NOROVIRUS IMMUNOBIOLOGY AND VACCINE DESIGN

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ABSTRACT

ANNA LOBUE: Norovirus Immunobiology and Vaccine Design

(Under the direction of Ralph S. Baric)

Noroviruses are a genus of 40+ diverse positive polarity RNA viruses that cause approximately 23 million cases of gastroenteritis annually in the United States alone. The lack of a cell culture system or small animal model in which to study these human pathogens has hindered development of norovirus vaccines since their discovery in 1972. Because noroviruses have a very low infectious dose and high transmissibility, vaccines would be beneficial for employees and patrons of institutionalized settings such as hospitals, nursing homes, and schools, where outbreaks frequently occur. To begin to unravel how norovirus exposure affects the adaptive immune response, we utilized Venezuelan equine encephalitis virus replicons as immune adjuvants and delivery vectors for norovirus antigens to demonstrate induction of B cell and T cell responses in protective immunity to noroviruses in mice. Norovirus-like particle (VLP) vaccination induces robust IgG and IgA responses in serum, feces, and tissues that can block norovirus binding to ABH histo-blood group antigen receptors in a strain-specific manner but have little cross-reactivity to additional norovirus strains. $CD4^+$ T cells are also activated following vaccination to produce large amounts of the anti-viral compound IFN-γ upon stimulation with homologous norovirus VLPs or peptides *in vitro*. To effect a broader immune response, we vaccinated mice with a cocktail of VLPs from multiple norovirus strains simultaneously resulting in cross-reactive receptor-

blocking antibody responses to heterologous strains not included in the vaccine composition. Furthermore, multivalent vaccination did not diminish specificity or quantity of antibody or T cell responses to individual vaccinating strains. Studies with the newly discovered murine norovirus (MNV) revealed that MNV VLP vaccination protects against MNV infection, and both humoral and cellular immunity are involved in clearance of the virus. Furthermore, adoptive transfer of serum but not $CD4^+$ or $CD8^+$ T cells from vaccinated mice completely protected immunodeficient mice from MNV infection, suggesting pre-existing antibodies can prevent establishment of acute infection. Vaccination with multiple human VLPs also provided significant protection against MNV infection in mice, advocating the development of multivalent human norovirus vaccines for cumulative protection against norovirus challenge.

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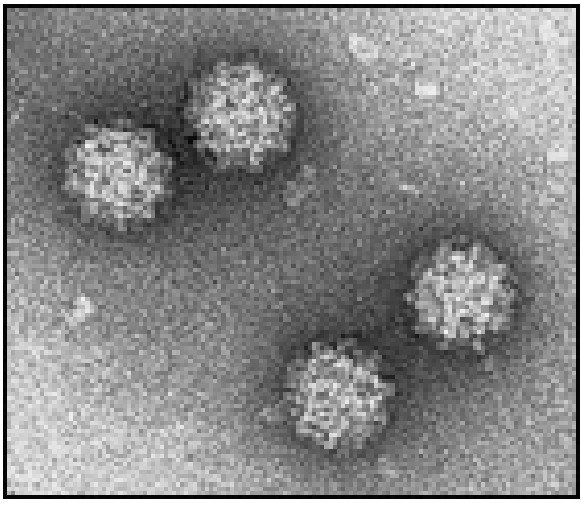
CHAPTER I.

Introduction

The *Caliciviridae* constitute a family of small (27-40 nm), non-enveloped, icosahedral viruses that infect a broad range of host species. These positive-sense single-stranded RNA viruses obtained their name from the Latin word *calix*, meaning chalice, due to the cup-shaped structures on the surface of intact capsids of several Calicivirus species, as seen by electron miroscopy (Figure 1.1). Caliciviruses are divided into four genera based on sequence homology of their ~7.5 kilobase genome (Figure 1.2). Norovirus and sapovirus species predominantly infect humans causing epidemic gastroenteritis; vesiviruses and lagoviruses are animal pathogens characterized by unique disease states. For example, feline calicivirus, a vesivirus, causes respiratory disease in cats, while rabbit hemorrhagic disease virus, a lagovirus, causes fatal symptoms per namesake in rabbits. Of the Calicivirus members, human noroviruses have the greatest impact in the medical and research fields due to the high incidence of norovirus outbreaks in susceptible populations, morbidity, and economic toll. First discovered in 1972 by Albert Kapikian and colleagues (122), noroviruses still remain largely

Figure 1.1.
Calicivirus electron micrograph (http://www.fli.bund.de/).



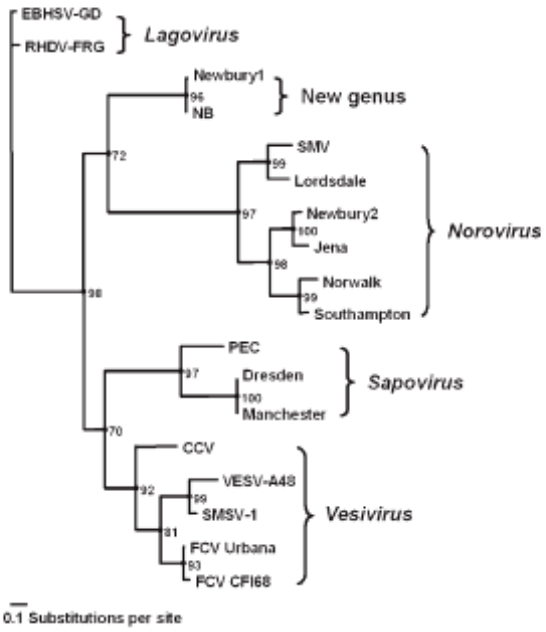
uncharacterized 35 years later due to the lack of an *in vitro* culture system or small animal model that has hindered in depth investigation into immune mechanisms, pathogenicity, replication, and vaccine development of these important pathogens.

Noroviruses

Genome, proteins, and replication

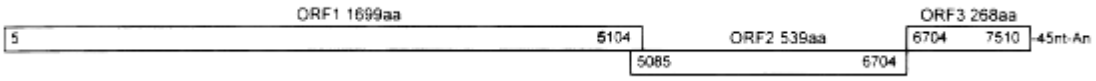
The linear RNA norovirus genome (7.5 kb) is composed of three open reading frames (ORFs) encoding both structural and non-structural genes (Figure 1.3). ORF1 comprises the first two-thirds (~5 kb) of the genome and encodes a polyprotein (200 kDa) that is proteolytically processed to yield the non-structural norovirus proteins required for viral replication. ORF 2 (1.8 kb) encodes the major structural capsid protein (57 kDa), which forms the outer surface of the norovirus virion, and ORF 3 (0.6 kb) encodes a minor basic structural protein (22 kDa) that has been suggested to function in packaging the genome into virions (75). ORF1 and ORF2 have a 17-20 amino acid overlap containing both the stop codon of ORF1 and the start codon of ORF2, creating a -2 frameshift. A single nucleotide overlap exists between the ORF2 stop and ORF3 start codons, which restores the reading frame of ORF3 to that of ORF1 (75). Genomic RNA is likely covalently linked at its 5' end to the virally expressed VPg protein

Figure 1.2. Phylogenetic tree of Calicivirus species based on capsid sequence (190).



and flanked at its 3' end by a polyadenylated tail (26, 95, 283). The polyprotein encoded by ORF1 is cleaved into six identified constituent replicase proteins by the self-expressed 3C-like viral cysteine proteinase. Cleavage products include, from N-terminus to C-terminus, an N-terminal

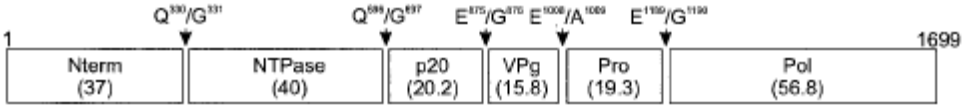
Figure 1.3. Norovirus genome organization (49).



protein (p48; 37-48 kDa), a 2C-like nucleoside triphosphatase (NTPase; 40 kDa), a 3A-like protein (p20; 20 kDa), a genome-linked viral protein (VPg; 16 kDa), a 3C-like proteinase (Pro; 19 kDa), and a 3D-like RNA-dependent RNA polymerase (Pol; 57 kDa) (232). The absolute function of the N-terminal protein p48 is not known; however, p48 expressed *in vitro* has been shown to colocalize with the Golgi apparatus in transfected cells and to potentially play a role in membrane rearrangement and intracellular protein trafficking (54, 59). Further proteolytic cleavage of the N-terminal protein by Pro or cellular caspase 3 may also occur and may be essential for replication, as seen with other calicivirus species (89, 147, 232, 238, 239). Activity of the norovirus NTPase was recently discovered when bacterially expressed protein was shown to bind and hydrolyze NTPs, similar to the picornavirus 2C protein, although its function does not appear to support DNA unwinding (208). VPg, which binds to the 5' end of the norovirus genome, plays a role in priming transcription and translation initiation of RNA, as suggested by its function in replication and its binding to translation initiation factors in both *in vitro* expression assays and following infection of cultured cells (43, 44, 225). Pro and pol have been more extensively studied than the aforementioned non-structural proteins, and their functions have been well characterized.

Pro resembles cellular chymotrypsin-like serine proteases (23) and contains amino acid sequence motifs resembling the picornavirus 3C protease. Pro catalytically cleaves the norovirus ORF1 polyprotein into 6 individual non-structural proteins at QG, EG, or EA sites (147, 148, 231, 238) (Figure 1.4). Stable precursors include a p20-VPg and Pro-Pol in addition to several less stable intermediates (17, 238), and the Pro-Pol precursor retains enzymatic activity (16). Pro amino acids His30, Glu54, and Cys139 have been identified as residues in the active site, which is located at the center of a deep cleft between the N- and C-

Figure 1.4. Cleavage sites for proteolytic processing of ORF 1 polyprotein into individual functional proteins (17).



terminal domains and is stabilized by hydrogen bonds between conserved residues (175, 237, 238). Furthermore, His157 is responsible for substrate binding (175). Pro is also able to inhibit cellular translation by severing the domains of cellular poly-A binding protein that bind translation initiation factors and RNA, respectively, as evidenced by addition of Pro to cell translation extracts. The Pol protein functions in viral genome replication by initiating and propagating RNA synthesis. Pol contains the amino acid motif glycine-aspartic acid-aspartic acid (GDD), which is common to many plus-strand RNA virus polymerase active sites, and has shown to be essential for Pol function *in vitro* (62, 132). Pol activity uses a VPg-priming mechanism for RNA synthesis from the genomic template that is dependent on the presence of the poly(A) tail; however, RNA synthesis from negative strand genomic templates are primer- and poly(A)-independent (62, 225). The presence of Mn^{2+} or Mg^{2+} cofactor is also required for Pol activity (62, 224).

Norovirus replication is currently poorly understood. It is believed genomic RNA acts as a template for negative strand synthesis, which in turn acts as a template for transcription of full-length positive strand RNA for packaging and subgenomic RNA for protein synthesis. A model for Pol activity in replication initiation of positive and negative strand RNA has been proposed by Rohayem *et al* (225). In positive genomic and subgenomic strand replication, Pol uridylylates VPg in the presence of polyadenylated RNA. Poly(U)-VPg then acts as a primer for initiation of negative strand synthesis. Subgenomically expressed proteins retain the first four nucleotides of genomic RNA—GTAA—indicating the transcription initiation site is conserved in subgenomic RNA as well (5). In contrast, replication of negative strand RNA by Pol is primer-independent. A poly(C) stretch is added to the 3' end of negative strand RNA by terminal transferase activity of Pol to initiate RNA synthesis (225). Studies with other animal caliciviruses suggest that N-terminal protein, NTPase, and p20 may be responsible for the formation of the viral replication complex (74, 121, 276); however, no studies with norovirus have supported these findings. While VPg may regulate transcription, this protein is not thought to be part of the replication complex in its mature form due to its strong basicity but could participate in a precursor form (43, 125). The cellular proteins La, hnRNP L, PTB, and PCBP-2, which play a role in hepatitis C and poliovirus translation, have also been found to bind synthetic norovirus RNA and may be used for viral translation and/or replication (83). Following replication, genomic RNA is packaged into viral particles from a signal suggested to originate from within ORF1 (5). While human noroviruses have not been cultured to date, murine noroviruses have a tropism for replication in dendritic cells *in vitro* and macrophages *in vivo* and *in vitro* (277).

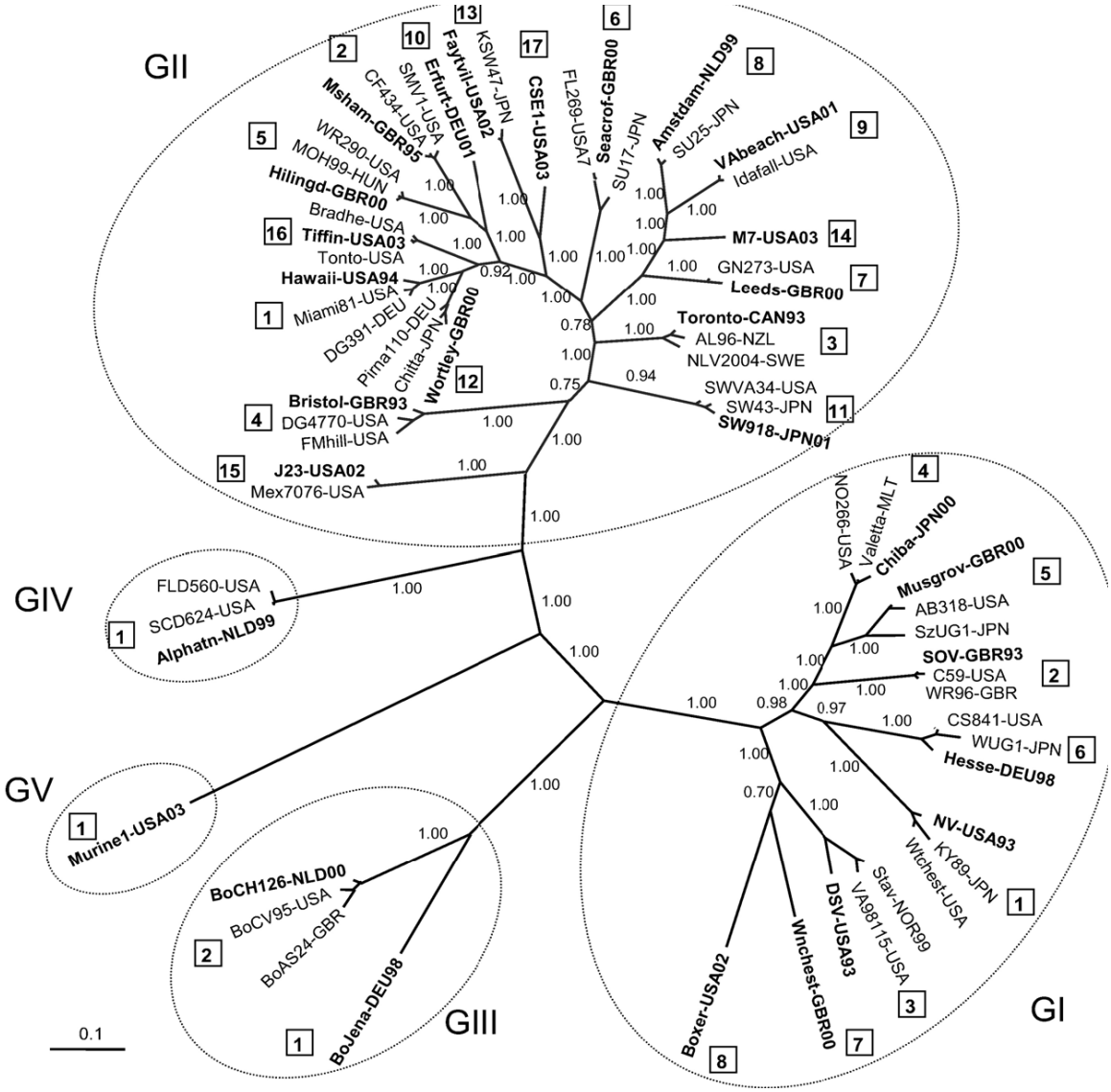
Phylogeny and nomenclature

Noroviruses constitute a genus of more than forty diverse virus strains that are divided into five genogroups based on sequence homology (55, 76, 124, 192). Genogroup I (GI), GII, and GIV viruses infect strictly human hosts, with the exception of porcine-specific virus within GII; GIII and GV viruses infect bovine and murine hosts, respectively. Each genogroup is subdivided into genoclusters. GI contains at least eight genoclusters, GII contains at least seventeen, GIII contains two, and GIV and GV contain one each, based on phylogenetic analysis proposed by Zheng et al (290). Genoclusters are designated numerically following identification of the genogroup to which they belong (i.e. GII.3). Distinct strains are further subdivided within a genocluster (Figure 1.5). Norovirus strains are commonly named for their location of initial identified outbreak (i.e. the Norwalk virus strain was first isolated from an outbreak in Norwalk, Ohio), and individual isolates routinely use *strain/year/country* nomenclature (i.e. NV/93/USA).

Analysis of representative complete norovirus genomes revealed 69-97% nucleotide homology between strains within a genogroup and 51-56% homology between strains in different genogroups (126), which is typified by highly conserved and highly variable regions. The ORF1/2 junction is the most highly conserved sequence in the norovirus genome maintaining 86-100% identity within a genogroup in the subsets of strains tested (120). ORF1 remains more conserved than ORF2 and 3, where specific cleavage sites within the polyprotein, as well as functional motifs within the Pol sequence, are nearly identical across norovirus species (17, 126, 228, 238). The structural proteins, on the other hand, are highly variable. Strains within a genogroup can differ by up to 40% in capsid amino acid

sequence while strains in different genogroups usually differ by >50% (75). ORF3 was found to differ

Figure 1.5. Phylogenetic tree representing norovirus genogroup classifications (290).



by up to 51% in nucleotide sequence within a genogroup, when just four strains were analyzed (230). While many attempts have been made to phylogenetically characterize

existing norovirus strains, no consensus region or strategy has been reached to focus these efforts, leading to a plethora of confusing and contradictory literature. Because ORF2 is the most highly variable genome segment distinguishing one strain from the next genetically and antigenically and because it is likely under the highest environmental pressure to evolve into new norovirus strains, we have chosen phylogenetic clustering based on the complete capsid sequence as a representative model for the purposes of this dissertation (Figure 1.5).

Disease and epidemiology

Norovirus infection in humans is characterized by vomiting (69%), diarrhea (66%), nausea (79%), low-grade fever (37%), and abdominal cramping (30%) (123). The incubation period for infection ranges from 24-48 hours, and manifestation of clinical symptoms usually lasts 12-72 hours, although individual cases can be prolonged (179). Viral shedding can last up to three weeks after resolution of symptoms resulting in enhanced opportunity for transmission. Noroviruses are responsible for more than 96% of all viral gastroenteritis cases and account for at least 23 million infections annually in the United States alone, as reported by the Centers for Disease Control and Prevention (166). Furthermore, noroviruses cause up to half of all outbreaks of gastroenteritis worldwide (6), as well as being the most common cause of sporadic diarrhea in community settings (220). All populations are susceptible to infection; however, the elderly are more susceptible, and outbreaks are common in retirement communities (110, 115). Furthermore, the very young, the elderly, and the immunocompromised have increased risk for severe disease (162, 172, 187). Infections are commonly food- and water-borne, although person-to-person transmission by direct contact, exposure to aerosols, or fecal-oral routes is predominantly responsible for amplification of

outbreaks (199). Many outbreaks occur in institutionalized settings such as schools, nursing homes, hospitals, and day care centers or in settings where human contact is unavoidable such as aboard cruise ships or in military barracks (Reviewed in (53) and (107). Persistence and frequency of outbreaks is heavily influenced by the very low infectious doses required for infection (<10 virions), as well as the high transmissibility and extreme stability of these viruses. Because of the characteristics highlighted above, noroviruses have been named class B select agents.

Noroviruses are cyclic in nature, both in the predominance of circulating strains and frequency of outbreaks (134). The GII.4 strains have been predominantly circulating since the 1990's, and recent studies indicate that evolution is occurring within this genocluster (25, 55, 84). Defining characteristics of this genocluster that allow its continued predominance are unknown. Outbreaks from other GII strains are also common albeit sporadic, while GI outbreaks occur even less frequently (55, 110, 272). Fluctuations in annual norovirus outbreak frequencies are likely primarily dependent on environmental factors and the emergence of new strains (261). The incidence of norovirus outbreaks during the winter of 2002-03 is the highest on record, as reported in the United States and Europe (2, 110, 261). While surveillance of norovirus outbreaks in previous decades has been rudimentary due to lack of advanced molecular assays to identify the etiological agents of gastroenteritis outbreaks or strain specificity, increased sensitivity of RT-PCR reagents and increased surveillance now allow stringent tracking of norovirus activity. These advances will allow identification of changing epidemiological trends in the future.

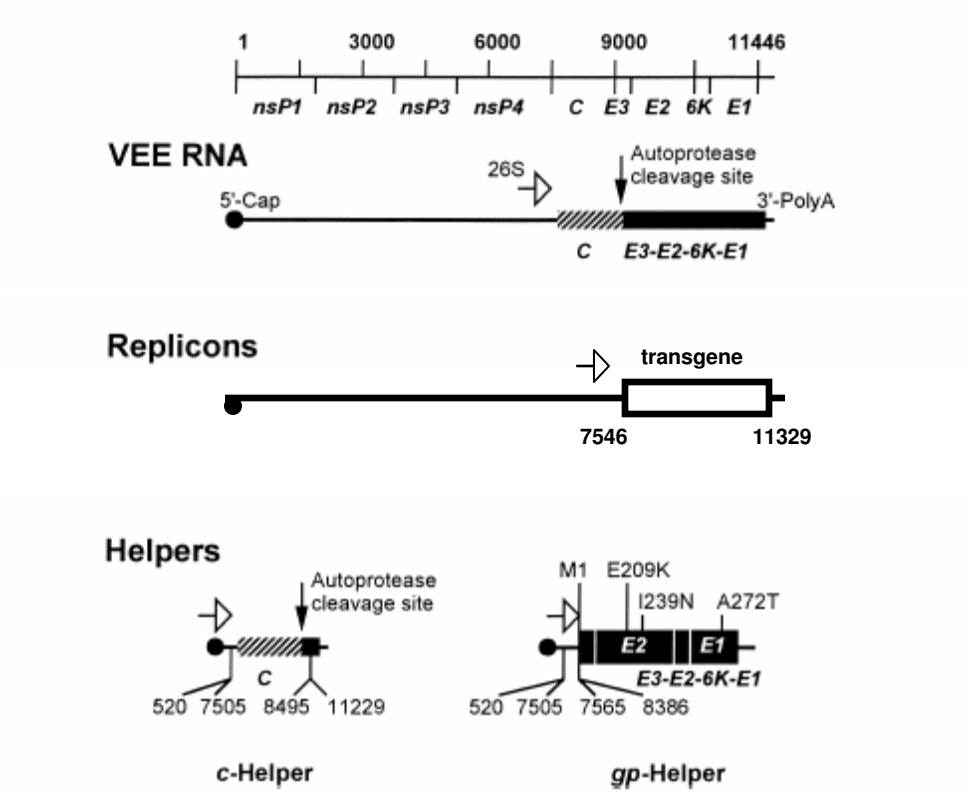
Capsid

Recombinant expression systems

Noroviruses cannot be cultured outside its human hosts, and native virus isolation is tedious and cumbersome. Because the intact capsid is the major immunogenic and antigenic determinant of the norovirus virion, recombinant expression systems have been developed to allow *in vitro* production of norovirus capsid protein that will self-assemble into virus-like particles (VLPs) in sufficient quantities to be characterized and utilized as immunological reagents. Baculovirus and Venezuelan equine encephalitis (VEE) replicon systems have been well characterized for production of VLPs (11, 116), which are morphologically and antigenically indistinguishable from native norovirus (11, 73). Baculovirus expression systems are implemented by cotransfecting wild-type baculovirus DNA and transfer vector DNA containing a cDNA copy of a norovirus ORF2 isolate. Baculovirus successfully expressing norovirus ORF2 following screening and plaque purification are used to reinfect Sf9 cells, and the expressed 38 nm VLPs are harvested on a sucrose cushion (116). The alternative VEE replicon system uses a methodology whereby norovirus ORF2 is directly cloned into the polyclonal site of the VEE plasmid under the control of an internal 26S promoter, replacing the structural genes of VEE. Transcripts of the replicon, helper VEE capsid, and helper VEE envelope glycoprotein are coelectroporated into baby hamster kidney (BHK) cells resulting in the packaging of VEE replicon particles (VRPs) that will undergo a single round of replication and express high levels of norovirus capsid protein that self-assembles into VLPs when infecting cells *in vitro* or *in vivo* (11) (Fig. 1.6). Additional methods using *E. coli* and yeast to express VLPs are not currently widely implemented for VLP production and are described elsewhere (252, 284). New methods using genetically

engineered plants such as tomatoes, potatoes, and tobacco for VLP production have also been described for the purpose of delivering VLP vaccines (105, 160, 246, 289).

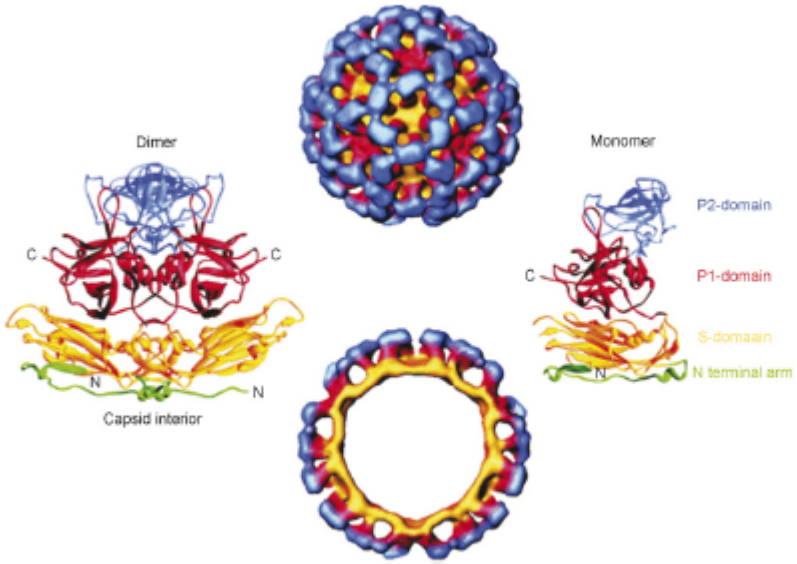
Figure 1.6. Depiction of VEE genome, RNA, replicons, and helpers (adapted from (214)).



Structure

Recombinant norovirus VLPs have been characterized by electron cryomicroscopy, x-ray crystallography, and computer imaging to reveal the structure of the intact norovirus capsid (212, 213). The capsid is composed of 180 capsid molecules organized into 90 dimers in a *T* = 3 icosahedral symmetry (213) (Figure 1.7). Capsid proteins have two distinct domains, the shell (S) domain and the protruding (P) domain, that are linked by a flexible hinge (212) (Figure 1.7). The S domain constitutes the first 125 residues of the capsid protein and forms

Figure 1.7. Cryo-electron microscopy and x-ray crystallography of NV VLPs (141).



the structural core of the intact capsid, maintaining its integrity by interactions with adjacent S domains (20). The P domain constitutes the remainder of the protein and is divided into two subdomains, P1 and P2. The P1 domain consists of residues 226-278 and 406-520 and functions as a stem region between the S and P2 domains. The P2 domain, consisting of residues 279-405, protrudes furthest from the capsid shell in an arch-like structure, forming the exterior of the intact capsid. The sequence of this domain is more hypervariable and contains receptor binding sites and a motif resembling an RNA binding domain (30, 212, 249, 263). During infection, immune pressure is exerted upon the distal regions of P2 as well as some surface exposed P1 residues, which can lead to novel patterns of evolutionary and structural changes in the protein (179).

Receptor binding

Norovirus VLPs have recently been shown to bind the ABH histo-blood group antigens (HBGAs) as putative receptors by hemagglutination of human red blood cells and attachment

to human saliva, gastroduodenal epithelial tissue, and synthetic HBGA carbohydrate chains (91, 92, 109, 159). HBGAs are a family of complex glycans that are expressed on the surfaces of red blood cells, gut and respiratory epithelia, and biological secretions in humans (reviewed in (158)). The ability of noroviruses to attach to HBGAs is dependent on specific residues in the distal P2 domain of capsid homodimers that have been identified by cocrystallization of recombinant P protein from the VA387 strain with synthetic A and B trisaccharides (30). It is likely that both strain-specific and non-specific interactions occur between virus and carbohydrate to stabilize binding. Strain-specific interactions include hydrogen bonding at residues corresponding to Thr344, Arg345, Asp374, and Gly442 of VA387 (30). Furthermore, Cao *et al.* and Tan *et al.* shared findings that Thr338 is essential for receptor binding due to conformational hydrogen bonding (30, 249). Additionally, the presence of Asp302 near the receptor binding site and a conserved C-terminal arginine cluster may also enhance the efficiency of receptor binding (249, 251). Long distance interactions between additional non-specific residues are likely necessary for further stabilization of virus-receptor interactions. Arg345 and Asp374 as well as peripheral stabilization sites are also conserved in heterologous Snow Mountain, Hawaii, and MOH genogroup II viruses. However, receptor binding interfaces for the Norwalk strain, a genogroup I virus that has also undergone crystallization of the P domain, are distinctly different from VA387. Such differences may explain why different norovirus strains exhibit diverse binding profiles to members of HBGA family.

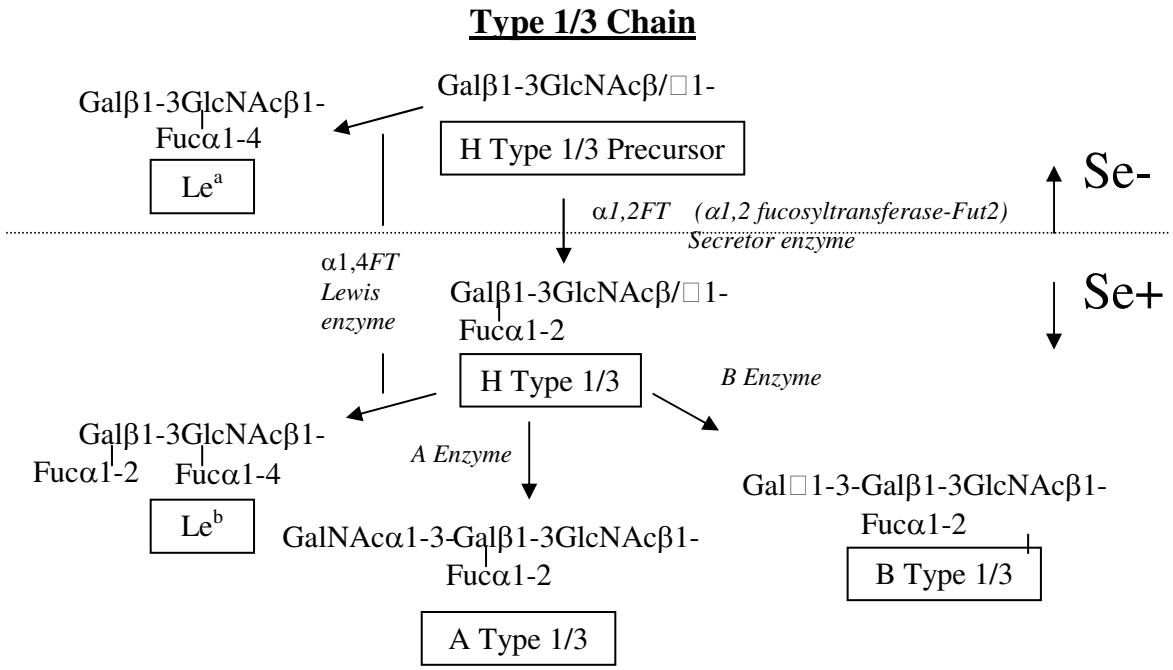
ABH histo-blood group antigens

Biochemistry

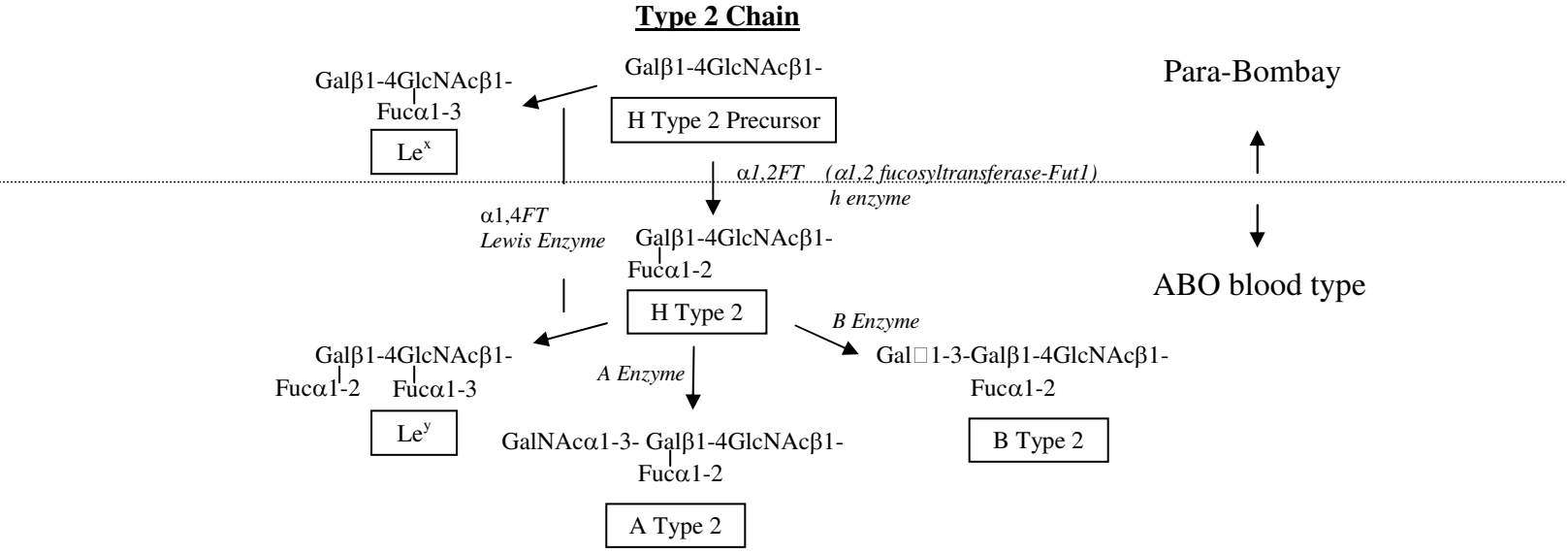
ABH histo-blood group antigens are carbohydrates moieties expressed on and secreted from mucosal tissues and red blood cells. HBGA expression is controlled by multiple genes that influence three biosynthetic pathways which lead to polymorphic ABO, Lewis, and secretor phenotypes (Figure 1.8). Synthesis begins with a disaccharide precursor (GalB1,3GlcNAc for the Type 1 pathway) to which monosaccharides are sequentially added by glycosyltransferases. The *FUT3* gene encodes a fucosyltransferase that adds fucose residues in α-1,3 or α-1,4 linkages to the precursor, leading to synthesis of a trisaccharide of the Lewis a (Le^a) phenotype, also synonymous with the non-secretor phenotype. A separate pathway, leading to the secretor phenotype, depends on the action of the *FUT2* gene, encoding a fucosyltransferase that adds residues to the precursor in an α-1,2 linkage, creating the H type 1 antigen. Further activity on the trisaccharide by FUT3 or the A and B enzymes lead to synthesis of tetrasaccharides Le^b, A type 1, and B type 1, respectively. The A and B enzymes add N-acetylgalactosamine or galactose in an α-1,3 linkage, respectively. Alternatively, the Type 2 pathway begins with the GalB1,4GlcNAc precursor and is also acted on by FUT2, FUT3, A and B enzymes in a parallel fashion, resulting in the production of H type 2, Le^x, Le^y, A type 2, and B type 2, respectively. The Type 3 precursor GalB1,3GalNAc is catalyzed by FUT2, resulting in the H type 3 product that can be further modified by the A and B

Figure 1.8. HBGA Type 1/3 (A) and Type 2 (B) synthesis pathways with enzymatic modifications by *FUT2* and *FUT3* genes (146).

A.



B.



enzymes. (Reviewed in (158)) The presence or absence of the *FUT2* and *FUT3* alleles in an individual can determine if that individual is susceptible to norovirus infection in a strain-specific manner (141).

Norovirus susceptibility

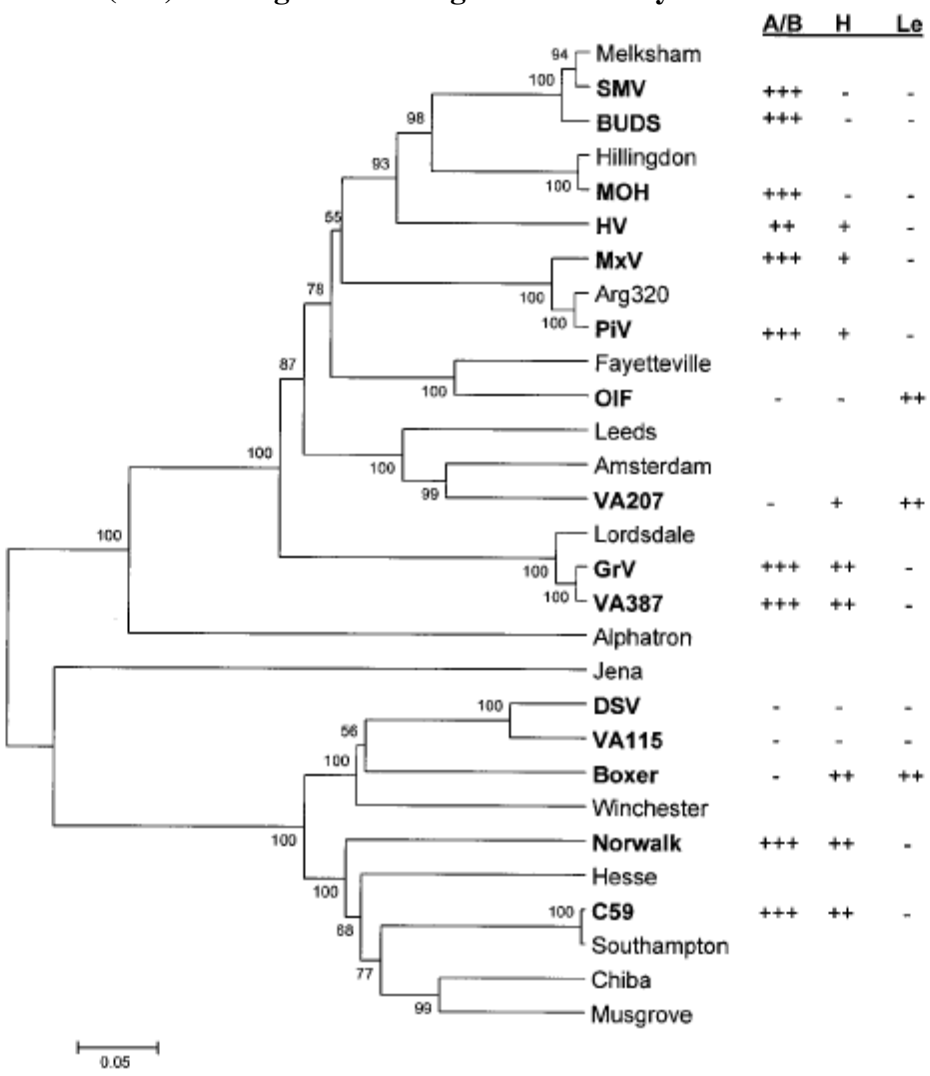
Recent outbreak investigations and human challenge studies following norovirus infection have lent strong evidence for HBGAs as the natural receptor for norovirus infection (94, 108, 146, 222). Similarly, expression of HBGAs and human susceptibility to infection appear to be directly linked. Early studies revealed that some individuals could not be infected with Norwalk virus upon challenge, that resistant individuals typically clustered in families, and that the presence of pre-existing antibody did not correlate with protection (119, 133, 203). Further studies indicated that individuals of the O blood type appeared to be more susceptible to Norwalk virus infection than other groups (108), and Norwalk VLPs bound to gastroduodenal epithelial cells from individuals who were positive for the *FUT2* allele and therefore exhibit a secretor-positive phenotype, while no binding occurred to cells from secretor-negative individuals (159). The *FUT2* gene encodes a fucosyltranferase responsible for generating the H type 1 and H type 3 antigens from disaccharide precursors, which are expressed on mucosal surfaces and have been shown to bind Norwalk VLPs (159). Furthermore, H type 1 and H type 3 antigens are further modified in individuals of A and B blood types by the A and B enzymes, which lead to differential HBGA phenotypes that have not been found to bind Norwalk VLPs. Inactivating mutations in the *FUT2* gene have been identified that lead to the non-secretor phenotype (193), and approximately 20% of European populations are homozygous recessive for the predominant Gly428Ala inactivating mutation.

These individuals, therefore, do not produce H type 1 or H type 3 antigens and appear to be genetically resistant to infection with Norwalk virus (146, 157, 159). A tiny subset of the population exhibits the Bombay phenotype, where HBGAs are entirely absent from cells and secretions (193). Additional studies with other norovirus strains have revealed that susceptibility to norovirus infection is differential and increasingly complicated compared to the paradigm shown with the Norwalk strain. For example, blood type and secretor status did not have any impact on susceptibility to infection with the GII.2 Snow Mountain norovirus strain (145). A separate study showed that individuals of the non-secretor phenotype had significantly lower antibody titers to GII.4 norovirus strains than secretors, indicating that non-secretors likely have a lower prevalence of infection to the predominantly circulating norovirus strains than secretors, but blood type was irrelevant to infection status (140). Additionally, Rockx *et al.* reported that individuals with blood type B appeared to have a lower incidence of infection with GI viruses (222). Although individual norovirus strains may only be capable of infecting specific subsets of the human population, the unique binding profiles of each strain may collectively allow nearly all individuals to be susceptible to a norovirus infection.

Norovirus binding profiles

Distinct norovirus strains have highly variable HBGA binding patterns. Carbohydrate binding specificities of norovirus strains have been reported (Figure 1.9), and the human infectivity spectrum based on polymorphism of *ABO*, *FUT2*, and *FUT3* has been deduced (141) (Figure 1.10). While genetically related strains can share binding

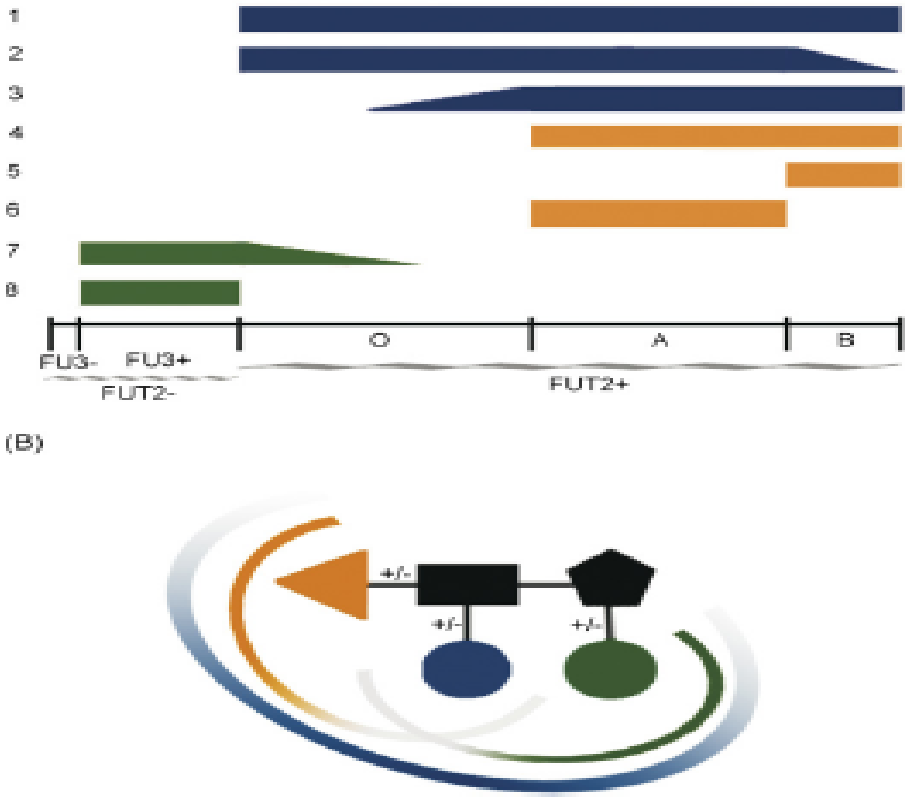
Figure 1.9. Norovirus VLP binding profiles to A/B, H, and Lewis carbohydrate chains (104). Strength of binding is indicated by +.



patterns, indicating that evolution of virus strains may be influenced by HBGA binding, specific binding profiles are not genogroup exclusive (104). Viruses can, however, be categorized into two binding profile groups: Those that bind A/B and/or H epitopes and those that bind Lewis and/or H epitopes. No strains have been shown to bind both A/B and Lewis epitopes simultaneously, although many strains from either group bind H epitopes. The H epitopes have been shown to be independent docking sites for virus attachment; however, due to their intermediate placement on the HBGA structure, they can act together

with the A/B or Lewis epitopes depending on the capsid binding site (104). Furthermore, supporting data from blockade studies with HBGA-typed saliva, synthetic HBGAs, and monoclonal anti-HBGA antibodies suggest H epitopes can cross-block binding of strains that also bind A/B or Lewis epitopes, but the latter two cannot cross-block each other (104). These data indicate that strains in the two binding profile groups have distinct binding sites on the viral capsid. A third group should be noted for which no HBGA ligands have been identified, such as the Desert Shield, VA115, and mouse norovirus (MNV-1) strains, suggesting additional alternative viral receptors may exist or the appropriate carbohydrate variations are not represented in saliva or are not biochemically available.

Figure 1.10. Human infectivity spectrum from eight identified HBGA binding specificities of norovirus strains dependent on FUT2 and FUT3 polymorphism of the individual (A) that are reflected on the HBGA monosaccharide (B) (141).



Norovirus infection models

Large animal models

Because no small animal model is susceptible to infection with human norovirus, investigations using primate species have been undertaken. Mangabey, rhesus, and pigtail macaques screened for naturally occurring anti-norovirus antibody yielded 53% and 58% seropositivity for GI and GII noroviruses, respectively (112). Likewise, chimpanzees were 92% seropositive for GI noroviruses, suggesting exposure and potential infections are occurring at substantial rates in non-human primates. One challenge study using common marmosets, cynomolgus and rhesus macaques, cotton top tamarinds, and chimpanzees found that, following inoculation with Norwalk and Grimsby virus strains, no animals developed diarrheal symptoms, and viral shedding in the feces had cleared after day four, although one of four rhesus macaques shed virus in stool for 19 days and developed IgG and IgM serum antibody responses (221). In two separate studies, however, newborn pigtail macaques and adult chimpanzees were able to be symptomatically and asymptomatically infected with Toronto and Norwalk virus, respectively, and both infections were transmissible to other primates (243, 282). Recently norovirus particles were also seen by electron microscopy in the feces of monkeys with diarrhea (269). Because primate infection studies thus far have yielded inconsistent findings and are very expensive to carry out, advanced norovirus infection studies are unlikely to occur using this model.

Noroviruses in genogroups II and III, have been shown to infect porcine and bovine species, respectively. Like human noroviruses, no cell culture system exists for these non-human strains. While 9-11% of cattle with diarrhea consistently test positive for bovine norovirus (169, 200), and antibody cross-reactivity may exist between bovine and some

human norovirus strains (14, 191), no pathology or mechanisms of infection have been determined in the bovine model. Porcine noroviruses have been detected in 0.4-2% of swine stools from large sampling groups (244, 262); however one study showed that 36% and 71% of pigs were seropositive for porcine norovirus in Japan and the United States, respectively (57). More than half of swine tested were also seropositive for human noroviruses. Furthermore, human VLPs can effectively bind to porcine buccal and intestinal tissues, binding is dependent on A/H expression phenotypes, and swine gastric mucin can effectively block human norovirus VLP binding to HBGAs or to porcine tissues (31, 258). Infection studies with a human GII.4 norovirus strain in gnotobiotic pigs revealed that animals developed mild diarrhea (48/65), were RT-PCR positive for human norovirus in rectal swabs (29/65), had infected duodenal and jejunal enterocytes by immunofluorescence and electron microscopy (18/31), and one animal developed intestinal lesions (1/7) (32). These findings suggest human noroviruses can replicate in a swine model and raise the question of zoonotic reservoirs for human noroviruses. However, large animal models are cumbersome and expensive, and researchers would benefit from a small animal model for study of norovirus infection and cultivation.

Murine norovirus

In 2003, the first murine norovirus, MNV-1, was discovered by Herbert W. Virgin's group at Washington University (124). The virus clusters in the unique murine norovirus-specific genogroup five (GV), and 15 distinct murine strains have been identified to date (98, 99, 124, 255). Furthermore, MNV appears to be the most common murine virus, infecting ~22.1% of laboratory mice tested (100). While MNV-1 asymptomatically infects wild-type mice and is

naturally cleared within one week of infection, other identified strains can cause persistent infections with prolonged viral shedding beyond eight weeks post-infection in some cases (99, 100, 124, 171, 255). MNV has furthermore been shown to specifically infect macrophages and dendritic cells (277). Kupffer cells, the resident macrophages of the liver, and cells primarily within the red pulp and marginal zone of the spleen in MNV-1 infected mice stained positively with MNV antisera, and bone marrow-derived macrophages and dendritic cells showed visible CPE when infected with MNV-1 *in vitro* (277). These findings lead to the subsequent discovery of the first *in vitro* cell culture system for a norovirus, where murine macrophage cell lines Raw 264.7, J774A.1, and WBC264-9C were able to support MNV infection and replication. Raw 264.7 cells have additionally been used to develop the first ever plaque assay for norovirus titer quantitation (277).

Using this information, questions about norovirus infection and pathology can now be readily answered. Following infection of Raw 264.7 murine macrophage-like cells, MNV virions could be seen intracellularly within or in close proximity to membranous vesicles after 12 hours, and vast intracellular membrane rearrangement had occurred after 24 hours (277). These findings suggest norovirus replication occurs in association with intracellular membranes. In wild-type 129 mice, MNV-1 was detectable in the small intestine, spleens, livers, and lungs of mice following oral inoculation with the virus, indicating that the virus is able to disseminate from the gastrointestinal tract following primary infection of the gut (171). No gastroenteritis or additional clinical symptoms were detectable; however, histopathological examination of tissues revealed an increased number of inflammatory granulocytes in the intestine and increased activation and hypertrophy of splenic white and red pulp cells, respectively (171). Immunodeficient mice lacking signal transducer and

activator of transcription 1 ($STAT1^{-/-}$) or receptors for interferons α, β, and γ ($IFN\alpha\beta\gamma R^{-/-}$) exhibited more severe pathology and fatal disease following MNV-1 infection, and mice lacking recombination-activating gene ($RAG^{-/-}$) exhibit persistent non-fatal infection following intracerebral inoculation (124, 171). Disease in $STAT1^{-/-}$ mice included encephalitis, meningitis, hepatitis, and pneumonia with high levels of viral RNA in the small intestine, brain, liver, lung, spleen, blood, and feces (124). Lethality was 100% in $STAT1^{-/-}$ and $IFN\alpha\beta\gamma R^{-/-}$ mice following intracerebral challenge; however per oral and intranasal challenge had variable lethalities in these mouse groups. Because wild-type mice do not show clinical signs of norovirus infection and because infection can be fatal in immunodeficient animals, the mouse is not a perfect representative model for studying human norovirus infection. However, the mechanism of norovirus infection and pathology in the mouse may well relate to that exhibited in human norovirus infection, and with newly identified culture, small animal, and reverse genetic (271) models we can learn exponentially more about noroviruses and their disease.

Norovirus immunology and vaccines

Human challenge and outbreak studies

Because no animal or cell culture model exists for human noroviruses, very little is known about the immune response elicited following infection. Norovirus outbreaks and human challenge studies provide the only available samples with which to study norovirus immunity in humans. Susceptibility studies have revealed that certain individuals are genetically

resistant to infection with specific norovirus strains (146); however, previous exposure history and the immune response likely play a central role in determining infection outcomes following norovirus challenge. Early human challenge and outbreak studies revealed that individuals with high serum or fecal antibody titers to Norwalk virus prior to challenge were more likely to become infected with the virus than individuals with low pre-existing antibody titers (13, 70, 119, 188). Most but not all individuals were resistant to subsequent infection with the same virus six months later; however, less than half maintained high antibody titers six months after secondary challenge (119). In long term immunity studies, individuals who were infected with Norwalk virus were all symptomatically reinfected 27-42 months later (203). However, some individuals without common pre-exposure factors who are genetically susceptible to Norwalk virus infection never became infected (119). These findings are contradictory in detailing the role of antibodies in preventing norovirus infection. Evidence for short-term but not long term immunity exists, but evidence for persisting long term immunity is much more controversial.

It is clear that the presence of preexisting serologic responses does not protect against norovirus challenge. These ambiguous data may be explained by the large number of circulating norovirus strains and the frequency of human exposure. Studies of seroprevalence to GI and GII strains have shown that at least 50% of children under age five are typically seropositive for norovirus exposure, which increases to 60-90% by age ten and reaches 100% by adulthood (42, 96, 97, 118, 176, 201). A cross-challenge study where volunteers infected with Norwalk virus were still susceptible to subsequent infection with Hawaii virus indicates that immunity to one strain is unlikely to confer cross-genogroup protection against infection with another strain (281). Cross-reactivity studies have revealed

that antibodies can recognize heterologous norovirus antigens, particularly within a genogroup (86, 145, 180, 219, 259). However, while homotypic antibodies from human antisera following infection can completely block VLP binding to HBGA receptors, heterotypic antisera to strains within the same genogroup are less able to do so (91, 219). Together, these findings suggest that cross-reactive antibodies are not likely to protect against heterologous norovirus infection; however, low to moderate antibody cross-reactivity between strains in combination with multiple exposures disguise the true impact of humoral immunity on resistance to subsequent norovirus challenge in the face of unknown exposure histories.

Cross-reactive antibody and T-cell responses in humans remain largely uncharacterized. A single study investigating the presence of T-cell activity following norovirus infection was described by Lisa Lindesmith and colleagues (145). Human peripheral blood monocytes (PBMCs) were harvested pre- and post-challenge with Snow Mountain virus and tested for activation following stimulation with homologous and heterologous VLPs. T helper 1 (T_H1) $CD4^+$ cells responded to homologous VLP stimulation with increased secretion of gamma-interferon (IFN-γ) and interleukin-2 (IL-2). IL-6 and IL-10 were not secreted above pre-challenge levels, indicating T_H2 cells may not be involved in norovirus immunity. PBMCs from both infected and uninfected volunteers could be stimulated with VLPs pre- and post-challenge suggesting previous norovirus exposure. Furthermore, cells from infected individuals post-challenge exhibited cross-stimulation by Hawaii VLPs but not Norwalk VLPs, indicating that T-cell cross-reactivity may be genogroup specific. (145) T-cell studies following infection with additional norovirus strains need to be conducted and collective cross-reactivity to multiple genoclusters in GI and GII

addressed. Antibody responses to noroviruses have been more thoroughly characterized, but only to a limited number of strains. Following exposure, individuals have been shown to mount serum IgG, IgA, IgM and/or salivary IgA antibody responses to the infecting norovirus strain (52, 70, 86, 145, 259). Individuals exhibiting symptoms following infection typically have elevated IgG and IgA antibody titers compared to asymptomatic individuals (70). On the contrary, cross-reactive antibodies sometimes exist following infection with heterologous strains, although serum responses are usually restricted to the IgG subclass and lower in magnitude than homotypic IgG (86, 219, 259). For example, following infection with Snow Mountain and Hawaii viruses, respectively, 6/15 and 2/12 individuals mounted mean cross-reactive IgG responses to Norwalk VLPs that increased 6.2-fold after infection; however, 15/20 individuals infected with the homologous Norwalk strain mounted responses almost 30-fold higher than pre-challenge titers (259). Another study showed that 3/9 volunteers challenged with Snow Mountain had a >4-fold increase in cross-reactive antibody to Hawaii VLPs (145). These responses indicate cross-reactivity within and across genogroups, the latter of which is not always detectable (145, 180). Furthermore, antibody cross-reactivity is usually detectable to strains within a genogroup (86) but can be highly variable depending on the antigenicity of distinct genoclusters. While little is currently known about this subject, outbreak studies by Noel *et al.* revealed that GI viruses may be more antigenically related than GII viruses (180). Antisera from four heterologous GI outbreaks retained cross-reactivity to Norwalk VLPs, with a 70% seroconversion rate, although viruses were ≤38% divergent in capsid amino acid sequence from the Norwalk strain. Only 16-46% of sera following heterologous GII infection seroconverted to intragenogroup Toronto or Hawaii antigens, suggesting these viruses are antigenically

distinct. All available literature on human antibody responses and cross-reactivity following norovirus infection are confined to human challenge studies comparing ≤3 strains or outbreaks. Many outbreak studies measure heterotypic but not homotypic antibody responses due to the lack of a recombinant antigen panel spanning many norovirus genoclusters that include the outbreak strain. Trends of increased antibody cross-reactivity within norovirus genogroups have emerged; however, confounding pre-exposure histories of infected individuals, increasing numbers of identified strains, and less-than-clear antigenic relationships between strains leave a muddled picture of homotypic and heterotypic norovirus antibody responses following human infection.

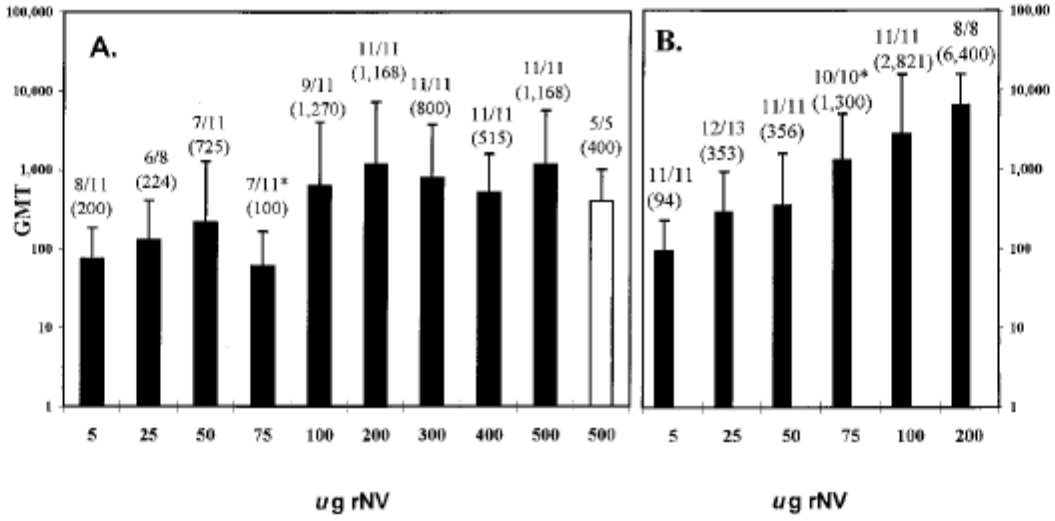
Recombinant vaccine studies

Norovirus VLPs have been used in vaccine trials for humans since 1996 with limited success. In Phase I clinical trials, VLPs are safe and immunogenic when administered orally but don't induce antibody titers as high as those achieved following actual infection (8, 9). Volunteers receiving two doses of 250 μg-2000 μg VLP mounted anti-VLP serum IgG responses in 70-90% of samples tested, but only 40% and ~30% of volunteers produced salivary and fecal IgA, respectively, regardless of dosage (247). Examination of cellular immune responses revealed that PBMCs proliferated and secreted IFN-γ when stimulated with homologous antigen 21 days after primary immunization; however, no proliferation or cytokine secretion was detectable by day 56 although volunteers received a booster between the two time points (247) . VLP vaccines have additionally been developed using transgenic potatoes, although overall immune responses were somewhat poor (246). Together, these data suggest that VLP

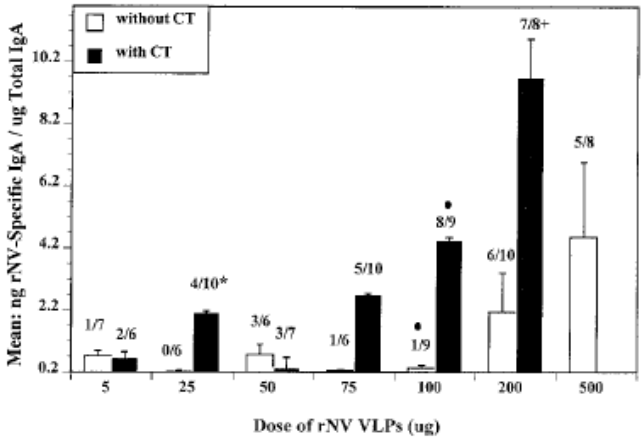
vaccines alone may not induce robust immune responses. Experiments examining the ability of VLP vaccines to protect individuals from norovirus infection have not been done.

Although no small model animal exists for norovirus infection, recombinant subunit vaccines can be used to induce norovirus immune responses in mice, providing an alternative model to study norovirus immunology. Mice have been shown to mount systemic, mucosal, and cellular responses following VLP vaccination by oral, intranasal, and subcutaneous administration (10, 80, 93, 105, 178, 207, 284, 289). While one study showed that oral immunization with as little as 5 μg of recombinant baculovirus-expressed NV VLP induced detectable, albeit very low, serum IgG responses, >200 μg VLP in up to four doses are required to consistently induce systemic and mucosal antibody responses (10). To circumvent the low immunogenicity of orally administered VLPs, adjuvants including cholera toxin (CT), the reduced toxicity CT mutant CT-E29H, heat-labile *Escherichia coli* toxin (LT), and the LT nontoxic mutant LT-R192G have been coadministered with norovirus subunit vaccines (8, 10, 178, 207). Serum IgG and mucosal IgA consistently increase with increasing VLP dosage, and the presence of adjuvant improves specific antibody responses in a dose-dependent manner (8, 10) (Fig. 1.11). In one vaccine study, three immunizations of 10 μg CT-E29H coadministered with 200 μg NV VLP increased fecal IgA responses from ~200 ng/ml to ~700 ng/ml specific IgA compared to mice immunized without adjuvant (207). While oral administration of norovirus vaccines would seem an obvious choice for these enteric pathogens, intranasal and subcutaneous vaccines have proved highly immunogenic by comparison. Intranasal administration of 10 μg NV VLP with or without LT-R192G adjuvant induced serum IgG geometric mean titers (GMT) of 90,447 and 9,123,

Figure 1.11. Murine serum IgG (A-B) and intestinal IgA (C) responses to oral administration of different doses of NV VLP in the absence (A) or presence (B) of adjuvant (10).

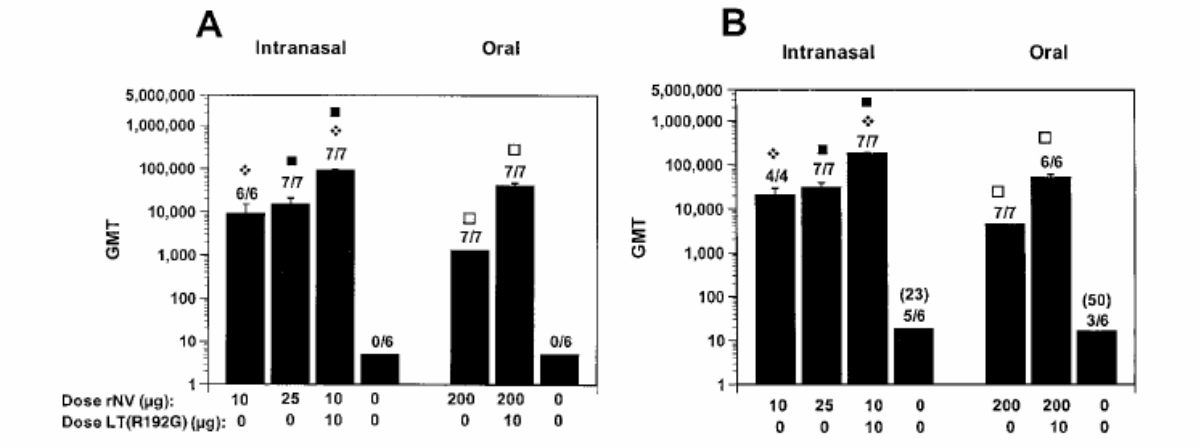


C.



respectively, after two immunizations (80). Fecal and vaginal IgA responses exhibited parallel trends. In contrast, oral administration of 200 µg NV VLP in the absence of adjuvant induced a serum IgG GMT of only 1,280, and no responses were detected in mice orally immunized with 10 µg VLP without adjuvant (80). Mice immunized intranasally with NV VLP and CT-E29H secreted high amounts of anti-NV IgA from lung, trachea, small intestine, and Peyer's patches, demonstrating immune induction occurs even in tissues not located near the site of

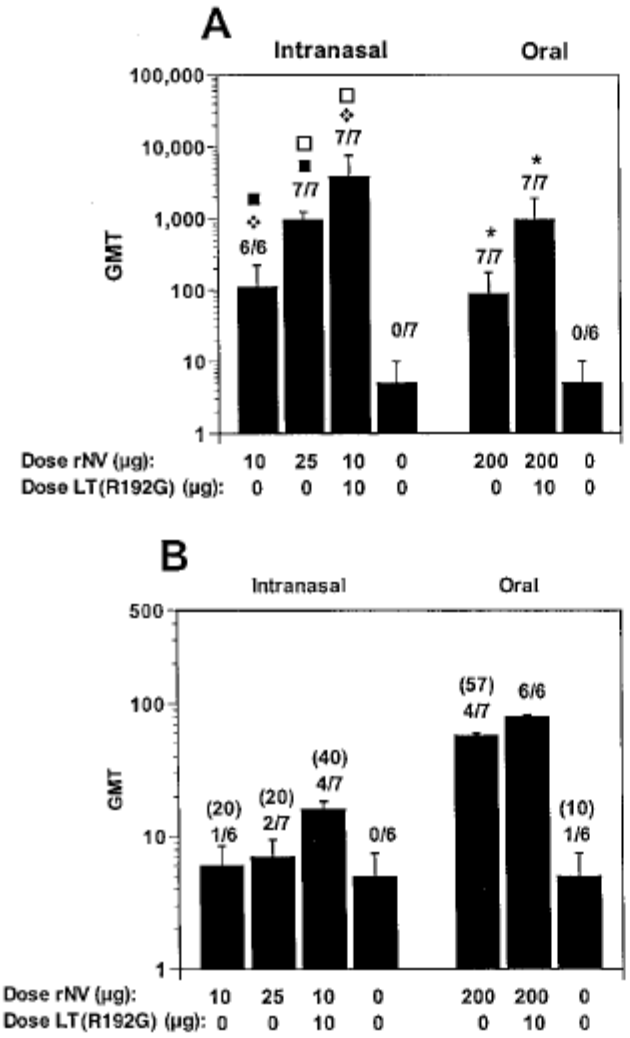
Figure 1.12. Murine serum IgG responses to NV VLPs following intranasal or oral administration in the absence or presence of adjuvant 36 (A) or 417 (B) days post-immunization (80).



immunization (207). Furthermore, serum IgG and fecal IgA responses were still detectable more than a year after intranasal vaccination with adjuvant or oral vaccination with or without adjuvant (80) (Fig. 1.12 and 1.13). Subcutaneous vaccination with VRPs that express norovirus VLPs *in vivo* and have inherent adjuvant activity also induced robust serum IgG and fecal IgA after two immunizations that remained strong 120 days post-vaccination (93).

