Chapter 9
Immunology of Norovirus Infection

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Abstract Noroviruses are the leading cause of epidemic non-bacterial gastroenteritis worldwide. Despite their discovery over three decades ago, little is known about the host immune response to norovirus infection. The purpose of this chapter is to review the field of norovirus immunology and discuss the contributions of outbreak investigations, human and animal challenge studies and population-based studies. This chapter will survey both humoral and cellular immunity as well as recent advances in norovirus vaccine development.

9.1 Introduction

9.1.1 Norovirus Disease Etiology

Noroviruses (NoV) are the major cause of epidemic gastroenteritis in the United States and a significant cause of severe diarrhea in young children in developing countries [1, 2]. NoV is also the most frequent cause of acute gastroenteritis after ingestion of raw shellfish [3, 4, 5]. NoV symptomatic infection causes vomiting, watery diarrhea, nausea, abdominal cramps, fever and general malaise. Gastroenteritis induced by NoV is self-limiting and rarely fatal. Fatality in children and the elderly is usually caused by severe dehydration after NoV infection [2, 6, 7].

9.1.2 Classification

NoV belong to the family *Caliciviridae*, genus *norovirus*, and are currently divided into five distinct genetic classifications called genogroups (GI-V). Genogroups are

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further subdivided into clusters, each categorized with a number and the name of the prototype strain. Each cluster is comprised of individual strains identified from various outbreaks and human and animal infections. GI, GII, and GIV affect humans, and currently there are 8 clusters for GI, and 17 clusters for GII [8]. It is not possible to determine the exact number of strains for each cluster and genogroup because new strains continue to be added.

9.1.3 Transmission

Transmission of NoV may occur via ingestion of fecal-contaminated food or water, exposure to contaminated fomites or aerosolized vomitus, and direct person-to-person contact [9, 10, 11, 12, 13, 14, 15, 16, 17]. In rare cases, transmission can occur through organ transplantation [16, 17]. A low infectious dose of less than 5 genomic copies (viral particles) could be enough to infect a healthy adult (Moe, C.L. et al., unpublished data). In certain symptomatic and asymptomatic individuals, virus can be shed for more than 3 weeks post-challenge or exposure [18, 19, 20]. In immunocompromised individuals, such as transplant recipients, NoV has been detected in stool specimens for up to two years after initial infection [16, 17, 21, 22, 23]. These individuals may be asymptomatic carriers of NoV and a possible reservoir for human NoV in a population.

9.1.4 Epidemiology

NoVs are the second most important cause of severe gastroenteritis in young children [24, 25], and may cause about 20% of endemic gastroenteritis in families [26]. Each year in the U.S., the public health impact of NoV is evidenced by the estimated 23 million infections that result in an estimated 50,000 hospitalizations and 310 fatal cases [27]. This number is probably a severe underestimate of the true burden of disease. In 2004, in the U.S., NoV was responsible for 48% of outbreaks among all reported gastroenteritis outbreaks and 79% of outbreaks among reported non-bacterial gastroenteritis outbreaks [28]. GI and GII strains cause the majority of human outbreaks. Analyses, based on published outbreak reports and national surveillance systems, suggests most outbreaks are associated with GII strains [29]. Within GII, cluster 4 (GII.4), “Bristol”, has been currently associated with most of the published outbreak reports among all the GI and GII clusters.

NoV are classified by the Centers for Disease Control and Prevention (CDC) and the National Institute of Allergy and Infectious Diseases (NIAID) as a Bioterrorism Category B Priority Pathogen based on their high transmissibility, low infectious dose, and serious public health and economic impact. No vaccine is currently available.

9.1.5 Overview

The purpose of this chapter is to review current advances in the field of NoV immunology. The reader is encouraged to read recent reviews on NoV immunology [30, 31, 32]. This chapter incorporates a synthesis of previous reviews and our recent understanding of NoV pathogenesis based on the development of new models of NoV disease.

At the onset, it is important to define key terms used throughout this chapter. NoV may be internalized, usually through the mucosal route, after active administration of NoV inocula to volunteers (*challenge*) or animals (*inoculation*) or passive contact of humans or animals with NoV, usually in outbreaks (*exposure*). Challenge, inoculation or exposure with NoV may or may not lead to infection. *Infection* with NoV is defined as replication of NoV in the host. NoV infection can be determined by detection of viral particles or viral RNA in the host or by the presence of viral nonstructural proteins in host cells. *Viral shedding* is defined as the release of quantifiable virus in host specimens such as feces and vomitus. NoV may be detected in these specimens by molecular detection techniques, such as various forms of reverse transcription PCR (RT-PCR: conventional PCR, real time PCR, quantitative real time PCR, etc.), antigen capture immunoassays (e.g. ELISA), or electron microscopy. Infection may or may not lead to *illness*. The terms *illness, disease*, and *symptomatic infection* will be used interchangeably. NoV immunology refers to the host immune response induced after challenge, inoculation, or exposure to NoV.

9.2 How to Study NoV Immunology

9.2.1 Population Studies

Until recently, there were few non-human models of NoV disease, and the majority of our understanding of NoV immunology came from human challenge studies and studies of human populations. Prospective, retrospective, and case-control studies were common study designs and often took advantage of existing community and clinical specimens collected from studies [33, 34, 35]. In addition, clinical specimens (e.g. saliva, sera, stool) taken from outbreak investigations continue to provide a wealth of information to our understanding of NoV immunology. The advantages of these approaches are that the results are more representative of *real* conditions than laboratory studies and are directly applicable to the human condition. Disadvantages include the difficulty in obtaining clinical specimens, limited control of study design and low or inadequate study power.

9.2.2 Human Challenge Studies

From the 1970s to the present, there have been over a dozen human challenge studies with NoV. In these studies, volunteers were enrolled in a clinical trial (after all

the necessary regulatory approvals were met) and studied under controlled clinical conditions, usually in a hospital setting. Volunteers were challenged with various preparations of NoV, clinical specimens collected, and their responses recorded. The advantages of human challenge studies include: direct applicability of results to the human condition, control of study design and study power, and ease of obtaining clinical specimens. The disadvantages include: difficulty in obtaining all the relevant regulatory and ethical reviews and approvals, logistical issues involved with human volunteers, and difficulty in obtaining adequate sample sizes (study power). Another disadvantage is that human volunteers often have a range of pre-challenge exposures that will affect their response to the challenge. The difficulty is two-fold: (1) volunteers have diverse pre-challenge exposure history and (2) we do not have adequate methods of measuring and characterizing their pre-challenge exposure history. Lastly, it is difficult to create approved NoV inocula for use in human challenge studies.

Four NoV clusters, with possibly varying strains, have been used in human challenge studies since the 1970s: Norwalk virus (GI.1), Montgomery County (GI.5) [36], Snow Mountain virus (GII.2) and Hawaii virus (GII.1) (classification determined from [8]). Other human challenge studies, prior to 1970, have used uncharacterized challenge agents and therefore it is unclear whether these challenge agents were only NoV or included other viruses (reviewed in [37, 38, 39]). Currently (i.e. 2007), two groups perform human challenge studies; these are Dr. Mary Estes' group at Baylor College of Medicine, in Houston, Texas, and Dr. Christine Moe's group at Emory University, in Atlanta, Georgia.

9.2.3 Non-Human Models

During the past decade, several new promising animal models have been developed that provide more tools for studying NoV pathogenesis and immunology. These models include the gnotobiotic (Gn) pig, Gn calves, non-human primates, mice, as well as other invertebrate models. All models shed virus and seroconverted to NoV antigens. Currently, both the Gn pig and murine models hold the most promise for a greater understanding of NoV immunology.

9.2.3.1 Gnotobiotic Pigs

In 2006, Dr. Linda Saif's lab developed a new model of human NoV disease that promises to advance the field of NoV immunology. This model utilizes gnotobiotic (Gn) pigs as an experimental animal model [40, 41, 42]. In this model, the investigators were able to infect the Gn pigs with a GII.4 human NoV after oral inoculation. Gn pigs have long been used to study human rotavirus pathogenesis and host immune responses and have the advantages of similar gastrointestinal physiology, mucosal immune responses and milk diet to that of infants [43, 44, 45, 46]. Other advantages of this model include susceptibility to human NoV infection, and similarity of symptoms with human disease, such as diarrhea and pathological changes

in the gastrointestinal system [40]. Additionally, Gn pigs have histo-blood group antigens, like humans, that influence susceptibility to NoV. The incubation period for GII.4 NoV in Gn pigs (12 to 48 hours) was similar to that observed in humans experimentally infected with Snow Mountain virus (SMV) (19 to 41 hours) [47], and the diarrhea was mild and of short duration (1 to 4 days in most pigs). However, there are some differences compared to human disease. Multiple passages of NoV through various pigs (up to 3 serial passages) seemed to reduce the duration of diarrhea, reduce the prevalence of Gn pigs that shed virus, and reduced the duration of viral shedding although the virus was still infectious; therefore, multiple passages in the Gn pigs may be required for more complete adaptation of the GII.4 NoV to this animal model. The human NoVs infect the proximal small intestine in both humans and in Gn pigs. However, infected humans reportedly exhibit inflammation in the duodenum and jejunum [48, 49, 50] whereas Gn pigs exhibit mild pathological changes in the duodenum, but not jejunum and ileum. Furthermore, only 1 of 7 inoculated pigs exhibited pathological changes [40].

One interesting observation was that intravenous administration of human NoV also consistently infected Gn pigs, and NoV was transiently detected in serum of orally inoculated pigs [40]. In humans, NoV viremia, i.e. systemic spread of the virus, has not yet been carefully examined; although it is hypothesized that NoV cannot be transmitted through blood products. However, based on the finding in pigs, re-examination of human NoV viremia is warranted.

9.2.3.2 Gnotobiotic Calves

In Dr. Linda Saif's lab, the Gn calf has also been used as an animal model to study pathogenesis and immune responses to viruses such as a human enteric coronavirus [51], rotaviruses [52] and NoV [53, 54]. Because Gn calves are raised on a milk diet and are free of microbes, their rumen does not develop and their gut physiology and mucosal immune responses (dominance of secretory IgA) remain similar to that of other monogastrics, including human infants. They provide an alternative animal model for the study of gastrointestinal viruses, such as the fastidious human NoV. Of importance, the occurrence of GIII NoVs in cattle also permits comparative studies of host-specific versus NoV adaptive host strains. In one model, the bovine GIII.2 NoV CV186-OH and the unassigned calicivirus genus, NB strain, infected the villous epithelial cells of the small intestine of Gn calves, especially the duodenum, causing cellular destruction and resulting in mild to severe diarrhea [55].

The Gn calves, like the Gn pigs, also serve as important animal models for the study of primary immune responses to NoV that are difficult to assess in adult volunteers due to the presence of pre-challenge antibodies to human NoVs. The immunogenicity of bovine NoV-like particle (VLP) and human VLP vaccines and the protection induced in Gn calves upon homologous viral inoculation have been evaluated [53]) and are further discussed in Sections 9.5.2 and 9.5.6 of this chapter.

9.2.3.3 Non-Human Primates

Non-human primates provided one of the earliest models of NoV disease. Several groups reported infection of non-human primates with human NoV [56, 57]. Similar to responses observed in adult human volunteers, newborn pigtail macaques reportedly began viral shedding after 24 hours, and it was reported that some shed virus in excess of 21 days [56]. Interestingly, the mother of one infected macaque that resided in the same cage also became ill with the same strain of human NoV. This finding suggested that horizontal transmission, probably by the fecal-oral route, had occurred, similar to NoV transmission in humans. Other investigators showed that inoculation of Rhesus macaques induced several weeks of viral shedding in one asymptomatic macaque [58]. Different non-human primate species exhibited varying clinical disease and shedding. Neither marmosets, cotton top tamarins, nor cynomolgus macaques developed diarrhea or clinical symptoms [58]. Both marmosets and tamarins shed virus for between 3 and 4 days post-inoculation, while cynomolgus macaques did not shed virus. Some non-human primates exhibited NoV-specific IgM and IgG (rapid rise by 14 days post inoculation); no NoV-specific IgA was detected in the saliva of any animal. The reported detection of IgG cross-reactive with human NoV in mangabey, pigtail, Rhesus, and chimpanzee species suggests that non-human primates may be naturally infected by NoV [59].

