure 17. IL-22/IL-18 treatment shortens duration of established diarrhea in young C57BL/6 mice.
(A and B) Seven-day old C57BL/6 mice were orally inoculated with RV as described in Methods. Three days after inoculation, at which time 100% of mice exhibited diarrhea, mice were treated with 50 µl PBS (vehicle) or 50 µl PBS containing 2 µg IL-22 plus 0.2 µg IL-18 by i.p. injection every day from 3-9 days p.i. and monitored for (A) incidence of diarrhea daily (Chi-square test, N=6,7, * P<0.01) and (B) duration of diarrhea after IL-22/IL-18 treatment (Student t-test, N=6, 7, *P<0.05). Asterisk indicates significant difference between untreated and flagellin-treated mice.
Supplementary Figure 18. Flagellin and IL-22/IL-18 treatment does not affect RV infection/replication significantly in cultured intestinal epithelial cells.
(A) Confluent monolayers of intestinal epithelial (HT-29) cells were pretreated with flagellin (100 ng/ml) 2 hours before infecting with rhesus RV at multiplicity of infection of 1.0. At indicated time points, cell lysates were analyzed for the RV antigen VP6, as an indicator of infection/replication, by SDS-PAGE immunoblotting. Data is representative of 5 experiments, which showed similar lack of effect of flagellin on RV infection/replication in vitro. (B) Polarized monolayers of Caco-2 cells, cultured on collagen-coated permeable supports, were basolaterally treated with recombinant human IL-22/IL-18 (100 ng/ml of each cytokine) 1 hour before infection with Rhesus RV at multiplicity of infection of 1.0. At indicated time points cell lysates were analyzed for the RV antigen VP6, as an indicator of infection/replication, by SDS-PAGE immunoblotting. Results are from a single experiment and representative of 3 experiments that showed similar results.
Supplementary Figure 19. Major remodeling of IEC gene expression in vivo by IL-22 but not IL-18.

RNA sequencing data were generated as described in Methods, and the function plotSmear of EdgeR package for R was used to generate plots of the log-fold-changes against log-cpm, for each gene of the mus musculus genome (mm10). Genes with significantly modified expression in the treated group compared to the control group were plotted in red, using exact test function of EdgeR which is conducting tagwise tests using the exact negative binomial test. The horizontal blue lines showed 2-fold changes (N= 3, significance determined as described in Methods).
Supplementary Figure 20. Processes and pathways altered in IL-22 and IL-22/IL-18 treated mice.
RNA sequencing data were generated as described in the full method. Genes with significantly increased expression in treated group compared to control group were determined using exact test function of EdgeR. The list of Ensembl gene IDs obtained was then summarized in biological process (upper panel) and pathway (lower panel) using Panther Classification System. (N= 3, significance determined as described in Methods).
Supplementary Figure 21. IL-22 and IL-22/IL-18 treatment induces cell death in RV-infected IEC.
Chronically RV-infected Rag1−/− mice were treated with 1 injection of PBS, PBS containing 10 µg IL-22, 1 µg IL-18 or 10 µg IL-22 plus 1 µg IL-18. At indicated time, the mice were sacrificed and intestinal epithelial cells IEC were prepared from small intestines. Whole cell lysates from above IEC were analyzed by SDS-PAGE immunoblotting with antibodies to cleaved caspase 3. Left panel shows results of 3 hours after different cytokines treatment and right panel shows results of indicated time points of IL-22/IL-18 treatment. Results are from a single experiment and representative of 3 experiments that showed similar results.
Appendix B: Figures of Specific Aim II