Studies into cellular immunity induced by VLP vaccination are limited. Following three oral immunizations with 200 μg NV VLP and CT-E29H, $CD4^+$ T cells but not $CD8^+$ T cells in murine Peyer's patches proliferated in response to *in vitro* restimulation with antigen; however, both T cell subsets responded to stimulation in the spleen (207). IFN-γ and IL-4 production were both detected by ELISpot assay of stimulated splenocytes from mice receiving adjuvant, but only IFN-γ was detected in mice immunized with VLP alone

Figure 1.13. Murine fecal IgA responses following intranasal or oral administration of NV VLPs 36 (A) or 417 (B) days post-inoculation (80).



suggesting T_H1 responses are induced by VLP immunization. A separate study showed that mice immunized twice intranasally with 10 μg Dijon171/96 VLPs and 10 μg LT adjuvant mounted mixed T_H1/T_H2 responses (178). Spleen, cervical lymph node, and mesenteric lymph node secreted IFN-γ, IL-2, IL-4, and IL-5 following *in vitro* restimulation with VLPs. No VLP immunizations without adjuvant were performed in this study, so it is unclear if the cytokine profiles are a result of immunization with VLP or adjuvant. Peyer's patches and CLN from mice intranasally or orally immunized with LT or LT-R192G had similar cytokine profiles following restimulation, although IL-2 and IL-5 but not IFN-γ were higher following intranasal immunization (178). While these mice exhibiting a mixed T_H1/T_H2 response had high levels of both IgG1 and IgG2a subtypes in sera, a third study showed that four oral immunizations with 200 μg NV VLP elicited a primarily IgG2b subclass response in serum, effective in complement activation and cell cytotoxicity (10). When CT was included as an adjuvant, the subclass IgG1 predominated, indicating a T_H2 response although low levels of IgG2a were also induced. Together, data indicate that VLP alone may induce a T_H1 biased $CD4^+$ T cell response; however, the addition of adjuvant can change the predominating cytokines and IgG subclasses produced.

Alternative safe and cost-effective immunization methods currently being developed include recombinant VLP expression from yeast and transgenic foods. Studies show that five administrations of raw yeast extract containing 1 mg each of yeast-expressed recombinant VA387 VLPs without adjuvant also resulted in high levels of anti-VA387 serum IgG and fecal IgA (284). Oral immunization with 4 doses of tobacco extract or dried tomato expressing a transgene for NV capsid protein (80 μg VLP) and adjuvanted with CT elicited

systemic and mucosal antibody responses in 100% of mice tested, while freeze-dried potato tuber immunizations required more VLP to elicit detectable responses in mice, and VLPs were less stable likely due to the freeze-drying process (160, 289). Such transgenic vaccination strategies appear to elicit lower antibody responses than direct VLP immunization, however. The use of VLP will likely prove instrumental in the development of norovirus vaccines. A report by Harrington *et al.* showed that immunization with NV capsid protein containing a defective particle formation mutation resulted in no detectable mucosal antibody response and lower systemic antibody responses exhibiting only partial blockade in receptor binding assays (93). Together, these data suggest adjuvanted intact VLP or vectored expression systems will likely be most effective in eliciting strong immune responses to noroviruses.

For norovirus vaccines to be effective, they must augment immune responses that are cross-reactive to additional norovirus strains. Very few recombinant vaccine studies have been undertaken to address this question. A single study showed that serum from mice immunized with NV contained antibodies that cross-reacted with NCFL, another GI strain (93). Hybridoma production with splenocytes or mesenteric lymphocytes following oral immunization with VLPs has resulted in isolation of monoclonal antibodies that can cross-react within and across genogroups (129, 253, 287). The CM54 monoclonal antibody that was generated by Southampton (SH) VLP immunization cross-reacted with other GI strains and bovine GIII strains, and an epitope within the shell domain was identified (14). Additional studies argue whether the placement of immunodominant epitopes are located primarily in the N- or C- terminus (90, 287). While monoclonal antibodies can be helpful for antigenic characterization, a panel of antisera generated against VLPs representing each

genocluster is needed to truly realize antigenic relatedness among norovirus strains. Furthermore, understanding this relatedness will be crucial in effective vaccine formulation.

Murine norovirus immunology

Since its discovery in March 2003, 15 distinct MNV strains have been identified to date (98, 99, 124, 255). All strains are closely related with only 13% variance at the nucleotide level between the most divergent strains, and genetic and serological tests confirm that all identified strains comprise a single genocluster and serotype (255). To understand the immunology of MNV resistance and clearance, studies with immunodeficient mouse strains have been undertaken. The innate immune response has been shown to be critical for MNV resistance, and knockout mice lacking the gene for STAT-1 (STAT-$1^{-/-}$) or Type I and Type II interferon receptors (IFN$\alpha\beta\gamma R^{-/-}$) were highly susceptible to MNV-1 infection (124, 270). In studies directly comparing STAT-$1^{-/-}$ and wild-type 129 mice, STAT-1 deficiencies were directly related to increased viral titers in the proximal intestine as early as 3 hours post-infection and dissemination to peripheral tissues by 24 hours post-infection (171). Mice deficient for STAT-1-dependent IFN signaling responded nearly identically to STAT-$1^{-/-}$ mice. However, RAG$1^{-/-}$ and RAG$2^{-/-}$mice, which have B- and T-cell deficiencies due to developmental arrest, did not have increased pathogenicity following per oral, intranasal, or intracranial infection but remained persistently infected (124). Combined, these data suggest that innate immunity may be required to control replication and dissemination of norovirus following infection, whereas adaptive immunity is required for viral clearance.

Norovirus immunology has been understudied in humans due to the lack of a small animal model in which to study human norovirus infection. Likewise, human norovirus

vaccines remain to be developed due to ineffectual pre-clinical trial options. Using the MNV infection model, new insight can be gained into the immune response following norovirus infection and innovative vaccine approaches tested.

CHAPTER II.

Multivalent norovirus vaccines induce strong mucosal and systemic blocking antibodies against multiple strains

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Abstract

Noroviruses are important agents of human gastroenteritis characterized by extensive sequence variation in the major capsid structural protein that likely encodes critical antigenic determinants of protective immunity. The lack of an infection model has limited detailed characterizations of viral antigenic relationships and identification of the essential components for protective immunity. This information would contribute to efficacious vaccine design against a broad array of norovirus strains. To understand the extent of heterotypic norovirus antibody specificity to inter- and intra-genogroup strains and its applicability to vaccine design, we collected sera from humans infected with different norovirus strains and from mice inoculated with alphavirus vectors expressing strain-specific recombinant norovirus-like particles (VLPs). We used VLPs that were assembled from Norwalk virus (NV), Hawaii Virus (HV), Snow Mountain virus (SM) and Lordsdale virus

(LV) as antigens to define and compare heterotypic antibody responses in humans and mice. We also examined if these heterotypic antibodies could block specific binding of ABH histo-blood group antigens, putative receptors for norovirus binding and entry, to norovirus VLPs. Furthermore, we examined the effect of multivalent inocula on the specificity, titer, and ligand blockade properties of systemic and mucosal norovirus-specific antibodies in mice. Our studies suggest that infection with one of several different genogroup I (GI) strains in humans induces heterotypic antibodies that block NV binding to ABH antigens, although comparable findings were not evident following infection with genogroup (GII) strains. Additionally, inoculating mice with vaccine cocktails encoding multiple norovirus VLPs enhances heterotypic and ligand attachment-blocking antibody responses against the LV strain not included in the cocktail. These data suggest that multivalent vaccination may provide better protection from a broader range of noroviruses than monovalent vaccination.

Introduction

Noroviruses, members of the *Caliciviridae* family, are a group of more than 40 extremely heterogeneous viruses whose study has been hindered since their discovery in 1972 due to the lack of an appropriate cell culture or animal model (122). Infection is typically characterized by self-limited vomiting and diarrhea, with symptoms prevailing for 12-60 hours (50). An estimated 23 million norovirus infections occur annually in the U.S. leading to an estimated 310 deaths in susceptible populations such as infants and the elderly, according to studies conducted by the Centers for Disease Control and Prevention and others (166, 170). Outbreaks commonly occur on cruise ships and in the military, nursing homes, child care centers, and schools due to the extreme stability, low infectious dose and high transmissibility of these viruses (3, 29, 68, 81, 111, 177). Public health risks, however, are likely underestimated as most norovirus infections are unrecognized. An effective vaccine would clearly benefit health care providers, food handlers, the military, the elderly, and additional personnel that work in institutionalized settings by decreasing the morbidity of the disease and transmission of the virus (4, 78, 153, 161, 198, 254).

Noroviruses have a single-stranded, positive-sense 7.5 kb RNA genome that is organized into three open reading frames (ORFs). ORF1 consists of non-structural genes encoding RNA-dependent RNA polymerase, 3C-like proteinase, and helicase proteins. The major and minor capsid proteins are expressed from ORF2 and ORF3, respectively. Noroviruses are divided into five distinguishable genogroups (GI-GV) based on genome sequence similarity; however, only virus strains from genogroups I-II are known to widely infect humans. Additional strains in the newly identified genogroup IV have also been detected in human stools (55). Noroviruses within a genogroup can differ by up to 40% in

capsid amino acid sequence and >50% between genogroups (71). Of the strains used in our studies, the Bristol-like GII.4 strains have been the predominant globally-circulating strains for the past decade (55, 84, 131), while the Hawaii-like GII.1 strains and the Snow Mountain-like GII.2 strains have variable reported disease prevalence and appear in sporadic outbreaks (63, 218, 279, 280). Outbreaks from the GI strains Norwalk, Desert Shield, and Chiba-like viruses occur more infrequently (55). While GII viruses cause person-to-person outbreaks, and GI viruses are more common in environmental transmission, endemic disease rates are unclear. Research efforts for the development of a multivalent vaccine against noroviruses have not yet been described. Although common epitopes of the antigenic capsid proteins may be shared both within and across genogroups, antibody responses in humans have not yet proven to be protective following successive exposures to multiple norovirus strains (58, 86, 87, 93, 119, 129, 173, 180, 202).

Specimens from human challenge studies and outbreak investigations have allowed characterization of strain-specific norovirus antibody responses in humans following viral challenge. Early studies on heterotypic antibody responses showed that some GII infected individuals mounted antibody responses capable of cross-reacting with the GI Norwalk virus strain (259), while others revealed little antibody cross-reactivity between genogroups (180). Heterotypic antibody characterizations previously reported by our lab have shown that following Snow Mountain virus (SM) challenge, serum IgG cross-reacted with Hawaii virus (HV) but not with Norwalk virus (NV) (145). However, the variable and unidentified pre-challenge exposure histories of humans have unknown effects on norovirus immunity but have been suggested to influence disease susceptibility (119).

Recombinant systems using Venezuelan equine encephalitis virus (VEE) replicon particles (VRPs) (11) or baculovirus vectors (116) expressing norovirus ORF2 have been developed and are capable of generating capsid proteins that self-assemble into virus-like particles (VLPs). While VLP reagents produced from baculovirus vectors expressing norovirus ORF2 + ORF3 have been reported to enhance the stability of VLPs (19), the ORF2 VLP reagents prepared in our laboratory using VEE VRPs are likewise indistinguishable from wild-type virus in their morphology, antigenicity, and ligand-attachment properties by transmission electron microscopy and solid-phase assay (11, 91-93). For these reasons, VEE VRPs are invaluable and relevant surrogates for wild-type virus in biochemical, immunogenicity, and vaccine design studies. We previously demonstrated that mice inoculated with VRPs expressing norovirus VLPs are capable of mounting robust homotypic antibody responses (93); however, further characterization of heterotypic antibody induction and its application to vaccine development are needed.

Recently, several norovirus strains have been shown to bind ABH histo-blood group antigens (HBGAs) (91, 92, 103, 109, 159). HBGAs are carbohydrate moieties expressed on red blood cells and mucosal surfaces such as the gut, the natural site of norovirus infection. While certain norovirus strains appear to require the presence of HBGAs to produce an active infection (145, 146), it is unclear whether these ligands serve as primary receptors or coreceptors for norovirus binding and entry. Various norovirus strains are capable of binding several different members of the HBGA family, suggesting that HBGA binding is a conserved and likely required step in norovirus infection. Furthermore, sera from both infected humans and inoculated mice block binding of norovirus VLPs to HBGAs in a strain-

specific manner. These results suggest that antigenic epitopes may be important in virus-ligand binding and should be considered in norovirus vaccine development strategies (91).

In this manuscript, we describe the first multivalent candidate vaccine against noroviruses. Using samples collected following both human infection with noroviruses and murine inoculation with VRPs expressing norovirus VLPs, we evaluated heterotypic antibody responses and assessed the ability of homotypic (against infecting strain) and heterotypic (cross-reactive to different strain) antibodies to block virus-ligand binding. Following murine inoculation with a cocktail of three or four VRPs expressing strain-specific VLPs, we demonstrate that sera not only contains antibodies specific for all components of the inoculum but also demonstrates enhanced heterotypic antibody titers and ligand blockade against novel strains. Our data suggest that cocktail-based vaccines may provide a means of protecting against a broad panel of noroviruses, including those not included within the antigen mix.

Materials & Methods

Cloning of Lordsdale isolate, a GII.4 norovirus.

The Lordsdale-like LV-NC1 ORF2 capsid clone was obtained by RT-PCR of RNA extracted from a fecal sample of an infected individual during a norovirus outbreak in North Carolina. A consensus cDNA was inserted into the multiple cloning site of the pVR21 VEE replicon vector (VRP), and its sequence was determined using external vector-specific primers. The ORF2 coding sequence was aligned with Norovirus strain 004, a GII.4 Bristol-like norovirus (181).

VLPs and VRPs. All VRPs were produced as described in Harrington *et al.* (93). VRP titers and efficient expression of recombinant protein were determined by immunofluorescence assay using human or mouse sera following respective viral challenge, as previously described (93). To produce VLPs, BHK cells grown in Opti-PRO serum-free medium (Gibco, Grand Island, NY) supplemented with gentamicin/kanamycin and 4mM L-glutamine (Gibco) were infected with VRPs at an MOI of at least 2 in phosphate-buffered saline (PBS; Sigma, St. Louis, MO) containing Ca^{2+} and Mg^{2+} and incubated at for 1 h at 37°C. PBS was then replaced with complete serum-free medium for 24-30 h at which time cells were pelleted and lysed with Triton X-100 (Sigma) (working solution: 100 µl Triton X, 1 complete mini protease inhibitor tablet (Roche, Indianapolis, IN) in 10 ml PBS) on ice. Lysates were applied to a filter-sterilized 40% sucrose cushion and ultracentrifuged at 100K x *g* for 1.25 h at 4°C. Lysate supernatant was discarded, and the entire sucrose fraction containing putative VLPs was collected and aliquoted. Production of full-length capsid protein was determined by SDS-PAGE and production of intact VLPs was confirmed by transmission electron microscopy. Protein concentration was determined by Bio-Rad Protein Assay (Bio-rad, Hercules, CA).

Murine vaccine regimen. Five to seven week-old male BALB/c mice were obtained from Charles River Institute (Wilmington, MA). Mice were allowed to acclimate for one week under constant conditions and were inoculated immediately thereafter by footpad injection (day 1) with 2.5 x 10^6 infectious units (IU) VRP in PBS (10 µl total) expressing NV (n=4), SM (n=4), HV (n=4) or LV (n=4) VLPs. Two additional groups of mice were primed with trivalent or tetravalent inocula consisting of equal concentrations of NV, SM, and HV (n=5) or NV, SM, HV, and LV-expressing (n=5) VRPs (with total VRP concentrations of 2.5 x 10^6

IU or 8 x 10^6 IU, respectively). Mice were boosted on day 23 with identical priming inoculum.

Serum samples. Serum samples were collected from both humans and mice. Pre-challenge and convalescent sera were obtained from human volunteers prior to and 2-4 weeks following human challenge studies where adult individuals were symptomatically infected with either NV (n=10) (91), SM (n=7) (145), or HV (n=2) (C. Moe, unpublished). We obtained informed consent from volunteers, and specimen collection and use is approved by the University of North Carolina Chapel Hill Institutional Review Board (IRB). Acute and convalescent human sera were also collected after the onset of symptoms and up to 5 weeks later, respectively, during outbreaks of Desert Shield-like virus (DS) (n=7) (C. Moe, unpublished), Chiba-like virus (DF) (n=4) (15), or Lordsdale-like virus (LV) (n=4) (C. Moe, unpublished) strains. Murine serum samples were collected by tail bleed on days 0, 14, and 35 days post-inoculation.

Serum IgG titers. Homotypic and heterotypic antibody titers were determined by standard indirect enzyme-linked immunosorbent assay (ELISA). Ninety-six-well high-binding round-bottom plates (Corning, Corning NY) were coated with 2 μg/ml VLPs expressed from VRPs encoding ORF2 derived from NV, SM, HV, or LV isolates for 4 h at RT and blocked overnight with 5% milk in PBS (Blotto) at 4°C. Human or mouse sera, diluted 1:50 or 1:100 in blotto, were added to wells in duplicate, and 2-fold serial dilutions were performed, followed by incubation for 2 h at 37°C. Plates were then incubated for 1 h with goat anti-mouse IgG or goat anti-human IgG with alkaline phosphatase (AP) conjugate (Sigma), developed with p-nitrophenyl phosphate (pNPP; Sigma), and the OD at 405 nm was measured (Bio-rad Model 680 microplate reader). All serum samples were tested against a

panel of NV, SM, HV, and LV VLPs for VLP-specific IgG content, and the VLP-specific IgG concentration was determined against a standard curve produced with a known mouse IgG standard (Pierce, Rockford, IL).

Lymphoid culture. Mice receiving either trivalent or control inoculum were sacrificed at 21 days post-boost, and spleen and gut tissues were harvested. The entire small intestines were harvested, and mesenteric fat, Peyer's patches, and gut contents were removed with forceps. Tissue was then dissected longitudinally and segmented. Gut tissue was washed 3x in Hank's balanced salt solution (HBSS; Gibco) containing 15mM Hepes (Gibco) and Ca^{2+}/Mg^{2+} followed by 2 additional washes in an identical buffer solution supplemented with 5mM EDTA, 10% fetal bovine serum (FBS; Gibco), and gentamicin (Gibco). Gut segments and spleens from each mouse were then washed once in complete RPMI 1640 media (Gibco) containing 15mM Hepes, 10% FBS, penicillin-streptomycin (Gibco), gentamicin, 2mM L-glutamine, and amphotericin B (Sigma) (modified from (39)) and placed individually in wells of 48-well flat-bottom plates. Tissues were incubated for 1 week at 37°C at which time supernatant was collected.

Supernatants were clarified by centrifugation, followed by isolation of gut IgG using an IgG separation column (Pierce), according to the manufacturer's directions. IgG eluate and retentant supernatant were then concentrated to volumes of 300 μl and 600 μl, respectively, using Centriplus centrifugal filtration devices with 100,000 molecular weight cut-off (Millipore, Bedford, MA), according to manufacturer's instructions. Samples were then used in IgG and IgA titer and blockade analysis assays. Spleen IgG was diluted 1:10 in blotto and gut IgG eluate at 1:4 for use in the IgG titer ELISA described above. Retentant supernatant was diluted 1:2 in blotto and screened for the presence of specific IgA in IgA

titer ELISAs otherwise identical to that described above for IgG. Total IgG and IgA were measured by sandwich ELISA where plates were coated with 2 μg/ml sheep anti-mouse Ig capture antibody (Chemicon, Temecula, CA) and blocked prior to addition of lymphoid culture supernatants (1:50) and standards otherwise identical to the ELISA described previously.

Antibody blockade assays. Serum antibody blockade of HBGA binding to VLPs was measured as described by Harrington *et al.*(91). Briefly, wells were coated with 2 μg/ml VLPs for 4 h at RT and blocked overnight with blotto at 4°C. Wells were then incubated with serial dilutions of 10% sera in blotto for 2 h; positive control wells received buffer alone. Wells were then incubated with H type 1-biotin or H type 3-biotin (Glycotech, Gaithersburg, MD) at a dilution of 1:50 (original concentration 1 mg/ml) in blotto for 4 h at 37°C. Following a final incubation with streptavidin-AP for 1 h, wells were developed with pNPP and the OD at 405 nm determined. Antibody blockade of HBGA binding in serum treated wells was determined as a percentage of the average HBGA binding value (OD_{405}) from positive control wells. For blockade assays performed using lymphoid culture-derived antibody from inoculated mice, an IgG concentration equal to that required for ligand blockade in corresponding sera samples was used (26 μg/ml), due to limited sample volumes, to determine efficient gut and spleen IgG binding blockade. Culture samples from control mice were measured for antibody blockade by 2-fold serial dilutions starting from 1:1.6, as described above.

Saliva binding assays. Saliva samples from a panel of individuals representative of each blood type and secretor status were tested for the ability to bind LV VLPs in an *in vitro* solid-phase binding assay, as described in (91). Briefly, microwells were coated with boiled saliva

from respective individuals followed by addition of LV VLPs. Binding of VLPs was detected with human anti-LV sera, goat anti-human IgG secondary antibody, and pNPP substrate. Results are represented as OD at 405 nm.

Statistics. Percentage ligand blockade by human pre-challenge or acute and convalescent serum from each sampling group and lymphoid antibody titers in experimental and control mice were individually compared using the student's t-test ($P \leq 0.05$). Statistical comparisons of serum IgG titers and 50% blockade serum concentrations between multiple groups having measurable values were performed using the One-way ANOVA. If groups were found to be statistically different, post-hoc analysis was performed using the Tukey HSD Test ($P \leq 0.05$). For 50% blockade analysis of groups containing samples not having a determinable endpoint value, categorical differences in the percentage of serum necessary to block (or not block) 50% ligand binding were determined using the One-way ANCOVA.

Results

Cloning and Characterization of Lordsdale Virus VLPs.

The LV-NC1 ORF 2 was cloned into the pVR21 VEE replicon vector, and the amino acid coding sequence exhibited 99.1% identity to the GII.4 Bristol-like LV004 strain (181). Intact LV VLPs were expressed from LV VRPs following infection of BHK cells, as determined by electron microscopy (Fig. 2.1a). The binding profile of LV VLPs to HBGAs was also determined by solid phase binding assay. VLPs bound strongly to the H type 3 and H type 1 antigens (net OD_{405} 1.64 and 0.51, respectively) without significant binding (net OD_{405} <0.2) to H type 2, the Lewis antigens, or H type 1 and H type 3 precursors (Fig. 2.1b), a profile that included fewer antigens than the related VA387 strain (104). LV VLPs were further tested

for the ability to bind saliva from individuals of all blood types and all secretor status. VLPs strongly bound saliva from 7 of 9 secretor-positive individuals regardless of blood type and did not appear to bind saliva from secretor-negative individuals (Fig. 2.1c), resembling binding patterns previously shown for related LV strains (103, 104).

Homotypic & heterotypic IgG responses in humans following norovirus infections. With the synthesis of the LV VLPs and our existing panel of NV, HV and SM VLPs (11, 91, 93), we measured homotypic and heterotypic serum antibody responses to each antigen following human norovirus infection with the NV (GI.1), DS (GI.3), DF (GI.4), HV (GII.1), SM (GII.2) or LV (GII.4) strain. Data are presented as median fold-increase in IgG in convalescent sera compared to that in matched acute or pre-challenge sera. Not surprisingly, homotypic sera from NV-infected individuals exhibited the highest IgG response against NV VLPs with a median fold-increase in IgG titer of 71.7 when compared to pre-challenge sera (Fig. 2.2a), consistent with earlier reports in the literature (93, 145, 180). Heterotypic serum antibody titers to NV VLPs were lower for all other infection groups; however, sera from infections caused by other GI noroviruses exhibited a trend of more robust heterotypic responses than sera obtained following GII infections. Both heterologous GI infections induced median fold-increases of 5.3 in IgG titer; GII infections induced median fold-increases from 1.8-2.9 (Fig. 2.2a). Similar results were found for homotypic and heterotypic IgG titers following GII infections: median homotypic titers to GII antigens increased 17.7-71.3-fold in convalescent sera, and heterotypic titers to GII antigens increased 2.4-8.5-fold. Heterotypic titers from GI infection groups increased 1.2-5.2-fold against GII antigens (Fig. 2.2b-d). Sera from each infection group contained higher homotypic IgG titers than

heterotypic titers to other norovirus antigens, while heterotypic antibody responses appeared higher within a genogroup than across genogroups. Neither observation was statistically significant, however, due to a high degree of heterotypic IgG variability between individuals in each infection group as well as between infecting strains (One-way ANOVA). However, in support of these observations we calculated percent seroconversion, defined as a >4-fold increase in IgG titer in convalescent or post-challenge sera compared to acute or pre-challenge sera, and found that 81% of GI infected individuals and 38.5% of GII infected individuals seroconverted to GI antigen. Likewise, 35% of GI infected individuals and 59% of GII infected individuals seroconverted to GII antigens (specified below each panel in Fig. 2.2).

Homotypic & heterotypic IgG responses in mice following inoculation with VRPs expressing norovirus VLPs. Given the complications in measuring heterotypic antibody responses in humans with unknown norovirus infection histories, we inoculated mice with VRPs expressing recombinant norovirus VLPs from either the NV, SM, HV, or LV strain to examine antigenic cross reactivity in a naïve host. Anti-norovirus serum IgG was analyzed by indirect ELISA and is presented as mean titer of post-boost IgG (μg/ml) per inoculation group (n=4) (Fig. 2.3). Pre-boost (day 14) IgG titers were consistently 10-28% of post-boost (day 35) titers. As seen with human sera, anti-norovirus IgG in the mouse was highly specific for homotypic VLPs (Fig. 2.3a-d) with titers reaching geometric means of 1099 μg/ml in VRP-NV inoculated mice, 1575 μg/ml in VRP-SM inoculated mice, 1463 μg/ml in VRP-HV-inoculated mice, and 658 μg/ml in LV VRP-inoculated mice on day 35. Heterotypic IgG responses were 10-40-fold less than the homotypic antibody responses.

Mean heterotypic responses, however, exhibited a trend of higher cross-reactivity within genogroups than across genogroups (Fig. 2.3a-d), with the individual heterotypic IgG response of SM VRP-inoculated mice being significantly more cross-reactive to HV VLPs than NV VLPs (One-way ANOVA, Tukey HSD, $P<0.01$).

Antibody blockade of norovirus VLP binding to ABH histo-blood group antigens. It has recently been shown that several norovirus strains from both GI and GII are capable of binding glycoconjugates of the ABH histo-blood group antigen family as putative ligands for cellular attachment (91, 92, 103, 109, 159). An antibody blockade assay, recently developed by Harrington *et al.* (91), measures the ability of antibody to block VLP binding to HBGAs. Because NV, the prototype GI norovirus, has been shown to bind strongly to the H type 1 antigen (91), and LV VLPs bind strongly to the H type 3 antigen (Fig. 2.1c), we investigated the ability of homotypic and heterotypic anti-norovirus serum IgG from humans and mice to block HBGA attachment to NV (Fig. 2.4) and LV VLPs (Fig. 2.5)

Pre-challenge or acute and convalescent serum samples collected from infected volunteers and outbreak cases were analyzed for blockade of NV VLP binding to synthetic H type 1 antigen (Glycotech) in blockade assays by serial dilution of 10% sera (Fig. 2.4a). While pre-challenge sera were unable to block H type 1 binding, convalescent sera from NV-infected volunteers ablated >90% of H type 1 binding to NV VLPs at an average concentration of 0.42% serum per volume. Interestingly, some samples from individuals infected with two GI strains, DS (GI.3) and DF (GI.4), which are distantly related to NV, were also able to completely ablate ligand attachment to NV VLPs, while other serum samples from the same outbreaks were unable to do so. The average blockade of all samples,

indicated in Fig. 4a, shows the average ability of DS samples to block >50% H type 1 binding at serum concentrations of 2.5% and DF samples to block 40% of binding at 5% serum concentrations. When the ability of individual serum samples to block 50% of ligand binding was compared, the concentrations of serum from individuals with heterologous GI infections were not significantly different from the concentrations of serum from individuals with NV infections in their ability to generate a 50% blockade response (One-way ANCOVA). Heterotypic antibodies following infection with GII strains, however, were entirely unable to block NV-H type 1 binding.

Previously, homotypic IgG blockade of VLP-ligand binding was demonstrated in mice following inoculation using the VEE-VRP system (91). Our data also clearly demonstrate that homotypic murine IgG eliminates NV VLP-H type 1 attachment (Fig. 2.4b). In agreement with results from our studies with human sera, heterotypic IgG from mice inoculated with VRPs expressing GII-derived VLPs SM, HV, or LV was unable to block H type 1 binding to NV VLPs (Fig. 2.4b).

Mean IgG titers from human and murine samples necessary for 50% and 90% blockade of ligand attachment (BT50/90) are shown in Fig. 4c. In humans, all NV serum samples exhibited BT50/90 values for H type 1 blockade, with <5.1 μg/ml NV IgG required for 50% blockade of H type 1 binding and <6.4 μg/ml required for 90% blockade. Five of seven DS serum samples had BT50/90 IgG titers, with mean antibody requirements being at least 2-fold and 3-fold higher, respectively, than titers necessary for blockade by homotypic NV IgG. Three of four DF serum samples showed BT50 values that were 1.2-fold higher than homotypic antibody requirements, and two of four DF samples showed BT90 values that were 1.7-fold higher than for homotypic IgG. Sera from GII-infected individuals,

however, were unable to block H type 1 attachment to NV VLPs in all samples tested. In murine sera, NV samples required 11.7 μg/ml IgG for 50% blockade of H type 1 attachment and 23.5 μg/ml for 90% binding blockade.

Homotypic and heterotypic sera from humans and mice were also analyzed for the blockade of H type 3 attachment to LV VLPs (Fig. 2.5). Convalescent human sera from LV-infected individuals completely blocked LV-H type 3 binding (Fig. 2.5a). However, convalescent human sera from all other infection groups, except HV, also partially ablated LV-H type 3 attachment (Fig. 2.5a). Upon analysis of matched pre-challenge or acute sera, we found that corresponding samples also partially or completely blocked H type 3 attachment (Fig. 2.5a) and were not significantly different from matched convalescent sera at any serum concentration tested (student's t-test, $P \leq 0.05$). These results suggest that previous exposure to the predominant GII.4 viruses is a likely cause of antibody specificity for LV epitopes and specific blockade of ligand binding. The LV and HV infection groups, however, did exhibit a significant change in blockade between pre-challenge or acute sera and convalescent samples. Sera following LV infection ablated >90% of H type 3 binding in 3 of 4 samples, whereas acute samples exhibited very minimal blockade ($P<0.05$ at all serum concentrations). Hawaii-specific sera, however, appeared to enhance H type 3 binding at low serum concentrations, a phenomenon not seen with matched pre-challenge sera ($P<0.05$ at 0.3-0.6% serum), in the two samples sets tested. Blocking titers of convalescent LV sera for 50% and 90% blockade of H type 3 binding were 2.4 μg/ml (4/4 samples) and 1.7 μg/ml (3/4 samples), respectively (Fig. 2.5c).

Sera from mice inoculated with VRPs expressing LV VLPs completely ablated H type 3 attachment (BT50=2.4 μg/ml; BT90=6.9 μg/ml (Fig. 2.5c)), while sera from other

inoculation groups did not block ligand binding regardless of genogroup (Fig. 2.5b). It is important to note here that, unlike human sera, sera from mice inoculated with replicons expressing Hawaii VLPs did not show antibody responses that enhanced LV VLP binding to H type 3. Because mice have no pre-exposure histories, our murine data likely reflect natural IgG blockade responses following primary exposure to norovirus antigens; however, multiple norovirus exposures may influence antibody specificity. To distinguish if multistrain exposure may be an alternative causative influence for the high blockade response by acute or pre-challenge sera to LV-ligand binding in humans, we tested for ablation of virus-ligand binding following multivalent inoculation in mice.

Mice inoculated with multivalent VRP vaccines mount cumulative antibody responses. Our data support the hypothesis that norovirus vaccines will likely require multivalent antigenic components to provide protection against this antigenically diverse group of viruses. To test the efficacy of such reagents, two groups of mice were vaccinated with a multivalent inoculum of VRPs expressing equivalent concentrations of three (NV, SM, HV) or four (NV, SM, HV, LV) heterologous VLPs but with varying total amounts of VRPs. Trivalent vaccines were composed of a total VRP amount equal to the total VRPs received by monovalently vaccinated mice. In tetravalent vaccines, each strain-specific VRP was administered at a dose equivalent to that received by monovalently vaccinated mice to determine if total VRP insult would proportionately alter the immune response. Sera were collected 12 days post-boost and analyzed for specific IgG to a panel of VLPs (NV, SM, HV, LV) (Fig. 2.6a). Mice receiving trivalent inoculum produced a robust antibody response to all three VLPs; those receiving tetravalent inoculum produced a robust response to all four

VLPs. IgG titers were similar to those elicited following inoculation with each VRP alone, as indicated in Fig. 2.6a, and total VRP amount per vaccination did not influence the intensity of the immune response, likely due to an already saturated immune insult with such large VRP quantities. No significant differences in mean IgG titers between sera from mice receiving individual versus multivalent inocula were seen for all four antigens, with the exception of higher NV-specific and SM-specific IgG responses in monovalent-inoculated mice than in mice inoculated with all four antigens (One-way ANOVA; $P<0.05$). Interestingly, mice receiving a trivalent vaccine also mounted a robust immune response to the fourth VLP (LV) that was not included in the inoculum and was not significantly different from responses mounted by the inoculation groups receiving the LV antigen. Furthermore, this heterotypic antibody response to LV VLPs was significantly greater than the heterotypic responses of mice inoculated with individual SM ($P\leq0.01$), NV ($P\leq0.05$) or HV VRPs ($P\leq0.01$; One-way ANOVA and Tukey HSD) and was also greater than the heterotypic responses from all three monovalently inoculated groups combined (Fig. 2.6b).

Blockade assays were also performed to measure antibody interference of HBGA attachment to NV and LV VLPs (Fig. 2.7), as described above. Sera from both multivalent inoculation groups blocked H type 1 binding to NV VLPs (Fig. 2.7a), and sera concentrations required for 50% blockade were not statistically different among the monovalent or multivalent groups. When sera were analyzed for blockade of H type 3 binding to LV VLPs, we found that sera from the multivalent inoculation group that received the LV antigen could efficiently block binding (Fig. 2.7b) and were likewise not statistically different from the monovalent LV inoculation group in sera concentration required for 50% binding blockade (One-way ANOVA). Importantly, sera from mice that did not receive LV antigen as an

inoculum component but mounted a robust heterotypic antibody response against LV antigen also blocked H type 3 attachment at high serum concentrations (Fig. 2.7b). This phenomenon was not seen after inoculation with VRPs to a single norovirus strain (Fig. 2.7c). Blockade ability was, however, significantly lower in the multivalent inoculation group not receiving LV antigen compared to groups that did ($P \leq 0.05$, One-way ANOVA and Tukey HSD). BT50 and BT90 values were calculated to further measure interference of ligand binding (Fig. 2.7d). Multivalent titers for H type 1 blockade were nearly equivalent to those achieved following monovalent inoculation with NV VRPs. BT50 values for interference of LV-H type 3 binding were 4-fold higher and BT90 values 3-fold higher in tetravalent-inoculated mice than monovalent-inoculated mice, although actual titers were similar to that seen for NV-H type 1 blockade. Furthermore, the trivalent vaccine that did not include the LV antigen was still able to induce BT50 values in mice that were roughly twice that necessary for blockade following a multivalent challenge that did include the LV antigen. Our data support the suggestion that potentially neutralizing heterotypic antibody responses can in fact be induced to novel antigenic strains.

3.6 Multivalent inoculum induces specific antibody secretion in multiple tissues.

To measure the level of heterotypic mucosal IgA and IgG responses at biologically relevant sites, whole gut and spleen tissues harvested and cultured from mice inoculated with a trivalent VRP inoculum expressing NV, SM, and HV VLPs were analyzed for antibody secretion (Table 2.1). Total antigen-specific IgG titers secreted from gut and spleen tissue and IgA titers secreted from the gut were determined for specificity to NV, SM, and HV VLPs. All mice demonstrated specific IgG secretion to all components of the inoculum in

the gut and spleen ($P \leq 0.01$) as well as IgA in the gut ($P \leq 0.05$ for 2/3 VLPs) compared to control mice that received parallel inoculation with VRPs expressing the influenza hemagglutinin gene or a PBS placebo (student's t-test). Control animals did not mount specific antibody responses to norovirus VLPs. Furthermore, when screened for ligand binding blockade, gut and spleen IgG blocked 100% of H type 1 binding to NV VLPs at similar IgG concentrations required for 100% blockade with serum IgG (Table 2.1). These results indicate that multivalent inoculation with VRPs expressing norovirus VLPs is capable of inducing an immune response that is evident at a relevant mucosal site in mice.

Discussion

Norovirus infections are associated with a significant disease burden, but the components of protective immunity in humans are unknown. The extensive antigenic diversity and complex serological relationships among strains challenge our understanding of strain variation on viral infection and pathogenesis. Unraveling the details of antigenic cross-reactivity is essential for developing effective vaccines. Following norovirus infection in humans, varying degrees of humoral and cellular immune cross-reactivity have been documented within and across genogroups (42, 129, 144, 145, 155, 180, 259, 286). However, human sera samples are limited to those collected from underrepresented outbreak samples and a limited number of human-challenge studies. Consequently, the biological significance of heterotypic immune responses has not been evaluated *in vitro*. In this study, we examined the impact of heterotypic immune responses on virus-ligand interactions in biochemical assays designed to measure ABH antigen binding using human and murine model systems and addressed the impact of multivalent vaccination.

In agreement with earlier reports, our studies demonstrate that humans mount moderate heterotypic antibody responses following norovirus exposure. We observed that the heterotypic responses to other members of the same genogroup were typically stronger than those between genogroups, but overall, the heterotypic IgG response was only about 5-10% of the measured homotypic IgG responses. Reports by Treanor *et al.* demonstrated that 6 of 15 individuals infected in a human challenge study with the SM virus seroconverted against the NV antigen (259), while our findings suggested a slightly lower heterotypic response with 2 of 7 SM samples displaying seroconversion against NV. Examining sera collected from norovirus outbreaks, Noel *et al.* noted very little cross-reactivity between genogroups but varying degrees of cross-reactivity within genogroups (180). Most humans have antibodies against noroviruses (152), but norovirus exposure histories are uncertain and can fluctuate drastically between individuals, making it difficult to decipher the exact degree of heterotypic immunity between strains.

Because mice cannot be productively infected with human noroviruses, they do not have pre-existing antibodies that complicate serologic comparisons. Mice can, however, mount robust immune responses to human norovirus antigens delivered by alphavirus replicon particles. Our data show that while homotypic antibody responses were singularly the most robust, heterotypic antibody responses were also mounted against all the strains we analyzed and were typically more robust within a genogroup than between genogroups, mirroring our findings following infection in humans. Furthermore, the murine footpad has proven to be a distinctly immunogenic site for VRP inoculation in comparative studies including subcutaneous, intradermal, and intramuscular inoculations (A. West, unpublished data). Because the murine footpad is closely related to the dermis, intradermal vaccination in

humans may be a preferred route to induce a similar immune response with VRPs. We are aware that inbred mouse strains likely recognize a subset of the total repertoire of norovirus epitopes recognized by human populations. Interestingly, norovirus VLPs supersaturated with hyperimmune murine sera were only able to bind 7-8% of corresponding human antibodies following NV, SM, or HV infection (data not shown), suggesting that many of the epitopes may be common in humans and mice. Overall, our data indicate that murine inoculation using recombinant VEE vectors may allow us to further characterize heterotypic immunity to multiple norovirus strains and help inform vaccine design strategies for humans.

Although we cannot truly define neutralizing antibodies without an animal model or cell culture system, our demonstration that noroviruses bind HBGAs as putative receptors has allowed us to further characterize the specificity of heterotypic antibodies in humans and mice. The HBGAs H type 1 and H type 3, glycoconjugates expressed on the surface of mucosal tissues, have been shown to strongly bind to NV and LV VLPs, respectively. Furthermore, antibodies generated against NV and LV following active infection in humans and generated against NV and LV VLPs following inoculation of mice completely block VLP binding to their HBGA ligands. Based on the hypothesis that antibodies that block virus-ligand interactions may neutralize infectivity (41, 102), antibody blockade experiments provide an obvious biological parameter to compare the immunogenicity between strains.

Acute and convalescent sera were available from different GI norovirus outbreaks (GI.3-Chiba-like and GI.4 Desert Shield-like strains) and from volunteers challenged with the GI.1 Norwalk virus strain. Our results showed that several human serum samples collected following infection with GI.3 and GI.4 strains were capable of completely blocking NV VLP binding to H type 1, while other corresponding samples were less able to do so.

Previous immunoprecipitation studies with sera from similar infections have indicated the presence of shared antigenic sites between capsid proteins as well as the retention of unique antigenic sites (144), which would explain our observation of varying degrees of antibody blockade even within the same infection groups. However, no sera from GII-infected individuals blocked the binding of H Type 1 antigen to NV VLPs, although our studies and others (259) demonstrate limited amounts of antibody cross-reactivity between genogroups. Our findings following murine inoculation with GII-derived VLPs agree with our findings in humans, although our current panel of GI-expressing replicons limits the scope of our overall study. Although our data suggest that GI infections in humans may induce antibody responses that can block attachment of heterotypic strains, additional studies are needed to confirm these findings. Interestingly, if GI infections induce a heterotypic blockade response, this may explain in part the noted lower prevalence of outbreaks associated with GI noroviruses (204).

LV and closely related GII.4 strains have been responsible for the majority of reported norovirus outbreaks in the last decade and have a worldwide distribution (181). When we analyzed heterotypic antibody blockade of LV VLP binding to the H type 3 ligand, we encountered several discrepancies between humans and mice. Following norovirus infection in humans, heterotypic antibodies appeared to partially block LV VLP-ligand binding regardless of the original infecting strain. Blockade potential, however, was also uniformly high in pre-immune sera, suggesting that the observed heterotypic blockade activity was due to an exposure to LV before the outbreak when the sera was collected or from heterotypic responses associated with exposure to common GII strains (84). Many pre-challenge and acute human sera samples also contained high antibody titers to LV VLPs.

When we compared our findings in humans to that in mice, it was clear that in animals with no norovirus exposure history, antibody responses to SM and HV provided no heterotypic antibody that would ablate LV VLP-ligand binding at any sera concentration tested. Our results suggest that antigenic epitopes of GII viruses vary greatly between strains, which may make it challenging to formulate a broadly protective vaccine.

Because heterotypic antibody responses did not block LV-H type 3 ligand interactions in mice, we tested the hypothesis that a cocktail of VLPs could provide a greater number of distinct cross-reactive epitopes in the mouse, providing for heterotypic blockade against novel strains. Cocktail VRP vaccines using multiple homologous proteins derived from different strains have not been developed and tested previously. One group of mice was inoculated with a cocktail of three VRPs derived from NV, SM, and HV, which likely represent a small subset of norovirus outbreaks. A second inoculation group added a fourth component of LV VRPs, which may represent a combined total of >70% of all norovirus outbreaks (134), depending on year-to-year variation. We found that, in both multivalent inoculation groups, robust antibody responses were induced to all components of the inoculum. Surprisingly, in the inoculation group not receiving the LV antigen as an inoculum component, a robust heterotypic IgG response to LV VLPs was also induced that was 2.5-5-fold higher than heterotypic IgG responses seen in mice monovalently inoculated with each VRP component. Importantly, antibody titers to LV in the inoculation group that did not include LV as a vaccine component were not significantly different compared to anti-LV titers in inoculation groups that did receive LV, supporting the hypothesis that multivalent vaccines might induce robust immunity against additional noroviruses not included in the vaccine mix. When serum samples were screened for blockade of NV and

LV VLP binding to their respective HBGAs, we found that both multivalent inoculation groups contained antibody capable of blocking both H type 1 and H type 3 binding to their respective VLPs. This finding experimentally supports the use of multivalent antigen cocktails as a plausible norovirus vaccine method. Unfortunately, animal models are not readily available to test whether cross-blocking antibody responses are protective against norovirus infection, although successful inoculation of rhesus macaques and chimpanzees have been reported with NV (221, 282). Our working hypothesis is that an increased amount of epitopes encountered by the immune system upon multivalent inoculation provides a broader set of cross-reactive epitopes, allowing for greater recognition of novel strains.

Throughout this study, we examined serum IgG responses in humans and mice because IgG is readily obtainable in high volumes from both species and because it contains high titers of specific antibody for further testing. Although the components of protective immunity and immune induction sites for clearance of norovirus infection are not currently known, the natural site of norovirus infection is the gut mucosa (159, 244), suggesting that mucosal immunity is likely critical for controlling infection. We found that trivalent VRP inoculation does indeed induce a multi-system immune response, as we were able to identify IgG specific for all three VLPs in the gut and spleen as well as IgA specific for all three antigens in the gut. Antigen-specific IgA and IgG were produced by resident lymphocytes present in intestinal tissue, as Peyer's patches were removed and intraepithelial lymphocytes disrupted prior to culture. Both IgG and IgA have separately been implicated in the efficient clearance of other viral as well as bacterial pathogens from the gut mucosa (12, 168, 217, 229), although the biological relevance of each antibody subtype in norovirus infection is not known.

We also tested the ability of IgG isolated from gut and spleen culture supernatants to effectively block binding of NV VLPs to its HBGA ligand and found that IgG from both tissues was able to completely block ligand binding at the lowest concentrations necessary for 100% binding blockade with sera, indicating that a specific and possibly neutralizing antibody response is induced in multiple tissues, including the biologically relevant gut, following inoculation with VRPs expressing norovirus VLPs in mice. Unfortunately, we were unable to isolate sufficient IgA to evaluate its VLP attachment blockade activity. Nevertheless, we have made substantial progress in the development of a candidate vaccine strategy that can induce broadly reactive and potentially neutralizing antibody responses at both the systemic and mucosal level to circumvent norovirus infections.

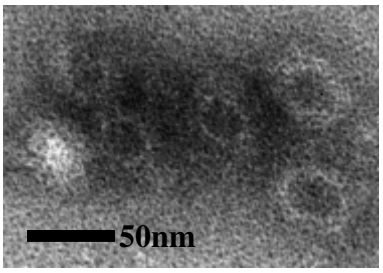
Acknowledgements

We would like to thank Victoria Madden and the UNC Microscopy Core Facility for providing technical transmission electron microscopy services, Martha Collier for packaging VEE replicon particles, Rhonda Roberts for statistical expertise, and Dr. Juan Leon for valuable comments.

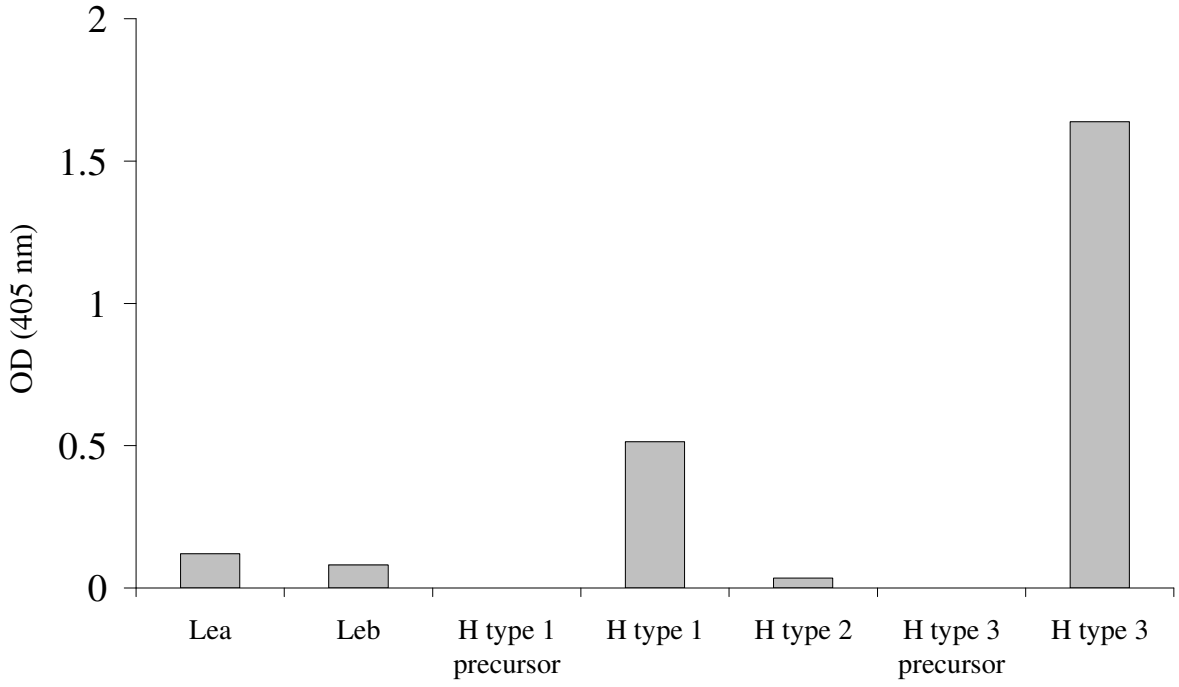
Funding for this research was provided by the U.S. Environmental Protection Agency (STAR grant R826139) and grants from the National Institutes of Health (RR00046 and AI056351).

Figure 2.1

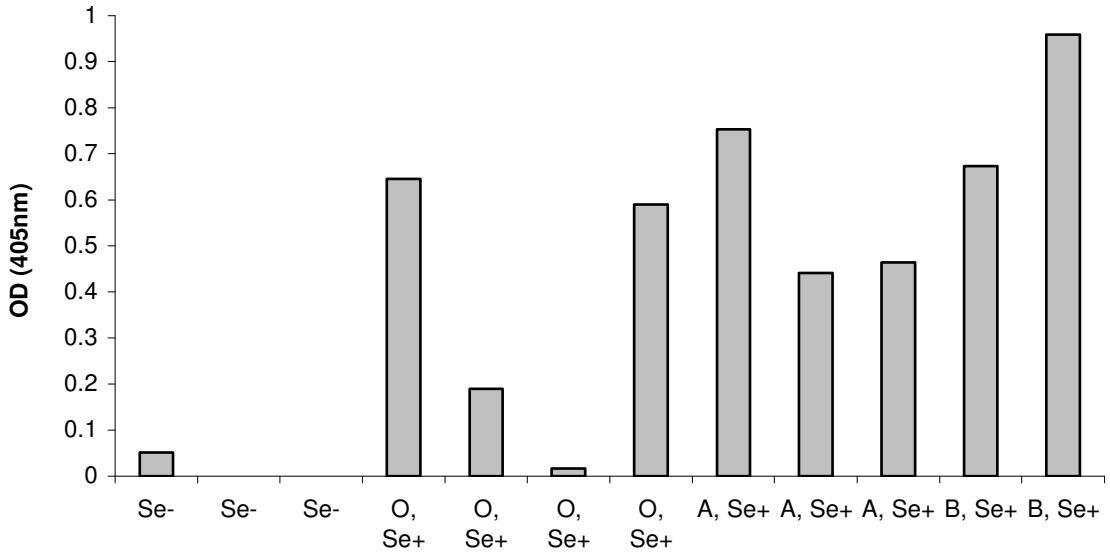
A.


50nm

B.


2
1.5
1
0.5
0
OD (405 nm)
Lea
Leb
H type 1 precursor
H type 1
H type 2
H type 3 precursor
H type 3

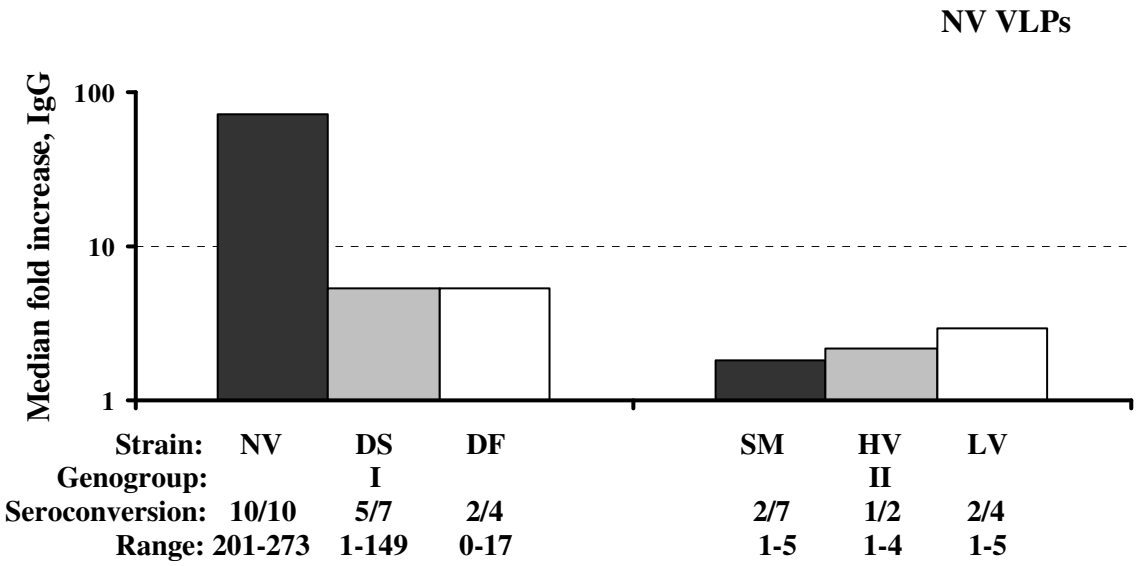
C.



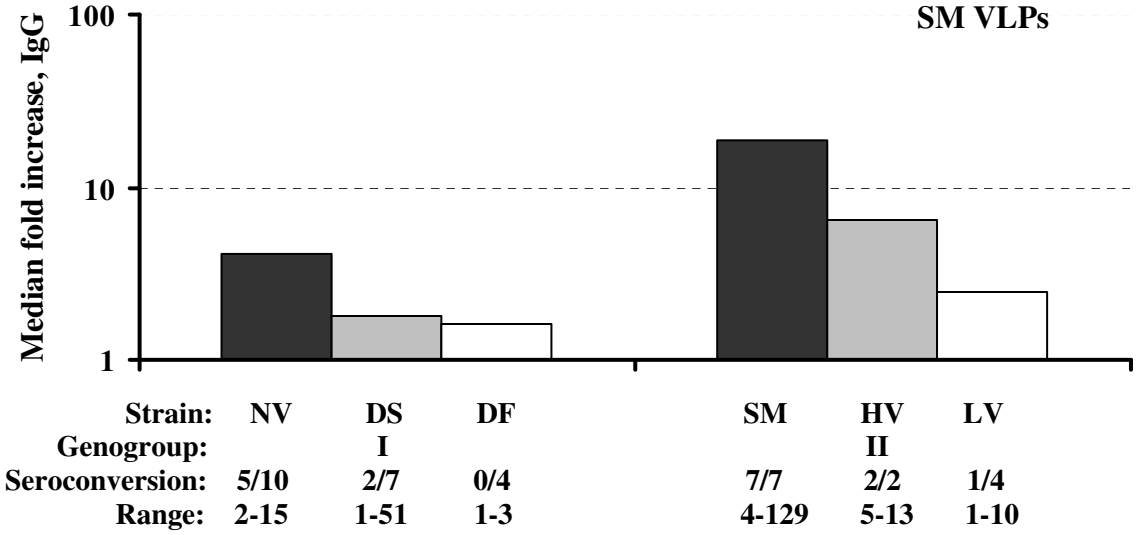
Characterization of LV-NC1 isolate. ORF2 of a norovirus isolate obtained following an outbreak in North Carolina (LV-NC1) was cloned into the pVR21 vector. Transmission electron microscopy revealed that VRPs expressing LV-NC1 ORF2 were able to form intact VLPs that were harvested following VRP infection of BHK cells (A). LV-NC1 VLPs exhibited a specific binding pattern to a panel of HBGAs, as determined by solid-phase binding assay (B). The ability of LV-NC1 VLPs to bind saliva from a representative panel of individuals of each blood type and secretor status was determined by solid-phase assay (C). Positive (Se+) and negative (Se-) sample designations indicate secretor status.

Figure 2.2

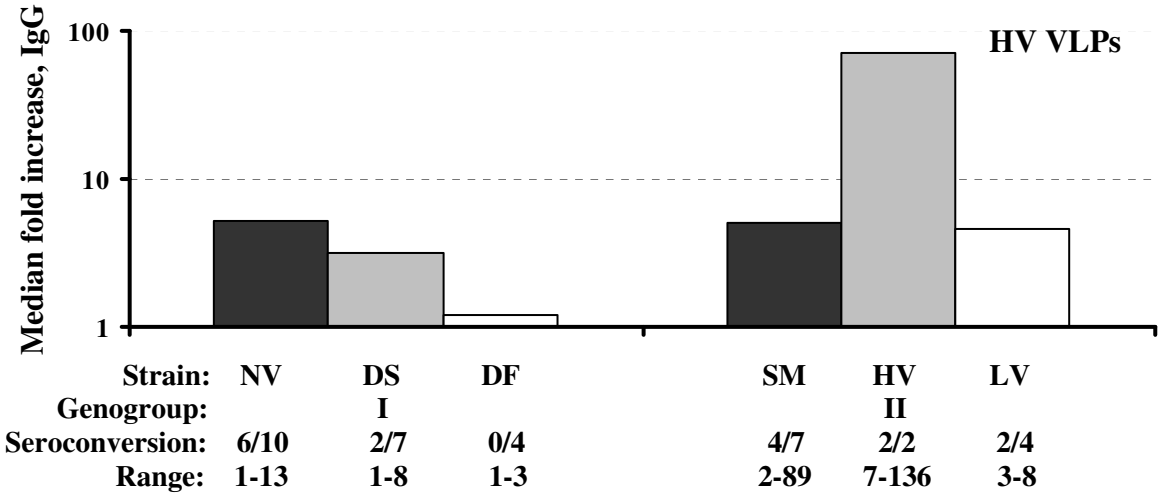
A.


NV VLPs
Median fold increase, IgG
100
10
1
Strain: NV DS DF SM HV LV
Genogroup: I II
Seroconversion: 10/10 5/7 2/4 2/7 1/2 2/4
Range: 201-273 1-149 0-17 1-5 1-4 1-5

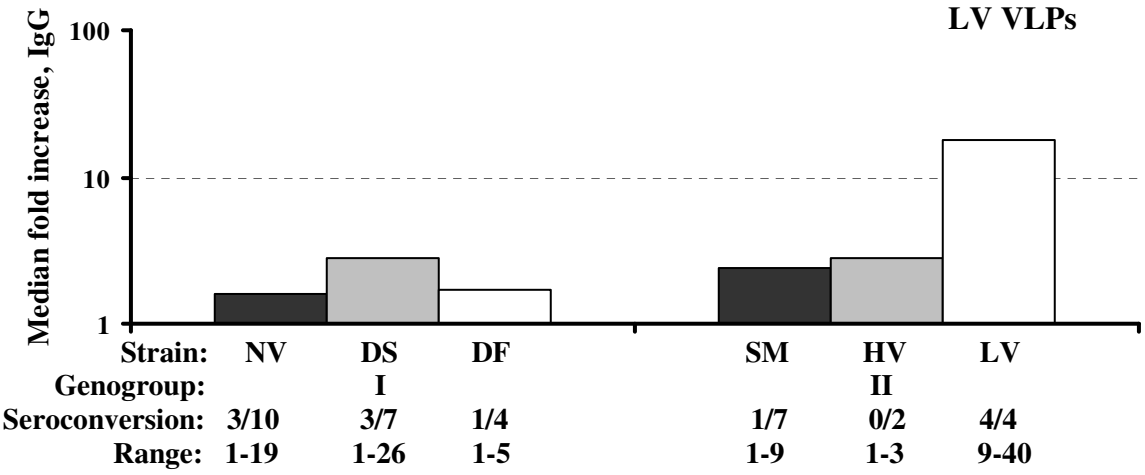
B.


SM VLPs
Median fold increase, IgG
100
10
1
Strain: NV DS DF SM HV LV
Genogroup: I II
Seroconversion: 5/10 2/7 0/4 7/7 2/2 1/4
Range: 2-15 1-51 1-3 4-129 5-13 1-10

C.


HV VLPs
Median fold increase, IgG
100
10
1
Strain: NV DS DF SM HV LV
Genogroup: I II
Seroconversion: 6/10 2/7 0/4 4/7 2/2 2/4
Range: 1-13 1-8 1-3 2-89 7-136 3-8

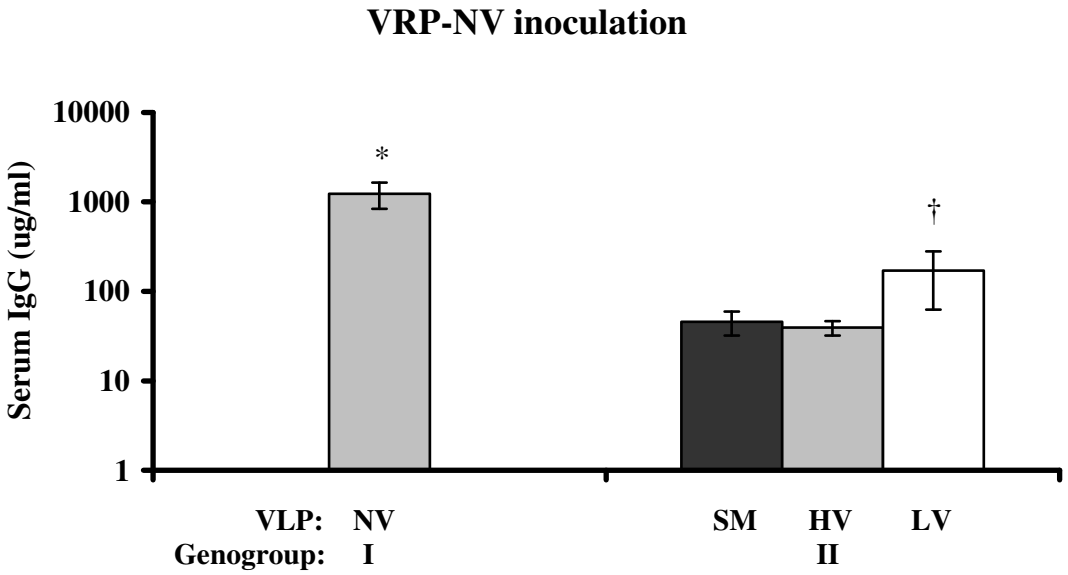
D.


LV VLPs
Median fold increase, IgG
100
10
1
Strain: NV DS DF SM HV LV
Genogroup: I II
Seroconversion: 3/10 3/7 1/4 1/7 0/2 4/4
Range: 1-19 1-26 1-5 1-9 1-3 9-40

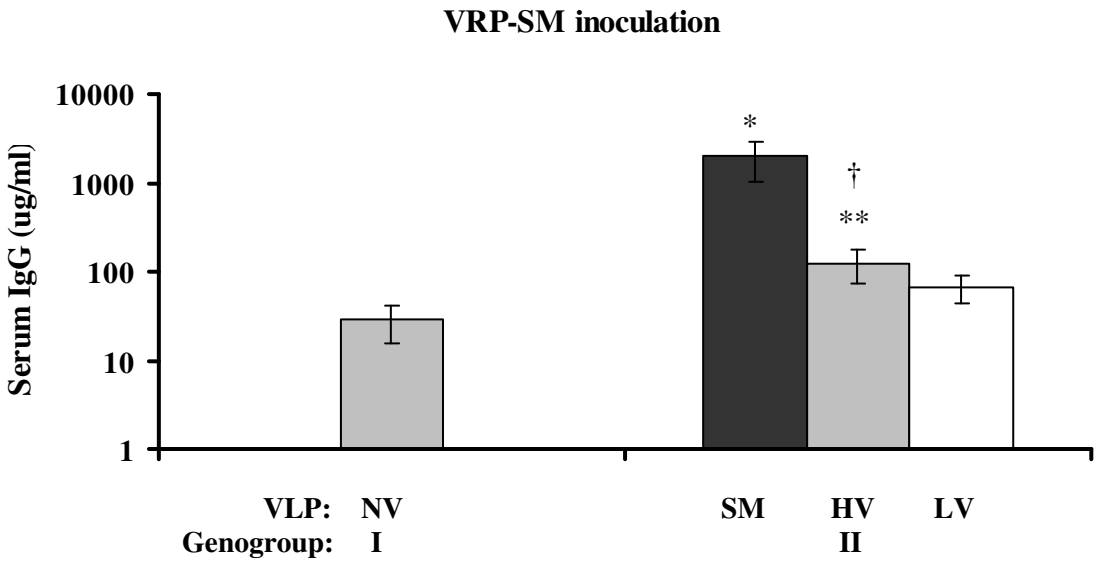
Homotypic and heterotypic serum IgG responses in humans following norovirus infection. Pre-challenge and convalescent serum samples were collected from volunteers that became infected following participation in norovirus challenge studies with either NV (n=10), SM (n=7), or HV (n=2). Acute and convalescent serum samples were collected from individuals infected during a LV (n=4), DF (n=4), or DS (n=7) outbreak. Infection groups (by strain) and respective norovirus genogroups are indicated below the x-axis. IgG titers from each infection group were measured using indirect ELISA for specificity to NV (A), SM (B), HV (C), or LV (D) VLPs. Antibody titers are represented as median fold-increase in convalescent serum IgG from pre-challenge IgG titers on a logarithmic scale. Values were not significantly different between any infection groups (One-way ANOVA). The number of samples per group that exhibited seroconversion to each antigen and the ranges of the fold-increase in IgG within each infection group are indicated below.