Advantages of this model are the physiologic similarity of non-human primates to humans, the ability to use human NoV to inoculate non-human primates, and the ability to control the immunologic exposure of the non-human primate if necessary. Disadvantages include finding access to a well-maintained primate facility, the high cost of the study and the effort to assure compliance with regulatory guidelines.

9.2.3.4 Mice

The murine NoV model is a recent exciting discovery by Dr. Herbert Virgin's group [60]. In this model, either immunocompromised or wild type mice can be infected with a mouse-specific NoV. This mouse-specific NoV was named murine NoV (MNV-1) and was classified as a Genogroup V virus. To date, MNV is known to infect only mice and is a common pathogen in mouse colonies [61]. Immunocompromised STAT 1 knockout mice, when inoculated with MNV-1, developed massive viremia and inflammation in all organs and died within 10 days post-inoculation (d.p.i.) [60]. In contrast, wild type adult (8 week) 129 mice, when inoculated with MNV-1, exhibited viremia, and virus in a few organs including liver, spleen, and proximal intestine. These mice were able to clear MNV-1 by 3 d.p.i. and did not die. Of five mice inoculated, all seroconverted to NoV antigen between 14 and 21 d.p.i. [60]. Infection by other wild type mouse strains, MNV-2, MNV-3, MNV-4, in juvenile (4 week) CD1 mice resulted in viral shedding in feces and the presence of virus in mesenteric lymph node, spleen, and jejunum up to 8 weeks post-inoculation ([62] and discussed in [63]).

The advantages of this model are that MNV-1 infected mice have some characteristics similar to infected humans. Mice can acquire the virus through the fecal oral route, similar to humans. Wild-type mice housed with persistently-infected

immunocompromised mice (RAG knockout mice) develop MNV-specific antibodies within 3 to 4 weeks of exposure (discussed in [63]). Interestingly, mice can also acquire the virus through the respiratory route. Immunocompromised mice, but not wild type 129 mice, develop gastric bloating and diarrhea among other diverse clinical signs. Other clear advantages are the wealth of immunologic tools and mouse strains available to study the immunology of infection in mice and the ease of maintaining and setting up experiments. One disadvantage of this model is that wild type mice are asymptomatic and do not show lesions in the small intestine upon macroscopic inspection as is seen in human disease. Also, mice do not have the emetic physiologic response, so this variable and route of transmission cannot be assessed. Additionally, whether mice have the corresponding histo-blood group antigens like humans and pigs that influence susceptibility to NoV is unclear and precludes the use of mouse models for such investigations. In addition, infection of some immunocompromised mice with MNV-1 is lethal and leads to disseminated viremia, unlike human hosts. For additional discussion of the strengths and weaknesses of the MNV model, please consult [63].

Murine models of MNV disease may not be limited to 129, CD1 and immunocompromised mice. As discussed, MNV is a prevalent pathogen of outbred mouse colonies and induces immune responses in these mice, as measured by seroconversion to NoV antigen [61]. Therefore, it is likely that improved MNV models will be used in the future for the study of NoV immunology.

9.2.3.5 Cell Culture

Since 2006, two novel cell culture systems for NoV infection have been developed. The first *in vitro* NoV cell system is based on MNV infection of tissue culture macrophages (RAW lineage) or tissue-derived cells such as dendritic cells or bone marrow derived cells [64]. This model will help investigators dissect the host cell-NoV interactions at the level of immune cells. The second model is based on human NoV infection (GI and GII viruses) of a three dimensional, organoid model of human small intestinal epithelium [65]. Both infection and at least limited replication (discussed in [66]) were demonstrated in this model. Whether this model can be adapted to other cells, such as immune cells of the macrophage or dendritic lineage is yet to be determined. These two models are likely only the beginning of in vitro models of NoV infection that will help our understanding of the relationship between the host immune system and NoV.

9.3 Population

9.3.1 Seroprevalence

Before discussing the various seroprevalence studies, it is important to point out that different studies used different sources and types of antigens. For example, older studies (prior to the ‘90s) used virus antigen purified from stool samples while

more recent studies (in the '90s to present) used recombinant VLP as antigen. In addition, different strains of NoV were used as antigen in these studies. These different antigens may have different cross-reactivity and binding affinities to human immunoglobulin in serum specimens. Therefore, while general trends on seroprevalence and epidemiology may be gleaned from these studies, the exact seroprevalence against NoV among the various studies are not comparable to each other.

Examination of age-specific antibody seroprevalence in a variety of populations provides another key to understanding the body's immunologic response to NoV. Pediatric seroprevalence is particularly insightful as it illustrates the role of maternal antibodies and varied rates of antibody acquisition in different environments. The seroprevalence of NoV antibody in infants less than 4 months of age is similar to the number of seropositive women of childbearing age in the same community suggesting that these infants possess maternal antibodies [67, 68, 69, 70, 71, 72]. In general, these maternal antibodies persist for several months before a decrease in seroprevalence occurs. This decrease is usually observed between 3 and 6 months of age, although Parker et al. suggests that maternal antibody may be detectable in infants up to 8 months of age [73]. Figure 9.1, collected from a representative set of studies, illustrates this trend. However, after 4 months of age, pediatric NoV seroprevalence shows variation across demographic lines. The contrasting rates of antibody acquisition in developed and developing countries is well documented and best understood within the context of pediatric seroprevalence. In several developing countries, including Bangladesh [74], Kuwait [68], rural Thailand [69], South Africa [33] and Mexico [75] children appear to acquire "primary infection" during or prior to their second year of life. In Bangladesh, 100% of four-year-old children were seropositive for NoV antibody and, in general, childhood seroprevalence reaches

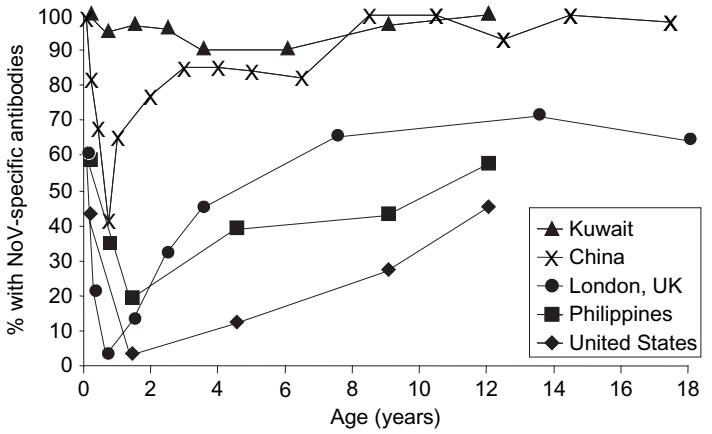

Fig. 9.1 Pediatric seroprevalence around the world. Data on seroprevalence in children ages 1 month to 18 years was collected from representative studies [67, 68, 70, 71]. The presence of maternal antibodies at birth (0 years) and subsequent decrease in seroprevalence between 3 months and 2 years is illustrated. Each data marker indicates the median age in years for the age range specified in the original report and seroprevalence value given for each age group in a population. Please see Section 9.3.1 for additional information regarding this figure

the maximum population-specific seroprevalence before age 5 in developing countries. In contrast, in developed countries, maternal antibodies decrease to low levels after 3 to 4 months, and the majority of children do not demonstrate subsequent serologic NoV response as a result of presumed exposure until age 12 [67, 76]. In Japan, NoV-specific seroprevalence is only 11% for children less than 6 years old while about 70% of adults are seropositive [76], and, in the United States, adult levels of NoV-specific seroprevalence are not reached until adolescence [67]. Seroprevalence data from Beijing, China, Hokkaido, Japan and Southeast Asia associate steep increases of childhood acquisition of antibody against NoV with daycare and elementary school attendance [70, 76, 77]. Ultimately, the varied patterns of age-specific antibody acquisition may provide insight into the environment and behavior associated with exposure to NoV.

Although the pattern of pediatric exposure to NoV is consistent, the pattern of adult seroprevalence of NoV appears to vary by populations. Greenberg et al. found that among 7 rural, urban, developed and developing countries there was no significant difference among adult seroprevalence [78]. However, examination of multiple seroprevalence studies indicates that there is variation among adult levels of seroprevalence. For example, an isolated Indian tribe in Ecuador, the Gabaro, was found to have no NoV antibodies in the late 1970s, while a Massachusetts cohort had a 50% NoV-specific seroprevalence in adulthood [79], and Kuwaiti adults had a 100% NoV-specific seroprevalence [68]. Table 9.1 illustrates adult seroprevalence from a representative number of international studies. While study design and method of

Table 9.1 Percent of adults* positive for NoV-specific antibody from representative regions

Region	Year of study	Percentage of Positive for NoV-specific antibody†	Reference
Beijing, China	1996–1997	100‡	[70]
Papua New Guinea	1979	100	[76]
Kuwait	1997**	100‡	[68]
Australian aborigines, Australia	1977	94	[71]
South Africa	1997**	93‡	[33]
Indonesia	1975–1976	90	[76]
Japan (*Hokkaido, Miyagi-ken, Saitama-ken, Kyoyo-fu, Fukuoka-ken*)	1984–1990	81	[76]
Dijon, France	2000–2001	78‡	[192]
Netherlands	1998–2001	74	[84]
United States	1974–1977	66	[78]
Singapore	1975	64	[76]
London, UK	1992	63	[71]
Massachusetts, USA	1975–1979	50	[79]
Ecuador (Gabaro)	1974–1977	0	[78]

* Adults are defined as individuals over 20 years, unless otherwise defined within the publication.
** Year of study was not available so the date the study was published was used instead.
† NoV-specific antibody for adults is defined as any isotype (IgG, IgA or IgM) seroprevalence for adults.
‡ Because no general adult prevalence value was given, the median adult prevalence value was calculated and rounded from published data.

antibody detection may account for some differences in the seroprevalence between populations, these findings also likely point to the roles of behavior and environment associated with exposure to NoV in a given population.

In general, certain behavioral and environmental characteristics can be linked to antibody acquisition against NoV in developing countries. First, lower socioeconomic status, poor standards of hygiene and limited access to potable water are found to be significantly associated with a higher NoV seroprevalence [35, 80, 81]. In agreement with these associations, a sero-epidemiological study of Chinese medical students found that students from rural towns had higher NoV-specific seroprevalence than students from urban settings [82]. Hygiene standards and socioeconomic status are often lower in rural settings. Other socio-behavioral factors associated with a higher NoV-specific seroprevalence include crowding within the home [35, 70, 80] and lower levels of maternal education [80]. In developed countries, the most common behavioral association with increased NoV-specific seroprevalence is childhood attendance at childcare centers or enrollment in elementary school [70, 76, 77, 80]. It is also notable that large outbreak situations are the most common pattern of infection in developed countries, indicating that high population density and common-source food or water contamination are important risk factors for infection [70, 83]. Additionally, there are a number of other factors associated with NoV-specific seroprevalence that are not specific to developed or developing countries including recreational water contact [80], consumption of sea food [80, 83], eating raw vegetables [80], blood type [82], and agricultural work [35, 84]. Ultimately, understanding the global patterns of seroprevalence and the associated risk behaviors will provide insight into the human immune response to NoV.

9.3.2 Immunology Learned from Outbreaks

Outbreaks have provided a wealth of information regarding the immune response of the host after NoV exposure and immunity against NoV. This information is supported and refined with data from human challenge studies that provide more controlled conditions (discussed in subsequent sections). For example, in outbreaks, several NoV-specific antibody isotypes have been identified including serum IgA [85], IgM [86], and IgG [87] as well as antibodies in saliva [88]. These NoV-specific antibodies are cross-reactive and bind to multiple NoV strains within various clusters within and across genogroups [87, 89]. In general, higher cross-reactivity is observed within clusters [89] and genogroups than across genogroups [6, 89]. As will be later discussed, pre-challenge serum antibodies, determined from acute serum specimens, did not provide protection from infection [90]. Lastly, outbreaks also show us that infection with one NoV strain does not protect from re-infection with a different strain [90].

Serology has been used in outbreaks to both diagnose and classify NoV strains. Multiple tests have been developed and used, including immune electron microscopy [91, 92], radioimmunoassay tests [93, 94, 95], isotype specific tests (e.g.

