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The Acute Serum Cytokine Response to Norovirus Infection and Illness

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The Acute Serum Cytokine Response to Norovirus Infection and Illness

By

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B.A., Yale University, 2010

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Abstract

The Acute Serum Cytokine Response to Norovirus Infection and Illness

By Kira L. Newman

Importance: Noroviruses (NoV) are a major cause of gastroenteritis worldwide. NoV have a low infectious dose and are environmentally persistent. NoV vaccine development has been limited, in part because human host immune factors that contribute to susceptibility to infection and symptoms are poorly understood.

Objective: This dissertation sought to determine whether immunocompetent adults develop NoV viremia and to describe the human serum cytokine response to experimental human NoV challenge and elucidate differences in immune activation as they corresponded to a variety of clinical response to NoV.

Methods: Using individuals from past NoV human challenge studies, we measured the frequency of NoV viremia among infected individuals. Combining two GI.1 NoV challenge studies, we assembled an age-matched population of 52 individuals, half of which had become infected post-challenge and of which had remained uninfected. We measured the concentrations of 16 serum cytokines and chemokines from pre-challenge and days 1-4 post-challenge for all subjects. We used a variety of statistical methods, including multivariable logistic regression and random effects models.

Results:
Study 1: We did not detect viremia in any of the 20 subjects or 38 corresponding serum samples.
Study 2: NoV infection post-challenge was associated with an elevation of Th1-type cytokines, Th2-type cytokines, and chemokines IL-8 and MCP-1, compared to individuals who remained uninfected post-challenge. Among infected individuals, most serum cytokines peaked on day 2 post-challenge, though IL-10 remained elevated through day 4 post-challenge.
Study 3: Symptomatic NoV infection was associated with greater elevation of Th1-type cytokines, Th2-type cytokines, and IL-8, compared to asymptomatic infection. Elevated daily viral RNA titer was associated with increased IFN-γ, IL-6, IL-8, and TNF-α. Symptoms were not significantly associated with elevated viral RNA shedding.

Conclusions: Though NoV infection of healthy adults was not associated with viremia, infected individuals exhibited brief and broad cytokine responses coincident with the development of symptoms. Symptomatic infection was associated with greater immune activation than asymptomatic infection, though it was not associated with greater viral burden. This suggests that NoV elicits a broad anti-viral response and that symptoms may be partially due to immune-mediated damage. Further work should investigate the pathogenesis of NoV symptoms due to their epidemiologic importance.

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CHAPTER 1: INTRODUCTION, AIMS, AND HYPOTHESES

Introduction

As reviewed in [1], Noroviruses (NoV) are the primary cause of gastroenteritis in developed countries and globally are a significant cause of severe diarrhea in children. NoV are highly transmissible, have a low infectious dose, persist despite common sanitation practices, and have a major economic impact. Because of these qualities, the CDC and NIAID-NIH classify NoV as Bioterrorism Category B Priority Pathogens. Attempts at vaccine development have hitherto failed to yield a commercially available vaccine, in part because very little is known about the impact of human host immune factors that contribute to NoV infection susceptibility. Animal model studies using murine NoV have yielded some insight, but critical differences between murine and human NoV, including cellular hosts and symptoms, make human NoV investigation essential for understanding.

Of special interest is that not all individuals are susceptible to NoV, and of those who do become infected post-exposure, only a subset exhibit clinical symptoms. There is evidence that genetics may contribute to infection susceptibility, but factors associated with asymptomatic response have not been explored. Data suggests that NoV-specific mucosal IgA production may protect genetically susceptible individuals, but NoV-specific mucosal IgA levels are not detected earlier than 3 days after infection. Therefore, it is likely that rapid, innate immune defenses protect the host during the first 24-48 hours after NoV challenge, even before detectable viral shedding occurs.

Dissertation Aims and Hypotheses

Overall hypothesis: differences in innate immunity are responsible for the variety in clinical response to NoV

Study 1:

Specific Aim: Quantify the frequency of NoV viremia among healthy adult volunteers infected with NoV in experimental challenge studies and assess its correlation with viral shedding.

Hypothesis: A subset of symptomatic NoV-infected adults exhibit NoV viremia, coincident with high concentrations of virus shed in stool.

Study 2:

Specific Aim: Analyze and quantify the cytokine and chemokine profiles of serum samples collected longitudinally from volunteers infected with NoV in experimental challenge studies.

Hypothesis: Clinically infected individuals exhibit a rapid cytokine and chemokine response that proceeds the detection of virus in stool or immunoglobulin in blood.

Study 3:

Specific Aim 1: Test the association between cytokine and chemokine profiles and the clinical manifestation of NoV infection.

Hypothesis: Cytokine and chemokine profiles of volunteers differ based on clinical manifestations and are associated with viral shedding.

Specific Aim 2: Assess the relationship between cytokine and chemokine profiles and NoV shedding in stool.

Hypothesis: Cytokine and chemokine profiles suggestive of high levels of immune activation are positively correlated with high concentrations of viral shedding.

Specific Aim 3: Test the association between the clinical manifestation of NoV infection and NoV shedding in stool.

Hypothesis: Symptomatic NoV infection is positively correlated with high concentrations of viral shedding.

CHAPTER 2: LITERATURE REVIEW: OF MICE AND MECHANISMS

Newman KL, JS Leon. Norovirus Immunology: Of Mice and Mechanisms. *European Journal of Immunology*. 2015 Aug 10. doi: 10.1002/eji.201545512. [Epub ahead of print].

Summary

Noroviruses (NoVs) are the most common cause of sporadic and epidemic gastroenteritis in the United States and Europe and are responsible for 20% of acute gastroenteritis worldwide. Over the past decade, the understanding of NoV immunology has grown immensely. Studies of the natural immune response to NoV in humans and animal models have laid the foundation for innovations in cell culture systems for NoV and development of new therapeutics. Evidence from animal models, NoV surrogates, observational human research, and human challenge studies suggest that the innate immune response is critical for limiting NoV infection but is insufficient for viral clearance. NoV may antagonize the innate immune response to establish or prolong infection. However, once a robust adaptive immune response is initiated, the immune system clears the infection through the action of T cells and B cells, simultaneously generating highly specific protective immunologic memory. We review here both the current knowledge on norovirus immunity and exciting new developments, with a focus on ongoing vaccine development work, novel cell culture systems, and advances in understanding the role of the gut microbiome. These changes reinforce the need for a better understanding of the human immune response to NoV and suggest novel hypotheses.

Introduction

Noroviruses (NoV) are the most common cause of sporadic and epidemic gastroenteritis in the United States and Europe across all age groups, and are estimated to be responsible for as much as 20% of all acute gastroenteritis worldwide [2-4]. Infection with this food-borne pathogen causes profuse vomiting and diarrhea, which is typically self-resolving, though among young children, the elderly, and the immunosuppressed, it may lead to more severe or protracted illness [5-8]. In low resource settings, such as many countries in Africa and southeast Asia, NoV and other causes of acute gastroenteritis (e.g. rotavirus, enteropathogenic E. coli) remain a major cause of morbidity and mortality, especially among children [9-13]. There is also growing recognition that NoV infection may be associated with some long-term sequelae, such as post-infection irritable bowel syndrome [14] and exacerbation of inflammatory bowel disease [15].

NoV infections are also a major economic concern. In the United States, food-borne NoV infection is estimated to cost over $2 billion annually in direct and indirect costs [16]. A similar study estimated that in the Netherlands NoV has the highest estimated annual costs of any food-borne pathogen [17]. The collective health and economic burden of NoV infection has led to increasing awareness of the need for better funding of NoV research [18].

In the past decade, there has been a dramatic expansion of NoV research and a corresponding increase in the understanding of NoV pathogenesis and immunology. In particular, there have been exciting recent advances in models of NoV infection and

understanding the cellular immune response to NoV. However until recently, the field has been limited by the lack of cell culture systems and small animal models of human NoV infection. Further, the broad diversity of NoV strains (currently over 30 genotypes of GI and GII NoVs [19]) continues to challenge researchers. Recent reviews have addressed advances in NoV detection [19], molecular virology [20, 21], animal models for NoV research [20], the role of histo blood group antigens (HBGA) in NoV infection [22], and vaccine and antiviral medication development [23-25]. Though some of these topics will be touched upon briefly, the focus of this review is to provide a detailed overview of NoV immunology.

2) NoV structure and models

The unenveloped positive sense RNA virus family of Caliciviridae is divided into five genera: Vesivirus, Lagovirus, Sapovirus, Nebovirus, and Norovirus [26, 27] .
The genus Norovirus is divided into six genogroups (GI-VI; though a seventh has been proposed [19]). Each genogroup contains many numbered genotypes, which are used in conjunction with genogroups, to designate strain types (e.g. GI.1 viruses belong to genogroup I, genotype 1). Humans can be infected by NoVs from genogroups I, II, and IV [19]. The other genogroups generally infect cattle (GIII), mice and rats (GV), and dogs (GVI) [19]. Though some humans have been found to carry antibodies to genogroup VI NoV [28], it is unknown whether this genogroup can cause symptomatic infection.

The NoV genome has three to four open reading frames (ORFs) and is capped on the 5’ end by the virally encoded protein VPg. ORF1 codes for non-structural proteins

(including Vpg), ORF2 codes for the NoV capsid protein (VP1), and ORF3 codes for a minor structural protein (VP2). Murine NoVs (MNV) have a fourth ORF (ORF4), which codes for virulence factor 1 [29]. Traditionally, NoVs have been classified using pairwise distances based on complete VP1 sequences [30], but, due to the diversity of novel NoV strains, ORF1 sequences should be included in order to better identify related strains [31].

Most NoV strains generally infect hosts in a species-dependent manner, which has hindered research on human NoV because of a lack of small animal models. However, many surrogate Caliciviridae have been used in lieu of human NoV (Table 1). Though these surrogate viruses are diverse, much like human NoV, the majority of research has been with specific canonical strains, such as Tulane virus and MNV-1.CW3 (Table 1).

Limitations of the existing in vivo models of human NoV infection include differences in cellular tropism, host-receptor targets, enteric pathology, and mechanism of infection. Until recently [32] in vitro models have faced similar challenges, including the absence of replicable cell culture systems for human NoV [33].

Although it has not yet been replicated or used in other studies, a recent study reported modest replication of human NoV in the human BJAB B-cell line in vitro [32]. The cell culture system built on an earlier discovery that some bacteria produce histo-blood group antigen (HBGA)-like substances on their surfaces, which can bind human NoVs [34]. HBGAs are present on the surface of erythrocytes and intestinal epithelial cells. NoVs bind HBGAs in a strain-dependent manner, with certain strains only binding to specific HBGAs [35]. HBGA-blocking antibodies have been shown to prevent NoVs from binding to HBGAs, which may reduce NoVs' ability to enter host cells (reviewed in

[22, 36]). Jones et al. [32] co-cultured BJAB cells with killed *Enterobacter cloacae*, a bacterium that expresses H-, A-, and B-type HBGAs on its surface [34], to grow GII.4-Sydney NoV. When NoVs bound HBGAs, it allowed for attachment to the surface of B cells, leading to infection. The BJAB model also sustained NoV replication when the cells were cultured with synthetic H antigen [32], suggesting that it may be possible to allow replication of other NoV strains through the addition of different HBGA to the culture. The researchers also found that NoV in unfiltered stool containing HBGA were able to pass through a polarized epithelial cell barrier to infect B cells in a separate compartment [32], which may be a potential mechanism of in vivo infection (Fig. 1).

There are multiple in vivo models of human NoV infection, but each has substantial limitations. For example, chimpanzees have been shown to be susceptible to GI.1 human NoV, have a similar immune system to humans, and shed virus for a similar duration (duration in GI.1-infected chimpanzees: 23 days [37]; in GI.1-infected humans: 17-28 days [38, 39]), but their infection is asymptomatic and regulatory challenges make widespread use prohibitive [37]. Gnotobiotic pigs and calves can be infected with human GII.4 NoV, and they develop gastrointestinal symptoms, but they shed virus for a shorter period of time (mean duration in GII.4-infected pigs and calves: 4 days [40, 41]; mean duration in GII.4-infected humans: 6+ days [42, 43]) and the financial and technical difficulties of working with these animals limits their availability [40, 44]. BALB/c mice deficient in Rag-γc also are susceptible to human NoV and are more widely available, but they require intraperitoneal inoculation and experience short and asymptomatic infection [45]. These difficulties have led to the use of human challenge studies as a major means of understanding NoV immunology (Table 2) [43, 46-50].

3) NoV infection

Human NoV infection can be symptomatic or asymptomatic. Based on human challenge studies and outbreak data, 15%-35% of all infected individuals are asymptomatic [38, 39, 43, 46, 51]. Symptom onset occurs 24-48 hours after exposure. Individuals are typically afebrile or have a low fever with vomiting and diarrhea [52]. In immunocompetent individuals, diarrhea is non-bloody, has low levels of lactoferrin (a marker of intestinal polymorphonuclear leukocyte inflammation) and few fecal leukocytes relative to bacterial diarrheas [53]. Diarrhea may be caused by epithelial barrier dysfunction and increased anion transport [54]. In immunocompetent individuals, symptoms generally resolve within 24-48 hours but intensity of symptoms may depend on NoV strains. Immunosuppressed individuals and neonates can have more severe and protracted symptoms, including fatal complications [6, 8].

Symptomatic and asymptomatic individuals both shed virus in stool at high levels for extended periods of time following infection [38, 39, 51]. Peak viral RNA titers reach 10^9-10^{12} genomic equivalent copies (GEC)/g stool in symptomatic individuals and may be 1-2 logs lower in asymptomatic individuals [38, 39]. A heterogeneity in the duration of shedding has been shown, with individuals shedding detectable levels of virus for a median of approximately 30 days, though durations as short as 5 or as long as 60 days have been reported in healthy adults [38, 39, 46, 51]. Immunocompromised individuals may be chronically infected and have been documented to shed NoV for years [55].

Histologically, human NoV infection causes alterations of the gut mucosa. Infected individuals may have small lesions of the duodenum with increased enterocyte apoptosis, flattened villi, crypt hypertrophy, mucosal inflammation, and disruption of the epithelial barrier function [54, 56, 57]. This is accompanied by neutrophil and mononuclear cell infiltration of the lamina propria and disruption of absorptive cells [56]. In the epithelium, there is a significant increase in perforin-producing $CD8^+$ intraepithelial lymphocytes (IELs) and a slight increase in $CD4^+$ IELs [54]. Histologic changes have been reported in both symptomatic and asymptomatic individuals, suggesting that asymptomatic individuals may have sub-clinical symptoms [56, 57]. Though the cellular tropism of human NoV remains unknown, the observed changes to epithelial cells and cells in the lamina propria following infection suggest that they may be possible sites of replication [56, 57].

Animal models of NoV infection display a broad range of gut histology, which partially mimic human infection (Table 1). For example, symptomatic AG129 mice infected with MNV-1.CW3 exhibit epithelial necrosis and mild lymphocytic infiltrate [58]. Some gnotobiotic calves and pigs infected with GII.4 NoV develop villous blunting in the duodenum, which appears similar to the pathological changes observed in humans[40, 44, 59]. In these animals, gut epithelial cells and cells in the lamina propria serve as targets for NoV infection [60, 61]. Rhesus macaques inoculated with Tulane Virus have lymphocytic infiltration of the lamina propria and moderate villous blunting [62]. Chimpanzees do not have histopathologic changes to the duodenum or jejunum following infection with GI.1 NoV [37].

4) Norovirus Immunity

Protection from NoV infection has both genetic and immunologic components. The genetic factors associated with protection from NoV infection are well reviewed [22, 63]. Briefly, secretor status is defined by the presence of functional fucosyltransferase 2 (FUT2) alleles, which express α1,2 fucosyltransferase 2, an enzyme that allows individuals with at least one functional FUT2 allele (i.e. secretors or secretor-positive) to express A, B, H-type 1, and Lewis b HBGAs antigens on their mucosal epithelial cells and in secretions [63]. Lack of functional α1,2 fucosyltransferase 2 (i.e. non-secretors or secretor-negative) has been associated with resistance to infection by certain strains of NoV [48, 64, 65]. Individuals who are homozygous recessive for inactivating mutations in the FUT2 gene (i.e. secretor negative) are largely resistant to infection by GI.1 NoV [48] and may also have decreased susceptibility to infection by other genogroups of NoV [43]. Overall, an estimated 70-80% of the population are secretors [66], though secretor genotype varies by ancestry [67].

Immunologic memory also factors into protection from NoV infection. Historically, broadly reactive NoV-specific IgG antibodies were not consistently associated with protection from subsequent NoV infection [68-70]. This may have been because in vitro testing by ELISA for NoV-specific antibodies did not correlate with in vivo ability to block interaction between NoVs and host cells [68] or because of confounding by genetic susceptibility [71]. Confounding by genetic susceptibility may occur because individuals who are genetically susceptible to NoV (e.g. secretor positive), compared to individuals who are genetically resistant, may be more likely to have high antibody titers. Specifically, individuals who are genetically susceptible to NoV,

compared to those who are resistant, likely have been repeatedly infected and therefore developed an adaptive immune response to NoV (i.e. higher titers of NoV-specific antibodies). Some studies have found that strain-specific NoV-blocking antibodies were associated with protection from NoV infection in a strain-dependent manner in humans [72-74] and in non-human primates [37]. For example, a study in chimpanzees suggests that strain-specific IgG may provide long-term resistance to infection by homologous NoV strains [37]. In addition to IgG antibodies, IgA antibodies may also help protect against NoV infection. An early salivary IgA antibody response to NoV challenge has been associated with protection from infection by GI.1 NoV [48]. This was further supported by a recent study by Ramani, et al. comprising a NoV challenge of human volunteers, which found that pre-challenge IgA-producing memory B cells significantly correlated with protection from infection by GII.4 NoV [75].

Researchers have also identified immunologic correlates of protection from symptomatic infection. One of the first described immunologic correlates of protection from symptomatic infection was HBGA-blocking antibodies [68]. HBGA are present on the surface of enterocytes in the mucosal epithelium of the gut. Though the mechanism of NoV entry into host cells is still unknown, HBGA-blocking antibodies prevent NoV from binding to HBGA, which may reduce NoV's ability to enter host cells (reviewed in [22, 36]). Another correlate of protection from symptomatic infection are hemagglutination inhibition (HAI) antibodies, a closely associated group of antibodies [76]. In addition, both HBGA-blocking and HAI antibodies have been proposed as surrogates for measuring virus-neutralizing antibodies, which has been supported by human [68, 76, 77] and non-human primate data [37]. Additional surrogates of protection from illness have

also been proposed. For example, Ramani, et al. found that pre-challenge salivary IgA antibodies and NoV-specific IgG memory B cells were significantly associated with protection from symptomatic NoV infection [75]. As vaccine development continues, the need for reliable correlates of immunity and symptomatic protection remain important areas of future research.

5) **Innate immunity to NoV**

Over the past decade, the understanding of NoV immunology has grown immensely. Studies of the natural immune response to NoV have led to observations that were important for growing NoV in cell culture and to developing therapeutics. Although some work has been conducted in humans, most of the evidence comes from animal models and human NoV surrogates. Based on this work, the innate immune response appears to play a critical role in limiting viral replication and initiating a memory-generating adaptive immune response to NoV (Fig. 1).

5.1 Mouse models

In mouse models of NoV, the innate immune response limits viral replication largely through interferon (IFN)-dependent pathways. MNV infects macrophages and dendritic cells [78], possibly in a manner dependent on microfold (M) cells (Fig. 1), an epithelial cell associated with Peyer's patches and involved in antigen sampling from the gut lumen [79]. In in vivo MNV infection, viruses are recognized by MDA-5, a Rig-I-like helicase, which initiates the innate immune response [80] (Fig. 2). This innate sensing leads to the production of type I and type II IFN by antigen presenting cells, which leads

to the production of pro-inflammatory cytokines through the STAT-1 pathway [81, 82] (Fig. 2). In primary macrophages isolated from wild-type mice, infection induces transcription factors interferon regulatory factor 3 (IRF-3) and IRF-7, which stimulate type I IFN production (IFN-α and IFN-β), though they are not necessary for governing their downstream antiviral effects [83].

This innate response leads to inhibition of viral replication. Type I and type II IFN have been shown to inhibit MNV translation in macrophages and dendritic cells, with type I IFN blocking viral replication in a double-stranded RNA-activated protein kinase (PKR)-independent manner and type II IFN (IFN-γ) arresting it in a PKR-dependent manner [84] (Fig. 2). In the absence of type I interferons, IFN-γ mediates antiviral activity against MNV by activating the autophagy protein (Atg) complex Atg5-Atg12/Atg16L1 (Fig. 2), which inhibits the MNV replication complex in the cytoplasm of infected macrophages but does not act through autophagy-associated degradation [85]. In the absence of IFN-y, type I IFNs may limit MNV replication through the activation of interferon-stimulated gene 15 (ISG15). ISG15 may act to impede an early stage in the MNV life-cycle, such as entry or uncoating [86] (Fig. 2), but IFNs alone are not enough to clear the infection [87]. However the innate response may not occur unimpeded. MNV carries an additional ORF4, encoding virulence factor 1 (VF1), which antagonizes the innate immune response [29]. VF1 delays up-regulation of CXCL10, ISG54, and IFN-β, and therefore enhances viral replication [29]. Interestingly, other NoVs (including human NoVs) do not share this gene, suggesting that human NoV may regulate the immune response through other mechanisms.

In some cases, the innate immune response may help with viral clearance or be exogenously stimulated to do so. Recent work has examined the role of IFN-λ, a type III IFN, in MNV infection. IFN-λ has been shown to be produced by cells in the mesenteric lymph nodes and Peyer's patches of healthy C57BL/6 control mice in response to infection by MNV-1.CW3 [87]. In contrast, MNV.CR6 infection of mice led to persistent infection and there was no initial induction of type III IFN production. However, treating the persistently MNV.CR6-infected mice with exogenous IFN-λ reduced viral persistence in the gut, eventually leading to viral clearance, without initiating an adaptive immune response [87]. Interestingly, antibiotic treatment of mice increased the antiviral effect of IFN-λ, suggesting that the bacterial microbiota may alter the innate immune response to viruses or its efficacy [88].