Figure 2.3

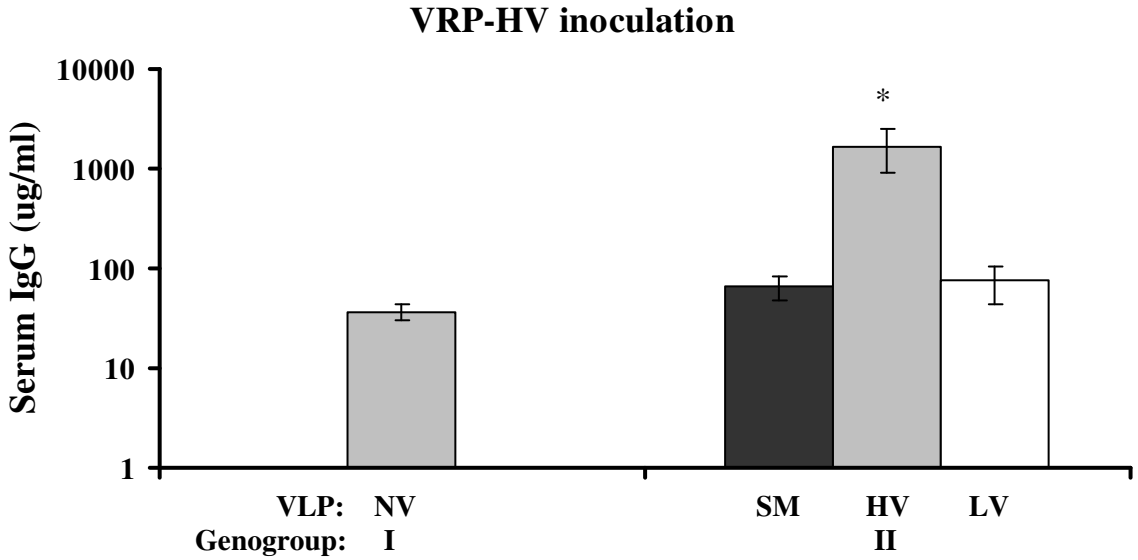
A.


VRP-NV inoculation
Serum IgG (ug/ml)
10000
1000
100
10
1
*
†
VLP: NV SM HV LV
Genogroup: I II

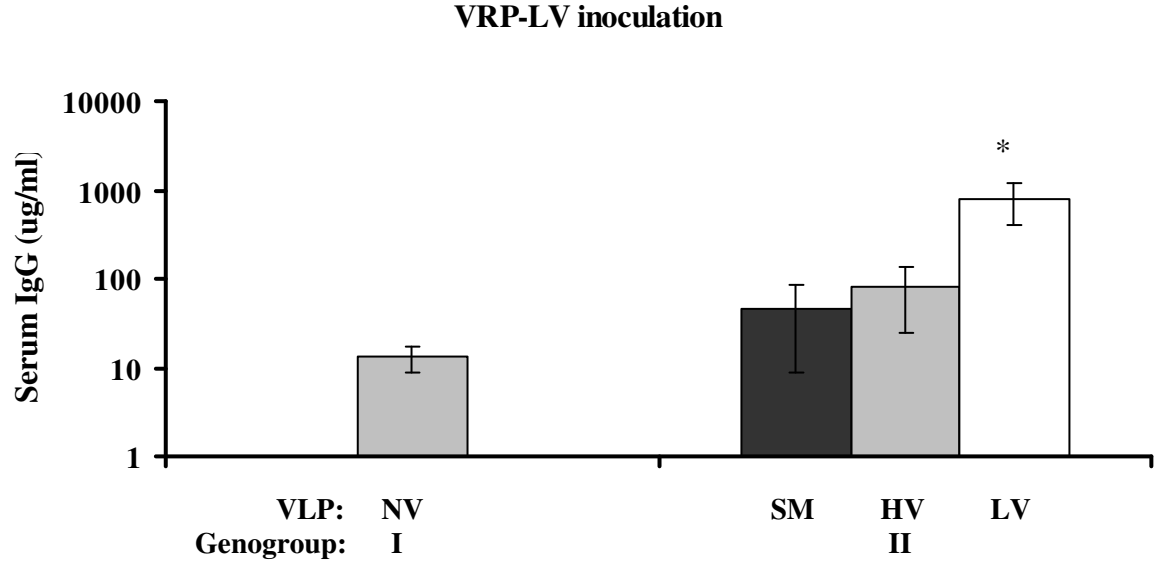
B.


VRP-SM inoculation
Serum IgG (ug/ml)
10000
1000
100
10
1
*
†
**
VLP: NV SM HV LV
Genogroup: I II

C.


VRP-HV inoculation
Serum IgG (ug/ml)
10000
1000
100
10
1
*
VLP: NV SM HV LV
Genogroup: I II

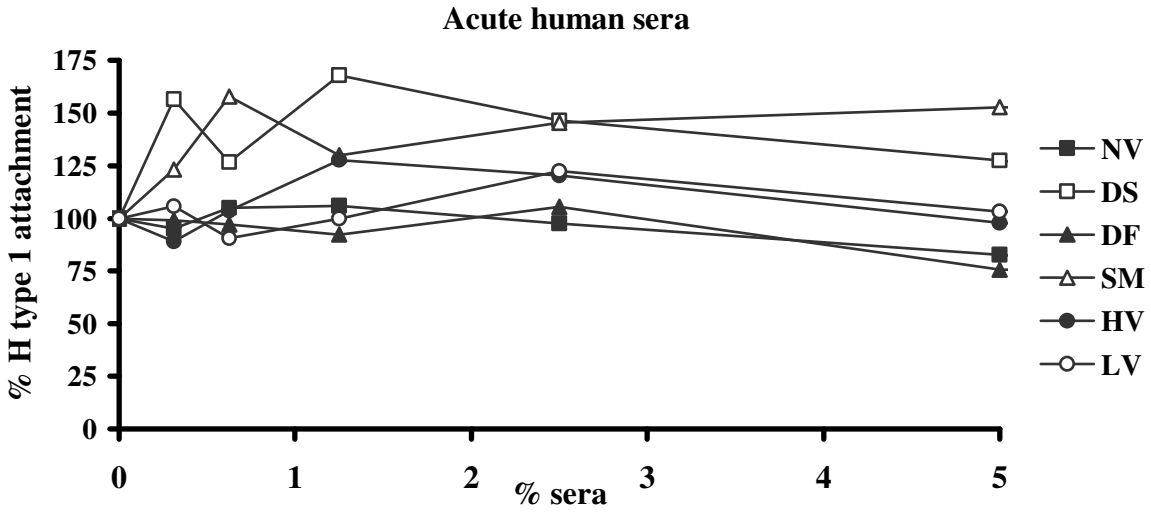
D.

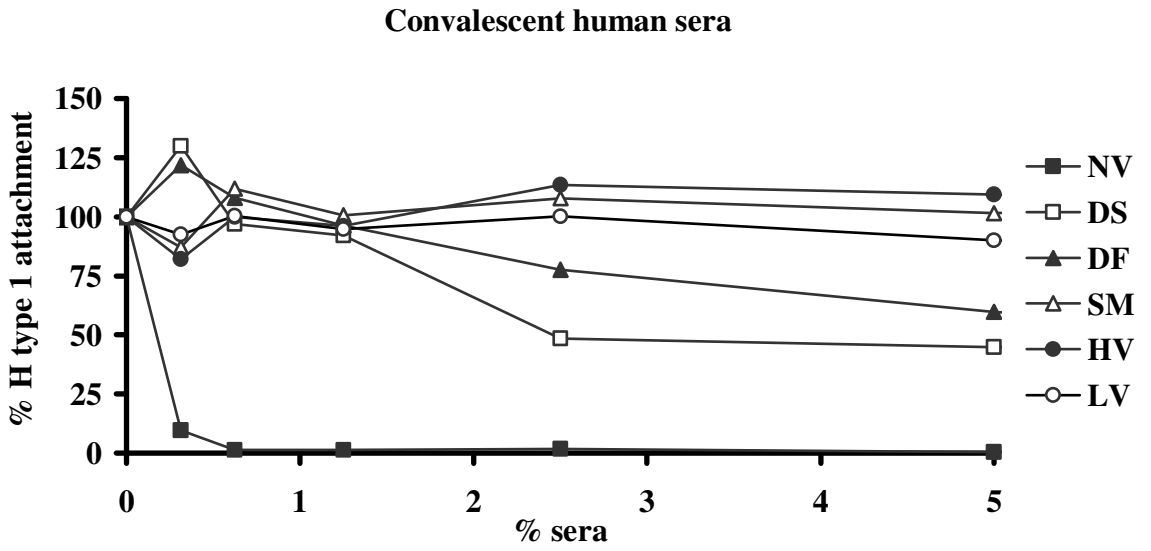

VRP-LV inoculation
Serum IgG (ug/ml)
10000
1000
100
10
1
*
VLP: NV SM HV LV
Genogroup: I II

Homotypic and heterotypic serum IgG responses in mice following inoculation with VRPs expressing norovirus VLPs. 6-week-old male BALB/c mice were primed and boosted with $2.5x10^6$ IU VRPs expressing NV, SM, HV, or LV VLPs in the footpad on days 1 and 23, respectively (n=4 per inoculation group). Serum was collected on day 35. IgG titers from inoculation groups VRP-NV (A), VRP-SM (B), VRP-HV (C), or VRP-LV (D) were measured using indirect ELISA for specificity to NV (GI), SM, HV, or LV (GII) VLPs, as indicated below the graph. Antibody titers are represented as the geometric mean serum IgG (μg/ml) on a logarithmic scale. All homotypic responses were significantly greater than heterotypic responses (*). Heterotypic responses that were significantly greater ($P \leq 0.05$) within a genogroup than between genogroups are indicated with **. Heterotypic responses that are significantly greater than others within a genogroup are indicated with † (One-way ANOVA, Tukey HSD). Bars show SD in each sample group. Average baseline (day 0) titers to each antigen are <1 ug/ml.

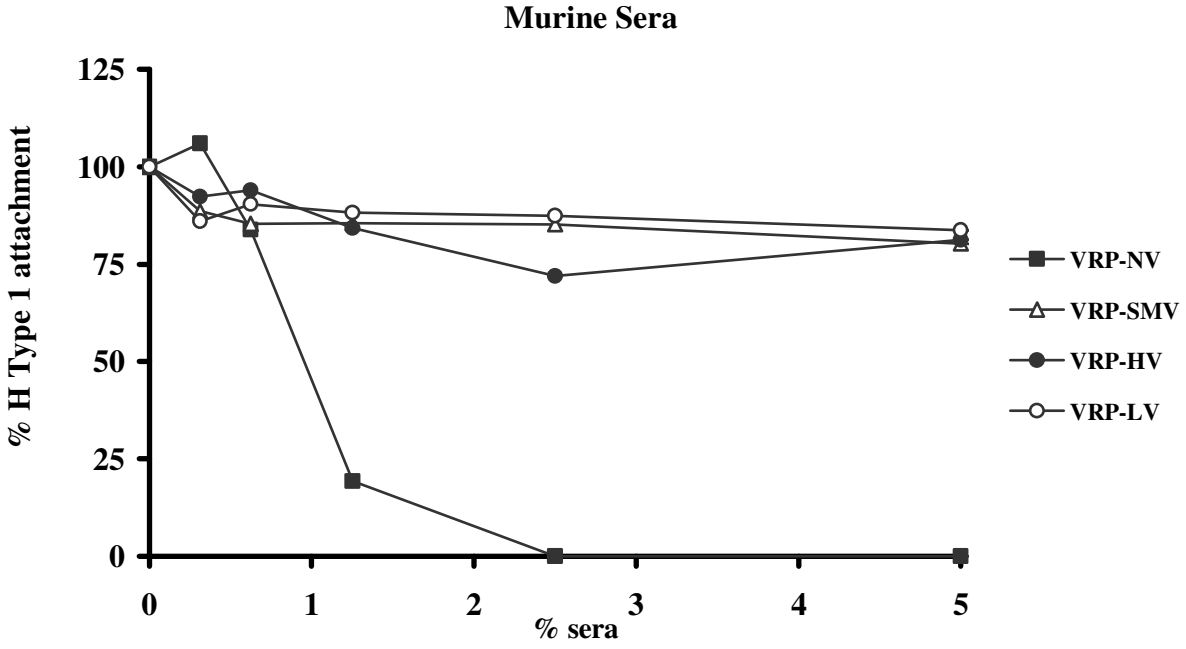
Figure 2.4

A.


Acute human sera
% H type 1 attachment
175
150
125
100
75
50
25
0
0
1
2
3
4
5
% sera
NV
DS
DF
SM
HV
LV


Convalescent human sera
% H type 1 attachment
150
125
100
75
50
25
0
0
1
2
3
4
5
% sera
NV
DS
DF
SM
HV
LV

B.



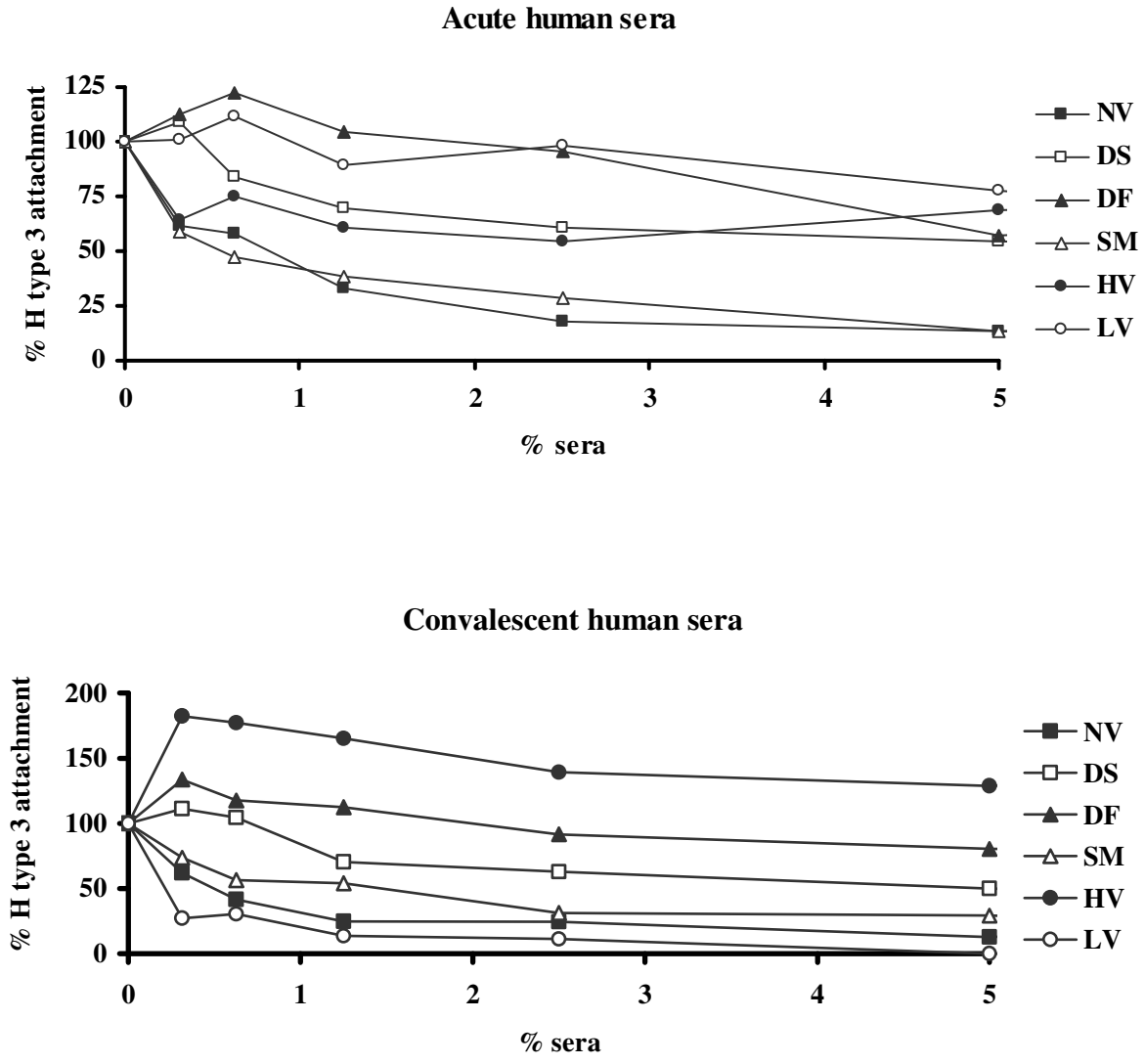
D.

		50% BT (μg/ml)	90% BT (μg/ml)
Human	**NV**	<5.1	<6.4
	DS	8.8	19.2
	DF	6.0	10.9
Mouse	**NV**	11.9	23.7
	NV2	5.8	N/A

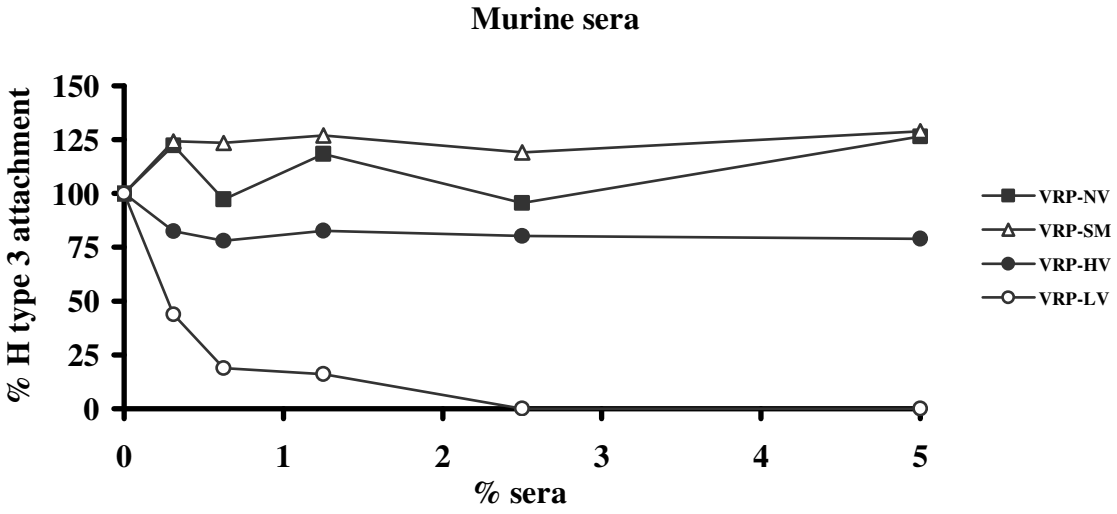
Homotypic and heterotypic serum IgG blockade of H type 1 antigen binding to NV VLPs in humans and mice. All pre-challenge or acute and convalescent sera collected from humans infected with NV, DS, DF, SM, HV, or LV (A; left and right panels, respectively) and from mice inoculated with VRPs expressing NV, SM, HV, or LV VLPs (B) were used in antibody blockade assays. Blockade of H type 1 binding was measured at OD_{405} and compared to control wells representing 100% H type 1 binding. Differences in blockade between matched human samples pre- and post-infection were significant following infection with NV at all serum concentrations ($P \leq 0.0001$, student's t-test)(A, right vs. left panels). Blockade of 50% binding by convalescent human NV sera was not significantly different from other GI infection groups (One-way ANCOVA; right panel A). Mean IgG titers (μg/ml) for 50% and 90% blockade of H type 1 binding (BT50/90) in measurable human convalescent sera samples and murine inoculation groups are presented in panel C.

Figure 2.5

A.


Acute human sera
% H type 3 attachment
125
100
75
50
25
0
0
1
2
3
4
5
% sera
NV
DS
DF
SM
HV
LV
Convalescent human sera
% H type 3 attachment
200
150
100
50
0
0
1
2
3
4
5
% sera
NV
DS
DF
SM
HV
LV

B.


Murine sera
% H type 3 attachment
150
125
100
75
50
25
0
0
1
2
3
4
5
% sera
VRP-NV
VRP-SM
VRP-HV
VRP-LV

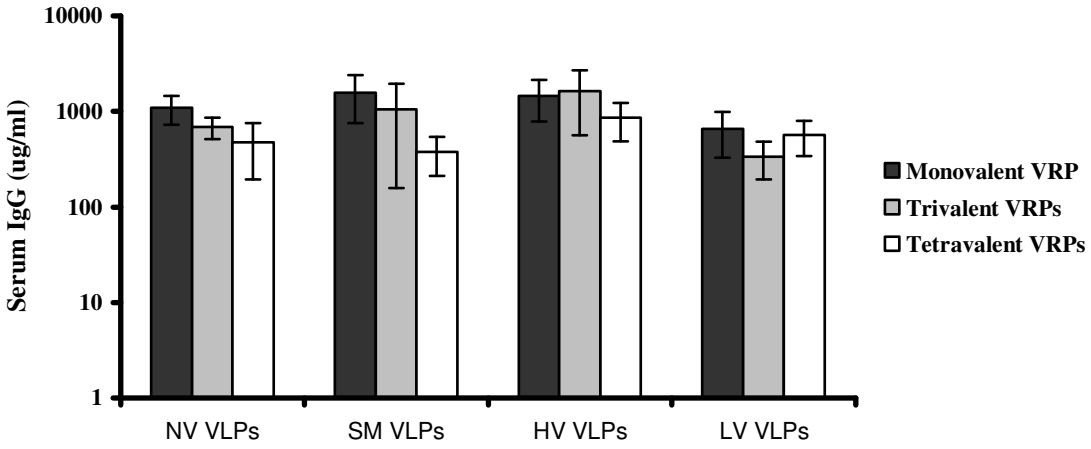
C.

	50% BT (μg/ml)	**90% BT (μg/ml)**
human	6.4	25.7
mouse	3.0	6.0

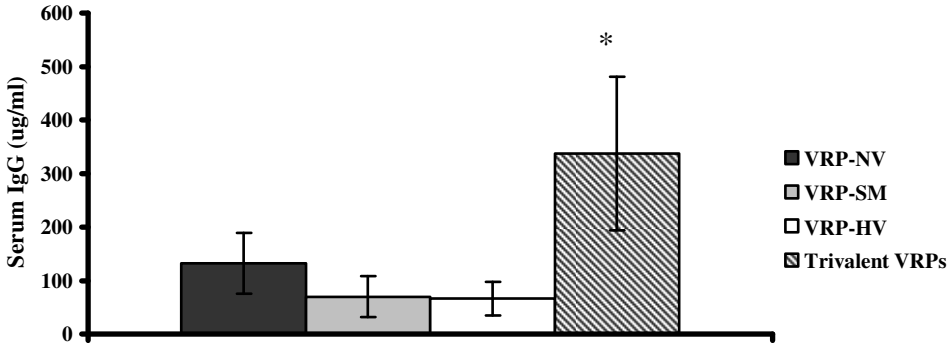
Homotypic and heterotypic serum IgG blockade of H type 3 antigen binding to LV VLPs in humans and mice. All acute or pre-challenge and convalescent sera collected from humans infected with NV, DS, DF, SM, HV, or LV (A; left and right panel, respectively) and from mice inoculated with VRPs expressing NV, SM, HV, or LV VLPs (B) were used in antibody blockade assays. Blockade of H type 3 binding was measured at OD_{405} and compared to control wells representing 100% H type 3 binding. Differences in blockade between matched human samples pre- and post-infection were significant following infection with LV at all serum concentrations ($P \leq 0.05$) and significantly lower following infection with HV at serum concentrations ≤0.6% ($P \leq 0.05$). Other sampling groups were not different (student's t-test)(A, left vs. right panels). Human convalescent LV sera and murine LV sera IgG titers (μg/ml) necessary for 50% and 90% blockade (BT50/90) of H type 3 binding are presented in panel C.

Figure 2.6

A.


10000
1000
100
10
1
Serum IgG (ug/ml)
NV VLPs
SM VLPs
HV VLPs
LV VLPs
Monovalent VRP
Trivalent VRPs
Tetravalent VRPs

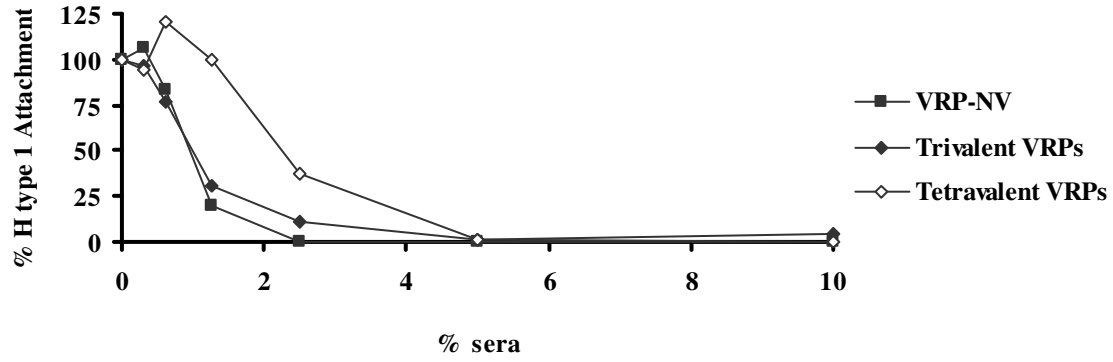
B.


600
500
400
300
200
100
0
Serum IgG (ug/ml)
*
VRP-NV
VRP-SM
VRP-HV
Trivalent VRPs

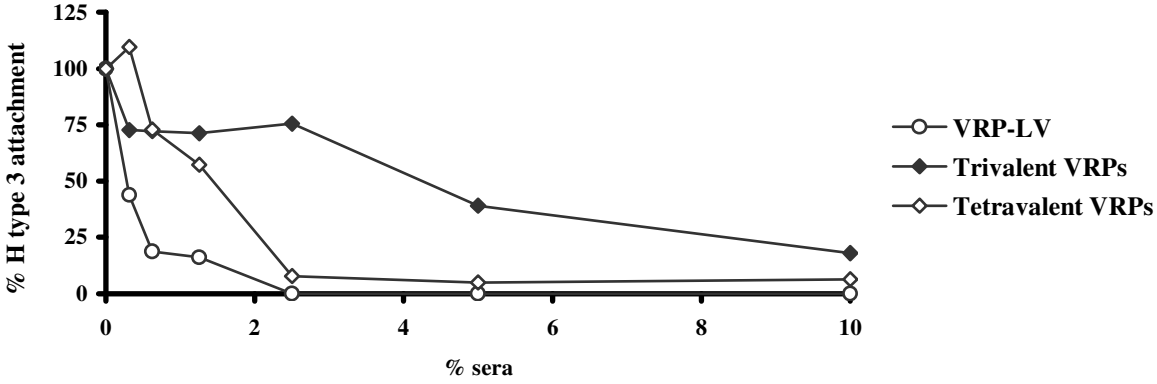
Serum IgG responses in mice following norovirus inoculation with a cocktail of VRPs expressing VLPs from multiple norovirus strains. 6-8 week-old BALB/c mice were primed and boosted with a total of $2.5x10^6$ IU VRPs expressing equivalent concentrations of NV, SM, and HV VLPs (trivalent VRPs; n=5), with $2.5x10^6$ IU each of NV, SM, HV, and LV VRPs (tetravalent VRPs; n=5), or with $2.5x10^6$ IU of the indicated strain-specific VRP (monovalent VRPs; n=4) in the footpad on days 1 and 23, respectively. Serum was collected on day 35. IgG titers from each inoculation group were measured using indirect ELISA for specificity to NV, SM, HV, or LV VLPs as indicated along the x-axis (A). Antibody titers are represented as μg/ml geometric mean serum IgG on a logarithmic scale. IgG titers induced by monovalent and tetravalent inocula were statistically different from one another in the NV and SM groups ($P \leq 0.05$); all other titers were not different (One-way ANOVA and Tukey HSD) (A). The heterotypic serum IgG titers to the LV antigen in mice receiving trivalent inoculum is significantly higher (*) than the heterotypic IgG titers induced by the individual components of the trivalent inoculum ($P \leq 0.05$, One-way ANOVA), as shown in panel B. Bars represent SD for each sample group. Average baseline (day 0) titers are <1 ug/ml.

Figure 2.7

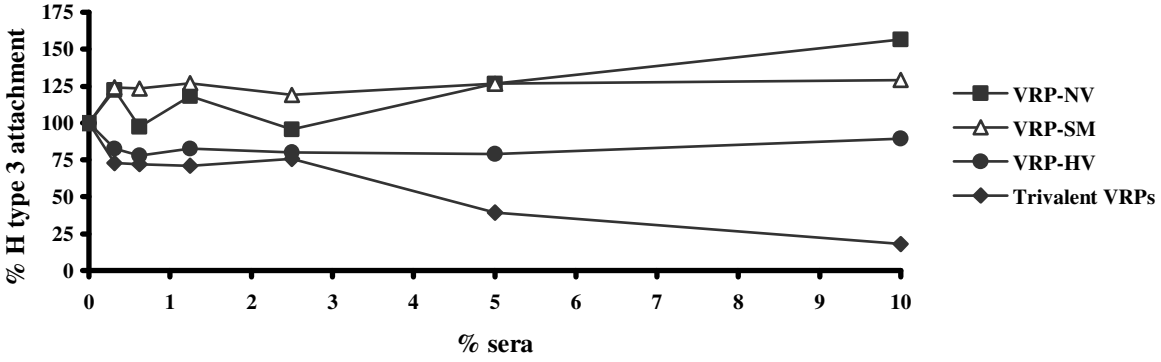
A.



B.



C.



D.

	H type 1			H type 3	
	50% BT	**90% BT**		**50% BT**	**90%BT**
NV	11.9	23.7	**LV**	3.0	6.0
3-pool	14.1	23.3	**3-pool**	35.1	36.8
4-pool	12.3	24.5	**4-pool**	14.3	15.7

Serum IgG blockade of ABH histo-blood group antigen binding to VLPs in mice inoculated with a VRP cocktail. Sera collected from mice following inoculation with VRPs expressing NV, SM, and HV (trivalent VRPs) or NV, SM, HV, and LV VLPs (tetravalent VRPs) were tested for blockade of H type 1 binding to NV VLPs (A) or H type 3 binding to LV VLPs (B) by antibody blockade assays. Monovalent and tetravalent inocula containing the LV antigen could block 50% H type 3 binding at significantly lower serum concentrations than trivalent inocula not containing the LV antigen ($P \leq 0.05$)(B). Blockade of H type 3 binding to LV VLPs by sera collected from mice receiving trivalent inoculum compared to mice receiving the individual components of the trivalent inoculum is represented in panel C. Panel D shows serum IgG titers (μg/ml) required for 50% and 90% blockade (BT50/90) of VLP-ligand binding.

TABLE 2.1. Norovirus-specific gut and spleen antibody secretion following lymphoid culture in mice inoculated with multivalent VRP vaccines.

Parameter[a]	Value [mean ± StD]	
	Pool[b]	Control[c]
Gut IgG (μg)		
HV VLP	12.5 ± 5.1	0.10 ± 0.12
SM VLP	7.6 ± 1.7	0.04 ± 0.07
NV VLP	9.9 ± 1.9	0.04 ± 0.06
Blockade[d]	≥100%	0-5.4%
Gut IgA (ng)		
HV VLP	726 ± 293	157 ± 89
SM VLP	1023 ± 214	338 ± 247
NV VLP	594 ± 497[*]	216 ± 149
Blockade	nt[e]	nt
Spleen IgG (μg)		
HV VLP	28.0 ± 12.7	0.57 ± 0.72
SM VLP	21.5 ± 7.8	0.26 ± 0.25
NV VLP	18.0 ± 11.3	0.29 ± 0.29
Blockade	≥100%	0%

[a] Gut and spleen lymphoid samples screened against HV, SM, and NV VLPs for specific antibody content and blockade of H type 1 binding to NV VLPs.
[b] Mice (n=3) inoculated with three VRPs (2.5×10^6 IU) expressing equivalent amounts HV, SM, and NV VLPs.
[c] Mice (n=3) inoculated with 2.5×10^6 IU control VRPs expressing HA (flu).
[d] See *Materials and methods* for protocol; control samples represent blockade at 30% serum concentrations
[e] Not tested due to limited sample volume.
[*] Not significant from control values.

CHAPTER III.

Mechanisms of GII.4 Norovirus Persistence in Human Populations

Adapted from PLOS Medicine, *in press*

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*authors who contributed equally to this work

Abstract

Background: Noroviruses are the leading cause of viral acute gastroenteritis in humans, noted for causing epidemic outbreaks in communities, the military, cruise ships, hospitals, and assistednliving communities. Worldwide, the majority of Norovirus outbreaks are caused by the GII.4 genocluster which was first recognized in the mid-1990's. The evolutionary mechanisms governing the persistence of the GII.4 viruses in human populations are unknown. Previous studies by our laboratory indicate that some Noroviruses readily infect individuals who encode a functional FUT2 gene and are secretor-positive because they express ABH histoblood group antigens (HBGAs), a highly heterogeneous group of related carbohydrates on mucosal surfaces. Individuals who encode defects in the FUT2 gene, are secretor-negative, don't express the appropriate ABH HBGA necessary for docking and entry and are resistant to Norwalk virus infection, These data argue that the FUT2 gene and other enzymes that regulate processing of the HBGA carbohydrates function as susceptibility alleles. However, secretor-negative individuals can be infected with other norovirus strains

and many secretor negative individuals have antibodies against human noroviruses. In this manuscript, we investigate the molecular mechanisms governing GII.4 susceptibility and persistence mechanisms in human populations.

Methods and Findings: Phylogenetic analysis of the GII.4 capsid sequences suggested a linear evolution over the last twenty years with serial replacements occurring every ~3-5 years. Five distinct evolutionary clusters were identified and representative ORF2 genes expressed as virus like particles (VLPs). Using salivary and carbohydrate binding assays, GII.4 VLP-carbohydrate ligand patterns changed over time and included carbohydrates regulated by human *FUT1*, *FUT2* and *FUT3* pathways, suggesting that strain sensitivity to human susceptibility alleles will vary. Variation in residues in the second ligand interaction domain, which stabilize carbohydrate binding, regulated carbohydrate binding patterns over time. Sites of variation also decorated the surface of P2 in the recently solved structure GII.4 capsid, suggesting that antigenic drift may occur over time. Antigenic relatedness of GII.4 VLPs was measured using time ordered murine and human sera and time-sensitive specific serologic and carbohydrate binding blockade responses were evident. These data suggest that the GII.4 noroviruses persist by recognizing new HBGA carbohydrate binding targets over time, which not only allows for escape from highly penetrant host susceptibility alleles, but simultaneously allows for immune driven selection in the receptor binding region to facilitate escape from protective herd immunity.

Conclusions: Our data suggest that the surface exposed carbohydrate ligand binding domain in the norovirus capsid is under heavy immune selection and likely evolves by antigenic drift in the face of human herd immunity. Variation in the capsid carbohydrate binding domain is tolerated because of the large repertoire of similar, yet distinct HBGA carbohydrate receptors

that are available on mucosal surfaces that could interface with the remodeled architecture of the capsid ligand binding pocket. The continuing evolution of new replacement strains suggests that, like influenza virus, vaccines can be targeted that protect against norovirus infections and that continued surveillance and reformulations of norovirus vaccines will be necessary every 3-5 years.

Introduction

The majority of norovirus outbreaks are caused by the GII.4 genotypes and pandemic spread was first recognized in the mid-1990's (181). During 1995-96, US95/96 was responsible for ~55% of the norovirus outbreaks in the USA and 85% of the outbreaks in the Netherlands (264). Between 2000 and 2004, US95/96 was replaced by two new GII.4 variants. In the USA, Farmington Hills (FH) (274) was ultimately associated with ~80% of norovirus acute gastroenteritis outbreaks (55). Simultaneously in Europe, a new GII.4 variant, GII.4b, emerged and caused outbreaks during the winter, spring and summer (151, 167, 209). In 2004, the Hunter GII.4 variant was detected in Australia, Europe and Asia (25, 138, 209), and may represent the next pandemic strain as explosive outbreaks of disease have recently been associated with this GII.4 genotype worldwide (25). Sakai represents a neoteric GII.4 outbreak strain associated with outbreaks in healthcare facilities in Southeast Asia (187) and may represent a unique geographically confined cluster. The evolutionary mechanisms governing the persistence and epidemic spread of the GII.4 viruses in human populations are unknown**.**

Expression of the Norovirus ORF2 protein produces virus-like particles (VLPs) and the Norwalk virus (NV) X-ray crystal structure indicates that dimer formation is required to form the higher order structure comprised of 180 subunits (212). The monomer has two domains known as the shell domain (S) which forms the inner core and the protruding domain (P), linked by a flexible hinge. P domain forms prominent protrusions that extend away from the structure (212) and is subdivided into P1 subdomains (residues 226-278 and 406-520) which functionally flank the P2 mostly surface exposed region (residues 279-405) (212).

Mutational analysis of the surface exposed P2 domain supports its role in HBGA binding (150, 248, 250), suggesting that it contains the determinants of strain specificity, receptor binding (34, 212, 249, 251, 263) and potential neutralizing antibody recognition sites (33, 150). More recently, the complex structures of the P-domain of a GII.4 virus, VA387, in complex with HBGA trisaccharide A- and B- antigens revealed a ligand interaction site in the P2 subdomain where specific capsid residues form a strong hydrogen bond network with the α-fucose group of the trisaccharide (30). A second interaction site on the VA387 P domain was predicted to stabilize binding and enhance ligand affinity by weak, long distance interactions with the β-Gal ring of the trisaccharide (30).

In this manuscript, we show that the GII.4 noroviruses are evolving linearly over time and map the antigenic variation onto the capsid structure. Using a time-ordered panel of GII.4 VLPs from 1987-2005, we demonstrate that specific changes in the 2nd interaction domain regulate carbohydrate binding patterns, which change over the 20 year interval. Using serum from human outbreaks in 1988 and 2000 and murine antisera, we use ELISAs and an *in vitro* carbohydrate blockade as a surrogate neutralization assay to demonstrate that the noted variation alters the serologic and blockade responses consistent with a model of antigenic drift. Our data suggest a model of molecular evolution in which norovirus GII.4 strains persist by evolving novel carbohydrate binding domains over time in response to immune driven selection and antigenic drift in the receptor binding regions of the P2 subdomain. The data suggest that vaccines are feasible but must be reformulated every few years as epidemic replacement strains emerge and sweep through human populations.

Materials and methods

Sequences and Sequence Analysis. Eighty-eight full-length GII.4 capsid amino acid sequences were downloaded from GenBank and aligned by ClustalXv1.83 (35) using the PAM distance matrix and default parameters. The alignment was refined manually, and variable sites were exported in table format and ordered by time. Columns that contained a single amino acid difference were removed as potential sequence errors. Five distinct clusters were identified and associated with outbreak years, and representative viruses from each cluster were selected, and analyzed by Bayesian inference of phylogeny using Mr. Bayesv3.1 under default settings (226). Trees were viewed using TreeViewPPC version 1.6.6 (195). The nucleotide sequences were aligned as codons using the program PAL2NAL (245) which aligned the corresponding nucleotide sequences based on the amino acid alignment. Positive selection analysis was conducted using HyPhy with the Fixed Effects Likelihood parameter (135, 211) under the TrN model.

VEE Replicon Particles (VRPs) and norovirus Virus-like Particles (VLPs). Capsid gene constructs for each of the representative strains were designed and synthesized as reported previously (46). Briefly, the ORF 2 genes of GII.4-1997 (LVNC1) (149) and GII.4-2002 were derived from RT-PCR products from outbreak stool samples collected in 1997 and 2004 (265) while the ORF2 genes of GII.4-1987, GII.4-2004 and GII.4-2005 were synthesized commercially by BioBasic (https://www.biobasic.com/index.php), and then inserted directly into the VEE replicon vector for the production of VRPs (VRP-GII.4-1987, VRP-GII.4-1997, VRP-GII.4-2002, VRP-GII.4-2002a, VRP-GII.4-2004, VRP-GII.4-2005) (11, 149). The VLPs were expressed in VRP-infected BHK cells, purified and visualized by negative staining EM (11, 149).

Carbohydrate Binding Assays. VLP binding to synthetic HBGAs was determined using Neutri-avidin coated plates (Pierce, Rockford, IL) treated with 10μg/ml biotinylated carbohydrate (Glycotech, Gaithersberg, MD) for one hour and washed with PBS-0.05% Tween 20 before the addition of 1-2μg/ml VLP for 1.5 hours at 37 ^{o}C. VLP binding was detected as described above. Blockade assays included serum pretreatment of the VLP for 1 hour at 37^{o}C before addition to the carbohydrate-bound plate. Assays using mouse antisera for blockade used rabbit polyclonal anti-GII VLP antisera followed by anti-rabbit IgG-AP (Sigma) for VLP binding detection. BT50 titers were defined as the lowest percentage of sera tested that blocked 50% of binding compared to levels determined in the absence of antibody pretreatment. Serum samples that did not reach a BT50 by the maximum % sera tested were assigned a BT50 value equal to 2X the maximum % sera tested for statistical analysis.

Serology. Samples from archived GII.4 outbreaks occurring in 1988 and 2000 were obtained from the Gastroenteritis & Respiratory Virus Laboratory Branch of the CDC, (Atlanta, GA). Any serum pair with a norovirus-positive stool sample or a ≥4-fold increase in anti-LV87 or LV97 IgG response between acute and convalescent samples (seroconversion) was further studied for cross reactivity and blockade ability. Mice were immunized with VRP constructs as described (149). Both human and murine VLP-reactive serum IgG was measured by ELISA (145, 146, 149) using VLP-binding detection methods as described. Human and mouse anti-VLP serum IgG was compared to a purified IgG of known concentration for quantitation.

Statistical Analysis. The Mann-Whitney 2-tail test was used to compare the median responses between groups for human samples. The One-way ANOVA was used to compare responses between murine immunization groups.

Results

Sequence Analysis and Bayesian Inference of Phylogeny

An amino acid multiple alignment of 88 full length GII.4 capsid sequences was generated and columns of heterogeneity were exported as a table, and ordered by time. Five distinct evolutionary clusters were identified in these analyses and representative strains were selected from each cluster and associated with outbreak dates such that the Camberwell cluster which ranges from 1987-1992 is represented by GII.4-1987, the Grimsby cluster from 1994 to 2001 is represented by GII.4-1997, the Farmington Hills cluster from 2002 to 2004 is represented by GII.4-2002, the newly recognized Hunter cluster (2004-2006) is represented by GII.4-2004, and the most recent Sakai cluster (2004-2006) is represented by GII.4-2005 (Figure 3.1A).

The extent of diversity among the clusters of GII.4 viruses is approximately 2%, with a total sequence identity of 92% occurring between the earliest cluster, Camberwell, and the extant clusters, Hunter and Sakai. While variation was noted in the S, P1, and P2 domains of the capsid, the majority of heterogeneity occurred within the P2 subdomain (Figure 3.1A). Of the two receptor interaction sites recently reported, site 1 is strictly conserved in all clusters, while the second site is highly variable at positions 393 through 395 (VA387 numbering) (Figure 3.1A). Of note, strains occurring after the GII.4-1997 cluster encode an inserted amino acid between positions 393 and 394 of VA387 (Figure

3.1A).

Four sites in P2 at positions 298, 372, 376, and 394/395 (site 394 in viruses of the GII.4-1987 and GII.4-1997 clusters and position 395 of all later clusters) were determined by HyPhy to be operating under positive selection. A second Farmington Hills isolate (GII.4-2002a) which differs from GII.4-2002 by a single amino acid difference in the P2 subdomain at positively selected residue 395 (Ala395 in GII.4-2002 and Thr395 in GII.4.2002a) was identified for further evaluation (Figure 3.1A). Analyses of the Bayesian phylogenetic tree in conjunction with the evolutionary profiles suggests that the GII.4 capsids evolved linearly over the last twenty years in a similar fashion as influenza (28). Bayesian inference confirmed that the evolution of each cluster was correlated with time, with the Camberwell cluster being near the root of the tree and the Hunter and Sakai clusters shown to have evolved linearly from the earlier clusters (Figure 3.1B). While some clusters persisted for ~8 years, new clusters appear to have evolved from subsequent populations every 3-5 years (Figure 3.1C), with the new strain eventually becoming the predominant strain. Taken together, these analyses suggest that the GII.4 viruses evolved linearly over the last twenty years, with intense heterogeneity within the P2 region of the capsid sequence facilitating the emergence of new predominant strains.

Evolutionary analyses showed that the ligand binding residues reported for site 1 (30) were strictly conserved in the GII.4 viruses, while one amino acid position in the second interaction site at position 393 was highly variable among the representative viruses. Further, three of the four P2 sites operating under positive selection occur near the two interaction sites, with position 395 being an important residue adjacent to the

second interaction site. We hypothesize that microevolution in site 2 alters receptor specificity, and we predict that GII.4-2002 and GII.4-2002a will have different binding characteristics, facilitated by a single amino acid difference in the P2 subdomain.

To further characterize the binding characteristics of the GII.4-2002 viruses as well as the other representative viruses, the Camberwell GII.4-1987, Grimsby GII.4-1997, Farmington Hills GII.4 2002 and 2002a, Hunter GII.4-2004 and Sakai GII.4-2005 ORF 2 sequences were inserted directly into the VEE replicon vector and all six replicons produced abundant 40nm VLPs following visualization by negative strain EM techniques (data not shown). Synthetic HBGA binding assays revealed differences in the patterns of carbohydrate binding for the five VLPs, where GII.4-1987 VLPs bound strongly to H type 3 and less well to LeY. Concordant with these findings, GII.4-1997 bound H type 3, but also bound efficiently to LeY, A and B. In contrast, GII.4-2002 bound strongly to LeY and less efficiently to H type 3, while GII.4-2002a, GII.4-2004 and GII.4-2005 did not bind any carbohydrate tested (data not shown). Concordant with the predicted remodeling of the receptor binding pocket, these data indicate that sequence variation in and around the second carbohydrate stabilizing domain of ORF 2 alters VLP structure and modulates HBGA binding patterns within a genotype, resulting in changes in VLP-carbohydrate ligand receptor binding over time.

Serologic Relationships among the GII.4 VLPs

Sera and stools were collected from infected subjects from GII.4 outbreaks during the 1988 and 2000 norovirus outbreak seasons. Both the acute and convalescent serum samples cross-reacted with each of the time-ordered VLPs, regardless of outbreak date.

Within both outbreaks, the reactivity to GII.4-1987 and GII.4-1997 VLP was equivalent. However, the 1988 outbreak convalescent sera titer to GII.4-2002 (P=0.02), GII.4-2004 (P<0.01) and GII.4-2005 (P<0.01) was significantly and proportionately reduced as compared to LV87 (Figure 3.2A). Comparison of the IgG titer across VLPS also demonstrated a significant negative trend in reactivity of 1988 outbreak convalescent sera and VLPs circulating at later times (P<0.001). Sera collected in 2000 no longer differed in reactivity to GII.4-2002, GII.4-2004, or GII.4-2005 compared to GII.4-1987, consistent with exposure to progressively different strains over the lifetime of individuals (Figure 3.2B).

Blockade Titer Varies Over Time

As a robust cell culture system is not available, we utilized a surrogate neutralization assay exploiting the ability of serum IgG to block norovirus VLP interaction with putative HBGA receptors (91, 149). Figure 2.3 shows the median % control binding of VLP in the presence of sera compared to the binding of VLP in the absence of antibody pretreatment. Although the acute serum samples reacted with GII.4-1987, GII.4-1997 and GII.4-2002 in the IgG EIA, none of the acute samples collected blocked the VLP-HBGA interactions, even at high serum concentrations (data not shown). However, convalescent serum collected in 1988 completely blocked GII.4-1987 and GII.4-1997 interaction with H type 3 but was significantly less able to block GII.4-2002 VLP interaction with LeY (P=.016) (Figure 3.3A). The median concentration of sera needed to block VLP-HBGA binding by 50% (BT50) was 0.13% for GII.4-1987 and GII.4-1997 interaction with H type 3 (Figure 3.3B). These titers were significantly different from the sera titer (>0.25%) needed to block GII.4-2002-LeY interaction

(P=0.03). Convalescent serum collected in the 2000 GII.4 outbreak completely and equivalently blocked GII.4-1987 and GII.4-1997-H type 3, and GII.4-2002-LeY interactions (data not shown). BT50 titers were 0.06%, 0.04% and 0.07% for each VLP respectively, indicating, no significant differences between any of the GII.4 VLPs (Figure 3.3B).

GII.4 Serologic Relationships Using Murine Sera

Analyzing human serum samples is complex as norovirus exposure histories are unknown and serologic relationships between strains are not well defined (149). Our data suggested a varying ability of serum collected from infected individuals to block the GII.4-2002 strain binding to LeY or H type 3, indicating that exposure history may affect antibody response to a current norovirus challenge, making it more complex to decode the antigenic relationship between the time-ordered GII.4 strains in humans. Further, our previous work demonstrated that animals immunized with a cocktail of norovirus VRPs developed blockade-competent IgG that not only blocked immunizing strains, but also weakly blocked HBGA-binding of unrelated strains that were not included in the immunizing cocktail (149). As mice are not susceptible to human norovirus infection, they provide a clean background in which to study antigenic relatedness between unique timeordered norovirus VLPs. Therefore, we immunized naive mice with VRPs encoding the variant ORF2 of each of the GII.4 strains and collected sera for testing IgG cross reactivity and blockade ability. As seen with human outbreak sera, antisera from mice immunized with VRP- GII.4-1987 or GII.4-1997 reacted equivalently to GII.4-1987 and GII.4-1997 VLPs and to a significantly lesser degree to GII.4-2002, GII.4-2004 and GII.4-2005 VLPs

(P<0.05), indicating that antigenic sites are maintained more completely in early GII.4 strains while becoming variable in later emergent strains (Figure 3.4). Immunization with VRP-GII.4-2002 elicited a strong homotypic response with weaker cross-reactivity to all of the other strains tested (P<0.01)). Interestingly, antisera from GII.4-2004 and GII.4-2005 immunized mice had uniformly low levels of heterotypic antibody to all strains tested compared to the homotypic response (P<0.01). Murine cross-reactive IgG data support the trend seen with human serum samples indicating clear serologic differences between the early and late GII.4 strains. To further test this hypothesis, blockade experiments were performed using mouse sera. Blockade experiments provide a biological measure of the ability of antiserum to block the interaction of a specific VLP with a carbohydrate ligand partner, a surrogate assay for neutralization (91, 149). GII.4-1987 and GII.4-1997 interaction with H type 3 was blocked by sera from mice immunized with VRPs expressing GII.4-1987 and GII.4-1997, weakly blocked by sera from GII.4-2004 and GII.4-2005, and minimally blocked by GII.4-2002 VRP-immunized mice (Figure 3.5A-C). GII.4-2002-LeY interaction was most completely blocked by antisera from mice immunized with VRPs expressing GII.4-2002, efficiently blocked with GII.4-2004 and GII.4-2005 sera, but not blocked with antisera from VRP- GII.4-1987 or GII.4-1997 immunized mice. Our inability to identify carbohydrates that bound GII.4-2004 and GII.4-2005 precluded the testing of the ability of sera from historic strains to block their binding to ligands. Median BT50 titers for blockade of GII.4-1987 and GII.4-1997 were equivalent for all immunization groups. Significantly more anti-GII.4-2002 sera (>5%) was needed to reach BT50 of GII.4-1987 (P<0.05) and GII.4-1997 (P<0.01) (Figure 3.5D), as compared to all other strains. Correspondingly, BT50 of GII.4-2002 binding to LeY required significantly more anti-GII.4-

1987 and GII.4-1997 sera (>5%) (P<0.01) compared to homotypic antibody BT50 at 0.31% serum (Figure 3.5D). None of the antisera generated to the GII.4 panel blocked NV-H type 3 interactions at any of the serum concentrations tested (data not shown). These data support the hypothesis that not only does antigenic drift occur in the capsid region of GII.4 norovirus strains over time, but that the variation significantly influences the ability of pre-existing herd immunity to neutralize exigent strains.

Discussion

Globally, noroviruses are the 2nd most important cause of severe viral gastroenteritis in young children, cause 20% of endemic diarrheal disease in families, cause travelers diarrhea in all ages and are especially virulent in the elderly (53, 107, 134). Our phylogenetic and evolutionary analyses in ORF2 suggest that the GII.4 viruses have evolved linearly over the last twenty years in a similar fashion as influenza, with serial replacements occurring every ~3-5 years, resulting in five distinct evolutionary clusters (Figure 3.1). Sites of heterogeneity predominantly occurred in the exposed P2 subdomain in and around the two carbohydrate interactions sites that form the receptor binding pocket (30, 150, 212). Site 2 was the most variable region in our model and changes in this region affected carbohydrate binding profiles. Our empirical studies suggest that escape from herd immunity may represent the selective force that drives antigenic variation within and around the receptor binding pocket on the surface of the GII.4 P2 domain of ORF2. Variation within the receptor binding domain in ORF2 variants is likely under strong co-selection to recognize variant HBGA carbohydrate receptors for docking and perhaps entry, allowing the GII.4 noroviruses to persist and simultaneously circumvent highly penetrant susceptibility alleles that are common

in human populations. Alternatively, as the current contemporary strains don't bind any carbohydrates tested, the receptor binding pocket may evolve to recognize other fucosylated carbohydrates or proteins for docking and entry.

In influenza, herd immunity, mediated primarily by neutralizing IgG antibodies (37), positively selects for antigenic variation in HA, although the exact effect of individual mutations on antigenicity is complex. Mutations may occur in one or more of five neutralizing epitopes or in the sialic acid binding site in the HA glycoprotein, thus selecting for replacement strains that circumvent antibody neutralization (28). Among noroviruses, the concept of herd immunity is controversial. Early human challenge studies suggested that strain-specific long-term immunity can be elicited following challenge, as 50% of volunteers did not become infected after multiple challenges with NV. However, this same study demonstrated that in some volunteers only short term immunity is evident (203, 281). In more recent studies, we and others have argued that long-term immunity is possible and that pre-exposure history may influence the duration of the immune response against individual strains (145, 146, 234). Although early mucosal IgA (146) and T cell (145) responses may include components of a long-term protective immune response in uninfected, challenged volunteers, the role of serum IgG in protective immunity remains controversial. Norovirus challenged volunteers or outbreak patients mount strong serum IgG antibody responses that block carbohydrate receptor-VLP interactions in a genogroup-specific manner in a surrogate neutralizing assay potentially representing a component of a long-term protective immunity (91, 149). However, IgG antibody levels are usually too low in pre-challenge sera, or in salivary or fecal samples for assaying by these methods. Importantly, the years following the

emergence of a new epidemic strain in Europe were characterized by decreased numbers of outbreaks, speculated to be associated with increased herd immunity (234). If herd immunity drives GII.4 norovirus evolution, these data predict that serologic relationships among temporal GII.4 epidemic strains should change over time.

Although GII.4-1987 and GII.4-1997 VLPs differed by seven amino acids, no significant differences in antibody reactivity were noted with sera derived from humans and experimentally immunized mice, suggesting that the few amino acid changes didn't significantly alter variation between the two strains. We speculate that pre-1995 Camberwell-like strains typically produced low level endemic disease and that these mutations promoted epidemic spread of the post 1996 Lordsdale/Grimsby strains in human populations, perhaps by allowing for more efficient binding with additional HBGA ligands on mucosal surfaces. The epidemic spread of the GII.4-1997-like strains in human populations may have subsequently allowed for higher levels of herd immunity and selected for more rapid antigenic changes in future strains. Influenza virus shows similar trends in that genetic variation oftentimes, but not always, tracks with antigenic variation, because some mutations result in disproportionately large antigenic changes (235). However, global serologic responses between GII.4-1987/1997 and GII.4-2002 demonstrated significant antigenic differences, reflecting the increased number of variant residues in this later serotype. Concordant with these findings, GII.4-2004 and GII.4-2005 epidemic strains were also serologically quite distinct from GII.4-1987 and GII.4-1997 and to a lesser extent distinct from GII.4-2002. Thus, epidemic replacement strain ORF2 capsid sequences were antigenically related yet distinct due to antigenic drift. At this time, surveillance data is not sufficiently robust to determine whether the emergence

of replacement epidemic strains drives earlier isolates to extinction as seen with influenza viruses or whether these strains continue to persist at low levels and cause sporadic endemic disease in previously unexposed, susceptible individuals.

All outbreak sera blocked carbohydrate binding of GII.4-1987 and GII.4-1997 VLPs, yet only 2000 sera could completely block binding of GII.4-2002 VLP. Interestingly, the mouse anti-GII.4-2004 and GII.4-2005 sera more efficiently blocked binding of GII.4-1987 and GII.4-1997 to H type 3 than GII.4-2002 sera. However, amino acids at positions 329, 355, and 365 in GII.4-2004 and GII.4-2005 are the same as GII.4-1987 and GII.4-1997, but not GII.4.2002, which implies that these sites may account for the cross blockade of anti-GII.4-2004 and anti-GII.4-2005 sera to GII.4-1987 and GII.4-1997 carbohydrate binding. These sites may also be important determinants of antigenic variation within the GII.4 genocluster. The GII.4-2004 and GII.4-2005 strains didn't bind any HBGA carbohydrates tested, suggesting that their carbohydrate ligands are either not represented within the panel of biotinylated HBGA carbohydrates available for testing or they utilize non-HBGA mediated pathways for entry. Thus, over time, it is reasonable to predict that noroviruses have the capacity to utilize the large number of related HBGAs as receptors. The potential plasticity in the carbohydrate binding site would likely accommodate sufficient amounts of antigenic drift to escape herd immunity, while simultaneously altering strain susceptibility to the many different human alleles that regulate HBGA expression.

Fucose ligand binding site 1 was strictly conserved in the GII.4 viruses, including paradoxically, exigent strains that do not bind saliva or any carbohydrate tested. In contrast, the secondary interaction site appears to facilitate receptor specificity as binding

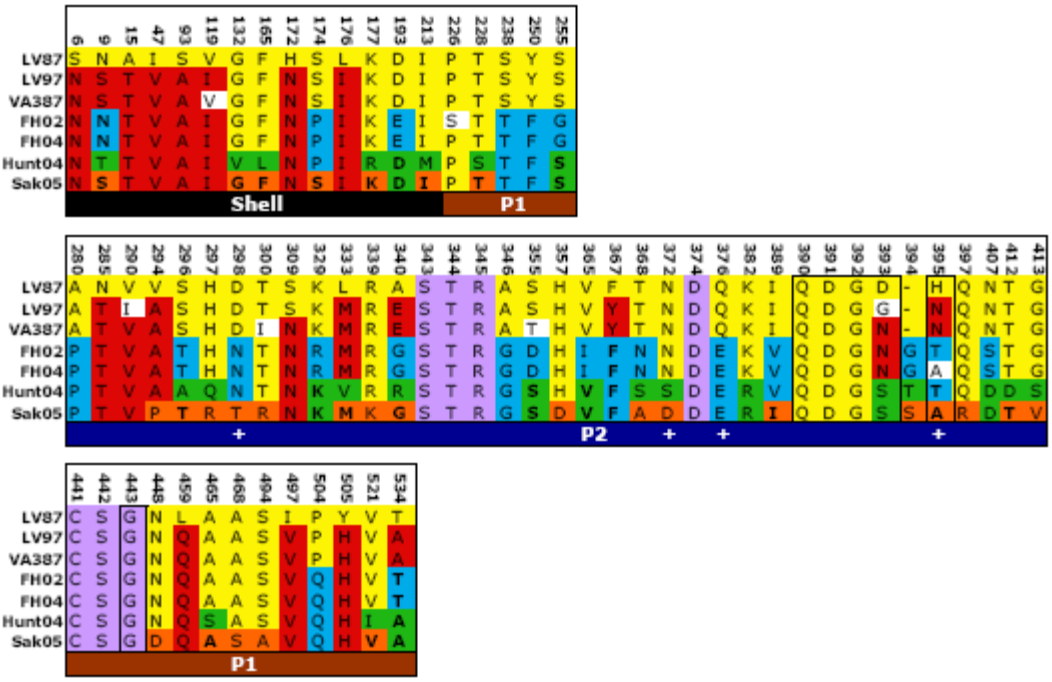
characteristics of the time ordered VLP panel varied extensively. In the secondary interaction site, positions 390, 391, 392 and 443 were conserved throughout the GII.4 strains while sites 393, 394 and 395 were variable. In two instances, binding characteristics could be directly correlated to residue changes within this region. First, based on our structural models, we predicted that carbohydrate binding would differ between the Camberwell cluster and the Grimsby cluster (including VA387) based primarily upon an Asp to Asn change at position 393 in site 2. In agreement with our hypothesis, binding between GII.4-1987 and GII.4-1997 was different. The substitution of an Asp at position 393 appears to sterically hinder or otherwise alter binding of the larger tri-saccharide moieties of A- and B- antigens, as the Camberwell representative VLP binds H type 3 and Le Y, but not A or B. In contrast, both GII.4-1997 and VA387 bind H type 3, Le Y, A, and B (113, 149); and they encode Gly and Asn at the 393 position, respectively. Interestingly, our data suggest that the primary impact of the mutations that occurred between the Camberwell and Grimsby clusters led to an expansion of receptor specificity as representative strains GII.4-1987 and GII.4-1997 were indistinguishable antigenically. In the second case, a Thr at position 395, as exhibited by GII.4-2002a, renders this VLP refractory to binding of any tested carbohydrate. Alanine at this position facilitates binding of LeY and H type 3 in GII.4-2002. These results are also in agreement with our hypothesis that microevolution in site 2 alters receptor specificity. Of note, HBGAs used to determine binding in this study were biotinylated and attached to Neutri-avidin coated plates. These synthetic HBGAs, although a useful reagent for determining binding, lack the complex structures often found *in vivo*. Larger polysaccharide moieties likely play a crucial role in receptor affinity and avidity, by interacting directly with the second interaction site.

The data presented in this manuscript provide support for the hypothesis that antigenic drift and receptor switching may function as one mechanism to maintain the GII.4 noroviruses in the presence of human herd immunity. Our data suggest that strain specific protective immunity is possible and that vaccines and immune prophylaxis must be formulated to protect against contemporary strains. As shown with influenza virus, new therapeutic formulations will be necessary every 3-5 years or so. Moreover, continued norovirus surveillance will be an essential component necessary for maintaining vaccine and drug effectiveness. At this time, it is unclear whether GII.4 noroviruses will continue to predominate as the major cause of epidemic gastroenteritis worldwide, or like influenza virus, undergo an antigenic shift to a variant GI or GII genocluster that is currently circulating at low levels in human populations, or a new strain introduced from zoonotic pools. However, important caveats must be considered when evaluating this work. While it is clear that the mucosal compartment has high concentrations of IgG, carbohydrate-VLP blockade assays use serum IgG while mucosal IgA and IgG responses may be more important in protective immunity (146, 149). Unfortunately, the mucosal antibody concentrations are usually not only insufficient for blockade studies, but mucosal antibodies were not obtained during the time-ordered norovirus outbreaks, preventing the testing of this possibility. In the absence of a robust cell culture model, blockade studies themselves represent a surrogate assay for neutralization, and it is likely that antibodies might neutralize virus infectivity by binding to regions distinct from the carbohydrate binding pocket or even outside of P2 and inhibit other steps in entry as shown with West Nile virus among others (189). Additional studies will be needed to prove these hypotheses and to determine if the evolutionary patterns are

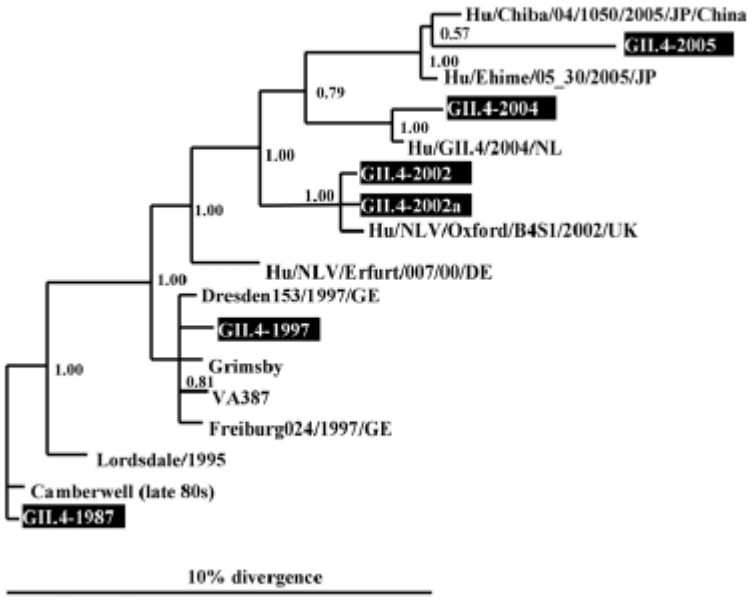
unique to the GII.4 noroviruses or represent a general evolutionary pattern of the Norovirus family. Our study, however, clearly articulates a predictive model for future empirical studies investigating the relationship between antigenic change, norovirus pathogenesis, vaccine design, and human disease.