IgG, IgM, IgA) [85, 88, 96], tests using serum and saliva specimens [88], western blots [97], enzyme immunoassays (e.g. ELISAs) using single [96] and multiple antigens [6, 98], and "blocking" antibody tests [93, 94, 99, 100]. In general, a fourfold rise in NoV-specific antibody levels (from samples collected at least after 2 weeks post challenge) compared to baseline (from samples collected before or temporally close to NoV challenge) suggests infection. Unfortunately, serology is not as specific as genomic methods (e.g. real time PCR, reverse transcriptase PCR, genomic based phylogeny and classification) to diagnose and classify NoV strains because of the presence of pre-challenge antibodies and cross-reactivity across strains. Serology may also not be as sensitive as genomic methods at diagnosing infections. For example, high levels of pre-challenge antibodies may mask a fourfold rise in antibody levels after exposure. Genomic methods should be used as the main diagnostic and classification tool for NoV strains, and serology should be used to complement existing results or as an aid in outbreak investigations when stool specimens or genomic methods are not available.

9.4 Individuals

9.4.1 Clinical Features of NoV Gastroenteritis

A subset of NoV exposed individuals may become infected. Within this subset, a further subset of infected individuals may experience symptoms of gastroenteritis. In volunteer studies, the time to first symptom, or incubation period, ranged from 10 to 51 hours for Norwalk virus (NV) challenge (reviewed in [38]) and 19 to 41 hours for Snow Mountain virus challenge [47]. Symptoms may last from a few hours to several days. In our experience, NV-infected volunteers usually exhibit symptoms from 1 to 2 days while Snow Mountain virus infected volunteers usually exhibit symptoms from 1 to 5 days (Leon, J.S. and Moe, C.L. unpublished data). Symptoms include nausea, vomiting, diarrhea, abdominal cramps, headache, fever, chills and myalgia. Diarrhea stools are non-bloody, watery, and do not contain mucus or fecal leukocytes [37]. In agreement with these manifestations, fecal lactoferrin, a sensitive marker for intestinal polymorphonuclear leukocyte inflammation, is generally not detected [101]. In rare cases, NoV has been associated with encephalopathy (NoV was also detected in stool, cerebrospinal fluid, and serum) [102], neck stiffness, disseminated intravascular coagulation, photophobia, reduced alertness [103] and sore throat [104]. Dehydration is often the most serious complicating factor and may induce death in young children and elderly individuals. There is a relationship between symptoms and NoV-specific antibodies and this will be discussed in Section 9.4.4.4. In an immunocompetent host, symptoms usually resolve after a few days. The severity of illness and manifestation of specific symptoms seems to depend on both the NoV strain and the individual.

NoV shedding varies with each host. In our challenge studies, we have observed that NoV shedding often continues beyond the cessation of symptoms. In

one extreme case, an immunocompetent individual exhibited symptoms until 4 days post-challenge and shed virus in their stool for beyond one month post-challenge (Leon, J.S. and Moe, C.L. unpublished data). Levels of viral shedding in stool may be as high as 10^{10} genomic copies per gram of stool (Leon, J.S. and Moe, C.L. unpublished data). These observations suggest that infected individuals could be asymptomatic NoV carriers and shed virus for many days beyond the cessation of symptoms. In contrast, we have observed NV-challenged individuals who seroconvert and do not shed detectable levels of NV in their stool, as evaluated by RT-PCR (Leon, J.S. and Moe, C.L. unpublished data).

Gastrointestinal function is altered after NV challenge. Challenged volunteers exhibited transient malabsorption of lactose, D-xylose, and fat [37]. Levels of small intestinal brush border enzymes, trehalase and alkaline phosphatase, were decreased 48 hours after NV challenge compared to baseline values [105]. Among Norwalk and Hawaii virus-challenged volunteers, gastric emptying was significantly delayed in those who exhibited illness compared those who did not develop illness [106]. Rates of gastric emptying returned to baseline levels usually once symptoms had resolved. Adenylate cyclase activity in the jejunum [107], gastric secretion of pepsin, hydrochloric acid, and intrinsic factor were not altered after NV challenge [106].

Human and animal models of disease suggest that the inflammation associated with NoV infection occurs in the small intestine. Jejunum biopsies of challenged volunteers exhibited histopathological lesions at the time of illness [49, 50, 105, 108]. The jejunum exhibited histological abnormalities five and six days after Hawaii and NV challenge and two days after symptom clearance [50, 108]. No abnormalities were observed two weeks [49, 105] and six to eight weeks after challenge [108]. Interestingly, these jejunum lesions were also observed 48 hours after challenge with Norwalk or Hawaii virus in some asymptomatic volunteers [50, 105, 108]. However, other asymptomatic volunteers exhibited no jejunum lesions 48 hours after NV challenge [48]. It is unlikely that these asymptomatic volunteers had no lesions because they were genetically resistant to NV infection, as some of these asymptomatic volunteers had previously been challenged and became ill after NV infection. Histological lesions were not observed in the gastric fundus, antrum, or rectal mucosa of volunteers challenged with NV [105, 109]. Based on this data, it is worth speculating that jejunum lesions are associated with NoV infection in the presence or absence of clinical illness.

The inflammation and lesion observed in the infected host had particular characteristics. The villi of the proximal small intestine exhibited blunting and broadening while the mucosa was histologically intact [49, 50, 105, 108]. Increased numbers of mononuclear cells and some polymorphonuclear leukocytes (neutrophil granulocytes) infiltrated the intercellular spaces between epithelial cells of the lamina propia [50, 108]. In the pig model, higher levels of apoptosis were observed among enterocytes in the small intestine of human NoV-inoculated pigs compared to mock-inoculated pigs [40]. In the mouse model, apoptosis of cells of the small intestine and spleen was only observed in STAT1 knockout mice but not in 129 mice infected with MNV [110]. Apoptosis has not yet been assayed in human biopsies.

There is much speculation about which cells are infected by NoV in humans. NoV was not detected by histology in any human biopsy of the small intestine taken after NoV challenge [48, 50, 105, 108]. However, animal models have provided several clues as to the identity of the target cell. In Gn pigs inoculated with human NoV, the NoV capsid protein was detected, via confocal immunofluorescent microscopy, in enterocytes in discrete areas of the villi of the duodenum and jejunum and in some cells in the ileum [40]. Positive enterocytes of these inoculated pigs were mainly located at the sides or tips of each villus. The viral capsid was also observed in deep areas of the duodenum. It was not clear whether the capsid protein was located in Brunner glands or crypts. To identify areas of viral replication, the nonstructural N-terminal viral protein was identified in some, but not all, enterocytes that expressed the capsid protein. The N-terminal protein was found in the apical end of enterocytes. By transmission electron microscopy, calicivirus-like particles of 25–40 nm in diameter were observed in cytoplasmic vesicles in enterocytes.

Other results were reported in studies in wild type mice and STAT1 knockout mice inoculated with MNV. To identify sites of MNV replication, the non-structural ProPol processing intermediate was detected in cells of the lamina propia in the villi within 24 hours post-inoculation with MNV in wild type 129 mice [110]. In STAT1 knockout mice, the ProPol protein was mainly detected in epithelial cells lining the villi, both the basolateral and apical regions by 12 hours post-inoculation. A few lamina propia cells exhibited the ProPol protein by 12 hours post-inoculation, but by 48 hours post-inoculation, the majority of cells expressing ProPol were in cells of the lamina propia and in the Peyer's patches. In contrast, very few epithelial cells expressed ProPol by 48 hours post-inoculation. The authors hypothesized that MNV initially seeds and replicates in epithelial cells in STAT1 knockout mice, but later replicates in the lamina propia and Peyer's patches.

In wild type 129 mice, infectious virus is first detected in the proximal small intestine 3 hours post-inoculation. MNV later spreads to peripheral organs including the spleen, lymph nodes, liver and lungs after 1 d.p.i. By 7 d.p.i., MNV levels were low or undetectable in all organs. In mice, the intestine may be the site of primary NoV seeding that later facilitates systemic spread [110]. Because MNV has been shown to replicate efficiently in wild type murine macrophages and dendritic cells [64], Wobus et al. hypothesized that infection of transepithelial dendritic cells in the lumen of the intestine may provide a pathway for initial NoV infection of the intestine [63]. In addition, we speculate that NoV-infected dendritic cells and macrophages could also be potential carriers of NoV infection to other peripheral organs including the spleen and lymph nodes.

9.4.2 Protective Immunity Against NoV Infection

There are two questions in the field of NoV immunology that are essential to the success of a NoV vaccine. The first question is whether short-term or long-term immunity can be induced and protect from future NoV infection. An effective vaccine

should be able to induce host immunity that protects from future NoV infection. The second question is whether protective homologous and heterologous immunity can be induced. An effective vaccine should induce protective immunity against antigens in the vaccine (homologous) and, ideally, against related antigens not present in the vaccine (heterologous). These questions have been incompletely addressed in three separate human NoV challenge-re-challenge studies [48, 111, 112]. These studies were unable to use the current definition of infection (detection of viral shedding) because molecular assays were not available at the time these studies were conducted. Instead, clinical symptoms were used as a marker of NoV infection. Because infected individuals do not always exhibit clinical symptoms, the results of these studies should be interpreted with caution. In addition, it is difficult to distinguish whether a positive immune response in a volunteer, especially to a cross-reactive antigen, may be the result of either a current or past norovirus infection.

Two general hypotheses can be derived from these studies (reviewed [30, 38, 113, 114]). The first hypothesis is that challenge or exposure to NoV induces "short-term immunity" (6 months or less) but not "long-term immunity" (beyond 2 years) that protects from subsequent re-challenge or re-exposure to the same strain. This is based on the observation that challenged volunteers who became ill initially were re-challenged, within 6–15 weeks with the *same* virus and did not become ill [112]. Illness after re-challenge was 0/5 volunteers for NV (GI.1), 0/6 volunteers for Montgomery County virus (GI.5), and 0/3 for Hawaii virus (GII.1). The inability of each strain to induce illness in the re-challenged host was not due to a genetic factor because each strain was able to induce illness in the initial challenge. Therefore, these results suggest that previous challenge or exposure to NoV may induce protective immunity (6–15 weeks) that protects from subsequent challenge or exposure to the same strain. In agreement with this hypothesis, challenge and re-challenge with the same strain of bovine NoV within 30 days induced protection from viral shedding and clinical signs in Gn calves (discussed in 5.6 and in [53]). A separate NV challenge study demonstrated that re-challenge 6 months after the initial challenge did not result in illness in some volunteers who had become ill in the initial challenge [111]. A third NV challenge study demonstrated that re-challenge after 27 and 42 months induced illness in all volunteers that initially developed illness upon first challenge [48]. Collectively, these observations suggest that challenge with NoV provides protection against subsequent illness if volunteers are re-challenged with NoV within 6 months, but not after 2 years. Whether re-challenge, between 6 months and 2 years, protects individuals from human illness has not yet been addressed.

The second hypothesis is that challenge with NoV can induce both protective homologous immunity against re-challenge with the same strain, shown above, and protective heterologous immunity against re-challenge with *certain* other strains. Heterologous immunity is thought to depend on immune reactivity to shared epitopes in the virus capsid between different NoV strains (shared epitopes will be discussed in Sections 9.4.4.2 and 9.4.4.3). This hypothesis is based on the observation that challenged volunteers who became ill and were re-challenged, within

6–13 weeks with a *different* virus, did not become ill [112]. Illness after Montgomery County virus (GI.5) re-challenge occurred in 0/8 volunteers initially challenged with NV (GI.1) and in 0/4 volunteers initially challenged with Hawaii virus (GII.1). Interestingly, illness after NV re-challenge occurred in 1/3 volunteers initially challenged with Montgomery County, suggesting that *complete* protection may have been in one direction, but that *incomplete* protection may occur in the other direction. Within GI NoVs, one Montgomery County challenged individual developed a significant rise of NV-virus-specific antibodies, suggesting antigenic relatedness between these viruses [112]. However, challenge and re-challenge between genogroups did not provide *complete* protection in either direction. For example, challenge with NV (GI) and re-challenge with Hawaii virus (GII) resulted in 3/6 ill volunteers while challenge with Hawaii virus and re-challenge with NV resulted in 2/3 ill volunteers. This result suggests that Norwalk and Hawaii virus are antigenically different which is confirmed by their classification into distinct genogroups [8] and the absence of antibody cross-reactivity in serologic studies [112, 115, 116]. This result is also in agreement with a study of two outbreaks involving some overlapping cases. One case who became ill upon infection with a Norwalk-like virus (GI) later became ill upon infection with a Hawaii-like virus (GII) 6 months later in another outbreak (classification of Norwalk and Hawaii virus was based on the reactivity of the individual's sera to NV and Hawaii antigen) [90]. Based on the challenge-re-challenge study [112], because some volunteers did not become ill upon re-challenge (NV-Hawaii, Hawaii-NV, and Montgomery County-NV challenge-re-challenge), it is still possible that heterologous immunity protected these individuals from illness, albeit to a lesser degree. It is also possible that while these volunteers were genetically susceptible to the initial challenge strain and all became ill, some volunteers could have been genetically resistant to the different re-challenge strain and therefore did not become ill.