Appendix B.1: Major Figures of Specific Aim II

Figure 5. IL-22 and IL-18 elicit distinct antiviral activities against mRV invasion.
(A) Chronically mRV-infected Rag-1⁻/⁻ mice were intraperitoneally (i.p) administrated with 200 µl PBS (vehicle), 10 µg IL-22, 1 µg IL-18 or 10 µg IL-22 plus 1 µg IL-18 at days 28 and 30 post inoculation as indicated with arrows. The abundance of mRV antigen was detected by enzyme-linked immunosorbent assay (ELISA), and the statistical significance of viral titers was determined by two-way analysis of variance (ANOVA) (n=4-5, P<0.0001). (B) 4 groups of adult C57BL/6J mice were challenged with a dose of either PBS, 2 µg IL-22, 1 µg IL-18 or both cytokines by means of intraperitoneal injection, 2 hours prior to mRV inoculation. Cytokines treatments were administered every other day from day 0 to 8 post infection. Experiment results shown among groups were significantly different (two-way ANOVA, n=4, P<0.0001). (C and D) Genetically modified mouse strains were orally inoculated with mRV. Cytokines were administrered to mice 2 hours prior to inoculation, and thereafter every other day till day 8 via i.p injection. IL-22⁻/⁻ mice were treated with IL-18 (C), while IL-18⁻/⁻ mice were treated with IL-22 (D). The difference between mice given PBS and cytokine was statistically significant for (C) and (D) (two-way ANOVA, n=5-8, P<0.0001).
Figure 6. Both IL-22 and IL-18-mediated antiviral pathway requires non-hematopoietic cell compartment.

Bone marrow chimeric mice from panel (A to D) were inoculated with mRV. Cytokines were given to mice 2 hours prior to inoculation, and thereafter every other day till day 8 via i.p injection. Feces were collected daily and assayed for mRV antigens by ELISA. Statistical evaluation was performed by two-way ANOVA. Lack of cytokines’ receptor expressed on non-hematopoietic cell compartment largely compromised the antiviral effect, as non-significant differences were discovered between PBS and cytokine-treated group (A) (two-way ANOVA, n=5-8, P=0.7715) and (C) (two-way ANOVA, n=6-8, P<0.0001), whereas chimeric mice groups (B) (two-way ANOVA, n=6-8, P<0.0001) and (D) (two-way ANOVA, n=6-8, P<0.0001) with consecutively expressed cytokines’ receptor on non-hematopoietic cells compartment remain their protection against mRV infection.
Figure 7. Accelerated proliferation rate and migration levels of IEC are correlated with debilitation of mRV infectivity.

(A and B) Adult C57BL/6 mice were i.p injected with PBS, or 10 µg IL-22, 2 µg IL-18 either alone or both, following BrdU administration 1-hour post cytokine treatment. All mice were sacrificed together post 16-hour BrdU administration. (A) Immunohistochemistry of anti-BrdU allowed visualization of positive BrdU-labeled IEC. (B) Sections were scored at least from 50 villus per group of mice (n=5). Distance of the foremost migrating cells along the crypt-villus axis were measured with ImageJ software. Results are presented as mean ± SEM. Statistical significance was evaluated by one-way ANOVA (****P<0.0001). (C) Adult C57BL/6 mice were i.p injected with PBS, or 10 µg murine EGF 2 hours prior to mRV inoculation on day 0, and thereafter every other day from day 2 to 8 p.i. Feces were collected from both groups of mice daily, and assayed for detection of RV antigen by means of ELISA. Levels of mRV shedding are shown as mean ± SEM. The difference between PBS and mEGF-treated groups of mice were significant (two-way ANOVA, N=5, P<0.0001).
Figure 8. IL-22 and IL-18 mediate the elevated frequency of cell extrusion and shedding

Adult mice were i.p given 10 μg IL-22, 2 μg IL-18, 10 μg IL-22 plus 2 μg IL-18 or 15 μg FliC, respectively. Following 8-hour post cytokine(s) or FliC treatment, small intestines and luminal content from small intestines, cecum and colon were collected from mice. (A) Immunohistochemistry of C57BL/6 small intestinal sections that were counterstained with DAPI allowed visualization of shedding cells from luminal side. (B to F) Luminal content from the different regions of the gastrointestinal (GI) tract was detected for host DNA level of 18s by qPCR. Luminal content was collected from various regions of GI tract in each panel: (B, C and F) small intestine, (D) cecum, (E) colon. (B to E) Adult C57BL/6 mice were i.p injected with either cytokines or FliC (administration of double doses of IL-22 plus IL-18 with 12 hours interval). Statistical significance was showed in panel (B), (C) and (D) (one-way ANOVA, n=5-15, *P<0.05, **P<0.01, ****P<0.0001), while significant difference was absent in group experiment (E). (F) The following strains of mice including C57BL/6, IL-22−/−, and IL-18−/− were subjected to 15 μg FliC treatment. Blockade of IEC shedding rate was commitment with the ablation of IL-22 or IL-18 signaling (one-way ANOVA, n=5, ***P<0.001; n.s., not significant).
Figure 9. Accelerated IEC apoptosis is concomitant with the treatment of IL-18.