5. 2) Gnotobiotic pigs

Gnotobiotic pigs are one of the few models with available data regarding their innate immune response to human NoV. Their response to NoV is similar to the response seen in mouse models of MNV. Most animals used are relatively young and may have less developed innate immune systems, rendering them more sensitive to low doses of NoV [89]. Gnotobiotic pigs have less robust neutrophil responses [90], fewer intraepithelial lymphocytes [91], and less developed Peyer's patches [92] with a preponderance of T cells rather than B cells [91]. Nevertheless, they still mount an innate immune response to human NoV, in the form of elevated serum IFN-α, intestinal IFN-α, and serum IFN-γ, though IFN-α does not appear until later in infection [41]. As in wild-type mice infected with MNV, this innate response is important for limiting viral replication in an IFN-dependent manner. Studies have also assessed gnotobiotic pigs

treated with simvastatin, a cholesterol-lowering medication that also inhibits IFN-α production and major histocompatibility complex (MHC) class II-dependent T-cell activation [59, 89]. These animals were more susceptible to NoV infection and shed virus for a longer duration and at higher levels [59, 89]. When gnotobiotic pigs which had not been administered simvastatin were treated with human IFN-α, they shed virus for a shorter duration and at lower levels than gnotobiotic pigs which were not treated with IFN-α or statins did. This result supports the role of IFN-α in controlling viral replication during NoV infection [59].

5.3) Humans

There are limited human data regarding the innate immune response to NoV. Past studies of humans challenged with Norwalk virus (NV—GI.1) and Hawaii virus (GII.1) did not show induction of detectable levels of IFN in sera, jejunal aspirates, or jejunal biopsy specimens taken 48 to 96 hours post-challenge [93]. However, a Snow Mountain virus (SMV—GII.2) challenge study reported a significant rise in serum IFN-γ and IL-2 levels 48 hours post-challenge in infected individuals as compared with that of uninfected controls [47]. This result was supported by a study of fecal cytokines in NoV-infected travelers; they exhibited significantly increased fecal IL-2 and IFN-γ levels in diarrhea specimens [53]. These conflicting data suggest that more research is needed to clarify the nature of the human innate immune response to NoV and whether it differs by strain.

6) **Adaptive immunity to NoV**

Though the innate immune response is important for blocking viral replication through early IFN production, its other key role is in initiating the adaptive immune response. Based on animal models , the adaptive immune response helps clear NoV infection and may generate immunologic memory to prevent reinfection (Fig. 1).

6.1) Mice

While the action of IFNs and the innate immune response inhibits viral replication, adaptive immunity seems critical for viral clearance. B cells and T cells seem necessary to clear NoV infection. B- and T-cell deficient RAG1 and RAG2 knockout mice have been shown to develop persistent or extended MNV infections with high viral RNA titers [81, 94]. B cells are hypothesized to help clear MNV infection through antibody production. In support of this hypothesis, B-cell deficient RAG1 knockout mice that received a transfer of B cells, incapable of producing NoV antibodies, did not clear MNV and were statistically no different from B-cell deficient RAG1 knockout mice [94]. However, RAG1 knockout mice that received infusions of MNV antibodies showed reduced MNV RNA titers [94]. Further, in a study of MNV capsid protein-vaccinated mice, broad T- and B-cell activation, including $CD4^+$ and $CD8^+$ T cells, was necessary for NoV resistance [95], indicating that T cells may also be necessary, alongside antibody-producing B cells, for clearing NoV infections. This multifaceted response is reflected in the serum cytokine response of wild-type mice orally administered MNV [96]. Ten days post-challenge, these mice were shown to have significantly elevated serum levels of IL-1α, IL-1β, IL-2, IL-6, IL-10, IL-12, IL-17-A, IFN-γ, TNF-α, G-CSF, and GM-CSF compared to unchallenged mice [96]. This systemic cytokine response

occurs in the absence of visible histopathologic changes in the gut [96], indicating that an immune response to NoV is not necessarily associated with NoV-induced pathology.

Recent work indicates that a suboptimal NoV-specific $CD8^+$ T-cell response results in persistent MNV infection [97]. Mice with lower levels of activated MNV-specific $CD8^+$ T cells were shown to have long-term MNV infections that they were unable to clear, whereas mice with higher levels of $CD8^+$ T-cell activation were able to clear the MNV infection [97]. Furthermore, RAG1 knockout mice that received a transfer of activated MNV-specific $CD8^+$ T cells had significantly reduced viral loads, indicating that $CD8^+$ T cells are another key component of the adaptive immune response that clears NoV infection [97]. However, to prevent successive NoV infection, antibodies and $CD4^+$ T cells are more important [98]. The presence of MNV-3-specific antibodies or $CD4^+$ T cells were each sufficient for partial protection from reinfection with MNV-3, but $CD8^+$ T cells alone did not provide protective immunity from infection with MNV-3. These situations may be similar to the chronic NoV infection observed in immunocompromised patients with impaired B- and T-cell responses, who are unable to clear the virus [55, 99-101]. Indeed, many transplant recipients are only cured of their NoV infections when their immunosuppressive medication dosing is relaxed [102].

In vitro work supports the importance of the Th1-type response in MNV infection and the production of IFNs in the immune response to NoV. MNV-1 infected murine RAW264.7 macrophages were shown to have a Th1-skewed response, with upregulation of CCL2, CCL3, CCL4, CCL5, CXCL2, CXCL10, and CXCL11 genes [103]. In vivo, some of these chemokines (e.g. CXCL10, CXCL11) can be induced by type I or type II

IFNs and are important for Th1-cell trafficking [104]. Thus, the early innate response may be critical for laying the foundation for later viral clearance.

Interestingly, different MNV strains induce different levels of protective immunity. In wild-type mice, MNV-3 has been shown to be associated with strong homotypic and heterotypic protection against re-challenge by MNV-3 or MNV-1 in a type I IFN-independent and B- and $CD4^+$ T-cell dependent manner [98]. MNV-3 induced significant serum IgG and mucosal IgA antibody responses that were reactive against antigens from both MNV-3 and MNV-1 [98]. However, MNV-1, a more virulent strain, did not induce heterotypic antibody production and led to lower levels of IgG and IgA antibody production compared with that following MNV-3 infection. One hypothesis for this strain-specific difference may be antagonism of the innate immune response. MNV-1 infected RAW264.7 cells exhibited lower levels of IFN-β, TNF-α, and MCP-1 transcripts [29, 98]. MNV-1 may also interfere with maturation of antigen presenting cells, leading to decreased T-cell activation [98]. Some of these effects may be related to MNV strain-specific differences in a protruding region of VP1, a capsid protein, as alterations in the sequences of the genes coding for the P2 region of VP1 have been found to be associated with differences in MNV strain virulence in STAT1 knockout mice [105], however others may be related to strain-specific differences in VF1 [98]. The presence of strain-related differences in MNV virulence and its impact on the immune response suggest that future work should consider whether strain-specific differences are present in human NoVs and impact the human immune response to NoV.

6.2) Gnotobiotic pigs and calves

NoV-infected gnotobiotic pigs and calves exhibit similar innate and adaptive cytokine and antibody responses to that in mice, and may serve as better models of primary NoV infection in humans because of immunologic and physiologic similarities to humans [90] and the ability to be infected with human NoVs (Table 1). NoV-infected gnotobiotic pigs show an early increase in IFN-γ [41], suggestive of an IFN-dependent innate anti-viral response. This is then followed by Th1 and Th2 activation. Specifically, infected animals develop significant elevations in serum IL-4, IL-6, and IL-10 between 2 and 8 days post-challenge [41]. They also exhibit persistently elevated serum IL-12 and intestinal IL-12 post-challenge [41]. Viral clearance in gnotobiotic pigs is associated with antibody responses in the form of low titers of NoV-specific serum IgG antibody [44] and detectable IgM, IgA, and IgG andtibodies in their intestines [41]. These levels of antibodies are positively correlated with the severity of diarrhea [41].

Gnotobiotic calves inoculated with human GII.4 NoV were shown to initially respond similarly to gnotobiotic pigs administered the same inoculum. Namely, the initial innate response appeared to be IFN-mediated, and was followed by serum and fecal cytokine elevations that suggested Th1 and Th2 activation [40]. NoV-infected gnotobiotic calves have an early increase in serum IFN-γ compared to uninfected animals [40]. Moreover, over the course of infection, infected gnotobiotic calves show increases in serum TNF-α, IL-4, IL-10, and IL-12 cytokine levels [40]. Fecal TNF-α, IFN-γ, IL-4, IL-10 and IL-12 were also elevated in infected calves as compared to controls. Adaptive immunity, through antibody responses following challenge, includes low titers of IgA, IgM, and IgG antibodies [40] and IgA- and IgG- secreting cells in the intestine and serum 28 days following challenge, with the highest concentration found in the intestine.

6.3) Humans

In humans, NoV infection leads to Th1-skewed T-cell activation in addition to a B-cell response. A study of five healthy volunteers with evidence of prior GII.4 infection (or infection by a strain that generated cross-reactive antibodies) showed that their peripheral blood mononuclear cells (PBMCs) expressed CD80, CD86, CD40, and HLADR activation markers, following GII.4 NoV virus-like particle (VLP) stimulation, indicating T-cell maturation [106]. Stimulation with GII.4 NoV VLPs also led to secretion of IFN-γ, IL-6, and TNF-α, suggesting Th1 and some possible Th2 activation [106]. NoV challenge study results have also shown in vitro cytokine responses by PBMCs collected 4 to 21 days post-challenge to be consistent with Th1 and some possible Th2 activation following stimulation with challenge study virus VLPs with significant elevations in IFN-γ, IL-2, and IL-5 and non-significant elevations in TNF-α, IL-4, and IL-10 [47, 107]. Depletion of $CD4^+$ and $CD8^+$ T cells in vitro has shown that $CD4^+$ T cells are largely responsible for IFN-γ production following in vitro VLP stimulation [47].These results are consistent with serum cytokine changes observed during challenge studies and observational research. Specifically, SMV-infected challenge study participants showed significant elevations in serum IFN-γ and IL-2 concentrations 2 days post-challenge [47]. Some other studies have also identified elevated serum IL-5, IL-6, IL-8, and MCP-1 in response to natural NoV infection [47, 108, 109]. These results indicate that human NoV infection leads to a Th1-driven response with possible Th2 involvement. This is further supported by challenge study and population studies indicating that, following NoV infection, IgG1 antibody predominates among IgG subclasses [47, 110].

As previously noted, immunocompromised patients can develop chronic NoV infections [55, 99-101]. Anecdotal evidence from these case reports suggest that T cells, in particular $CD4^+$ cells, and B cells may be important for NoV clearance [101, 111]. In one case report, an HIV positive individual with AIDS and a chronic NoV infection showed a decrease in NoV RNA titer after successful initiation of an antiretroviral regimen and subsequent increase in $CD4^+$ cell count [111]. In a case series, T cell recovery following pediatric hematopoietic stem cell transplant was correlated with clearance of chronic NoV infection [101]. Other reports of immunocompromised patients have suggested that intravenous or enteral immunoglobulin may be a potential therapy for chronic NoV infection, though its efficacy is unproven [99, 100]. However, B cells alone do not appear sufficient in immunocompromised patients to prevent chronic NoV infection, as evidenced by a case of an individual with normal serum concentrations of IgG, IgA, and IgM antibodies but with severely compromised T-cell function who developed a chronic NoV infection lasting over two years [112].

In humans, NoV infection has been shown to induce increases in IgA, IgG, and IgM antibodies [75, 107, 113-115], the kinetics of which have been summarized in greater detail elsewhere [116]. IgA is one of the earliest antibodies produced at detectable levels and begins to rise around day 5 post-challenge in serum and as early as day 2 post-challenge in saliva [46, 48]. Strain-specific fecal IgA antibody levels are associated with lower viral RNA titer in stool and less severe illness [75]. NoV infection also results in IgM antibody production [115]. IgG antibodies begin to develop around day 7 post-challenge, with 100% of infected individuals exhibiting IgG sero-response 14 days post-challenge [46]. Some of these antibodies may prevent reinfection with the same or related

strain, but given the patterns of cross-reactivity and broad within-genogroup diversity, antibody-mediated protection derived from a single NoV infection may not extend to the entire genogroup of related NoVs, particularly for GII NoVs [47, 107]. For example, GII.1 and and GII.2 infections sometimes generated serum IgG antibodies that were cross-reactive in vitro against GI.1 capsid antigens, but the antibody reactivity was lower than in individuals who were infected with a GI.1 NoV, and they did not generally produce cross-reactive serum IgA or IgM antibodies [117]. To date, challenge studies and epidemiologic research provide evidence both for and against the hypothesis that prior infection protects from subsequent infection with a heterologous strain (across genotype or genogroup) [118-120].

Interestingly, there is conflicting evidence regarding the duration of protection from symptomatic infection by homologous strains. Epidemiologic modeling suggests that duration of immunity may range from 4 to 8 years [121]. One challenge study found complete protection against symptomatic infection by a homologous strain when rechallenge took place 6 to 14 weeks later [118], though in a different study, homologous rechallenge 8 weeks after an earlier symptomatic infection led to a second symptomatic infection [122]. The only studies to consider longer timeframes have found some individuals who initially became ill were not protected from symptomatic infection when rechallenged with a homologous strain 6 months later [70] and no individuals who became ill in an earlier challenge were protected from infection when rechallenged 27 to 42 months later [70, 122].

Overall, the evidence from animal models, NoV surrogates, observational human research, and human challenge studies suggest that while the innate immune response is

critical for limiting the severity of NoV infection, it is insufficient for viral clearance. NoV may antagonize the innate immune response in order to establish or prolong infection, but once a robust adaptive immune response is initiated, the immune system clears the infection through the action of both T cells and B cells, simultaneously generating immunologic memory, which is highly specific and may be short-lived.

7) Frontiers in NoV immunology

NoV immunology is in the midst of exciting transformations, thanks to vaccine development work, novel cell culture systems, and advances in understanding the role of the gut microbiome. These changes reinforce the need for better understanding of the human immune response to NoV and suggest novel hypotheses regarding pathogenesis and protection.

7.1) Vaccine development

One of the most significant public health-related developments in NoV immunology is the clinical testing of VLP-based vaccines [42, 123]. A NoV vaccine is most needed in developing parts of the world, such as in many African and southeast Asian countries, where the burden of diarrheal disease remains highest [9-13]. The most recent vaccine iteration is a bivalent vaccine GI.1 and GII.4 NoV VLPs expressing VP1. This bivalent vaccine has proven effective in reducing the severity of illness. However, it has not been associated with a significant reduction in infection rate in healthy adults challenged with a GII.4 strain following vaccination with VLP-based vaccine or placebo [42]. An earlier monovalent formulation of the vaccine with GI.1 NoV VLPs showed a

reduction in the rates of infection and illness among healthy adults challenged with GI.1 NoV following vaccination [123]. VLP vaccines for NoV have been shown to be immunogenic when administered through a variety of different methods [124-127], generating NoV VLP-specific serum IgG and IgA antibodies [126, 127], antibodies in serum blocking the interaction between vaccine-strain NoV VLPs and HBGAs [127], and an expansion in the number of antibody-secreting cells as early as 7 days post-vaccination [125, 126].

In addition to the difficulty in establishing protective immunity from infection, there are some additional questions regarding the vaccine's long-term prospects (reviewed in [128]). Some key issues are that NoVs are highly diverse, and the lack of strong host heterotypic protection from prior NoV infection [118] may foretell difficulties in creating any single vaccine with broad protection. Total NoV-specific IgG antibody concentration is not consistently associated with protection from infection [68], suggesting that cross-reactive antibodies may not confer complete protection. Because these vaccine studies challenged individuals with NoV relatively soon after vaccination [42, 123], it was not possible to measure the duration of protection to these vaccines. Lastly, in areas that would benefit most from a vaccine, many individuals may be malnourished. Malnourished mice have been shown to develop less protective immunity following NoV infection, including weaker antibody responses and higher viral RNA titers, [129], suggesting that malnourished, NoV-infected humans may form a similar response. Therefore, it will also be critical to address the immunogenicity of a NoV vaccine under similar circumstances. Nevertheless, there are plans to complete field trials

in adults, and safety trials in children, with VLP-based vaccines in the near future (ClinicalTrials.gov NCT02142504 and NCT02153112).

7.2) NoV and the microbiome

In addition to vaccine development work, researchers have begun to investigate the interplay between NoVs and the microbiome in vivo. There is evidence that NoV infection might alter the composition of the gut microbiome [129-131]. A subset of travelers infected with NoV were shown to have significantly reduced numbers of *Bacteriodetes* and increases in *Proteobacteria* compared to uninfected controls [131]. The increase in intestinal *Proteobacteria* was driven by increases in non-enteropathogenic *Escherichia coli* during NoV infection. Studies in mice have been equivocal with regard to the response of the gut microbiota to MNV infection. One study reported that C57BL/6 mice did not have disruption of the gut microbiota following MNV-1 or MNV-4 infection [130]. A later study found that healthy C57BL/6 mice did show alterations in the composition of their gut microbiota following MNV-1 infection but malnourished mice did not [129]. Non-malnourished mice infected with MNV-1 showed a significant reduction in the proportion of *Bacteriodetes*, as was seen in humans [131], and an increase in *Firmicutes*.

Beyond descriptive findings, there is a growing body of knowledge related to the complex interplay between NoV and the microbiome. Research in mice indicates that gut bacteria may play a critical role in NoV infection [88]. Mice treated with broad-spectrum antibiotics were largely resistant to persistent infection by MNV CR6. No single antibiotic was solely responsible for resistance, and resistance depended on intact innate

immune responses, particularly IFN-λ, STAT1 and IRF3 [88]. The work suggests that commensal bacteria, which had been largely reduced by treatment with antibiotics, may counteract the innate immune response to NoV, limiting its efficacy in preventing infection. A second hypothesis is that commensal bacteria may even help NoV enter target cells, a hypothesis supported by the BJAB culture system for human NoV, which relies on the presence of an enteric bacterium for infection to occur [32] (Fig. 1). There is also a growing appreciation for the impact of NoV infection on the immune response to other microbes. MNV has been shown to suppress the human immune response rather than simply evade it [29, 98], and in the presence of underlying genetic and microbiome factors, may lead to inflammatory illness [132, 133]. Mice deficient in Atg16L1, an autophagy gene that is associated in humans with with Crohn's disease, when infected with MNV CR6, were shown to develop a Crohn's-like illness mediated by TNF-α and IFN-γ, but they did so only when commensal bacteria were present [132]. When treated with broad-spectrum antibiotics, the mice were protected from developing a Crohn's-like illness, suggesting that normal gut microbiomes may become a nidus for inflammatory disease following an infectious trigger in individuals who are genetically more susceptible to inflammatory conditions [132]. NoVs may be one such trigger. In a similar set of experiments, Basic *et al*. found that IL-10 knockout mice with normal gut microbiota developed mucosal inflammation following MNV infection, but germ-free knockout mice did not develop illness following MNV infection [133], suggesting that NoV infection may be an inciting event for inflammatory gastrointestinal illness among susceptible individuals.

The effect of NoV infection on gut homeostasis may be even more complicated. NoV infection in the presence of specific genetic and environmental factors may lead to illness, but in the absence of an intact bacterial microbiome, MNV has been shown to promote the healthy development of mucosal immunity [134]. Specifically, germ-free mice experimentally infected with MNV CR6 developed apparently healthy small intestines with more normal levels of $CD4^+$ and $CD8^+$ T-cells compared with that of uninfected germ-free mice and more comparable serum antibody levels and markers of cellular function, such as Paneth cell granules, lysozyme expression, and IFN-γ expression [134]. In antibiotic-treated mice, MNV infection also prevented intestinal injury by *Citrobacter rodentium* and enhanced the immune response [134]. Based on this work, it appears that NoV may play a commensal role under certain conditions.

8) Conclusions

Despite huge advances since the identification of NoV over 40 years ago, major questions remain unanswered. The cellular tropism of NoV remains unknown. There are no small animal models that mimic human NoV pathogenesis and there is still no in vitro infectivity assay, though the novel cell culture system may be one possible option. The role of antibodies in resistance to NoV infection remains an open question. The pathogenesis of human NoV infection and causes of symptoms are ill-understood. As vaccine studies advance, researchers continue searching for optimal surrogates and correlates of protection. Studies of interactions between NoV and the human microbiota

are still in their infancy. The field of NoV immunology remains open to innovation and discovery to prevent and treat this major cause of illness worldwide.

9) Acknowledgements

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Figures

Figure 1. Proposed schematic of norovirus infection in the gut and immune response. Noroviruses (NoV) may infect multiple cell types in the gut, and this may vary between humans and animal models. There are three major hypothetical infection pathways shown. 1) NoV may infect monocytes and other cells associated with Peyer's patches in a microfold (M) cell-dependent manner, 2) NoV may infect epithelial cells directly, or 3) NoV may be transported across the epithelium, perhaps through interactions with commensal gut bacteria, where they may infect lymphoid cells. Infected cells produce IFNs. IFNs inhibit viral replication in infected cells and may stimulate $CD4^+$ T-cell differentiation and activation, which may lead to $CD8^+$ T-cell activation and B-cell maturation. This schematic is not intended to make suggestions on the host location of $CD4^+$ and $CD8^+$ differentiation and activation following NoV infection because these data are unknown. Cytokines may lead to inflammation of the gut epithelium and recruitment of neutrophils and monocytes. B cells produce NoV-specific antibodies that are critical for clearing the infection. NoV infection also generates memory B cells, which may protect against future infection by the same strain.