In one challenge-re-challenge study, two observations contradict the aforementioned hypotheses. The first observation was that NV challenge induced illness in two individuals but did not induce illness upon NV re-challenge *three years* later [111]. This observation suggests that long-term immunity (beyond two years) can be induced after one NoV challenge. These two individuals developed seroconversion to NV after re-challenge. It could be argued that these individuals were not protected because they seroconverted which may indicate viral shedding. However, seroconversion does not always indicate viral shedding and seroconversion can still occur in a protected host. Therefore, "long-term" immunity may have protected these two challenged-re-challenged human volunteers from clinical disease. In comparison, initial inoculation of calves with bovine NoV induced diarrhea, viral shedding, and seroconversion, and subsequent challenge 30 days later with bovine NoV failed to significantly increase IgA or IgG titer consistent with lack of diarrhea or detectable viral shedding in these "protected" calves (discussed in Section 9.5.6) [51].

The second observation that contradicts the current hypotheses regarding NoV immunity was that one individual who had undetected pre-challenge NV-specific IgG titers did not become ill, seroconvert, or increase NV-specific IgG after three

separate challenges (spaced 6 months to 3 years apart) but seroconverted, without illness, *after the fourth challenge* [111]. Genetic resistance to NV in this individual would explain the results after the first three challenges. However, the current hypothesis is that genetically resistant individuals do not respond immunologically to NoV [117]. If this hypothesis is valid, and the individual was genetically resistant, then they could not have responded immunologically by seroconversion after the fourth challenge. Therefore, either the individual was genetically resistant and this hypothesis is invalid or the individual was genetically susceptible and this hypothesis is still valid. A second explanation for this observation could be that resistance to infection in this individual was mediated by a non-genetic, non-serum immune mechanism, such as a local mucosal immune mechanism, and that after four repeated immunological insults, the serum immune mechanism became activated and the individual seroconverted. Because no additional data is available on this individual, it is difficult to determine a definitive mechanism for this observation.

Lastly, it is important to acknowledge the contributions of early investigators like Parrino et al., who proposed several hypotheses that have been confirmed two and three decades later. For example, Parrino et al. [48] stated, "Our data suggests that serum antibody by immune electron microscopy reflects infection in susceptible persons but does not appear to play a uniformly protective part in Norwalk-agent illness. . .. One hypothesis that could explain our findings is that local antibody rather than circulating antibody may determine clinical response after challenge with Norwalk agent." This hypothesis is based on the observation that levels of pre-challenge NV-specific titers did not correlate with illness in challenged-re-challenged volunteers with NV. We and many other groups have also observed this finding (discussed in Section 9.4.4.4). In addition, our group has found that "local antibody", in the form of mucosal IgA, was associated with protection to infection [117]. A similar association between induction of fecal IgA antibodies by wild type NoV (one inoculation) or NoV VLPs (2 or 3 immunizations), using LT(192G) as adjuvant, and complete or partial protection, respectively against homologous GIII NoV inoculation at post-inoculation day 30 was reported for Gn calves by Han et al. [53]. Parrino et al. [48] also stated that, "A second hypothesis that could explain our findings is based upon a genetic control of susceptibility to Norwalk-agent infection." This hypothesis is based on the observation that some individuals remained illness-free upon challenge and re-challenge, after 31–24 months, with NV. We and others have also identified genetic factors mediating resistance (discussed in 4.3) to Norwalk virus infection.

9.4.3 Genetics and Immunity

Though the focus of this chapter is on NoV immunology, a brief mention will be made of general trends in our understanding of how host genetic factors influence NoV infection and therefore impact NoV-specific immune responses. For a more in depth review of the association between genetics and NoV infection, the reader is encouraged to read the following excellent reviews [31, 114, 118, 119].

The current model of susceptibility and resistance to NoV infection states that, within a human population, there will be individuals who are genetically resistant to infection with *specific* NoV strains [117]. The mechanism of this resistance is thought to be due to the absence of a NoV receptor or binding molecule in genetically resistant individuals. These genetically resistant individuals, when exposed to NoV, do not provide any binding or internalization site for NoV, their cells do not become infected, and they do not develop illness. This mechanism is similar to that postulated for the CCR5 receptor in HIV infection [120, 121]. Presumably, because these individuals do not become infected, their immune system does not "see" the virus, and they do not mount an immune response to this specific NoV strain. The absence of immune response in genetically resistant individuals has not been rigorously tested but is supported by current evidence from human challenge and outbreak studies [117, 122, 123].

In contrast, it is thought that individuals who are genetically susceptible can become infected with the virus because they have a NoV receptor or binding site [117]. More recently, we have found that not all of these "genetically susceptible" individuals become infected upon challenge with NoV. We propose that not all of these "genetically susceptible" individuals become infected because they possess a "protective" immune response that protects them from infection. As discussed throughout this chapter, the existence of a protective NoV immune response is uncertain.

Host genetic factors thought to be important for susceptibility and resistance include the histo blood group antigens (HBGA). HBGAs are complex carbohydrates present on the surfaces of mucosal epithelium and red blood cells of the genitourinary, respiratory, and digestive tracts and are also present as free oligosaccharides in saliva, intestinal contents, breast milk and blood. Three families of HBGAs associated with NoV infection are thought to be the Lewis, secretor and ABO families (reviewed in [119]). Secretor status, one of the HBGA families, is associated with resistance to certain GI and GII NoV infections in challenge, endemic, and outbreak studies [117, 122, 123, 124], but seems to be NoV strain specific [125]. Because secretor status seems to affect susceptibility to NoV infection, it also appears to affect the levels and prevalence of NoV-specific antibodies. In 105 plasma specimens from Swedish blood donors, secretor negative individuals and Le^{a+b-} individuals had significantly lower GII.4 VLP-specific antibody titers and were more often antibody negative than secretor positive and Le^{a-b+} individuals [122]. Secretor negative individuals did not develop significant NV VLP-specific antibody response after NV challenge [117].

The ABO blood antigens, also members of the HBGA family, are also associated with resistance to infection. In general, blood type O individuals seem to be at increased risk of NV infection while blood type B individuals seem to be at decreased risk of infection. In agreement with this hypothesis, in two separate NV challenge studies, blood type O individuals were at a significantly increased risk of NV infection compared to other blood types [117, 126]. In one of these NV challenge studies, individuals who had the B antigen (B and AB blood phenotypes) were at a significantly decreased risk of infection and illness [126] while in the other NV

challenge studies, investigators found a decreased risk of infection in blood type B individuals but this risk did not reach statistical significance [117]. In addition, in an outbreak study, blood type B was also associated with a significant decreased risk of infection from another GI virus [127].

By influencing susceptibility to infection, ABO status may also influence NoV-specific antibody levels. Though not statistically significant, one report found a trend of higher GII.4 VLP-specific antibody titers in individuals with blood group O and lower antibody titers in individuals with blood group B in 105 plasma specimens from Swedish blood donors [122]. In a study of a GI virus outbreak, individuals with blood group B were significantly less likely to acquire NV VLP-specific IgG and individuals with blood group A were significantly more likely to acquire NV VLP-specific IgG [127]. Taken together, these results suggest that an individual's ABO status affects their risk of infection and therefore their development of NoV-specific antibodies.

Care must be taken when interpreting the results of these studies and others like them. Recent models suggest that each NoV strain or cluster has a different pattern of HBGA binding than others and the ability of a NoV strain to bind to a host HBGA may affect its infectivity and ability to induce strain-specific antibodies. NoV strain binding patterns must take the ABO, secretor, and Lewis families into account [119, 128, 129]. One model suggests that most of the binding patterns can be sorted into two groups: the A/B and the Lewis (non-secretor) binding groups [119, 128]. In agreement with this model, the strain-specific antibody titers and prevalence of individuals from a Chinese military medical university were associated with the specific strain binding patterns [82]. Of note, the patterns of NoV-HBGA binding do not clearly match the genetic classification of NoVs although strains with the same or closely related binding patterns seem to be clustered [119, 128]. Overall, these findings indicate that a single strain cannot bind to all human HBGA variants although, collectively, NoV can almost cover the diversity of human HBGAs [118]. Interestingly, certain pig HBGAs (A, H) that correspond to the human counterparts can also bind human NoV VLPs and are associated with infection, viral shedding and seroconversion to NoV VLPs in pigs [41].

9.4.4 Humoral Immunology

Given our recent models of host genetics and NoV infection, the subsequent humoral and cellular immunology sections must be read with the understanding that the role of host genetics was not known at the time the majority of these studies were published.

There are two current questions about the role of the humoral immune response after NoV challenge or exposure: (1) How and what antibodies are generated after NoV challenge or exposure; (2) What role do NoV-specific antibodies play in infection and disease? These questions are addressed in the subsequent sections.

9.4.4.1 Antibody Isotypes and IgG Subclasses

Humans

Lindesmith et al. suggested that infection, *and not just exposure* (or challenge), with NoV is essential for activation of a NoV-specific antibody response even in volunteers with pre-challenge NoV-specific antibodies [117, 125]. This hypothesis is supported by data from various challenge studies where individuals with pre-challenge antibodies, when challenged with NoV, remained uninfected and did not seroconvert to NoV antigens [19, 111, 117, 125]. The exception to this hypothesis is in the case of NoV vaccination, where oral administration of high doses of NoV antigen, without infection, is sufficient to induce NoV-specific antibodies (reviewed in [32]). Therefore this hypothesis can be modified to state that, infection with NoV or vaccination with high doses of NoV antigen is necessary for activation of a NoV-specific antibody response.

In general, increases in NoV-specific antibody isotypes are associated with infection (viral replication in the host), but not necessarily challenge, inoculation or exposure (internalization of the virus) because some hosts may not become infected upon challenge, inoculation or exposure. Because increases in NoV-specific antibodies are usually induced after infection, they can be used as markers of infection. This is observed across various strains [117, 124], antibody isotypes, IgA, IgG, and IgM [88, 116, 130], in both volunteer studies [19, 117] and outbreak situations [6, 87, 131]. NoV-specific IgD and IgE levels have not been assessed. In animal models, seroconversion to antibody isotypes is also associated with infection, which may or may not be associated with illness. For example, wild type mice infected with MNV seroconvert to MNV antigen but do not exhibit illness [60, 63]. Interestingly, in Gn pigs inoculated with human NoV, seroconversion had a stronger correlation with illness (diarrhea) than with viral shedding [40].

NoV-specific IgG subclasses are generated after NoV challenge or exposure. One study of 132 Swedish serum specimens found that all IgG subclasses against NV were detected in varying degrees [132]. IgG1 predominated in all age groups, followed by IgG4, IgG3 and IgG2 had the lowest prevalence (3%). Similar findings were observed in a Snow Mountain virus challenge of volunteers: Snow Mountain virus-specific IgG1 was common while IgG2 was rarely detected [125]. Other antibody isotype subclasses (e.g. IgA1, IgA2) have not yet been assessed.

In general, a NoV-specific antibody rise should not be relied on as a diagnostic tool because of the high incidence of false positives perhaps due to NoV cross-reactivity [87]. In addition, not all NoV-infected individuals (defined as infected because they shed virus) exhibit a rise in NoV-specific antibodies (Leon, J.S. and Moe, C.L., unpublished data). However, serology data may be used to complement an outbreak investigation that uses RT-PCR (the gold standard) as a diagnostic test [133].