Both infected and uninfected adult C57BL/6 mice were i.p given PBS, 10µg IL-22, 2 µg IL-18 or 10 µg IL-22 plus 2 µg IL-18. Administration of cytokine(s) to mRV-infected mice was on day 3 post mRV inoculation. Following 6-hour PBS or cytokines treatment, a small portion of the proximal jejunum as well as the whole luminal content from the small intestines were collected from the mice, while the rest of the small intestine was harvested to isolate the IEC. (A and B) the abundance of host DNA level of 18s from luminal content was quantified by q-PCR (one-way ANOVA, n=5, *P<0.05). (C and D) Whole cell lysates from IEC were analyzed by SDS-PAGE immunoblotting for detection of cleaved caspase 3. (E and F) Immunohistochemistry of TUNEL allowed visualization of apoptotic cells along the crypt-villus axis, and cell nucleus were counterstained with DAPI. (G and H) Sections were scored at least from 30 villus per group of mice, and enumerated for TUNEL-positive cells (Student’s t test, n=5, *P<0.05, ****P<0.0001).
Figure 10. Administration of IL-18 rapidly releases the replicating virus into the luminal side.

mRV-infected adult C57BL/6 mice were i.p injected with PBS, 10μg IL-22, 2 μg IL-18 or 10 μg IL-22 plus 2 μg IL-18 on day 3 post virus inoculation. Following 6-hour PBS or cytokines treatment, the mRV genome were extracted from the isolated small intestinal epithelial cells as well as the whole luminal content. The abundance of virus genome is reflected by NSP3 RNA levels, meanwhile the efficiency of viral replication is represented as the excess copy number of NSP3 (+) RNA strand over complimentary NSP3 (-) RNA strand. (A and B) The overall mRV genome and efficacy of virus replication in small intestinal epithelial cells. (C to F) The overall mRV genome and efficacy of virus replication in luminal content from small intestine (Student’s t test, n=5-10, *P<0.05, ****P<0.0001).
Supplementary Figure 22. IL-22 and IL-18 couldn’t alter RV infection in cultured IEC.
The basolateral side of the polarized Caco-2 cells were stimulated with 0.5 μg/ml IL-22 plus 0.25 μg/ml IL-18, 1.5 hours prior to expose to Rhesus Rotavirus (RRV) infection. Trypsin-pretreated RRV was added to the upper chamber of the Transwell plates and allowed for adsorption at 37°C for 40 min before washed with serum-free medium (SFM). The presence of IL-22 and IL-18 were maintained constant throughout the experiment. Cell lysates were collected at indicated time points and analyzed for the virus antigen VP6 and phosphorylation of STAT3. Administration of IL-22 and IL-18 successfully induced phosphorylation of STAT3, however, cytokine treatment didn’t alleviate RRV infection (Student’s t test, n=5, *P<0.05).
Supplementary Figure 23. Flagellin-mediated changes of cell subpopulations along intestinal villus-crypt axis.

(A to D) C57BL/6J, TLR5^/-/NLRC4^/-, IL-22^/-, and IL-18^/- mice were treated with PBS ± flagellin (20 µg) via intraperitoneal injection. Following 24-hour PBS or flagellin administration, IEC were isolated from the small intestines. Debris and doublets were excluded by sequential gating on SSC-width vs. SSC-area, followed by FSC-width vs. FSC-area. Dead cells were excluded from alive ones based on succinimidyl esters (NHS ester)-Alexa Fluor 430. The isolated IEC (CD326^+CD45^-) were separated for CD26, a marker enriched in cell subpopulation that are susceptible to rotavirus infection, and CD44, is highly expressed in cells that are resistant to rotavirus infection. (A) Scatter plots using CD26 and CD45 to quantitate the percentage of IEC subsets (CD326^+CD45^-) in each condition from different mice strains. The difference of IEC (CD44^+CD26^-) subsets between the PBS and flagellin groups was statistically significant in WT C57BL/6j, while nonsignificant in TLR5^/-/NLRC4^/-, IL-22^/- and IL-18^/- groups (Student’s t test, n=5-10, *P<0.05).
Supplementary Figure 24. IL-22 and flagellin-mediated virus control is independent of STAT3 signaling.