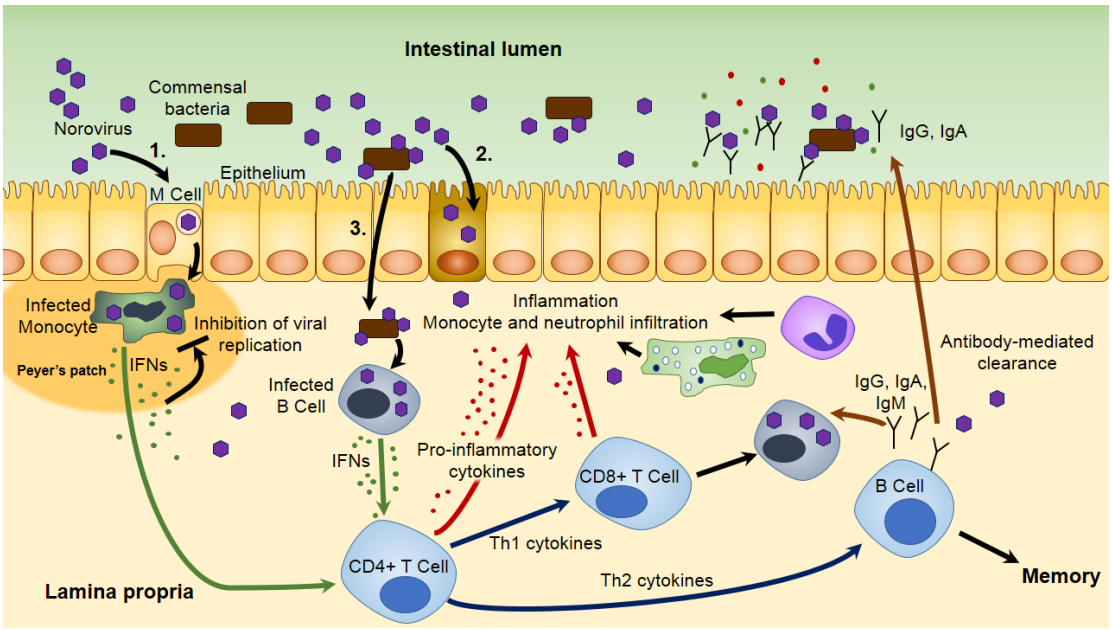
Intestinal lumen
Commensal bacteria
Norovirus
1.
2.
3.
Epithelium
M Cell
IgG, IgA
Infected Monocyte
Inhibition of viral replication
Peyer's patch
IFNs
Infected B Cell
Inflammation
Monocyte and neutrophil infiltration
Antibody-mediated clearance
IgG, IgA, IgM
IFNs
Pro-inflammatory cytokines
CD8+ T Cell
B Cell
Th1 cytokines
CD4+ T Cell
Th2 cytokines
Lamina propria
Memory

Figure 2. Proposed schematic of intracellular immune response to murine norovirus (MNV) infection. MNV may infect macrophages or dendritic cells in the gut. Infected cells sense MNV via MDA-5 and produce IFNs through this or the STAT-1 pathway. Type I IFNs inhibit MNV uncoating via ISG15 and may inhibit replication complex formation via an Atg complex. Type II IFNs block MNV translation via RNA-activated protein kinase (PKR). VF1-related immune suppression by MNV is not included in this schematic.

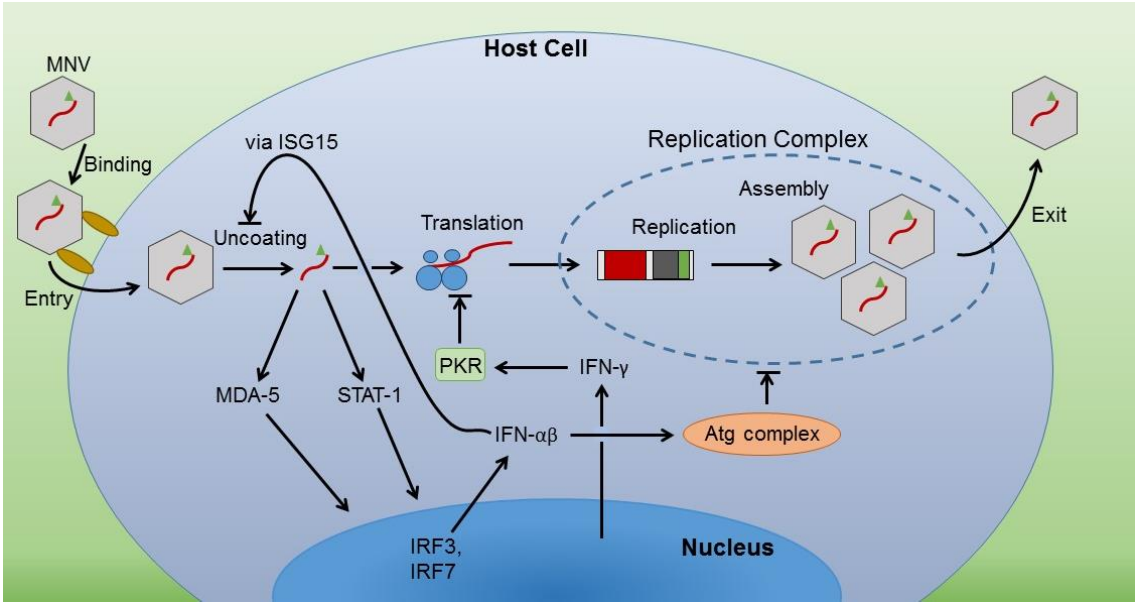

Table 1. Surrogate viruses and model systems used to study human NoV.[a]

Virus	Common lab strains	Family/ genus	Enteric virus	Host receptors, co-receptors	Hosts	Symptoms[b]	Infection duration in host	Cell tropism	Cell culture system	Animal models
Human NoV	GI.1 NW, GII.1 HI, GII.2 SMV, GII.4 Sydney	Caliciviridae/ Norovirus	Yes	HBGA [35], heparin sulfate [135]	Human	Vomiting, diarrhea	1-4 weeks	Unknown, possibly gut epithelial [56, 57, 136] and B cells [32]	Human B cell line BJAB co-cultured with killed HBGA-like antigen expressing bacteria [32]	Chimpanzee [37], Gn pig [44], Gn calf [40], mouse [45]
Feline calicivirus (FCV)	F9, Urbana strain, 255	Caliciviridae/ Vesivirus	No	JAM-1 [137], sialic acid [138]	Cat	Pneumonia, stomatitis, gingivitis, lameness	Acute-chronic[c]	Mucosal epithelial cells,macrophages [139]	Crandell-Reese feline kidney cells [140]	Cat
Murine NoV (MNV)	MNV-1.CW1, MNV-1.CW3, MNV-3, MNV.CR6, MNV-4	Caliciviridae/ Norovirus	Yes	Sialic acid [141], glycoproteins [142]	Mouse	None	Acute-chronic[c]	Dendritic cells, macrophages [78]	Murine macrophage cell line RAW264.7 [78]	Mouse
Bovine NoV	GIII.1, GIII.2	Caliciviridae/ Norovirus	Yes	αGalactose epitope [143]	Cow	Diarrhea	1-3+ days (GIII.1) and 1-3 weeks (GIII.2) [61, 144]	Gut epithelial cells and lamina propria (GIII.1) [61], unknown for GIII.2 [144]	None	Calf, Gn calf [61, 144]
Porcine sapovirus (Porcine enteric calicivirus)	Cowden strain	Caliciviridae/ Sapovirus	Yes	α2,3- and α 2,6-linked sialic acids on o-linked glycoproteins [145]	Pig	Diarrhea	1-7+ days	Gut epithelial cells [60]	Pig kidney cell line LLC-PK with added bile acids [146, 147]	Piglet, Gn pig [60]
Recoviruses (Rhesus Enteric Caliciviruses)	Tulane Virus	Caliciviridae/ Recovirus	Yes	HBGA [148]	Rhesus macaque	Diarrhea, fever	8-10 days	B cells [62]	Monkey kidney cell line LLC-MK2 [149]	Rhesus macaque
Bovine nebovirus (Bovine enteric caliciviruses)	Nebraska, Newbury1	Calicivirus/ Nebovirus	Yes	Unknown	Cow	Diarrhea, anorexia	3-7 days	Unknown	None	Calf, Gn calf [150]

[a]Abbreviations: NoV, norovirus; NW, Norwalk virus; HI, Hawaii virus; SMV, Snow Mountain virus; HBGA, histo blood group antigens; Gn, gnotobiotic

[b]Symptoms in immunocompetent host.

[c]Short (acute) or long (chronic) duration of infection varies based on virus strain.

Table 2. Summary of published human NoV challenge study data collection and results.[a]

Ref.	Year	Strain	Intervention	n	Infected	Symptomatic	Rechallenge/ reinfection	Histology	Serology	Viral shedding
[151]	1971	NV	None	16	10[b]	10	No/NA	No	No	No
[152]	1972	NV	None	7	6[b]	6	No/NA	No	Yes	No
[153]	1972	NV	Heat, ether, acid, filtration	103	35[b]	35	Yes/No	No	No	No
[56]	1973	NV	None	15	12[b]	12	No/NA	Yes	No	No
[154]	1973	NV	None	7	4[b]	4	No/NA	Yes	No	No
[118]	1974	NV, HI, MC	None	52 (NV), 23 (HI), 18 (MC)	31 (NV), 12 (HI), 10 (MC)[b]	31 (NV), 12 (HI), 10 (MC)*	Yes/Yes	No	No	No
[93]	1975	HI	None	7	4[b]	4	No/NA	Yes	Yes	No
[155]	1975	NV	None	15	9[b]	9	No/NA	Yes	No	No
[122]	1977	NV	None	12	6[b]	6	Yes/Yes	Yes	Yes	No
[69]	1979	NV	None	38	18[b]	18	No/NA	No	Yes	No
[156]	1982	SMV	None	12	9[b]	9	No/NA	No	Yes	No
[157]	1985	NV	Chlorinated water	32	17[b]	17	No/NA	No	Yes	No
[158]	1988	HI	None	10	8[b]	8	No/NA	No	Yes	No
[70]	1990	NV	None	42	27[b]	25	Yes/Yes	No	Yes	No
[159]	1994	NV	None	50	41	28	No/NA	No	Yes	Yes
[48]	2003	NV	None	55 Se+, 22 Se-	34 (all Se+)	22 (all Se+)	No/NA	No	Yes	No
[47]	2005	SMV	None	15	9	7	No/NA	No	Yes	No
[38]	2008	NV	None	not reported	16	11	No/NA	No	Limited	Yes
[160]	2008	NV	None	80 Se+, 28 Se-	40 (all Se+)	24 (all Se+)	No/NA	No	No	No
[123]	2011	NV	Monovalent VLP vaccine	43 vaccinated, 41 placebo	23 vaccinated, 32 placebo	14 vaccinated, 27 placebo	No/NA	No	Yes	No
[49]	2011	NV	Hydrostatic pressure processing[c]	44 in four arms	13	9	No/NA	No	Yes	Yes
[50]	2011	NV	Persistence in water	13	10	10	No/NA	No	Yes	Yes
[43]	2012	GII.4	None	23 Se+, 17 Se-	16 Se+, 1 Se-	12 Se+, 1 Se-	No/NA	No	Yes	Yes
[42]	2014	GII.4	Bivalent VLP vaccine	50 vaccinated, 48 placebo	27 vaccinated, 30 placebo	13 vaccinated, 16 placebo	No/NA	No	Yes	Yes

[a]Abbreviations: NV, Norwalk virus (GI.1); HI, Hawaii virus (GII.1); MC, Montgomery County virus (GI.5); SMV, Snow Mountain virus (GII.2); VLP, virus-like particle; Se+, secretor positive; Se-, secretor negative; NA, not applicable; Ref., reference. [b]Study did not use genomic testing for infection status. [c]Study included four arms—placebo and three processing conditions.

CHAPTER 3: STUDY 1: IMMUNOCOMPETENT ADULTS FROM HUMAN NOROVIRUS CHALLENGE STUDIES DO NOT EXHIBIT NOROVIRUS VIREMIA

Newman KL, Z Marsh, AE Kirby, CL Moe, JS Leon. Immunocompetent adults do not exhibit norovirus viremia. *Journal of Virology*. 2015 Jul 1;89(13):6968-9. doi: 10.1128/JVI.00392-15. Epub 2015 Apr 22.

Noroviruses (NoV) are among the leading causes of acute gastroenteritis worldwide [161]. There is also limited knowledge of the pathogenesis of human NoV infection. Children, immunocompromised adults, and gnotobiotic pigs and calves used as models of human NoV infection all can develop NoV viremia [40, 44, 162-165], suggesting that NoV infection may not be limited to the gut. However, no data on NoV viremia in immunocompetent adults exists.

To determine whether immunocompetent adults develop NoV viremia, we tested samples from NoV-infected adults who participated in two challenge studies, which are described fully elsewhere [47, 49]. Based on our power calculations, we chose 38 serum samples from 20 subjects (13 Norwalk virus (NV)-infected, 7 Snow Mountain virus (SMV)-infected). On average, samples were from day 4.6 post-challenge, the same as the average day of peak viral shedding in stool. NV-infected subjects had a median of 3 consecutive samples tested per subject (range 1-3), selected from samples taken during the peak of viral shedding in stool. SMV-infected subjects had 1 sample tested per subject, selected from the day of peak viral shedding in stool. Our sample was large enough to detect an absence of viremia with over 95% power, alpha=0.05, and assuming a null hypothesis of at least 15% of subjects with viremia, a lower rate than that seen in pediatric NoV or gnotobiotic models [40, 44, 162, 163]. Serum samples were taken on the days of greatest viral shedding in stool, as quantified by RT-PCR [39], because a past

study identified higher fecal NoV load in children with viremia, leading us to assume that days of highest NoV shedding in stool would be most likely to be associated with viremia [163]. All samples were stored at -80° C prior to testing.

Viral RNA was extracted from 140 µl of 20% stool suspension or 140 µl of serum using QIAamp Viral RNA Mini kit (Qiagen Valencia, California), following the manufacturer's instructions. A stool sample with known high NoV titer, a NoV-seeded serum sample, and 140 µl molecular-grade water were used as positive and negative extraction controls. *In vitro*-transcribed NV and SMV standards were used for quantification [39]. RT-qPCR was performed with a One-step RT-qPCR kit (Qiagen Valencia, California), as described in Kirby, et al. 2014, using NV and SMV specific primers and probes whose sequences have been previously published [39, 166]. All reactions were performed in duplicate on a Bio-Rad CFX96 system (Bio-Rad Hercules, California). Amplification data was collected and analyzed with the Bio-Rad CFX Manager software (Bio-Rad Hercules, California). We repeated RNA extraction and PCR testing on 10 samples to verify our findings. We also performed a dilution series of NoV in serum samples to determine the limit of detection for our assay, which we estimated to be approximately 1×10^1 genomic equivalence copies (GEC) per reaction or 6×10^2 GEC per ml of serum.

NoV was not present at detectable levels in any samples. The subjects had a mean age of 28 years (SD=9.5), 50% were female, 44% were Caucasian, and 39% were African American. Their stool median peak viral titer was 1.2×10^8 genomic equivalence copies/g stool (interquartile range (IQR) $1.6 \times 10^7 – 6.9 \times 10^8$) and their median day of peak NoV shedding in stool was day 4 post-challenge (IQR 4 - 5). These data indicate

that an adult can shed high levels of NoV in their stool yet still have no detectable NoV in their blood. This result suggests that an adult with a healthy immune system is capable of preventing NoV from crossing from the gastrointestinal tract into the bloodstream, and viremia may be associated with primary infection. Some limitations of this work include the lack of a true NoV positive serum sample (such as one from a child or immunosuppressed individual) to use as a positive control, a long sample storage time, and the possibility that NoV may be present but below the limit of detection. This report warrants further investigation into the mechanisms by which NoV infects the blood stream of susceptible populations yet remains absent in the blood of healthy adults.

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CHAPTER 4: STUDY 2: HUMAN NOROVIRUS INFECTION AND THE ACUTE SERUM CYTOKINE RESPONSE

Newman KL, CL Moe, AE Kirby, WD Flanders, CA Parkos, JS Leon. Human norovirus infection and the acute serum cytokine response. *Clinical and Experimental Immunology*. 2015 Jul 14. doi: 10.1111/cei.12681. [Epub ahead of print].

Summary

Noroviruses (NoV) are the most common cause of epidemic gastroenteritis worldwide. The acute immune response to NoV in humans is poorly understood, hindering research on prevention and treatment. To elucidate the acute immune response and test for cytokine predictors of susceptibility to infection, serum samples from two human NoV challenge studies were tested for 16 cytokines. Subjects who became infected (n=26) were age-matched with subjects who remained uninfected following NoV challenge (n=26). Samples were tested from pre-challenge and days 1-4 post-challenge. Cytokine responses were compared between infected and uninfected groups. Overall, infected individuals exhibited an elevation in Th1 and Th2 cytokines, as well as chemokines IL-8 and MCP-1, compared to uninfected individuals (all $p<0.05$). Most cytokines peaked on day 2 post-challenge in infected subjects, and TNF-α, IL-8, and IL-10 remained elevated through day 3. The only cytokine significantly elevated among infected subjects through day 4 post-challenge was IL-10 ($p=0.021$). Pre-challenge cytokine concentrations were not predictive of infection status post-challenge. There were no significant changes in serum cytokines among NoV-challenged subjects who remained uninfected. These results suggest that NoV infection elicits a Th1 type response with some Th2 activation. Persistent elevation of IL-10 among infected subjects is consistent with activation of adaptive immune responses, such as B-cell expansion, as well as down-regulation of Th1

cytokines. This study presents the first comprehensive description of the acute cytokine response to GI.1 NoV in humans.

Introduction

Noroviruses (NoV) comprise seven genogroups in the positive-sense RNA virus family *Caliciviridae* [167, 168]. NoV are responsible for 18% of gastroenteritis worldwide [2]. Despite this broad impact, human NoV immunology is poorly understood [107, 169], in part because until recently, there was no small animal model or reliable cell culture system for human NoV [32, 45]. Furthermore, there are few prospective experimental studies on the acute human innate and cellular immune response to NoV.

The genetic determinants of NoV infection (e.g. secretor blood group antigens) are relatively well characterized, but many aspects of protective innate and cellular immunity have not been described [36, 65]. Histologic studies [54, 57, 122, 169] and in vitro tests of peripheral blood mono-nuclear cells [47] suggest cytotoxic and CD8+ T-cell activation with some cells sensitized to NoV challenge antigens, but little work has been done in vivo to describe the cytokine response to NoV infection. In particular, there is a gap in understanding the acute immune response to NoV. Prior studies have shown that adaptive immunity is critical for clearing the infection [94], but there is a gap in the knowledge about early pathogenesis of NoV infection. Key cytokines for consideration include acute response-related pro-inflammatory cytokines (e.g. IL-1, IL-6, and IL-12), chemokines involved in neutrophil and monocyte recruitment (e.g. IL-8 and MCP-1), and Th1- and Th2-related cytokines (e.g. IFN-γ, IL-2, and TNF-α and IL-4, IL-5, and IL-10, respectively) involved in the cellular immune response and initiation of adaptive immune mechanisms.

The goal of this study was two-fold. The first goal was to describe serum cytokine responses to NoV infection, including the temporal trends in serum cytokines during the

acute phase of human NoV infection. The second goal was to use cytokine concentrations pre-challenge to predict infection post-challenge. Human serum samples from two prior NoV challenge studies that used the same Norwalk virus (GI.1) inoculum were analyzed for a broad panel of relevant cytokines [49, 50]. The results have important implications for determining the role of specific cytokines in NoV infection.

Materials and methods

Population and samples

The samples tested for this study were collected from subjects involved in two separate NoV challenge studies, described previously [49, 50]. However, these studies could used the same inoculum preparation, were conducted at the same institution, and used very similar protocols. Key differences between these two challenge studies (i.e. inoculum dose) were accounted for in the modeling strategy of this study. Briefly, the first study enrolled healthy secretor-positive adult volunteers, who were challenged with Norwalk virus 8FIIb inoculum at Emory University Hospital's Clinical Interaction Site, part of the Atlanta Clinical and Translational Science Institute Clinical Interaction Network, between May 2006 and December 2006. Volunteers ingested filtered groundwater artificially seeded with 6.5×10^7 genomic equivalent copies (GEC) of NoV inoculum, which had been incubated at room temperature in the dark for different set lengths of time. Prior to challenge, serum and stool samples were collected. During the first 4 days post-challenge, serum samples were collected daily, as were all stool samples.

The second study enrolled healthy secretor-positive adult volunteers, who were also challenged with Norwalk virus 8FIIb inoculum at Emory University Hospital's Clinical Interaction Site between February 2008 and September 2009. They were randomized into control and intervention groups and administered oysters seeded with 1 x 10^4 GEC of virus, which had been treated with high hydrostatic pressure processing for 5 minutes (intervention) or left untreated (control). Pre-challenge stool and serum samples were collected before challenge. During the first 4 days post-challenge, serum samples were collected daily, as were all stool samples.

The Emory University Institutional Review Board approved both studies, and both were registered on Clinical Trials.gov (identifiers NCT00313404 and NCT00674336). All subjects consented to the future use of all biological specimens from the studies. All specimens were stored at -80°C.

Of the initial participants in both NoV challenge studies, a total of 26 became infected as defined by a NoV-positive stool or emesis sample tested by RT-PCR (limit of detection: 3,570 GEC/g stool) [39, 50]. These 26 were pair-matched by age to 26 of the challenge study participants who remained uninfected following NoV challenge. Some infected subjects from one challenge group were matched with uninfected subjects from a different challenge group because within the same group there were no uninfected individuals within 3 years of age of the infected individual. The total sample size for this study was 52 participants (13 from the 2006 trial, 39 from the 2008-2009 trial). Because the focus of this study is the acute immune response, only serum samples from pre-challenge and days 1-4 post-challenge were included, for a total of five longitudinal samples per subject.

Inoculum

The two NoV challenge studies included participants who ingested water or shellfish seeded with 8FIIb NoV inoculum. The water samples were seeded with identical amounts of NoV and stored at room temperature in the dark for varying lengths of time. They showed no evidence of titer attenuation over time, as measured by RT-PCR [50]. Therefore, for these subjects, the regression models did not control for study arm. The shellfish samples were seeded with identical amounts of NoV and treated under varying processing conditions. The inocula showed some evidence of attenuation for some treatments. For subjects from this study, all adjusted models controlled for study arm as a surrogate for dose.

Detection of viral shedding

NoV presence was measured by RT-PCR for all stool and emesis samples at all time points during the original challenge studies. A subject with a positive sample at any time point, even after day 4 post- exposure, as determined in the original or subsequent studies, was defined as infected.

Detection of cytokines

Serum samples from pre-challenge (pre-challenge), and days 1-4 post-challenge (five total samples) were analyzed from all study subjects. Serum was collected during the studies, processed, and stored at -80°C until testing. Samples were tested by a commercial laboratory (EMD Millipore Corporation Discovery and Development Solutions) using a Milliplex human cytokine 16-plex assay for IFN-α2, IFN-γ, IL-1a, IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, MCP-1, TNF-α, and

TNF-β. Samples were run in duplicate with controls. Standard curves were generated using recombinant cytokines and calculated using a five parameter logistic model for each cytokine. Lower limits of detection (LLOD) were 3.2 pg/mL for IFN-γ, IL-10, IL-12p70, IL-1b, IL-2, IL-5, IL-6, IL-8, MCP-1, and TNF-α; 16.0 pg/mL for TNF-β; and 80.0 pg/mL for IFN-α2, IL-12p40, IL-1ra, IL-1a, and IL-4. Values below LLOD were assigned the value of the LLOD.