Figure 3.1

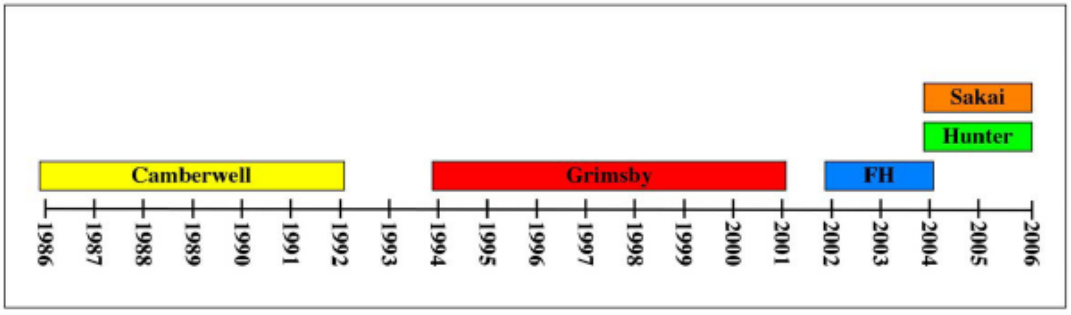
A.



B.


Hu/Chiba/04/1050/2005/JP/China
0.57
GII.4-2005
1.00
Hu/Ehime/05_30/2005/JP
0.79
GII.4-2004
1.00
Hu/GII.4/2004/NL
1.00
GII.4-2002
1.00
GII.4-2002a
Hu/NLV/Oxford/B4S1/2002/UK
1.00
Hu/NLV/Erfurt/007/00/DE
1.00
Dresden153/1997/GE
GII.4-1997
Grimsby
0.81
VA387
Freiburg024/1997/GE
1.00
Lordsdale/1995
Camberwell (late 80s)
GII.4-1987
10% divergence

C.


Sakai
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Camberwell
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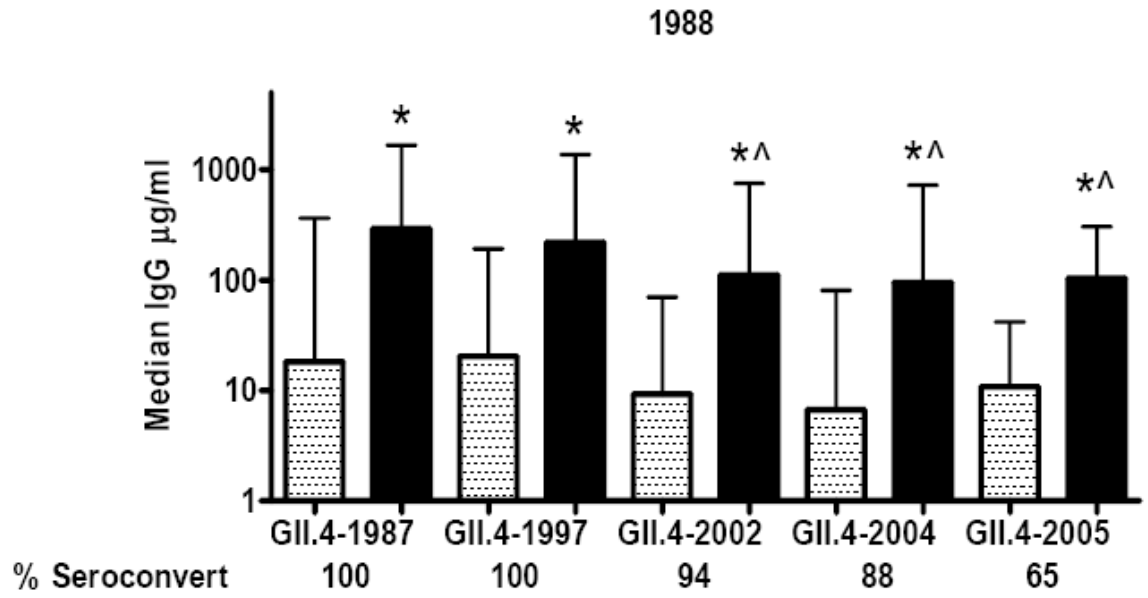
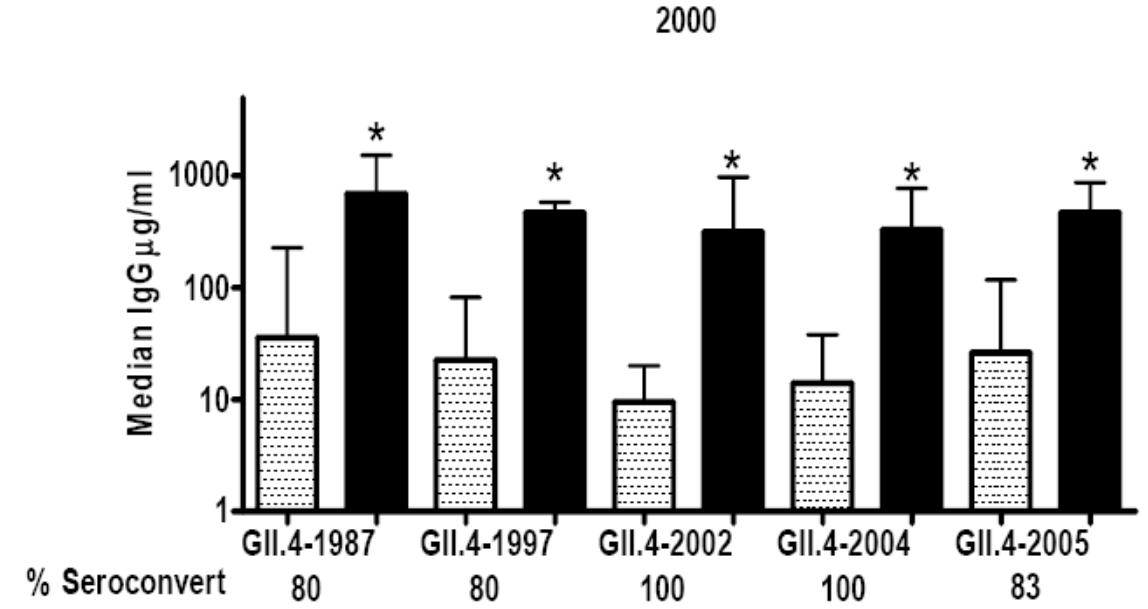
Evolutionary analysis of representative GII.4 strains from 1987 to present. **Panel A.** Five distinct evolutionary patterns were observed in the mutational profiles of the GII.4 sequences, and these are represented by: GII.4-1987, GII.4-1997, GII.4-2002, GII.4-2004, and GII.4-2005. Amino acids present in the late 1980s Camberwell cluster (GII.4-1987) are highlighted in yellow, changes that occurred to form the Grimsby (GII.4-1997) cluster are noted in red, changes associated with the Farmington Hills (GII.4-2002) cluster are blue, changes specific to the Hunter cluster (GII.4-2004) are green and substitutions important for the Sakai cluster (GII.4-2005) cluster are orange. The P2 region is highlighted in dark blue beneath the amino acids, with the N-terminal and C-terminal flanking regions of heterogeneity noted in black for the Shell domain and brown for P1. Lavender sites represent residues that hydrogen bond to the ligand at site 1, framed residues have been predicted to interact in a second stabilization domain. Amino acids operating under positive selection within P2 are shown with a plus sign in the dark blue row marking the P2 domain. Residues that are not colored represent single amino acid changes that were seen in other strains in the cluster. Strain VA387 is included for comparison, as it is a Grimsby-like virus with a solved crystal structure of the capsid. Bold residues represent amino acids which reverted to a residue from a previous cluster. **Panel B.** Phylogenic reconstruction of the GII.4 capsid sequence. Seventeen sequences selected to represent the five clusters were optimally aligned with ClustalX and the alignment was used to generate a phylogenetic by Bayesian Inference using Mr. Bayes3.12. Node confidence is presented as posterior probabilities. This tree shows a linear evolutionary relationship among the five clusters indicated by the representative sequences (shown in white text on black background). This observation supports the hypothesis that the GII.4 strains are evolving via episodic antigenic drift. **Panel C.** Analysis of the representative clusters over time. Although the sequence information available from GenBank is sporadic and does not provide enough information to completely reconstruct the evolution and emergence of the GII.4 strains, our data suggest that episodic antigenic drift facilitates linear replacement of epidemic GII.4 strains every 3-5+ years. A comparison of the span of each cluster indicates that each ranges from 3-8 years, with overlap likely occurring between clusters. This suggests that epidemic strains emerge from one cluster and eventually out-compete with the extant strain to evolve a new cluster. There are not enough sequences to fully establish the cluster boundaries and the extant clusters are likely still evolving. Yellow, sequences from Camberwell (GII.4-1987) cluster; red, sequences from Grimsby cluster (GII.4-1997); blue, sequences from the Farmington Hills (GII.4-2002) cluster; green, sequences from the Hunter cluster (GII.4-2004); and orange, sequences from the Sakai cluster (GII.4-2005).

Figure 3.2

A.


1988
Median IgG µg/ml
1000
100
10
1
*
*
*^
*^
*^
GII.4-1987
GII.4-1997
GII.4-2002
GII.4-2004
GII.4-2005
% Seroconvert
100
100
94
88
65

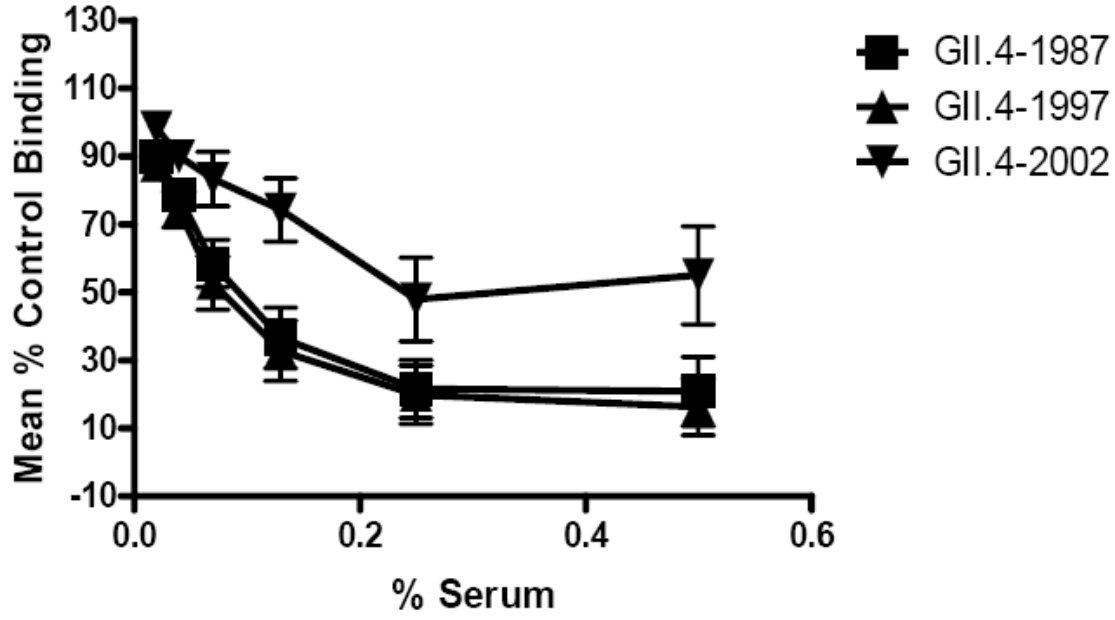
B.


2000
Median IgG µg/ml
1000
100
10
1
*
*
*
*
*
GII.4-1987
GII.4-1997
GII.4-2002
GII.4-2004
GII.4-2005
% Seroconvert
80
80
100
100
83

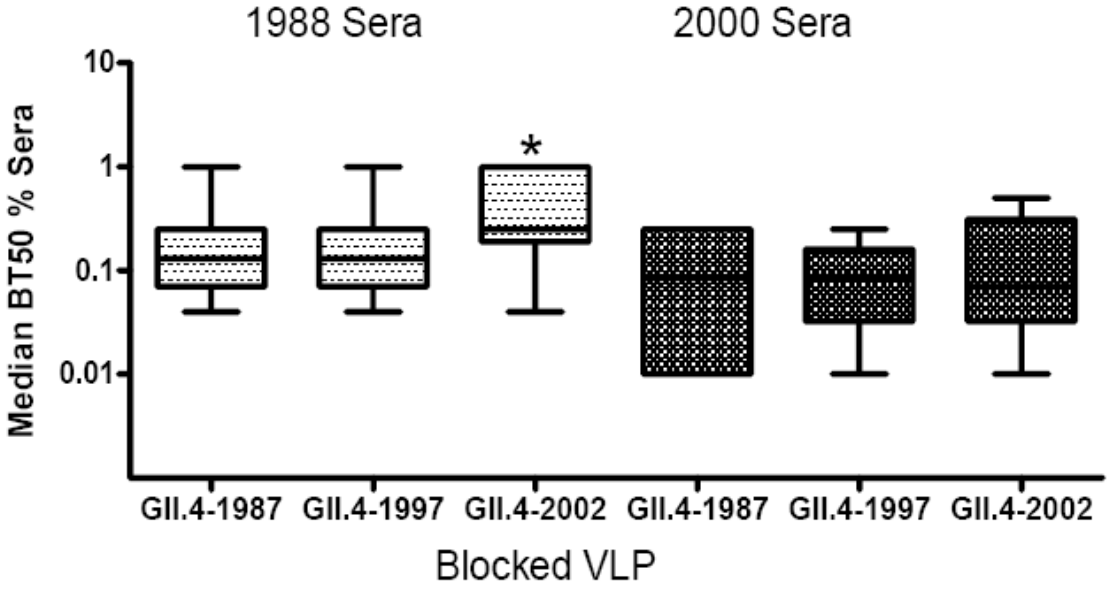
Anti-GII.4 VLP IgG titers. The median geometric mean titer of anti-VLP IgG (⌠g/ml) for acute (dotted bars) and convalescent (solid bars) serum samples was assayed for the 1988 (**Panel A**), and 2000 (**Panel B**) GII.4 outbreaks and the percentage of subjects who seroconverted to each VLP determined. * Significant increase in titer between acute and convalescent samples. ^ Significant difference between convalescent titers compared to LV87. Error bars represent the range of the data.

Figure 3.3

A.

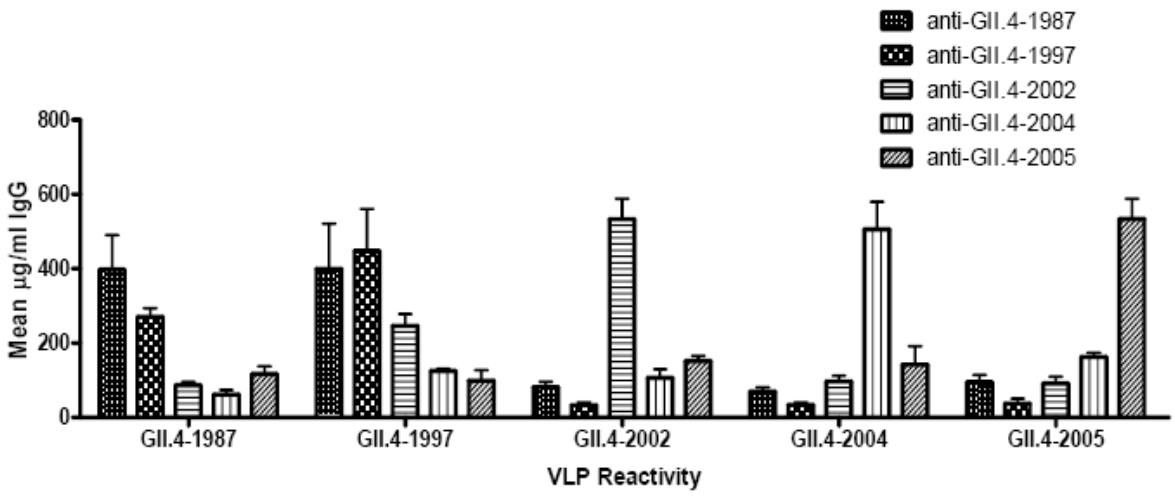

Mean % Control Binding
130
110
90
70
50
30
10
-10
0.0
0.2
0.4
0.6
% Serum
GII.4-1987
GII.4-1997
GII.4-2002

B.


1988 Sera
2000 Sera
Median BT50 % Sera
10
1
0.1
0.01
*
GII.4-1987
GII.4-1997
GII.4-2002
GII.4-1987
GII.4-1997
GII.4-2002
Blocked VLP

Blockade of GII.4 VLPs binding to HBGA by outbreak sera. Convalescent serum collected from a GII.4 outbreak in 1988 (**Panel A**) were assayed for blockade of GII.4-1987 and GII.4-1997 interaction with H type 3, or GII.4-2002 interaction with LeY bound to avidin-coated plates and the median % control binding calculated compared to the no-serum control. Error bars represent SEM. The box plot (**Panel B**) shows the median % sera needed for BT50 for each outbreak and each VLP; the whiskers show the 25-75 percentiles and the error bars represent the minimum and maximum. Outbreak sera BT50 responses significantly different from GII.4-1987 or GII.4-1997 are marked with an *.

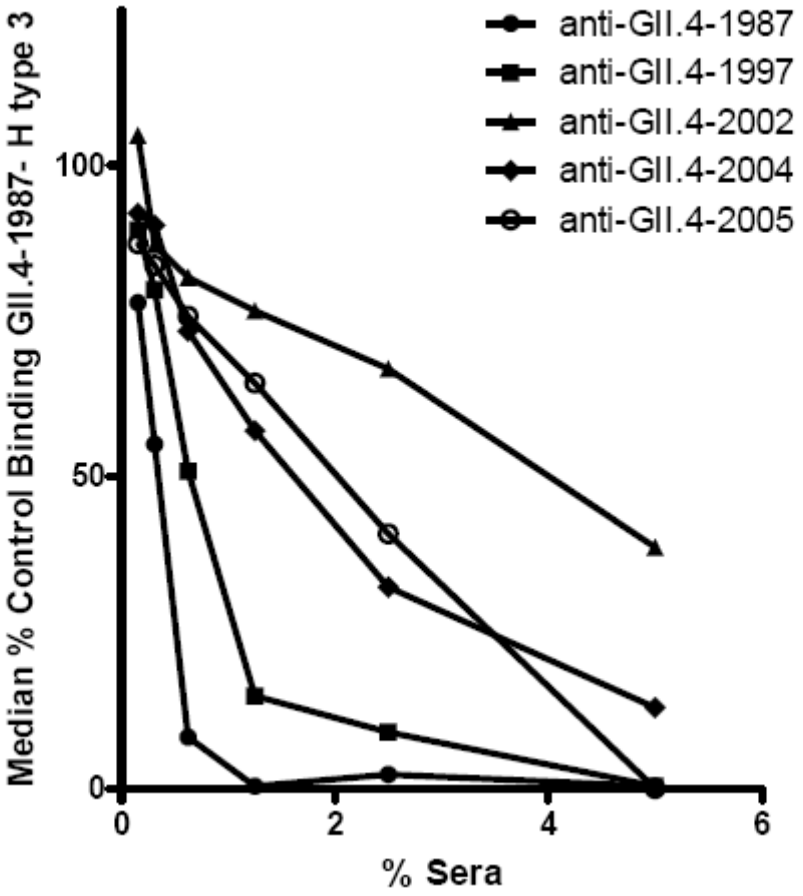
Figure 3.4



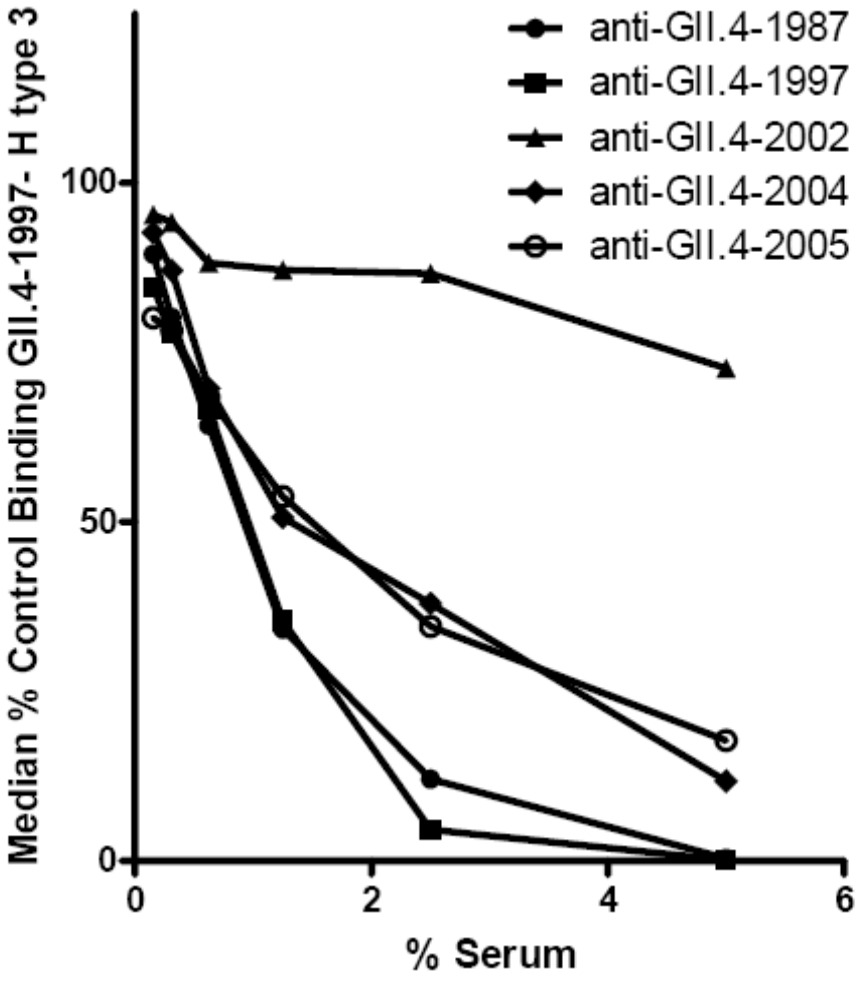
Murine antisera cross-reactivity to GII.4 VLPs. Mice were immunized by footpad inoculation on day 1 and day 21with 2.5 x 10^6 IU VRPs expressing GII.4-1987, GII.4-1997, GII.4-2002, GII.4-2004, GII.4-2005 ORF2 (N=4 per inoculation group). Antisera were collected on day 35 and analyzed for homotypic and heterotypic IgG responses to each VLP by ELISA. Antibody titers are represented as geometric mean µg/ml serum IgG.

Figure 3.5

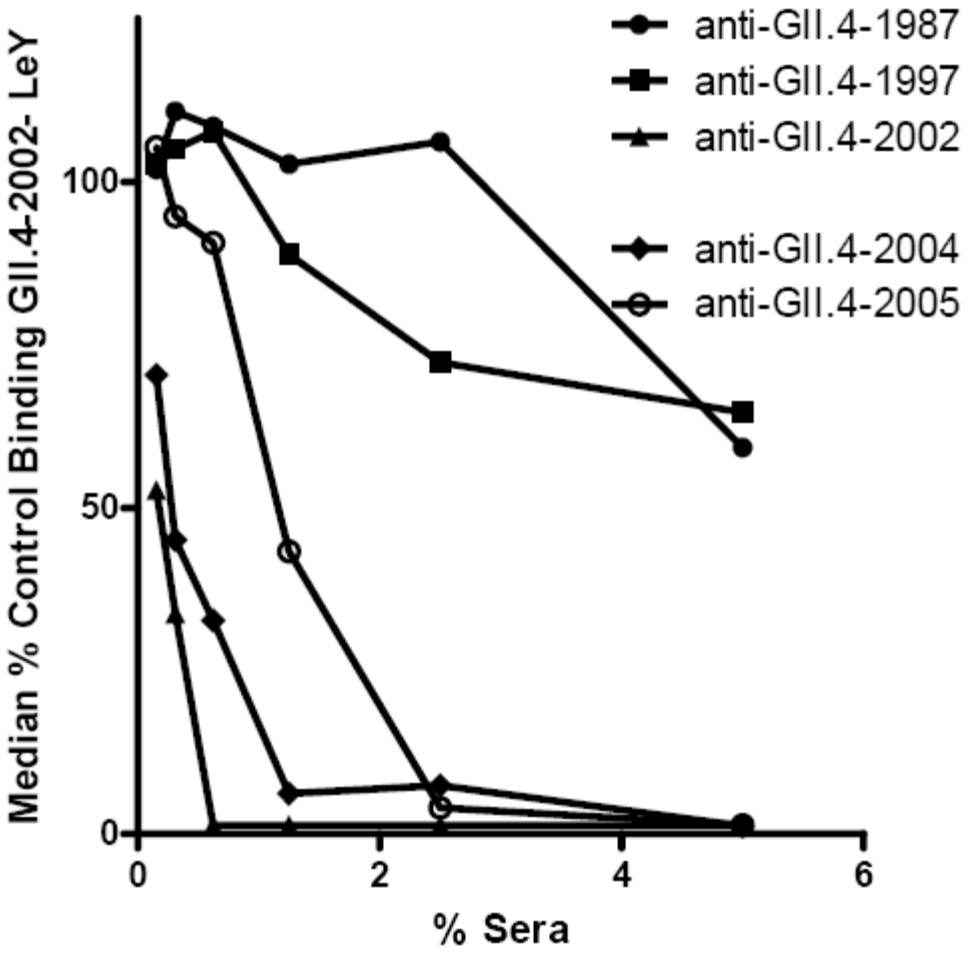
A.


anti-GII.4-1987
anti-GII.4-1997
anti-GII.4-2002
anti-GII.4-2004
anti-GII.4-2005
Median % Control Binding GII.4-1987- H type 3
100
50
0
0
2
4
6
% Sera

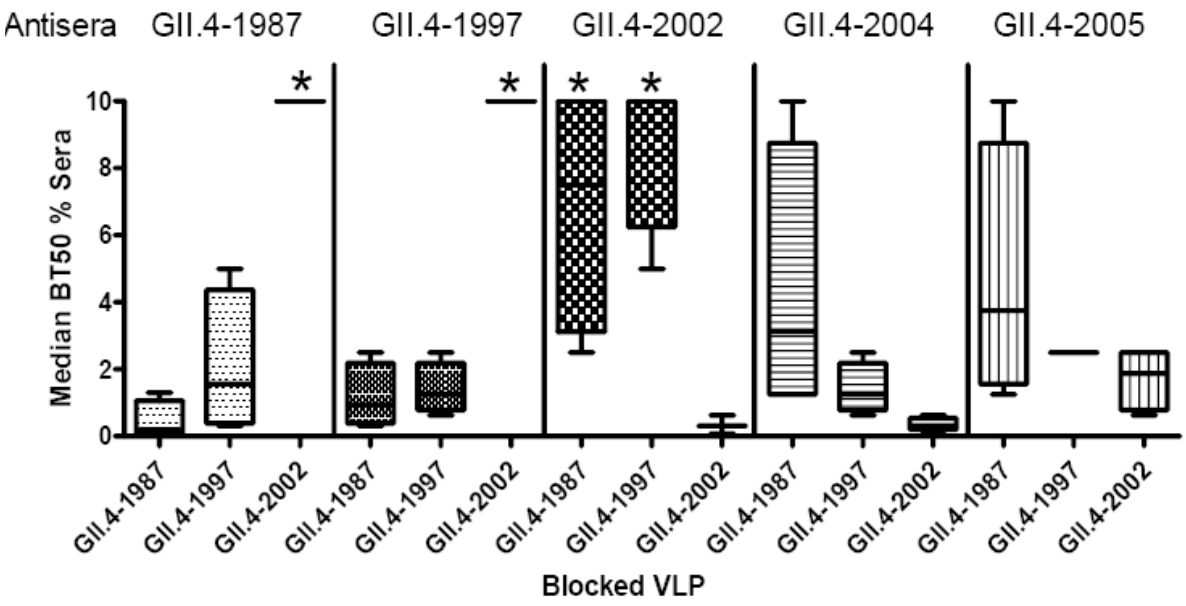
B.


anti-GII.4-1987
anti-GII.4-1997
anti-GII.4-2002
anti-GII.4-2004
anti-GII.4-2005
Median % Control Binding GII.4-1997- H type 3
100
50
0
0
2
4
6
% Serum

C.


anti-GII.4-1987
anti-GII.4-1997
anti-GII.4-2002
anti-GII.4-2004
anti-GII.4-2005
Median % Control Binding GII.4-2002- LeY
100
50
0
0
2
4
6
% Sera

D.



Murine antisera blockade of GII.4 VLP binding to HBGAs. Antisera collected from mice immunized against each GII.4 ORF2 were assayed for blockade of GII.4-1987 (**Panel A**) and GII.4-1997 (**Panel B**) interaction with H type 3, or GII.4-2002 (**Panel C**) interaction with LeY bound to avidin-coated plates and the median % control binding calculated compared to the no-serum control. The box plot (**Panel D**) shows the median % sera needed for BT50 for each antisera and each VLP; the whiskers show the 25-75 percentiles and the error bars represent the minimum and maximum. Antisera groups that did not block 50% VLP-HBGA binding at the highest serum concentration tested (5%) was assigned an arbitrary value of 10%. VLP requiring significantly less serum for BT50 compared homologous sera are designated with an *.

CHAPTER IV.

Identification of unique norovirus $CD4^+$ T cell epitopes in Norwalk virus and Farmington Hills virus capsid sequences

Abstract

The mechanisms governing norovirus immunity in humans are unknown. Homotypic antibody responses following norovirus infection have been characterized; however, T cell responses remain largely undefined. We immunized mice with alphavirus vectors expressing Norwalk virus or Farmington Hills virus-like particles to examine T cell responses following norovirus antigen exposure. Depletion of splenic $CD4^+$ or $CD8^+$ T cells followed by stimulation with norovirus VLPs revealed a predominantly $CD4^+$ T cell response. $CD4^+$ T helper 1 (T_H1) and T_H2 cell-specific cytokine profiles were examined, and splenocytes secreted high amounts of interferon-γ (IFN-γ) following stimulation, indicating a T_H1-mediated response. Surprisingly, stimulation with norovirus VLPs from heterologous strains also resulted in high IFN-γ secretion, suggesting T cell responses may be cross-reactive between norovirus strains. Stimulation of splenocytes with overlapping peptides from complete libraries of the NV or FH capsid proteins further revealed specific amino acid sequences containing T cell epitopes that were conserved within genoclusters and/or genogroups. Identification of unique strain-specific epitopes will allow detailed studies into the cross-reactive responses elicited by genetically related and unrelated norovirus strains and provide insight into T cell mediated norovirus immunity.

Introduction

Norovirus infection is characterized by the induction of both humoral and cellular immune responses. Humoral immunity in humans following norovirus infection has been described in detail to a limited number of norovirus strains (52, 70, 86, 145, 259). Humans mount specific antibody responses to the infecting strain, which bears limited cross-reactive yet undefined epitopes to other strains within or across genogroups (180, 259). Short-term immunity following homologous norovirus challenge has been documented, but long term immunity remains controversial (119, 203). Furthermore, no studies have demonstrated cross-protection following heterologous norovirus challenge to date (281). While susceptible individuals can become reinfected with multiple norovirus strains throughout their lifetime, the mechanism of short-term protection and the impact of previous exposures on susceptibility to reinfection remain unknown.

The role of T cells in controlling norovirus infection also remains largely undefined and unexplored. A single comprehensive study detailing immune responses in Snow Mountain virus-infected individuals revealed that $CD4^+$ T_H1 cells can be stimulated by VLPs to secrete IFN-γ and IL-2 (145). Furthermore, heterologous stimulation from VLPs derived from different norovirus strains within but not across genogroups also induced significant IFN-γ production compared to uninfected individuals (145). Vaccination of humans with VLPs also results in short-term induction of IFN-γ (247).

Because norovirus infection studies in humans are confounded by previous exposure histories, the use of inbred mice maintained in pathogen-free environments allow for the study of norovirus immune responses on a naive background. While mice cannot be infected with human norovirus strains, VLP vaccines induce comparable immune responses that can

be measured and studied. Mice immunized orally or intranasally with VLP vaccines in the presence of adjuvant similarly induced $CD4^+$ IFN-γ responses in Peyer's patches and spleen (178, 207). Induction of $CD8^+$ T cells and secretion of the T_H2 cytokine IL-4 were separately noted; however, it is unclear if these responses were influenced by the presence of VLPs or coadministered vaccine adjuvants (178, 207).

To advance our understanding of the scope of the cellular immune response, we immunized mice with VEE replicon particles (VRPs) expressing norovirus VLPs derived from the Norwalk (GI.1) or Farmington Hills (GII.4) strains and analyzed splenocytes for T cell subset activation, cytokine secretion, heterologous VLP stimulation and epitope identification.

Materials and methods.

VRP immunization. VRPs were produced as described in Harrington *et al.* (93). 6 week old BALB/c mice (N=4/experiment) were immunized with 2.5 x 10^6 VRP expressing Norwalk virus (NV) VLPs, Farmington Hills virus (FH) VLPs, influenza hemagglutinin (HA) or a short noncoding sequence (null VRP) at day 0 and 21. Sera and tissue were harvested on day 42.

Splenocyte stimulation. Spleens were harvested three weeks post-boost. Individual splenocytes were obtained by manual separation, filtration, and lysis of red blood cells. $CD4^+$ and $CD8^+$ T cells depletions were performed using QuadroMACS magnetic bead separation (Miltenyi) per manufacturer's instructions. Cells were cultured at 1 x 10^6 cells/well in 96 well cell culture treated plates in 100 μl complete RPMI media containing one of the following: VLP (1 μg/ml), inactivated MNV or VRP (1 μg/ml), individual NV or

FH peptides or peptide pools (1 μg/ml each), Concanavalin A (5 μg/ml), or no stimulant. Splenocytes were cultured for 48 h at which time supernatants were harvested and stored at -80° C. VLPs were produced as described in LoBue *et al.* (149). Complete overlapping 15mer (+5) peptide libraries spanning the entire NV and FH capsids were synthesized at the UNC peptide synthesis core facility. Lyophilized peptides were resuspended in PBS; insoluble peptides were further dissolved in 1:1 acetonitrile:PBS.

ELISA and cytometric bead array. Sera samples were tested for IgG subtype reactivity to NV or FH VLPs by indirect ELISA as previously described (149). Purified IgG1 (Sigma) or IgG2a (Sigma) were used as assay standards. IFN-γ concentrations in splenocyte culture supernatants were determined by EIA (BD Pharmingen); TNF-α, IL-2, IL-4, and IL-5 concentrations were determined using the T_H1/T_H2 cytokine cytometric bead array (BD Pharmingen) per manufacturer's instructions. T-tests were performed to measure statistical differences between sample groups of two. All other statistics were performed using One-way ANOVA and Tukey post-test.

Sequence alignments. Stimulatory sequences identified using 15-mer peptides within the NV and FH capsids were aligned with sequences representing the entire VLP panel using Vector NTI software.

Results

$CD4^+$ T cells exhibit heterologous VLP stimulation.

Comprehensive T cell cross-reactivity analyses have not been reported across norovirus strains, which can differ genetically by >50% in the capsid amino acid sequence (75). Because human samples from heterologous outbreaks with which to conduct these

studies are scarce, we have cloned the antigenic ORF2 capsid gene from multiple heterologous strains into alphavirus vectors that, when expressed, form VLPs that can be simultaneously used as antigenic reagents and to induce immune responses in mice. Cellular immune responses following norovirus immunization with VRP are also unknown.

Previous reports describe $CD4^+$ T_H1-like immune induction following norovirus infection in humans and VLP immunization in mice (145, 178). To determine if $CD4^+$ or $CD8^+$ T cells are responsible for IFN-γ secretion following immunization with VRP-NV, splenocytes were depleted of $CD4^+$ or $CD8^+$ T cells. Depleted cell suspensions were stimulated with NV VLPs, and unstimulated cells were cultured in parallel. Supernatant was then collected, IFN-γ secretion measured by EIA, and background IFN-γ levels from unstimulated cultures subtracted. Splenocytes depleted of $CD8^+$ T cells secreted significantly higher amounts of IFN-γ than $CD4^+$ depleted splenocytes ($P<0.001$; Fig. 4.1), suggesting IFN-γ secretion following VRP immunization are mediated by $CD4^+$ cells.

To determine if T cell responses could be induced by heterologous VLP stimulation, splenocytes from VRP-NV immunized mice were harvested and cultured with homologous NV VLPs or GI VLPs derived from Southampton (SH), Chiba (CV), or Desert Shield (DS) strains, GII VLPs derived from Snow Mountain (SM), Hawaii (HV), Lordsdale (LV), Toronto (TV), or M7 strains. Unstimulated cells were used as negative controls. Supernatants were harvested after 48 h and tested for IFN-γ levels by EIA. All GI VLPs and the one GII VLP induced significantly more IFN-γ secretion than unstimulated controls ($P<0.01$; Fig. 4.2). Stimulation with other GII VLP did not induce IFN-γ levels different from stimulation with GI VLPs or negative controls. Interestingly, heterologous GI stimulation was not different from homologous NV VLP stimulation. These data suggest

that cross-reactive T cell responses to human norovirus strains can be induced following norovirus vaccination, with highest cross-reactivity to genetically related strains within a genogroup.

To determine if additional T_H1 cytokines are also induced following VLP stimulation, supernatants from VRP-NV splenocyte cultures stimulated with NV VLP, heterologous GI VLPs (CV), heterologous GII VLPs (HV) were pooled and analyzed for secretion of T_H1/T_H2 cytokines TNF-α, IFN-γ, IL-2, IL-4, and IL-5 by CBA. Homologous stimulation with NV VLPs resulted in secretion of the T_H1 cytokines TNF-α (420 pg/ml), IFN-γ (1975 pg/ml), and IL-2 (150 pg/ml) that were 2.7-, 8.4-, and 7.7-fold higher, respectively, than unstimulated controls (Table 4.1). The T_H2 cytokine IL-4 was induced to much lower levels (26.8 pg/ml) but was higher (3.4-fold) than unstimulated controls. IL-5 secretion was not different between NV VLP and unstimulated cultures. Fold-increase in cytokine levels following VLP stimulation compared to unstimulated controls closely mirrored those reported in human challenge studies from infected individuals compared to matched pre-challenge controls (145). Heterologous VLP stimulation induced cytokine profiles equivalent to homologous stimulation. These data support previous findings that noroviruses induce T_H1-type responses following antigen exposure.

To rule out non-specific stimulatory effects that may be induced by immunization with VRPs or stimulation with VLPs, mice were additionally immunized with VRP expressing the irrelevant hemagglutinin (HA) protein, and unimmunized mice were treated in parallel. Splenocytes were stimulated with NV VLPs, UV-inactivated MNV (i-MNV), or i-VRP and tested for IFN-γ secretion by EIA. Splenocytes from VRP-NV immunized mice secreted significantly higher amounts of IFN-γ following VLP stimulation than VRP-HA or

unimmunized controls (P<0.001; Fig. 4.3). However, control VRP-HA cultures also mounted a stimulatory response to NV VLPs compared to unimmunized mice (P<0.05), and both VRP immunized groups secreted equivalent and significant IFN-γ responses following stimulation with i-VRP (P<0.001), which acts as a non-replicating control for VEE-specific antigen stimulation, compared to unimmunized cultures. These data suggest that VRP immunization results in specific IFN-γ responses to the VRP antigens and non-specific stimulation to VLPs.

Stimulation with overlapping peptide libraries facilitates identification of T cell epitopes.

Because stimulation with VLPs did not reveal distinct cross-reactivity profiles to heterologous norovirus strains, we generated overlapping peptide libraries reconstituting the capsid protein of the Norwalk (GI.1) and Farmington Hills (GII.4) strains. To determine if overlapping peptides could induce IFN-γ responses, we immunized mice with VRP-NV, VRP-FH, or null VRP and harvested splenocytes. Splenocytes from unimmunized mice were treated in parallel. Peptide libraries were sequentially divided into 5 overlapping pools, as shown in Table 4.2. Splenocytes were stimulated with individual peptide pools composed of 1 μg/ml each peptide and supernatants analyzed for IFN-γ by EIA or for additional T_H1/T_H2 cytokines TNF-α, IL-2, IL-4, and IL-5 by CBA.

Splenocytes from VRP-NV immunized mice secreted significantly higher levels of IFN-γ following stimulation with NV peptide pool 1 (amino acids 1-105; Table 4.2) compared to other NV peptide pools or controls (P<0.001; Fig. 4.4a). Immunization with null VRP, which does not express a transgene from its internal promoter, did not result in

splenocyte stimulation by NV peptides (Fig. 4.4a). Sequential overlapping peptide pools consisting of two peptides each from NV peptide pool 1 were then used to stimulate splenocytes from VRP-NV immunized mice (Fig. 4.4b). Pools containing NV peptide 9 were positive for IFN-γ stimulation compared to all other overlapping peptides ($P<0.01$). Individual and sequentially overlapping peptides flanking peptide 9 were used to confirm this finding (Fig. 4.4c). These data suggest a stimulatory $CD4^+$ T cell epitope resides in the shell domain of the NV capsid within sequence FDLSLGPHLNPFLLH spanning amino acids 81-95 (Fig. 4.4d).

Splenocytes from VRP-FH immunized mice secreted significantly higher levels of IFN-γ following stimulation with FH peptide pool 5 (amino acids 431-540; Table 4.2) compared to other FH peptide pools ($P<0.001$; Fig. 4.5a). Cultures from mice immunized with null VRP or unimmunized controls were not stimulated by FH pool 5 ($P<0.001$; Fig. 4.5a). Sequentially overlapping pools and individual peptides within FH peptide pool 5 were then used to stimulate splenocytes from VRP-FH immunized mice as described above (Fig. 4.5b-c). Overlapping peptides 45-46 induced significantly higher levels of IFN-γ than peptides 46-47 ($P<0.01$) and all other overlapping groups ($P<0.001$; Fig. 4.5b). Peptides 46-47 and 47-48 also induced higher levels than all remaining overlapping groups ($P<0.001$). Upon closer evaluation, stimulation with individual or overlapping peptides containing peptide 46 induced significant IFN-γ responses compared to individual peptides 45 or 48 ($P<0.05$) but not peptide 47 (Fig. 4.5c). Stimulation with individual or overlapping peptides containing peptide 47 also induced IFN-γ secretion that was elevated above peptides 45 or 48 stimulation but was not significant. Together, these data suggest that peptides 46 and 47

spanning amino acids 461-485 of sequence CLLPQEWVQHFYQEAAPAQSDVALL in the P1 capsid domain contain 1-2 distinct T cell stimulatory epitopes (Fig. 4.5d).

Supernatants from original peptide pool stimulations analyzed by CBA also revealed elevated levels of TNF-α. NV cultures stimulated with NV pool 1 and FH cultures stimulated with FH pool 5 secreted TNF-α 2-fold and 4-fold, respectively, over unstimulated pools, verifying a T_H1-type response to peptide stimulation (Table 4.3). Levels of IL-2 or the T_H2 cytokines IL-4 and IL-5 were not elevated compared to unstimulated controls. Supernatants from null VRP and unimmunized control cultures were not stimulated by any norovirus peptide pools (data not shown). Sera from VRP-NV and VRP-FH immunized mice were also tested for IgG subclass responses to NV or FH VLPs, respectively. IgG1 and IgG2a titers were not different from one another in either immunization group (Figure 4.6).

Stimulatory sequences are conserved within genoclusters and within genogroup I.

Stimulatory sequences identified within the NV and FH capsids by overlapping peptide stimulation were aligned with corresponding sequences representing our panel of VLPs. Alignments revealed that sequences from the two NV-like strains tested within the GI.1 genocluster were completely conserved (Fig. 4.7A). Furthermore, the stimulatory sequence was also highly conserved (86.7%) across all other GI sequences tested but was only 46.7% conserved among GII sequences.

Stimulatory FH sequences were highly conserved (88%) within the GII.4 genocluster among six strains tested, and the LV97, FH02, and FH04 sequences, representing three sequential evolutionary strains, were identical. However, sequences to additional strains within GII (60%) or in GI (40%) were much less conserved (Fig. 4.7B).

Not surprisingly, cross-stimulation of NV-immune splenocytes with stimulatory FH pool 5 and FH-immune splenocytes with stimulatory NV pool 1 did not result in IFN-γ secretion (Fig. 4.8), suggesting epitopes are not conserved across genogroups due to lack of sequence identity.

Discussion

The components of protective immunity are unknown. Understanding the mechanisms governing norovirus immunity is of utmost importance for development of norovirus vaccines and phophylactics. Two vaccine strategies for norovirus immune induction are currently being evaluated: 1) Oral or intranasal administration of VLPs, and 2) VRP expression of VLP *in vivo* following subcutaneous administration. Oral administration of VLP vaccines in humans, while safe and immunogenic, have been shown to induce very weak humoral and cellular immune responses to norovirus compared to norovirus infection (247). In contrast, VRPs induce strong mucosal, cellular, and humoral immune responses to the expressed transgene, including norovirus VLP (45, 93, 256).

The emergence of literature documenting T cell responses following norovirus infection in humans or immunization in mice suggests that the cellular immune response is a potentially important component in noroviruses vaccine design. Findings from our group and others support $CD4^+$ T_H1 T cells as likely candidates involved in the cellular immune response to norovirus challenge [(145, 178, 207, 247) and Table 4.1]. Our results did not show that VRP vaccination elevated serum IgG2a antibody subclass responses over IgG1 levels (Fig. 4.6), which can be reflective of a T_H1 response; however, T_H1 cytokines IFN-γ and TNF-α but not T_H2 cytokines were elevated in splenocyte culture supernatants following

stimulation with norovirus VLPs or peptides (Fig. 4.2, 4.4, 4.5, Table 4.1 and 4.3). Previous work reported by our group also showed that VRPs induce IgA and IgG responses at mucosal surfaces that can block receptor binding (149). Together these data suggest that norovirus vaccination by VRP may be a promising strategy for individuals in high exposure risk environments such as hospitals and primary care facilities.

To facilitate vaccine design, we have begun to characterize T cell responses to homologous and heterologous norovirus capsid peptides following VRP immunization in the mouse. While we cannot predict if murine and human T cell epitopes are conserved across species, we can determine murine-specific epitopes and their relative conservation across genetically related norovirus strains. This information will likely be useful in determining if previous exposure or vaccination to heterologous strains can confer T cell-mediated protection upon subsequent challenge.

Our results indicate that T cell responses following VRP vaccination to one norovirus strain are cross-reactive to heterologous norovirus VLPs, with highest cross-reactivity to genetically related strains within a genogroup (Fig. 4.1). These data support previous findings by Lindesmith *et al.* showing that peripheral blood mononuclear cells (PBMCs) from humans infected with a GII strain cross-reacted to stimulation with VLPs from another GII strain but less so to GI VLPs (145). Because unknown pre-exposure histories in humans can skew cross-reactivity profiles and are highly variable between individuals, we used norovirus-naïve mice to provide a clearer view of T cell cross-reactivity between strains. We were surprised to discover, however, that intra-genogroup VLPs were able to stimulate IFN-γ secretion to the same degree as homologous VLPs from the immunizing strain, and cross-reactivity by inter-genogroup VLPs was also present. However, splenocyte cultures from

mice immunized with VRP expressing an irrelevant protein were also stimulated by norovirus VLP (Fig. 4.3). UV-inactivated MNV (i-MNV), which consists of intact capsid particles not produced by the VRP expression system, were used as a control for VLP stimulation. Because VRP-HA cultures were stimulated by VLP but not i-MNV and because cultures from all VRP immunized mice were stimulated with i-VRP, we believe background stimulation by VRP antigens, including VLPs produced using the VRP expression, may be skewing cross-reactivity results. The impact of VRP immunization independent of norovirus antigen delivery is beyond the scope of our research; however, it has been documented that $CD4^+$ T cells are activated and IFN-γ produced following alphavirus infection in mice (22, 77, 227), suggesting residual VRP-induced IFN-γ secretion could also occur. In addition, strains within norovirus genogroups can still differ by up to 40% in capsid amino acid sequence; therefore, we would not expect equivalent stimulation to heterologous GI VLPs in NV immune mice.

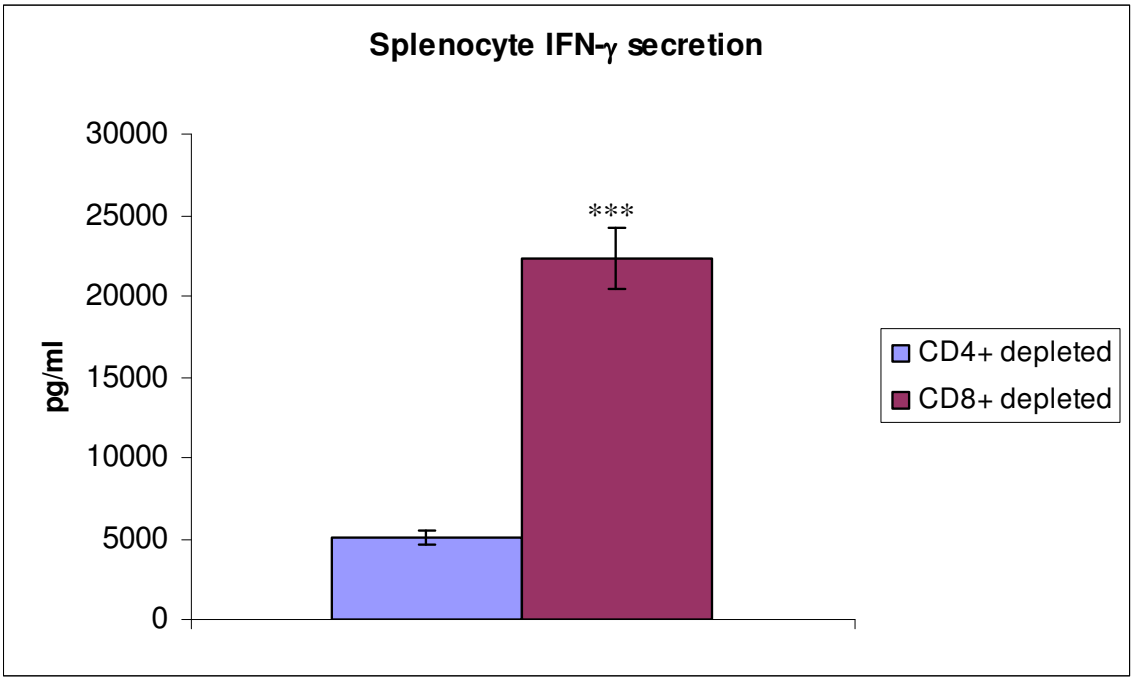
To circumvent the potential problem of background VLP cross-reactivity induced by the VRP vector, we turned to synthetically produced overlapping peptides reconstituting norovirus capsid sequences. Peptides can be used not only to determine cross-reactivity between strains but also to identify specific T cell epitopes, making them particularly advantageous. Stimulation with peptides resulted in robust IFN-γ secretion from identifiable pools (Fig. 4.4A and 4.5A); however, cross-reactivity was not evident between NV and FH strains (Fig. 4.8). Stimulatory sequences were located in different domains of the capsid for NV and FH. The amino sequence FDLSLGPHLNPFLLH located in the shell domain was found to be stimulatory to NV-immune splenocytes, and the sequence

CLLPQEWVQHFYQEAAPAQSDVALL located in the P1 domain was found to be stimulatory to FH-immune splenocytes.

Alignments of capsid sequences of additional GI.1 and GII.4 strains to the stimulatory sequences within NV and FH, respectively, revealed high homology to additional strains (100% and 88%, respectively), suggesting T cell epitopes are conserved within a genocluster and are likely to activate a memory T cell response upon subsequent challenge with genetically related strains. In contrast, additional GI sequences outside the GI.1 genocluster were also highly conserved (86.7%) compared to the stimulatory NV sequence whereas heterologous GII sequences outside the GII.4 genocluster were not highly conserved (60%) to the stimulatory FH sequence. These findings may explain why GII strains are more globally predominant than GI strains, assuming previous exposure lends some level of immunity upon subsequent challenge. Alternatively, presumed higher variability within the GII.4 cluster and heterologous GII sequences may simply be the result of a higher number of strains included in the alignment compared to GI. Likewise, the shell (S) domain, where the stimulatory NV sequence resides, is typically more highly conserved across strains than the latter part of the P1 domain (88), where the stimulatory FH sequence resides. Sequences were not conserved across genogroups, which is supported by our findings that stimulatory NV peptides did not stimulate FH-immune splenocytes and vice versa (Fig. 4.8). Identification of the exact T cell epitopes within stimulatory peptide sequences and heterologous immunization followed by epitope stimulation of lymphocytes will determine the extent of true cross-reactive T cell responses. Defining specific epitopes for T cell activation on distinct norovirus capsids will reveal unknown antigenic and immunogenic relationships among norovirus strains. Epitopes for $CD4^+$ T cell activation in mice may be

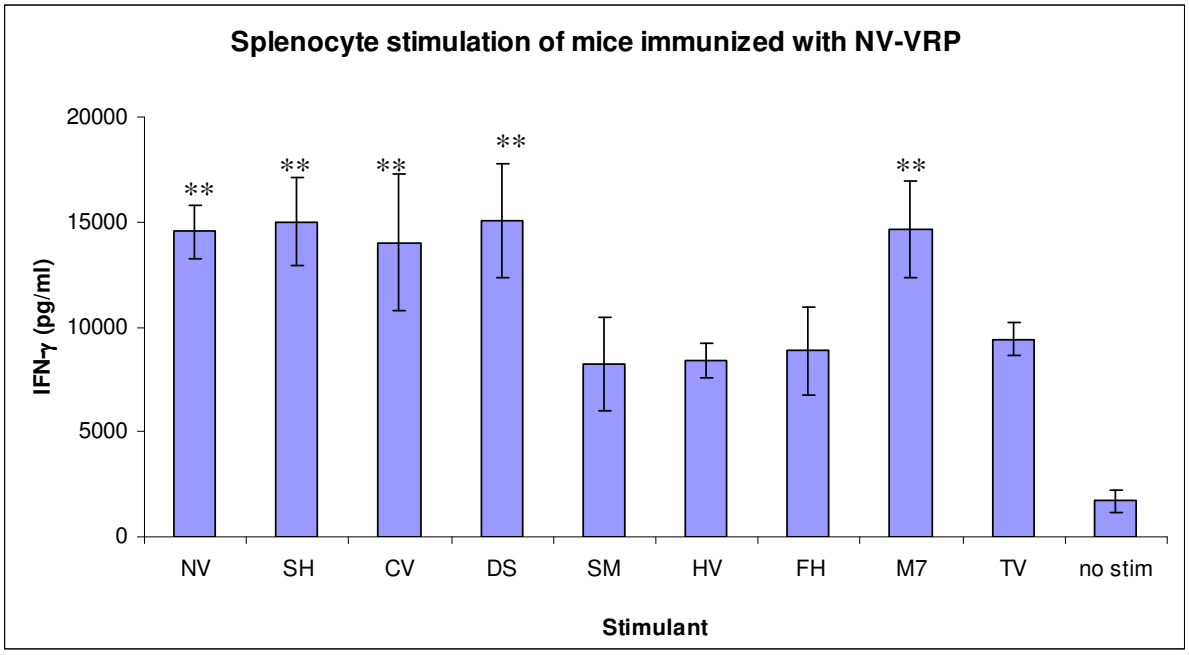
different than that in humans; however, the mechanism(s) of protection following infection are likely similar. The newly developed mouse norovirus challenge model will allow continued investigation into the effect of the T cell response following homologous or heterologous norovirus exposure as well as a system in which to evaluate the efficacy of preventative therapies triggering T cell-specific responses. These preliminary studies will provide previously unattainable answers to questions that can bridge to important issues in human medicine.

Figure 4.1



CD4$^+$ and CD8$^+$ depleted splenocyte stimulation. CD4$^+$ or CD8$^+$ T cells were depleted from splenocyte preparations from mice immunized with VRP-NV using marker-specific depleting antibodies conjugated to magnetic beads (Miltenyi). Depleted splenocytes were stimulated with NV VLP for 48 h and supernatants analyzed for IFN-γ by EIA. CD8 depleted cultures secreted significantly more IFN-γ than CD4 depleted cultures ($P<0.001$). CD8 depleted cultures secreted significantly more IFN-γ upon stimulation with NV VLPs than unstimulated controls ($P<0.01$). Background IFN-γ secretion from unstimulated cultures was subtracted from total values.

Figure 4.2



Splenocyte stimulation with norovirus VLP panel. Splenocytes from mice immunized with VRP-NV were stimulated with 5 μg/ml VLP from one of nine norovirus strains from GI or GII. After incubation for 48 h, culture supernatants were analyzed for IFN-γ by EIA (BD Pharmingen). GI strains NV, SH, CV, and DS and GII strain M7 stimulated IFN-γ secretion significantly more than unstimulated cultures ($P<0.01$).

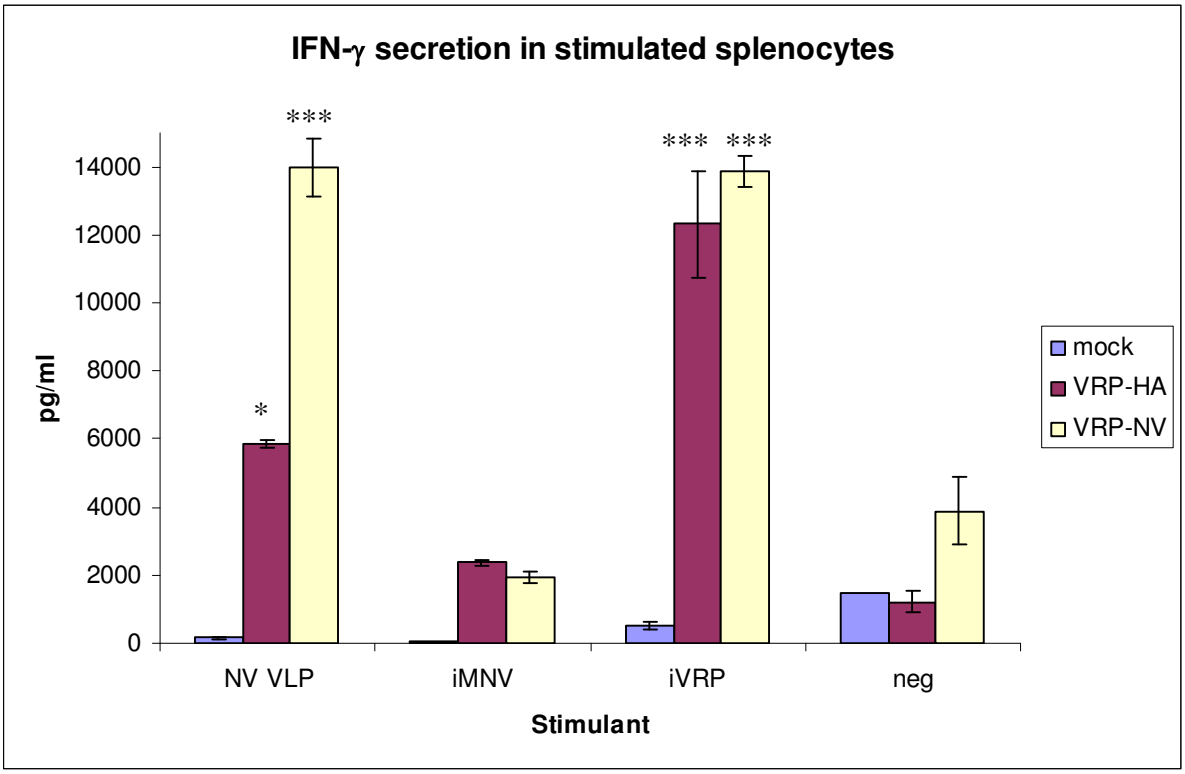
Table 4.1. NV immune stimulated splenocyte cytokine profile by cytometric bead array.

	TNF-α		IFN-γ		IL-2	
Stimulant	pg/ml	fold-Increase*	pg/ml	fold-increase	pg/ml	fold-increase
NV VLP	419.6	2.7	1974.9	8.4	149.2	7.7
CV VLP	398.4	2.6	2172.1	9.3	219.4	11.3
HV VLP	336.5	2.2	1263.7	5.4	144.7	7.4
mock	155.8	1.0	233.9	1.0	19.5	1.0

	IL-4		IL-5	
Stimulant	pg/ml	fold-increase	pg/ml	fold-increase
NV VLP	26.8	3.4	284.5	1.3
CV VLP	26.5	3.3	315.3	1.4
HV VLP	28.4	3.6	315.2	1.4
mock	7.9	1.0	224.5	1.0

***fold increase in cytokine levels over mock stimulation**

Figure 4.3



Splenocyte stimulation controls. Splenocytes harvested from unimmunized mice or mice immunized with VRP-NV or VRP-HA were stimulated with 5 μg/ml NV VLP, UV-inactivated MNV (i-MNV), or i-VRP for 48 h. Culture supernatants were analyzed for IFN-γ by EIA. Cultures from VRP-NV immunized mice secreted significantly more IFN-γ than VRP-HA immunized mice or mock controls upon stimulation with VLP ($P<0.001$). IFN-γ levels from VLP stimulated cultures were also significantly higher than levels following i-MNV stimulation or no stimulation in VRP-NV cultures. VLP stimulated cultures from HA immunized mice had significantly higher levels than mock immunized mice ($P<0.05$). i-VRP stimulated cultures were not different between HA and NV immunized mice and were significantly higher than mock immunized mice ($P<0.001$). IFN-γ levels in NV cultures were not different following VLP or i-VRP stimulation, but levels in HA cultures were higher following i-VRP stimulation than VLP stimulation ($P<0.001$).

Table 4.2

Norwalk virus and Farmington Hills virus capsid peptide library sequences.

Capsid domains*	Amino acids
S	1-125
P1	226-278
	406-520
P2	279-405

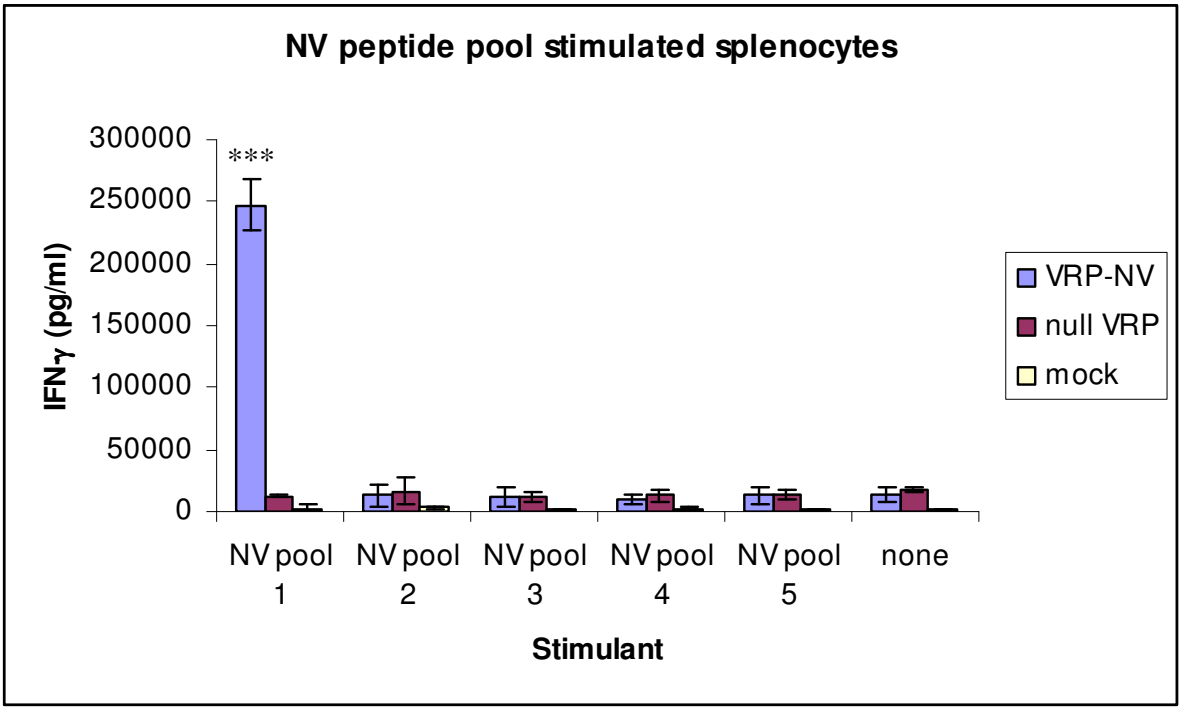
NV peptide pools	Amino acids
1	**1-105**
2	91-215
3	201-305
4	291-405
5	391-525

FH peptide pools	Amino acids
1	1-115
2	101-235
3	221-345
4	331-445
5	**431-540**

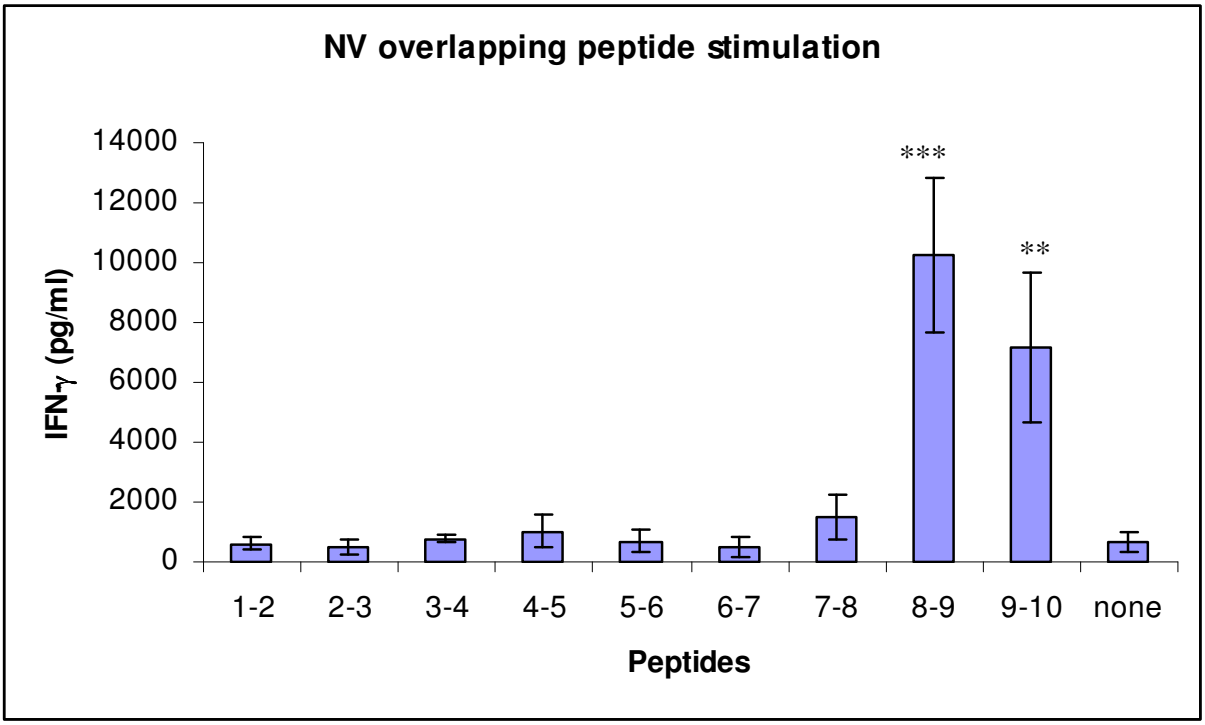
*As described in (20).