There seems to be a general temporal order of the appearance of NoV-specific antibody isotypes, IgG, IgM, and IgA. This order and the relative levels vary depending on the individual and may be confounded by pre-challenge NoV-specific

antibodies due to previous NoV infection. The synthesis presented below is based on data from a few human challenge studies and has not been rigorously addressed. General trends and ranges of these NoV-specific isotypes are illustrated in Fig. 9.2. In general, in studies of human volunteers challenged with NoV, NoV-specific serum IgG, IgM and IgA increases are first observed after 5 days [86, 130, 134, 135]. In general, NoV-specific IgG levels reach their maximum at 3 weeks post-challenge, although some data suggests it may take up to 7 weeks for NoV-specific IgG levels to peak. Peak NoV-specific IgA and IgM levels occur earlier than IgG, generally during the second week of observation [86, 134, 135]. NoV-specific IgG levels tend to be higher than NoV-specific IgM levels that tend to be higher than NoV-specific IgA levels [86]. Furthermore, NoV-specific serum IgG levels tend to last longer at substantially elevated levels compared to NoV-specific IgM and IgA levels and maintain a plateau near their peak value for up to 100 days or more and decline thereafter. NoV-specific serum IgM levels revert to pre-challenge levels between 2 and 4 months [86, 130, 134], while NoV-specific serum IgA levels seem to persist for 1–2 months and then revert back to pre-challenge levels by 3 months [86, 130]. In some individuals, NoV-specific IgA may persist for 15 months after challenge [86]. Notably, several individuals failed to mount an IgG, IgA or an IgM response

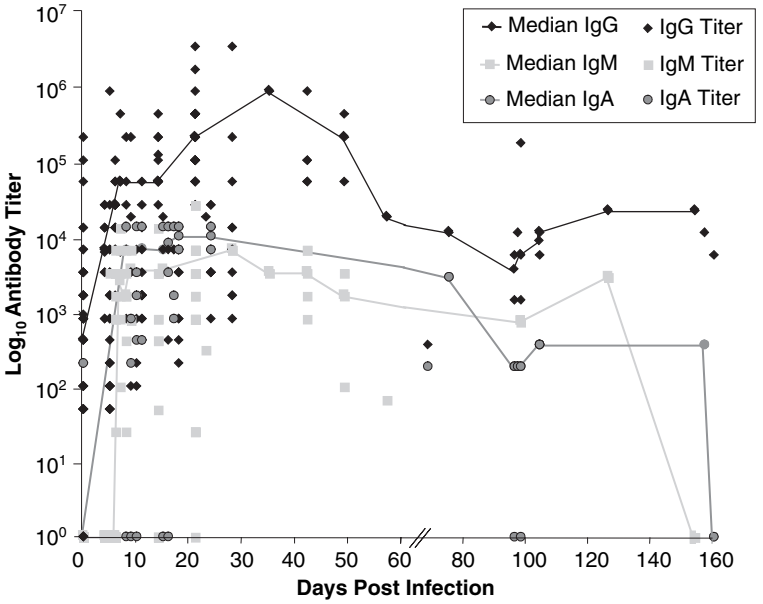

Fig. 9.2 Trends of antibody isotypes after infection. Data on titers was collected from various human challenge studies and outbreaks [86, 130, 134, 135]. The range of antibody isotypes can be observed from the individual symbols, as indicated in the figure legend, that correspond to antibody isotype titers of individuals. Symbols with lines indicate median titers for the range of titers at each specific day post-challenge. The two parallel lines in the X axis represent a break in scale. Please see Section 9.4.4.1 for additional information regarding this figure

after challenge [130, 135], perhaps because they were genetically resistant and not infected. Because of these trends, NoV-specific IgM and IgA seroconversion could be *cautiously* used as a marker of recent infection. In general, NoV-specific salivary IgA and IgG are induced one week after challenge and peak 2 weeks after initial challenge [117]. Some individuals may exhibit a rapid rise in NoV-specific salivary IgA prior to 5 days post-challenge. NoV-specific salivary IgG and IgA may persist for up to 40 days post-challenge (Leon, J.S. and Moe, C. L. unpublished data) but longer time-points post-challenge have not been reported. NoV-specific fecal IgA antibodies can be detected by two weeks post-challenge and may persist for up to two months [136]. Repeated infections may prolong the duration and magnitude of the antibody response [134].

The degree of correlation among the antibody isotypes has not been adequately addressed. In general the correlation between levels of serum IgG, IgA, and IgM seem to be high, but the degree of correlation has not yet been statistically analyzed [86, 116, 130]. We found that volunteers challenged with NV ($n = 77$) had NV-specific serum and saliva IgA and IgG levels that were significantly correlated with each other and had Pearson correlation coefficients between 0.5 and 0.8 (Leon J.S. and Moe. C. L. unpublished data). Because animal studies can use naïve hosts for inoculation, the duration of antibodies and degree of correlation between antibody isotypes obtained from animal studies will be clearer than the data collected from human studies.

Gnotobiotic Pigs

In the human NoV (HuNoV) HS66-inoculated pigs, although IgM, IgA and IgG antibodies were elicited at low titers, 65% of the HuNoV-HS66-inoculated pigs seroconverted, and 64% coproconverted (fourfold rise in HS66-specific fecal antibodies) with either IgA or IgG antibodies [42]. Positive associations were found between disease severity (diarrhea score) and serum and intestinal IgA and IgG antibody titers, suggesting that disease severity may reflect the intensity of intestinal stimulation, inducing increased titers of both local and systemic antibodies.

Low numbers of both IgA and IgG antibody-secreting cells (ASC) were elicited in the HuNoV-HS66-inoculated pigs. Similar numbers of IgA and IgG ASC were detected both locally (intestine) and systemically (spleen and blood) at 21 or 28 d.p.i., and the local ASC responses strongly correlated with systemic ASC responses after HuNoV-HS66 infection [42].

When we compared these results to that of Gn pigs infected with another human enteric virus, the virulent-Wa human rotavirus (HRV) [44], we observed that the HRV elicited higher numbers (10-fold) of IgA and IgG ASC in the intestine of the Gn pigs at 21 d.p.i. than the HuNoV-HS66 at 28 d.p.i.; however, similar numbers of IgA and IgG ASC were induced in spleen and blood in both HRV and HuNoV-infected pigs at 21 or 28 d.p.i. This higher level of intestinal ASC responses in the HRV-infected pigs is probably a consequence of the higher, and longer lasting, intestinal viral replication and diarrhea observed, and also of the more extensive intestinal lesions caused by the HRV in the Gn pigs [45].

9.4.4.2 Mapping of NoV antigenic sites

Because the main NoV target of the host immune response is the capsid protein, multiple groups have attempted various mapping strategies including genomic [17, 137], proteomic [138], monoclonal antibodies [139, 140], and crystallographic studies [141, 142] to identify antigenic, receptor binding, and cell binding regions. A single capsid protein has two main domains, the shell domain (S, aa residues 1–225) and the protruding domain (P) that is subdivided into two subdomains, P1 (aa residues 226–278 and 406–530) and P2 (aa residues 279–405) [143]. The distally located P2 subdomain is a large insertion in the P1 subdomain and protrudes more than the P1 subdomain. A comparison of capsid proteins from various calicivirus suggests that the S domain is well conserved, the P1 subdomain is moderately conserved, and the P2 subdomain is highly variable [142]. Chronic human infection with NoV induces the least number of mutations in the S domain, more in the P1 domain, and the majority in the P2 domain, suggesting that the P2 domain has important functions in host-pathogen interaction and pathogen or viral evasion [17]. Conserved regions in both the S domain and P1 domain may contribute to antibody cross-reactivity across clusters and genogroups [137]. For example, epitopes in the S domain from GI and GIII capsid proteins [144] or from GIII and GII VLPs [145] could bind the same monoclonal antibody derived from immunization of mice with NoV VLPs. The majority of monoclonal antibodies induced after immunization with NoV capsid protein targeted the S domain and cross-reacted with epitopes on GI and GII NoV capsid proteins [146]. One epitope in the P1 domain (aa 457–530) is common to GI viruses [139, 147]. The hypervariable P2 domain seems to contain multiple determinants for antigenicity, cell binding, and host specificity [138, 140, 148, 149, 150, 151, 152, 153] (reviewed in [114, 119]). With the development of in vitro NoV-specific antibody blocking assays, additional epitopes important for NoV neutralization and blocking will be identified.

9.4.4.3 NoV-Specific Antibody Cross-Reactivity

In general, infection with NoV or immunization with NoV antigens will induce a cross-reactive antibody response, most likely due to the conserved epitopes discussed in the preceding Section 9.4.4.2. These NoV-specific antibodies are cross-reactive and bind to multiple NoV strains within various clusters and across genogroups [87, 89]. In general, higher cross-reactivity is observed within clusters [89] and genogroups than across genogroups [6, 89]. In addition, the prevalence of cross-reactive antibodies after challenge, inoculation, exposure or immunization is usually lower than 100% in human and animal models. Because NoV infection induces cross-reactive antibodies, serologic assays are not useful for classification of NoV strains. In addition, the ability of serologic assays to detect cross-reactivity also depends on the quality and nature of the antigens used in the detection assay (e.g. fully formed VLPs versus recombinant capsid proteins that do not form VLPs). The cross-reactive nature of the immune response against NoV can be utilized to design effective vaccines that protect against antigens not present in the vaccine (discussed

in Section 9.5.4) and may eventually be helpful in the design of broad serologic assays for the detection of NoV-specific antibodies in populations.

9.4.4.4 Contribution of NoV-Specific Antibodies to Infection and Illness

The exact contribution of antibodies to clinical NoV infection and disease is currently unknown. One of the main questions is whether antibodies are associated with protection from infection and disease or whether they are merely a marker of infection and disease. Various reports contradict each other in stating that pre-challenge NoV-specific antibodies are associated with a *decreased* risk of infection or illness [74, 154, 155, 156], are associated with an *increased* risk of infection or illness [19, 79, 116, 117, 122, 157], or are not associated with infection or illness [90, 115]. This contradiction may be due to differences in host populations, infectious NoV strains, varied study definitions of "infection", or the presence of host genetic confounders.

The majority of papers examining this question have focused on pre-challenge serum NoV-specific IgG antibodies. However after adjusting for secretor status in volunteers challenged with one virus strain, NV, (NV, n = 77), the presence of pre-challenge serum NV-specific IgG antibodies were associated with an *increased risk* of infection [117]. This association was also observed for all volunteers without adjustment for any confounders. Pre-challenge NV-specific saliva IgA was not associated with infection. In agreement with this finding, infected volunteers from another NV challenge study (n = 50) exhibited significantly higher pre-challenge NV-specific IgG than uninfected volunteers [19]. In another NV challenge study using similar volunteers (n = 38), a higher pre-challenge NV-specific fecal IgA antibody geometric mean titer was found in ill compared to well volunteers and in infected versus uninfected volunteers [157]. In these two studies, secretor status was unknown and therefore the effect of secretor status on the probability of becoming infected and having an antibody response was not taken into account. In another study, we challenged volunteers with a NoV genotype II (GII) virus, Snow Mountain (SMV) (n = 15) [125]. We did not observe any association between the presence of pre-challenge Snow Mountain-specific IgG or IgA and infection.

Interestingly, in our NV challenge study, 74% of secretor negative volunteers (genetically resistant) had pre-challenge NV-specific serum IgG. These secretor negative volunteers, although resistant to NV, may have been susceptible to other NoV strains and previously infected with other strains of NoV. Infection with these other strains may have induced cross-reactive IgG to NV antigen in these secretor negative volunteers.

Our results indicate that while pre-challenge NoV-specific serum IgG may not be associated with protection, rapidly induced NoV-specific salivary IgA may be associated with protection and may indicate a NoV-specific memory response. In our NV human challenge study, a portion of genetically susceptible individuals (secretor positive) was resistant to NV infection, suggesting that a memory immune response or other mechanism also protected them from NV infection [117]. The

majority of the uninfected group (60%) exhibited a rapid rise in NV-specific salivary IgA titer *before* 5 days post-challenge. In contrast, the majority of the infected group (83%) exhibited a rise in NV-specific salivary IgA titer only *after* 5 days post-challenge. The rapid immunological response to NV challenge in secretor-positive, uninfected individuals suggests that acquired immunity may explain the difference in those secretor-positive volunteers who developed infection after challenge and those who did not. Therefore, the timing of the NV-specific mucosal IgA may be a good predictor of the risk of infection among genetically susceptible volunteers. At the same time, 6 out of the 15 uninfected volunteers did not develop a NV-specific salivary IgA response suggesting that other unidentified factors may also be associated with protection. In another one of our studies, where volunteers were challenged with Snow Mountain virus ($n = 15$), volunteers were infected regardless of their secretor status or blood group type [125]. Because we could not adjust for a genetic component (if there was one), we could not assess the effect of immunity independently and examine if the timing of salivary antibodies affected infection. Instead, we found Snow Mountain virus (SMV) infection increased SMV-specific IgG levels in serum and SMV-specific IgA levels in saliva after 14 or 21 days.