Statistical analysis

The data were analyzed using SAS 9.4 (Cary, NC, USA). When possible, age and inoculum were controlled for in adjusted analyses. Because the exact amount of infectious NoV in the inocula for study arms with attenuation was unknown, inoculum was controlled for using a categorical variable for each study and study arm. The Wilcoxon signed rank test was to test for the significance of the unadjusted difference between pre-challenge and post-challenge serum cytokine levels. Conditional logistic regression was used to evaluate the association between pre-challenge and post-challenge fold changes in serum cytokine levels (i.e. ratio between post-challenge and pre-challenge cytokine concentrations), controlling for inoculum and age. To summarize the differences between infected and uninfected individuals responses over time while accounting for correlation between time points, a mixed linear model was used to test the association between $\log_{10}$ cytokine concentration and day post-challenge, stratified by infection status with a random effect by subject, autoregressive correlation between different time points taken for the same individual, and fixed effect for inoculum. The parameter estimates for the effect of each day on $\log_{10}$ cytokine concentration for each stratum were averaged and Student's t-test was used to compare infected and uninfected

subjects cytokine responses over time. Conditional logistic regression adjusted for inoculum and age was also used to assess whether three measures of early cytokine levels or responses could be used as predictors of post-challenge infection. The measures assessed were pre-challenge $\log_{10}$ cytokine concentration, pre-challenge to day 1 post-challenge $\log_{10}$ cytokine change, and pre-challenge to day 1 post-challenge fold change. All cytokines were modeled individually. Differences between groups were considered statistically significant at $p < 0.05$.

Results

To determine the acute serum cytokine response to GI.1 NoV infection, serum cytokine responses in individuals who became infected following experimental challenge were compared to those who remained uninfected after challenge, as determined by RT-PCR detection of GI.1 NoV RNA in stool or emesis samples. Immune responses may differ with age, therefore the infected and uninfected subjects were matched for age (Table 1). The study population was relatively young, with a mean age of 26.7 years and a median age of 25 years. There was also a similar distribution of sex and race between infected and uninfected subjects selected for the study, though neither was a matching factor. Some uninfected subjects exhibited symptoms, including fever and diarrhea, which is consistent with past NoV challenge studies [49]. All infected individuals had higher cumulative shedding than the inoculum dose (data not shown), reducing the likelihood of misclassification of infection status because of detection of inoculum post-challenge in the absence of infection. Serum samples were available for pre-challenge through day 4 post-challenge (five time points) and all samples were tested for 16 serum

cytokines. Two subjects did not have samples for day 3 and values for these samples were imputed using the average of neighboring observations for the same subject. A total of 10 subjects had invalid test results for one or more cytokine tested (29 total observations). Using imputation, all but one of these invalid results was estimated to be below the LLOD.

Studies of serum cytokine levels in humans often have high rates of observations below the LLOD [170]. Overall of the 16 cytokines tested, nine had greater than 50% of observations above the LLOD, and four had greater than 75% of observations above the LLOD (Table 2). In general, infected subjects had more samples above the LLOD.

To assess whether serum cytokines exhibited a change in concentration following NoV challenge, the Wilcoxon signed rank test was used, stratifying by infection status. Among infected individuals, there were significant increases in IL-2, IL-10, MCP-1, and TNF-α in the days post-challenge (Figure 1, all $p < 0.05$). Among uninfected individuals, there were no significant changes in serum cytokine concentrations post-challenge compared to pre-challenge. Based on conditional logistic regression models controlling for age and inoculum, there were no significant differences between infected and uninfected individuals' changes in serum cytokine concentrations between pre-challenge and day 1 post-challenge (data not shown). However, infected individuals were significantly more likely to have increased IFN-γ, IL-6, IL-8, IL-12p70, MCP-1, and TNF-α between pre-challenge and day 2 post-challenge compared to uninfected individuals (Table 3). Infected individuals were also significantly more likely to have increased levels of TNF-α, IL-8, and IL-10 from pre-challenge to day 3 post-challenge (all $p<0.05$; data not shown). At day 4 post-challenge, the only cytokine significantly

elevated over pre-challenge levels was IL-10, which was significantly elevated in infected individuals (OR of infection based on a two-fold increase in IL-10 from pre-challenge to day 4 post-challenge=3.92, p=0.049; data not shown).

Differences between infected and uninfected individuals over time

Overall trends in serum cytokine response were analyzed using mixed models to account for correlation within subject over time (Figure 2). For infected individuals compared to uninfected individuals, there were statistically significant fold changes across the four days post-challenge from pre-challenge levels in most serum cytokines measured (i.e. IFN-α2, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, MCP-1, TNF-α, and TNF-β, Figure 2), with all but TNF-β significantly higher in infected individuals compared to uninfected individuals. All cytokines peaked at day 2 post-challenge among infected subjects, with the exception of IL-1ra, but many were elevated by day 1 post-challenge (i.e. IL-2, IL-6, IL-8, MCP-1, and TNF-α) or remained elevated through day 4 post-challenge (i.e. IL-10) (Figure 2).

Pre-challenge predictors of infection

To determine if pre-challenge serum cytokine concentrations or early cytokine responses could predict infection status following challenge, the associations were estimated between infection status and three measures of serum cytokines: $\log_{10}$ serum cytokine concentration, $\log_{10}$ difference between pre-challenge and day 1 post-challenge serum cytokine concentrations, and pre-challenge to day 1 post-challenge fold change (i.e. ratio between pre-challenge and day 1 post-challenge concentrations). Across these measures, the only significant predictor of infection status was pre-challenge to day 1

post-challenge $\log_{10}$ change in MCP-1 concentration (data not shown). An increase of 1 $\log_{10}$ over this time period was associated with an OR of infection of 5.74 (p=0.018). No other measures of early cytokine response were statistically significant predictors of infection.

Discussion

This study found overall significant elevation in IFN-a2, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, MCP-1, TNF-α, and TNF-β in infected subjects compared to uninfected subjects. Among infected individuals, these cytokines peaked on day 2 post-challenge. The results suggest that NoV infection elicits a Th1- and Th2-type response. There also was persistent elevation of IL-10 among infected subjects, suggestive of an evolving early adaptive immune response and down-regulation of Th1 cytokines. This study represents the first comprehensive description of the dynamics of serum cytokines in the acute time period following NoV challenge, including the largest number of cytokines simultaneously described for NoV infection (Table 3).

We found that before challenge, individuals who became infected following NoV challenge were similar to individuals who did not become infected with regard to all 16 cytokines tested. From these similar pre-challenge features, differences began to emerge as early as 24 hours post-challenge and persisted through day 4 post-challenge. As previously documented with human Snow Mountain strain (SMV; GII.2) infection, there was a significant Th1 response (i.e. IFN-γ, IL-2, and TNF-α) with significant elevation of one Th2-related cytokine (Table 3) [47]. In this study, some Th2 and other cytokines (e.g.

IL-4, IL-5, IL-6, IL-8, and IL-10) were also significantly elevated following NoV challenge in infected individuals.

The cytokine responses also resembled results from animal studies (Table 3) but differed in the timing and magnitude of cytokine response [40, 41]. These results suggest that the immune response by gnotobiotic animals to human NoV may be substantially different from the response in humans, perhaps due to the gnotobiotic animals' immature immune systems [171] or because of the use of a different NoV inoculum.

Initial response: IL-8, MCP-1

We found that IL-8 levels increased in infected subjects by day 1 post-challenge and remained elevated through day 3 post-challenge. IL-8 elevation has been identified in prior field studies of NoV infection (Table 3) [108, 109]. IL-8 is a powerful chemoattractant for neutrophils [172, 173] and intraepithelial lymphocytes [174, 175], and a key mediator of the immune response to other gastrointestinal pathogens [176]. Histologic findings from previous human NoV challenge studies show increases in granulocyte and monocyte cells in the lamina propria of the small intestine 12-48 hours after challenge in infected subjects [56]. Based on the rise in IL-8 alongside an increase in MCP-1 and a later rise in IL-6, two monocyte chemoattractants, these observations are consistent with neutrophil recruitment acting in concert with monocyte response to NoV infection. Earlier work found that GI and GII NoV infection is associated with elevated fecal MCP-1, suggesting local monocyte activation [109], but this study is the first to show elevated serum MCP-1, suggesting a systemic response as well.

TNF-α and symptoms

In this study, the peak serum TNF-α concentration at 48 hours post-challenge corresponded with the time most NoV-infected individuals were symptomatic. TNF-α increases cellular permeability, leading to edema mucosal damage [177]. Elevated TNF-α has also been associated with NoV infection in past human and animal studies (Table 3) [40, 109]. In some gastrointestinal infections, TNF-α is associated with symptoms [178-180]. The association between TNF-α and NoV symptoms is an important area for future research.

IL-6

IL-6 is a multi-functional cytokine with strong pro-inflammatory effects. It is associated with damage to the intestinal mucosa [56] and increased frequency of diarrhea [108], suggesting a role in pathogenesis and clinical severity of illness. In this study, IL-6 was significantly elevated on day 2 post-challenge. This confirms some clinical studies' identification of the association between IL-6 and NoV infection but contrasts with the results from a SMV challenge study (Table 3) [47, 108, 109] and suggests possible strain-related differences in immune response. This study reported a median maximum serum IL-6 level that was higher than prior studies have identified [108, 181], but this discrepancy may be the result of differences in sample collection.

IL-10 persistently elevated

Though IL-10 peaked on day 2 post-challenge, it was significantly elevated through day 4 post-challenge. This suggests an ongoing role in NoV infection beyond the acute time period. IL-10 is involved in the Th2 response and B-cell development [182], so its elevation may be associated with the development of NoV-specific antibodies,

which begin to be detectable around or before day 7 post-challenge [75, 126]. Elevated IL-10 in the context of NoV infection has been described in prior studies of human NoV (Table 3). IL-10 may also play an anti-inflammatory role in down-regulating Th1 cytokine production. The significant elevation of IL-10 in infected subjects following the elevation of Th1 cytokines supports the conclusion that NoV elicits a Th1-type response. Furthermore, IL-10-deficient mice exhibit mucosal inflammation and epithelial barrier dysfunction following MNV-challenge, whereas wildtype mice do not [133]. It is possible that IL-10 plays a similar protective role in NoV-challenged humans.

Strengths and Limitations

This study used rigorously collected human NoV challenge study data, which provided a detailed, longitudinal dataset. It is the largest collection of human NoV challenge subjects yet studied for serum cytokine response. It represents broadest panel of cytokines yet examined at one time for human NoV infection. Some limitations of this study are that it may have been underpowered to detect some changes in serum cytokines, the exposure history of subjects prior to NoV challenge was unknown, and there is the possibility that the duration of sample storage may have caused cytokine degradation [183]. An additional limitation of the study is that though RT-PCR is the gold-standard for NoV testing, there may have been individuals who were misclassified as uninfected because they shed virus at levels below the limit of detection.

Conclusion

Although NoV remains a major cause of morbidity worldwide, some aspects of the immune response continue to be poorly understood. To develop effective vaccines

and prophylaxis, it is important to understand the human acute immune response to NoV. This study demonstrated a Th1- and Th2-type response to GI.1 NoV infection, early elevation of chemokines IL-8 and MCP-1, and ongoing elevation of IL-10. This work confirms earlier findings of the increase in MCP-1 associated with NoV infection, suggesting macrophage and dendritic cell involvement in NoV infection. These findings buttress the existing findings from MNV models and enhance the depth of knowledge regarding the human cytokine response to NoV. Differences between the results of this study and those of other human and animal work may be attributable to strain-related variability in immune response. Future work should consider the interplay between the immune response and both clinical outcomes and viral shedding to understand NoV pathogenesis and help develop clinical and public health strategies to reduce NoV transmission.

Acknowledgements

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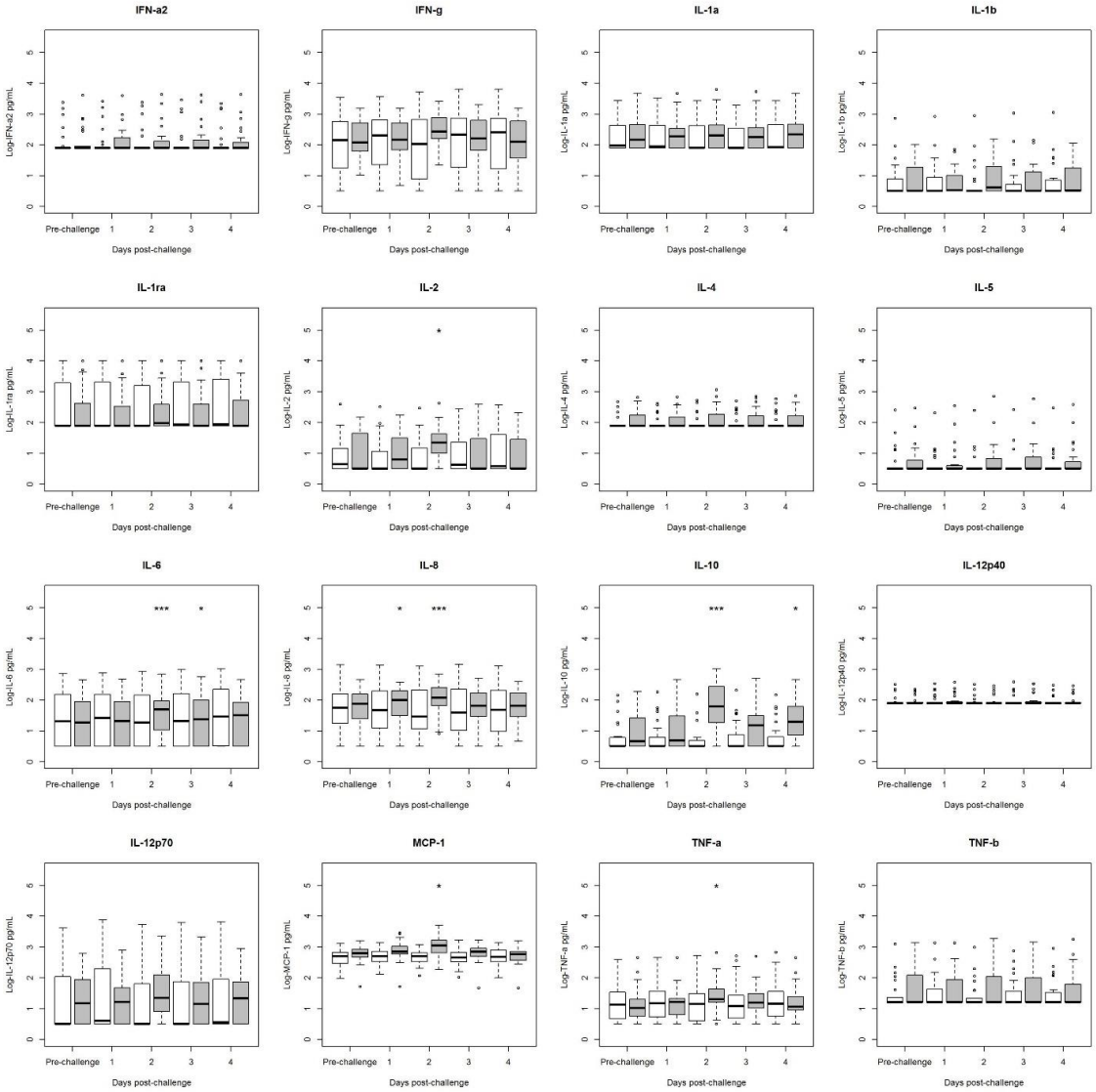
Figures

Figure 1: Comparison of pre-challenge to post-challenge serum cytokine concentrations in norovirus-challenged individuals, stratified by infection status (infected n=26, uninfected n=26). Values below the lower limit of detection (LLOD) were assigned to the value of the LLOD. Significance of change from pre-challenge value was tested using Wilcoxon test and is denoted by an asterisk above the relevant category. Interquartile ranges (IQRs) for infected individuals (gray boxes) and for uninfected individuals (white boxes) are shown. Dark lines indicate median values. Whiskers indicate most extreme value that is no more than 1.5 times the IQR away from the bound of the IQR. Circles indicate outliers. *$p<0.05$, **$p<0.01$, ***$p<0.001$


IFN-a2
Log-IFN-a2 pg/mL
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IL-1a
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IL-10
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IL-12p40
Log-IL-12p40 pg/mL
IL-12p70
Log-IL-12p70 pg/mL
MCP-1
Log-MCP-1 pg/mL
TNF-a
Log-TNF-a pg/mL
TNF-b
Log-TNF-b pg/mL
Pre-challenge
Days post-challenge

Figure 2. Mixed linear model results for the association between $\log_{10}$ cytokine change from pre-challenge to post-challenge by day, with fixed effects for inoculum dose and day and a random effect by individual subject. Solid line: infected individuals, dashed line: uninfected individuals. Points represent estimates of the effect of day on change in cytokine concentration. Error bars indicate one standard error. P-value indicates significance of overall elevation in cytokine concentration across days.

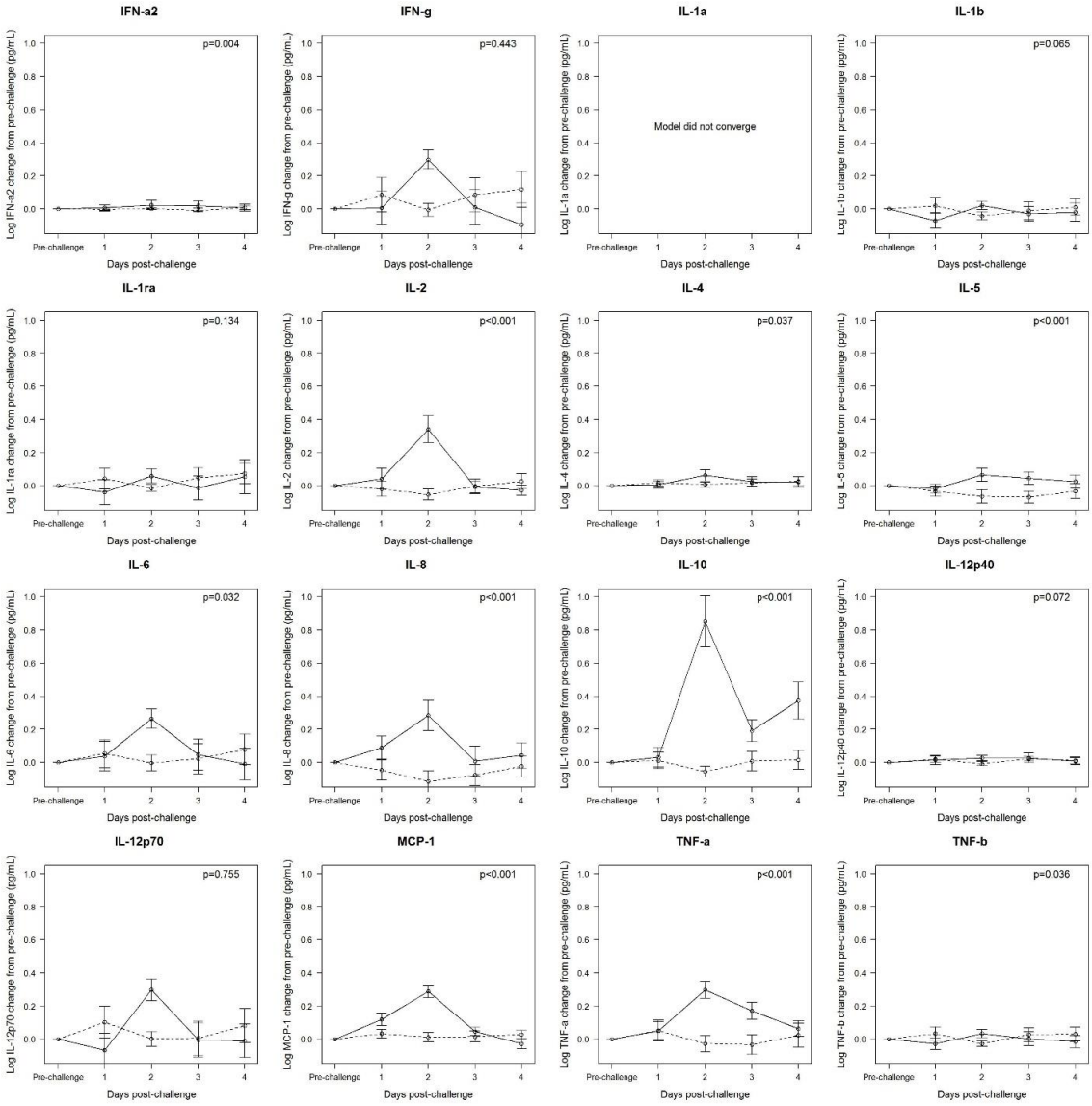

Table 1: Characteristics of norovirus-challenged individuals included in analytic sample

	Total (n=52)	Infected (n=26)	Uninfected (n=26)
Age (mean, SD)	26.7 (7.6)	26.7 (7.5)	26.7 (7.9)
Female	32 (61.5%)	17 (65.4%)	15 (57.7%)
Race			
White	25 (48.1%)	15 (57.7%)	10 (38.5%)
Black	21 (40.4%)	8 (30.8%)	13 (50.0%)
Other	6 (11.5%)	3 (11.5%)	3 (11.5%)
AGE symptoms[a]	19 (33.9%)	17 (65.4%)	2 (7.7%)
Modified Vesikari score (mean, SD)	2.6 (2.4)	4.1 (2.4)	1.1 (0.9)

Abbreviations: SD, standard deviation; AGE, acute gastroenteritis.

[a]AGE symptoms defined as diarrhea (3 or more or >= 400g loose stools in 24 hours) or emesis during days 1-4 post-challenge.

Table 2. Serum cytokine concentrations (pg/mL) of norovirus-challenged individuals pooled across days, stratified by infection status[a].