Figure 4.4

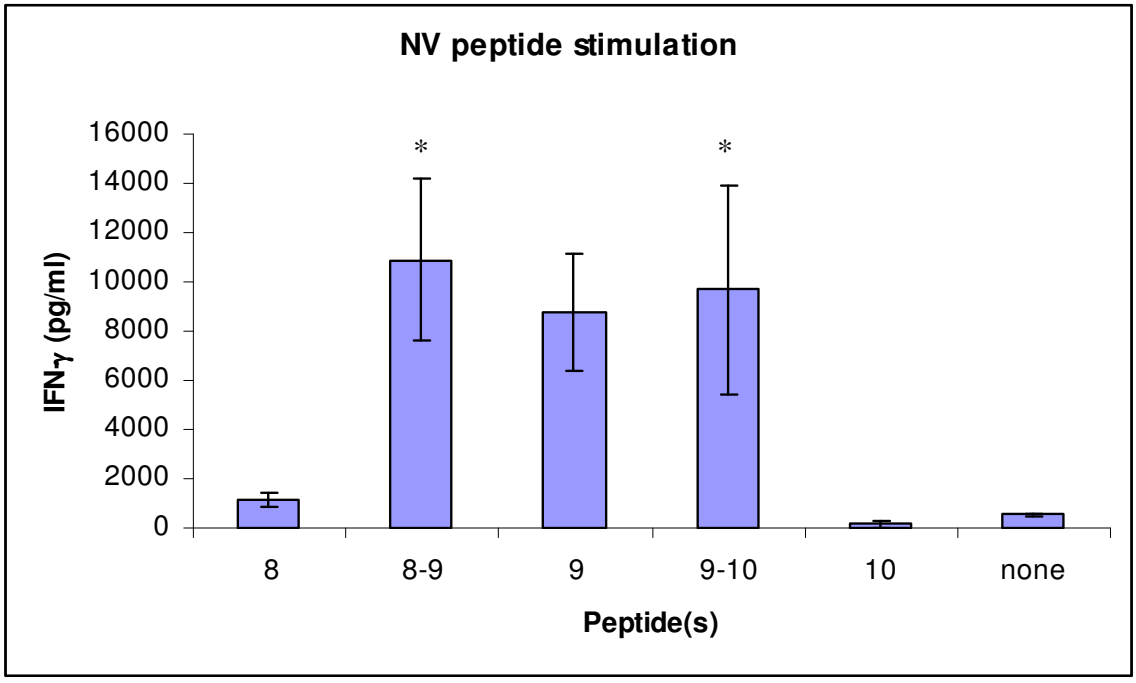
A.


NV peptide pool stimulated splenocytes
300000
250000
200000
150000
100000
50000
0
IFN-γ (pg/ml)
***
NV pool 1
NV pool 2
NV pool 3
NV pool 4
NV pool 5
none
Stimulant
VRP-NV
null VRP
mock

B.


NV overlapping peptide stimulation
14000
12000
10000
8000
6000
4000
2000
0
IFN-γ (pg/ml)
***
**
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
none
Peptides

C.



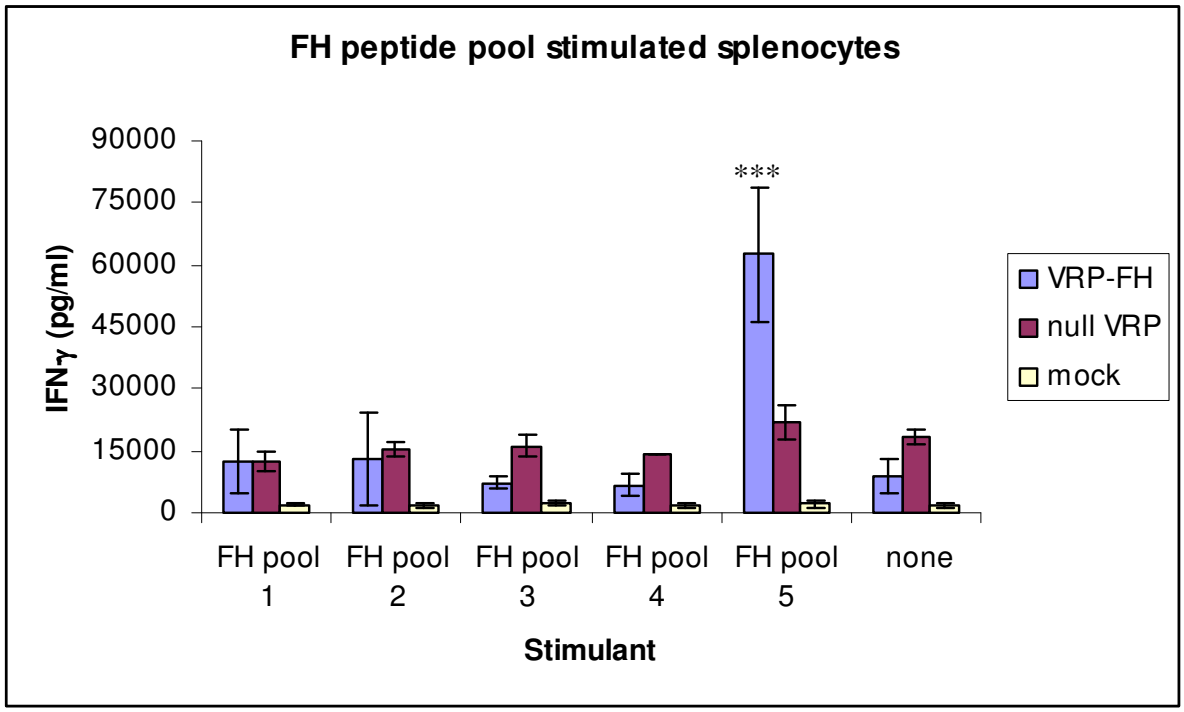
D.

NV peptide	Amino acids	Sequence	Domain
8	71-85	SPNNTPGDVLFDLSL	S
9	81-95	**FDLSLGPHLNPFLLH**	S
10	91-105	PFLLHLSQMYNGWVG	S

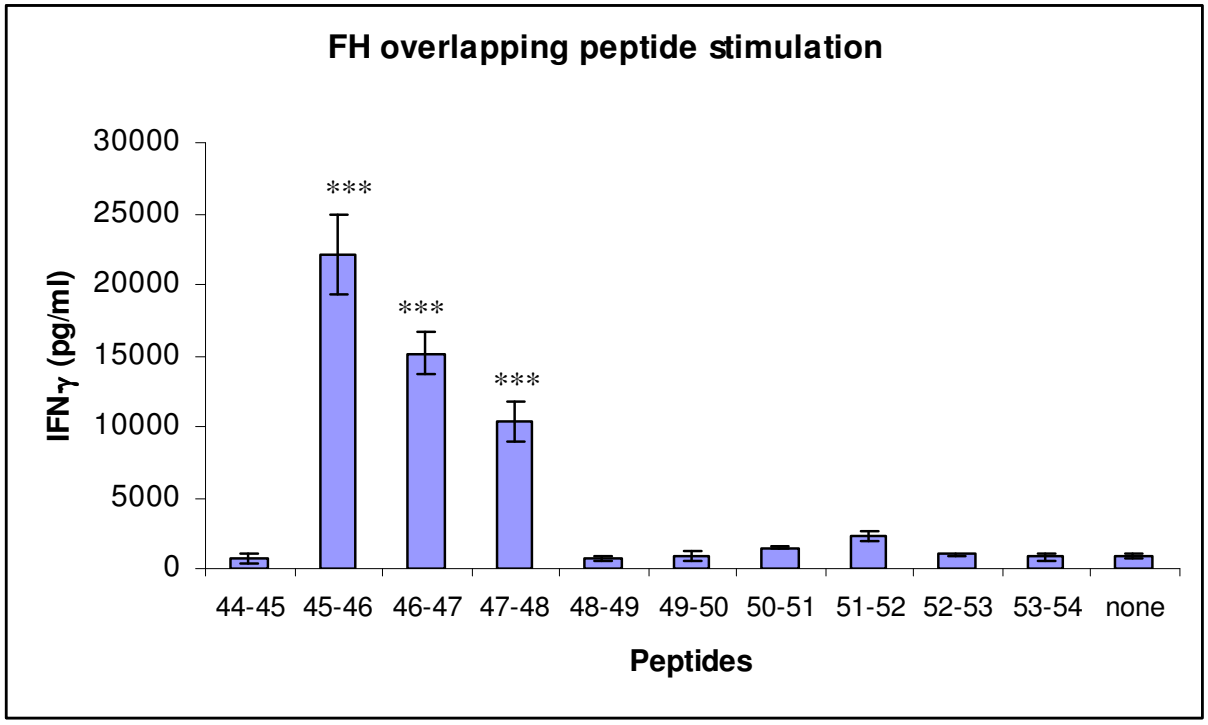
Norwalk capsid peptide stimulation. Splenocytes from mice immunized with VRP-NV, null VRP, or no immunogen were stimulated with overlapping peptide pools of 10-11 peptides spanning the entire capsid and supernatants tested for IFN-γ (A). Overlapping sets of peptides from stimulatory pools were used to stimulate splenocytes from VRP-NV immunized mice to identify stimulatory peptides (B). Individual and overlapping peptides were then used to identify peptides containing stimulatory sequences (C). Stimulatory peptide sequences are shown in (D). Peptides in NV pool 1 (A) stimulated IFN-γ secretion significantly more than other pools ($P<0.001$). Overlapping peptides 8-9 and 9-10 stimulated IFN-γ secretion significantly more than all other overlapping pairs ($P<0.01$; B) and more than peptide 8 or 10 alone ($P<0.05$; C).

Figure 4.5

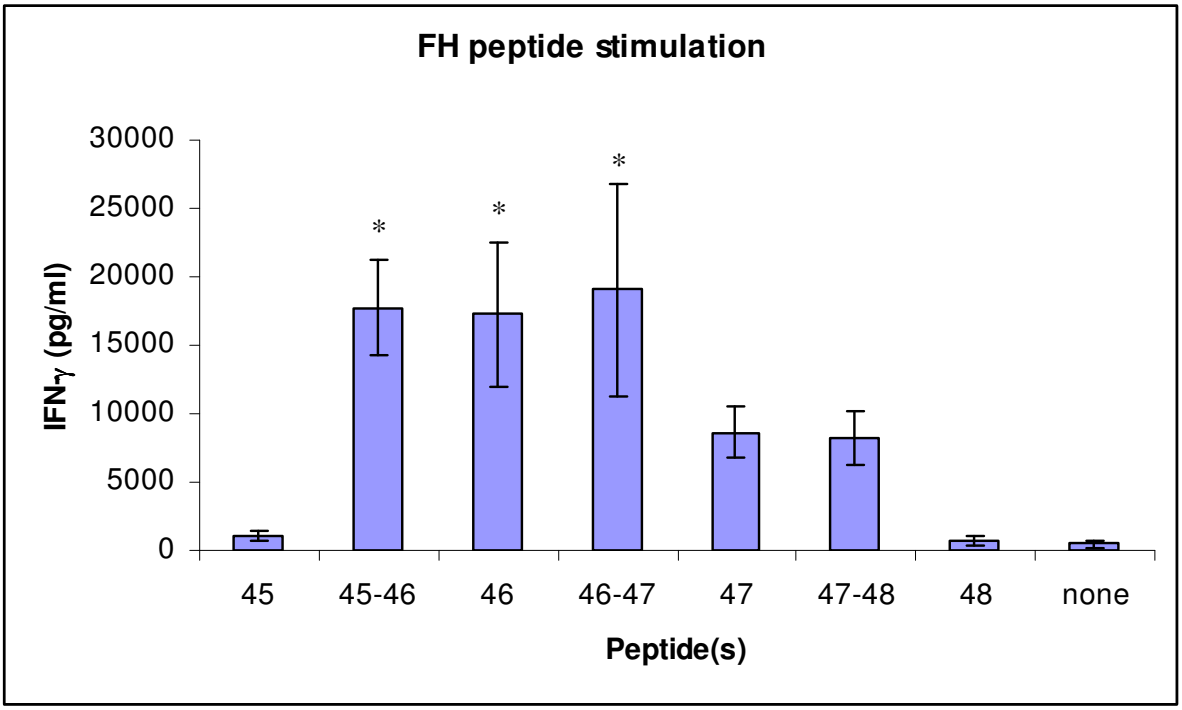
A.


FH peptide pool stimulated splenocytes
90000
75000
60000
45000
30000
15000
0
IFN-γ (pg/ml)
***
VRP-FH
null VRP
mock
FH pool 1
FH pool 2
FH pool 3
FH pool 4
FH pool 5
none
Stimulant

B.


FH overlapping peptide stimulation
30000
25000
20000
15000
10000
5000
0
IFN-γ (pg/ml)
***
***
***
44-45
45-46
46-47
47-48
48-49
49-50
50-51
51-52
52-53
53-54
none
Peptides

C.



D.

FH peptide	Amino acids	Sequence	Domain
45	451-465	CSGYPNMNLDCLLPQ	P1
46	461-475	**CLLPQEWVQHFYQEA**	P1
47	471-485	**FYQEAAPAQSDVALL**	P1
48	481-495	DVALLRFVNPDTGRV	P1

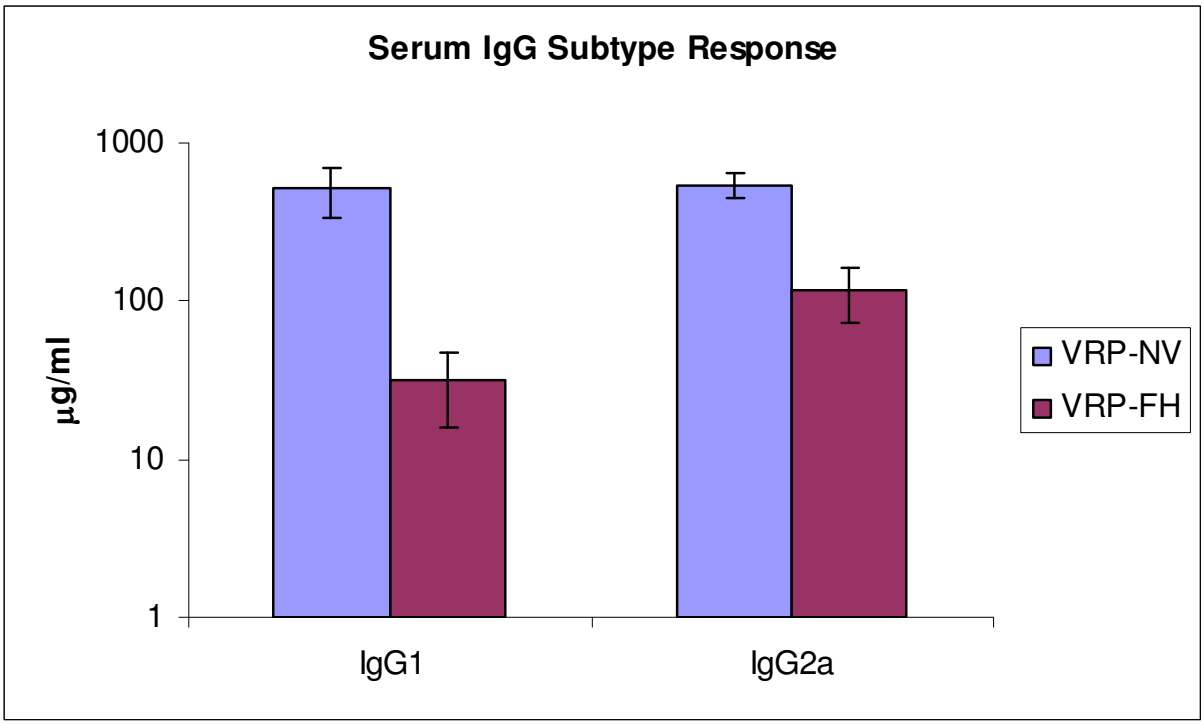
Farmington Hills capsid peptide stimulation. Splenocytes from mice immunized with VRP-FH, null VRP, or no immunogen were stimulated with overlapping peptide pools of 10-11 peptides spanning the entire capsid and supernatants tested for IFN-γ (A). Overlapping sets of peptides from stimulatory pools were used to stimulate splenocytes from VRP-NV immunized mice to identify stimulatory peptides (B). Individual and overlapping peptides were then used to identify peptides containing stimulatory sequences (C). Stimulatory peptide sequences are shown in (D). IFN-γ was significantly higher following stimulation with FH pool 5 than other pools ($P<0.001$; A). Overlapping peptides 45-46 induced increased IFN-γ secretion compared to peptides 46-47 ($P<0.01$) and all other overlapping peptides ($P<0.001$; B). Peptides 46-47 and 47-48 induced increased IFN-γ secretion to all other overlapping peptides ($P<0.001$; B). Individual and overlapping peptide stimulations containing peptide 46 were significantly higher than peptide 45 or 48 stimulation alone ($P<0.05$; C).

Table 4.3

Cytometric bead array of pooled stimulated splenocyte supernatants from immunized mice for T_H1 and T_H2 cytokine secretion.

Vaccine	Stimulant	Cytokine (pg/ml)			
VRP-NV		TNF-a	IL-2	IL-4	IL-5
	N1	497	531	24	178
	N2	248	636	19	183
	N3	332	690	22	176
	N4	315	673	22	166
	N5	259	557	25	156
	none	264	954	19	199
VRP-FH	F1	299	322	14	98
	F2	256	252	14	93
	F3	208	221	15	97
	F4	227	245	15	94
	F5	823	200	18	92
	none	208	394	13	148

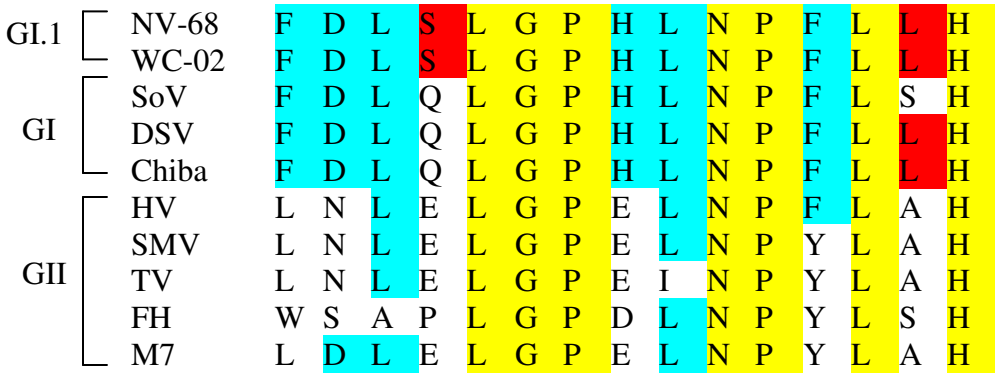
Figure 4.6



Serum IgG subtype response. Sera from mice immunized with VRP-NV or VRP-FH were tested for specific IgG1 and IgG2a to NV or FH VLPs, respectively, by ELISA. IgG1 and IgG2a levels were not different from one another within each immunization group.

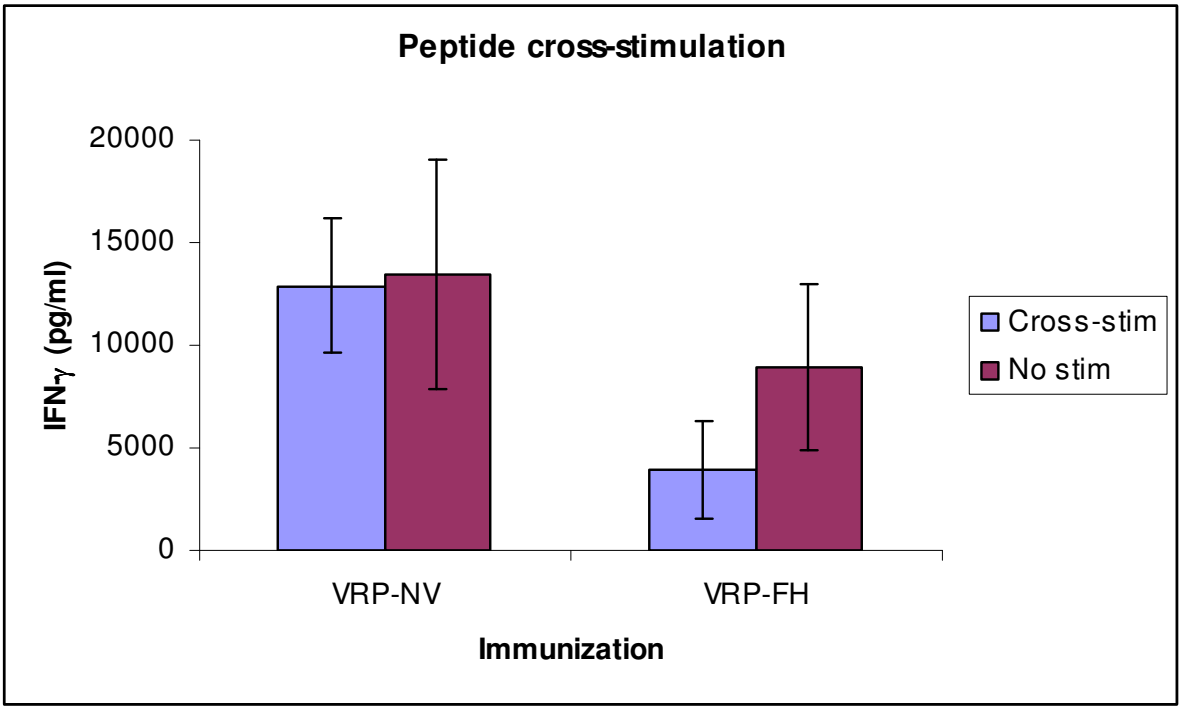
Figure 4.7

A.


GI.1
NV-68 F D L S L G P H L N P F L L H
WC-02 F D L S L G P H L N P F L L H
GI
SoV F D L Q L G P H L N P F L S H
DSV F D L Q L G P H L N P F L L H
Chiba F D L Q L G P H L N P F L L H
GII
HV L N L E L G P E L N P F L A H
SMV L N L E L G P E L N P Y L A H
TV L N L E L G P E I N P Y L A H
FH W S A P L G P D L N P Y L S H
M7 L D L E L G P E L N P Y L A H

Alignments of stimulatory NV and FH capsid peptide sequences. Identified stimulatory sequences within the NV (A) and FH (B) capsids were aligned with corresponding sequences from other GI and GII strains. =amino acid completely conserved across genogroups; = amino acid completely conserved within genogroup; =amino acid completely conserved within genocluster; =variable.

Figure 4.8



Cross-stimulation of NV and FH immune splenocytes to stimulatory peptide pools. NV-immune splenocytes were stimulated with FH pool 5 and FH-immune splenocytes were stimulated with NV pool 1 and IFN-γ levels in supernatant measured by EIA.

CHAPTER V.

Vaccination against mucosal and lymphatic norovirus infection requires B cells, CD4 T cells, CD8 T cells

Adapted from two submitted papers

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Abstract

Human noroviruses cause greater than 90% of epidemic non-bacterial gastroenteritis worldwide. Despite the importance of these pathogens, the nature of norovirus immunity and applicability of vaccination has not been demonstrated in part due to the lack of culture systems and small animal models for human noroviruses. Using the murine norovirus system, we define both antibody- and T cell-mediated mechanisms responsible for efficient clearance of primary norovirus infection and demonstrate both short term and long term protective immunity generated by live virus vaccination. Furthermore, vaccination with the capsid protein using the VEE replicon vector system is effective and tissue specific for the effects of $CD4^+$ T cells, $CD8^+$ T cells, and B cells. Vaccination with the capsid protein of human Lordsdale virus (GII) provided partial protection against MNV challenge. Perforin, but not interferon-γ, was required for the $CD8^+$ T cell-mediated clearance of MNV infection. Adoptively transferred polyclonal anti-MNV sera or neutralizing anti-MNV monoclonal

antibodies (mAbs) were also sufficient to reduce the level of MNV infection both systemically and in the intestine. These studies prove the practicality of vaccination against mucosal norovirus infection, identify tissue-specific requirements for vaccination and clearance, and suggest that there may be conserved epitopes between human and murine noroviruses.

Introduction

More than 90% of epidemic nonbacterial gastroenteritis worldwide can be attributed to human noroviruses (56, 94, 196, 197, 285, 288). Infection is most commonly transmitted by the fecal-oral route (75), and symptomatic infection is characterized by nausea, vomiting and/or diarrhea lasting 24-48 hours (281). Non-epidemic, community acquired infections may be more indolent than epidemic infections, with symptoms lasting longer than one week (220). There are reports that some infected individuals shed virus for longer periods after clearance of symptoms than others, but the basis for this is not known (51, 53, 69, 172, 188, 220).

Despite the significant costs and morbidity due to human norovirus infections, no vaccine is currently available. This is in part due to our incomplete understanding of the nature of norovirus immunity. Adaptive immunity is likely involved in control of primary infection as immunocompromised individuals have been documented to shed virus for prolonged periods (64, 128, 179). The potential to vaccinate against these and related viruses has been demonstrated in gnotobiotic piglets and rabbits (18, 156, 242), and human challenge studies demonstrate short-term but not long term protection against homologous but not heterologous viral challenge (50, 203, 281). Because human noroviruses are extremely

genetically heterogeneous, lack of cross-protection poses a challenge for vaccine development.

In the absence of a cell culture system for human noroviruses, virus-like particles (VLPs) that assemble when the viral capsid protein is expressed have been important tools for evaluating norovirus immune responses (47, 49, 72, 85, 114, 116, 117, 129, 142, 182). In VLP vaccination studies, high doses of VLPs administered intranasally (i.n.), or perorally (p.o.), with or without adjuvants, induced mucosal IgA and serum IgG in human volunteers, calves, pigs or experimental mice (9, 10, 80, 91, 109, 207, 240-242, 247). Antisera from infected human volunteers and experimentally vaccinated mice are also able to block binding of Norwalk and Lordsdale VLPs to ABH Histo-blood group antigens (91, 109, 149). The presence of antibodies that block norovirus receptor binding suggests that antibodies may exert a protective effect against infection or promote resolution of symptoms. Additionally, inoculating mice with vaccine cocktails comprised of multiple norovirus VLPs enhances the production of heterotypic blocking antibodies against strains not included in the cocktail (149). However, no formal assessment of the physiologic importance of antibodies in norovirus infection has been undertaken to date.

Studies into the cellular immune response have shown that peripheral blood mononuclear cells (PBMCs) from Snow Mountain virus (GII) infected individuals produce T cell effector cytokines IL-2 and interferon γ (IFN-γ) when stimulated with VLPs from viruses in the same genogroup *in vitro* (145). Other studies in human volunteers, gnotobiotic pigs and calves, and mice using Norwalk Virus and HuNoV-HS66 VLPs show cytokine production as well as proliferation of $CD4^+$ and $CD8^+$ T cells from murine Peyer's patches and spleens after *in vitro* restimulation (207, 240-242, 247). Together, these studies show that

norovirus infected individuals and VLP vaccinated mice develop B cell and T cell responses but do not define the importance of these responses for clearance of primary infection or resistance to re-challenge. These studies in total reflect the potential for vaccination, but leave open important questions about vaccine efficacy, longevity of immune responses, mechanisms of adaptive immunity, and the potential for cross-protection between distantly-related noroviruses.

The identification of the first murine norovirus (MNV) and its routine propagation in cultured cells provides a facile animal model for studies of norovirus immunity and pathogenesis (124, 277). MNV is an efficient enteric virus that, like its human counterparts, is spread by fecal-oral transmission (278). The MNV genome encodes three open-reading frames with ORF1 encoding a polyprotein that is cleaved into individual non-structural proteins in a manner similar to the polyprotein of human noroviruses (238), demonstrating the conserved nature of norovirus replication mechanisms. The MNV capsid, like that of human strains, consists of 90 dimers of the capsid protein arranged in a spherical capsid (127). Studies into MNV pathogenesis reveal an important role for IFN-mediated innate immunity and STAT-1 dependent immunity in resistance to acute infection (124, 171). In addition, the importance of adaptive immunity in control of MNV infection is suggested by the observation that RAG1-/- mice develop persistent MNV infection whereas wild type mice can clear infection (124, 171, 255).

MNV infected mice generate a significant neutralizing antibody response (99, 100, 124, 206, 255, 270), and neutralizing mAbs have been identified (277). Cellular immune responses, however, have not been investigated. The MNV1.CW3 strain, which is cleared from intestine, spleen, liver, mesenteric lymph nodes (MLN) and feces within seven days of

infection (124, 171, 255), provides a challenge model to study vaccine efficacy and the adaptive immune mechanisms responsible for clearance of virus following primary infection.

We show here that vaccination with either live MNV or Venezuelan equine encephalitis (VEE) replicon particles (VRPs) expressing the MNV capsid protein protects against re-challenge for up to six months. Both clearance of primary infection and effective vaccination require the concerted efforts of $CD4^+$ T cells, $CD8^+$ T cells, and B cells. However, the role of each cell type is tissue-specific, and requirements for immunity in the ileum differ from those in the lymphatic system.

Materials and methods

Viruses, viral stocks, VRPs, and plaque assays. MNV strain MNV1.CW3 or MNV1.CW1 were used in all virus infections (255, 277). Two non-synonymous mutations distinguish the genomes of MNV1.CW3 and MNV1.CW1 (277). To generate concentrated virus stocks, RAW 264.7 cells were infected in VP-SFM media (Gibco) for 2 days at a multiplicity of infection (MOI) of 0.05. Supernatants were clarified by low-speed centrifugation for 20 min at 3,000 rpm. Virus was concentrated by centrifugation at 4°C for 3 h at 27,000 rpm (90,000 *g*) in a SW32 rotor. Viral pellets were resuspended in PBS and titered on RAW 264.7 cells as previously described (277). Type I Lang reovirus was kindly provided by Dr. Terrence S. Dermody (Vanderbilt University, Nashville, TN). Plaque assays were performed as previously described (277). Tissues were harvested into sterile, screw-top 2-ml tubes containing 500 mg of 1-mm zirconia/silica beads (BioSpec Products, Bartlesville, OK) and stored at −80 °C. To determine viral titers,1 ml of complete DMEM was added to each

sample on ice and homogenized using a MagNA Lyser (Roche) prior to plaque assay. The limit of detection was 20 PFU/ml.

All VRPs were produced as previously described (93). Briefly, ORFs 1, 2 and 3 from MNV1.CW3 and ORF2 from Lordsdale virus (LV) and Chiba virus (CV) were individually cloned into VRP expression vectors. Following infection of BHK cells with VRPs for 24 h, culture supernatants were harvested and cells lysed. Proteins were separated by SDS-PAGE and analyzed by western blot with polyclonal rabbit anti-MNV serum. VRP titers and efficient expression of recombinant protein were determined by immunofluorescence assay using mouse antisera following inoculation with respective antigens. Cell lysates from MNV ORF2, CV, and LV VRP-infected cultures were further purified to obtain VLPs as described (149).

Cell cultures and antibodies. RAW 264.7 cells (ATCC, Manassas, VA) were maintained as previously described (277). Monoclonal antibodies (MAbs) YTS191.1 specific to CD4 (38), H35 specific to CD8 (236), 9BG5 specific to the reovirus type 3 hemagglutinin (27), anti-MNV mAbs A6.1, A6.2 and H6.1 and SFR3-DR5 (ATCC HB-151 (216)) were produced in INTEGRA Celline CL1000 flasks (Integra Biosciences, Ijamsville, MD) as previously described (163). The titer of polyclonal anti-MNV antibody in serum was determined using ELISA (149).

Mice, inoculations, and infections. All mice were bred and housed at Washington University School of Medicine or the University of North Carolina at Chapel Hill in accordance with all federal and university policies. Wild type C57BL6/J, B6 RAG1-/-, IFNγ -/-, perforin -/-, MHC Class II -/- and B-cell -/- (μMT) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). $K^{b-/-}x\ D^{b-/-}x\ \beta_2\text{-}m^{-/-}$ *(MHC Class I x* $\beta_2\text{-}m^{-/-}$*)* mice were a generous

gift of Dr. Ted Hansen (154). Senescent wild type C57BL6/J mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and aged to 14 months. Wild type mice were tested by ELISA for the presence of MNV antibody prior to experiments. All mice used in these studies were seronegative.

Mice used in vaccination studies were immunized with $3x10^7$ plaque forming units (PFU) of MNV1.CW1, MNV1.CW3, or control Type I Lang reovirus p.o. in 25µl of DMEM containing 10% fetal bovine serum (Hyclone, Logan, UT) or with 2.5 x 10^6 infectious units (IU) of each VRP expressing MNV1.CW3 ORF1, ORF2, or ORF3 (individually or in groups of 2-3 VRPs), CV ORF2, LV ORF2, or HA VRP in 10µl or 50µl volume by footpad inoculation on day 0 and boosted on day 21. Control mice were inoculated with PBS in parallel. Mice were then challenged with MNV1.CW3 at specified times after boost and tissues harvested three days post-challenge. RAG1-/- and all splenocyte donor mice were infected with $3x10^6$ PFU of MNV1.CW3 p.o. in 25µl of cDMEM. All other mice were infected with $3x10^7$ PFU MNV1.CW3 p.o. RAG1-/- mice were inoculated at 4-6 weeks of age, and aged wild type mice were inoculated at 14 months of age. All other mice were inoculated at 6-10 weeks of age. In RAG1-/- mice, a one inch section of the small intestine immediately distal to the pylorus of the stomach (duodenum/jejunum) and a one inch section of the small intestine immediately proximal to the cecum (distal ileum) were harvested. In all other mice the distal ileum and three mesenteric lymph nodes (MLN) were harvested. The duodenum/jejunum was not harvested from wild type mice since viral titers could not be detected at this site.

Adoptive and passive transfer studies. Spleens were harvested from mice and single cell suspensions were generated. Cells were counted and diluted in RPMI-1640 media (Sigma, St.

Louis, MO) supplemented with 10% characterized fetal calf serum (HyClone), 100 U penicillin/ml, 100 µg/ml streptomycin, 10 mM HEPES (N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid), 1mM sodium pyruvate and 2 mM L-glutamine (cRPMI). Cells were injected into persistently infected RAG1-/- mice by intraperitoneal (i.p.) injection in 0.5 ml cRPMI. Immune and control anti-serum was obtained from either immunized or control mice, filtered (0.2µm), heat fixed for 30 min at 55ºC and stored for use. Serum and mAbs were passively transferred into recipient mice i.p.

Statistical methods. All data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA). Viral titer data were analyzed with the nonparametric Mann-Whitney test. All differences not specifically stated to be significant were insignificant ($P > 0.05$).

Results

Construction of norovirus capsid protein-expressing VRPs.

VEE based VRPs expressing MNV1.CW3 ORF1, ORF2 and ORF3 as well as ORF2 of the human noroviruses Lordsdale (LV) and Chiba (CV) were produced for use in vaccination experiments. Western blots of ORF1 VRP infected cell lysates revealed 77 kDa and 19 kDa proteins, which correspond in size to MNV proteinase-polymerase precursor protein and cleaved 3C-like proteinase (Fig.5.1A) (239). Culture supernatants contained a 57 kDa protein, which corresponds in size to the MNV RNA-dependent RNA polymerase. ORF2 VRP infected cell lysates and supernatant both contained the 58 kDa capsid protein. ORF3 VRP infected cell lysates contained the 22 kDa ORF3 protein. Denatured MNV, LV, or CV VLPs purified from cells infected with VRPs expressing each respective ORF2 were

used to examine the potential cross-reactivity of capsid proteins from different noroviruses by western blot. Hyper-immune polyclonal rabbit antisera to MNV (277) cross-reacted at low levels with CV (GI.4) and LV (GII.4) VLPs.

Short term live virus and subunit vaccination against MNV

We first determined whether we could detect short term immunity to homologous MNV challenge and if proteins encoded by specific MNV ORFs could elicit effective immunity. After oral inoculation with MNV1.CW3, wild type mice exhibit detectable viral titers in the ileum and the MLN three to five days post infection; virus is cleared by day seven. Wild type mice were therefore primed orally and boosted three weeks later with MNV1.CW3, the closely related strain MNV1.CW1, or reovirus Type I Lang as an unrelated virus control, in live-virus vaccines (275). Additional mice were vaccinated subcutaneously and boosted three weeks later in the footpad with VRPs expressing MNV ORF1, MNV ORF2, or MNV ORF3, either separately or in combination. Controls for VRP vaccination included PBS or VRPs expressing hemagglutinin (HA) from a mouse adapted influenza A virus. Two weeks post-boost, mice were orally challenged with MNV1.CW3 and organs harvested three days later for titration of virus (Fig. 5.2A). Prior infection with either MNV1.CW1 ($p=0.0002$) or MNV1.CW3 ($p=0.0009$) significantly decreased MNV1.CW3 replication in the ileum compared to mice infected with reovirus (Fig. 5.2B). Similar results were observed in the MLN following MNV.CW1 ($p=0.0001$) and MNV.CW3 ($p=0.0003$) vaccination (Fig. 5.2B). ORF2 VRP vaccines (alone or in combination with other VRPs) also protected against MNV1.CW3 in both distal ileum ($p=0.005$) and MLN ($p=0.02$) compared to the HA VRP control group. ORF1 VRPs alone or in combination with ORF3 VRPs had a

slight but statistically significant effect on MNV1.CW3 levels in the distal ileum and MLN (Fig. 5.2B). ORF3 VRPs alone did not confer significant protection (Fig. 5.2B). Together these data show that vaccination with either live virus or ORF2 VRPs can confer short term protection against MNV challenge.

We then compared efficacy of vaccination with homologous versus heterologous ORF2 proteins. Mice were vaccinated with VRPs expressing ORF2 from CV (genogroup GI.4) or LV (genogroup GII.4) and challenged with MNV1.CW3. Vaccination with LV capsid partially protected against MNV infection in the distal ileum ($p=0.0007$) but not the MLN (Fig. 5.2B). No significant reduction in MNV titers was seen after immunization with CV capsid (Fig. 2B). Protection after LV ORF2 VRP vaccination did not correlate with generation of cross-reactive serum IgG as measured by ELISA (Fig.5.1B), despite cross-reactivity observed by western blot using hyper-immune rabbit antisera generated against the MNV capsid (Fig.5.1A). Fecal extracts collected at 1-4 weeks post-immunization yielded no measurable homotypic or heterotypic IgG or IgA (data not shown). These data show that there is measurable immunologic cross protection between Lordsdale virus and MNV in the distal ileum, but that this does not correlate with the production of cross-reactive antisera.

Vaccination can occur in aged mice

Because the elderly are more susceptible than younger adults to human norovirus infections (2), we determined whether increased age altered vaccine efficacy in mice. Prior work has shown that mice older than 1 year of age have diminished vaccine responses to SARS virus antigens (46). We therefore compared vaccine efficacy in eight week old (adult) and aged (14 month old) mice. Adult and aged mice were vaccinated with MNV ORF2

VRPs or HA VRPs and challenged with MNV1.CW3 as outlined in Fig. 5.2A. In contrast to studies using SARS virus antigens, aged mice responded as well as adult mice to MNV ORF2 vaccination as indicated by decreased MNV1.CW3 titers after challenge in both the distal ileum and MLN (Fig. 5.2C). Interestingly, despite this protective effect, sera from vaccinated aged mice had significantly lower anti-MNV ORF2 IgG compared to adult mice (Fig. 5.1C). These data again indicate that protection against MNV infection does not always correlate with serologic responses, raising the possibility that T cells play a fundamentally important role in vaccination against MNV.

Protective effect of live MNV vaccine does not wane over 6 months

We next determined whether protection conferred by MNV1.CW3 or MNV ORF2 VRPs was long lived. Wild type mice were primed and boosted as in Fig. 5.3A with MNV1.CW3 or MNV ORF2 VRPs. Control vaccinations included HA VRPs, reovirus TIL or PBS. Mice were then challenged with MNV1.CW3 two, four, 14, or 24 weeks later and tissues harvested three days post-challenge for MNV titration. We observed complete protection against ileal MNV1.CW3 infection two weeks post-boost after vaccination with either MNV1.CW3 ($p=0.0001$) or ORF2 VRPs ($p<0.0001$) compared to reovirus TIL or HA VRP controls (Fig. 5.3B). While vaccination with either MNV1.CW3 of ORF2 VRPs was effective at controlling MNV1.CW3 replication in MLN, live virus vaccination was more effective than ORF2 VRP vaccination ($p=0.0003$) (Fig. 5.3B). Live virus vaccines conferred full protection against MNV1.CW3 replication in both the distal ileum and the MLN at 4, 14 and 24 weeks after vaccination. In contrast, vaccination with ORF2 VRPs, while statistically significant, was less effective than vaccination with MNV1.CW3 at these later time points

(Fig. 3B). This data shows that both live virus and subunit vaccines induce long term protection against MNV infection. However, live virus may provide more complete protection against reinfection.

Mechanisms responsible for vaccination by live virus and ORF2 VRPs

We next determined the immunologic mechanism(s) responsible for effective vaccine responses. We vaccinated B cell -/-, MHC Class I x β2M -/- (130), and MHC Class II -/- mice immunodeficient in B cells, CD8 T cells (154, 205), and CD4 T cells (79), respectively, with MNV1.CW3 or ORF2 VRPs (Fig. 5.4A). PBS, HA VRPs, and reovirus TIL served as vaccination controls. Mice were challenged with MNV1.CW3 orally two weeks post-boost, and tissues were harvested and analyzed for viral titers three days later.

Live MNV vaccination induced significant protection against MNV challenge in both the distal ileum and the MLN of B cell -/-, MHC Class II -/- and MHC Class I x β2M -/- mice ($p < 0.05$; Fig. 5.4B). However, there was considerable variation in the efficacy of vaccination in distal ileum and MLN among immunodeficient mice species. In both B cell -/- and MHC Class I x β2M -/- mice, vaccination with live virus resulted in complete protection in the distal ileum but only partial protection in the MLN (Fig. 5.4B). In MHC Class II -/- mice, partial protection was elicited in both tissues (Fig. 4B). In contrast, wild type mice were completely protected in both tissues. These data demonstrate that complete protection after vaccination with live virus requires the concerted action of B cells, MHC Class II, MHC Class I, and β2M, consistent with the tissue specific roles for B cells, CD4 T cells, and CD8 T cells in the development of complete protection against MNV infection. Less variation was observed after vaccination with ORF2 VRPs between the distal ileum and

MLN, which was partially protective in B cell -/- and MHC Class II -/- in both tissues (Fig. 5.4B). There was no protection in either tissue after vaccination of MHC Class I x β2M -/- mice with ORF2 VRPs (Fig. 5.4B), suggesting that protection by VRPs critically depends on either classical or non-classical CD8 T cells. We can conclude from these experiments that all major aspects of adaptive immunity are required for optimally effective responses to either live virus or ORF2 VRP vaccination.

CD8 and CD4 T cells are important for clearance of primary MNV infection.

The experiments above support the hypothesis that CD4 T cells, CD8 T cells, and B cells are important for optimal vaccine responses to MNV. We, therefore, asked if the same cell types are required for clearance of acute infection. To determine the role of T cells in clearance of acute MNV infection, we inoculated wild type, MHC Class II-/-, and MHC Class I x β_2-m$^{-/-}$ mice orally with MNV1.CW3 and measured viral titers in the distal ileum and MLN three, five, seven and 21 days post infection (Fig. 5.5B-C). We detected no significant difference in viral titer between MHC Class I x β_2-m$^{-/-}$ mice and wild type mice three and five days post infection, indicating that MHC Class I was dispensable in MNV infection at early time points (Fig. 5.5B). However, at seven days post-infection, MHC Class I x β_2-m$^{-/-}$ mice had significant levels of MNV titers in both the distal ileum ($p=0.0002$) and the MLN ($p<0.0001$) compared to wild type mice, which had completely cleared the infection (Fig. 5.5B). MHC Class I x β_2-m$^{-/-}$ mice eventually cleared MNV infection, as demonstrated by a lack of detectable virus by 21 days post infection, suggesting CD8 T cells are important for efficient clearance of MNV, but are not required for eventual clearance of MNV infection.

In contrast to MHC Class I x β_2-m$^{-/-}$ mice, MHC Class II-/- mice had higher MNV titers in the ileum than wild type mice both three ($p=0.0002$) and five ($p=0.0058$) days after infection (Fig. 5.5C). At seven days post infection, minimal viral titers remained, and at eight days post infection, both MHC Class II-/- and wild type mice had cleared the infection from the distal ileum. In MLN, viral titers in wild type and MHC Class II-/- were not significantly different at days three and five post-infection. However, there was a small, but statistically significant increase in titer in the MLN of MHC Class II-/- compared to wild type mice at seven days post infection ($p=0.0402$; Fig. 5.5C). By eight days post infection, MLN infection was cleared. Together these data indicate that CD4 T cells are necessary for control of acute MNV infection but, like CD8 cells, are not required for eventual clearance of MNV infection.

The availability of persistently infected RAG1-/- mice allowed us to determine the role of CD4 and CD8 T cells in clearance of MNV infection using adoptive transfer. Both immune splenocytes and intraepithelial lymphocytes (IEL) can control murine rotavirus and reovirus enteric infection (48, 165, 184, 186). We therefore transferred splenocytes from MNV-immune wild type mice to persistently infected RAG1-/- mice and followed the clearance of MNV from ileum and duodenum/jejunum. Transfer of $1x10^7$ immune, but not non-immune, splenocytes into persistently infected RAG1-/- recipients significantly reduced MNV titer in the duodenum/jejunum ($p<0.0001$) and distal ileum ($p<0.0001$) six days post transfer (Fig. 5.6B) and was maintained through 16 days post-transfer (data not shown). These data show that adoptively transferred immune splenocytes are sufficient to clear persistent MNV infection in the intestine of RAG1-/- mice.

To define which cells in immune splenocytes were required for MNV clearance, CD4 or CD8 T cells were depleted from splenocytes transferred into RAG1-/- recipients. To determine the extent of depletion *in vivo*, we monitored CD4 and CD8 T cell populations in the spleens of RAG1-/- recipients. Significant numbers of CD4 and CD8 T cells could be detected in spleens by flow cytometry analysis six days post transfer of undepleted splenocytes (Fig. 5.6A). Administration of depleting antibodies led to effective depletion of the appropriate T cell populations (Fig. 5.6A).

Control antibody did not alter the capacity of immune splenocytes to decrease MNV titer in the duodenum/jejunum ($p<0.0001$) or distal ileum ($p<0.0001$) (Fig. 5.6B). Depletion of either CD4 ($p=0.0042$) or CD8 ($p=0.0002$) T cells individually led to a significant increase in MNV titers in duodenum/jejunum compared to control depletion (Figure 5.6B). Combined depletion of both CD4 and CD8 T cells from transferred immune splenocytes caused an additional significant increase in MNV titers when compared to CD4 depletion alone ($p=0.02$) or CD8 depletion alone ($p=0.03$). In the distal ileum, depletion of CD4 T cells ($p=0.0003$) or CD8 T cells ($p<0.0001$) led to a significant increase in MNV titers (Figure 5.6B). However, the depletion of both CD4 and CD8 T cells from transferred immune splenocytes did not have an additive effect. This data demonstrate that both immune CD4 and CD8 T cells are necessary for clearance of persistent MNV infection from the intestine.

Perforin has a role in clearance of MNV infection

Two major effector mechanisms for the antiviral effects of T cells are the production of IFNγ and perforin-mediated cytolysis (reviewed in (267)). To address which activity is important in viral clearance, we adoptively transferred immune splenocytes from IFNγ-/- or

perforin-/- mice into persistently infected RAG1-/- mice and determined their capacity to clear intestinal MNV infection. Immune splenocytes from IFNγ-/- mice cleared MNV infection as effectively as splenocytes from wild type mice (Fig. 5.6B). However, immune splenocytes from perforin-/- mice were significantly less effective at clearing intestinal MNV infection than cells from either wild type or IFNγ-/- mice in the duodenum/jejunum ($p=0.0003$) and distal ileum ($p=0.0075$; Fig. 5.6B). Perforin-deficient immune splenocytes did, however, retain some capacity to clear MNV infection in the duodenum/jejunum ($p=0.0086$) and distal ileum ($p=0.0001$) compared to transfer of non-immune cells. These data suggest that perforin is critical for efficient clearance of MNV infection from the intestine, but it is not likely to be the only mechanism driving viral clearance.

B cells are required to control early mucosal MNV replication and long term clearance in mesenteric lymph nodes but not intestine.

We have previously demonstrated that RAG1-/- mice, which are deficient in both T cells and B cells, develop a chronic persistent MNV infection (124), whereas wild type mice efficiently clear infection. This indicates that adaptive immunity is important for MNV clearance. To evaluate the role of B cells in the control of primary MNV infection we compared MNV titers in B cell deficient μMT mice and wild type B6 mice in the distal ileum and the mesenteric lymph nodes (MLN) after p.o. infection. At three and five days post infection μMT mice had significantly higher virus titers compared to the wild type controls in the distal ileum ($p=0.002$ and $p=0.014$, respectively) and also in the MLN ($p=0.009$ and $p=0.0004$, respectively; Fig. 5.7). The majority of both wild type B6 and μMT mice cleared ileal infection by day seven post-infection, with only two of ten μMT mice having any

measurable ileal titer at this time point (Fig. 5.7). In the MLN, wild type mice cleared infection by day seven, while titers in μMT mice decreased approximately 100-fold from their peak at five days post-infection. However, MLN titers in μMT mice remained detectable at days seven ($p=0.0004$), ten ($p=0.045$), and 21 ($p=0.045$) post-infection, and were statistically significant compared to wild type mice (Fig. 5.7). These data show that B cells are important in the control of MNV infection at days three and five in both the distal ileum and MLN. However, while B cells were dispensable for clearance of MNV in the distal ileum, they were required for clearance of infection from MLN.

MNV specific polyclonal sera and IgG are sufficient to limit MNV replication in the intestine and spleen

To determine if the lack of antibodies in μMT mice are directly responsible for clearance of virus, we tested the ability of passively transferred anti-MNV antibody to limit viral replication. To obtain MNV specific polyclonal antibody we mock immunized or MNV immunized wild type mice and obtained non-immune and immune serum 35 to 42 days post infection. We transferred 500μl of serum via i.p. injection into persistently infected RAG1-/- mice and measured virus titers in the distal ileum and spleen six days post-transfer. In the distal ileum, transfer of immune serum led to a modest but significant decrease in the levels of MNV titer compared to non-immune serum transfers ($p= 0.013$; Fig. 5.8A). Similar results were obtained in the duodenum/jejunum (data not shown). In the spleen, the transfer of immune serum had a much more significant effect, where levels of MNV were reduced to below the level of detection in half of the mice examined ($p<0.0001$). This demonstrates that

MNV immune polyclonal serum is sufficient to reduce MNV titers in the intestine and spleen.

Even though IgA is classically thought of as the main antibody isotype present at the mucosal surface (reviewed in (174)), there are reports of IgG antibody controlling mucosal viral infection (139, 183, 273). We therefore wanted to determine if IgG antibodies specific for MNV capsid could reduce MNV titers. We obtained the anti-MNV capsid monoclonal antibodies (mAb) A6.1, A6.2 and H6.1, all of IgG2a isotype, that had been previously shown to neutralize MNV *in vitro* ((127, 277) & unpublished data). We administered 500µg of each mAb via i.p. injection into persistently infected RAG1-/- mice. In the distal ileum, each mAb significantly reduced MNV titers compared to the 9BG5 mAb control ($p=0.0002$; Fig. 5.8B). In the spleen, mAbs A6.1, A6.2 and H6.1 were also able to significantly reduce the levels of MNV titers ($p=0.0002$, $p=0.0004$ and $p=0.0207$, respectively; Fig. 5.8B). This demonstrates that mAbs of IgG isotype directed against MNV capsid are able to control MNV infection in the distal ileum and spleen.

Discussion

Noroviruses are a significant public health problem throughout the world; however, there is no licensed vaccine for human noroviruses. The study of immunity to noroviruses has been limited by the lack of an immunologically manipulable small animal model in which to study protection and immunity to live noroviruses, as well as the mechanisms involved in clearing norovirus infection from the intestine. The discovery of MNV and the development of murine models present the opportunity to examine immunity to a norovirus and its individual components.

The two to three day time frame of resolution of clinical symptoms of human norovirus infection has been largely thought to be due to innate immune mechanisms, and indeed previous studies in STAT1 and IFNαβγ receptor deficient mice support this conclusion. However, the present study demonstrates that adaptive immune cells also had roles that were relevant in three to seven days post-infection. B cells and T cells may be important not only in clearing a primary norovirus infection, but also in preventing subsequent infections. Given that cross-reactivity between noroviruses has been demonstrated for T cells and antibodies within the same genogroup (145), effector cells from a previous norovirus infection may be important in combating additional norovirus challenge. Our data convincingly showed both arms of the immune system as important in both the natural history and the generation of memory effector cells in norovirus infection. This demonstrates that vaccination strategies against noroviruses must develop mechanisms to target T cells and/or B cells and boost their ability to function more effectively.

In this report, we demonstrated that protective short-term and long-term immunity were generated to MNV by vaccination with live virus or VRPs expressing ORF2, the viral capsid protein. This is also the first study to delineate the importance of ORF2 but not ORF1 and ORF3 proteins in generating effective immunity to noroviruses. While protection associated with ORF2 vaccination was not complete in all experiments, it lead to significant reduction in viral loads even in long term vaccination studies. Our demonstration of long term immunity to MNV is the first demonstration of long term immunity to a norovirus. Previous challenge studies with the human Norwalk virus strain did not demonstrate long term immunity (203); therefore, immunity generated by vaccination against MNV in contrast to human noroviruses must be further dissected. In particular, in the continued absence of a

cell culture system for human noroviruses, VLPs derived from human strains from different genogroups will be instrumental in determining if cross-protection exists between MNV and other noroviruses. Furthermore, VLPs may allow us to determine if subunit or live-attenuated virus is a better vaccine strategy.

In the distal ileum, B cells, MHC Class I, and β2M were not required for viral clearance following vaccination with live virus; however, partial clearance still occurred following vaccination in MHC Class II -/- mice (Fig. 5.4B). Furthermore, virus was consistently cleared from the distal ilea of both B cell -/-, MHC Class I -/-, and MHC Class II -/- immunodeficient mice following acute MNV infection (Fig. 5.5B and 5.7). In contrast, vaccination in the MLN was sensitive to the loss of any subset of adaptive immune cells, and B cells were particularly important in generating protective immunity following vaccination (Fig. 5.4B) or in clearance of acute infection (Fig. 5.7). These findings suggest that each arm of the adaptive immune response is important in efficient clearance of MNV infection, and effective vaccination likely requires a full complement of T cells and B cells to confer complete protection from MNV infection in multiple tissues.

Immune CD4 and CD8 T cells derived from MNV immune wild type mice were able to clear persistent MNV infection in the intestine of RAG1-/- mice in adoptive transfer experiments. Similar experiments with rotavirus and reovirus infections show that control of these viruses is also dependent on the presence of T cells (61, 66, 266). However, in contrast to the requirement for both CD4 and CD8 T cells that we observed in MNV infection, rotavirus and reovirus each rely more heavily on either CD4 or CD8 T cell responses. In rotavirus, the predominant protective role of T cells is due to TCRαβ+ CD8 T cells (48, 60, 61, 164, 185), though CD4 T cells appear to play a specific role in rotavirus VP6 protein

mediated immunity (165). In reovirus, immunodepletion experiments have shown that CD4 T cells are more important than CD8 T cells in reovirus immunity, though part of the CD4 T cell effect is likely related to B cell activation (266).

We have demonstrated that T cells are important in norovirus immunity and shown that the T cell effector molecule perforin, but not IFNγ, had a role in clearance of MNV infection (Fig. 5.6B). It is interesting that we did not observe a requirement for IFNγ in clearance of MNV infection. Data from human norovirus studies show that CD4 and CD8 T cells are able to proliferate and produce IFNγ (145, 207, 247). However, our finding that perforin may be important in norovirus immunity appears consistent with data from reovirus infection studies, where lymphocyte populations in the intestine have been demonstrated to express perforin, in addition to other T cell effector molecules FasL and TRAIL post-reovirus infection (21, 82). Because the effector role of perforin is unequivocally linked to granzyme-mediated pathways (reviewed in (143, 267)), it is likely that one or more of the known granzymes will be demonstrated as having a role in clearance of MNV infection in future studies as well. Overall, the difference between MNV and other enteric viruses in the importance of CD4 and CD8 T cells highlights the fact that similar organ tropisms for viruses may not always predict the manner in which they are individually controlled by the immune system.

CD4 and CD8 T cell subsets as well as B cells had different tissue specific roles in vaccination and clearance of MNV replication. Following vaccination, MHC Class I x β_2-$m^{-/-}$ mice were completely protected from MNV challenge with live virus in the distal ileum, but not in the MLN (Fig. 5.4B). In genetically deficient mice, CD4 T cells were important in the distal ileum at early time points post infection, with no effect in the MLN (5.5C). The

distal ileum of B cell deficient mice was cleared of MNV infection by seven days after infection, but the MLN was not. Thus clearance of ileal MNV infection appears to be more dependent on each individual immune cell subset than clearance of infection from intestinal lymphatic tissue. The reason for this is not clear. It is possible that MNV infection in the lymphatic system is inaccessible to specific cell subsets or antibodies. Passive transfer of mAbs successfully limited MNV infection in the intestine of RAG -/- mice; however, as RAG1-/- mice do not have normal mesenteric lymph nodes, adoptive and passive transfer experiments shed no light on the role of antibodies or T cells in lymphatic infection. Alternatively, because viral loads in the MLN are typically higher per gram of tissue weight than in the ileum, individual immune cell subsets may be insufficient to clear infection and instead require the concerted effect of multiple cell types.

The role of B cells in control of MNV infection appears to be primarily due to the production of anti-viral antibody. Data supporting this conclusion include decreased mucosal MNV infection following passive transfer of polyclonal antisera into immunodeficient mice (Fig. 5.8A) and the lack of viral clearance in the MLN of B cell deficient μMT mice (Fig. 5.7). We further found that IgG mAbs can significantly decrease MNV infection at mucosal sites (Fig. 5.8B). Together these data argue that systemic IgG has a significant effect on mucosal norovirus infection, although the specific types of IgG induced and epitopes targeted by vaccination to alter mucosal infection remains an open question.

In conclusion, this report includes the first demonstration of short and long term protective immunity against norovirus infection using both live virus and VLP-based vaccines. We showed that B cells, CD4 and CD8 T cells are all required for generation of complete and efficient protective immunity against MNV challenge, although protective

immunity can still be generated in the absence of individual subsets. Additionally, a combination of studies using immunodeficient mice, lymphocyte depletion, and passive and adoptive transfers revealed that antibody, CD4, and C8 T cells each had important and tissue specific roles in clearance of norovirus infection from the murine intestine and MLN, with the role of CD8 T cells partially dependent on the effector molecule perforin.

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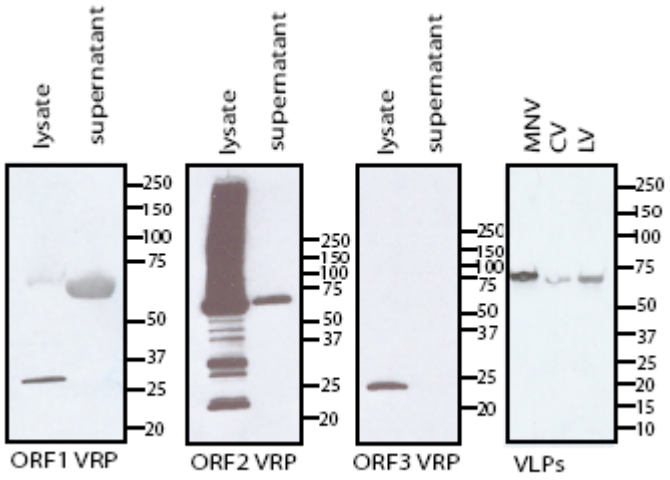
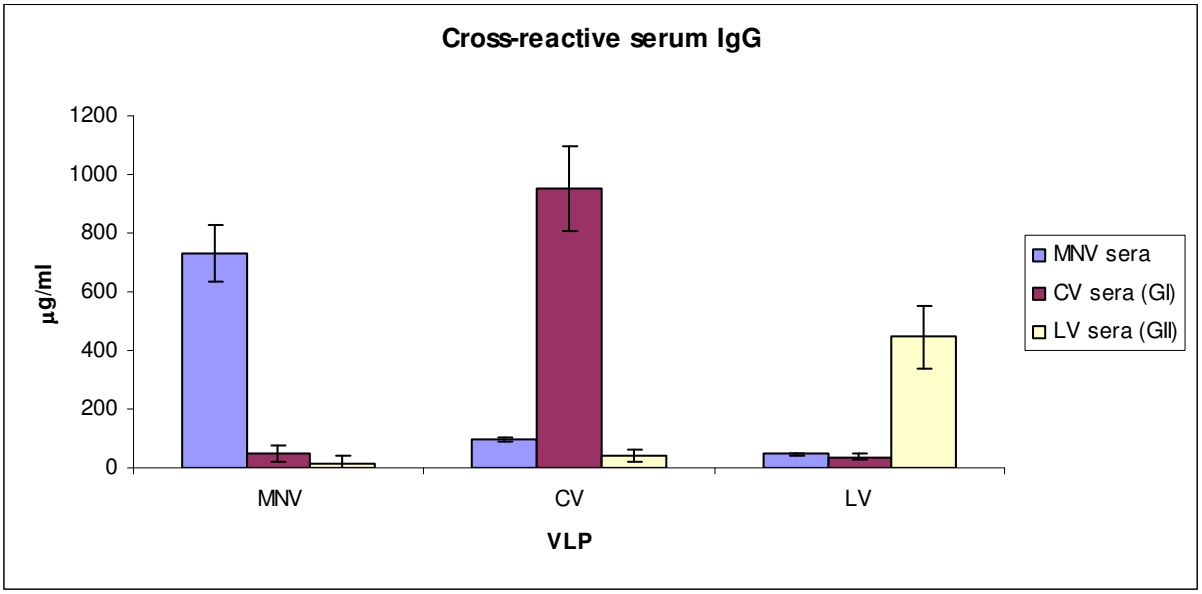
Figure 5.1

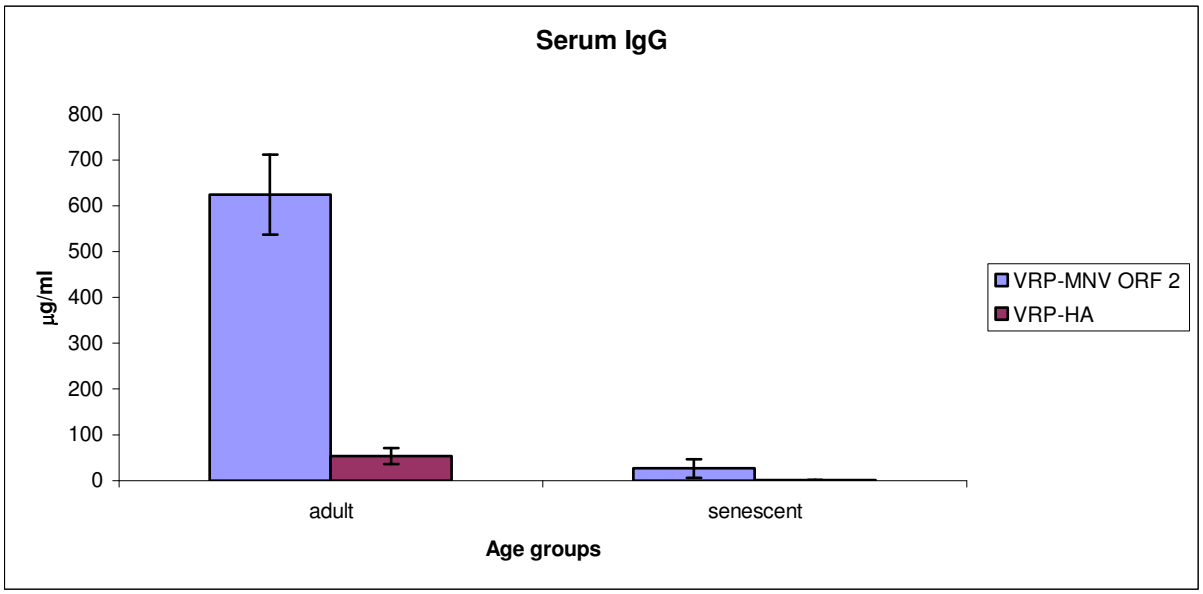
A.


lysate
supernatant
lysate
supernatant
lysate
supernatant
MNV
CV
LV
250
150
100
75
50
37
25
20
ORF1 VRP
ORF2 VRP
ORF3 VRP
VLPs

B.