In summary, the majority of the reports indicate that presence of pre-challenge serum (and fecal) antibodies suggest prior infection and therefore susceptibility to future infection. Individuals who are genetically resistant to infection do not mount an immune response to NoV and therefore have low or no NoV-specific antibodies. As an additional confounder, there may be individuals who are genetically resistant to infection with some NoV strains, for example, strain A, but not others, such as strain B. These individuals, in general, will have lower NoV A-specific antibody levels, but once infected with strain B, may have higher NoV A and B specific antibody levels due to heterologous and homologous cross-reactivity. The timing of other antibodies, such as NoV-specific salivary IgA, may be associated with protection. However, there still may be other types of unidentified antibodies (e.g. mucosal, isotypes, IgG4) that may be associated with protection.

NoV-specific antibodies have also been shown to be associated with clinical illness. As discussed above, the presence of NoV-specific pre-challenge antibodies has been associated with increased risk of infection. Because infection may lead to symptoms, it is not surprising that the presence of pre-challenge antibodies (serum IgG, IgA, IgM, fecal IgA) has been associated with increased risk of developing symptoms [79, 116] and viral excretion [19]. Similarly, because infection may lead to both symptoms and rise of NoV-specific antibodies, the development of NoV-specific antibodies (serum IgG, IgA, IgM), as expected, was also associated with symptoms [79, 115, 130] and viral excretion [19]. In the NV challenge study reported by Cukor et al. [134], the association between NV-specific serum IgM and symptoms was significant ($RR = 3.8$, 95% CI [1.1–12.5], $p < 0.01$) (Leon, J.S. and Moe, C.L. unpublished data). Few reports have examined the association between NoV-specific antibodies and certain symptoms. One NV challenge study found a significant association between rise of the NV-specific titers and vomiting and nausea, headache or body ache, vomiting, and vomiting and diarrhea [19] while an

epidemiologic study of Brazilian children found an association between NV-specific IgG seroconversion and diarrhea or vomiting [34].

9.4.4.5 NoV "Neutralizing" or "Blocking" Antibodies

To determine whether antibodies contribute to protection from NoV infection, it is necessary to determine whether NoV-specific antibodies can prevent infection. One direct way to assess their contribution in vivo is through passive transfer studies where serum or purified NoV-specific antibodies are transferred to recipients that will be challenged or exposed to NoV and then monitor them for infection and illness. In humans, epidemiological studies may address this by examining the association between transfer of maternal NoV-specific antibodies to children and children's risk of infection. In animal models, NoV-specific antibodies may be passively transferred to recipients to assess whether these antibodies prevent infection or diminish the duration or severity of infection in the recipient. Currently, both the pig and murine models are best suited for this approach. In unpublished observations, passive transfer of MNV1-reactive polyclonal serum was able to delay MNV1-induced lethality in immunocompromised mice (RAG2/STAT1) after intraperitoneal transfer suggesting that NoV-specific antibodies may protect the host from NoV infection and disease (discussed in [63]).

An indirect way to assess the ability of NoV-specific antibodies to protect from infection is to measure their ability to "block" NoV infection in vitro. Because, until recently, there was no in vitro model of NoV infection it was not possible to detect these "blocking" or "neutralization" antibodies. Instead, over the past 3 decades, groups have attempted to detect antibodies that can competitively prevent NoV binding to other antibodies [79, 96, 158, 159], other cells [153], or putative NoV receptors like the HBGA family [160, 161]. These assays have been adapted for the detection of various NoV antibody isotypes [134] and NoV specificities [86, 162, 163]. Antibodies that block binding of NoV to other antibodies have been associated with increased risk of infection and illness, as discussed in Section 9.4.4.4. Antibodies that block binding of NoV VLPs to tissue culture cells were obtained from a monoclonal antibody raised against NV VLP, but not sera from NV-infected individuals [153]. These antibodies identified site(s) of the P2 domain on VP1 important for binding of tissue culture cells to VLPs [140]. Antibodies that block binding of NoV VLPs to HBGA structures are present in infected individuals [100, 161], mice [160, 161], and pigs [41] and block HBGA binding in a strain-specific manner. These assays have not yet been used in human challenges studies to investigate the associations between these antibodies and infection, symptoms, viral shedding and other clinical parameters.

In 2004, NoV-specific antibodies that effectively block NoV from infecting cells have been detected in an in vitro tissue culture assay [64]. This in vitro assay takes advantage of the ability of MNV1 infection to form plaques in tissue culture murine macrophage cell monolayers. Through this assay, Thackray et al. demonstrated that MNV-specific antibodies induced after infection with 5 genetically distinct MNV

strains were all equally effective at blocking MNV1 infection of RAW cells in a dose dependent manner. These results suggest that murine MNVs collected from research mouse colonies probably comprise only 1 serogroup [164]. In addition, these two reports are a few examples of the protective role of NoV-specific antibodies [64, 164]. In the coming years, other similar in vitro assays, perhaps based on the Gn pig model, will increase our understanding of the protective role of antibodies in human NoV infection.

9.4.5 Cellular Immunology

Little is known about the immune cells, cytokines, and chemokines associated with NoV infection and disease. Limited data on immune cells and serum immune factors and their role in NoV infection and disease comes from human challenge studies and murine, Gn pig and calf models. The recent introduction of these animal models will exponentially advance our understanding of the cellular immune response against NoV.

9.4.5.1 Innate Immunity

Immunity against NoV can be divided into innate and adaptive immunity. Innate immune protection seems to be mediated by downstream mediators of the STAT1 response, including the family of interferons, α, β, and γ. When the STAT-JNK pathway was deleted in mice, intracranial murine NoV (MNV) inoculation killed all STAT1 knockout mice [60]. Intracranial MNV inoculation of IFN-αβγ receptor knockout mice also killed all mice suggesting that deletion of *all* the interferon receptors renders a mouse susceptible to death from NoV inoculation. Neither, IFN-αβ receptor or IFN-γ receptor knockout mice died after MNV inoculation suggesting that *any* receptor is sufficient for survival. However, these mice had similar and persistent levels and distribution of NoV as their inoculated STAT1 counterparts suggesting that *other* factors are important for viral clearance. Knocking out other important IFN anti-viral mediators, such as inducible nitric oxide synthase (iNOS) and protein kinase RNA-activated (PKR), in mice also did not result in lethality. Interestingly, while intracranial inoculation killed all susceptible mice, intranasal and perioral inoculations were less lethal than intracranial inoculation. Other innate mucosal factors may mediate protection in these mice after inoculation. Taken together, these results suggest that innate immunity, specifically IFN or STAT1-dependent immune responses may be responsible for the rapid control of NoV infection in humans but not viral clearance [63].

STAT1 and the interferons seem to be necessary for the control of virus replication. Inoculated wild type mice that have STAT1-dependent immune responses have lower levels of virus in their intestine and peripheral organs than inoculated STAT1 knockouts or infected IFN-αβγ receptor knockout mice [110]. This low viremia in wild type mice was accompanied by detectable levels of IFN-α in sera and intestinal homogenates. In addition, bone marrow derived macrophages or dendritic cells from

wild type mice exhibited lower MNV replication rates than STAT1, IFN-αβγ receptor knockout, and IFN-αβ receptor knockout mice [64]. This lower replication rate was accompanied by secretion of IFN-α in MNV-infected wild type bone marrow derived macrophages and dendritic cells [64]. Interestingly, infected macrophage or dendritic cells from IFN-γ receptor knockout, iNOS knockout, and PKR knockout mice had similar MNV replication rates as wild type suggesting that IFN-γ iNOS, and PKR do not affect MNV replication rates [64]. These results suggest that STAT1, the combined IFN-αβγ receptors, and the combined IFN-αβ receptors are important in the control of viral replication. Therefore, it is likely that both IFN-α and β are also important in the control of viral replication, but direct experiments to demonstrate this have yet to be done. In contrast, the IFN-γ receptor, PKR, and iNOS have no significant effect, compared to wild type, in the control of viral replication in macrophages and dendritic cells. Based on these results, Mumphrey et al. proposed two mechanisms for the role of STAT1 and IFNs: (1) IFN-induced host factors directly inhibit viral replication at the primary site of entry and (2) IFN responses limit NoV dissemination and replication in secondary tissues [110]. Interestingly, human challenge with NV or Hawaii virus did not induce detectable levels of interferon in sera, jejunal aspirates, or jejunal biopsy specimens taken 48–96 hours post-challenge [165].

9.4.5.2 Adaptive Immunity

Mice

If the STAT1 pathway, through the IFNs, inhibits viral replication and dissemination but not clearance, then other factors must be responsible for viral clearance. Experiments by Karts et al. showed that MNV inoculated RAG1 and RAG2 knockout mice, mice that lacked B and T cells, did not die [60]. However, these mice exhibited a persistent viremia throughout all their organs up to 90 d.p.i. Based on these results, the authors hypothesize that the adaptive immune response, in contrast to the STAT1-dependent innate immune response, was not required for protection against lethal MNV infection. Instead, the components of the adaptive immune response, specifically B and/or T cells, were required to contain and clear murine NoV infection [63]. These studies also showed that NoV can continuously replicate in tissues for long periods of time without causing severe morbidity or lethality. These results may explain the persistent viral shedding observed in immunocompromised patients, which may last up to two years in some cases [16, 17, 21, 22, 23]. These immunocompromised patients have impaired B and T cell responses that may not be able to clear replicating virus. One group found that reducing immunosuppression in transplant recipients was essential for clearing human calicivirus infection [22].

Humans

In humans, information on the adaptive immune system after human NoV challenge comes from our human challenge study performed with Snow Mountain virus

(SMV) [125] and one case-control study of American students with diarrhea after visiting Mexico [101]. In our human challenge study, we demonstrated that peripheral blood mononuclear cells (PBMCs) from challenged volunteers respond to Snow Mountain virus-like particles (VLPs) in vitro by secreting cytokines 8–21 days post-challenge [125]. Among all secreted cytokines assayed, IFN-γ and IL-2 exhibited the highest levels and prevalence among all specimens. Post-challenge PBMCs also secreted TNF-α, IL-4, IL-5 and IL-10 cytokines (at least fourfold level above baseline) in a few volunteers. There was no significant difference in pre-challenge and post-challenge PBMCs stimulated with SMV VLP for any cytokine in either uninfected or infected volunteers, perhaps due to small sample size. Interestingly, IFN-γ was detected in 91% of pre-challenge PBMCs after SMV VLP stimulation suggesting previous NoV exposure and immunological memory in these volunteers. Sera was also assayed for cytokine levels, and infected volunteers exhibited a significant rise in IFN-γ and IL-2 levels at 2 days post-challenge; no difference was observed for IL-6 and IL-10. There was no difference in IFN-γ, IL-2, IL-6 or IL-10 from post-challenge serum specimens compared to pre-challenge serum specimens for uninfected volunteers. These results suggest that: (1) challenged individuals respond to NoV antigen with secretion of anti-inflammatory and inflammatory cytokines, (2) serum cytokines can be quickly induced by 2 days post-challenge and (3) challenged individuals probably had pre-challenge NoV exposure.

Results similar to the human challenge study were found in the case-control study of American students with diarrhea [111]. Students that shed NoV (n = 7, 6 GI and 1 GII strain), in the absence of other detectable pathogens, had significantly higher levels of inflammatory cytokines, IFN-γ and IL-2, in their diarrhea specimens than students who had no detectable pathogens (n = 19). Interestingly, a group of students that had a NoV and enterotoxigenic *E. coli* (ETEC) co-infection (n = 10, 8 GI and 2 GII strains), exhibited the aforementioned cytokines and a significant and large increase in secreted IL-10 in their diarrhea specimens compared to students who had no detectable pathogens. It is important to note that cytokine secretion in this study was not antigen-specific. One advantage of this study is that fecal cytokines probably better reflect the gut mucosal immune response during NoV infection than serum cytokines. These results suggest that symptomatic NoV infection is associated with an inflammatory gut response, while symptomatic co-infection with ETEC is associated with both an inflammatory and anti-inflammatory gut response.