	All			Infected			Uninfected		
Cytokine	Median	IQR	% above LLOD	Median	IQR	% above LLOD	Median	IQR	% above LLOD
IFN-α2	80.0	80.0-80.0	24.8%	80.0	80.0-133.0	27.9%	80.0	80.0-80.0	21.7%
IFN-γ	186.5	40.4-656.1	90.3%	182.0	67.9-629.3	98.4%	188.3	18.2-669.5	82.2%
IL-1ra	80.0	80.0-999.3	54.3%	80.0	80.0-389.1	73.6%	80.0	80.0-1,993.9	34.9%
IL-1a	163.4	80.0-433.2	24.7%	179.8	80.0-433.2	28.5%	80.2	80.0-433.55	21.1%
IL-1b	3.2	3.2-10.3	58.9%	3.3	3.2-18.45	70.2%	3.2	3.2-7.2	48.1%
IL-2	4.6	3.2-30.1	47.8%	7.5	3.2-38.2	48.0%	3.6	3.2-17.6	47.6%
IL-4	80.0	80.0-99.9	54.7%	80.0	80.0-170.9	59.4%	80.0	80.0-80.0	50.0%
IL-5	3.2	3.2-3.9	38.4%	3.2	3.2-6.0	48.1%	3.2	3.2-3.2	28.7%
IL-6	23.9	3.2-120.2	52.5%	27.8	3.2-93.0	55.5%	21.4	3.2-161.1	49.6%
IL-8	66.5	20.7-198.1	25.4%	81.7	34.2-198.0	28.9%	45.0	11.5-202.4	21.9%
IL-10	4.4	3.2-29.5	26.8%	17.0	3.2-50.3	33.9%	3.2	3.2-6.2	19.7%
IL-12p40	80.0	80.0-80.0	70.7%	80.0	80.0-85.0	69.5%	80.0	80.0-80.0	71.9%
IL-12p70	6.1	3.2-89.8	92.2%	19.5	3.2-86.6	96.9%	3.2	3.2-96.0	87.6%
MCP-1	612.8	397.1-832.5	100.0 %	693.5	510.8-944.3	100.0 %	495.1	329.4-673.1	100.0 %
TNF-α	14.3	6.0-27.2	85.3%	15.1	8.3-25.2	87.6%	14.0	4.9-36.2	82.9%
TNF-β	16.0	16.0-58.5	37.2%	16.0	16.0-100.5	35.7%	16.0	16.0-33.4	38.8%

Abbreviations: IQR, inter-quartile range; LLOD, lower limit of detection; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor.

[a]Values below LLOD included in estimates as LLOD value.

Table 3. Table comparing results from this study to prior studies of cytokine responses to human NoV infection. An 'x' indicates significant elevation compared to uninfected controls. Gray cells indicate no testing was conducted for that cytokine.

Study	This study[a]	Lindesmith 2005 [12]	Long 2011 [22]	Chen 2012 [37]	Souza 2008 [18]	Souza 2007 [19]
NoV genogroup	GI.1	GII.2	Mixed	Mixed	GII.4	GII.4
Subjects	Human	Human	Human	Human	Gn Calf	Gn Pig
Study design	Challenge	Challenge	Observational cohort	Cross-sectional	Challenge	Challenge
Cytokine						
IFN-α						x
IFN-α2	ns					
IFN-γ	x	x	ns		x[b]	x
IL-1ra	ns					
IL-1a	ns					
IL-1b	ns					
IL-2	x	x				
IL-4	ns	ns	ns		x[b]	x
IL-5	ns	x	x			
IL-6	x		ns	x		x
IL-8	x		x	x		
IL-10	x	ns	ns		x[b]	x
IL-12					x[b]	x
IL-12p40	ns					
IL-12p70	x					
MCP-1	x		x			
TNF-α	x	ns	ns		x[b]	
TNF-β	ns					

Abbreviations: ns, not significant; NoV, norovirus; Gn, gnotobiotic.

[a]Modeled using conditional logistic regression conditional on inoculum dose and adjusted for age.

[b]Cytokine concentrations were elevated compared to controls but no statistical testing was conducted.

CHAPTER 5: STUDY 3: NOROVIRUS IN SYMPTOMATIC AND ASYMPTOMATIC INDIVIDUALS: COMPARISON OF THE ACUTE CYTOKINE RESPONSE

[Submitted to Clinical and Experimental Immunology on September 10, 2015]

Abstract

Noroviruses (NoV) are the most common cause of epidemic gastroenteritis worldwide. Our goal was to compare the serum cytokine responses and viral RNA shedding of asymptomatic and symptomatic NoV-infected individuals. We tested serum samples from infected subjects (n=26; 19 symptomatic, 7 asymptomatic) from two human challenge studies of GI.1 NoV for 16 cytokines and stool samples for NoV RNA. While symptomatic and asymptomatic groups had similar patterns of cytokine responses, symptomatic subjects exhibited greater elevations of Th1 and Th2 cytokines and IL-8 post-challenge compared to asymptomatic subjects (all $p<0.001$). Daily viral RNA titer was positively associated with daily IFN-γ, IL-6, IL-8, and TNF-α concentration (all $p<0.05$). Symptoms were not significantly associated with daily viral RNA titer, viral shedding duration, or cumulative shedding. Symptomatic individuals, compared to asymptomatic, have greater immune system activation, but they do not have greater viral burden, suggesting that symptoms may be immune-mediated in NoV infection.

Introduction

Norovirus (NoV) is one of the leading infectious causes of diarrhea worldwide, and is considered responsible for an estimated 18% of all cases [2]. Though it often causes a brief and self-limited illness, among vulnerable populations, such as the elderly or individuals who are immunosuppressed, NoV illness can be more protracted and have severe outcomes [184-186].

NoV infection can be symptomatic or asymptomatic; in human challenge studies approximately 30% of NoV (GI.1 Norwalk) challenged individuals are asymptomatic [38, 39]. Though symptomatic infection is of great concern both for individual and public health reasons [187, 188], the causes of symptomatic NoV infection are poorly understood. In part, the lack of understanding is the result of a paucity of available animal models of NoV infection, none of which fully recapitulates symptoms observed in humans (reviewed in [1]).

Recent advances in NoV vaccine development suggest that though the vaccine candidates only provided modest protection from infection, they demonstrated a greater reduction the incidence of symptoms and in their severity [42, 123]. The mechanism of this protection is unknown. In order to develop more effective vaccines to target both symptom prevention and protection from infection, it is important to better understand potential immunologic drivers of symptoms.

NoV infection leads to a rapid human immune response that is characterized by elevation in serum cytokines 24-48 hours post-infection [189]. This occurs around the same time as the development of symptoms, leading to the hypothesis that symptomatic

infection is caused by immune-mediated damage or direct viral activity. There is limited evidence of the association between viral load and symptoms, with some data, from two challenge studies, suggesting that symptomatic individuals have higher viral RNA titers in stool than asymptomatic individuals [38, 39] and conflicting data, from outbreak studies, indicating that symptomatic and asymptomatic individuals shed virus at similar levels [51].

In this study, we describe the serum cytokine response to NoV infection in symptomatic and asymptomatic individuals who became infected following experimental challenge. The goals were two fold. The first was to assess whether symptoms were associated with greater immune responses to NoV infection, might indicate immune-mediated damage as a cause of symptoms. The second was to determine whether symptoms were associated with greater viral burden in a pooled analysis of GI.1 NoV challenge studies, potentially indicating virus-mediated damage leading to illness.

Methods

Population and samples

The study population was comprised of individuals from two separate NoV challenge studies, described in [49, 50]. Both studies enrolled healthy secretor-positive adults and challenged them with Norwalk virus 8FIIb inoculum. The first study was conducted between May and December of 2006 at Emory University Hospital's Clinical Interaction Site, part of the Atlanta Clinical and Translational Science Institute Clinical Interaction Network. Volunteers were challenged with 6.5×10^7 genomic equivalent

copies (GEC) of the NoV inoculum, which was seeded into water and left in the dark at room temperature to incubate for varying time periods. Stool samples were collected prior to challenge, during a 4-day inpatient stay during the challenge study, and at planned intervals until the end of shedding post-challenge.

The second study was a randomized trial of NoV inactivation methods and was conducted between February 2008 and September 2009 at Emory University Hospital's Clinical Interaction Site. Volunteers were assigned to different control or intervention groups and consumed an oyster that had been seeded with 1×10^4 GEC of the NoV inoculum and then treated with high hydrostatic pressure processing or left untreated as a control. Stool and serum samples were collected prior to challenge, during a 4-day inpatient stay during the challenge study, and at planned intervals until the end of shedding post-challenge

All subjects in both studies consented to allow for future use of all biological specimens. All specimens were stored at -80° C. Emory University's Institutional Review Board approved both studies, and they were registered on ClinicalTrials.gov (identifiers NCT00313404 and NCT00674336).

Stool and emesis samples were tested by quantitative real-time PCR (RT-qPCR) to determine whether individuals had become infected. Infection was defined as having at least one sample test positive by RT-qPCR for NoV RNA following NoV challenge. Between the two studies, a total of 26 individuals became infected with NoV following challenge. Of the 26 who were infected, 19 developed symptoms and 7 remained asymptomatic (symptom measurement described below).

Detection of viral shedding

NoV RNA was quantified by RT-qPCR in all stool and emesis samples from the 2008-2009 study by Kirby, et al. [39] and from the 2006 study by Newman, et al. [189]. Samples from all time points were tested as described [39]. In brief, stool was suspended at a 20% concentration in sterile water and then combined with an equal volume of Vertrel XF (DuPont, Wilmington, DE) to facilitate extraction. The suspension was incubated at 4° C for 2-18 hours then centrifuged at 13,000g for 10 minutes. The QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) was used according to the manufacturer's protocol to extract viral RNA from 140 µl of the aqueous phase. All extractions were also conducted with a known NoV-positive extraction control and a NoV-negative extraction control of sterile water.

Extracted RNA was stored at -20° C then quantified using qRT-PCR with the QiagenOne-Step RT-PCR kit (Qiagen) on a Bio-Rad CFX96 system (Bio-Rad Hercules, CA) with Norwalk Virus-specific primers and probes (NVKS1, NVKS2, and NVKS3) and *in vitro*-transcribed Norwalk virus standards [39, 190] and a limit of detection of 3570 GEC/g stool. Amplification data was analyzed, and concentrations were calculated with the Bio-Rad CFX Manager (Bio-Rad Hercules). All samples were tested in duplicate. Average copy number is reported. Samples that had inconsistent results between duplicates (e.g. difference in cycle threshold values >3) or failed to amplify in either duplicate were retested. All samples were run with successful positive and negative controls. The positive controls were a stool sample known to be positive for NoV RNA and a PCR control of known NoV RNA. The negative controls were a stool sample

known to be negative for NoV RNA and sterile water controls for both extraction and PCR.

Daily titers were calculated based on the average viral RNA titer per gram of stool of all samples collected that day. For days when a stool sample was not available, daily titer was calculated as the average of the daily titers from the prior and subsequent day, as long as the prior and subsequent day had NoV-positive stool samples. If either day did not have a positive stool sample, shedding on the day without an available sample was assumed to be below the limit of detection. Peak viral RNA titer was defined as the highest titer measured for an individual over the entire course of infection. Duration of shedding was calculated as the days from the first positive stool or emesis sample until the midpoint date between the date of the last positive sample and the date of the first negative sample followed by only negative samples. If the last sample available was positive, the date of that sample was regarded as the end of shedding. Cumulative shedding during the first five days post-challenge was calculated by multiplying the GEC/g stool or emesis for each sample, as measured by RT-qPCR, by the weight of each sample and summing the estimated GEC in the sample over the total number of samples collected during the first five post-challenge days, during which all samples were collected and none were lost.

Gastrointestinal symptoms and scoring

Clinical symptoms of infection were assessed during the challenge studies and scored as previously reported [39]. Symptomatic infection was defined as the presence of detectable NoV RNA in stool with a) diarrhea alone or b) emesis plus one additional

symptom (i.e. abdominal cramps, nausea, oral temperature ≥37.6°C, myalgia, chills, fatigue, or headache) or c) at least one of the following symptoms: nausea, abdominal cramps, headache, chills, myalgia, fatigue, or emesis. All symptoms except for fever (oral temperature ≥ 37.6°) were self-reported. Asymptomatic infection was defined as having a NoV positive stool sample but failing to meet the prior definition for symptomatic infection. Severity of infection was assessed using the 17-point modified Vesikari score according to previously described methods [39, 123] and was calculated for all individuals included in the study.

Detection of cytokines

For all but one subject, five serum samples from pre-challenge (pre-challenge) and days 1-4 post challenge were analyzed for cytokines. A sample from day 2 post-challenge was missing for one subject, and so only four samples were analyzed for that individual. Samples were sent to an outside contractor (EMD Millipore Corporation Discovery and Development Solutions) and tested using a Milliplex human cytokine 16-plex assay for IFN-α2, IFN-γ, IL-1a, IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, MCP-1, TNF-α, and TNF-β, as previously described [189]. The assay was run with duplicates of all samples and controls. Concentrations were calculated using a five parameter logistic model for each cytokine and standard curves from recombinant cytokines, as per EMD Millipore standard protocols. The assay's lower limits of detection (LLOD) were 3.2 pg/mL for IFN-γ, IL-10, IL-12p70, IL-1b, IL-2, IL-5, IL-6, IL-8, MCP-1, and TNF-α; 16.0 pg/mL for TNF-β; and 80.0 pg/mL for IFN-α2, IL-12p40, IL-1ra, IL-1a, and IL-4. All values below LLOD were assigned the value of the LLOD.

Missing data

Cytokine values for missing serum samples (n=1, i.e. 16 cytokine test results) and invalid cytokine test results (n=20) were imputed as the average of the concentrations of the same cytokine from the day before and after the missing value for the same subject. A total of 2 subjects had invalid test results for one or more cytokine tested (20 total invalid results). Using imputation, these invalid results were estimated to be below the LLOD because the observations for the same subject and cytokine before and after the test were below the LLOD.

Statistical analysis

Cytokines and symptoms

To investigate the association between serum cytokine response and symptoms at individual time points, the significance of unadjusted differences between pre-challenge and post-challenge serum cytokine levels in symptomatic and asymptomatic individuals were tested using the Wilcoxon signed rank test because the data was not normally distributed. To investigate the association between serum cytokine response and symptoms during the first four days post-challenge, we used a mixed linear model to test the association between $\log_{10}$ cytokine concentration (outcome) and day post-challenge (exposure), stratifying by symptom status (yes/no), with a subject-specific random effect, and an autoregressive correlation structure to account for correlation between time points for each individual. The parameter estimates for the effect of day on cytokine concentration were then averaged to create a single measure of cytokine change over

time. Student's t-test was used to compare these measures of cytokine change between symptomatic and asymptomatic individuals.

Cytokines and viral RNA shedding

To assess the relationships between serum cytokine response (outcome, i.e. fold change in serum cytokine level from pre-challenge) and daily viral RNA titer (exposure, i.e. $\log_{10}$ GEC/g stool) during the first four days post-challenge, a mixed effects model was used. It included fixed effects for the effect of day post-challenge and for viral RNA titer each day and a subject-specific random effect. To examine whether pre-challenge serum cytokine levels were predictive of duration of viral shedding, we used unadjusted Kaplan-Meier survival curves to test the association between cytokines divided into three categories (below LLOD, above the LLOD and below the median pre-challenge serum cytokine level, and above the LLOD and above the median pre-challenge serum cytokine level) and time to the end of viral shedding.

Symptoms and viral RNA shedding

To investigate the association between symptoms and viral shedding, Wilcoxon signed rank tests with a normal approximation and Kaplan-Meier survival analysis were used. The associations between symptoms and three viral shedding parameters (as described above) were assessed: peak viral RNA titer, cumulative shedding during the first five days post-challenge, and duration of viral shedding. Associations between symptoms and peak viral RNA titer and cumulative shedding were assessed using the Wilcoxon test because the data were not normally distributed. The association between

symptoms and the duration of viral shedding was assessed using a Kaplan-Meier survival analysis of time from the onset of shedding until the end of detectable shedding.

All analyses were conducted using SAS 9.4 (Cary, NC). P-values of <0.05 were considered significant.

Results

Among the 26 individuals who had stool or emesis samples that tested positive for NoV by RT-PCR following experimental challenge, 19 developed symptoms and seven remained asymptomatic (Table 1). Subjects were relatively young (mean age=26.9 years). All individuals who were symptomatic reported feeling nausea, even if they did not vomit or have diarrhea.

Studies of human cytokine responses can often have many values measured below the LLOD [170] Therefore, we examined the distribution of each cytokine measured (Table 2). Three cytokines measured (IFN-γ, IL-8, and MCP-1) had fewer than 5% of observations below the LLOD, and two additional cytokines (IL-10 and TNF-α) had fewer than 30% of observations below the LLOD. For many cytokines, asymptomatic individuals had a greater percentage of observations below the LLOD compared to symptomatic individuals (Table 2). Generally, the cytokines had a wide range of observed concentrations.

Cytokines and symptoms

To assess whether there was a difference between pre-challenge and post-challenge cytokine concentrations within symptomatic and asymptomatic individuals, we compared pre- and post-challenge concentrations using a Wilcoxon test. Most cytokine concentrations increased post-challenge among symptomatic and asymptomatic individuals, though the changes were not generally significant (Figure 1). The only statistically significant increases were for IL-10, which exhibited significant elevation from pre-challenge levels among symptomatic individuals on days 2 and 4 post-challenge; MCP-1, which exhibited significant elevation from pre-challenge levels among symptomatic individuals on day 2 post-challenge; and TNF-α, which exhibited significant elevation from pre-challenge levels among symptomatic and asymptomatic individuals on day 2 post-challenge (Figure 1).

To compare the overall changes in cytokine concentrations between symptomatic and asymptomatic individuals following NoV challenge, controlling for day post-challenge, we used a mixed linear model stratified by symptom status. We tested this model for the difference between the estimated parameters for symptomatic and asymptomatic individuals using a t-test. In the adjusted model, symptomatic and asymptomatic individuals remained similar in their pattern of serum cytokine response to NoV, though symptomatic individuals generally had higher concentrations of serum cytokines (Figure 2). Symptomatic individuals had significantly greater changes in serum IFN-γ, IL-1b, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF-α, and TNF-β during the first four days post-challenge, compared to asymptomatic individuals (Figure 2). Asymptomatic individuals had significantly greater changes in IFN-α2, IL-5, and IL-12p40 concentrations post-challenge compared to symptomatic individuals, though the

magnitude of the difference in change was relatively small compared to the differences observed for other cytokines.

Cytokines and viral RNA shedding

To better understand the association between serum cytokine concentrations and NoV shedding, we used a mixed model to test the association during the first four days post-challenge while controlling for post-challenge day. Based on the mixed model for the association between $\log_{10}$ cytokine change from pre-challenge and $\log_{10}$ daily viral RNA titer (independent variable), increased viral RNA titers were significantly associated with elevated levels of IFN-γ (beta=0.009, standard error (SE)=0.004, p=0.035), IL-6 (beta=0.007, SE=0.003, p=0.023), IL-8, (beta=0.017, SE=0.008, p=0.041) and TNF-α (beta=0.009, SE=0.005, p=0.046) post-challenge (data not shown). In addition, we investigated whether pre-challenge cytokine levels were predictive of duration of viral shedding, and we found that no pre-challenge serum cytokines were significant predictors of duration of viral shedding (data not shown).

Symptoms and viral RNA shedding

We then investigated the associations between symptoms and viral shedding. We tested the unadjusted association between symptoms and viral shedding using the log rank test and Kaplan-Meier curves (Figure 3). Though symptomatic individuals tended to shed virus for a longer time than asymptomatic individuals (25.3 days vs. 19.3 days, respectively, Table 1 and Figure 3), the difference was not significant (p=0.2855). We also examined the association between peak viral titer and cumulative shedding with symptoms during the first five days post-challenge using the Wilcoxon test. Symptomatic

individuals did not appear to have higher peak viral titers than asymptomatic individuals (mean peak titer 1.7 x 10^9 GEC/g stool vs 2.0 x 10^9 GEC/g stool, respectively, p=0.119). Similarly, symptomatic individuals did not appear to have meaningfully different levels of cumulative shedding during the first five days post-challenge than asymptomatic individuals (mean cumulative shedding 1.3 x 10^{11} GEC among symptomatic vs 1.5 x 10^{11} GEC among asymptomatic, p=0.215, Table 1).

Discussion

The study goals were to assess the relationships between symptoms and serum cytokine response and between symptoms and shedding. This study found that while both symptomatic and asymptomatic individuals showed similar cytokine response patterns overall, symptomatic individuals had significantly higher serum cytokine levels for IFN-γ, IL-1b, IL-1ra, IL-2, IL-4, IL-8, IL-10, IL-12p70, TNF-α, and TNF-β compared to asymptomatic individuals. Higher daily NoV titers were associated with higher levels of serum IFN-γ, IL-6, IL-8, and TNF-α. Symptomatic infection was not significantly associated with greater duration of viral shedding, higher peak viral titers, or greater cumulative shedding in the first five days post-challenge compared to asymptomatic infection.

Though prior studies have documented the immune responses to NoV challenge in infected individuals compared to uninfected individuals [47, 189], but this is the first study to our knowledge that compares the responses of symptomatic and asymptomatic individuals. This study found that individuals who became symptomatic during infection

had higher levels of Th1-type and some Th-2 type cytokines as well as IL-8 than asymptomatic individuals (Figure 2). Studies of rotavirus gastroenteritis have found that serum TNF-α is significantly associated with diarrhea incidence when compared to healthy controls and controls with non-viral diarrhea [191, 192]. Other viral infections, including hepatitis B and dengue virus, induce different pro-inflammatory cytokine gene expression in symptomatic compared to asymptomatic individuals [193, 194]. In the case of dengue, symptomatic individuals had higher expression of many pro-inflammatory cytokine genes compared to asymptomatic individuals, suggesting that some of dengue's most severe outcomes may be partially caused by an inflammatory response [194]. Indeed, cytokines are thought to cause diarrhea in other infections without primary gastrointestinal tropism (e.g. influenza) [195, 196]. The presence of elevated serum cytokines in symptomatic NoV infection thus suggests that symptoms may be immune-mediated in NoV infection.

This study also found that high daily levels of viral shedding were significantly associated with elevated same-day levels of IFN-γ, IL-6, IL-8, and TNF-α. Though it is impossible to establish causality, as other studies of cytokine levels and viral shedding have found [197], this may indicate that individuals with poor viral control (i.e. high viral titers) mount greater pro-inflammatory responses in an attempt to limit viral replication. Murine NoV research suggests that IFN-γ and other Th-1 cytokines are important for limiting viral replication [81, 82]. However, murine NoV is able to suppress Type-1 IFN production [29]. In our study, we observed no significant elevation of IFN-α2, a Type-1 IFN, post-challenge, suggesting that human NoV may be able to act similarly. Therefore, this may indicate that individuals with high viral titers because of poor initial viral control

may be responding with elevated pro-inflammatory cytokines in order to limit viral replication, possibly despite NoV inhibition of the immune response.