Cross-reactive serum IgG
µg/ml
1200
1000
800
600
400
200
0
MNV
CV
LV
VLP
MNV sera
CV sera (GI)
LV sera (GII)

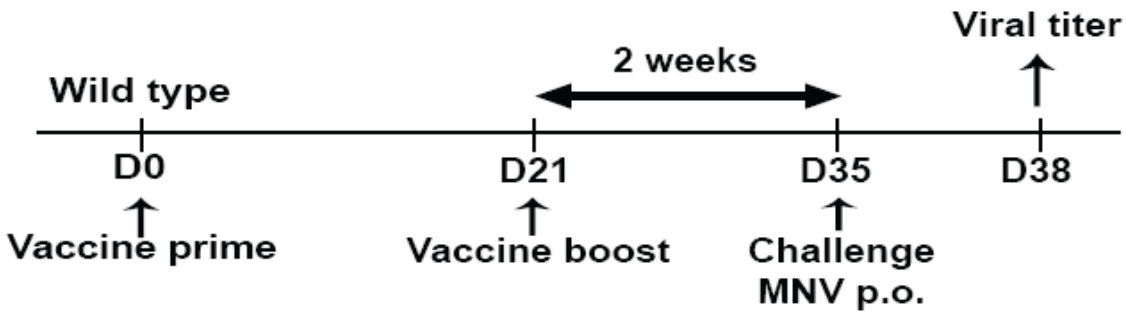
C.



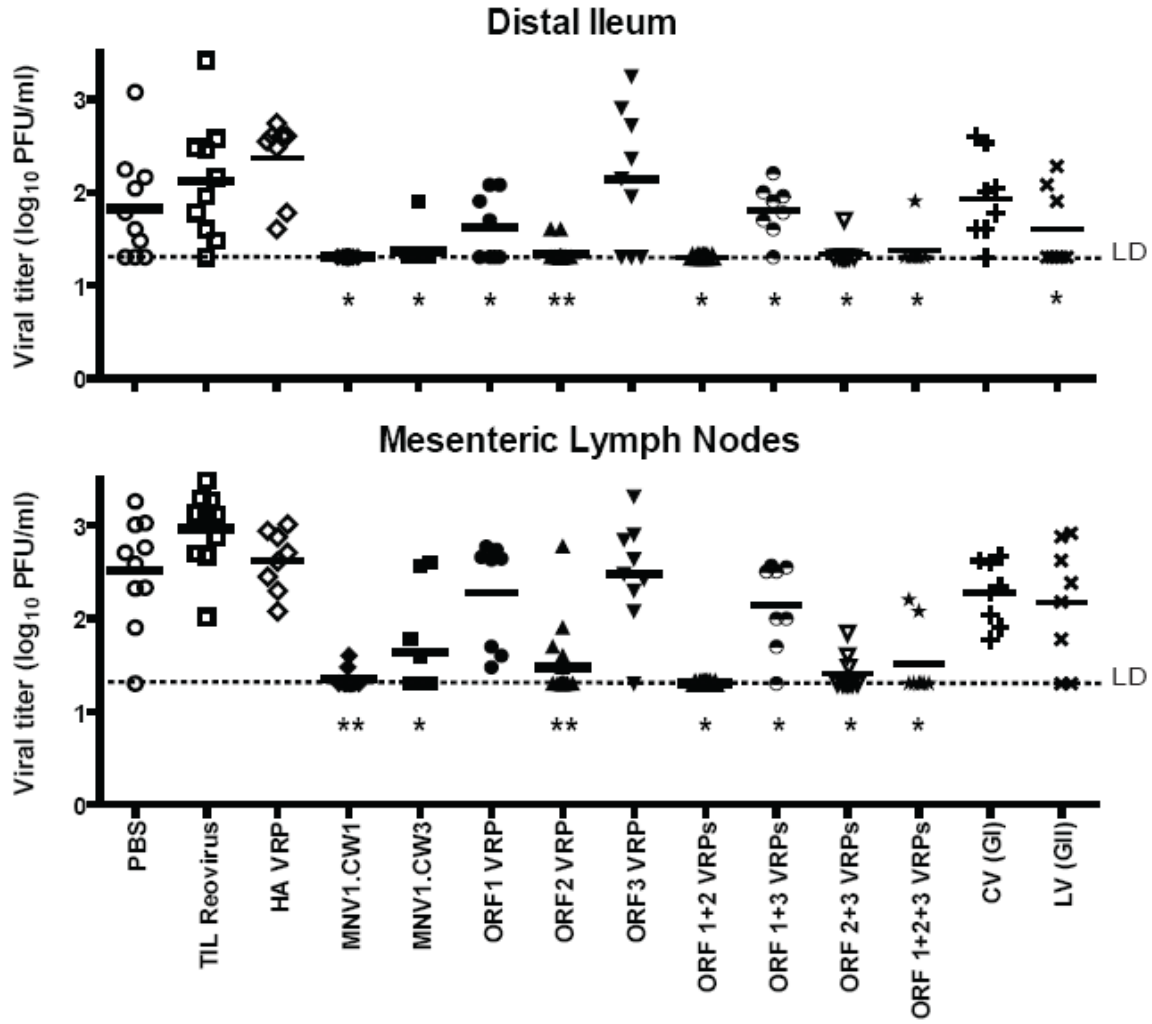
VRP protein expression and serum IgG responses in immunized mice.
(A) MNV protein expression from culture supernatants and cell lysates of BHK cells infected with VRPs expressing MNV1.CW3 ORF1, ORF2, or ORF3. Chiba virus (CV) and Lordsdale virus (LV) VLPs were analyzed for cross-reactivity with MNV rabbit polyclonal antisera by western blot. **(B)** Sera from mice immunized with VRPs expressing ORF2 from MNV1.CW3, CV, or LV tested for cross-reactivity to MNV1.CW3, CV, and LV VLPs by ELISA. **(C)** Serum anti-MNV antibody measured by ELISA from 8 week old and 14 month old mice after MNV1.CW3 challenge.

Figure 5.2

A.


Viral titer
2 weeks
Wild type
D0
D21
D35
D38
Vaccine prime
Vaccine boost
Challenge
MNV p.o.

B.


Distal Ileum
Viral titer (log10 PFU/ml)
LD
Mesenteric Lymph Nodes
Viral titer (log10 PFU/ml)
LD
PBS
TIL Reovirus
HA VRP
MNV1.CW1
MNV1.CW3
ORF1 VRP
ORF2 VRP
ORF3 VRP
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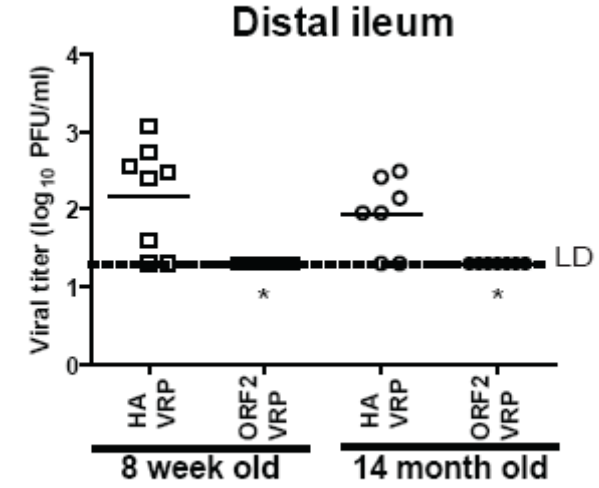
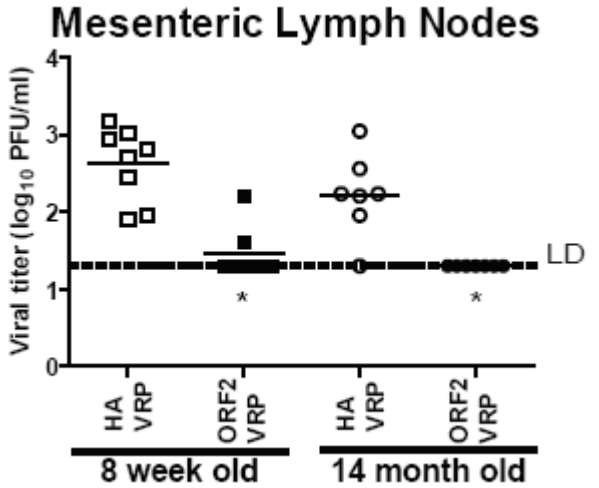
CV (GI)
LV (GII)

C.

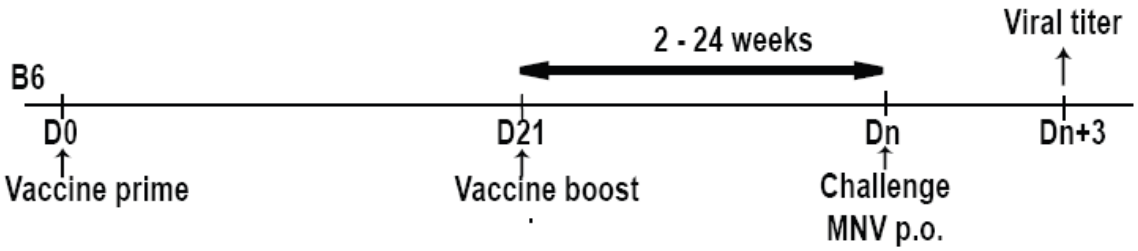




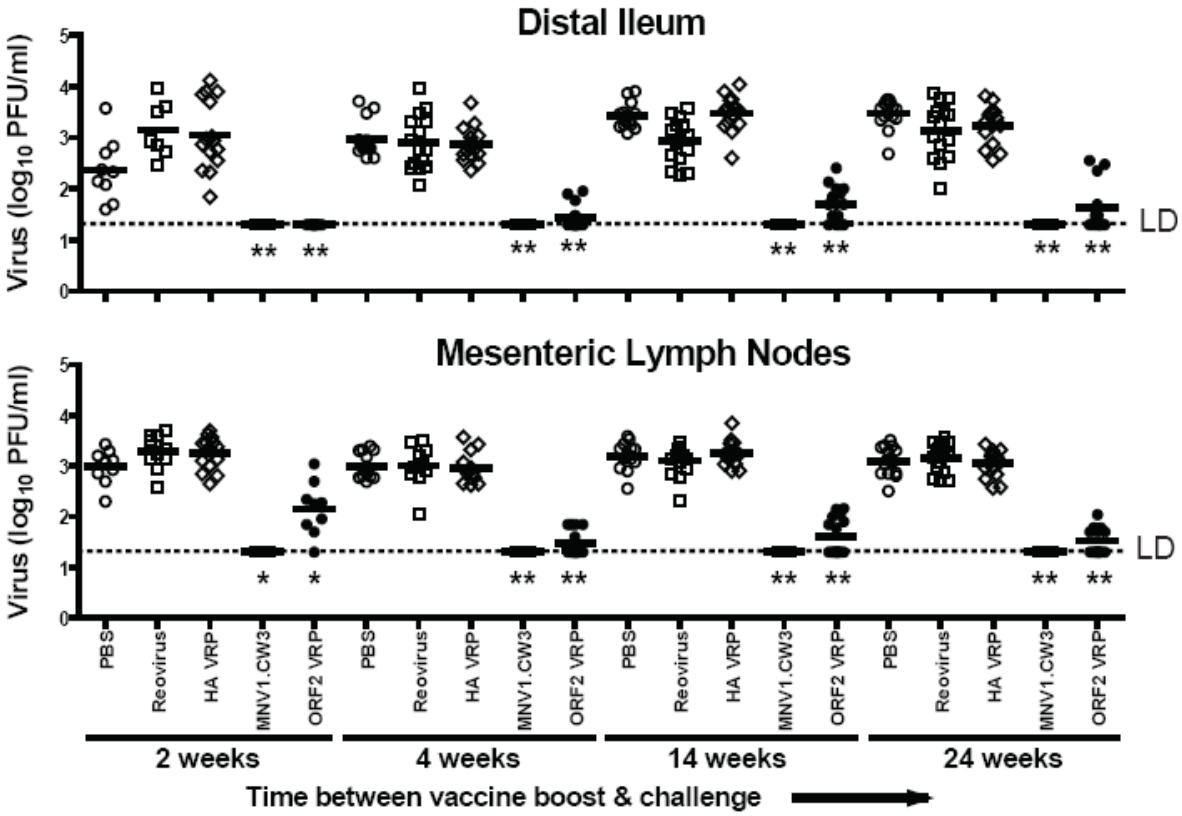
Short term vaccination against MNV using live MNV strains and VRPs expressing ORF1, ORF2 and ORF3 from MNV and ORF2 from Chiba virus and Lordsdale virus. **(A)** Vaccination protocol used in short term vaccination. **(B)** Viral titers in distal ileum and MLN of wild type mice after MNV1.CW3 challenge following vaccination with the indicated vaccines. **(C)** Viral titers in distal ileum and MLN of 8 week old or 14 month old wild type mice immunized with MNV1.CW3 ORF2 VRP or HA VRP and challenged with MNV1.CW3. These data are pooled from 2 independent experiments with 3-5 mice per group in each experiment. (**) indicates $p<0.0001$, (*) indicates $p<0.05$. LD indicates the limit of detection.

Figure 5.3

A.


2 - 24 weeks
Viral titer
B6
D0
D21
Dn
Dn+3
Vaccine prime
Vaccine boost
Challenge
MNV p.o.

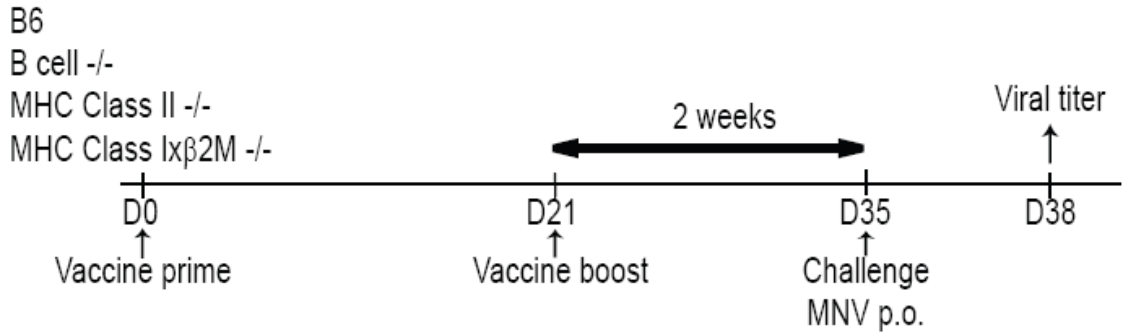
B.



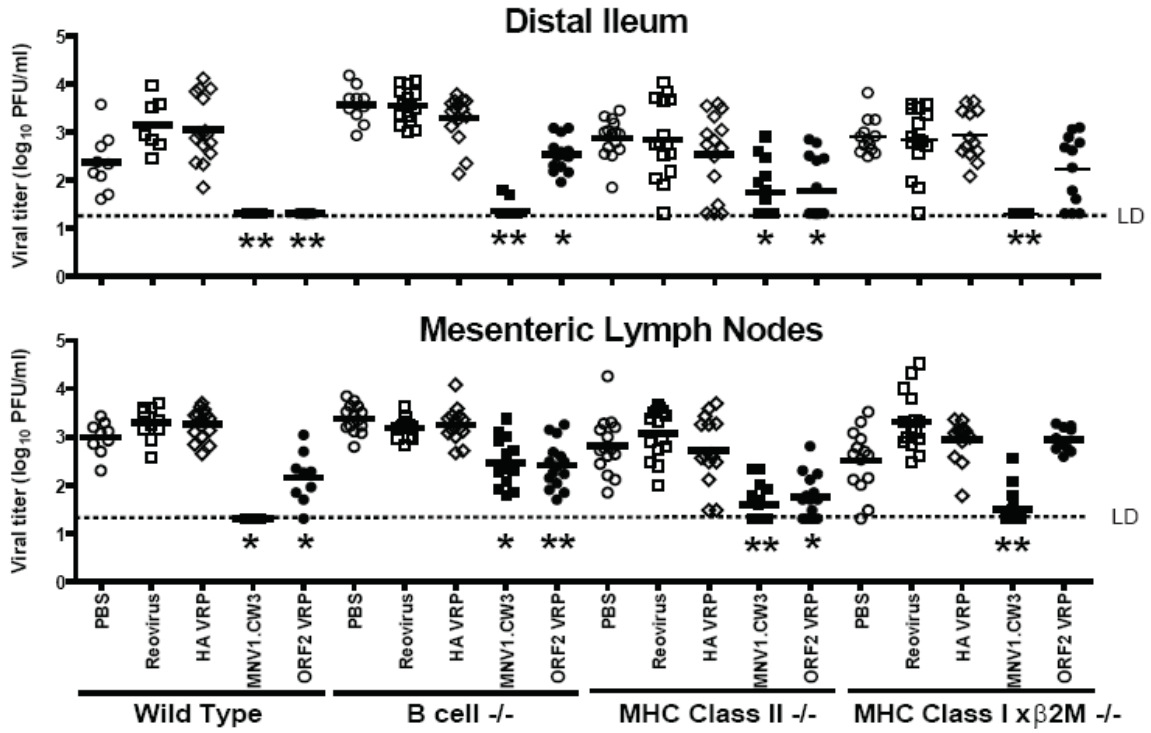
Long term vaccination against MNV using a live MNV strain and ORF2 VRPs.
(A) Vaccination protocol used in long term vaccination. **(B)** Viral titers in distal ileum and MLN of wild type mice after MNV1.CW3 challenge following vaccination with indicated vaccines. These data are pooled from 3 independent experiments with 5 mice per group in each experiment. (**) indicates $p<0.0001$, (*) indicates $p<0.05$. LD indicates the limit of detection.

Figure 5.4

A.


B6
B cell -/-
MHC Class II -/-
MHC Class Ixβ2M -/-
2 weeks
Viral titer
D0
D21
D35
D38
Vaccine prime
Vaccine boost
Challenge
MNV p.o.

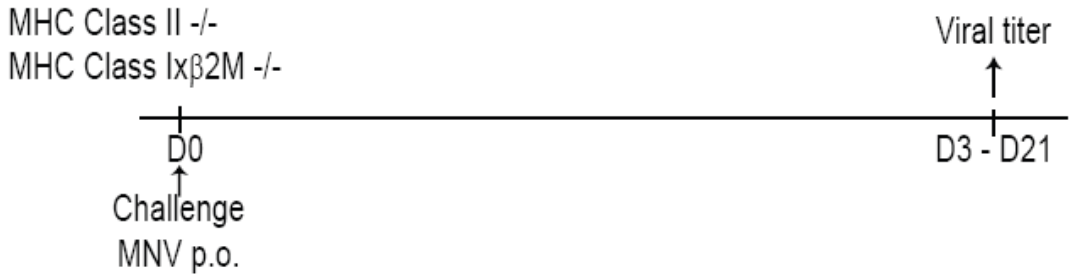
B.


Distal Ileum
Mesenteric Lymph Nodes
Viral titer (log10 PFU/ml)
LD
PBS
Reovirus
HA VRP
MNV1.CW3
ORF2 VRP
Wild Type
B cell -/-
MHC Class II -/-
MHC Class I xβ2M -/-

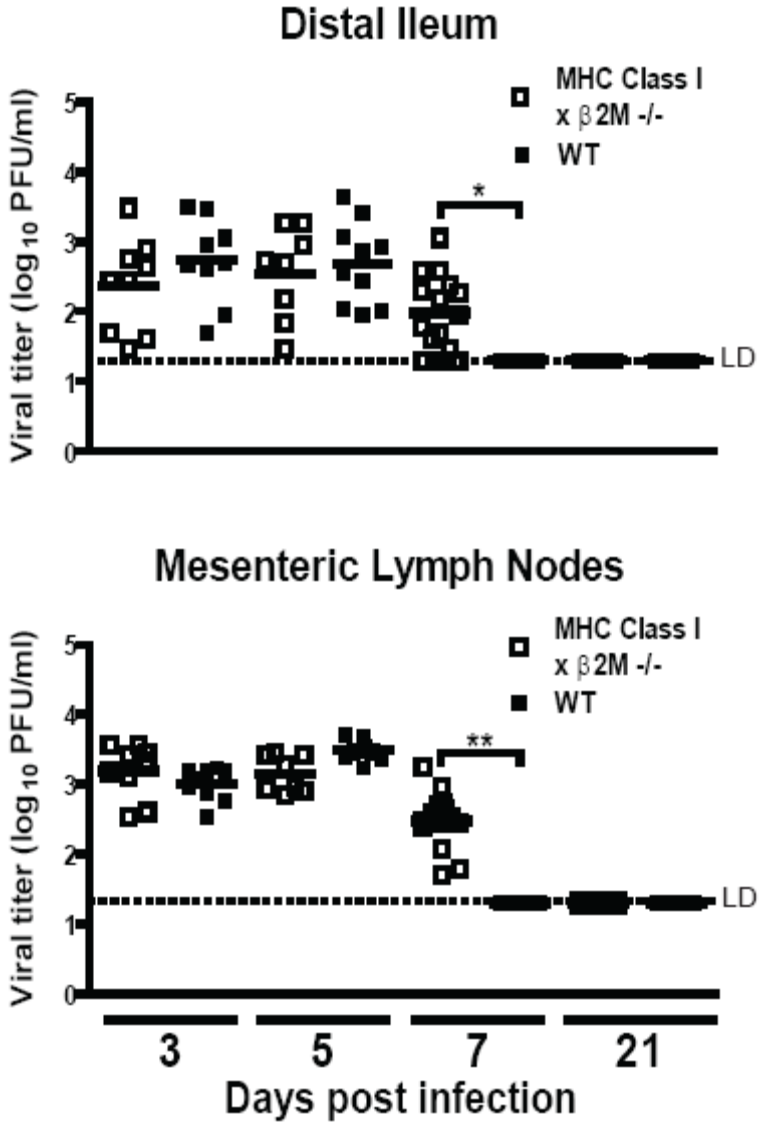
Complete short term protection against MNV infection requires MHC Class II, MHC Class I, β2M and B cells. **(A)** Vaccination protocol used in short term vaccination using immunodeficient mice. **(B)** Viral titers in distal ileum MLN of B cell -/-, MHC Class II -/- and MHC Class I x β2M -/- mice after MNV1.CW3 challenge following short term vaccination with the indicated vaccines. These data are pooled from 3 independent experiments with 3-5 mice per group in each experiment. (**) indicates $p<0.0001$, (*) indicates $p<0.05$. LD indicates the limit of detection.

Figure 5.5

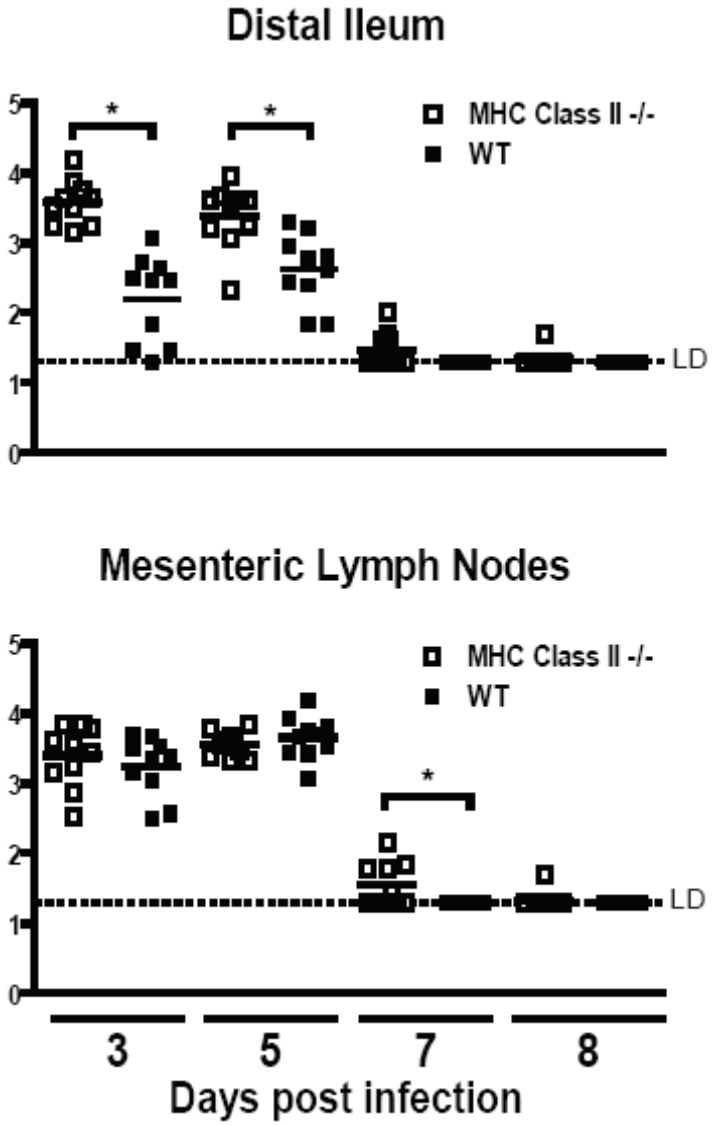
A.


MHC Class II -/-
MHC Class Ixβ2M -/-
Viral titer
D0
D3 - D21
Challenge
MNV p.o.

B.


Distal Ileum
MHC Class I
x β2M -/-
WT
Viral titer (log10 PFU/ml)
*
LD
Mesenteric Lymph Nodes
MHC Class I
x β2M -/-
WT
Viral titer (log10 PFU/ml)
**
LD
3
5
7
21
Days post infection

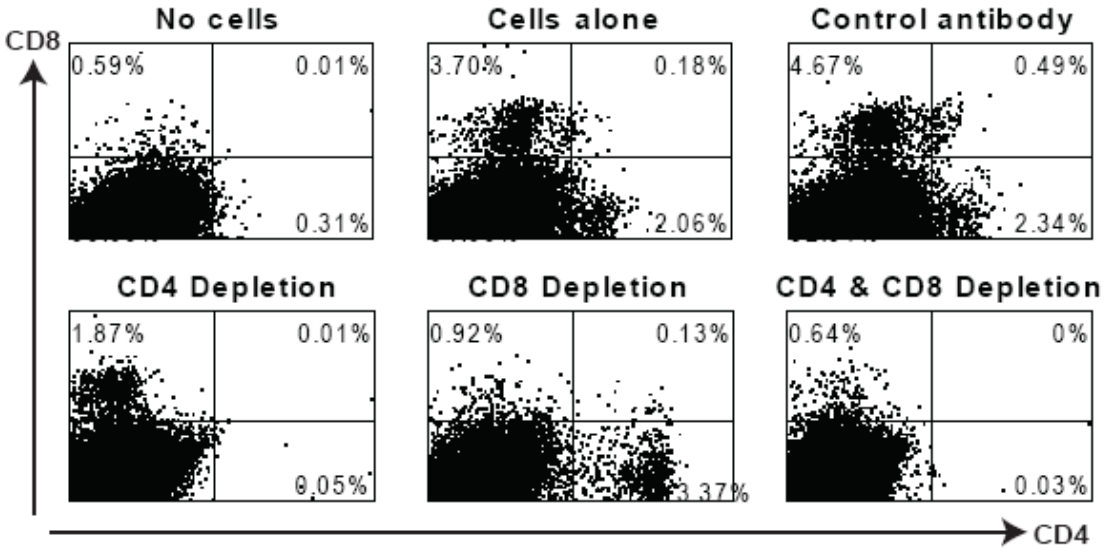
C.



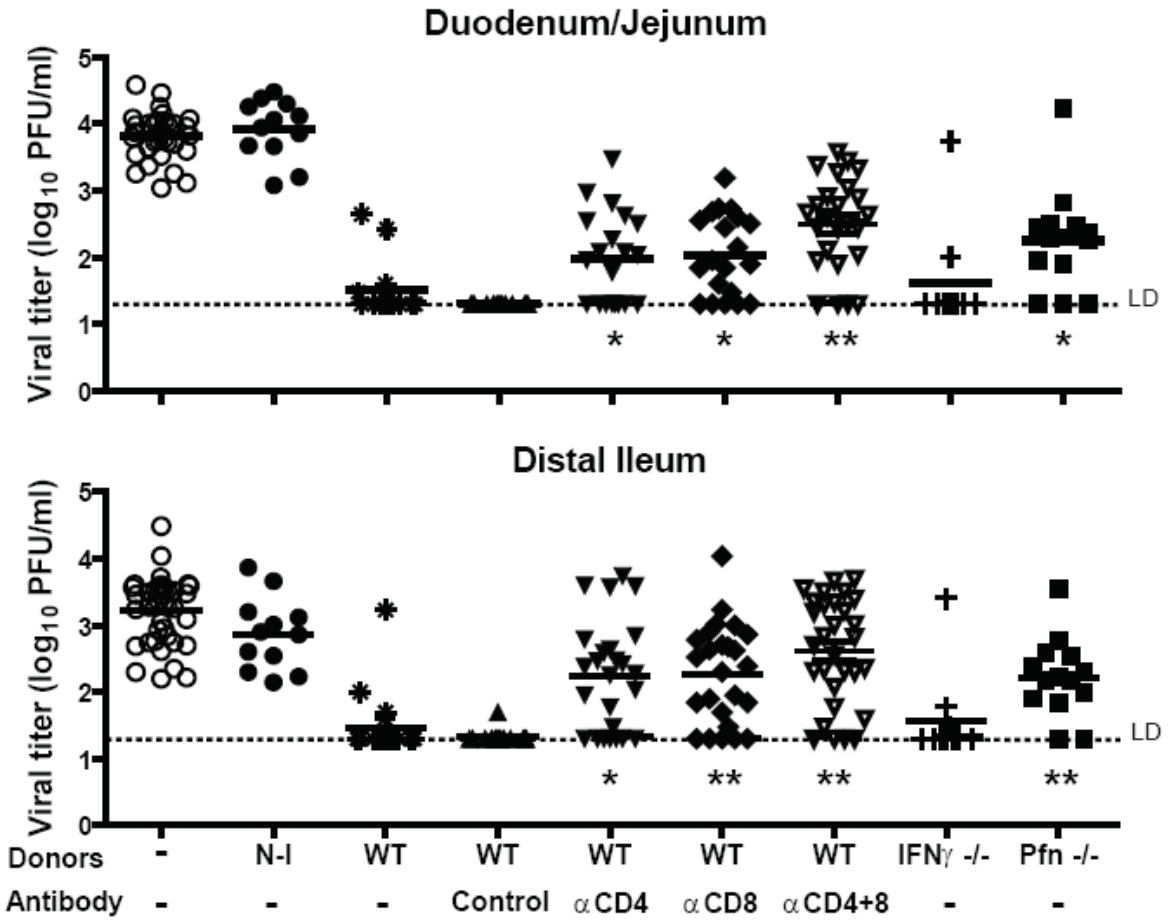
MHC Class II limits early MNV replication, and deficiency in MHC Class II or MHC Class I & β_2m delays MNV clearance. **(A)** Protocol of challenge used in experiments in this figure. Viral titers in distal ileum and MLN of wild type and MHC Class I x β_2-m$^{-/-}$ mice **(B)** and MHC Class II-/- mice **(C)** after infection with MNV1.CW3. These data are pooled from 3 independent experiments with 3-5 mice per group in each experiment. (**) indicates $p<0.0001$, (*) indicates $p<0.05$. LD indicates the limit of detection.

Figure 5.6

A.

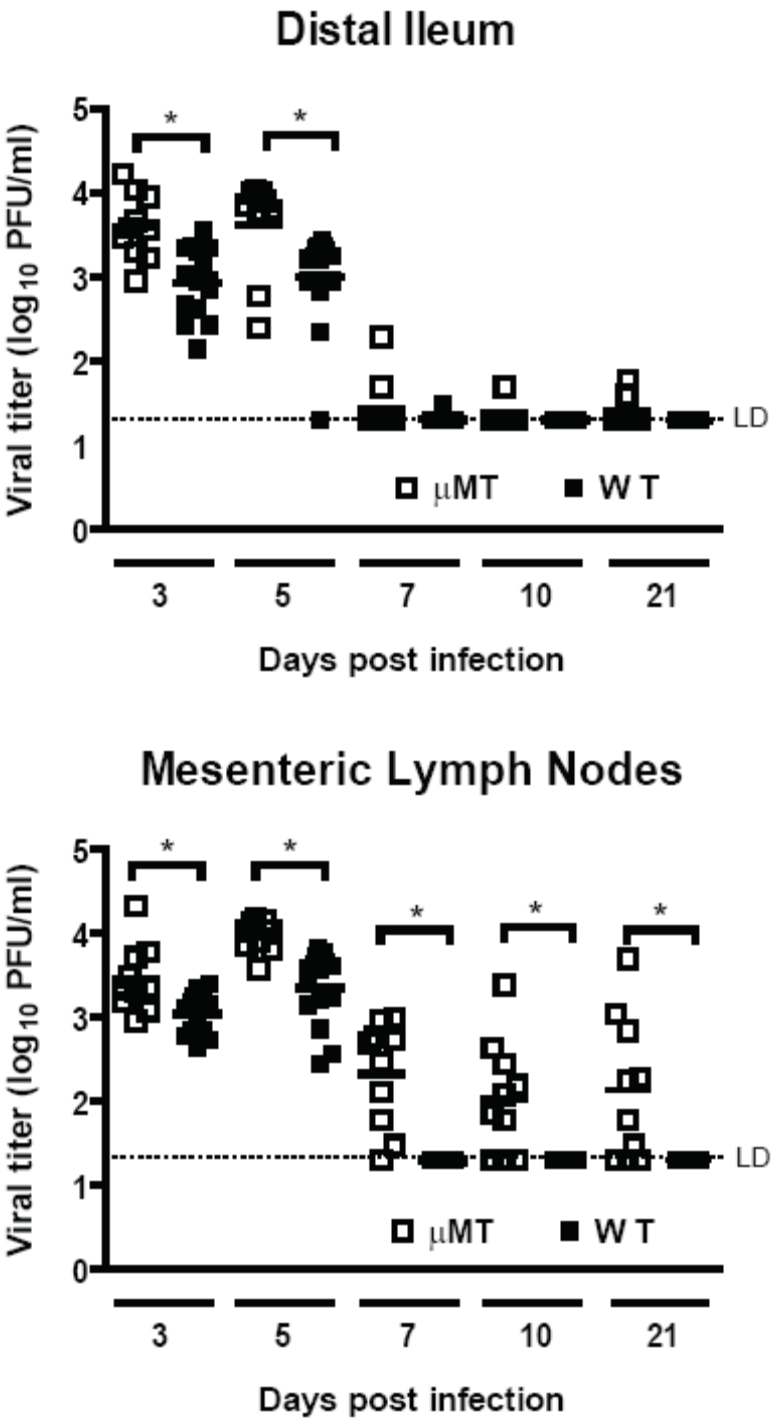

No cells
Cells alone
Control antibody
CD8
0.59%
0.01%
0.31%
3.70%
0.18%
2.06%
4.67%
0.49%
2.34%
CD4 Depletion
CD8 Depletion
CD4 & CD8 Depletion
1.87%
0.01%
0.05%
0.92%
0.13%
3.37%
0.64%
0%
0.03%
CD4

B.



Immune CD4 and CD8 T cells are both required and perforin plays a role in clearance of persistent MNV infection in RAG1-/- recipients. (A) Representative flow cytometric analysis of splenocytes harvested from RAG1-/- recipient mice 6 days post transfer of splenocytes. (B) Viral titers in duodenum/jejunum and distal ileum 6 days after adoptive transfer of medium alone, wild type non-immune splenocytes, wild type immune splenocytes, wild type immune splenocytes with or without depleting antibodies or immune splenocytes from IFNγ -/- or perforin -/- (Pfn -/-) mice . These data are pooled from 3 independent experiments with 3-5 mice per group in each experiment. (**) indicates $p<0.0001$, (*) indicates $p<0.05$. LD indicates the limit of detection.

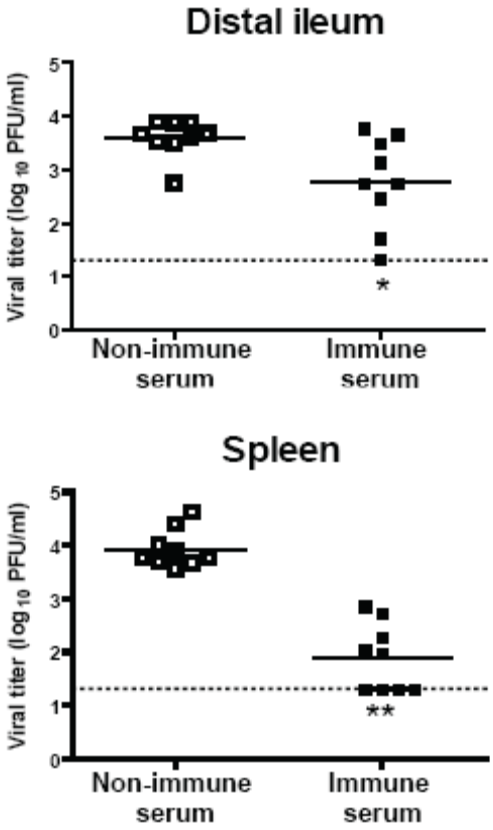
Figure 5.7


Distal Ileum
Viral titer (log_{10} PFU/ml)
5
4
3
2
1
0
*
*
LD
μMT
WT
3
5
7
10
21
Days post infection
Mesenteric Lymph Nodes
Viral titer (log_{10} PFU/ml)
5
4
3
2
1
0
*
*
*
*
*
LD
μMT
WT
3
5
7
10
21
Days post infection

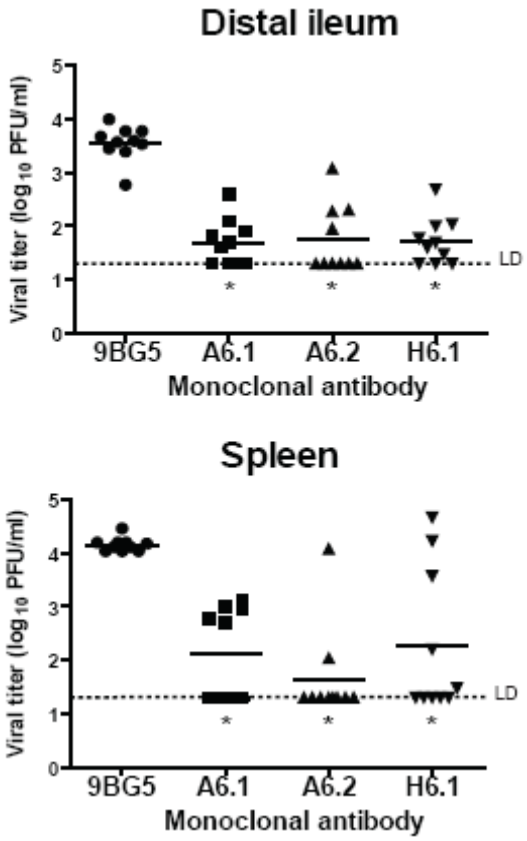
B cells limit MNV replication and are required for MNV clearance in the distal ileum and mesenteric lymph nodes. Viral titers in distal ileum and MLN of B6 and μMT mice. These data are pooled from at least 2 independent experiments with 5 mice per group in each experiment and each symbol indicates a sample from an individual mouse. (*) indicates $p<0.05$. LD indicates the limit of detection.

Figure 5.8

A.


Distal ileum
Viral titer (log 10 PFU/ml)
0
1
2
3
4
5
*
Non-immune serum
Immune serum
Spleen
Viral titer (log 10 PFU/ml)
0
1
2
3
4
5
**
Non-immune serum
Immune serum

B.



Immune serum and neutralizing IgG monoclonal antibodies reduce MNV infection from intestine and spleen of RAG1-/- mice. Viral titers in distal ileum and spleen of persistently infected RAG1-/- recipients 6 days after passive transfer of either immune serum (A) or neutralizing monoclonal antibodies specific for capsid protein (B). These data are pooled from 3 independent experiments with 3 mice per group in each experiment. (**) indicates $p<0.0001$, (*) indicates $p<0.05$. LD indicates the limit of detection.

CHAPTER VI.

Alphavirus adjuvanted norovirus VLP vaccines: Heterologous, humoral and mucosal immune responses protect from murine norovirus challenge

Abstract

The development of an effective norovirus vaccine likely requires the capacity to protect against infection with multiple norovirus strains. Advanced recombinant genetic systems and the recent discovery of a mouse-tropic norovirus strain provide robust model systems for vaccine efficacy studies. We coadministered multivalent VLP vaccines with alphavirus adjuvant particles to mice and evaluated homotypic and heterotypic humoral and cellular protective immunity to human and murine norovirus strains. Multivalent VLP vaccines induced robust IFN-γ and receptor-blocking antibody responses to heterologous human strains not included in the vaccine composition. Furthermore, vaccination with or without MNV VLP resulted in significantly reduced viral loads following MNV challenge. Passive transfer of sera from mice monovalently vaccinated with MNV VLP to immunodeficient mice completely protected against MNV infection; however, adoptive transfer of purified $CD4^+$ or $CD8^+$ cells did not influence viral loads in murine tissues. Together, these data suggest humoral immunity induced by multivalent norovirus vaccines may protect against heterologous norovirus challenge.

Introduction

Noroviruses are responsible for at least 23 million global infections in the United States annually (166), although this number is likely under a drastic underestimate. Effective vaccines are needed to control widespread norovirus outbreaks; however, immunity to noroviruses remains a controversial topic, as short-term but not long-term immunity could protect against re-infection in human challenge studies (119, 203). Human norovirus vaccine research has been hampered by the lack of a small animal model or *in vitro* culture system for identifying key components of protective immunity. The manipulation of recombinant genetic systems, however, has allowed for *in vitro* production of norovirus antigens from multiple strains that can be delivered as norovirus vaccines to small animals to generate immune responses (10, 80, 93, 105, 178, 207, 284, 289). Furthermore, the recent discoveries of murine norovirus strains that can replicate *in vitro* and *in vivo* provide alternative avenues for vaccine development (124, 277).

Previous studies with norovirus-like particle (VLP) vaccination have shown that humoral and cellular immune responses can be generated to human norovirus strains in both humans and mice (8, 9, 178, 246, 247). Furthermore, antibody responses following infection with norovirus or immunization with VLP can block ABH histo-blood group antigen (HBGA) binding to VLPs in a strain specific manner (91). HBGAs are carbohydrates ubiquitously expressed on mucosal tissues and red blood cells that have been implicated as natural receptors for norovirus binding and entry, suggesting blockade of HBGA interactions with VLPs may prevent norovirus infection (159). Alternatively, $CD4^+$ T cell responses following norovirus infection in humans or VLP vaccination in mice are also induced and have been characterized by secretion of Type II interferons (IFN-γ) upon stimulation with

VLPs (145, 178). Presently, however, the components of protective immunity and the impact of multiple exposures in norovirus immunity are unknown.

The norovirus family consists of 40+ genetically diverse strains that can differ by up to 40% in capsid amino acid sequence between strains within a genogroup and by >50% between genogroups (75). Most studies into norovirus immunity have focused on individual strains; however, a limited number have shown that antibody responses to one norovirus strain have little cross-reactivity to other strains both within and across genogroups (86, 145, 180, 219, 259). Not surprisingly, human challenge studies have shown that infection with one norovirus strain does not prevent infection with another strain (281). Efficacy of norovirus vaccines, however, is dependent on protection from multiple circulating strains. Our group previously addressed this problem by showing that multivalent immunization with VEE replicon particles (VRPs) expressing norovirus VLPs from three genetically distinct strains induced antibody responses that blocked receptor binding to a heterologous VLP not included in the vaccine composition (149). This is the first data suggesting that administration of multiple VLPs may be a successful vaccination strategy for protection against more than one norovirus strain, including those not incorporated in the vaccine. However, the key strains necessary for eliciting a broad-based immune response to multiple noroviruses still requires more detailed studies including homologous and heterologous cross-challenge in experimental animals.

To induce robust immune responses to noroviruses, some VLP vaccines have been coadministered with known biological adjuvants (8, 10, 80, 178, 207). Previous studies from our group have alternatively used the VRP as a delivery vaccine vector for VLP expression *in vivo* (93). We believe this model is advantageous because mammalian cells are permissive

to VRP infection and VLPs are assembled *in vivo* in large quantities, with the caveat that different vaccine formulations are needed for each norovirus strain. However, a recent finding from Thompson *et al*. showed that VRPs have inherent adjuvant activity when uncoupled from transgene expression function (256). This approach has enhanced safety features associated with uncoupling VRP infectivity from transgene protein expression function and the ability to administer multiple VLPs simultaneously with a common adjuvant However, it is not clear if this approach provides for protective immune responses.

The concurrent discovery of murine noroviruses (MNV) provides a workable model in which to test the efficacy of multivalent vaccine formulations as well as the mechanism of protection (124, 277). Upon peroral inoculation, MNV infects the murine intestine as the primary site of replication followed by establishment in multiple peripheral tissues as secondary sites. In this study, we evaluate in detail the immune responses elicited to human and murine norovirus strains within and across genogroups following multivalent vaccination with norovirus VLPs coadministered with null VRP adjuvants. These experiments establish the null VRP adjuvant as a robust strategy for eliciting high levels of protective humoral and cellular immunity against noroviruses.

Materials and methods

VLPs and VRPs. VRPs expressing norovirus ORF 2 were cloned and produced as described in (11). Null VRP were kindly provided by the Carolina Vaccine Institute (UNC). Norovirus VLPs were produced and purified as described in (149) and visualized by EM to insure appropriate particle size and structure. VLPs used in vaccination experiments were further concentrated by centrifugation at 3000 x *g* in Centricon tubes (Millipore) O/N at 4º C.

VLPs used in ELISpots were produced by direct electroporation of pVR21-NoV ORF2 RNA to avoid potential structural VEE protein contamination.

Vaccination. 6 week old BALB/c mice (Charles River) were vaccinated via footpad inoculation with monovalent or multivalent norovirus VLP vaccines containing 2 μg of each VLP alone or in conjunction with 10^5 null VRP or 1 μg CpG DNA (Invivogen). Mice used in VLP titration experiments received VLP doses of 0.02 μg, 0.2 μg, 2 μg, or 10 μg Norwalk virus (NV) VLP coadministered with null VRP. Other monovalent vaccination groups received NV (GI.1), , Lordsdale-like (LV; GII.4), or MNV-1 (GV) VLPs. Multivalent groups received genogroup I (GI)-specific VLPs representing Southampton (SH; GI.2), Desert Shield (DS; GI.3), and Chiba (CV; GI.4) strains with or without NV VLPs; GII-specific VLPs representing Hawaii (HV; GII.1), Toronto (TV; GII.3), and M7 (GII.13) strains with or without LV VLPs; or complete VLP cocktails containing all GI and GII VLPs with or without NV and LV VLPs or all GI and GII VLPs with or without MNV VLPs (GV) (Table 6.1). Mice were vaccinated and boosted at days 0 and 28. Donor mice for adoptive transfers were vaccinated a third time on day 52.

MNV infection. MNV-1 CW.3 was kindly provided by Dr. H.W. Virgin (Washington University School of Medicine). To generate virus stocks, murine macrophage-like Raw 264.7 cells (UNC TCF) cultured in complete DMEM (Gibco) were infected with MNV at an MOI of 0.1 and incubated for 36 hours. Supernatant was then collected, clarified by centrifugation at 13,000 x *g* for 15 min (Beckman), and ultracentrifuged for 3 h at 100,000 x *g* over a 5% sucrose cushion to pellet purified virus. Pellets were resuspended in PBS, aliquotted, and stored at -80º C until use. Virus stocks were titered by plaque assay as

previously described (277). Mice used in MNV challenge experiments were infected with 3 x 10^7 pfu MNV-1 CW.3 in 30 μl total volume per orally on d 42.

Sera, feces, and tissue samples. Animals were euthanized and distal ileum, spleen, mesenteric lymph node (MLN), and sera were harvested from mice used in MNV challenge experiments on d 45 and stored at -80º C. Tissues were resuspended in 1 ml complete DMEM and disrupted with silica/zirconia beads (Biospec Products) using the MagnaLyser homogenizer (Roche) at 6000 rpm for 30 s. Sera and fecal samples from all other mice were collected on d 42. Ten fecal pellets per mouse were resuspended in 1 ml PBS containing 10% goat serum and 0.01% Kathon fecal inactivator (Supeleco) and homogenized by vortexing for 20 min. Solid material was then removed by centrifugation for 20 min, and fecal extracts stored at -20º C.

ELISA and HBGA binding blockade assays. ELISAs for serum IgG antibody cross-reactivity to NoV VLPs and binding assays for serum antibody blockade of HBGA binding were performed as previously described (149). IgG subtype ELISAs were performed as described using purified IgG1 (Sigma) or IgG2a (Sigma) as standard controls and anti-IgG1-AP (Southern Biotech) and anti-IgG2a-AP (Southern Biotech) as secondary antibodies, respectively. To quantitate specific antibody in fecal extracts, 96-well high binding plates (Costar) were coated with 2 μg VLP or serially diluted mouse IgG or IgA standard for 4 h at RT and blocked overnight in blocking buffer (Sigma) at 4° C. Fecal extracts diluted 1:2 in blocking buffer were 2-fold serially diluted and incubated in wells containing VLP for 2 h at RT. Wells were then incubated with anti-mouse IgG-HRP or IgA-HRP (Southern Biotech) for 2 h and developed with OPD tablets (Sigma) dissolved in 1:1 0.1 M sodium citrate and 0.1 M citric acid and 0.02% hydrogen peroxide for 30 min in the dark. Reactions were

stopped with 0.1 M sodium fluoride and read at OD_{450} (Biorad Model 680). IFN-γ EIA (BD Pharmingen) was performed per manufacturer's instructions. Splenocyte culture was conducted as described for ELISpots. Culture supernatants were clarified by centrifugation, diluted 1:2 in assay diluent, followed by two-fold serial dilutions for use in the assay.

ELISpots. Prewetted multiscreen 96-well filtration plates (Millipore) were coated overnight with rat anti-mouse IFN-γ antibody AN18 (Mabtech) at a concentration of 5μg/ml in 100 μl PBS at 4°C. Plates were then blocked for >1 h with complete RPMI 1640 media (10% FBS, 1mM non-essential amino acids, 1mM sodium pyruvate, 100 ug/ml penicillin-streptomicin) at 37°C. Spleens from vaccinated mice were harvested and individual splenocyte suspensions obtained by manual disruption, filtration with a 100 μm cell strainer, and lysis of red blood cells. 5×10^5 splenocytes were seeded onto plates and stimulated with NV VLPs or LV VLPs at 1 μg/ml or Concanavalin A at 5 μg/ml (MP Biomedicals) in duplicate in 100 μl media for 48 h at 37°C under 5% CO_2. Plates were then washed with PBS/0.05% Tween, incubated with 100 μl biotinylated anti-mouse IFN-γ antibody at 1ug/ml (Mabtech) in PBS-0.5% FBS for 2 h at 37°C, washed, and incubated with 100 μl streptavidin-alkaline phosphatase (Mabtech) diluted 1:1000 in PBS-0.5% FBS for 1 h. Plates were washed and developed with 100 μl BCIP/NBT (Promega) as substrate in AP Buffer (0.5 M Tris, 0.5 M NaCl, 0.025 M $MgCl_2$). Spots counts were analyzed by Zellnet Consulting (Fort Lee, New Jersey). Wells containing confluent areas were assigned compensatory values using the equation [total spot number = spot count + 2 x (spot count x %confluence /[100% - %confluence])].

Passive and adoptive transfers. Eight wild-type mice were immunized as described above, and unimmunized controls were treated in parallel. Sera and spleens from immune and

control groups were harvested on d 56. Spleens from respective immunization groups were pooled and single-cell splenocyte suspensions obtained by manual disruption through a 100 μm cell-strainer. Splenocyte suspensions were resuspended in MACS buffer (PBS pH 7.2, 0.5% BSA, 2 mM EDTA), divided in half, and $CD4^+$ or $CD8^+$ cells purified, respectively, by magnetic bead sorting using the QuadroMACS purification system (Miltenyi) per manufacturer's protocol. For adoptive transfers, 5 x 10^6 $CD4^+$ or $CD8^+$ cells from immune or non-immune mice were administered in a total volume of 500 μl i.p. to Scid C.B.17 mice (Jackson Laboratories) (N=6/recipient group). For passive transfer of sera, immune or non-immune serum samples were equivalently pooled, diluted 1:2 in PBS, and administered i.p. to Scid mice at 200 μl per mouse. Scid mice were challenged with 3 x 10^7 pfu MNV.CW3 twenty-four hours post-transfer, and tissues were harvested three days post-infection. Tissue samples were processed as described above.

FACS. Whole and purified splenocyte suspensions from adoptive transfer groups were set aside for FACS analysis. 5 x 10^5 cells per tube were blocked with anti-FcκII/III (1:500; eBioscience) in 100 μl FACS buffer (HBSS + 2% FBS) for 20 min on ice. Cells were then pelleted, resuspended in 100 ul FACS buffer, and stained with anti-B220 conjugated to FITC (1:400), APC (1:400), or biotin (1:800) as single color controls for staining or cocktails containing anti-CD3-FITC (1:200), anti-CD4-Biotin (1:1000), and anti-CD8-APC (1:800). Cells were incubated for 45 min on ice, pelleted, and resuspended in 100 μl FACS buffer with avidin-PerCP (1:400) for 45 min on ice. Samples were then washed and resuspended in 500 μl PBS. All antibodies were obtained from eBioscience (San Diego, CA). FACS analysis was performed by the UNC Flow Cytometry Core Facility.

Real-time PCR. RNA was Trizol-extracted from VLPs and subjected to RT-PCR using random hexamers for amplification. Real-time PCR was performed using non-structural VEE-specific primers provided by Ande West (Carolina Vaccine Institute, UNC).

Statistics. All statistics comparing two groups were performed using the two-tailed t-test; all statistics comparing multiple groups were performed using One-way ANOVA and Tukey's post-test in GraphPad software. Single asterisks (*) are representative of P values <0.05, double asterisks (**) are representative of P<0.01, and triple asterisks (***) are representative of P<0.001.

Results

Null VRP adjuvants induce robust systemic and mucosal antibody responses in monovalent VLP vaccines.

To determine effective VLP concentrations for subsequent vaccinations, mice were immunized twice with a VLP titration series consisting of 10 μg, 2 μg, 0.2 μg, or 0.02 μg NV VLP coadministered with 10^5 IU null VRP. Fecal IgA, fecal IgG, serum IgG, and serum blockade of receptor binding were evaluated (Fig. 6.1)**.** Measurable IgA and IgG were detected in fecal extracts of all mice receiving 0.2-10 μg VLP (Fig. 6.1a). Antibody titers increased in correlation with increasing amounts of VLP administered, and IgG titers were consistently higher than IgA. Serum antibody was also significantly higher following vaccination with all VLP concentration >0.02 μg (P<0.05; Fig. 6.1b) and blocked H type 3 receptor binding increasingly effectively with increased VLP concentration (Fig. 6.1c). From these data, we concluded that 2 μg VLP elicited a robust humoral immune response in

rodents, and as such, all subsequent multivalent vaccine experiments were performed at this dose.

To compare the effect of null VRP adjuvant activity to that of an FDA-approved adjuvant for human vaccination, we immunized mice with 2 μg NV VLP or LV VLP alone or in conjunction with either 10^5 IU null VRP or 1 μg CpG DNA. Serum antibody responses to NV or LV VLPs, respectively, were significantly higher following null VRP vaccination than CpG vaccination ($P<0.01$ and $P<0.001$, respectively), and both adjuvant groups induced significantly higher responses than VLP alone ($P<0.001$; Fig. 6.2a). Sera from groups vaccinated with NV VLP but not LV VLP blocked NV VLP binding to H type 3, and adjuvanted groups blocked binding with lower sera concentrations than groups receiving VLP alone (6.2b). Parallel results were obtained for blockade of LV VLP binding to H type 3 following LV VLP vaccination, respectively (Fig. 6.2c). Percentage of sera necessary for blockade of 50% (BT50) and 90% (BT90) H type 3 binding are shown in Table 6.2. BT50 and BT90 values were significantly lower in adjuvanted than non-adjuvanted sera ($P<0.05$).

Multivalent vaccines induce enhanced cross-reactive and receptor-blocking antibody responses.

To determine the effect of multivalent VLP vaccination coadministered with null VRP or CpG adjuvants on homotypic and heterotypic antibody responses and receptor blockade, we vaccinated mice with pools of VLPs (2 μg/VLP) alone or coadministered with null VRP or CpG adjuvants. Mice received multivalent immunizations consisting of genogroup I (GI) VLPs, GII VLPs, or both GI and GII VLPs. GI VLPs are derived from the NV (GI.1), SoV (GI.2), DS (GI.3), and Chiba (GI.4) strains, and the GII VLPs are derived

from the LV (GII.4), HV (GII.1), TV (GII.3), and M7 (GII.13) strains. VLP vaccine formulations and acronyms are summarized in Table 6.1. NV VLPs were excluded from GI-specific (GI-) and complete GI/GII (GI-/GII-) multivalent vaccine formulations to allow determination of heterotypic antibody blockade of receptor binding to NV VLPs compared to vaccines containing the NV antigen. LV VLPs were likewise excluded GII-specific (GII-) and complete (GI-/GII-) vaccine formulations Serum IgG responses following vaccination with the complete cocktail of GI/GII VLPs (GI+/GII+) coadministered with null VRP adjuvants resulted in robust antibody responses to NV and LV VLPs, respectively, that were significantly higher than in groups lacking adjuvant ($P<0.001$; Fig. 6.3a). Furthermore, antisera following GI-/GII- vaccination still mounted strong cross-reactive IgG responses to NV and LV VLPs, supporting our previous findings (149). GI-/GII- VLP pools coadministered with null VRP induced significantly higher heterotypic responses to NV and LV VLPs than GI-/GII- VLP vaccination without adjuvant ($P<0.05$). However, GI-/GII- heterotypic antisera reactivity to NV and LV VLPs was significantly less than homotypic GI+/GII+ antisera ($P<0.05$). Evaluation of antisera blockade of H type 3 binding to VLPs revealed that GI+/GII+ antisera completely blocked H type 3 binding to both NV and LV VLPs with increased blockade in groups receiving adjuvant (Fig. 6.3b-c). Significantly less sera was required to attain BT90 values following GI+/GII+ vaccination with adjuvant than without adjuvant (Table 6.2). Furthermore, GI-/GII- antisera following null VRP vaccination contained cross-reactive antibodies that partially ablated H type 3 binding to both NV and LV VLPs. BT50 serum concentrations were significantly higher following GI-/GII- null VRP vaccination than GI+/GII+ null VRP vaccination in NV VLP-H type 3 blockade ($P<0.05$); however, they were not significantly different in LV VLP-H type 3 blockade.

Also, BT50 concentrations were significantly lower following GI-/GII- null VRP vaccination than GI-/GII- VLP vaccination without adjuvant ($P<0.001$ in NV blockade and $P<0.05$ in LV blockade). These data suggest that multivalent vaccines coadministered with null VRP adjuvants efficiently induce cross-reactive and receptor-blocking IgG responses to heterologous strains that cannot be attained following monovalent vaccination.

We performed an additional study where mice were vaccinated with genogroup-specific VLP pools in conjunction with null VRP adjuvant. Groups of mice received immunizations of all four GI VLPs (GI+), all four GII VLPs (GII+) or three genogroup-specific VLPs lacking NV or LV VLPs, respectively (GI- and GII-; Table 6.1). Serum IgG responses comparing genogroup-specific vaccination to monovalent or multi-genogroup VLP vaccines are shown in Fig. 6.4a. Cross-reactive responses of monovalent NV antisera to LV VLP and vice versa are shown as controls. All monovalent or multivalent vaccines containing NV or LV VLPs, respectively, induced highly reactive IgG responses to NV or LV VLPs that were not significantly different from one another. Genogroup-specific or multi-genogroup VLP pools lacking NV and/or LV, respectively, mounted cross-reactive responses that were not significantly different from one another and were only significantly less than homotypic monovalent responses ($P<0.01$) but not homotypic multivalent responses. Blockade profiles from each genogroup-specific vaccination group uphold findings discussed above whereby multivalent genogroup-specific vaccines lacking target antigens mount intermediate blockade responses (Fig. 6.4 b & c) with BT50 values significantly higher than homotypic values ($P<0.05$) but significantly lower than heterotypic monovalent values ($P<0.01$; Table 6.2). Furthermore, increasing the number of VLPs in the vaccine composition did not significantly change homotypic antibody titers or blockade of

receptor binding. Increasing genogroup-specific VLP vaccines to include VLPs from both genogroups appeared to moderately increase cross-reactive responses to both NV and LV VLPs, respectively. Increasing the amount of null VRP administered from 10^5 IU to 10^6 IU per vaccine did not enhance cross-reactive receptor-blockade responses (data not shown).

Complete cross-reactivity profiles from all null VRP antisera groups to the entire panel of VLPs is shown in Fig. 6.5. Obvious trends that emerge are significantly low cross-reactivity to additional VLPs following monovalent vaccination with NV or LV ($P<0.001$), although slightly increased cross-reactivity exists to VLPs within a genogroup; low cross-reactivity to strains in opposite genogroups following GI and GII vaccination ($P<0.05$); enhanced cross-reactivity to heterologous NV or LV strains within a genogroup following GI- and GII- vaccination, respectively; and cumulative cross-reactivity to heterologous NV and LV strains following complete VLP vaccination. These results suggest cross-reactivity induced by multivalent vaccination is likely genogroup-specific; therefore, vaccines must contain both GI and GII strains to induce a cumulative cross-reactivity to the majority of human norovirus strains.

Because noroviruses are enteric pathogens and the likely site of neutralization is the gastrointestinal tract, we also analyzed NV-specific IgG and IgA content in fecal extracts from monovalent and multivalent vaccination groups coadministered with no adjuvant, CpG or null VRP (Table 6.3). NV-reactive and total IgG and IgA content were determined, and percentages of NV-specific subtype antibody were calculated. Significantly more total IgA than IgG was present in fecal extracts ($P<0.001$); however, significantly more IgG than IgA was specific for NV VLPs as a percentage of total IgG than total IgA ($P<0.001$). Vaccination with null VRP adjuvant induced significantly more total IgG but not IgA compared to CpG

(P<0.05) or VLP alone (P<0.01). A similar trend was seen by increasing the number of VLPs administered, although values were not significant. Monovalent vaccination with null VRP induced significantly higher homotypic NV-specific IgA responses than multivalent vaccination (P<0.05); multivalent vaccination induced higher homotypic IgG responses than monovalent vaccination, although not significantly. Multivalent GI-/GII- null VRP vaccination induced significantly lower NV-specific IgG (P<0.05) but not NV-specific IgA than GI+/GII+ vaccination. Percentages of NV-specific IgG were equivalent in monovalent and GI+/GII+ groups receiving adjuvant and contributed a substantial amount to total measurable IgG; percentages of NV-specific IgA were miniscule. LV-specific responses following monovalent and multivalent LV VLP vaccination were lower and more variable (data not shown). These data suggest multivalent null VRP vaccination induces a predominantly IgG subtype response in feces. Fecal extracts from unimmunized controls were able to block receptor binding equally as well as from vaccinated samples; therefore, neutralization capacity of these antibodies could not be determined (data not shown).

Null VRP vaccines induce stimulation of T helper 1 cells.

Previous literature has reported that $CD4^+$ T helper 1 (T_H1) cells are activated following norovirus infection and IFN-γ produced (145). To evaluate activation of T_H1 responses following monovalent and multivalent null VRP vaccination, we harvested splenocytes from vaccinated mice and stimulated cultures with NV or LV VLPs. We performed IFN-γ ELISpots on splenocytes from unimmunized mice or mice immunized with NV or LV VLPs without adjuvant; NV VLPs, LV VLPs, GI+/GII+ VLP pools, or GI-/GII- VLP pools coadministered with null VRP; or null VRP alone (Fig. 6.6a). Splenocytes from

mice immunized with VLP alone did not secrete IFN-γ upon stimulation with NV or LV VLPs. In contrast, splenocyte cultures from all mice immunized with null VRP contained significantly higher numbers of IFN-γ-secreting cells following stimulation with NV or LV VLP ($P<0.01$) than VLP alone or unimmunized controls. Furthermore, the numbers of IFN-γ-secreting cells following NV or LV VLP stimulation were not different from one another in any null VRP vaccine group, regardless of presence or number of VLPs in the vaccine composition. Unstimulated cultures did not secrete IFN-γ, ruling out non-specific stimulation. To exclude the possibility of VLP contamination during production with structural VRP particles, which have similar densities as VLPs and may cosediment during purification, we produced all VLPs used in stimulations by direct electroporation of VEE non-structural vector RNA containing the norovirus capsid sequence without VEE helper RNAs. Conversely, reverse-transcription PCR followed by real-time PCR of NV and LV VLP preparations with non-structural VEE-specific primers were positive, revealing that VLPs encapsidate VEE non-structural RNA upon assembly (data not shown). This finding explains the high number of IFN-γ secreting cells upon VLP stimulation in cultures from mice vaccinated with null VRP alone. Furthermore, we cannot determine from our ELISpot data if cross-stimulation with NV and LV VLP exists in null VRP vaccinated mice. Because ELISpots measure the number of IFN-γ secreting cells and not total amounts of IFN-γ produced, we stimulated parallel splenocyte cultures with NV or LV VLPs and harvested culture supernatants to circumvent this issue. EIA of culture supernatants from each group again showed high IFN-γ secretion in all groups vaccinated with null VRP; however, coadministration of NV VLP or LV VLP induced secretion of significantly more IFN-γ upon stimulation with homologous NV or LV VLPs, respectively, than vice versa ($P<0.05$; Fig.

6.6b). This data suggests that norovirus strain-specific stimulation is in fact induced; however, the variability between groups in this experiment does not allow for null VRP background compensation or analysis of cross-reactivity to norovirus VLPs.