In our human challenge study, we also assessed whether cellular cross-reactive responses occurred after Snow Mountain virus (GII) challenge [125]. PBMCs from SMV infected volunteers were assayed for their cytokine response to Hawaii (GII) and NV (GI) antigen in vitro. Stimulation with Hawaii VLPs induced significantly higher levels and prevalence of cytokines in PBMCs compared to stimulation with NV VLPs. Hawaii VLP stimulation of PBMCs also induced significantly higher levels of IFN-γ and IL-2 compared to NV VLP stimulation of PBMCs. IL-10 (fourfold levels above baseline) was also detected in both Hawaii and NV VLP stimulation of PBMCs while TNF-α was only detected in Hawaii VLP stimulation of PBMCs.

We also investigated the relative roles of $CD4^+$ and $CD8^+$ cells on IFN-γ secretion after SMV VLP stimulation. PBMCs from 5 volunteers (both infected and

uninfected) were depleted of either $CD4^+$ or $CD8^+$ cells and then stimulated with SMV VLPs. $CD4^+$ depleted PBMCs exhibited significantly lower IFN-γ levels (82–97% lower) compared to undepleted or $CD8^+$ depleted PBMCs after SMV VLP stimulation. $CD8^+$ depleted PBMCs from three infected volunteers exhibited no significant difference in IFN-γ levels compared to undepleted PBMCs. Interestingly, $CD8^+$ depleted PBMCs from two infected volunteers exhibited *higher* IFN-γ levels compared to undepleted PBMCs. In summary, we hypothesized that in PBMCs, $CD4^+$ cells are the primary source of SMV-specific IFN-γ secretion and that $CD8^+$ cells may regulate IFN-γ secretion in $CD4^+$ cells.

Gnotobiotic Pigs

Recently, the intestinal and systemic antibody titers, ASC responses, innate Type-I IFN (IFN-α), pro-inflammatory (IL-6), Th1 (IL-12 and IFN-γ), Th2 (IL-4) and Th2/T-reg (IL-10) cytokines and cytokine-secreting cell (CSC) profiles were evaluated in Gn pigs after oral inoculation with GII.4 HuNoV (HS66 strain) and compared to mock-inoculated controls [42].

In serum of the HuNoV-HS66-inoculated Gn pigs, delayed (10 and 12 d.p.i.) (2.2-fold over controls) innate (IFN-α) and low and early (4 d.p.i.) pro-inflammatory (IL-6) cytokine responses were observed. A balanced serum Th1/Th2 response was also observed with persistently higher levels of IL-12 at most d.p.i., and a transient, but significantly higher peak of IFN-γ was seen at 2 d.p.i. (2.5-fold over controls). This early peak of IFN-γ was similar to that observed in the serum of human volunteers orally challenged with a GII HuNoV, Snow Mountain virus (SMV) [125]. The Th2 (IL-4) and Th2/T-reg (IL-10) cytokines were only detected in the serum of the HuNoV-HS66-inoculated pigs at low concentrations and were significantly elevated above controls earlier in infection (2 to 8 d.p.i.).

In intestinal contents (IC) of the HuNoV-HS66-inoculated pigs, only innate (IFN-α) (early at 2 and 8 d.p.i. and later at 21 d.p.i.) and Th1 (IL-12) (later at 28 d.p.i.) responses were significantly higher compared to controls. Failure to detect significantly elevated concentrations of the other cytokines in IC compared to those found in serum may merely reflect the instability of these cytokines in IC. The local and systemic cytokine-secreting cell (CSC) responses were characterized by low numbers of pro-inflammatory (IL-6) CSC detected early in the gut and later systemically (in blood), and a generally more biased Th1 (IL-12 and IFN-γ) CSC response, both locally and systemically in the HuNoV-HS66-inoculated pigs.

In the HuNoV-HS66-infected Gn pigs, the low pro-inflammatory (IL-6) responses to viral infection, low levels of the anti-inflammatory IL-10 in serum and lack of significantly elevated numbers of IL-10 CSC could be due to the low levels of viral replication, mild pathology and low inflammation in the gut [40].

9.4.5.3 Other Immune Cells Involved in NoV Infection

Infection is also accompanied by inflammation in the intestine in mice and humans. In mice, granulocytes were observed 24 hours after infection [110]. In humans,

duodenojejunal biopsies taken 48 hours after challenge showed monocyte and neutrophil infiltration of the lamina propia and epithelial cells in several volunteers 48 hours after infection [48, 50]. Murine NoV infection also increases the number of B cells and macrophages in the spleen after 72 hours [110].

Murine NoV can infect several immune cells in vitro including bone marrow derived murine macrophages and dendritic cells [64]. Murine NoV also seems to infect macrophages in vitro in immunodeficient mice as several cells co-stained with ProPol and F4/800, a macrophage lineage marker [166]. In STAT1 knockout mice, murine NoV seeds and replicates in intestinal epithelial cells but replicates predominantly in the lamina propia and Peyer's patch cells at later times post-infection [110]. Little to no viral replication was observed in wild type mice; it is unclear whether epithelial cell infection was prevented in wild type hosts. Wobus et al. hypothesized that infection of transepithelial dendritic cells in the lumen of the intestine may provide a pathway for NoV infection [63].

9.4.6 Maternal Factors Involved in NoV Protection

Human milk can protect children from infectious diseases through a number of mechanisms. To date, human milk is thought to protect the infant from NoV infection through two mechanisms (reviewed in [118, 119]). One mechanism is through transfer of NoV-specific antibodies from mother to child. Epidemiologic studies have suggested the presence of high titers of maternal NoV-specific antibodies in infants up to 8 months of age [67, 68, 69, 70, 71, 72, 79]. The ability of maternal milk to block NoV binding in a radioimmunoassay in the late 1970s indicated the presence of NoV-specific antibody. However, it was not until recently that NoV-specific IgA has been identified in human maternal milk [167, 168]. About 13% of human milk specimens, from mothers in Chiba City, Japan, had NoV-specific IgA that bound to different and multiple NoV clusters, including those within GI and GII. The prevalence of NoV-specific IgA to certain clusters varied depending on the clusters (e.g. antibodies to GII.6 had a prevalence of 13.6% while antibodies to GI.8 had a prevalence of 0.8%) [167]. In addition, the prevalence of NoV-specific IgA to certain clusters also depended on the mothers' HBGA makeup, specifically her secretor status [168].

A second mechanism of NoV protection in neonates is through decoy receptors. These decoy receptors in human milk are thought to bind NoV, prevent NoV from binding to host gastrointestinal cells, and therefore prevent NoV infection in infants. These decoy receptors are glycoproteins that correspond to the bile salt stimulated lipases and a milk fraction including the mucins MUC1 and MUC4 [169]. The ability of these glycoproteins to protect from NoV infection may depend on the mother's HBGA makeup, the infant's HBGA makeup and the HBGA binding pattern of the NoV strain [118]. In agreement with this model, two groups found a concordance between the mother's secretor status and Lewis phenotype and the ability of their milk to inhibit the binding of different NoV strains [168, 169]. Interestingly, not all

of the mother's HBGAs were expressed in their milk specimens [168]. It is likely that, in the future, additional protective factors against NoV will be identified in mother's milk.

9.5 Vaccine Efforts

A NoV vaccine will be superior to other public health interventions at reducing morbidity and mortality associated with NoV illness. Clean water, hygiene and sanitation are effective public health interventions at reducing diarrhea morbidity and mortality associated with most enteric pathogens [170, 171]. However, developed countries, like the U.S. and Japan, that generally have clean water, good sanitation and hygiene practices, still have a high rate of outbreaks associated with NoV. This suggests that, for enteric pathogens that are highly infectious, these measures are not sufficient to prevent transmission. Therefore, an effective NoV vaccine is a necessary intervention to reduce the public health burden of this disease, especially in developed countries. To date, there is no commercially available NoV vaccine, but much progress has been made in the past decade to develop a vaccine. This section summarizes these advances, and the reader is encouraged to read other reviews on this topic [30, 32, 172, 173, 174, 175].

9.5.1 NoV VLPs are Immunogenic

Initial studies in mice have shown that oral administration or subcutaneous immunization with virus-like particles (VLPs) alone induce NoV-specific immune responses in mice. Both CD1 and Balb/c mice were fed recombinant NV VLPs produced by a bacculovirus expression vector. Increasing doses of NV VLPs induced higher NoV-specific serum IgG and fecal IgA and a higher prevalence of seroconversion in both strains of mice [176]. Among all NV-specific IgG isotypes, NV-specific IgG2b levels were the highest levels observed after VLP administration. In agreement with this study, oral administration of NV VLPs to Balb/c mice induced high levels of NV-specific serum, fecal, and vaginal antibodies, NV-specific T cell proliferation, IFN-γ and IL-4 secretion, and NV-specific $CD4^+$ and $CD8^+$ proliferation [177, 178]. In a separate study, Balb/c mice were fed NV VLPs from a Venezuelan equine encephalitis (VEE) replicon expression system [179]. In contrast to the Ball et al study [176], NV-specific IgM and IgG levels were low or non-existent [179]. However, footpad inoculation of Venezuelan equine encephalitis virus replicating particles (VEE-VRP) expressing NV antigen induced robust levels of NV-specific serum IgG, IgM and fecal IgA.

In humans, oral administration of NV VLPs, produced in a bacculovirus expression system, induced NV-specific immune responses. Various doses of VLPs induced serum NV-specific IgA antibody secreting cells, seroconversion and NV-specific IgA and IgG [180, 181]. NV-specific IgA was detected in saliva, stool,

semen, and vaginal washes. Three doses of VLPs, 250 μg, 500 μg and 2000 μg, were administered to different groups of volunteers, and no significant differences were observed in either levels of antibodies, seroconversion rates, or antibody secreting cells among the various volunteer groups. PBMCs harvested from the groups receiving 250 μg and 500 μg of VLPs at day 21 post-immunization, proliferated and secreted IFN-γ in response to NV VLP antigens. IFN-γ secretion was not detected in PBMCS collected 56 days post-immunization. Interestingly, PBMCs harvested from the group receiving 2000 μg did not develop any cell-mediated response at either day 21 or 56 post-immunization. The authors could not explain this finding, but it is possible that a high VLP antigen dose could have induced oral tolerance to this antigen, similar to that observed in other systems (reviewed in [182]). No IL-4 was detected from any of the stimulated PBMCs. Ingestion of VLPs was safe in humans and did not induce any symptoms different in severity or prevalence than that seen in placebos (e.g. headache, malaise, nausea).

9.5.2 Adjuvants Enhance NoV VLPs' Immunogenicity

In general, adjuvants administered with NoV VLPs enhanced their immunogenicity in mice. Administration of cholera toxin together with oral feeding of NV VLPs to Balb/c mice and CD1 mice induced higher levels of NV-specific antibodies, a higher response at lower VLP immunization doses, and a higher rate of seroconversion [176]. In a similar study, a modified cholera toxin administered to mice that received oral NV VLPs also boosted the levels of NV-specific IgA present in various organs including the lungs, trachea, small intestine, and Peyer's patches [177]. The authors observed higher NV-specific IgG, IgA, and IgA antibody secreting cells, higher T cell proliferation and IFN-γ and IL-4 secretion and higher $CD4^+$ and $CD8^+$ stimulation in spleens and Peyer's patches. Interestingly, in this study, administration of bicarbonate also had a mild "adjuvant" effect. Another mucosal adjuvant, mucosal *Escherichia coli* heat-labile toxin, LT and its nontoxic mutant, LT(R192G), also enhanced the magnitude and duration of NV-specific IgG and IgA antibody responses after oral and intranasal administration of NV VLPs to Balb/c mice [178, 183]. Mice inoculated with these two adjuvants developed NV-specific IFN-γ, IL-2, IL-4, and IL-5 secretion in splenocytes, lymph node cells, and Peyer's patches [183]. Lastly, oral administration of a raw material from a yeast cell lysate containing a GII.4 VLP induced NoV-specific serum IgG, fecal IgA and blocking antibodies to histo-blood group putative receptors. The raw yeast material could have served as an adjuvant although this was not tested [184]. Purified GII.4 VLP intramuscular administration with Ribi adjuvant also induced NoV-specific immunity [184].