In addition, this study found no significant association between symptomatic infection and viral shedding as measured by duration of shedding, peak titer, or cumulative shedding. This result is different from the results of an earlier analysis by Kirby, et al., which examined individuals from the 2008-2009 challenge study, which was included in the present analysis, and found that in that population there was a significant association between peak titer and symptomatic infection and cumulative shedding in the first seven days post-challenge and symptoms [39]. The present study analyzed a larger population by combining the 2008-2009 challenge study with a 2006 challenge study conducted at the same institution, with the same sample and symptom data collection protocols, and using the same NoV inoculum. It not only differs from the Kirby, et al. study in using an expanded population but also in the use of five day cumulative shedding versus seven day. However the population is still relatively small, and therefore may be underpowered to detect a true association, especially in the presence of the high degree of variability observed. Evidence from other studies is mixed, with some suggesting an association between symptoms and higher levels of viral shedding [38] and others showing no significant association [51]. However, the Atmar, et al. study did not test for the statistical significance of the difference between symptomatic and asymptomatic individuals, whereas the study by Teunis, et al. did. Overall, this study's finding that there was no association between viral control and symptoms supports the hypothesis that symptoms may be the result of an individual susceptibility to NoV symptoms or that symptoms may be caused by immune-mediated mechanisms, such

as cytokine storm, rather than the hypothesis that symptoms are the result of direct viral damage. However, it is also possible that this study was underpowered to detect a true association.

As a result of this study's findings and prior NoV and immunologic research, we propose a schematic for potential causes of symptomatic NoV infection (Figure 4). Histologic studies of NoV-infected individuals [54] and select animal models [40, 44, 82] indicate that NoV infection causes damage to the gut mucosa, particularly the epithelium. This damage may lead to immune activation, or the presence of virus alone may stimulate immune activation. Both hypotheses are supported by the finding from this study that daily viral RNA titer is associated with multiple pro-inflammatory serum cytokines and chemokines. The immune response to NoV has been found to be critical for limiting viral replication and eventually clearing infection [81, 94, 95]. However, there may also be immune-mediated damage, as is seen in other enteric infections [198].

The cause of symptoms is ill understood, and we suggest three possible hypotheses. First, symptoms may be the direct result of immune-activation. Elevated serum cytokines have been shown in rotavirus infection to be associated with symptomatic illness [191] and high levels of serum cytokines and immune activation may cause severe symptoms, including vomiting and diarrhea, as seen in infections leading to cytokine storm [199]. Second, symptoms may be the result of physiologic damage and may be associated with greater viral load and possibly with direct viral activity. This seems plausible, however, the degree of physiologic damage observed in NoV histologic specimens is relatively minor [54], with no detectable viremia in immunocompetent adults [200], and in this study, viral load as assessed by peak titer and cumulative

shedding was not significantly associated with symptomatic illness. Third, we suggest that symptoms may be related to all the three central variables (i.e. viral load, damage, and immune activation) and can potentially be modified by additional factors known to modify immune response, such as genetics [48], immunocompetence [101], prior exposure to similar strains [107], physiologic factors (e.g. comorbidities, microbiome) [88], or coinfection [201]. Future research should consider these hypotheses when investigating the causes of symptomatic NoV infection and the role of vaccines in preventing symptoms.

Strengths and limitations

Key strengths of this research include the analysis of a wide range of serum cytokines and use of a large, longitudinal cohort of NoV-challenged individuals, including many asymptomatic individuals. An additional strength is the use of robust statistical methods that account for correlated longitudinal data. However, there are limitations to this work. As with many challenge studies, there are relatively few individuals included, which limited its statistical power, and pre-challenge exposure history could not be assessed. Due to the nature of the study, it is impossible to assess causality, and there is the possibility that during the time that samples were stored, some of the cytokines may have degraded. An additional limitation is the potential for measurement error when assessing viral shedding. Viral titers may depend on stool volume, and individuals with similar absolute levels of intestinal viral shedding may have different calculated titers if the virus is diluted in a greater amount of stool shed by some individuals (e.g. individuals with high fiber diets may have more copious stool output than individuals with low fiber diets). We have attempted to avoid some of this error by

also considering cumulative shedding, however, this may also be biased in cases of fecal retention or constipation.

Conclusions

Symptomatic NoV infection is a major cause of morbidity and mortality worldwide, and the causes of symptomatic infection continue to be poorly understood. To help develop better prophylaxis, including vaccines, against both symptomatic and asymptomatic NoV infection, it is important to understand the immunologic basis of NoV-related illness. This study found that symptomatic individuals had significantly higher levels of serum cytokines post-challenge than asymptomatic individuals, though they did not have significantly higher viral titers, longer durations of viral shedding, or greater amounts of viral shedding in the five days post-challenge. These findings suggest that symptoms may be partially driven by an immune-mediated mechanism rather than depend on viral burden. However, there are likely additional factors involved in symptom development. Future vaccine candidates should capitalize on the growing understanding of symptomatic infection and leverage existing gains in enhancing protection against severe gastroenteritis.

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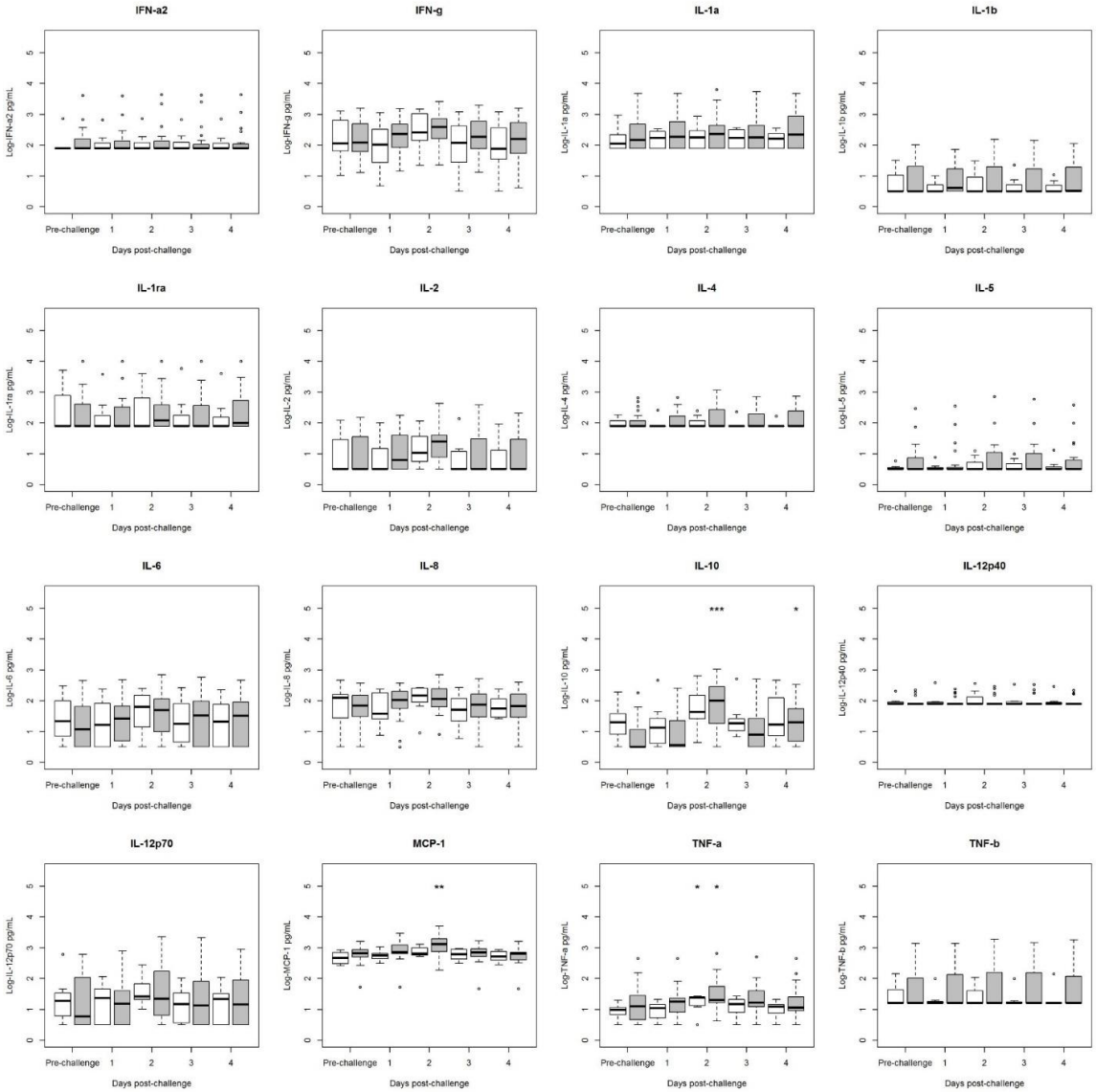
Figures

Figure 1. Comparison of pre-challenge to post-challenge serum cytokine concentrations in infected humans from two norovirus challenge studies, stratified by symptom status (symptomatic n=19, asymptomatic n=7). Values below the lower limit of detection (LLOD) were assigned to the value of the LLOD. Significance of change from pre-challenge value was tested using Wilcoxon test and is denoted by an asterisk above the relevant category. Interquartile ranges (IQRs) for symptomatic individuals (gray boxes) and for asymptomatic individuals (white boxes) are shown. Dark lines indicate median values. Whiskers indicate most extreme value that is no more than 1.5 times the IQR away from the bound of the IQR. Circles indicate outliers. *$p<0.05$, **$p<0.01$, ***$p<0.001$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IL-12p70
Log-IL-12p70 pg/mL
MCP-1
Log-MCP-1 pg/mL
TNF-a
Log-TNF-a pg/mL
TNF-b
Log-TNF-b pg/mL
Pre-challenge
Days post-challenge

Figure 2. Mixed linear model results for the association between $\log_{10}$ serum cytokine concentration change from pre-challenge to post-challenge by day, with fixed effects for day and a random effect by individual subject. Solid line: symptomatic individuals, dashed line: asymptomatic individuals. Points represent estimates of the effect of day on change in cytokine concentration. Error bars indicate one standard error. P-value indicates significance of overall elevation in cytokine concentration across days.

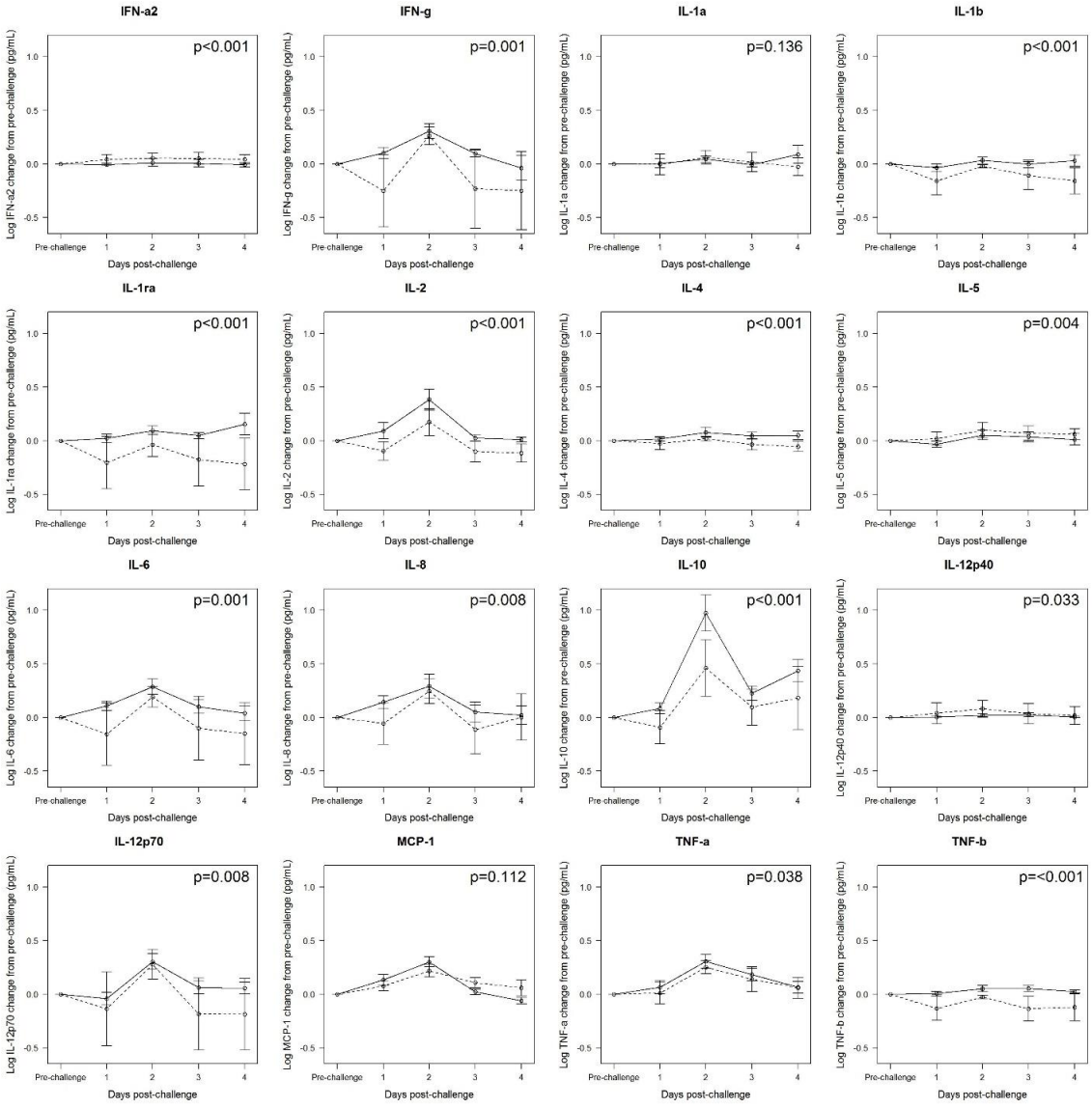

Figure 3. Kaplan-Meier survival curves for association between symptom status and duration of viral RNA shedding (days) among NoV infected individuals. Solid line indicates symptomatic individuals; dashed line indicates asymptomatic individuals.

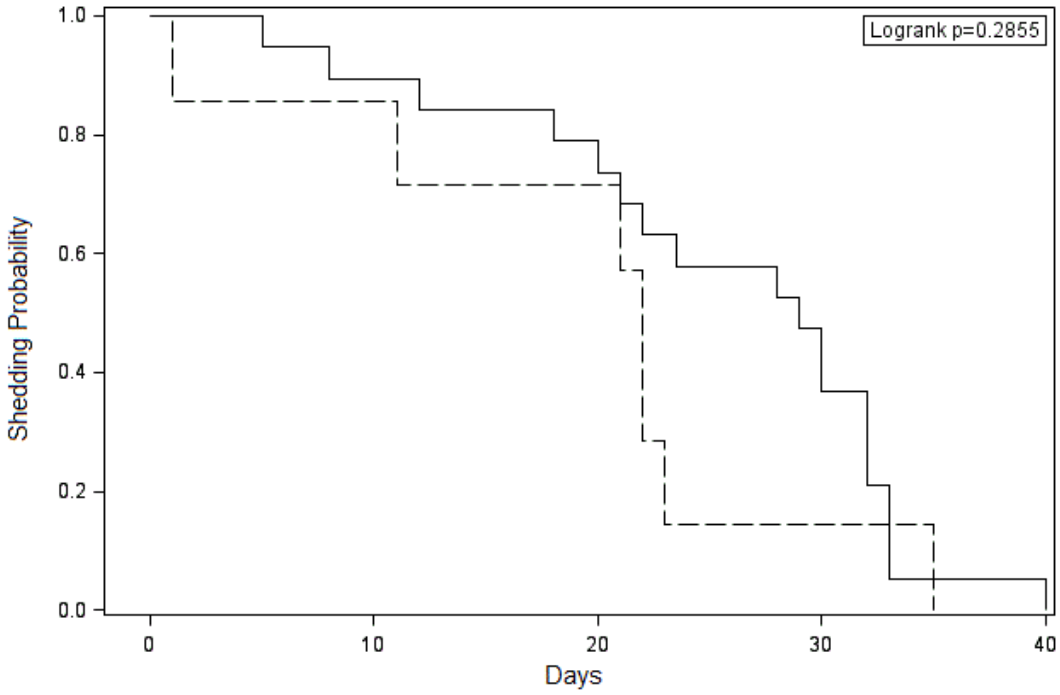

Figure 4. Proposed schematic of potential causes of symptomatic NoV infection. Solid lines indicate stimulation. Bar at the end of an arrow indicates inhibition. Dotted lines indicate hypothesized causes.

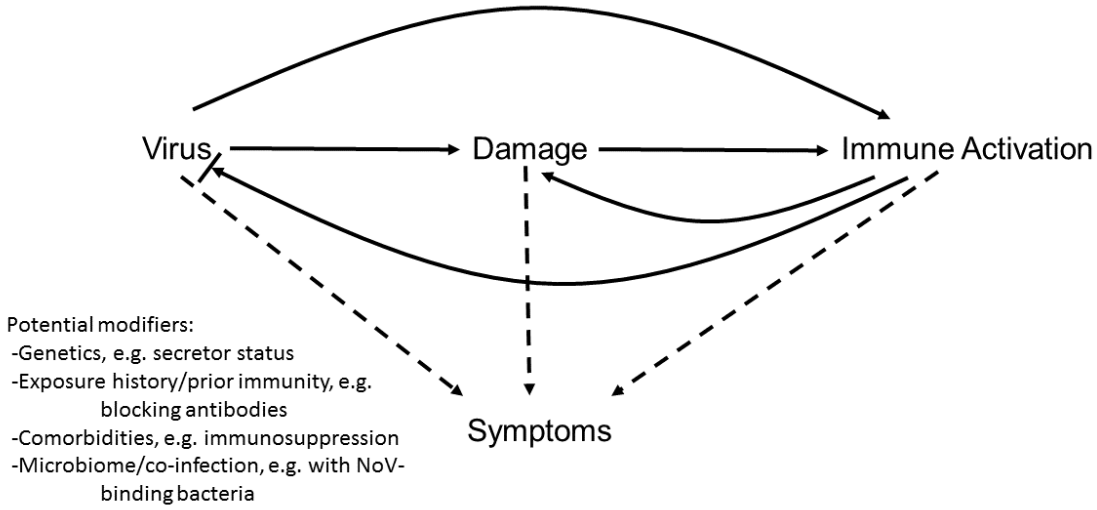

Table 1. Characteristics of norovirus-infected individuals included in study.

Characteristic	All (n=26) Mean (SD)	Symptomatic (n=19) Mean (SD)	Asymptomatic (n=7) Mean (SD)
Age (years)	26.9 (7.5)	27.1 (5.8)	25.6 (11.6)
Female (n, %)	16 (61.5%)	11 (57.9%)	5 (71.4%)
Modified Vesikari Score	4.1 (2.4)	5.3 (1.8)	1 (0)
Duration of shedding (days)	23.7 (9.8)	25.3 (9.2)	19.3 (10.0)
Cumulative shedding in first 5 days (log_{10} GEC)	11.1 (11.3)	11.1 (11.2)	11.2 (11.5)
Mean peak viral RNA titer (log_{10} GEC)	9.3 (9.5)	9.2 (9.5)	9.3 (9.7)

Table 2. Serum cytokine concentrations (pg/mL) of norovirus-infected individuals pooled across days, stratified by symptom status[a].

	All		Symptomatic		Asymptomatic	
Cytokines	**Median (IQR)**	**% below LLOD**	**Median (IQR)**	**% below LLOD**	**Median (IQR)**	**% below LLOD**
IFN-α2	80.0 (80.0-138.3)	71.5%	80.0 (80.0-136.8)	70.5%	80.0 (80.0-166.8)	74.3%
IFN-γ	183.7 (67.8-634.6)	1.5%	240.0 (79.0-592.7)	0.0%	118.5 (56.8-936.5)	5.7%
IL-1a	3.2 (3.1-16.5)	40.8%	185.2 (80.0-503.1)	40.0%	3.2 (3.2-7.5)	42.9%
IL-1b	177.2 (80.0-412.8)	52.3%	3.2 (3.2-20.8)	50.5%	163.4 (80.0-316.7)	57.1%
IL-1ra	80.0 (80.0-393.0)	52.3%	88.1 (80.0-407.5)	47.3%	80.0 (80.0-390.2)	65.7%
IL-2	6.7 (3.2-36.6)	45.4%	7.0 (3.2-39.6)	43.2%	3.2 (3.2-17.5)	51.4%
IL-4	80.0 (80.0-170.3)	71.5%	80.0 (80.0-188.2)	68.4%	80.0 (80.0-80.0)	80%
IL-5	3.2 (3.2-6.3)	66.2%	3.2 (3.2-7.5)	64.2%	3.2 (3.2-4.0)	71.4%
IL-6	27.8 (3.2-94.7)	30.8%	32.4 (3.2-91.3)	30.5%	21.1 (3.2-96.9)	31.4%
IL-8	81.7 (33.2-200.0)	3.8%	85.2 (35.2-196.5)	4.2%	64.5 (26.5-222.3)	2.9%
IL-10	17.0 (3.2-50.3)	26.2%	11.6 (3.2-50.3)	32.6%	19.2 (6.9-50.4)	8.6%
IL-12p40	80.0 (80.0-85.8)	72.3%	80.0 (80.0-80.0)	77.9%	80.0 (80.0-94.5)	57.1%
IL-12p70	19.5 (3.2-85.3)	32.3%	15.4 (3.2-111.6)	33.7%	21.8 (3.2-46.0)	28.6%
MCP-1	695.6 (510.6-949.5)	0.0%	711.2 (517.5-1,062.5)	0.0%	603.5 (415.5-804.1)	0.0%
TNF-α	15.2 (8.2-25.2)	12.3%	16.8 (9.0-38.9)	11.6%	12.0 (5.9-20.2)	14.3%
TNF-β	16.0 (16.0-100.7)	64.6%	16.0 (16.0-207.0)	62.1%	16.0 (16.0-19.2)	71.4%

Abbreviations: IQR, inter-quartile range; LLOD, lower limit of detection; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor.

[a]Values below LLOD included in estimates as LLOD value.

CHAPTER 6: RESEARCH SUMMARY, STRENGTHS, LIMITATIONS, PUBLIC HEALTH IMPLICATIONS, FUTURE DIRECTIONS

Research Summary

The research relied on prior human NoV challenge studies. Though the detailed methods of these studies have not been included, it is important to acknowledge their centrality in this work. Human challenge studies represent the only source of controlled, prospective data on human NoV infection in healthy adults. As such, they are valuable resource.