Because T_H1 responses correlate with serum IgG2a subclass responses, we used this alternative evaluation to determine induction of T_H1 cell responses by multivalent VLP vaccination. Serum samples from mice vaccinated with monovalent or multivalent VLP vaccines alone or in conjunction with CpG or null VRP adjuvants were analyzed for IgG1 and IgG2a subclass specificity to NV and/or LV VLPs (Fig. 6.7). Monovalent and multivalent vaccination with NV and/or LV VLPs induced IgG2a titers that were slightly increased when coadministered with CpG and significantly increased when coadministered with null VRP compared to VLP alone ($P<0.05$). Heterotypic IgG2a responses to NV and LV VLPs following GI-/GII- vaccination were lower than homotypic responses, and titers were not different in CpG and null VRP recipient groups. IgG1 titers were not significantly different in VLP versus adjuvant groups but maintained uniform levels of reactivity to NV and LV VLPs that were significantly lower than IgG2a titers in null VRP recipient groups ($P<0.05$), although a spike in NV-specific IgG1 levels appeared to occur following monovalent and multivalent VLP vaccination with CpG. Increasing the number of VLPs in NV or LV null VRP vaccines from one to four to eight VLPs did not change specific IgG1 or IgG2a responses to NV or LV VLPs, respectively. Together, these data suggest null VRP vaccines induce IgG2a responses specific for NV and/or LV antigens, which may correlate to a T_H1-type response. Furthermore, CpG and null VRP adjuvants induced cross-reactive IgG2a to NV and LV VLPs in the GI-/GII- vaccine group, implying T_H1 cross-reactivity to additional strains may also occur.

Multivalent VLP vaccines coadministered with null VRP result in decreased viral load following MNV challenge.

To determine if monovalent and multivalent vaccines can protect against norovirus challenge, we utilized the MNV infection model. Mice were immunized with monovalent MNV VLP vaccines or multivalent VLP vaccines consisting of eight human VLPs with MNV VLPs (Hu+/MNV+) or without MNV VLPs (Hu+/MNV-; Table 6.1). Each was administered alone or in conjunction with CpG or null VRP adjuvants, similar to the human strain vaccines described previously in this paper. Mice were then challenged with MNV three weeks after secondary immunization, and spleens, mesenteric lymph nodes (MLN), and distal ileums were harvested three days later. Tissue homogenates were analyzed for viral titers by plaque assay. Monovalent and MNV+/Hu+ vaccination with or without adjuvant induced complete protection from MNV infection in the spleen, with significantly lower viral titers than vaccination with null VRP alone ($P<0.001$; Fig. 6.8a). Hu+/MNV- vaccination did not completely protect against MNV infection in the spleen; however, viral loads were significantly lower in Hu+/MNV- groups coadministered with null VRP adjuvant compared to those vaccinated with null VRP alone ($P<0.05$). Viral loads in MLN and distal ileum were not significantly reduced following monovalent or multivalent VLP or CpG vaccination compared to unvaccinated controls. Null VRP administration, however, significantly reduced viral loads compared to controls following monovalent and Hu+/MNV+ vaccination in both MLN ($P<0.001$) and distal ileum ($P<0.05$). Hu+/MNV- vaccination coadministered with null VRP significantly reduced viral loads in the distal ileum as well ($P<0.05$). MNV, Hu+/MNV+, and Hu+/MNV- antisera all contained MNV-reactive serum IgG following null

VRP vaccination, where MNV and Hu+/MNV+ responses were equivalent and significantly higher than the cross-reactive response in Hu+/MNV- groups ($P<0.001$; Fig. 6.8b). These findings show that multivalent VRP vaccines can successfully protect against spread of norovirus infection to some peripheral tissues and can reduce viral loads in primary and additional secondary sites of replication even without the presence of homologous MNV antigen in the vaccine composition using the MNV infection model. These results lend strong support for the development of multivalent human norovirus vaccines.

Humoral immunity protects against acute MNV infection.

To determine the mechanism of protection induced by null VRP vaccines, we vaccinated immunocompetent wild-type mice monovalently with MNV VLPs coadministered with null VRP and passively transferred antisera or adoptively transferred purified $CD4^+$ or $CD8^+$ splenocytes into immunodeficient Scid mice. Unimmunized mice were treated in parallel as controls. $CD4^+/CD3^+$ and $CD8^+/CD3^+$ T cells from immune and non-immune spleens were each found to be ≥90% pure by FACS analysis (Fig. 6.9a-d). After 24 h, Scid mice were infected with MNV, and tissues were harvested three days later. Because Scid mice do not support a competent adaptive immune system and have underdeveloped immune organs, MLNs were not analyzed. Adoptive transfers of immune $CD4^+$ or $CD8^+$ did not prevent establishment of MNV infection in the spleen compared to transfer of non-immune cells, as determined by plaque assay (Fig. 6.10a). Passive transfer of antisera, however, was able to completely protect immunodeficient mice from MNV infection in the spleen in all mice tested, whereas transfer of non-immune sera had no effect on viral titers ($P<0.001$). Significant MNV-specific antibodies were found to be circulating

in both donor wild-type mice and recipient Scid mice compared to non-immune controls ($P<0.001$; Fig. 6.10b). Plaque assays on distal ileum of immune and non-immune mice did not result in measurable viral titers, indicating immunodeficient mice may support differential infection than wild-type mice. Additional studies must be conducted to determine if viral titers are present in duodenum, jejunum, and proximal ileum following MNV infection in Scid mice. Nonetheless, these data clearly indicate that humoral immunity induced by monovalent null VRP vaccination can prevent establishment of acute MNV infection and provide further support for the development of null VRP vaccines in humans.

Discussion

Multivalent vaccination has become a popular tool in generating cross-reactive immunity to heterologous strains of bacterial and viral pathogens. Cattle immunized with bivalent adjuvanted vaccines containing two viral subtypes of killed bovine viral diarrheal virus produced neutralizing antibody and IFN-γ responses to both strains (210). Mice vaccinated simultaneously with VEE replicon particles (VRPs) expressing three different cowpox proteins survived infection and were protected from clinical symptoms better than monovalently vaccinated mice (257). In humans, adjuvanted multivalent streptococcal peptide vaccines from six serotypes are in phase I clinical trials (136), and multivalent vaccines containing 26 serotypes are in development (101). Of particular significance, effective quadrivalent human papilloma virus (HPV) VLP vaccines are currently available (reviewed in (233)), which are as effective as monovalent vaccines at inducing seroconversion (65). Multivalent vaccination is not without its drawbacks, however. Immune interference was reported in one study where diphtheria and tetanus toxoids had

reduced immunogenicity when coadministered with pertussis toxoid (260). Furthermore, multivalent vaccines do not necessarily elicit cross-reactive immunity to additional heterologous strains (36). This is the first study to address the efficacy of multivalent norovirus VLP vaccines using a codelivered adjuvant as well as the first study to address the efficacy of null VRP adjuvants as vaccine components in a small animal infection model. In this study, null VRPs represent a novel vaccine adjuvant that should not only be safe for use in human vaccine trials (45) but also generates higher immune induction to coadministered antigens than the human-approved CpG DNA adjuvant.

We have systematically designed and tested the efficacy of monovalent and multivalent norovirus VLP vaccines coadministered with null VRP adjuvants in generating cross-reactive and receptor-blocking antibody responses, T cell responses, and protection from heterologous MNV challenge. These findings are supported by evidence showing that: 1) Immunodeficient mice were completely protected against MNV infection following transfer of antisera from wild-type mice following monovalent MNV VLP vaccination coadministered with null VRP adjuvant, most likely by antibody-mediated neutralization. 2) Increasing the number of antigens in the vaccine composition did not significantly blunt the immune response to the original antigens. 3) VLP vaccines lacking target antigens induced strong cross-reactive antibody responses to heterologous strains that partially blocked receptor binding to these strains. 4) VLP vaccines lacking target antigens significantly reduced viral loads in murine tissues following heterologous viral challenge. Although multivalent vaccination did not provide protection from heterologous MNV infection, a significant reduction in viral load may be tightly correlated with reduction of clinical disease, as seen with HIV, respiratory syncytial virus, or human papilloma virus infections (1, 24,

67), or transmission following infection. However, one study recently showed no differences in viral load in symptomatic and unsymptomatic norovirus infected individuals (194). Overall, our data provides strong support and justification for development of multivalent VLP/null VRP vaccines against highly heterogeneous noroviruses.

Alphavirus replicon particles (VRP) are single hit vectors which traditionally express high concentrations of transgene in infected tissues, and the dogma has argued that co-expression is essential for vaccine efficacy (214). Alternatively, the adjuvant activity of VRPs lacking a transgene has been clearly documented (256). Our data clearly show that co-administration of VLPs with null VRP adjuvants induces significant systemic, mucosal, and cellular immune responses. The mechanism by which null VRPs function as adjuvants is not known, but stimulation of immune cells by the single round of viral RNA replication these replicons undergo in mammalian cells is a likely possibility. Of importance, the safety of VRPs as vaccine vectors has long been questioned due to the presence of functional Venezuelan equine encephalitis genes and BL3 requirements for safety testing (215). Despite this, the safety record for VRPs and other alphavirus replicon vectors is robust, and VRPs have been approved for use in human clinical trials for HIV gene expression (45). One potential new concern, however, is evidence that VLP preparations expressed using this system may efficiently package VEE replicon RNAs. By uncoupling VLP transgene expression from VRPs in vaccines, additional safety benefits are realized that prevent the inadvertent design of new chimeric pathogens capable of self-replication, packaging and release. While helper virus contamination has been recognized in Epstein-Barr virus and adeno-associated virus vector systems, for example (40, 268), no evidence of chimeric virus generation has been reported. Although extensive safety trials have not been published for

null VRPs, these adjuvants retain safety features of the original vector but are a more likely candidate for extensive use in clinical trials due to these encouraging features.

Previous work from our lab has shown that antibodies from both infected humans and VRP vaccinated mice can block HBGA binding to homologous norovirus strains (91, 149). We also published an original study showing that VRP vaccination can generate intermediate cross-reactive receptor-blocking antibodies to heterologous norovirus strains when multiple vectors are administered simultaneously (149). While this study laid the groundwork for the research presented here, only four VLPs were available to us at that time. This work utilizes a representative panel of eight human VLPs that together account for ≥95% of all norovirus infections, including the predominantly circulating GII.4 strains. Furthermore, we are now able to address inter-genogroup versus intra-genogroup cross-reactivity following multivalent vaccination, which was not possible with the single GI VLP previously available to us. Our findings obviate a clear discrepancy in cross-reactivity between genogroups. Monovalent vaccines generated very low cross-reactive antibody responses to all heterologous strains, although strains within a genogroup elicited a slightly higher cross-reactive response. However, multivalent genogroup-specific vaccination elicited strong cross-reactive and intermediate receptor blocking antibody responses to other genogroup-specific strains but in no way enhanced cross-reactivity to strains between genogroups. This result is most likely caused by a cross-reactive epitope repertoire that is greater when multiple, closely related but distinct antigens are included in the vaccine composition compared to a single antigen. The addition of strains from both genogroups in our cumulative VLP vaccines did not detract from either genogroup-specific response but rather accentuated cross-reactivity and receptor blockade to inter-genogroup strains. Antisera from multivalent vaccination groups could also

block receptor binding of evolutionarily distinct GI.1 (NV genocluster) and GII.4 (LV genocluster) VLPs (data not shown), suggesting blockade responses may be genocluster rather than strain specific and may be of particular importance when applied to GII.4 norovirus vaccine design. Previous studies in multivalent vaccination to *Neisseria* and *Streptococcus* species induced cross-reactive antibodies that could neutralize heterologous serotypes (7, 106); however, multivalent HIV envelope vaccines failed to induce cross-reactive neutralizing antibody responses (36). Together, these data support the rationale for including multiple norovirus strains from different genogroups in a comprehensive norovirus vaccine.

Multivalent human VLP vaccines coadministered with null VRP and lacking the NV and LV components elicited intermediate receptor blockade responses to NV and LV VLPs *in vitro* in our surrogate neutralization assay. No receptor for MNV has been identified to date; however, multivalent mouse VLP vaccines coadministered with null VRP lacking the MNV VLP component lent intermediate protection from MNV challenge *in vivo*. We can speculate that these two findings are reflective of one another and intermediate protection is conferred to heterologous strains following multivalent vaccination. However, several additional factors must be considered when evaluating the vaccine design of this study that may impact protective outcomes. VLP vaccination lacking adjuvant imparted specific but significantly lower receptor blocking responses *in vitro* and elicited no protection from MNV challenge in the ileum and MLN of infected mice, which can be explained by poor immune responses against unadjuvanted VLP vaccines (8, 10). In contrast, CpG adjuvanted vaccines resulted in significant antibody induction to homologous and heterologous antigen; however, null VRP adjuvants imparted significantly more protection following MNV challenge. While

serum antibody reactivity and blockade were not significantly different following CpG and null VRP adjuvanted vaccination, fecal IgG and IgA as well as the serum IgG2a subtype were significantly higher in null VRP vaccinated mice, which may explain this discrepancy. Unmethylated CpG DNA adjuvant activity is the result of innate immune activation through Toll-like receptor (TLR)-9, which promotes antigen-specific adaptive immune responses (137, 223). The mechanism of null VRP adjuvant activity is not known but may be linked to RNA replication. The protective effects of null VRP adjuvant vaccination may, therefore, be linked to differential mechanisms of immune induction or a higher activation of antibody and T cell responses in selective tissues that may not be represented by serum IgG responses.

Preparation of VLP reagents may be a caveat to our vaccine design. We surmise that concentration by centrifugation causes some VLPs to lose particle structure A previous study by Harrington *et al.* showed that antisera following vaccination with norovirus capsid proteins containing a mutation that does not allow for particle assembly only partially blocked receptor binding (91). Because we achieve complete blockade in our receptor binding assays, significant structure is not lost; however, it may determine complete protection versus partial protection in the MNV infection model. Because we observed only partial protection in some tissues in monovalent and multivalent MNV vaccination experiments adjuvanted with null VRP (Fig. 6.8), we boosted wild-type donor mice a third time with unconcentrated VLPs prior to performing passive transfer experiments. The use of unconcentrated VLP, an additional immune boost by a third immunization, or both likely resulted in complete protection from challenge following passive transfer of antisera (Fig. 6.10) but not in the original vaccination experiment. We also showed in our original VLP titration experiments that vaccination with 10 μg of VLP induces better receptor blockade

than the 2 μg administered in the experiments presented here. We were unable to increase this concentration in multivalent vaccines due to the finite volume that can be administered to a mouse footpad and the concentrations we can achieve with individual VLP preparations; however, higher concentrations of VLP may be required to induce an entirely protective state. We can test these hypotheses by vaccinating monovalently with higher VLP concentrations or multivalently by considering different routes of administration.

We chose to use Scid mice for our transfer experiment as they are the most commercially available immunodeficient mouse crossed on the BALB/c background. We observed that Scid mice did not maintain MNV infection in the same tissues that have been documented for multiple strains of wild-type and immunodeficient mice crossed on the C57BL/6 (discussed in Chapter V). While the MLN is underdeveloped in immunodeficient strains, and therefore, does not support MNV infection like immunocompetent mice, we were surprised to find the distal ileum did not contain significant MNV titers following infection of non-immune recipient mice in our transfer experiment. Additional sites of MNV replication in this strain need to be determined for future experiments. We have observed from our work with the MNV infection model in wild-type mice that the MLN typically maintains the highest levels of infection, followed by the distal ileum. The spleen consistently maintains the lowest level of infection (unpublished observations). Although the spleen was the only tissue in which we could observe viral loads in our transfer experiment, the high viral titers found in non-immune sera-recipient mice compared to the complete lack of viral titers found in the immune sera-recipient mice lend strong support that pre-existing antibody can prevent acute MNV infection, although we would have preferred to observe this finding in additional tissues. Both donor and recipient mice had robust circulating anti-MNV

IgG titers. While Scid mice can exhibit T and B cell "leakiness" at 12+ weeks of age that can confound transfer data, we used mice 6 weeks of age to avoid this problem. Therefore, we can conclude that humoral immunity is the likely mechanism of protection following MNV VLP vaccination.

Overall, our data suggest that increased antibody cross-reactivity to heterologous norovirus strains following multivalent VLP vaccination coadministered with null VRP adjuvant can significantly decrease viral loads upon challenge. Unfortunately, mice don't develop clinical disease making it impossible to determine if this reduction in viral load corresponds to reduced morbidity. Homologous vaccination induced antibodies that completely blocked receptor binding and were able to completely protect against infection in transfer experiments. Human VLP vaccines containing GII.4 components are widely needed to prevent frequent norovirus outbreaks; however, multivalent vaccines containing multiple GI and GII components may be crucial in preventing other isolated outbreaks and emergence of new predominant strains.

Acknowledgements

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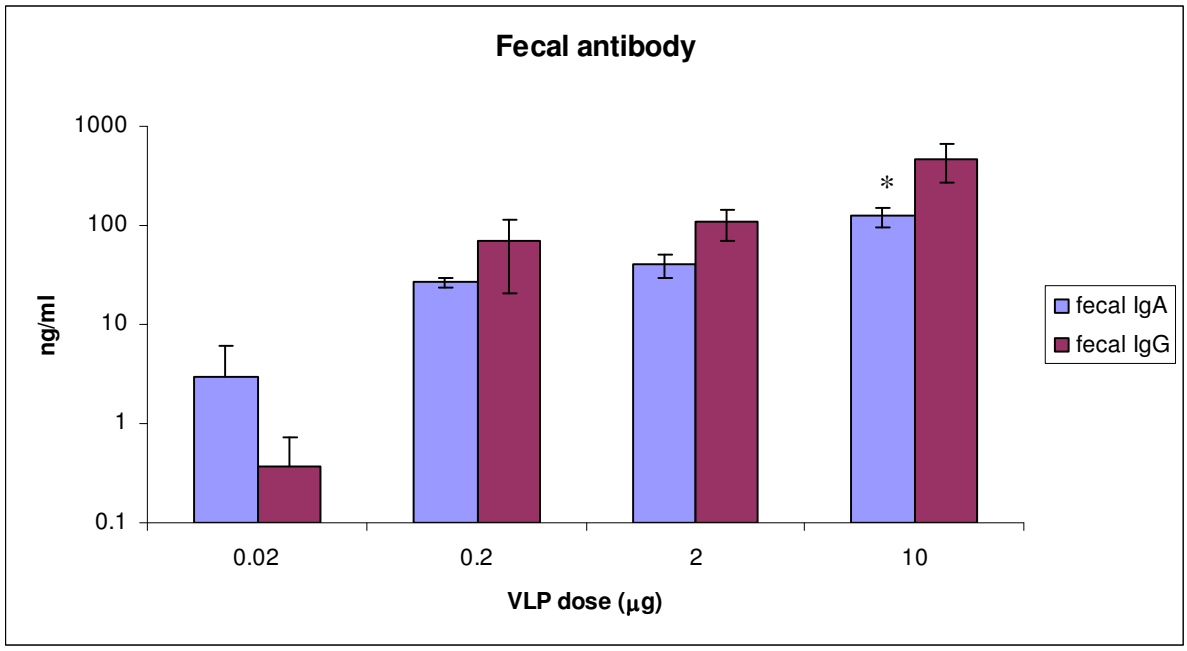
Table 6.1

VLP vaccination chart.

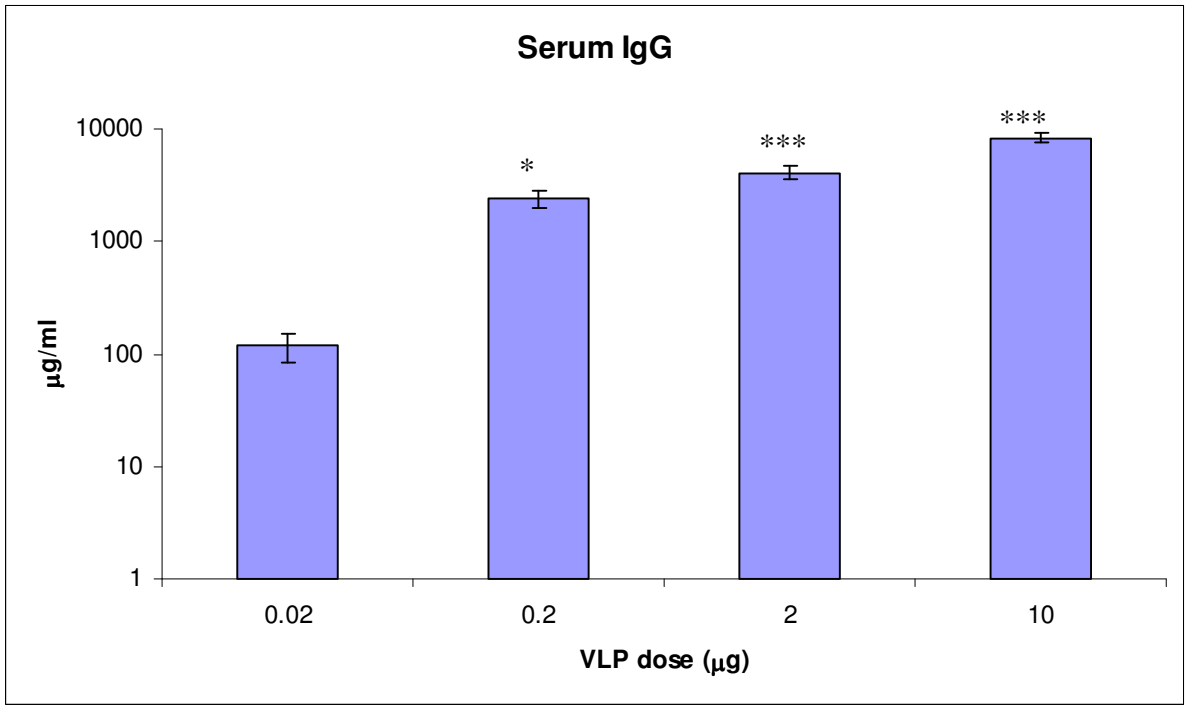
Name	Genogroup(s)	Type	VLP(s) in vaccine composition
NV	**GI**	**Monovalent**	**NV**
GI+		**Multivalent**	**NV, SoV, DSV, Chiba**
GI-		**Multivalent**	**SoV, DSV, Chiba (-) NV**
LV	**GII**	**Monovalent**	**LV**
GII+		**Multivalent**	**LV, HV, TV, M7**
GII-		**Multivalent**	**HV, TV, M7 (-) LV**
GI+/GII+	**GI/GII**	**Multivalent**	**all GI/GII**
GI-/GII-		**Multivalent**	**all GI/GII (-) NV/LV**
MNV	**GV**	**Monovalent**	**MNV**
Hu+/MNV+	**GI/GII/GV**	**Multivalent**	**all GI/GII, MNV**
Hu+/MNV-	**GI/GII**	**Multivalent**	**all GI/GII (-) MNV**

Figure 6.1

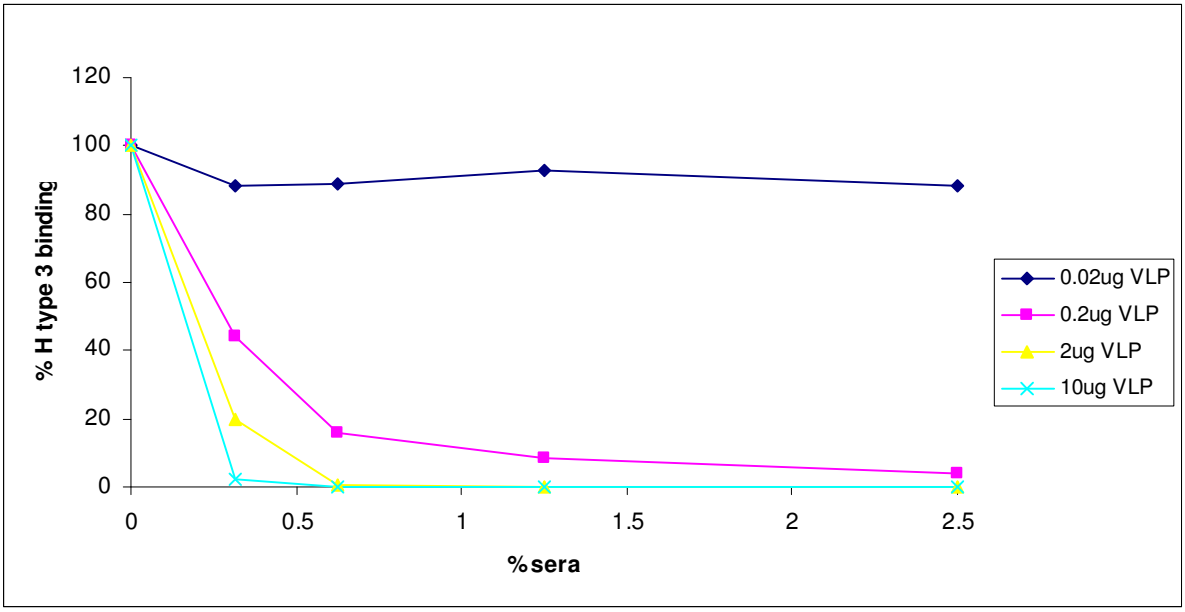
A.


Fecal antibody
1000
100
10
1
0.1
ng/ml
*
0.02
0.2
2
10
VLP dose (µg)
fecal IgA
fecal IgG

B.


Serum IgG
10000
1000
100
10
1
µg/ml
*
***
***
0.02
0.2
2
10
VLP dose (µg)

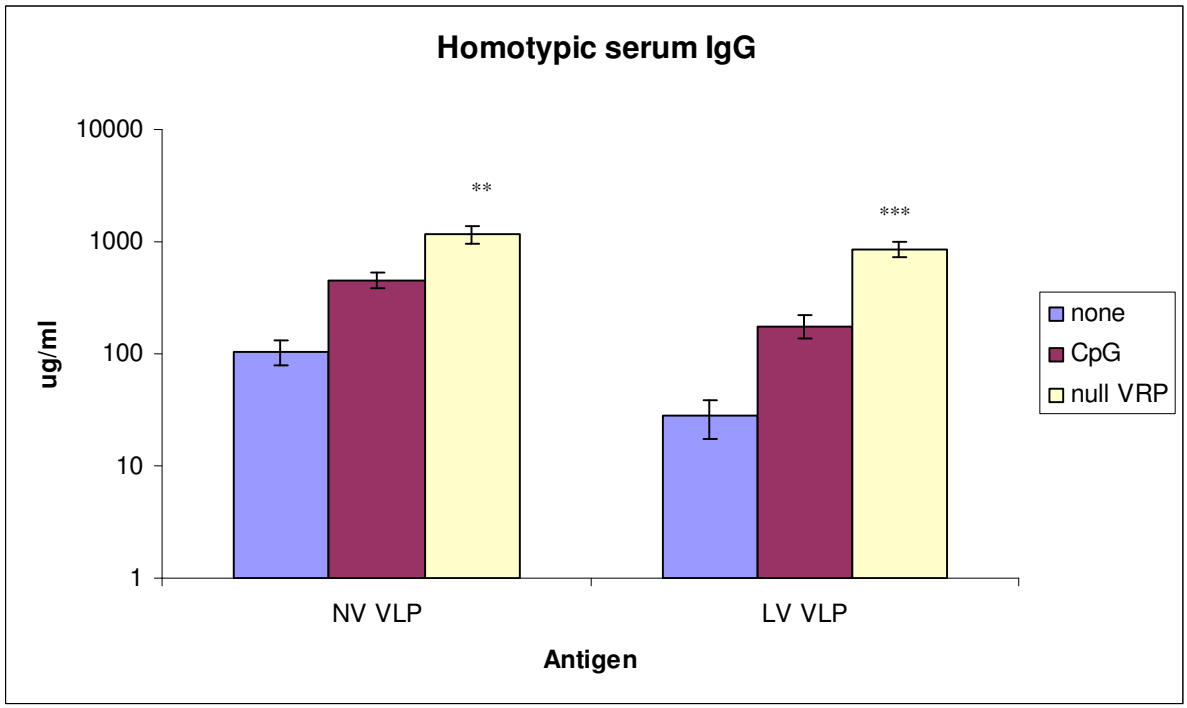
C.



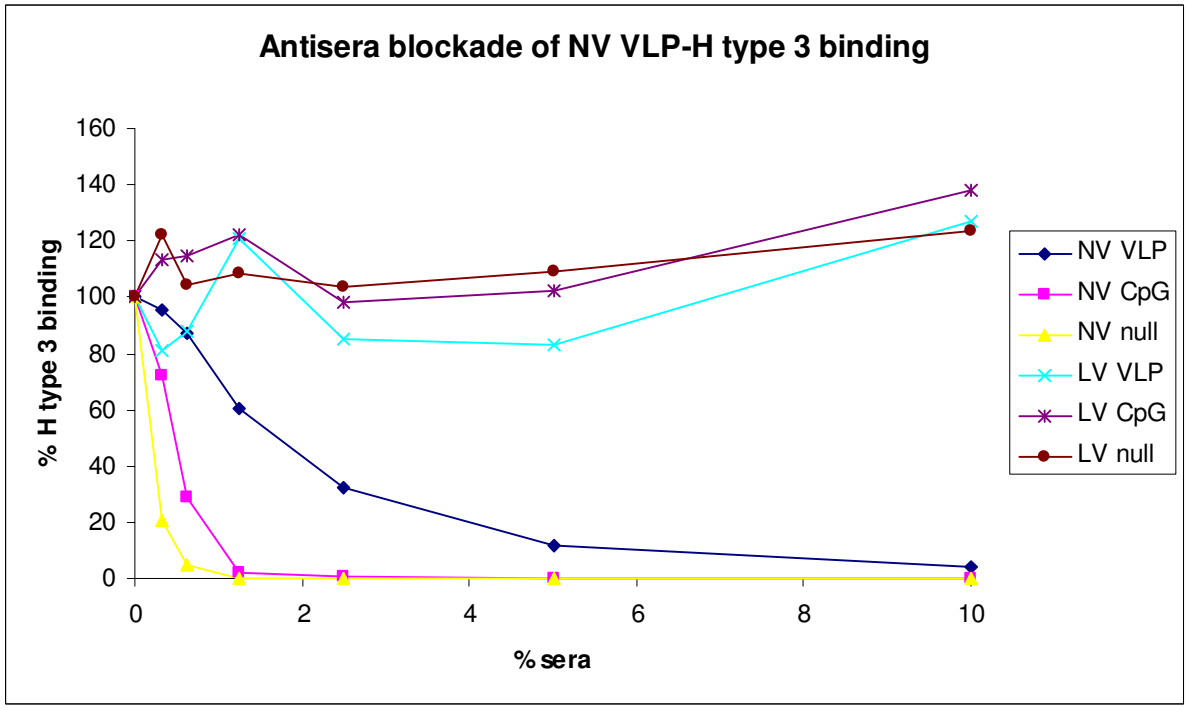
VLP titration. Mice were immunized with NV VLP at doses of 0.02 μg, 0.2 μg, 2 μg, and 10 μg. Sera and feces were harvested and anti-NV antibody quantitated by ELISA (A-B). Sera were also tested for interference of H type 3 binding to NV VLPs (C).

Figure 6.2

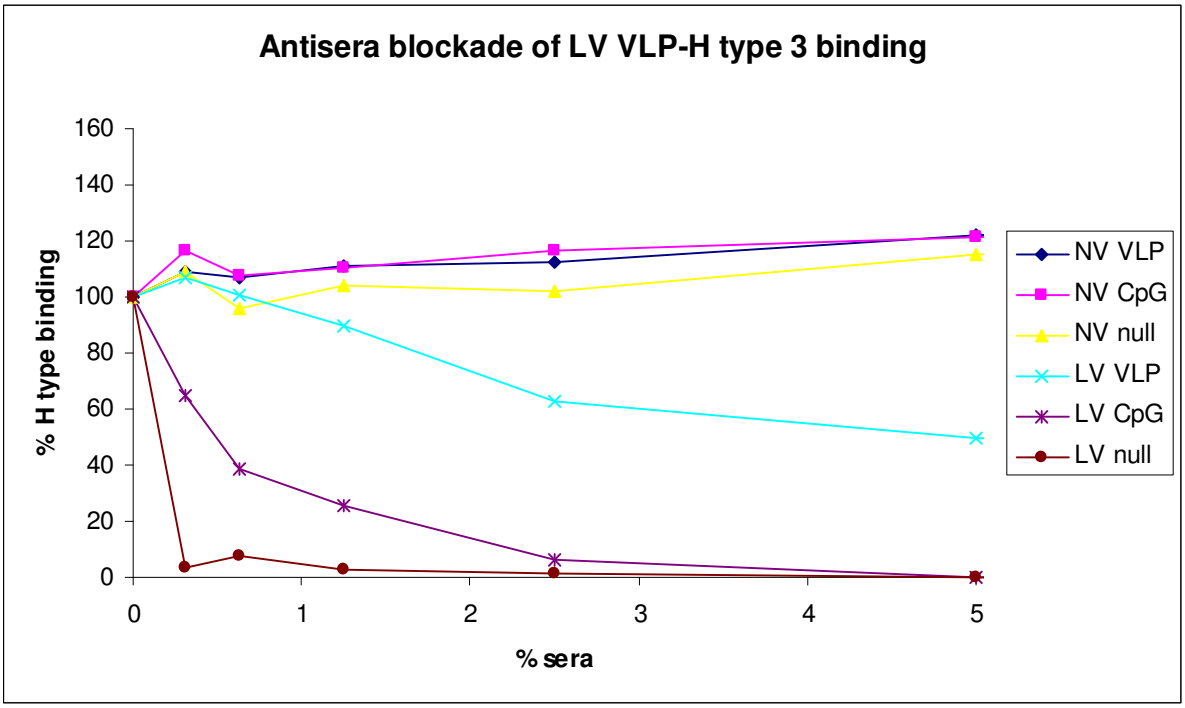
A.


Homotypic serum IgG
10000
1000
100
10
1
ug/ml
**
***
none
CpG
null VRP
NV VLP
LV VLP
Antigen

B.


Antisera blockade of NV VLP-H type 3 binding
160
140
120
100
80
60
40
20
0
% H type 3 binding
0
2
4
6
8
10
% sera
NV VLP
NV CpG
NV null
LV VLP
LV CpG
LV null

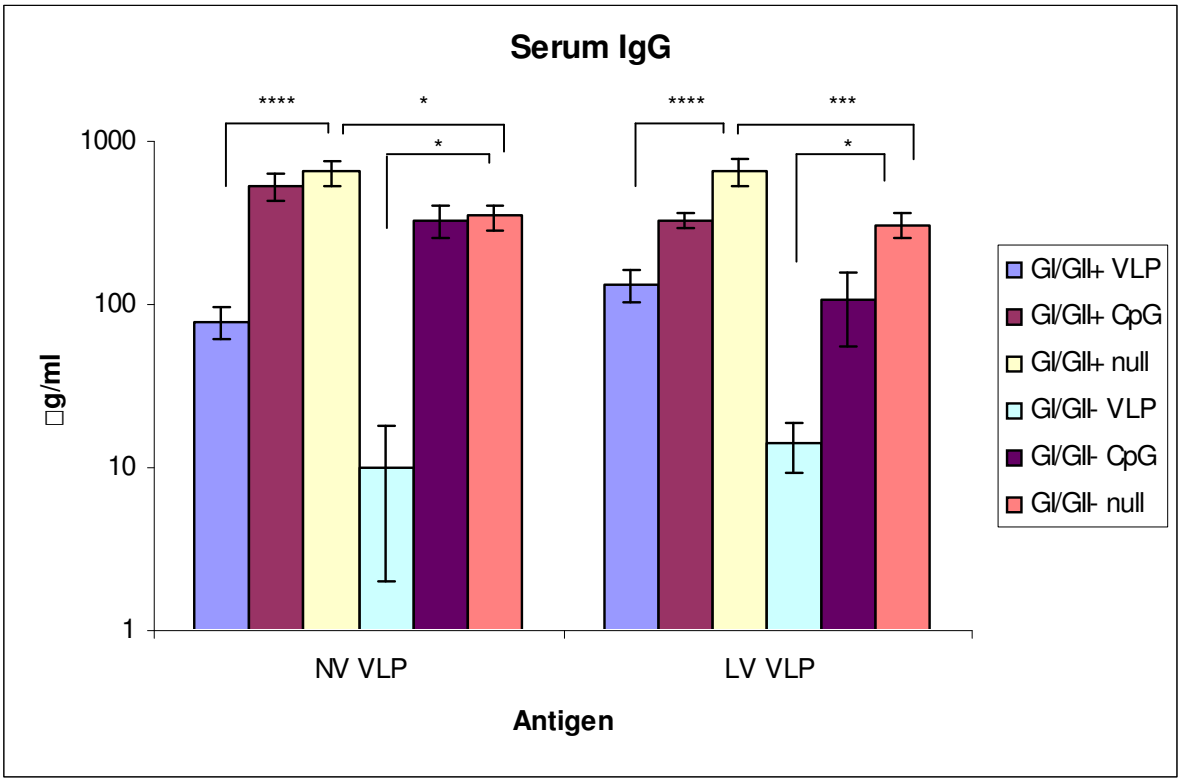
C.



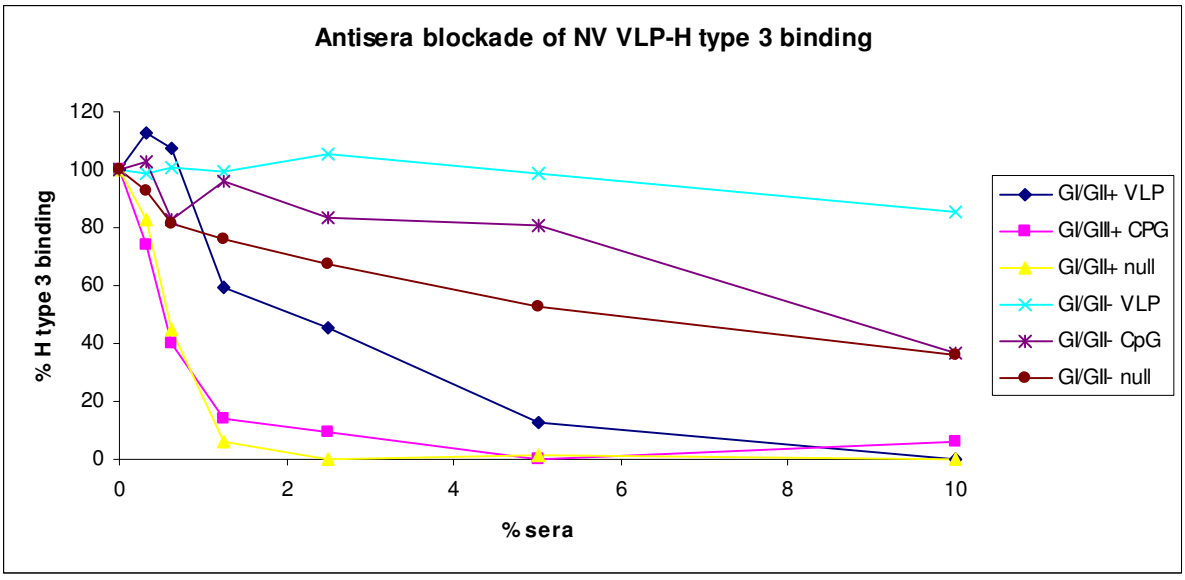
Homotypic antibody responses following monovalent vaccination with and without adjuvant. Sera from mice immunized with NV or LV VLP alone or in conjunction with CpG or null VRP adjuvants were analyzed for anti-NV or anti-LV IgG, respectively, by ELISA (A). Serially diluted antisera were also tested for blockade of H type 3 binding to NV VLPs (B) and LV VLPs (C).

Figure 6.3

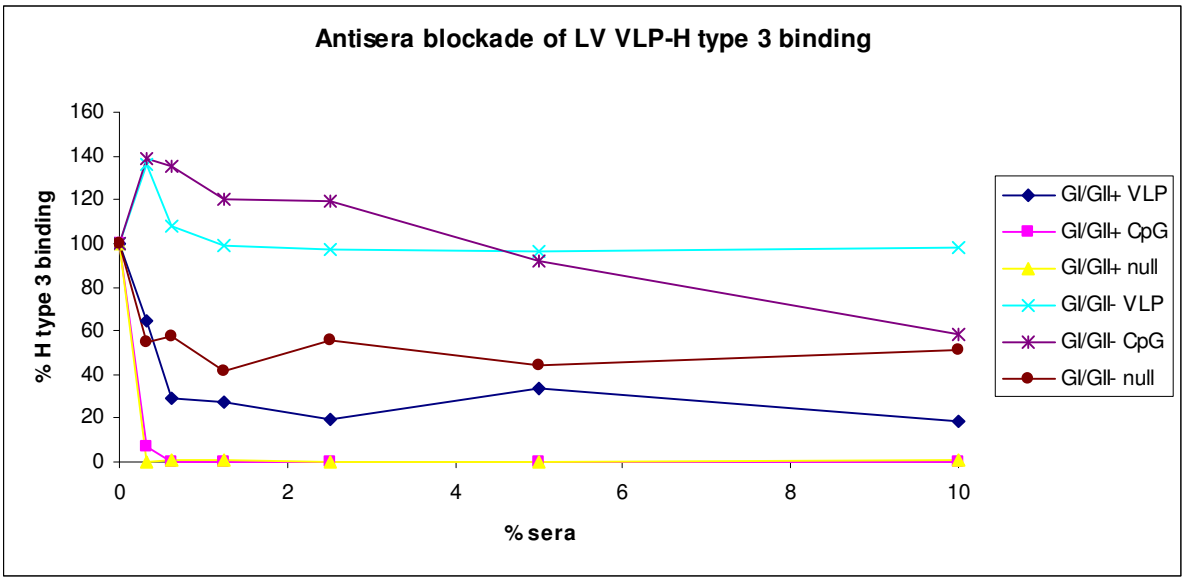
A.


Serum IgG
****
*
*
****
***
*
1000
100
10
1
□g/ml
NV VLP
LV VLP
Antigen
GI/GII+ VLP
GI/GII+ CpG
GI/GII+ null
GI/GII- VLP
GI/GII- CpG
GI/GII- null

B.


Antisera blockade of NV VLP-H type 3 binding
% H type 3 binding
120
100
80
60
40
20
0
0
2
4
6
8
10
% sera
GI/GII+ VLP
GI/GIII+ CPG
GI/GII+ null
GI/GII- VLP
GI/GII- CpG
GI/GII- null

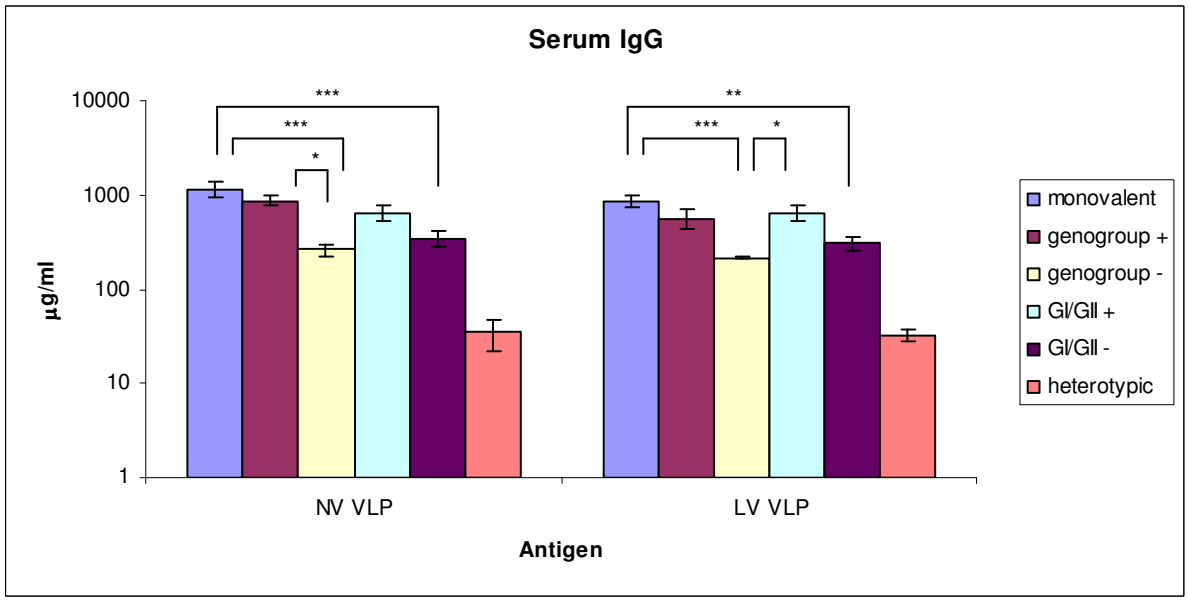
C.



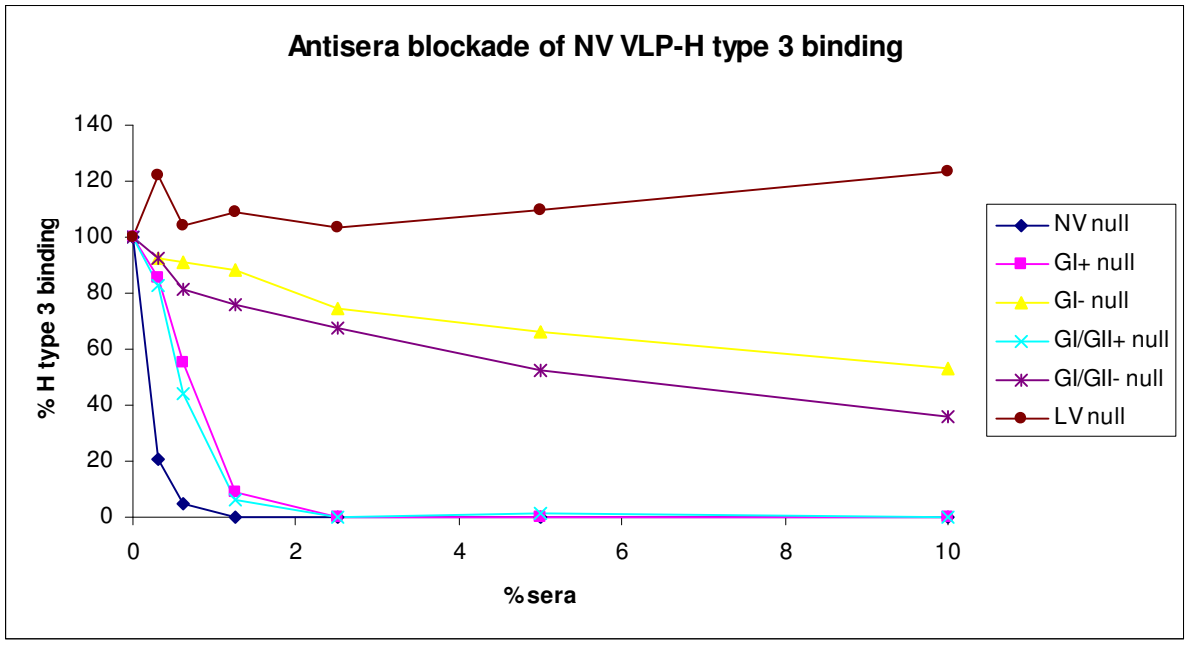
Antibody responses following multivalent vaccination with or without adjuvant. Sera from animals immunized with multivalent VLP vaccines either alone or in conjunction with CpG or null VRP adjuvant were analyzed for IgG reactivity to NV or LV VLPs (A). GI+/GII+ groups received NV and LV VLPs as a vaccine component; GI-/GII- groups did not. Serially diluted sera were also tested for interference of H type 3 binding to NV VLPs (B) and LV VLPs (C). Significance between null VRP groups are noted with (*) (A).

Figure 6.4

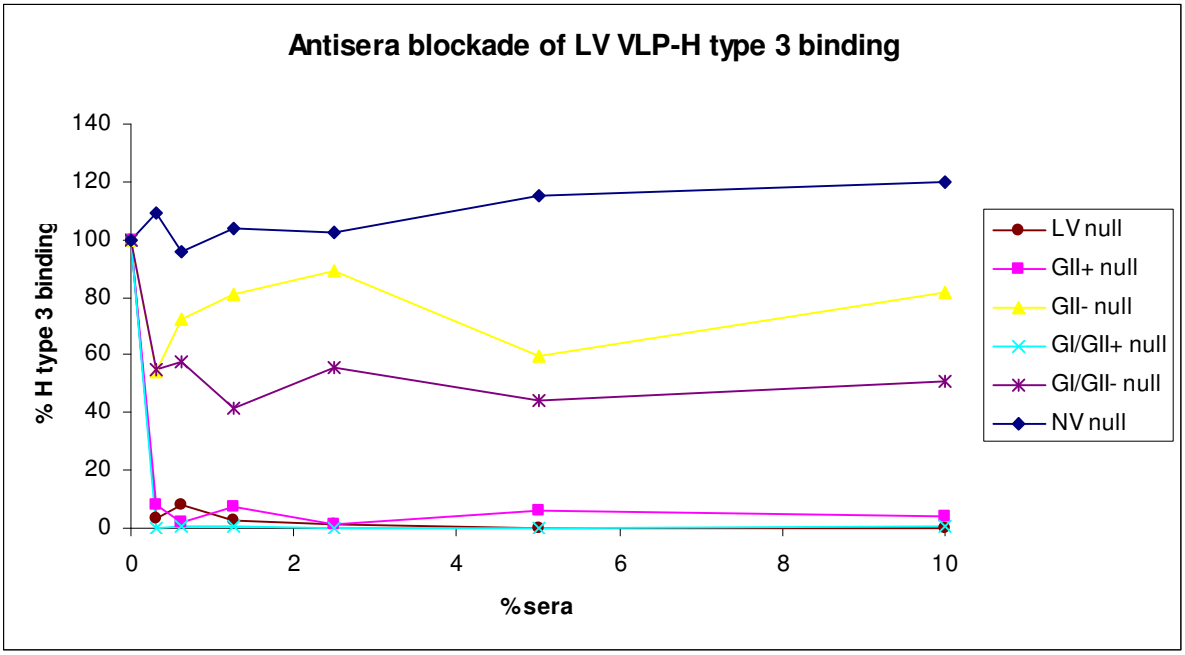
A.


Serum IgG
10000
1000
100
10
1
µg/ml
***
***
*
**
***
*
NV VLP
LV VLP
Antigen
monovalent
genogroup +
genogroup -
GI/GII +
GI/GII -
heterotypic

B.


Antisera blockade of NV VLP-H type 3 binding
140
120
100
80
60
40
20
0
% H type 3 binding
0
2
4
6
8
10
%sera
NV null
GI+ null
GI- null
GI/GII+ null
GI/GII- null
LV null

C.



Null VRP adjuvant coadministered with monovalent, genogroup-specific, and cumulative VLP cocktail vaccines. Sera from animals immunized with null VRP and monovalent, genogroup-specific multivalent, cumulative multivalent, or heterotypic monovalent VLP vaccines with or without NV or LV VLPs as a vaccine component were analyzed for IgG reactivity to NV or LV VLPs (A). Serially diluted sera were also tested for interference of H type 3 binding to NV VLPs (B) and LV VLPs (C).

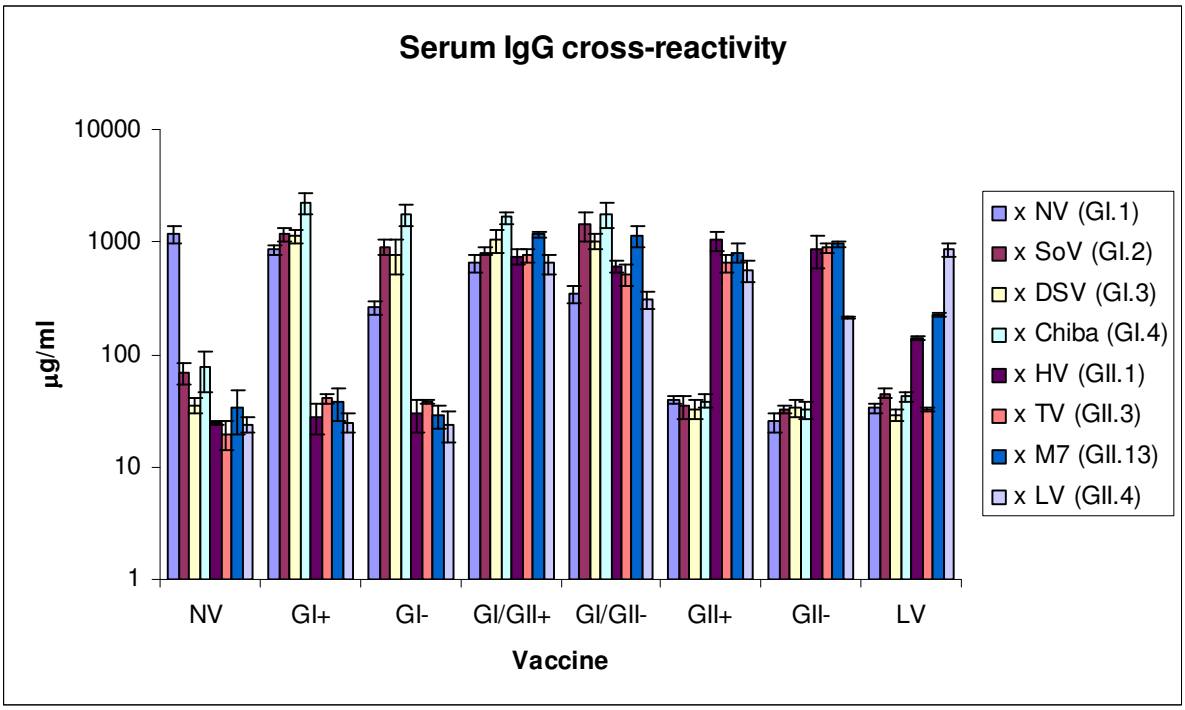
Table 6.2

Average percent sera for blockade of 50% (BT50) and 90% (BT90) H type 3 binding.[†]

	NV VLP-H type 3		LV VLP-H type 3	
Vaccine	BT50 (range)	BT90 (range)	BT50 (range)	BT90 (range)
NV VLP	2.2 (0.6-5)	6.9 (1.3-20)	N/A	N/A
NV CpG	0.5 (0.2-0.6)	1.4 (0.6-2.5)	N/A	N/A
NV null	0.2 (0.2-0.6)	0.4 (0.2-1.3)	N/A	N/A
LV VLP	N/A	N/A	6.3 (2.5-10)	12.5 (5-20)
LV CpG	N/A	N/A	1.0 (0.2-2.5)	2.0 (0.6-5)
LV null	N/A	N/A	0.2 (--)	0.4 (0.2-1.3)
GI+ null	0.8 (0.6-1.3)	1.7 (1.3-2.5)	N/A	N/A
GI- null	12.5 (10-20)	N/A	N/A	N/A
GII+ null	N/A	N/A	0.2 (--)	0.3 (0.2-0.6)
GII- null	N/A	N/A	N/A	N/A
GI+/GII+ VLP	2.9 (1.3-5)	7.7 (1.3-10)	1.5 (0.2-5)	17.5 (10-20)
GI+/GII+ CpG	0.6 (0.2-1.3)	1.8 (0.6-2.5)	0.2 (--)	0.3 (0.2-0.6)
GI+/GII+ null	0.8 (0.6-1.3)	1.7 (1.3-2.5)	0.2 (--)	0.2 (--)
GI-/GII- VLP	N/A	N/A	N/A	N/A
GI-/GII- CpG	8.0 (0.2-20)	18 (10-20)	17.5 (10-20)	N/A
GI-/GII- null	7.1 (2.5-10)	N/A	8.8 (2.5-20)	N/A

[†] Sera that blocked H type 3 binding at the lowest concentration tested were assigned a BT value half the lowest serum concentration tested (0.2). Sera that could not block H type 3 binding at the highest concentration tested were assigned a BT value twice the highest serum concentration tested (20). Antisera groups with no BT values within the range of detection were designated N/A.

Figure 6.5



Serum IgG cross-reactivity profile. Antisera from mice immunized with each monovalent or multivalent VLP vaccine coadministered with null VRP adjuvant were analyzed for cross-reactivity to the VLP panel.

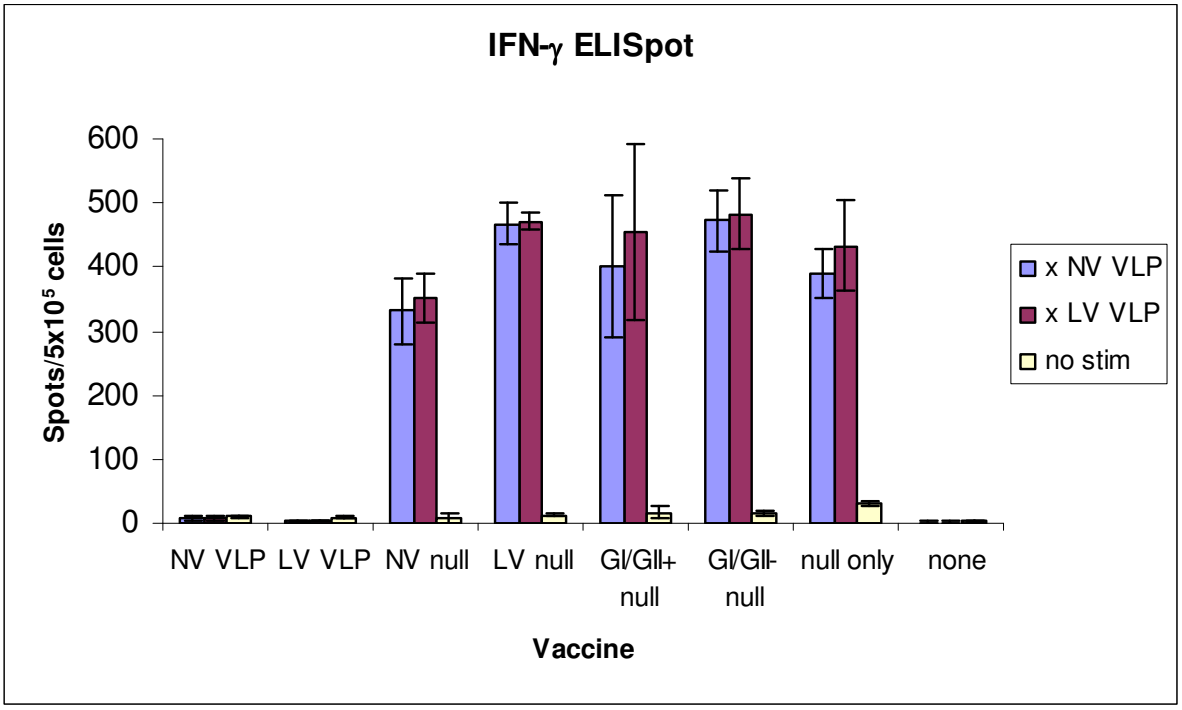
Table 6.3

Anti-NV IgG and IgA in fecal extracts.

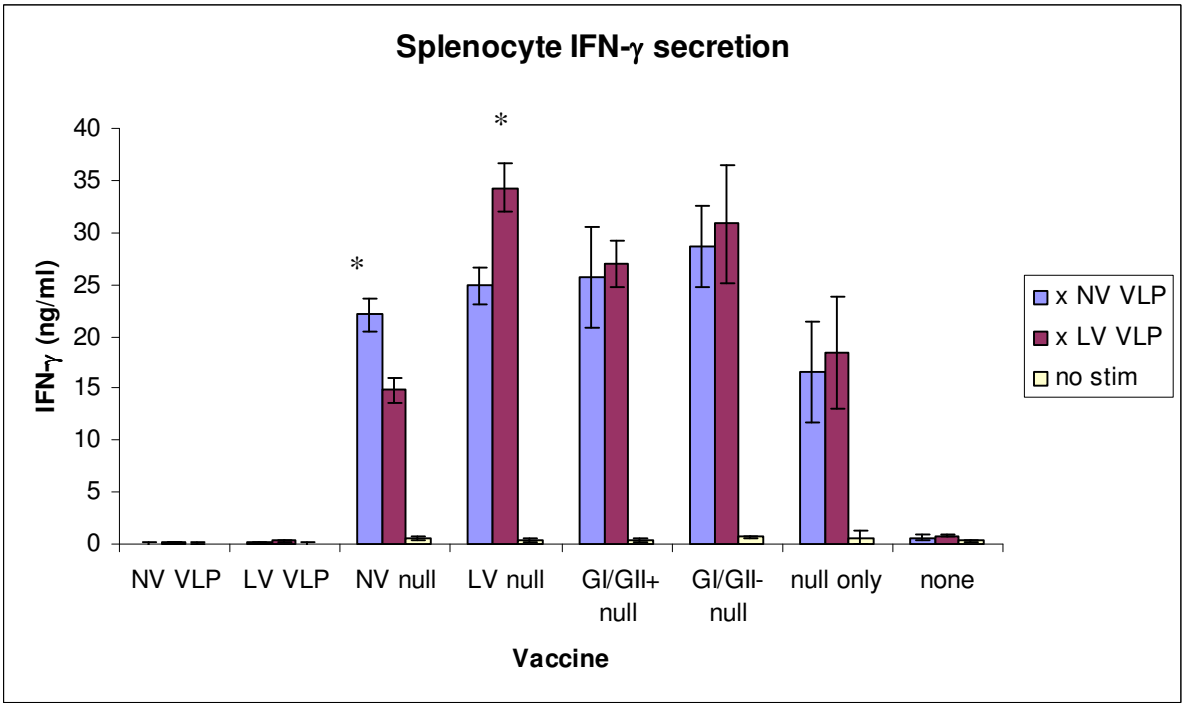
Vaccine	Anti-NV IgG ± SEM (ng/ml)	Total IgG ± SEM (ng/ml)	Anti-NV/ total IgG (%)	Anti-NV IgA ± SEM (ng/ml)	Total IgA ± SEM (μg/ml)	Anti-NV/ total IgA (%)
VLP						
NV	0.5 ± 0.3	62.3 ± 12.6	0.8	2.5 ± 1.2	47.3 ± 10	5.3E-03
GI/GII+	2.4 ± 0.7	174.6 ± 79.1	1.4	0.7 ± 0.1	25.7 ± 4.6	2.7E-03
GI/GII-	0.5 ± 0.2	110.7 ± 30.6	0.5	1.1 ± 0.7	30.6 ± 2.6	3.6E-03
CpG						
NV	12.2 ± 6.7	94.5 ± 9.7	12.9	7.2 ± 2.2	56.5 ± 2.0	1.3E-02
GI/GII+	34.9 ± 8.0	316.0 ± 203.8	11.0	3.2 ± 1.9	30.6 ± 8.1	1.0E-02
GI/GII-	10.4 ± 6.6	315.8 ± 6.0	3.3	1.8 ± 0.8	31.3 ± 1.5	5.8E-03
null VRP						
NV	44.1 ± 9.3	254.0 ± 54.9	17.4	68.1 ± 32.2	44.0 ± 4.7	1.5E-01
GI/GII+	88.1 ± 47.9	504.6 ± 267.4	17.5	10.4 ± 8.7	27.5 ± 1.6	3.8E-02
GI/GII-	9.1 ± 4.2	318.3 ± 50.6	2.9	7.7 ± 2.4	31.5 ± 7.6	2.4E-02

Figure 6.6

A.

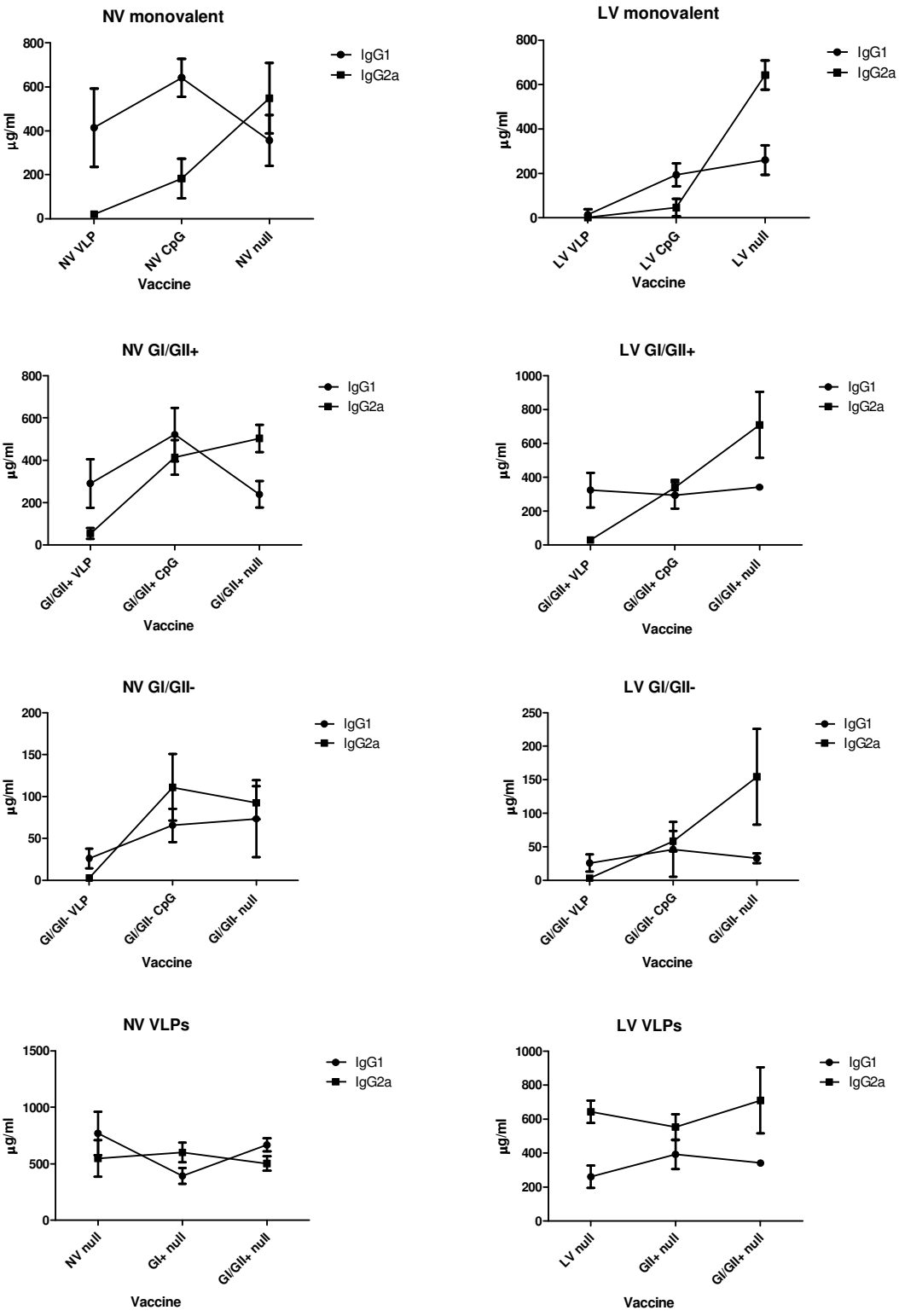

IFN-γ ELISpot
Spots/5x10^5 cells
600
500
400
300
200
100
0
NV VLP
LV VLP
NV null
LV null
GI/GII+ null
GI/GII- null
null only
none
Vaccine
x NV VLP
x LV VLP
no stim

B.