(A to C) IECs from the ileum of STAT3ΔIEC and STAT3flox/flox were isolated and assessed for expression of total Stat3 and phophos-Stat3 (Tyr705) by Western Blot. Stat3 was specifically depleted from Stat3ΔIEC mice compared to STAT3flox/flox littermate controls, and IL-22-activated phosphorylation of STAT3 was abrogated in Stat3ΔIEC as well. (D) STAT3ΔIEC and STAT3flox/flox were both inoculated with mRV, and feces were collected daily and assayed for mRV antigens by ELISA. Lack of Stat3 signaling didn’t increase the susceptibility to mRV infection. (E to G) PBS, FliC or IL-22 were given to mice on day 0, 2 hours prior to mRV inoculation, and thereafter every other day from day 2 to 8 post infection via i.p infection. Administration of FliC fully protected STAT3flox/flox mice against mRV invasion (E), Whereas FliC (F) or IL-22 (G) largely remained their protection against mRV in mice lacking of STAT3 signaling (two-way ANOVA, n = 5-6, P < 0.0001).
REFERENCE


27. Y. Wang, J. B. Mumm, R. Herbst, R. Kolbeck, Y. Wang, IL-22 Increases Permeability of Intestinal Epithelial Tight Junctions by Enhancing Claudin-2 Expression. *Journal of immunology* 199, 3316-3325 (2017); published online EpubNov 1 (10.4049/jimmunol.1700152).


10 family. The Journal of biological chemistry 284, 20869-20875 (2009); published online EpubJul 31 (10.1074/jbc.M109.002923).

35. S. Nordlander, J. Pott, K. J. Maloy, NLRC4 expression in intestinal epithelial cells mediates protection against an enteric pathogen. Mucosal immunology 7, 775-785 (2014); published online EpubJul (10.1038/mi.2013.95).


46. J. Q. Jiang, X. S. He, N. Feng, H. B. Greenberg, Qualitative and quantitative characteristics of rotavirus-specific CD8 T cells vary depending on the route of infection. Journal of virology 82, 6812-6819 (2008); published online Epub Jul (10.1128/JVI.00450-08).


A. Berard, K. M. Coombs, Mammalian reoviruses: propagation, quantification, and storage. *Current protocols in microbiology* Chapter **15**, Unit15C 11 (2009); published online EpubAug (10.1002/9780471729259.mc15c01s14).


71. F. A. Carvalho, I. Nalbantoglu, J. D. Aitken, R. Uchiyama, Y. Su, G. H. Doho, M. Vijay-Kumar, A. T. Gewirtz, Cytosolic flagellin receptor NLRC4 protects mice against mucosal
and systemic challenges. *Mucosal immunology* 5, 288-298 (2012); published online EpubMay (10.1038/mi.2012.8).


73. M. Vijay-Kumar, F. A. Carvalho, J. D. Aitken, N. H. Fifidara, A. T. Gewirtz, TLR5 or NLRC4 is necessary and sufficient for promotion of humoral immunity by flagellin. *European journal of immunology* 40, 3528-3534 (2010); published online EpubDec (10.1002/eji.201040421).


79. B. Chassaing, R. E. Ley, A. T. Gewirtz, Intestinal epithelial cell toll-like receptor 5 regulates the intestinal microbiota to prevent low-grade inflammation and metabolic syndrome in mice. *Gastroenterology* 147, 1363-1377 e1317 (2014); published online EpubDec (10.1053/j.gastro.2014.08.033).


117. M. Gac, J. Bigda, T. W. Vahlenkamp, Increased mitochondrial superoxide dismutase expression and lowered production of reactive oxygen species during rotavirus infection. Virology 404, 293-303 (2010); published online EpubSep 1 (10.1016/j.virol.2010.05.018).

118. A. Khanna, Interleukin-18, a potential mediator of inflammation, oxidative stress, and allograft dysfunction. Transplantation 91, 590-591 (2011); published online EpubMar 15 (10.1097/TP.0b013e31820d3b02).