Study 1: Serum samples were available for the days that corresponded with times of peak viral shedding. However, upon testing these samples (n=38) we did not detect viremia in any of the 20 subjects. Given that all control conditions in the experiment worked, we believe this result to be indicative that NoV infection does not lead to viremia in immunocompetent adults. Viremia in immunocompromised individuals and pediatric cases may indicate that these populations exhibit different immune responses or have different levels of protection from NoV invasion of or extraversation into the blood.

Study 2: This study used a population of 52 age-matched individuals who were experimentally challenged with GI.1 NoV and of whom 26 became infected and 26 remained uninfected post-challenge. Individuals who became infected with NoV post-challenge had elevated Th1-type cytokines, Th2-type cytokines, and chemokines IL-8 and MCP-1, compared to individuals who remained uninfected post-challenge. Among infected individuals, most serum cytokines peaked on day 2 post-challenge, though IL-10 remained elevated through day 4 post-challenge. This indicates that individuals who remained uninfected post-challenge did not exhibit detectable immune activation and

may have been protected through other mechanisms, such as pre-existing antibodies, absence of cellular receptors, or others . However, it is possible that individuals who remained uninfected post-challenge exhibited local immune responses that were sufficient to neutralize the inoculum dose or otherwise limit its infectivity.

Study 3: This study used the 26 infected individuals from Study 2, of whom 19 were symptomatically infected and 7 were asymptomatically infected. Symptomatic NoV infection was associated with greater elevation of Th1-type cytokines, Th2-type cytokines, and IL-8, compared to asymptomatic infection. Elevated daily viral RNA titer was associated with increased IFN-g, IL-6, IL-8, and TNF-a. Symptoms were not significantly associated with elevated viral RNA shedding. This suggests that symptoms may be the result of immune activation as well as potentially due to damage by NoV. The lack of an association between higher viral burden and greater likelihood of symptoms suggests that NoV may not act directly to elicit symptoms, or the study may be underpowered to note a significant association.

Research Strengths

There are many strengths to this dissertation. This dissertation contains the first published study, to our knowledge, to test for NoV viremia in NoV-challenged immunocompetent adults. It also has the largest study of human serum cytokine response to NoV challenge. Lastly, this dissertation includes the first study to explicitly examine immunologic factors associated with symptomatic infection.

Some strengths are intrinsic to the challenge study data itself. Challenge studies represent the only source of human samples of NoV-infected adults with controlled longitudinal follow-up throughout the course of infection. The studies benefit from being able to study the human immune response directly and from the multiple available time points at which to immune response could be measured and for which corresponding symptom and viral shedding data was available.

Studies 2 and 3 had additional strengths in having tested for the broadest range of serum cytokines assayed in any study of human NoV conducted to date. The range allowed for the study of a broad variety of immune responses. They also tested for cytokines both before infection and at multiple post-infection time points, which is unique to these studies. Lastly the studies were strengthened by the use of robust statistical methods that accounted for correlated data.

Research Limitations

There are some limitations to this work, many of which stem from the limitations of challenge studies. The studies had small sample sizes, though they were relatively large compared to earlier studies of the immune response of NoV-challenged individuals. This limited the statistical power of this dissertation. In addition, symptoms aside from fever, vomiting, and diarrhea were self-reported. In addition, the prior exposure histories of the challenge study participants were unknown.

Due to the nature of the studies, it is impossible to assess causality in the relationship between cytokine response and viral titer. It is also possible that during the time that samples were stored between collection and testing, some of the cytokines may

have degraded. An additional limitation is the potential for measurement error when assessing viral shedding. Viral titers may depend on stool volume, and individuals with similar absolute levels of intestinal viral shedding may have different calculated titers if the virus is diluted in a greater amount of stool shed by some individuals (e.g. individuals with high fiber diets may have more copious stool output than individuals with low fiber diets). Finally, the relative homogeneity of the study population with regard to age and health status may limit generalizability to some of the most vulnerable populations—children, the elderly, and the immunosuppressed.

Research Conclusions

Though NoV infection of healthy adults was not associated with viremia, infected individuals exhibited brief and broad cytokine responses coincident with the development of symptoms. Symptomatic infection was associated with greater immune activation than asymptomatic infection, though it was not associated with greater viral burden. This suggests that NoV elicits a broad anti-viral response and that symptoms may be due to immune-mediated damage.

Public Health Implications

The findings in this dissertation provide a greater understanding of the acute immune response to NoV and its role in the development of symptomatic infection. As vaccine development continues, this understanding is important for helping guide development of an effective vaccine to prevent symptomatic illness. The public health burden of NoV infection is due to the symptoms it causes, therefore, the development of a vaccine that provides protection against gastroenteritis would decrease morbidity and mortality due to NoV.

Furthermore, the finding that symptomatic and asymptomatic individuals shed virus at similar levels during the first five days of infection helps inform epidemiologic modeling of NoV. Models of NoV infection should therefore place weight both on the behaviors of symptomatic and asymptomatic individuals as well as their viral titers when attempting to understand or describe epidemic and endemic NoV spread. Because the viral titers between symptomatic and asymptomatic individuals were similar, they may both contribute to viral propagation, though perhaps through different actions. In addition, models of NoV infection in populations require rigorously obtained data on basic parameters, such as viral shedding duration and titer, and this dissertation adds to the available data.

Research Future Directions

This work serves as a starting point for research in three different directions. These include better understanding the causes and effects of NoV viremia in vulnerable populations, continued work elucidating the mechanisms of protection for individuals who do not become infected following NoV challenge, and efforts to characterize the pathogenesis of NoV symptoms and the role of the immune response.

First, the finding that immunocompetent adults infected with NoV post-challenge do not exhibit viremia suggests that future work should consider the mechanism by which viremia occurs in immunosuppressed individuals and children infected with NoV. Important avenues of future research along these lines include obtaining longitudinal serum and stool samples from viremic individuals to describe the duration of viremia. Additionally future epidemiologic work should assess whether immunocompetent adults with severe NoV infection (i.e. those requiring intravenous fluids or hospital admission)

exhibit viremia. These two studies would help determine whether viremia is a transient feature of infection in immunosuppressed and pediatric populations. If viremia is not transient, it is possible that viral presence in the blood may be associated with specific immunologic processes. Given the increased vulnerability of these populations, it is important to understand the factors that may predispose them to harmful sequelae. Studying immunocompetent adults with severe NoV symptoms may also help to clarify whether viremia is associated with worse symptoms.

Second, our study failed to identify immunologic correlates of protection against NoV infection following challenge. Serum cytokines pre-challenge did not predict individuals who would become infected post-challenge. Future research should examine individuals in the hours post-challenge to ensure that uninfected individuals do not exhibit a rapidly resolving serum cytokine response. Additional work should consider testing a broader array of immune parameters, including pre-challenge blocking antibodies and the mucosal immune response. However, the absence of a good animal model or cell culture system serves as an impediment to a sizable amount of the necessary work to understand the role of prior exposure in protecting against NoV challenge.

Lastly, it is important to continue to work towards understanding the causes of NoV symptoms. Symptomatic infection is responsible for a greater proportion of NoV spread, making it important to control symptoms. The current vaccine candidate shows poor protection against infection but mild protection against severe symptoms. If the vaccine could be optimized to protect against symptoms, it would be successful in mitigating the major economic and public health burden caused by NoV infection, since

these are largely due to lost productivity and morbidity from symptomatic infection. Gnotobiotic animal models of NoV may be a useful starting point for understanding the role that vaccination can play in protection from symptoms. These models exhibit mild symptoms that may be similar to primary infection. One limiting factor is their lack of immunecompetence. Given that the vaccine candidate provides some symptomatic protection, there may be an important role of immune memory or antibodies in preventing NoV gastroenteritis, and gnotobiotic animals' immune systems may be insufficient to investigate this. However, there are ongoing human vaccine studies which should consider identification of surrogates of symptom protection to be a high priority.

Ultimately, NoV will be difficult if not impossible to eradicate. Therefore, the ideal goal is to develop therapeutic options and prophylaxis that will allow for coexistence. NoV infection has few long-term sequelae. Therefore, prevention of acute illness can be prioritized because of its benefit to quality of life and the likely additional effect of reducing overall transmission.

REFERENCES

1. Newman, K.L.L., J. S., *Norovirus immunology: Of mice and mechanisms.* European Journal of Immunology, 2015.
2. Ahmed, S.M., et al., *Global prevalence of norovirus in cases of gastroenteritis: a systematic review and meta-analysis.* Lancet Infect Dis, 2014. **14**(8): p. 725-30.
3. Payne, D.C., et al., *Norovirus and medically attended gastroenteritis in U.S. children.* N Engl J Med, 2013. **368**(12): p. 1121-30.
4. Verhoef, L., et al., *The estimated disease burden of norovirus in The Netherlands.* Epidemiol Infect, 2013. **141**(3): p. 496-506.
5. Trivedi, T.K., et al., *Hospitalizations and mortality associated with norovirus outbreaks in nursing homes, 2009-2010.* JAMA, 2012. **308**(16): p. 1668-75.
6. Schwartz, S., et al., *Norovirus gastroenteritis causes severe and lethal complications after chemotherapy and hematopoietic stem cell transplantation.* Blood, 2011. **117**(22): p. 5850-6.
7. Siebenga, J.J., et al., *High prevalence of prolonged norovirus shedding and illness among hospitalized patients: a model for in vivo molecular evolution.* J Infect Dis, 2008. **198**(7): p. 994-1001.
8. Bagci, S., et al., *Clinical characteristics of viral intestinal infection in preterm and term neonates.* Eur J Clin Microbiol Infect Dis, 2010. **29**(9): p. 1079-84.
9. Murray, C.J., et al., *Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010.* Lancet, 2012. **380**(9859): p. 2197-223.

10. Lozano, R., et al., *Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010.* Lancet, 2012. **380**(9859): p. 2095-128.

11. Walker, C.L., et al., *Global burden of childhood pneumonia and diarrhoea.* Lancet, 2013. **381**(9875): p. 1405-16.

12. Liu, L., et al., *Global, regional, and national causes of child mortality in 2000-13, with projections to inform post-2015 priorities: an updated systematic analysis.* Lancet, 2015. **385**(9966): p. 430-40.

13. Lanata, C.F., et al., *Global causes of diarrheal disease mortality in children <5 years of age: a systematic review.* PLoS One, 2013. **8**(9): p. e72788.

14. Zanini, B., et al., *Incidence of post-infectious irritable bowel syndrome and functional intestinal disorders following a water-borne viral gastroenteritis outbreak.* Am J Gastroenterol, 2012. **107**(6): p. 891-9.

15. Khan, R.R., et al., *Gastrointestinal norovirus infection associated with exacerbation of inflammatory bowel disease.* J Pediatr Gastroenterol Nutr, 2009. **48**(3): p. 328-33.

16. Gastanaduy, P.A., et al., *Burden of norovirus gastroenteritis in the ambulatory setting--United States, 2001-2009.* J Infect Dis, 2013. **207**(7): p. 1058-65.

17. Belliot, G., et al., *The Burden of Norovirus gastroenteritis: an important foodborne and healthcare-related infection.* Clin Microbiol Infect, 2014.

18. Head, M.G., J.R. Fitchett, and R. Atun, *Systematic analysis of funding awarded for norovirus research to institutions in the United Kingdom, 1997-2010.* J R Soc Med, 2014. **107**(3): p. 110-5.

19. Vinje, J., *Advances in Laboratory Methods for Detection and Typing of Norovirus.* J Clin Microbiol, 2014.

20. Karst, S.M., et al., *Advances in norovirus biology.* Cell Host Microbe, 2014. **15**(6): p. 668-80.

21. Karst, S.M., S. Zhu, and I.G. Goodfellow, *The molecular pathology of noroviruses.* J Pathol, 2015. **235**(2): p. 206-16.

22. Tan, M. and X. Jiang, *Histo-blood group antigens: a common niche for norovirus and rotavirus.* Expert Rev Mol Med, 2014. **16**: p. e5.

23. Arias, A., et al., *Progress towards the prevention and treatment of norovirus infections.* Future Microbiol, 2013. **8**(11): p. 1475-87.

24. Ramani, S., R.L. Atmar, and M.K. Estes, *Epidemiology of human noroviruses and updates on vaccine development.* Curr Opin Gastroenterol, 2014. **30**(1): p. 25-33.

25. Rocha-Pereira, J., J. Neyts, and D. Jochmans, *Norovirus: Targets and tools in antiviral drug discovery.* Biochem Pharmacol, 2014.

26. Clarke, I.N.E., M.K.; Green, K.Y.; Hansman, G.S.; Knowles, N.J.; Koopmans, M.K.; Matson, D.O.; Meyers, G.; Neill, J.D.; Radford, A.; Smith, A.W.; Studdert, M.J.; Thiel, H.-J.; Vinjé, J., *Caliciviridae*, in *Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses.*, A.M.Q. King, Adams, M.J., Carstens, E.B. and Lefkowitz, E.J., Editor. 2012, Elsevier: San Diego. p. 977-986.

27. Carstens, E.B., *Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2009).* Arch Virol, 2010. **155**(1): p. 133-46.

28. Mesquita, J.R., et al., *Presence of antibodies against genogroup VI norovirus in humans.* Virol J, 2013. **10**: p. 176.

29. McFadden, N., et al., *Norovirus regulation of the innate immune response and apoptosis occurs via the product of the alternative open reading frame 4.* PLoS Pathog, 2011. **7**(12): p. e1002413.

30. Zheng, D.P., et al., *Norovirus classification and proposed strain nomenclature.* Virology, 2006. **346**(2): p. 312-23.

31. Kroneman, A., et al., *Proposal for a unified norovirus nomenclature and genotyping.* Arch Virol, 2013.

32. Jones, M.K., et al., *Enteric bacteria promote human and mouse norovirus infection of B cells.* Science, 2014. **346**(6210): p. 755-9.

33. Papafragkou, E., et al., *Challenges of culturing human norovirus in three-dimensional organoid intestinal cell culture models.* PLoS One, 2013. **8**(6): p. e63485.

34. Miura, T., et al., *Histo-blood group antigen-like substances of human enteric bacteria as specific adsorbents for human noroviruses.* J Virol, 2013. **87**(17): p. 9441-51.

35. Huang, P., et al., *Noroviruses bind to human ABO, Lewis, and secretor histo-blood group antigens: identification of 4 distinct strain-specific patterns.* J Infect Dis, 2003. **188**(1): p. 19-31.

36. Tan, M. and X. Jiang, *Norovirus gastroenteritis, carbohydrate receptors, and animal models.* PLoS Pathog, 2010. **6**(8): p. e1000983.

37. Bok, K., et al., *Chimpanzees as an animal model for human norovirus infection and vaccine development.* Proc Natl Acad Sci U S A, 2011. **108**(1): p. 325-30.

38. Atmar, R.L., et al., *Norwalk virus shedding after experimental human infection.* Emerg Infect Dis, 2008. **14**(10): p. 1553-7.

39. Kirby, A.E., et al., *Disease course and viral shedding in experimental Norwalk virus and Snow Mountain virus infection.* J Med Virol, 2014.

40. Souza, M., et al., *Pathogenesis and immune responses in gnotobiotic calves after infection with the genogroup II.4-HS66 strain of human norovirus.* J Virol, 2008. **82**(4): p. 1777-86.

41. Souza, M., et al., *Cytokine and antibody responses in gnotobiotic pigs after infection with human norovirus genogroup II.4 (HS66 strain).* J Virol, 2007. **81**(17): p. 9183-92.

42. Bernstein, D.I., et al., *Norovirus Vaccine Against Experimental Human GII.4 Virus Illness: A Challenge Study in Healthy Adults.* J Infect Dis, 2014.

43. Frenck, R., et al., *Predicting susceptibility to norovirus GII.4 by use of a challenge model involving humans.* J Infect Dis, 2012. **206**(9): p. 1386-93.

44. Cheetham, S., et al., *Pathogenesis of a genogroup II human norovirus in gnotobiotic pigs.* J Virol, 2006. **80**(21): p. 10372-81.

45. Taube, S., et al., *A mouse model for human norovirus.* MBio, 2013. **4**(4).

46. Atmar, R.L., et al., *Determination of the 50% human infectious dose for Norwalk virus.* J Infect Dis, 2014. **209**(7): p. 1016-22.

47. Lindesmith, L., et al., *Cellular and humoral immunity following Snow Mountain virus challenge.* J Virol, 2005. **79**(5): p. 2900-9.

48. Lindesmith, L., et al., *Human susceptibility and resistance to Norwalk virus infection.* Nat Med, 2003. **9**(5): p. 548-53.

49. Leon, J.S., et al., *Randomized, double-blinded clinical trial for human norovirus inactivation in oysters by high hydrostatic pressure processing.* Appl Environ Microbiol, 2011. **77**(15): p. 5476-82.

50. Seitz, S.R., et al., *Norovirus infectivity in humans and persistence in water.* Appl Environ Microbiol, 2011. **77**(19): p. 6884-8.

51. Teunis, P.F., et al., *Shedding of norovirus in symptomatic and asymptomatic infections.* Epidemiol Infect, 2014: p. 1-8.

52. Glass, R.I., U.D. Parashar, and M.K. Estes, *Norovirus gastroenteritis.* N Engl J Med, 2009. **361**(18): p. 1776-85.

53. Ko, G., et al., *Fecal cytokines and markers of intestinal inflammation in international travelers with diarrhea due to Noroviruses.* J Med Virol, 2006. **78**(6): p. 825-8.

54. Troeger, H., et al., *Structural and functional changes of the duodenum in human norovirus infection.* Gut, 2009. **58**(8): p. 1070-7.

55. Schorn, R., et al., *Chronic norovirus infection after kidney transplantation: molecular evidence for immune-driven viral evolution.* Clin Infect Dis, 2010. **51**(3): p. 307-14.

56. Schreiber, D.S., N.R. Blacklow, and J.S. Trier, *The mucosal lesion of the proximal small intestine in acute infectious nonbacterial gastroenteritis.* N Engl J Med, 1973. **288**(25): p. 1318-23.

57. Schreiber, D.S., N.R. Blacklow, and J.S. Trier, *The small intestinal lesion induced by Hawaii agent acute infectious nonbacterial gastroenteritis.* J Infect Dis, 1974. **129**(6): p. 705-8.

58. Rocha-Pereira, J., et al., *The viral polymerase inhibitor 2'-C-methylcytidine inhibits Norwalk virus replication and protects against norovirus-induced diarrhea and mortality in a mouse model.* J Virol, 2013. **87**(21): p. 11798-805.

59. Jung, K., et al., *The effects of simvastatin or interferon-alpha on infectivity of human norovirus using a gnotobiotic pig model for the study of antivirals.* PLoS One, 2012. **7**(7): p. e41619.

60. Flynn, W.T., L.J. Saif, and P.D. Moorhead, *Pathogenesis of porcine enteric calicivirus-like virus in four-day-old gnotobiotic pigs.* Am J Vet Res, 1988. **49**(6): p. 819-25.

61. Otto, P.H., et al., *Infection of calves with bovine norovirus GIII.1 strain Jena virus: an experimental model to study the pathogenesis of norovirus infection.* J Virol, 2011. **85**(22): p. 12013-21.

62. Sestak, K., et al., *Experimental inoculation of juvenile rhesus macaques with primate enteric caliciviruses.* PLoS One, 2012. **7**(5): p. e37973.

63. Rydell, G.E., et al., *Susceptibility to winter vomiting disease: a sweet matter.* Rev Med Virol, 2011. **21**(6): p. 370-82.

64. Thorven, M., et al., *A homozygous nonsense mutation (428G-->A) in the human secretor (FUT2) gene provides resistance to symptomatic norovirus (GGII) infections.* J Virol, 2005. **79**(24): p. 15351-5.

65. Hutson, A.M., et al., *Norwalk virus infection associates with secretor status genotyped from sera.* J Med Virol, 2005. **77**(1): p. 116-20.

66. Ferrer-Admetlla, A., et al., *A natural history of FUT2 polymorphism in humans.* Mol Biol Evol, 2009. **26**(9): p. 1993-2003.

67. Currier, R.L., et al., *Innate Susceptibility to Norovirus Infections Influenced by FUT2 Genotype in a United States Pediatric Population.* Clin Infect Dis, 2015. **60**(11): p. 1631-8.

68. Reeck, A., et al., *Serological correlate of protection against norovirus-induced gastroenteritis.* J Infect Dis, 2010. **202**(8): p. 1212-8.

69. Blacklow, N.R., et al., *Immune response and prevalence of antibody to Norwalk enteritis virus as determined by radioimmunoassay.* J Clin Microbiol, 1979. **10**(6): p. 903-9.

70. Johnson, P.C., et al., *Multiple-challenge study of host susceptibility to Norwalk gastroenteritis in US adults.* J Infect Dis, 1990. **161**(1): p. 18-21.

71. Larsson, M.M., et al., *Antibody prevalence and titer to norovirus (genogroup II) correlate with secretor (FUT2) but not with ABO phenotype or Lewis (FUT3) genotype.* J Infect Dis, 2006. **194**(10): p. 1422-7.

72. Malm, M., et al., *High Serum Levels of Norovirus Genotype-Specific Blocking Antibodies Correlate with Protection from Infection in Children.* J Infect Dis, 2014.

73. Nurminen, K., et al., *Prevalence of norovirus GII-4 antibodies in Finnish children.* J Med Virol, 2011. **83**(3): p. 525-31.

74. Ryder, R.W., et al., *Evidence of immunity induced by naturally acquired rotavirus and Norwalk virus infection on two remote Panamanian islands.* J Infect Dis, 1985. **151**(1): p. 99-105.

75. Ramani, S., et al., *Mucosal and Cellular Immune Responses to Norwalk Virus.* J Infect Dis, 2015.

76. Czako, R., et al., *Serum hemagglutination inhibition activity correlates with protection from gastroenteritis in persons infected with Norwalk virus.* Clin Vaccine Immunol, 2012. **19**(2): p. 284-7.

77. Harrington, P.R., et al., *Binding of Norwalk virus-like particles to ABH histo-blood group antigens is blocked by antisera from infected human volunteers or experimentally vaccinated mice.* J Virol, 2002. **76**(23): p. 12335-43.

78. Wobus, C.E., et al., *Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages.* PLoS Biol, 2004. **2**(12): p. e432.

79. Gonzalez-Hernandez, M.B., et al., *Efficient Norovirus and Reovirus Replication in the Mouse Intestine Requires Microfold (M) Cells.* J Virol, 2014. **88**(12): p. 6934-6943.