In addition to increasing the magnitude of NoV-specific immune responses, adjuvants may also affect the type of the immune response. For example, NV-specific IgG2b was the highest IgG subtype induced in mice orally immunized with NV VLPs. In contrast, NV-specific IgG1 was the highest IgG subtype in-

duced in mice orally immunized with NV VLP and cholera toxin [176]. Similarly, levels of NV-specific secretion of IFN-γ were higher than IL-4 in mice orally immunized with VLPs. In contrast, levels of NV-specific secretion of IL-4 were higher than IFN-γ in mice orally immunized with VLPs and modified cholera toxin [177].

Gn calves were immunized with bovine GIII NoV VLPs in the presence of various adjuvants [53]. Intramuscular immunization with the oil adjuvant ISA50V induced the highest VLP-specific serum IgG antibody titers compared to intranasal VLP immunization with ISCOM or LT (R192G) or oral immunization with LT (R192G), but failed to prevent diarrhea or viral shedding post-inoculation in the Gn calves. However, only the LT (R192G) co-administered intranasally with VLPs induced fecal IgA antibodies to bovine NoV (BoNoV) in the calves and partial protection (delayed and shortened diarrhea and shedding of 1–2 days) compared to controls (8–9 days diarrhea). In contrast to the murine studies described above, LT (R192G) given orally with VLPs induced low or non-existent NoV-specific antibodies in the serum of immunized calves. However, the LT (R192G) administered orally with VLPs induced BoNoV-specific fecal IgA antibodies in Gn calves.

9.5.3 Immunization Route Affects NoV VLPs' Immunogenicity

In general, in mice, intranasal administration of VLPs seems to induce a stronger and longer immune response against NoV than oral feeding of VLPs. Balb/c mice that were immunized by the intranasal route with NV VLPs had higher levels of NV-specific serum, fecal (mucosal SIgA was not assayed so IgA could have been serum derived), and vaginal IgG and IgA than oral immunization [178, 183]. Levels induced by intranasal immunization persisted longer than levels induced by oral immunization. It is possible that the use of appropriate adjuvants for oral immunization would enhance the responses seen after this route of immunization. In mice, other investigators used intramuscular and intranasal immunization routes but did not directly compare them with each other or with oral routes [177, 184]. In a separate study in mice, oral immunization was superior to intraperitoneal or subcutaneous immunization (without adjuvant) at generating NoV-specific hybridomas, suggesting that oral immunization induced a higher number of antibody-producing cells than the other tested routes 2[185]. Lastly, one group successfully infected mice with Venezuelan equine encephalitis virus replicating particles (VEE-VRP) expressing various NoV antigens in the footpad [160, 179]. Mice mounted high levels of serum and gut NoV-specific IgA and IgG after infection. Differing immunization routes also induced different types of humoral and cellular immune responses [183, 185].

9.5.4 Cross-Protection After Immunization with NoV VLPs

Because of the diversity of NoV strains, an effective NoV vaccine needs to protect against NoV strains that were not present in the vaccine. Cross-protection can be

indirectly assayed by determining whether cross-reactive humoral and cellular responses are present against various antigens or *directly* assayed by immunizing a host against one antigen or infecting a host with a NoV strain and later determining whether challenge with a different strain results in decreased or no disease/infection. It is important to note that only direct assays of cross-protection, and not indirect assays of cross-protection, will indicate whether immunization or infection with an antigen/NoV strain will protect from future challenge with a different NoV strain. Cross-protection was indirectly observed in humans and animals after the induction of cross-reactive antibodies and cellular responses after immunization with NoV VLPs and after infection with NoV, as previously discussed in Sections 9.4.2, 9.4.4, and 9.4.5. Cross-protection was directly observed in early human challenge studies where volunteers were initially challenged with NV (GI.1) or Hawaii virus (GII.1) and did not become ill when later challenged with Montgomery County virus (GI.5) [112] (classification of Montgomery County virus determined from [36]), as discussed in 4.2. In general, it seems as though cross-reactivity and protection is preferentially genogroup specific. For example, challenge with GII antigens induced stronger immune responses against other GII antigens than GI antigens [115, 116, 125].

The ability of vaccine VLPs to induce NoV cross-reactive antibodies was investigated in one study where Balb/c mice were inoculated with infectious Venezuelan equine encephalitis virus (VEE) replicon particles (VRPs) expressing Norwalk, Hawaii, Snow Mountain, or Lordsdale antigen. These mice developed homotypic antibodies to the specific antigens they received as well as heterotypic antibodies to other antigens (Norwalk, Hawaii, Snow Mountain, and Lordsdale). As previously discussed, the levels of the cross-reactive antibodies were highest among genogroups than across genogroups [160]. Interestingly, naïve Balb/c mice immunized with a trivalent VEE-VRP cocktail containing Norwalk, Hawaii, and Snow Mountain antigen and *lacking* Lordsdale antigen, generated levels of Lordsdale-specific IgG of similar magnitude as Balb/mice immunized with VEE-VRP cocktails *containing* Lordsdale antigen. In addition, the levels of the heterotypic Lordsdale-specific serum IgG after trivalent immunization were significantly higher than combining each level of the heterotypic Lordsdale-specific serum IgG after monovalent (Norwalk, Hawaii, Snow Mountain antigen) immunization. This exciting finding suggests that NoV cocktail vaccines may protect against NoV strains not in the original immunization and therefore may be useful against new NoV variants. The new murine and Gn pig models will facilitate the testing of these multivalent vaccines against both homotypic and heterotypic infections.

9.5.5 VLP Vaccine Delivery Vehicles

In addition to evaluating the vaccination route and adjuvants used, it is also important to evaluate the delivery vehicles used to administer the vaccine to humans. To date, three general types of delivery vehicles have been proposed: purified VLPs,

VLPs in unicellular organisms, and VLPs in plants. High yields of VLPs can be purified from baculovirus [180], yeast [184], and Venezuelan equine encephalitis vectors [160]. VLPs could be administered as part of a unicellular organism like in yeast and *Lactobacillus casei*, but these VLP-expressing organisms have not been tested in human volunteers [184, 186]. VLPs have also been expressed in tomatoes [187], potatoes [188, 189], and tobacco [189]. All three NV VLP-expressing plants induced NV-specific antibodies when fed to mice [187, 189]. In CD1 mice, inoculation with tobacco expressing NV VLPs induced higher levels of NV-specific serum IgG and fecal IgA than inoculation with potato expressing VLPs [189]. Additionally, tomatoes expressing NV VLP still retained their ability to induce NV-specific antibodies after they were freeze-dried [187]. Of the three plants, only potatoes expressing NV VLP have been tested in human volunteers. Feeding of raw potatoes expressing NV VLP to volunteers was safe and induced no symptoms other than nausea in volunteers receiving wild type potatoes or potatoes expressing NV VLP [188]. Potatoes expressing NV VLP induced NV-specific serum IgG, IgA, and IgM, fecal IgA and seroconversion. The rise in NV-specific IgG titers persisted until day 61, the latest time point measured, while NV-specific IgM levels decreased to pre-immunization levels by day 28 [188]. For additional reviews on plants that express NoV VLPs, please consult the following articles [32, 174, 175, 190].

Purified VLPs, VLPs in unicellular organisms and VLPs in plants have advantages and disadvantages. They are all easy to administer by the oral route, but purified VLPs have the highest likelihood of approval for immunization via non-oral routes. VLPs in unicellular organisms and plants may have higher acceptance by consumers, than purified VLPs, because they can be eaten and administered in foods that are commonly consumed (e.g. yogurts, beer, raw fruits and vegetables). In contrast, purified VLPs may be easier to standardize and assay for efficacy because there are less external factors that may affect the host immune system (e.g. other plant or unicellular proteins). Ultimately, there are several promising vaccine delivery vehicles available once a NoV vaccine has been shown to protect human volunteers from infection.

9.5.6 VLP Vaccines can Partially Protect from NoV Infection

Currently, there is only one study that demonstrated *partial* protection from NoV challenge after VLP immunization. This study addressed whether immunization with bovine NoV VLPs would protect Gn calves from infection with virulent bovine NoV (BoNoV) using BoNoV strain CV186-OH for both the VLP vaccine and challenge. This BoNoV is classified as GIII.2 NoV and has 45–50% amino acid sequence identity to GI NoV and 43–46% amino acid identity to GII NoV [53]. BoNoV VLPs were used to immunize 4- to 5-day-old Gn calves via the intramuscular, oral or intranasal routes in two or three doses. BoNoV VLPs were inoculated with different adjuvants including immunostimulating complexes (ISCOM), *E. coli* LT toxin (mLT, R192G) or oil (ISA50V). Immunized calves were then challenged

with virulent BoNoV 20 days after immunization. As a positive control, a separate group of Gn calves were orally inoculated with one dose of virulent BoNoV (9×10^5 RT-PCR detectable units/calf) and orally re-inoculated with the same dose of virulent BoNoV 20 days after initial inoculation.

In this positive control group of Gn calves, initial inoculation with virulent BoNoV induced diarrhea and viral shedding. Protection was evident (no diarrhea or viral shedding) upon subsequent re-inoculation with virulent BoNoV, suggesting that one dose of virulent BoNoV is sufficient to induce protective immunity from BoNoV infection and disease. Examination of the immune response of these calves revealed that both BoNoV-specific serum and fecal IgA and IgG antibody responses were induced after initial inoculation. Low titers of serum BoNoV-specific IgA antibodies were detected at 10 and 20 days after initial inoculation and at 10 days post-re-inoculation (30 days after the initial inoculation). The highest fecal NoV-specific IgA antibody GMT (1,280) was detected at 10 days post inoculation. Titers were maintained through 20 days post-inoculation and did not increase post-re-inoculation. Serum BoNoV-specific IgG antibodies reached a geometric mean titer (GMT) of 400 and 2,560 at 10 and 20 days after inoculation, respectively. Fecal BoNoV-specific IgG antibody titers decreased from a GMT of 240 to 30 between 10 and 20 days after inoculation. Fecal BoNoV-specific IgG antibody titers then increased 6-fold after re-inoculation.

In contrast, Gn calves that were intranasally immunized with two or three doses of bovine VLPs administered with LT (R192G) and then were orally challenged with virulent BoNoV were *partially* protected showing a delayed onset of diarrhea and a shorter period of diarrhea and viral shedding. This immunization regime induced lower BoNoV-specific serum IgA and IgG antibody titers and no BoNoV-specific fecal IgG compared to inoculation with virulent BoNoV. These immunized calves were the only vaccinated group that developed NoV-specific fecal IgA antibody responses. Gn calves immunized intramuscularly with two doses of the BoNoV VLPs (250 μg/dose, 10 days apart) emulsified with oil adjuvant were not protected from diarrhea or infection after challenge, although they developed higher or similar serum IgA and IgG antibody titers, compared to calves that received virulent BoNoV. NoV-specific fecal IgG was also induced after the second dose of this vaccine (GMT 80). However, no NoV-specific fecal IgA was detected in these calves.

These data indicate that BoNoV-specific mucosal (fecal) IgA antibody responses are associated with protection of Gn calves against BoNoV infection and disease, but BoNoV-specific fecal and serum IgG antibody responses are not. Other factors, such as innate immunity and cellular immune responses may also contribute to protection. Similarly, in human volunteers orally challenged with NV, mucosal (salivary) IgA antibody responses were associated with protection against NV infection [117]. However, some individuals who were susceptible to NV and who did not have strong salivary IgA antibody responses were also resistant to infection, suggesting that other factors may be necessary to confer protection against NV infection.

The Gn calf study also indicated that virus-specific, short-term protective immunity exists after BoNoV infection, similar to human NoV infection [48, 112, 191].

Whether long-term protection can be induced by BoNoV infection has yet to be determined. Based on similarities shared between BoNoV and HuNoV infections and immune responses, investigation of innate and cell-mediated immune responses in Gn calves could provide additional information essential to understand immunity against NoV infections and for future vaccine development against NoV.

9.6 Conclusions

This is an exciting time for NoV immunology research. The advent of new animal models of disease and in vitro assays promises to provide new breakthroughs in our understanding of pathogenesis and immunity. Seroepidemiology studies are identifying risk factors and differences in immunity across diverse populations worldwide. The current clinical trials of NoV vaccines may lead to the development of commercially available vaccines against NoV that will reduce the substantial morbidity and mortality associated with these infections.

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