80. McCartney, S.A., et al., *MDA-5 recognition of a murine norovirus.* PLoS Pathog, 2008. **4**(7): p. e1000108.

81. Karst, S.M., et al., *STAT1-dependent innate immunity to a Norwalk-like virus.* Science, 2003. **299**(5612): p. 1575-8.

82. Mumphrey, S.M., et al., *Murine norovirus 1 infection is associated with histopathological changes in immunocompetent hosts, but clinical disease is*

prevented by STAT1-dependent interferon responses. J Virol, 2007. **81**(7): p. 3251-63.

83. Thackray, L.B., et al., *Critical role for interferon regulatory factor 3 (IRF-3) and IRF-7 in type I interferon-mediated control of murine norovirus replication.* J Virol, 2012. **86**(24): p. 13515-23.

84. Changotra, H., et al., *Type I and type II interferons inhibit the translation of murine norovirus proteins.* J Virol, 2009. **83**(11): p. 5683-92.

85. Hwang, S., et al., *Nondegradative role of Atg5-Atg12/ Atg16L1 autophagy protein complex in antiviral activity of interferon gamma.* Cell Host Microbe, 2012. **11**(4): p. 397-409.

86. Rodriguez, M.R., et al., *ISG15 functions as an interferon-mediated antiviral effector early in the murine norovirus life cycle.* J Virol, 2014. **88**(16): p. 9277-86.

87. Nice, T.J., et al., *Interferon lambda cures persistent murine norovirus infection in the absence of adaptive immunity.* Science, 2014.

88. Baldridge, M.T., et al., *Commensal microbes and interferon-lambda determine persistence of enteric murine norovirus infection.* Science, 2015. **347**(6219): p. 266-9.

89. Bui, T., et al., *Median infectious dose of human norovirus GII.4 in gnotobiotic pigs is decreased by simvastatin treatment and increased by age.* J Gen Virol, 2013.

90. Meurens, F., et al., *The pig: a model for human infectious diseases.* Trends Microbiol, 2012. **20**(1): p. 50-7.

91. Pabst, R. and H.J. Rothkotter, *Postnatal development of lymphocyte subsets in different compartments of the small intestine of piglets.* Vet Immunol Immunopathol, 1999. **72**(1-2): p. 167-73.

92. Pabst, R., et al., *Postnatal development and lymphocyte production of jejunal and ileal Peyer's patches in normal and gnotobiotic pigs.* Immunology, 1988. **64**(3): p. 539-44.

93. Dolin, R., et al., *Viral gastroenteritis induced by the Hawaii agent. Jejunal histopathology and serologic response.* Am J Med, 1975. **59**(6): p. 761-8.

94. Chachu, K.A., et al., *Antibody is critical for the clearance of murine norovirus infection.* J Virol, 2008. **82**(13): p. 6610-7.

95. Chachu, K.A., et al., *Immune mechanisms responsible for vaccination against and clearance of mucosal and lymphatic norovirus infection.* PLoS Pathog, 2008. **4**(12): p. e1000236.

96. Park, K., K.E. Cha, and H. Myung, *Observation of inflammatory responses in mice orally fed with bacteriophage T7.* J Appl Microbiol, 2014.

97. Tomov, V.T., et al., *Persistent enteric murine norovirus infection is associated with functionally suboptimal virus-specific CD8 T cell responses.* J Virol, 2013. **87**(12): p. 7015-31.

98. Zhu, S., et al., *Identification of immune and viral correlates of norovirus protective immunity through comparative study of intra-cluster norovirus strains.* PLoS Pathog, 2013. **9**(9): p. e1003592.

99. Chagla, Z., et al., *Chronic norovirus infection in a transplant patient successfully treated with enterally administered immune globulin.* J Clin Virol, 2013. **58**(1): p. 306-8.

100. Ronchetti, A.M., et al., *Norovirus-related chronic diarrhea in a patient treated with alemtuzumab for chronic lymphocytic leukemia.* BMC Infect Dis, 2014. **14**: p. 239.

101. Saif, M.A., et al., *Chronic norovirus infection in pediatric hematopoietic stem cell transplant recipients: a cause of prolonged intestinal failure requiring intensive nutritional support.* Pediatr Transplant, 2011. **15**(5): p. 505-9.

102. Bok, K. and K.Y. Green, *Norovirus gastroenteritis in immunocompromised patients.* N Engl J Med, 2012. **367**(22): p. 2126-32.

103. Waugh, E., et al., *Characterization of the chemokine response of RAW264.7 cells to infection by murine norovirus.* Virus Res, 2014. **181**: p. 27-34.

104. Groom, J.R. and A.D. Luster, *CXCR3 ligands: redundant, collaborative and antagonistic functions.* Immunol Cell Biol, 2011. **89**(2): p. 207-15.

105. Strong, D.W., et al., *Protruding domain of capsid protein is necessary and sufficient to determine murine norovirus replication and pathogenesis in vivo.* J Virol, 2012. **86**(6): p. 2950-8.

106. Ponterio, E., et al., *Pattern of activation of human antigen presenting cells by genotype GII.4 norovirus virus-like particles.* J Transl Med, 2013. **11**: p. 127.

107. Lindesmith, L.C., et al., *Heterotypic humoral and cellular immune responses following Norwalk virus infection.* J Virol, 2010. **84**(4): p. 1800-15.

108. Chen, S.M., et al., *The significance of serum and fecal levels of interleukin-6 and interleukin-8 in hospitalized children with acute rotavirus and norovirus gastroenteritis.* Pediatr Neonatol, 2014. **55**(2): p. 120-6.

109. Long, K.Z., et al., *Vitamin A modifies the intestinal chemokine and cytokine responses to norovirus infection in Mexican children.* J Nutr, 2011. **141**(5): p. 957-63.

110. Hinkula, J., et al., *Antibody prevalence and immunoglobulin IgG subclass pattern to Norwalk virus in Sweden.* J Med Virol, 1995. **47**(1): p. 52-7.

111. Wingfield, T., et al., *Chronic norovirus infection in an HIV-positive patient with persistent diarrhoea: a novel cause.* J Clin Virol, 2010. **49**(3): p. 219-22.

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

113. Iritani, N., et al., *Humoral immune responses against norovirus infections of children.* J Med Virol, 2007. **79**(8): p. 1187-93.

114. Rockx, B., et al., *Characterization of the homo- and heterotypic immune responses after natural norovirus infection.* J Med Virol, 2005. **77**(3): p. 439-46.

115. Cukor, G., N.A. Nowak, and N.R. Blacklow, *Immunoglobulin M responses to the Norwalk virus of gastroenteritis.* Infect Immun, 1982. **37**(2): p. 463-8.

116. Leon, J., et al., *Immunology of Norovirus Infection*, in *Immunity Against Mucosal Pathogens*, V. M, Editor. 2008, Springer: New York. p. 219-262.

117. Treanor, J.J., et al., *Subclass-specific serum antibody responses to recombinant Norwalk virus capsid antigen (rNV) in adults infected with Norwalk, Snow Mountain, or Hawaii virus.* J Clin Microbiol, 1993. **31**(6): p. 1630-4.

118. Wyatt, R.G., et al., *Comparison of three agents of acute infectious nonbacterial gastroenteritis by cross-challenge in volunteers.* J Infect Dis, 1974. **129**(6): p. 709-14.

119. Parra, G.I. and K.Y. Green, *Sequential gastroenteritis episodes caused by 2 norovirus genotypes.* Emerg Infect Dis, 2014. **20**(6): p. 1016-8.

120. Saito, M., et al., *Multiple norovirus infections in a birth cohort in a Peruvian Periurban community.* Clin Infect Dis, 2014. **58**(4): p. 483-91.

121. Simmons, K., et al., *Duration of immunity to norovirus gastroenteritis.* Emerg Infect Dis, 2013. **19**(8): p. 1260-7.

122. Parrino, T.A., et al., *Clinical immunity in acute gastroenteritis caused by Norwalk agent.* N Engl J Med, 1977. **297**(2): p. 86-9.

123. Atmar, R.L., et al., *Norovirus vaccine against experimental human Norwalk Virus illness.* N Engl J Med, 2011. **365**(23): p. 2178-87.

124. Ball, J.M., et al., *Recombinant Norwalk virus-like particles given orally to volunteers: phase I study.* Gastroenterology, 1999. **117**(1): p. 40-8.

125. El-Kamary, S.S., et al., *Adjuvanted intranasal Norwalk virus-like particle vaccine elicits antibodies and antibody-secreting cells that express homing receptors for mucosal and peripheral lymphoid tissues.* J Infect Dis, 2010. **202**(11): p. 1649-58.

126. Tacket, C.O., et al., *Humoral, mucosal, and cellular immune responses to oral Norwalk virus-like particles in volunteers.* Clin Immunol, 2003. **108**(3): p. 241-7.

127. Treanor, J.J., et al., *A Novel Intramuscular Bivalent Norovirus Virus-Like Particle Vaccine Candidate-Reactogenicity, Safety, and Immunogenicity in a Phase 1 Trial in Healthy Adults.* J Infect Dis, 2014.

128. Atmar, R.L. and M.K. Estes, *Norovirus vaccine development: next steps.* Expert Rev Vaccines, 2012. **11**(9): p. 1023-5.

129. Hickman, D., et al., *The effect of malnutrition on norovirus infection.* MBio, 2014. **5**(2): p. e01032-13.

130. Nelson, A.M., et al., *Murine norovirus infection does not cause major disruptions in the murine intestinal microbiota.* Microbiome, 2013. **1**(1): p. 7.

131. Nelson, A.M., et al., *Disruption of the human gut microbiota following Norovirus infection.* PLoS One, 2012. **7**(10): p. e48224.

132. Cadwell, K., et al., *Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine.* Cell, 2010. **141**(7): p. 1135-45.

133. Basic, M., et al., *Norovirus triggered microbiota-driven mucosal inflammation in interleukin 10-deficient mice.* Inflamm Bowel Dis, 2014. **20**(3): p. 431-43.

134. Kernbauer, E., Y. Ding, and K. Cadwell, *An enteric virus can replace the beneficial function of commensal bacteria.* Nature, 2014. **516**(7529): p. 94-8.

135. Tamura, M., et al., *Genogroup II noroviruses efficiently bind to heparan sulfate proteoglycan associated with the cellular membrane.* J Virol, 2004. **78**(8): p. 3817-26.

136. Murakami, K., et al., *Norovirus binding to intestinal epithelial cells is independent of histo-blood group antigens.* PLoS One, 2013. **8**(6): p. e66534.

137. Makino, A., et al., *Junctional adhesion molecule 1 is a functional receptor for feline calicivirus.* J Virol, 2006. **80**(9): p. 4482-90.

138. Stuart, A.D. and T.D. Brown, *Alpha2,6-linked sialic acid acts as a receptor for Feline calicivirus.* J Gen Virol, 2007. **88**(Pt 1): p. 177-86.

139. Monne Rodriguez, J.M., et al., *Alveolar macrophages are the main target cells in feline calicivirus-associated pneumonia.* Vet J, 2014. **201**(2): p. 156-65.

140. Kreutz, L.C., B.S. Seal, and W.L. Mengeling, *Early interaction of feline calicivirus with cells in culture.* Arch Virol, 1994. **136**(1-2): p. 19-34.

141. Taube, S., et al., *Ganglioside-linked terminal sialic acid moieties on murine macrophages function as attachment receptors for murine noroviruses.* J Virol, 2009. **83**(9): p. 4092-101.

142. Taube, S., et al., *Murine noroviruses bind glycolipid and glycoprotein attachment receptors in a strain-dependent manner.* J Virol, 2012. **86**(10): p. 5584-93.

143. Zakhour, M., et al., *The alphaGal epitope of the histo-blood group antigen family is a ligand for bovine norovirus Newbury2 expected to prevent cross-species transmission.* PLoS Pathog, 2009. **5**(7): p. e1000504.

144. Jung, K., et al., *Pathogenesis of GIII.2 bovine norovirus, CV186-OH/00/US strain in gnotobiotic calves.* Vet Microbiol, 2014. **168**(1): p. 202-7.

145. Kim, D.S., et al., *Both alpha2,3- and alpha2,6-linked sialic acids on o-linked glycoproteins act as functional receptors for porcine sapovirus.* PLoS Pathog, 2014. **10**(6): p. e1004172.

146. Chang, K.O., et al., *Bile acids are essential for porcine enteric calicivirus replication in association with down-regulation of signal transducer and activator of transcription 1.* Proc Natl Acad Sci U S A, 2004. **101**(23): p. 8733-8.

147. Parwani, A.V., et al., *Serial propagation of porcine enteric calicivirus in a continuous cell line. Effect of medium supplementation with intestinal contents or enzymes.* Arch Virol, 1991. **120**(1-2): p. 115-22.

148. Farkas, T., et al., *Genetic diversity and histo-blood group antigen interactions of rhesus enteric caliciviruses.* J Virol, 2010. **84**(17): p. 8617-25.

149. Farkas, T., et al., *Characterization of a rhesus monkey calicivirus representing a new genus of Caliciviridae.* J Virol, 2008. **82**(11): p. 5408-16.

150. Smiley, J.R., et al., *Characterization of an enteropathogenic bovine calicivirus representing a potentially new calicivirus genus.* J Virol, 2002. **76**(20): p. 10089-98.

151. Dolin, R., et al., *Transmission of acute infectious nonbacterial gastroenteritis to volunteers by oral administration of stool filtrates.* J Infect Dis, 1971. **123**(3): p. 307-12.

152. Kapikian, A.Z., et al., *Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis.* J Virol, 1972. **10**(5): p. 1075-81.

153. Dolin, R., et al., *Biological properties of Norwalk agent of acute infectious nonbacterial gastroenteritis.* Proc Soc Exp Biol Med, 1972. **140**(2): p. 578-83.

154. Agus, S.G., et al., *Acute infectious nonbacterial gastroenteritis: intestinal histopathology. Histologic and enzymatic alterations during illness produced by the Norwalk agent in man.* Ann Intern Med, 1973. **79**(1): p. 18-25.

155. Widerlite, L., et al., *Structure of the gastric mucosa in acute infectious bacterial gastroenteritis.* Gastroenterology, 1975. **68**(3): p. 425-30.

156. Dolin, R., et al., *Detection by immune electron microscopy of the Snow Mountain agent of acute viral gastroenteritis.* J Infect Dis, 1982. **146**(2): p. 184-9.

157. Keswick, B.H., et al., *Inactivation of Norwalk virus in drinking water by chlorine.* Appl Environ Microbiol, 1985. **50**(2): p. 261-4.

158. Treanor, J.J., H.P. Madore, and R. Dolin, *Development of an enzyme immunoassay for the Hawaii agent of viral gastroenteritis.* J Virol Methods, 1988. **22**(2-3): p. 207-14.

159. Graham, D.Y., et al., *Norwalk virus infection of volunteers: new insights based on improved assays.* J Infect Dis, 1994. **170**(1): p. 34-43.

160. Teunis, P.F., et al., *Norwalk virus: how infectious is it?* J Med Virol, 2008. **80**(8): p. 1468-76.

161. Ahmed, S.M., et al., *Global prevalence of norovirus in cases of gastroenteritis: a systematic review and meta-analysis.* Lancet Infect Dis, 2014. **14**: p. 725-730.

162. Frange, P., et al., *Prevalence and clinical impact of norovirus fecal shedding in children with inherited immune deficiencies.* J Infect Dis, 2012. **206**(8): p. 1269-74.

163. Fumian, T.M., et al., *Quantitative and molecular analysis of noroviruses RNA in blood from children hospitalized for acute gastroenteritis in Belem, Brazil.* J Clin Virol, 2013. **58**(1): p. 31-5.

164. Lemes, L.G., et al., *Prospective study on Norovirus infection among allogeneic stem cell transplant recipients: Prolonged viral excretion and viral RNA in the blood.* J Clin Virol, 2014.

165. Takanashi, S., et al., *Detection, genetic characterization, and quantification of norovirus RNA from sera of children with gastroenteritis.* J Clin Virol, 2009. **44**(2): p. 161-3.

166. Bae, J. and K.J. Schwab, *Evaluation of murine norovirus, feline calicivirus, poliovirus, and MS2 as surrogates for human norovirus in a model of viral persistence in surface water and groundwater.* Appl Environ Microbiol, 2008. **74**(2): p. 477-84.

167. Vinje, J., *Advances in Laboratory Methods for Detection and Typing of Norovirus.* J Clin Microbiol, 2015. **53**(2): p. 373-381.

168. Green, K.Y., *Caliciviridae: The Noroviruses,* in *Fields Virology,* D.M.H. Knipe, P., Editor. 2013, L.W.W.

169. Leon JS, S.M., Qiuhong W, Smith ER, Saif LJ and Moe CL., *Immunology of Norovirus Infection,* in *Immunity Against Mucosal Pathogens*, V. M, Editor. 2008, Springer: New York. p. 219-262.

170. Wong, H.L., et al., *Reproducibility and correlations of multiplex cytokine levels in asymptomatic persons.* Cancer Epidemiol Biomarkers Prev, 2008. **17**(12): p. 3450-6.

171. Wagner, R.D., *Effects of microbiota on GI health: gnotobiotic research.* Adv Exp Med Biol, 2008. **635**: p. 41-56.

172. Kucharzik, T., et al., *Acute induction of human IL-8 production by intestinal epithelium triggers neutrophil infiltration without mucosal injury.* Gut, 2005. **54**(11): p. 1565-72.

173. Baggiolini, M. and I. Clark-Lewis, *Interleukin-8, a chemotactic and inflammatory cytokine.* FEBS Lett, 1992. **307**(1): p. 97-101.

174. Roberts, A.I., M. Bilenker, and E.C. Ebert, *Intestinal intraepithelial lymphocytes have a promiscuous interleukin-8 receptor.* Gut, 1997. **40**(3): p. 333-8.

175. Ebert, E.C., *Human intestinal intraepithelial lymphocytes have potent chemotactic activity.* Gastroenterology, 1995. **109**(4): p. 1154-9.

176. Casola, A., et al., *Rotavirus infection of cultured intestinal epithelial cells induces secretion of CXC and CC chemokines.* Gastroenterology, 1998. **114**(5): p. 947-55.

177. Neurath, M.F., *New targets for mucosal healing and therapy in inflammatory bowel diseases.* Mucosal Immunol, 2014. **7**(1): p. 6-19.

178. Peterson, K.M., et al., *Association between TNF-alpha and Entamoeba histolytica diarrhea.* Am J Trop Med Hyg, 2010. **82**(4): p. 620-5.

179. Nicholls, S., et al., *Cytokines in stools of children with inflammatory bowel disease or infective diarrhoea.* J Clin Pathol, 1993. **46**(8): p. 757-60.

180. Greenberg, D.E., et al., *Markers of inflammation in bacterial diarrhea among travelers, with a focus on enteroaggregative Escherichia coli pathogenicity.* J Infect Dis, 2002. **185**(7): p. 944-9.

181. Chen, S.M., et al., *Diagnostic performance of serum interleukin-6 and interleukin-10 levels and clinical predictors in children with rotavirus and norovirus gastroenteritis.* Cytokine, 2012. **59**(2): p. 299-304.

182. Sabat, R., et al., *Biology of interleukin-10.* Cytokine Growth Factor Rev, 2010. **21**(5): p. 331-44.

183. de Jager, W., et al., *Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays.* BMC Immunol, 2009. **10**: p. 52.

184. Green, K.Y., *Norovirus Infection in Immunocompromised Hosts.* Clin Microbiol Infect, 2014.

185. Gustavsson, L., et al., *Excess mortality following community-onset norovirus enteritis in the elderly.* J Hosp Infect, 2011. **79**(1): p. 27-31.

186. Desai, R., et al., *Severe outcomes are associated with genogroup 2 genotype 4 norovirus outbreaks: a systematic literature review.* Clin Infect Dis, 2012. **55**(2): p. 189-93.

187. Sukhrie, F.H., et al., *Nosocomial transmission of norovirus is mainly caused by symptomatic cases.* Clin Infect Dis, 2012. **54**(7): p. 931-7.

188. Zelner, J.L., et al., *Linking time-varying symptomatology and intensity of infectiousness to patterns of norovirus transmission.* PLoS One, 2013. **8**(7): p. e68413.

189. Newman, K.L., et al., *Human norovirus infection and the acute serum cytokine response.* Clin Exp Immunol, 2015.

190. Liu, P., et al., *Quantification of Norwalk virus inocula: Comparison of endpoint titration and real-time reverse transcription-PCR methods.* J Med Virol, 2010. **82**(9): p. 1612-6.

191. Jiang, B., et al., *Cytokines as mediators for or effectors against rotavirus disease in children.* Clin Diagn Lab Immunol, 2003. **10**(6): p. 995-1001.

192. Azim, T., et al., *Rotavirus-specific subclass antibody and cytokine responses in Bangladeshi children with rotavirus diarrhoea.* J Med Virol, 2003. **69**(2): p. 286-95.

193. Lian, J.Q., et al., *Expression profiles of circulating cytokines, chemokines and immune cells in patients with hepatitis B virus infection.* Hepat Mon, 2014. **14**(6): p. e18892.

194. Yeo, A.S., et al., *Lack of clinical manifestations in asymptomatic dengue infection is attributed to broad down-regulation and selective up-regulation of host defence response genes.* PLoS One, 2014. **9**(4): p. e92240.

195. Reisinger, E.C., et al., *Diarrhea caused by primarily non-gastrointestinal infections.* Nat Clin Pract Gastroenterol Hepatol, 2005. **2**(5): p. 216-22.

196. Wang, J., et al., *Respiratory influenza virus infection induces intestinal immune injury via microbiota-mediated Th17 cell-dependent inflammation (vol 211, pg 2397, 2014).* Journal of Experimental Medicine, 2014. **211**(13): p. 2683-2683.

197. Mehta, S.K., et al., *Reactivation of latent viruses is associated with increased plasma cytokines in astronauts.* Cytokine, 2013. **61**(1): p. 205-209.

198. Navaneethan, U. and R.A. Giannella, *Mechanisms of infectious diarrhea.* Nat Clin Pract Gastroenterol Hepatol, 2008. **5**(11): p. 637-47.

199. Martina, B.E., *Dengue pathogenesis: a disease driven by the host response.* Sci Prog, 2014. **97**(Pt 3): p. 197-214.

200. Newman, K.L., et al., *Immunocompetent adults from human norovirus challenge studies do not exhibit norovirus viremia.* J Virol, 2015. **89**(13): p. 6968-9.

201. Osborne, L.C., et al., *Coinfection. Virus-helminth coinfection reveals a microbiota-independent mechanism of immunomodulation.* Science, 2014. **345**(6196): p. 578-82.