Splenocyte IFN-γ secretion
IFN-γ (ng/ml)
40
35
30
25
20
15
10
5
0
*
*
NV VLP
LV VLP
NV null
LV null
GI/GII+ null
GI/GII- null
null only
none
Vaccine
x NV VLP
x LV VLP
no stim

IFN-γ secretion following VLP stimulation. Splenocytes from mice immunized with monovalent or multivalent VLP vaccines with or without null VRP adjuvant, with null VRP alone, or with no antigen were stimulated with NV VLPs, LV VLPs, or media alone and tested for IFN-γ secreting cells by ELISpot (A) or IFN-γ secretion in supernatant by EIA (B).

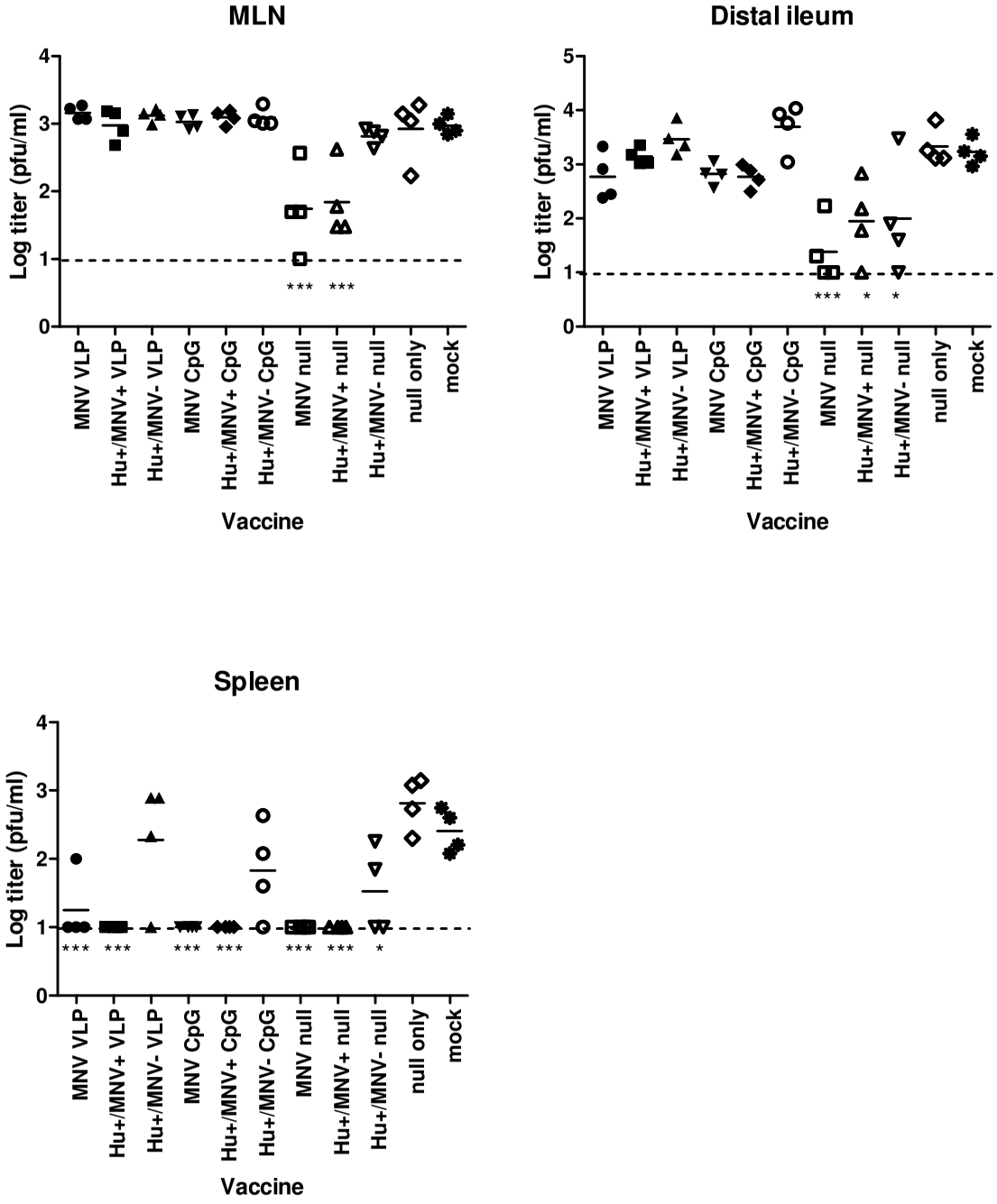
Figure 6.7


NV monovalent
LV monovalent
NV GI/GII+
LV GI/GII+
NV GI/GII-
LV GI/GII-
NV VLPs
LV VLPs
IgG1
IgG2a
µg/ml
Vaccine
NV VLP
NV CpG
NV null
LV VLP
LV CpG
LV null
GI/GII+ VLP
GI/GII+ CpG
GI/GII+ null
GI/GII- VLP
GI/GII- CpG
GI/GII- null
GI+ null
GII+ null

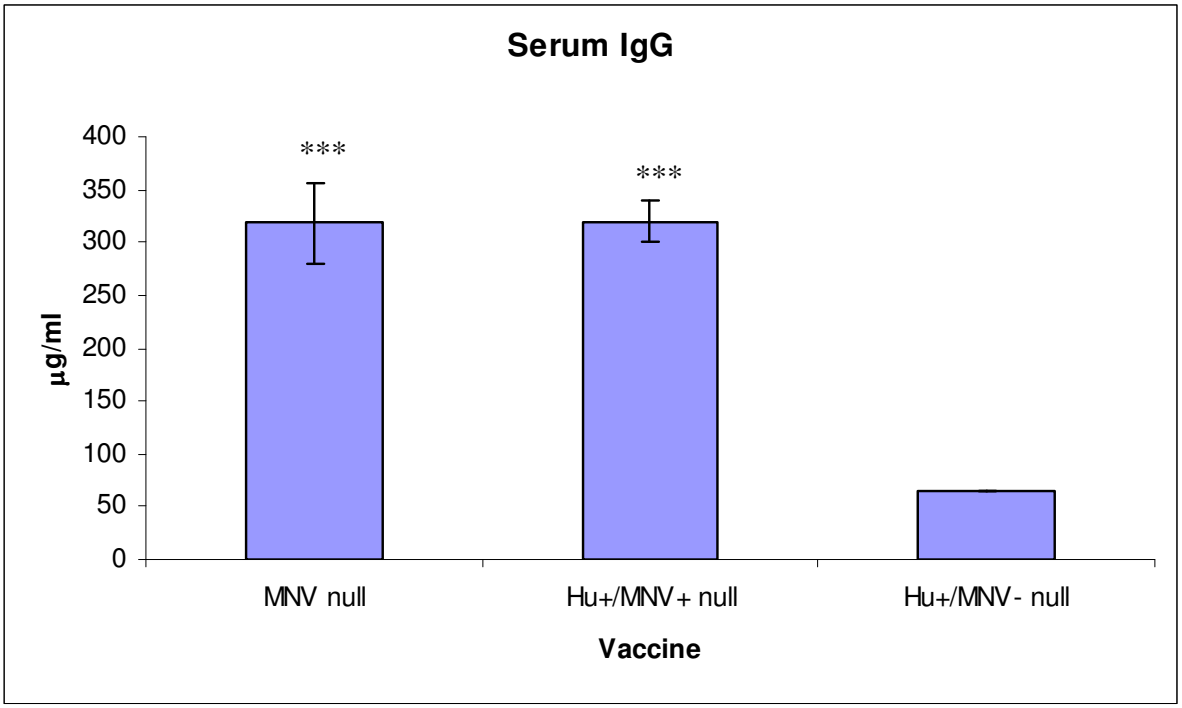
IgG subtypes. Mice immunized with monovalent (A-B) and multivalent vaccines with (C-D) or without (E-F) NV or LV VLPs were analyzed for IgG1 and IgG2a serum antibody subtype responses. Subtype responses to increasing amounts of VLPs are shown in (D).

Figure 6.8

A.


MLN
Log titer (pfu/ml)
Vaccine
MNV VLP
Hu+/MNV+ VLP
Hu+/MNV- VLP
MNV CpG
Hu+/MNV+ CpG
Hu+/MNV- CpG
MNV null
Hu+/MNV+ null
Hu+/MNV- null
null only
mock
Distal ileum
Spleen

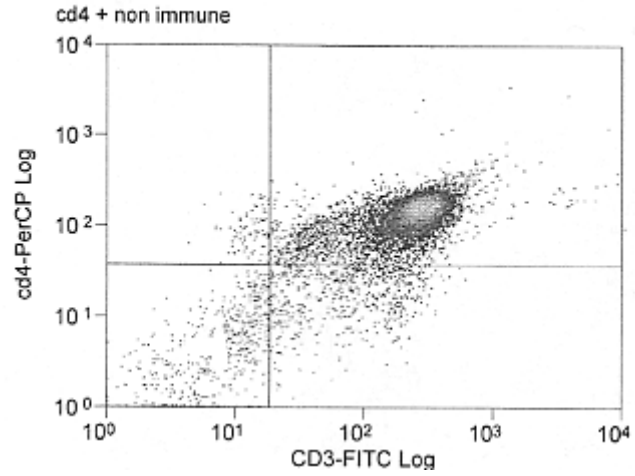
B.



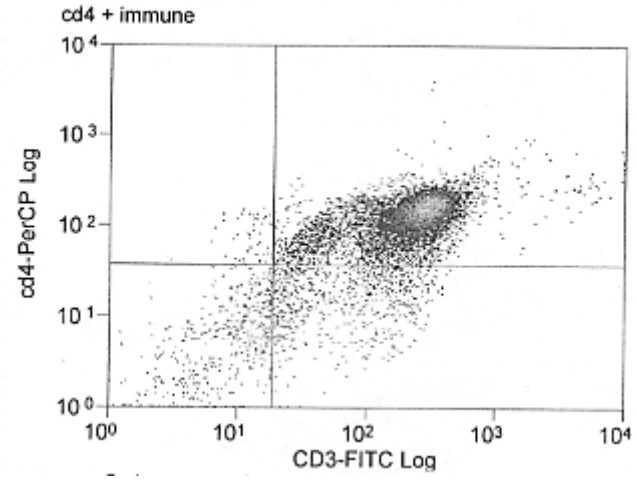
MNV vaccination and challenge. Mice immunized with monovalent MNV VLP or multivalent VLPs +/- MNV VLP were challenged with MNV and tissues harvested three days post-infection. Plaque assays were performed on homogenized spleen, MLN, and distal ileum (B). Serum IgG reactivity to MNV VLPs was determined by ELISA (B).

Figure 6.9

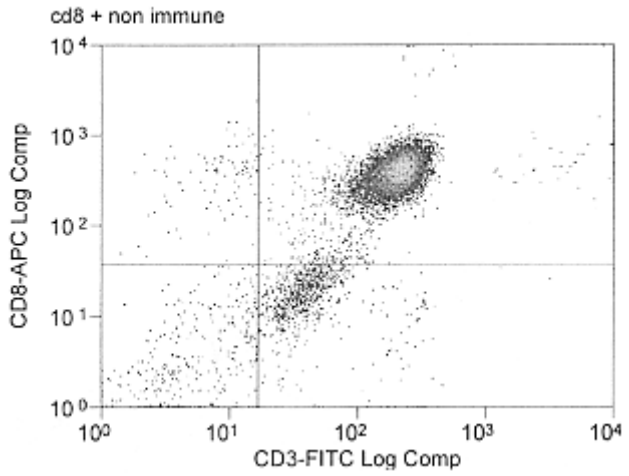
A.


cd4 + non immune
cd4-PerCP Log
CD3-FITC Log
10 4
10 3
10 2
10 1
10 0
10⁰
10¹
10²
10³
10⁴

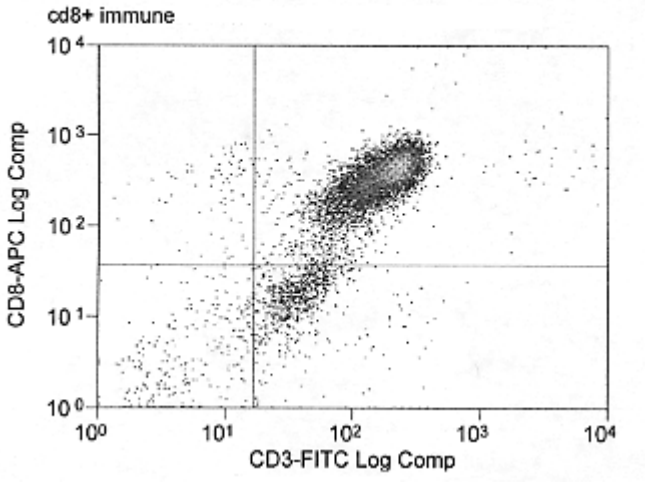
B.


cd4 + immune
cd4-PerCP Log
CD3-FITC Log
10 4
10 3
10 2
10 1
10 0
10⁰
10¹
10²
10³
10⁴

C.



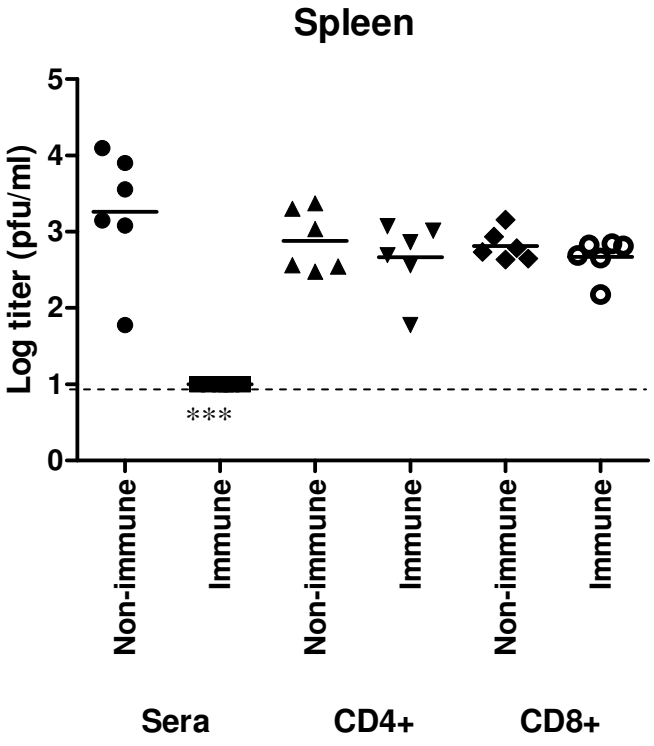
D.



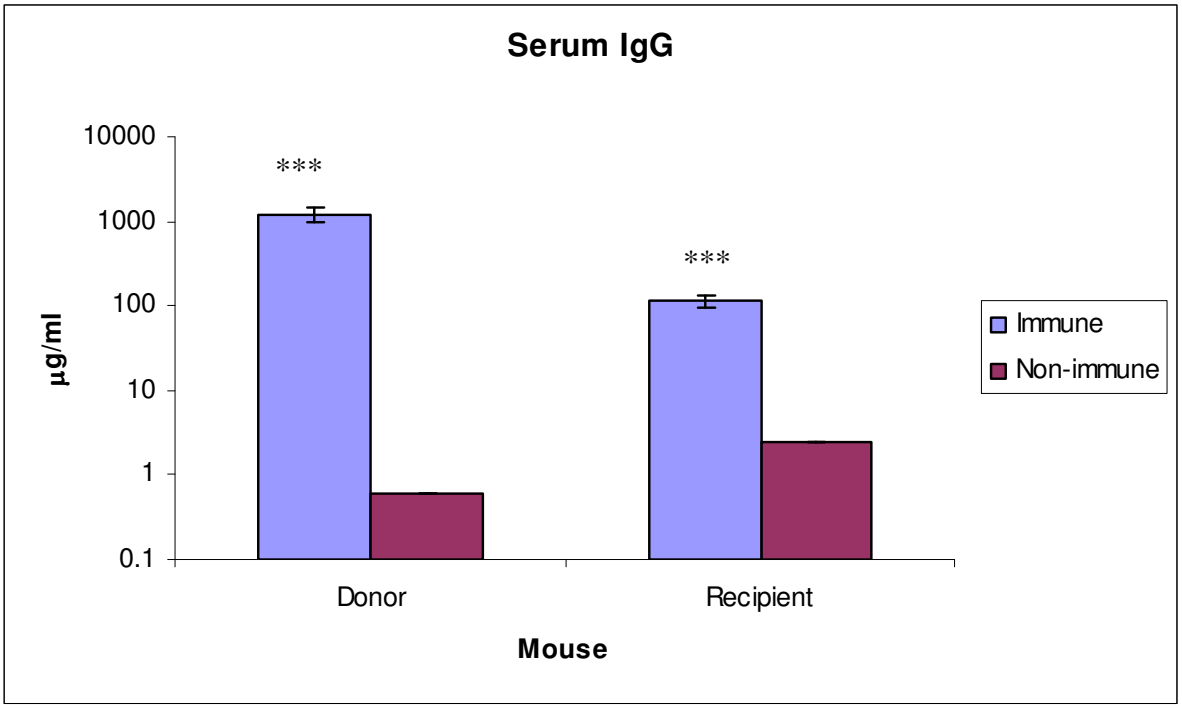
FACS analysis. $CD4^+$ and $CD8^+$ cells purified from non-immune (A,C) and immune (B,D) splenocytes were verified for $CD3^+$ and $CD4^+$ (A-B) or $CD8^+$ (C-D) expression by FACS (A-D).

Figure 6.10

A.


Spleen
Log titer (pfu/ml)
5
4
3
2
1
0
***
Non-immune
Immune
Non-immune
Immune
Non-immune
Immune
Sera
CD4+
CD8+

B.



Adoptive transfers. Wild-type mice were immunized three times with MNV VLP coadministered with null VRP. Unimmunized controls were treated in parallel. Two weeks after final boost, sera, $CD4^+$ and $CD8^+$ splenocytes were harvested and purified. Sera, $CD4^+$, or $CD8^+$ were passively or adoptively transferred to Scid knockout mice. Twenty-four hours post-transfer, mice were infected with MNV1 and tissues harvested 3 days post-infection. Spleens were evaluated for MNV titers by plaque assay (A). Distal ilea did not support MNV infection in any transfer recipients (data not shown). MNV-specific serum IgG in serum donor and recipient mice were measured by ELISA (B). Non-immune mice had no detectable MNV antibody and were assigned values half the lower limit of detection per assay.

CHAPTER VII.

Conclusions

This dissertation thoroughly characterizes B cell and T cell immunity induced by vaccination with norovirus VLPs expressed by or coadministered with VEE replicon particles in the mouse. Experiments conducted *in vitro* correspond with findings from *in vivo* studies, painting a well-defined picture of the shared impact of both antibody and T cell mechanisms in homotypic and cross-reactive immune responses following vaccination. Importantly, VLP vaccines are also able to protect against mouse norovirus challenge, providing the first proven effective vaccine against norovirus infection.

The work presented here began as a small research project investigating the cross-reactive responses of antibodies following monovalent or multivalent VRP vaccination with four human norovirus strains. Thanks to the cloning work of Lisa Lindesmith, our VRP and VLP panel grew to include 18 strains from 10 genoclusters in 3 genogroups over the last four years, which allowed the detailed characterization of cross-reactive antibody responses from very closely genetically related strains within a genocluster to inter-genogroup inter-species strains. The discovery of the adjuvant activity of the VRP by Joseph M. Thompson (256) allowed us to study the effects of multivalent vaccines comprising VLPs representing all 10 genoclusters in the VLP panel with ease and efficiency. The concurrent discovery of the mouse norovirus by Skip Virgin's group at Washington University and the optimization of techniques to study adaptive immunity by Karen Chachu lead to a beautiful collaboration

testing the efficacy of these vaccines in a mouse model. Each of these contributions was instrumental in bringing my dissertation project to its mature status, and I recognize them accordingly.

The immune response following norovirus infection is highly undercharacterized. Antibody and T cell responses have been documented following norovirus infection in humans and following norovirus VLP vaccination in humans and mice (10, 145, 247). Induction of immune responses following VLP vaccination suggests the capsid protein is immunogenic, and antibody specificity for norovirus VLPs and T cell stimulation with VLPs following infection suggests the capsid is antigenic as well (145, 247). Because the capsid composes the outer surface of the norovirus virion that is exposed to the internal environment during infection, we can hypothesize that the capsid is the primary target of the humoral immune response for antibody neutralization. However, previous studies into the immune response to ORF1 and ORF3 had not been undertaken heretofore. Our studies into the protective effect of vaccination with VRP expressing norovirus ORF1, ORF2, and ORF3 solidified our hypothesis where ORF1 and ORF3 offered little to no protection against MNV challenge in the mouse model. In contrast, ORF2 conferred significant protection against MNV infection. These important findings support ongoing research into VLP subunit vaccines for protection against noroviruses.

Effective vaccines must also activate the branch of the adaptive immune system responsible for virus neutralization. Extensive studies into the mechanisms of the immune response elicited following MNV infection have been carried out in knockout mice by Karen Chachu at Washington University. These findings, summarized in Chapter V, clearly indicate that both B cells and T cells are instrumental in controlling MNV infection. Mice

immunodeficient for B cells (μMT mice) did not clear MNV from the mesenteric lymph nodes (MLN) at all time points tested through 21 days post-infection (Fig. 5.7), and passive transfer of immune antisera from wild-type mice or monoclonal antibodies to MNV both significantly reduced MNV titers in persistently infected $RAG^{-/-}$ mice (Fig. 5.8). Together these data support anti-MNV antibody responses as critical for controlling MNV infection. Parallel studies in mice deficient in $CD4^+$ or $CD8^+$ T cell production were also tested for clearance of MNV infection. Both cell subsets were also clearly important in clearance of MNV, as immunodeficient mice cleared virus more slowly than wild-type mice (Fig. 5.5), and *in vitro* depletion of $CD4^+$ and/or $CD8^+$ cells from immune wild-type splenocyte suspensions prior to adoptive transfer into persistently infected $RAG1^{-/-}$ mice also resulted in significantly higher viral loads compared to transfer of undepleted control preparations (Fig. 5.6). Although T cells were not required for eventual clearance of MNV, they played a significant role in controlling viral replication at early time points post-infection. Furthermore, infection of IFN-γ and perforin knockout mice revealed that MNV may be cleared by T cells using a perforin-mediated mechanism (Fig. 5.6). These data conclude that both B cells and T cells are involved in the adaptive immune response following MNV infection. Cell subsets likely act in a coordinated fashion in viral clearance and can compensate for one another if one subset is compromised.

We have shown that MNV ORF2 expressed from VRP protect against MNV infection. However, the mechanism of adaptive immunity induced by vaccination may be different than the mechanism induced following natural infection. To characterize the efficacy of MNV ORF2 vaccines and their mechanism of action, we conducted vaccination experiments over prolonged periods of time and in knockout mice. Results conclusively

showed that vaccination of wild-type mice significantly protects against MNV infection through 24 weeks post-vaccination (Fig. 5.3). Vaccination of B cell$^{-/-}$, MHC class I$^{-/-}$, and MHC class II$^{-/-}$ knockout mice with VRP-MNV ORF2 additionally induced significantly reduced viral loads in all three immunodeficient groups post-infection; however, no knockout group was able to completely protect against MNV infection as well as wild-type mice (Fig. 5.4), suggesting B cells and T cells may use a combined mechanism following vaccination. In contrast, a separate experiment described in Chapter VI showed that antisera from wild-type mice vaccinated with MNV VLPs coadministered with null VRP and passively transferred to immunodeficient Scid mice completely protected against acute MNV infection, whereas adoptively transferred purified CD4^{+} or CD8^{+} splenocytes did not protect. This finding suggests humoral immunity is integral in preventing MNV infection. It is possible that VRP used as a vaccine vector to express VLPs induces a different immune mechanism than VRP coadministered with VLP as an adjuvant or that knockout mice bred on different genetic backgrounds respond differently. However, because both antibodies and T cells appear to be associated with viral clearance following norovirus infection, an ideal norovirus vaccine would activate both arms of the immune system.

We recognize that the immune response induced following MNV infection or vaccination may not entirely mirror that seen following norovirus infection in humans, although human infection studies have also revealed activation of both humoral and cellular immunity (145, 247). Studying human immunity, however, is much less straightforward because humans can be infected with multiple norovirus strains that are extremely genetically diverse, and understanding how one norovirus infection impacts subsequent norovirus infections is additionally important. In Chapter II and Appendix 1 we showed

comprehensive antibody cross-reactivity profiles among norovirus strains where strains in different genogroups exhibit very low cross-reactivity and more genetically related strains in the same genogroup exhibit slightly heightened (but still quite low) cross-reactivity. Additional findings that no antisera can block receptor binding to heterologous VLPs in *in vitro* assays may explain why individuals can have multiple norovirus infections with different strains even after previous exposure (203, 281). Cross-reactivity between limited numbers of norovirus strains and homotypic antibody blockade of receptor binding has been documented repeatedly (86, 91, 145, 180, 219, 259); however, our data shows the most comprehensive antibody cross-reactivity and blockade profile to date.

We are also the first group to compare cross-reactive antibody responses to strains within the same genocluster. The GII.4 noroviruses currently cause approximately 80% of all norovirus outbreaks worldwide (55, 264). To attempt to explain their continued circulation over the last decade, we created a time-ordered panel of GII.4 VLPs spanning three decades. Multiple alignments of published sequences revealed clusters of mutations that coincided with circulating strains during established outbreak periods, and bioinformatic analysis showed that evolution of the capsid sequence was occurring at specific sites structurally important for receptor binding (Fig. 3.1). VLPs representing each evolutionarily distinct strain and expressed by VRPs to generate antisera were tested for cross-reactive and receptor blocking antibody responses. Our results indicated that strains were in fact antigenically distinct: Antisera responses were strain-specific with limited cross-reactivity, binding profiles to HBGAs expanded and contracted with inclusion of specific mutations in the P2 binding domain and peripheral sites, and blockade of receptor binding was variable and attributable to identifiable mutations by bioinformatics prediction. Assuming blocking

receptor binding yields a virus noninfectious, we were able to conclude that antibodies generated in humans or mice against earlier strains were likely not protective to later circulating GII.4 strains as they did not prevent HBGA binding to contemporary VLPs (Fig. 3.3 and 3.5). The continued evolution of GII.4 norovirus capsids mirrors that seen with influenza where new antigenically distinct strains emerge every 2-3 years and could explain escape from herd immunity one would expect to see with a continually circulating virus. Additional studies with time-ordered VLPs from the GI.1 and GII.2 genoclusters described in Appendix 2 revealed that not all genoclusters exhibit continued evolution. GI.1 VLPs isolated more than three decades apart exhibited identical antibody cross-reactivity and receptor blockade profiles (Fig. A2.1), suggesting this genocluster is not evolving and may explain the low prevalence of GI outbreaks. GII.2 VLPs, on the contrary, showed an intermediate evolutionary pattern where an archaic strain cross-reacted with one of two contemporary strains, one contemporary strain cross-reacted with the other, and one contemporary strain was altogether antigenically distinct (Fig. A2.2). This data supports evolution within the GII genogroup and may explain their increased prevalence compared to GI strains. Overall, evolution within norovirus clusters likely dictate emergence of new predominant strains into populations naïve to the selected mutations.

Cross-reactive T cell responses to noroviruses are ambiguous. While several reports have stated that IFN-γ is secreted in response to VLP stimulation of T cells following norovirus infection or VLP vaccination in humans and mice (145, 178, 247), only a single study broached the subject of cross-reactivity (145). Lindesmith *et al.* observed that $CD4^+$ PBMCs from individuals infected with Snow Mountain virus (SMV) mounted significant IFN-γ responses upon stimulation with homologous SMV VLPs or heterologous

intragenogroup Hawaii VLPs (145). Norwalk VLPs, derived from a different genogroup, did not stimulate PBMCs. In contrast, we reported in Chapter V that IFN-γ knockout mice were still able to combat MNV infection whereas perforin -/- mice were less able to do so, indicating that perforin but not IFN-γ may be involved in the anti-norovirus immune mechanism. While these findings in the mouse are appealing, studies with human strains remain the focus of cross-reactivity studies. Unfortunately, we learned that using the VRP expression system for production of VLPs lead to VEE non-structural RNA contamination of the particles, which in turn lead to VEE-specific T cell activation following VLP stimulation of cells from VRP vaccinated mice (Fig. 4.3 and 6.6). This finding makes identification of cross-reactive VLPs nearly impossible using our system, although our experiments clearly show that IFN-γ is abundantly produced following VLP stimulation. To circumvent this problem, we turned to peptide stimulation using overlapping 15-mer pools reconstituting the complete Norwalk and Farmington Hills capsid sequences synthesized independently of VRP. This approach will not only allow us to identify cross-reactivity between strains but also to identify specific epitopes within the capsid sequence for T cell stimulation. Our results show that the NV sequence FDLSLGPHLNPFLLH in the shell domain contains one potential NV-specific epitope, and the FH sequence CLLPQEWVQHFYQEAAPAQSDVALL in the P1 domain contains 1-2 potential FH-specific epitopes based on IFN-γ secretion following peptide stimulation of splenocytes (Fig. 4.X). We additionally found that these stimulatory sequences to NV- or FH-immune cells were not cross-reactive to one another. Additional studies to identify the exact epitope within these stimulatory sequences and multiple alignments to determine conservation of epitopes across strains within genoclusters and genogroups must be performed. Furthermore,

peptide stimulation of human immune cells must also be conducted as epitopes may not be conserved across species. However, using a small animal model to mimic immune responses likely seen in humans suggests that norovirus capsid peptides will also induce IFN-γ secretion following human immune cell stimulation and that specific epitopes can be identified. These findings could be instrumental in vaccine design for anti-norovirus T cell activation and will teach us how antigenically conserved genetically related strains are in cellular immunity.

Identifying strains that generate cross-reactive immune responses to other strains is very important for formulation of cumulative vaccines. Our original finding in Chapter II showed that administering a VRP cocktail expressing three heterologous VLPs to mice induced enhanced antibody cross-reactivity as well as enhanced cross-reactive receptor blockade to strains not included in the vaccine composition. We followed up on these results by designing a comprehensive study into the effects of monovalent versus multivalent vaccination with our expanded panel of VLPs with particular emphasis on cross-reactive and receptor blockade responses within and across genogroups and the protective effect of these vaccines in the mouse model. We substituted VRP expression vectors with non-coding VRP adjuvants, which we coadministered with VLPs, to capitalize on the availability of a single common adjuvant for vaccine formulation. Our results described in Chapter VI showed that multivalent genogroup specific vaccines induce cross-reactive and receptor blocking antibody responses to strains within the common genogroup but not across genogroups (Fig. 6.4). Inclusion of VLPs from both genogroups expands cross-reactivity to additional GI and GII strains and enhances the cross-blockade response (Fig. 6.4). Furthermore, splenocytes were also activated to produce IFN-γ upon homologous and heterologous stimulation with

VLP in both monovalent and multivalent vaccine recipients, although specific cross-reactivity could not be determined due to background stimulation induced by VRP (Fig. 6.6). These *in vitro* data support use of multivalent vaccines for enhanced protection against cumulative norovirus challenge. To test the efficacy of multivalent vaccines in an *in vivo* infection model, we vaccinated mice with multivalent human VLP vaccines with or without the MNV VLP component. Our results showed that MNV VLP coadministered with VRP adjuvant in monovalent or multivalent vaccines significantly reduced viral loads post-MNV challenge in all tissues tested (Fig. 6.8). Furthermore, multivalent vaccines lacking MNV VLP also reduced viral loads in all tissues (Fig. 6.8), suggesting cross-reactive protection. Because protection was not absolute, additional studies using increased VLP concentrations must be performed to optimize vaccine formulation. We performed an additional experiment where antisera, purified $CD4^+$ cells, or purified $CD8^+$ cells were adoptively transferred from monovalently vaccinated wild-type mice into immunodeficient Scid mice prior to MNV challenge. Mice receiving antisera were completely protected from MNV infection whereas those receiving purified T cells were not (Fig. 6.10). Our results not only revealed that the mechanism of protection induced by monovalent null VRP vaccines is likely antibody-mediated but also that complete protection can be attained with these vaccines. Passive transfer of antisera from multivalently vaccinated mice must be performed to determine if this mechanism is upheld and if cross-reactive antibody can elicit a protective effect in immunodeficient mice.

The data presented in this dissertation provide insight into the mechanisms of adaptive immunity induced by norovirus infection and norovirus VRP vaccination. Norovirus infection and VRP vaccination induce both B cell- and T cell-mediated responses;

however, null VRP vaccines may mediate protection in a T cell independent manner. We have shown that low levels of cross-reactive antibody are induced following monovalent vaccination and are not likely to protect against heterologous norovirus challenge. However, multivalent vaccination induced antibody responses that are cross-reactive and potentially cross-protective against heterologous norovirus strains. Further characterization of multivalent norovirus vaccines in the mouse model and subsequent clinical trials in humans will reveal the efficacy of these novel norovirus vaccine candidates.

APPENDIX 1
Chapter II.

Materials and methods.

Southampton virus (SoV; GI.2), Desert Shield virus (DSV; GI.3), Chiba virus (CV; GI.4), Toronto virus (TV; GII.3), M7 virus (GII.13), mouse norovirus-1, (MNV-1; GV), GI.1 Westchester, and GII.2 Buds and Ina capsid genes were cloned into pVR21 and VRPs produced as described (11). Mice were primed and boosted with each individual strain, and sera were collected on day 35 for use in ELISA and receptor binding blockade assays as described in Chapter II. Additional groups of mice received heterologous monovalent and/or multivalent VRP prime-boost regimens on day 1 and 42. Prime-boost VRP vaccination groups included HV-HV, HV-SM, HV-NV, HV-cocktail (NV, SM, and HV), cocktail-cocktail, cocktail-HV, cocktail-SM, and cocktail-NV. Sera were collected on day 0, 14, 28, pre-boost, and day 3, 7, 14, and 21 post-boost and ELISAs performed to determine strain-specific antibody responses.

Statistics were performed using One-way ANOVA and Tukey's post-test in GraphPad Prism software. Significance is designated with * ($P<0.05$), ** ($P<0.01$), or *** ($P<0.001$).

Results.

Intermediate heterotypic antibody responses are induced to additional norovirus strains within a genogroup but not across genogroups.

Following immunization with our panel of VRPs expressing norovirus VLPs, antisera to each individual strain were tested for antibody cross-reactivity to our panel of VLPs. Our

results supported previous findings from Chapter II. Homotypic antibody responses in mice were significantly more robust to the immunizing VLP than to heterologous VLPs ($P<0.001$); however, clear heterotypic trends emerged whereby cross-reactive antibodies recognize VLPs within a genogroup better than VLPs in different genogroups that were significant in many cases (Fig. A1.1a-b). Antisera cross-reactivity to our panel of VLPs following immunization with GI strains is shown in Fig. A1.1a (Beachfront Avenue!); antisera cross-reactivity following GII immunization is shown in Fig. A1.1b. Antisera blockade of NV VLP or LV VLP binding to H type 3 was also tested (Fig. A1.1c-d). As previously noted, no heterologous antisera group could block binding, regardless of cross-reactive IgG titers.

An additional group of mice were immunized with MNV VLPs, which constitute the murine-specific GV cluster. MNV antisera were tested for cross-reactivity to our panel of human VLPs (Fig. A1.2a), and our panel of human antisera were tested for cross-reactivity to MNV VLPs (Fig. A1.2b). Not surprisingly, very low cross-reactivity existed between these intergenogroup strains, although MNV antisera reacted significantly more to M7 VLPs than the rest of the panel (Fig. A1.2a). Furthermore, MNV antisera could not block NV VLP or LV VLP binding to its H type 3 receptor (Fig. A1.2c-d).

Multivalent vaccination but not heterologous monovalent vaccination elicits enhanced antibody responses.

In Chapter II, we analyzed cross-reactivity of human antisera to heterologous VLPs. Overall cross-reactivity to heterologous VLPs was lower than to the infecting strain; however, a single sample from an individual infected with SM cross-reacted more strongly to HV VLPs than to SM VLPs. To investigate if previous exposure to one strain can lead to a

higher cross-reactive response to that strain following subsequent heterologous exposure, we primed mice with one strain, boosted with a heterologous strain, and analyzed antisera cross-reactivity to both VLPs pre- and post-boost compared to mice primed and boosted with homologous antigen. In analyzing homologous prime and boost with HV VRP, antisera IgG specific for HV VLPs peaks and plateaus at approximately 1 mg/ml 14 days post-prime (Fig. A1.3a). After HV VRP boost, specific IgG levels increased to >2 mg/ml, plateaued, and began to drop by day 21 post-boost. In contrast, mice primed with HV VRP followed by boost with SM or NV VRPs (Fig. A1.3b-c) lead to weaker serum IgG responses to HV, SM, and/or NV VLPs post-boost (400-800 μg/ml IgG) compared to homologous boost with HV. Responses to both priming and boosting strains were equivalent at day 21 post-boost. However, reactivity to SM or NV VLPs at day 14 post-boost was not significantly different from reactivity to SM or NV VLPs 14 days post-prime with each antigen, respectively (data not shown), suggesting heterologous immunization may affect IgG responses to the immunizing antigen independent of previous exposures although pre-existing antibody titers are also boosted.

Our results in Chapter II described strongly-reactive antibody responses to each vaccine component following multivalent vaccination in mice. Because heterologous prime and boost regimens did not induce substantial antibody responses to either immunizing strain, we primed mice with HV VRP or multivalent VRP vaccines and boosted with multivalent VRP vaccines or individual VRP components, respectively, to determine the effect of multivalent vaccination on IgG responses to individual strains (Fig. A1.4). Mice immunized with HV VRP and boosted with multivalent vaccines composed of NV, SM, and HV VRPs mounted a strong serum IgG response to HV VLPs but not to NV or SM VLPs

(Fig. A1.4a). However, priming with multivalent VRPs followed by boosting with identical multivalent VRPs or one of the three VRP components lead to very strong serum IgG responses to the boosting strain(s) (Fig. A1.4b-e). Boosting with identical multivalent vaccines (Fig. A1.4b) resulted in specific IgG responses to each strain to levels mirroring that following homologous monovalent vaccination (Fig. A1.3a). Boosting with individual VRPs resulted in specific IgG levels of 3.5-4.5 mg/ml to the boosting strain (Fig. A1.4c-e), which are nearly twice that seen following homologous monovalent vaccination (Fig. A1.3a). These data reveal that multivalent vaccination results in very robust antibody responses upon re-exposure with any vaccine component. Boosting with heterologous VRP not included in the multivalent VRP prime did not mount strong cross-reactive responses to the boosting strain (data not shown), suggesting that one immunization with multivalent VRPs is not sufficient to induce heightened cross-reactivity to heterologous strains.

Discussion.

Chapter II discussed the levels of antibody cross-reactivity induced among four norovirus strains following VRP vaccination in the mouse. Our increased panel of VRPs and VLPs has allowed further characterization of cross-reactivity among genetically distinct noroviruses, and our results support our original findings that more antisera cross-reactivity exists between strains within a genogroup than between genogroups following monovalent VRP immunization. Analysis of nine human strains in separate genoclusters within GI and GII showed a more delineated pattern in cross-reactivity within a genogroup as well as more statistical significance in cross-reactive responses compared to inter-genogroup cross-reactivity. These results are not surprising, as one would expect more genetically related

strains to induce more cross-reactive antibodies. The low cross-reactivity of GI and GII antisera to the GV MNV VLP and vice versa corroborate our findings. Nonetheless, no additional antisera contained cross-reactive antibodies that could block NV VLP or LV VLP binding to the H type 3 receptor, regardless of genogroup origin of the vaccinating strain, reiterating that strains are in fact antigenically distinct.

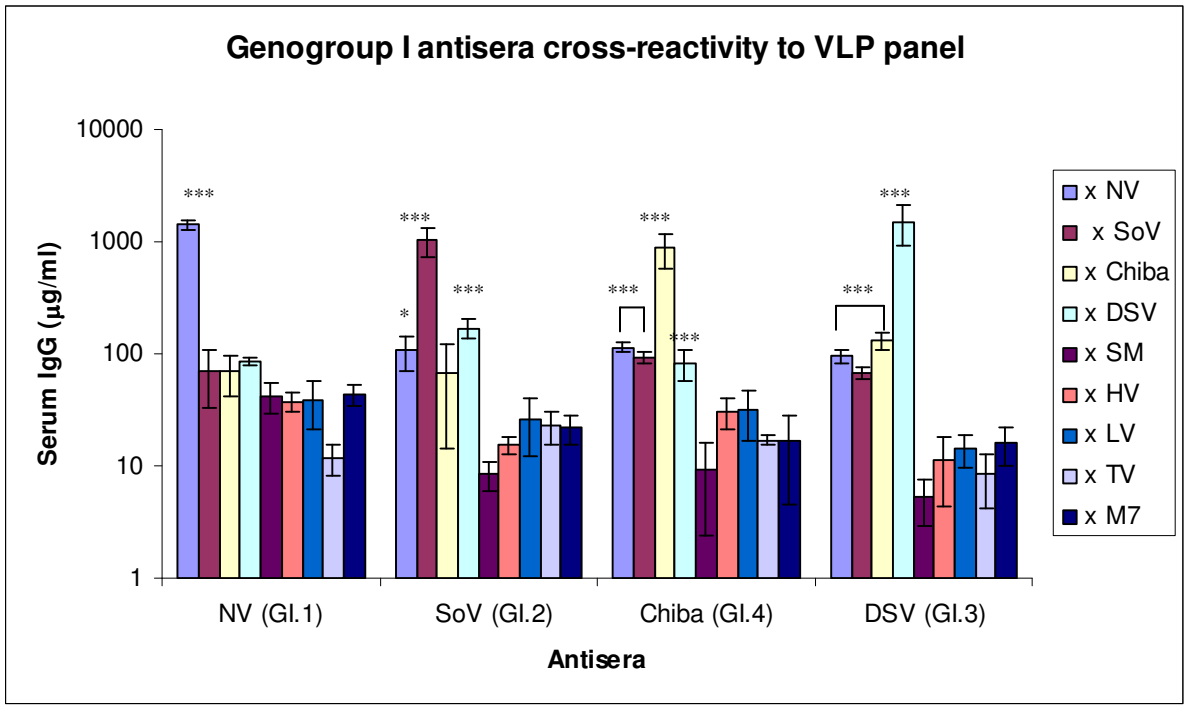
In our studies in human antisera cross-reactivity in Chapter II, one human antisera sample reacted more strongly to heterologous HV VLPs than to homologous SM VLPs, representing the infecting strain. We were curious if pre-exposure history to heterologous strains can change specific antibody responses following exposure to a different strain. Original antigenic sin (OAS) is defined as a state in B cell immunology where memory cells recognize epitopes on a related but genetically distinct strain following a previous infection and become activated to produce antibodies against the original strain, which may or may not be protective against the current strain. We hypothesized that OAS could explain the continued circulation of noroviruses through repeatedly exposed populations, the predominance of the GII.4 strains, and the capacity for individuals to have multiple norovirus infections within a short time period. To address this issue, we immunized mice sequentially with two heterologous strains. Compared to homologous immunization, heterologous prime-boost lead to low serum IgG to both the priming and boosting strains at all time points following the boost. While the IgG reactive for the priming strain was in fact boosted by the heterologous immunization, levels of IgG reactive for the boosting strain resembled IgG levels following primary immunization, suggesting the IgG response to the heterologous boost may be independent of the prime. Because VRP induced robust IgG levels to the immunizing strain only following homologous prime and boost, additional studies into the

potential for OAS would likely need to use an immunization schedule composed of a prime and boost with the same homologous strain, followed by a secondary booster with a heterologous strain to conclusively determine the effect of multiple exposures.

In Chapter II, multivalent norovirus vaccination was shown to induce strong antibody responses to each component of the vaccine composition that were not different from antibody responses following monovalent vaccination with each individual strain. We performed an additional study to determine the effect of re-exposure to individual antigens on antibody responses. Mice primed with HV VRP and boosted with a multivalent pool including HV VRP mounted a strong serum IgG response to HV VLPs but not to the other pool antigens, mirroring our results above suggesting a prime and boost with homologous antigen are required to attain high specific antibody levels. Interestingly, boosting with a multivalent vaccine following monovalent priming increased the HV-specific response to levels nearly twice that of homologous monovalent or multivalent vaccination. Additional experiments replicated this finding, where multivalent VRP prime followed by monovalent boost with each of the vaccine components resulted in stronger IgG responses to the boosting antigen than homologous prime-boost. Together, these findings suggest that multivalent vaccination leads to very strong antibody responses following re-exposure to a vaccinating strain, even after primary immunization.

Figure A1.1.

A.



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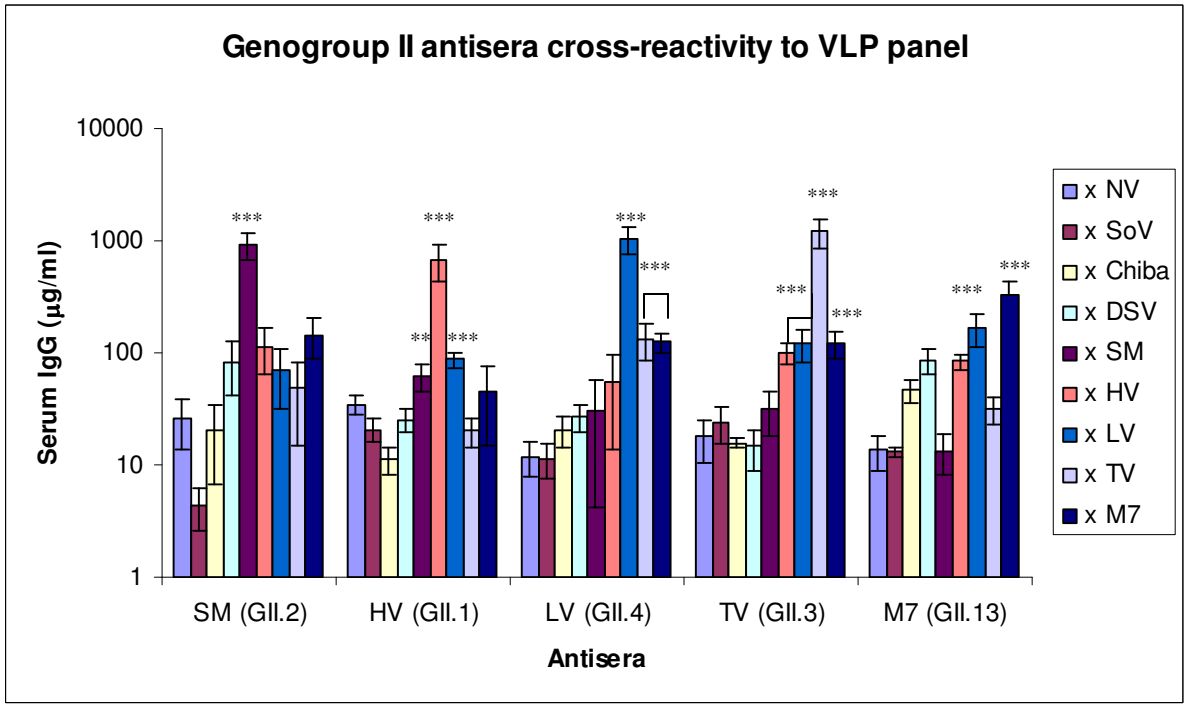

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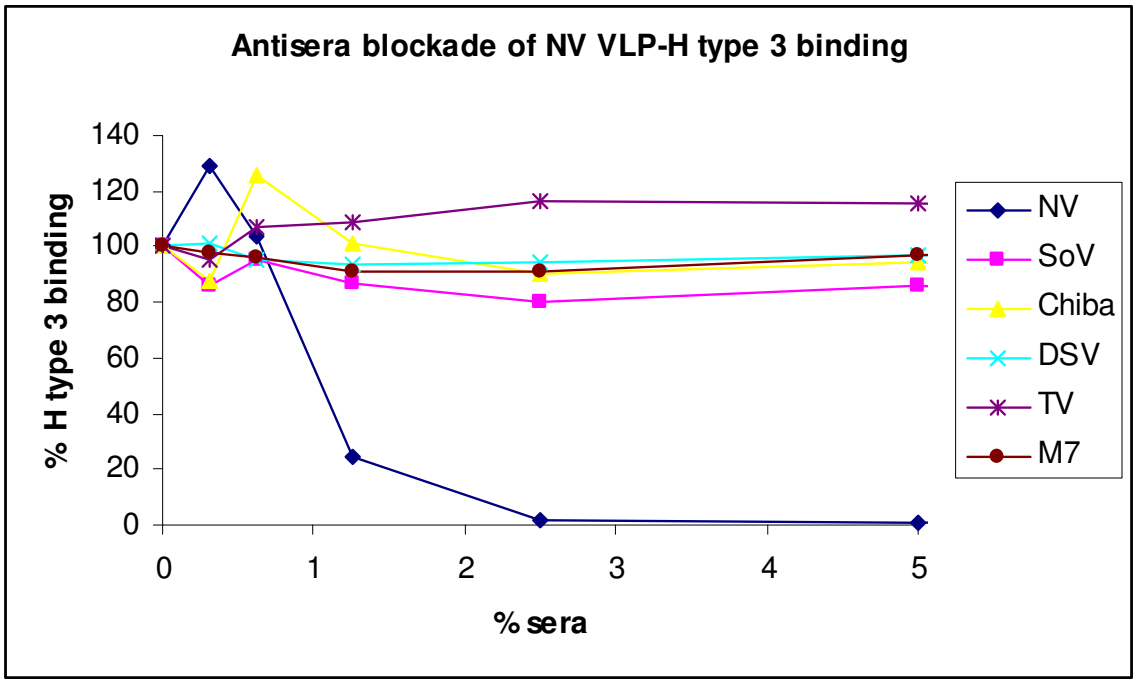
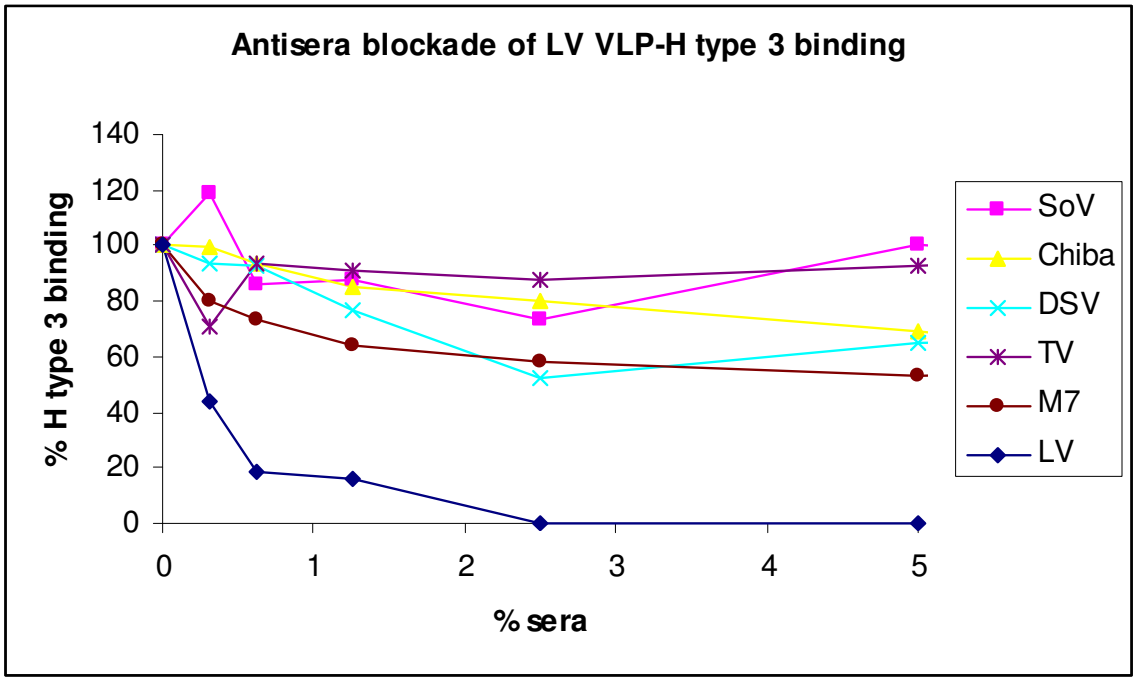


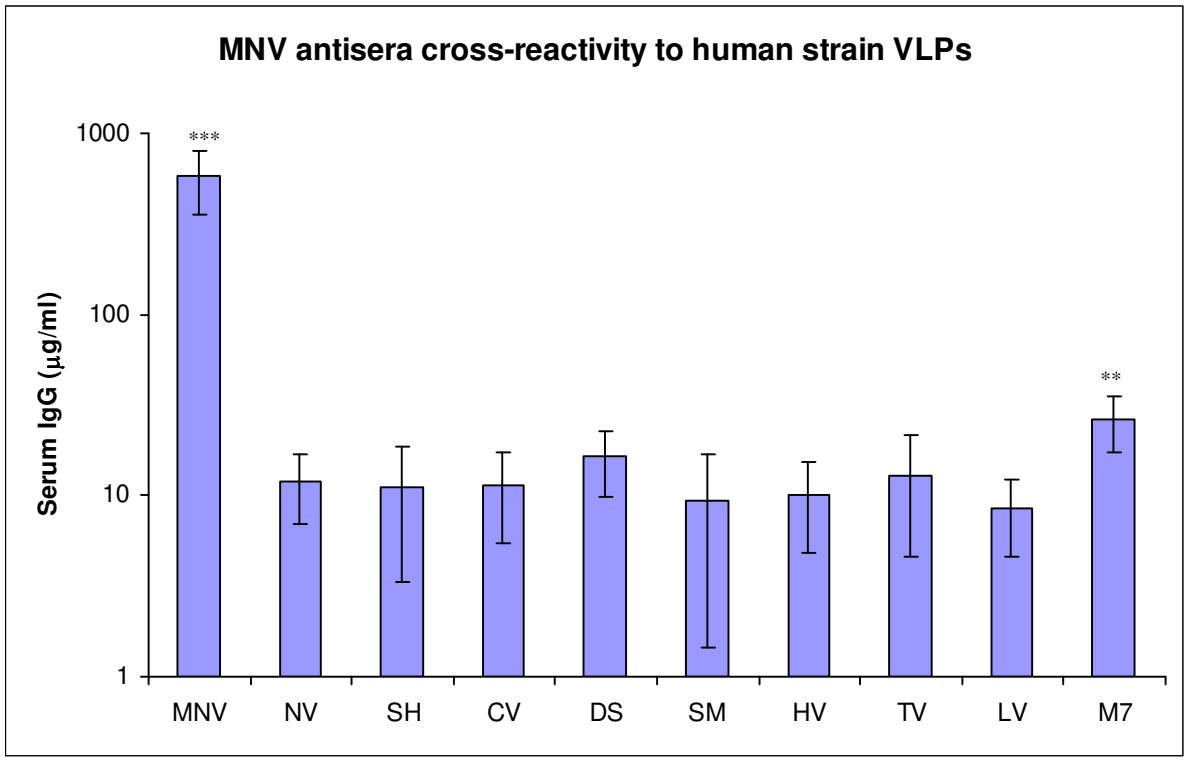
D.



Cross-reactive antibody responses to human strains within and across genogroups. Antisera from mice vaccinated with each human norovirus strain from our panel of VRPs were tested for cross-reactivity to our panel of VLPs by ELISA (A-B) and blockade of NV VLP-H type 3 and LV VLP-H type 3 binding (C-D).

Figure A1.2

A.



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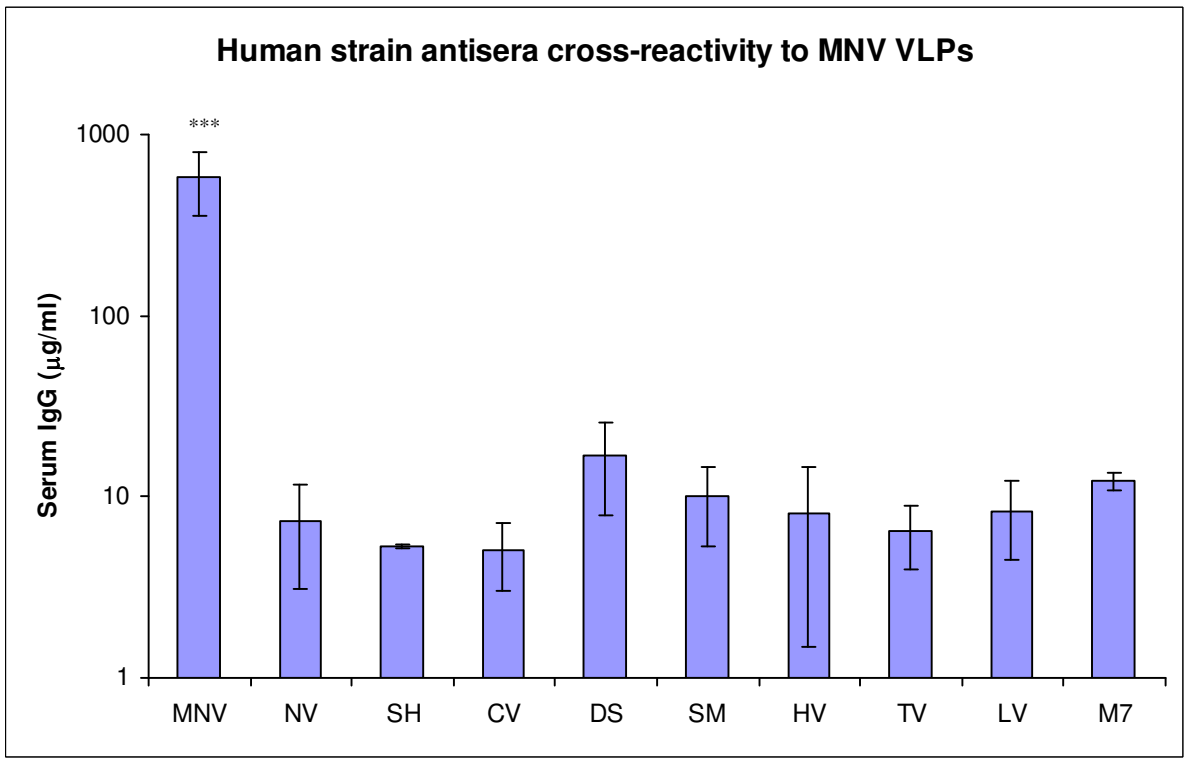

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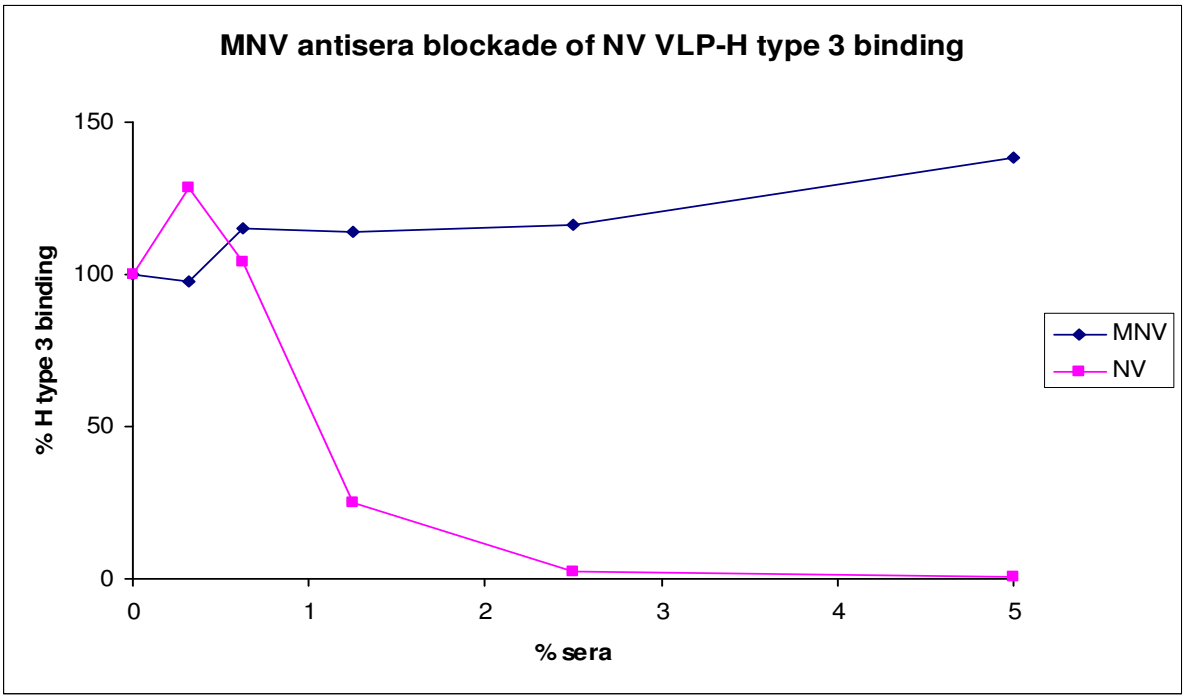

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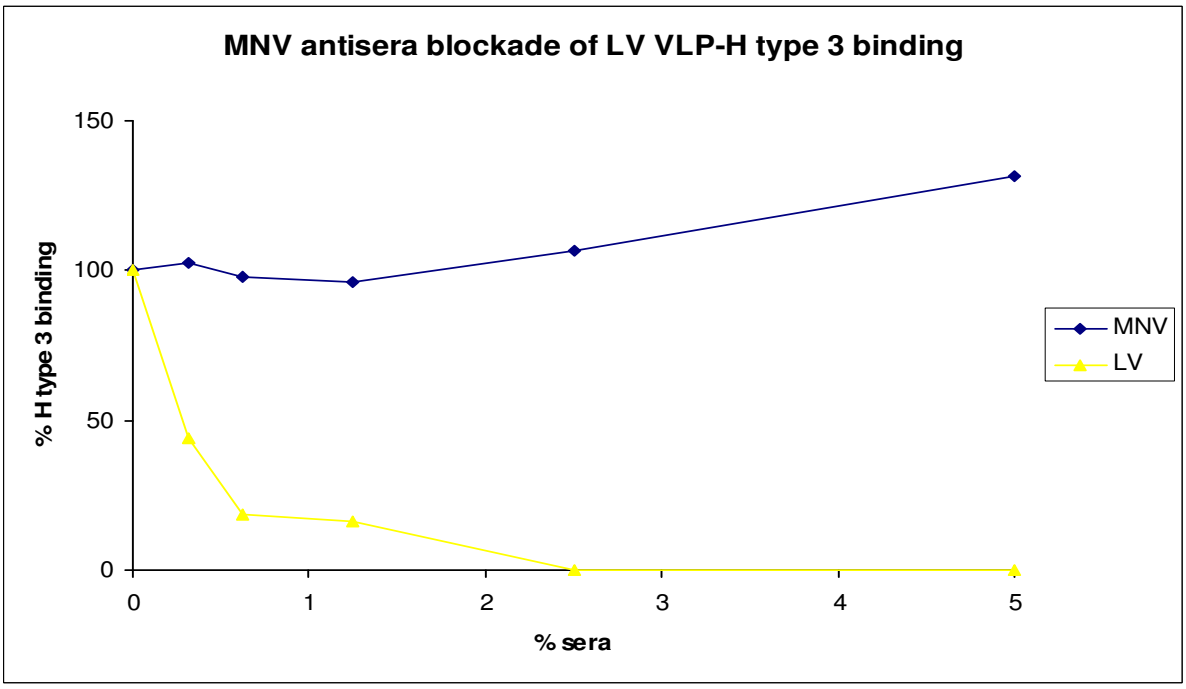
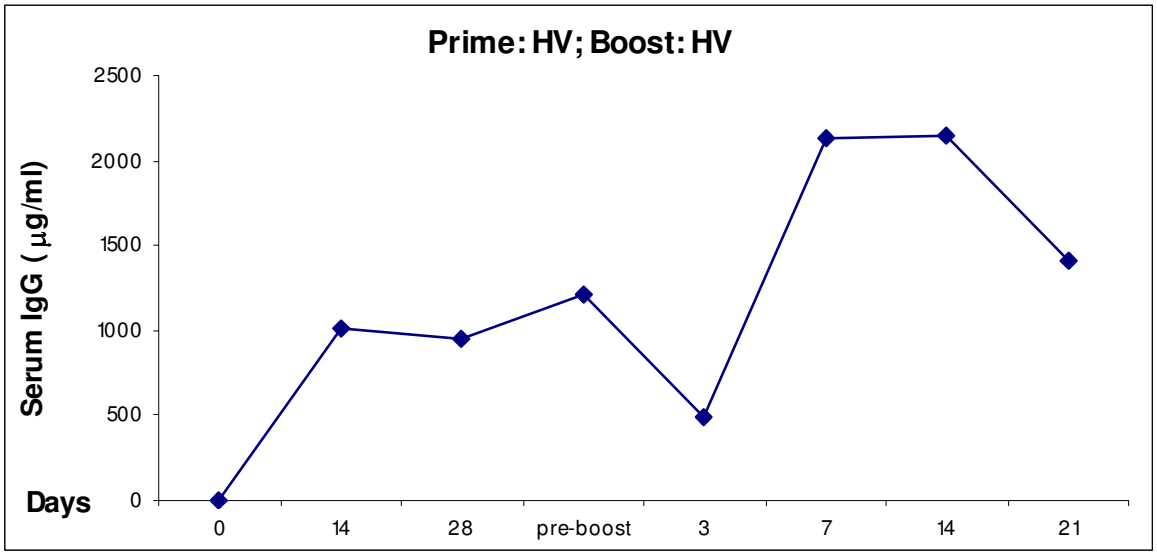


Mouse norovirus cross-reactive antibody response. Mice immunized with VRP-MNV (A) or our panel of human strain VRPs (B) were tested for cross-reactive serum IgG to our panel of human VLPs or MNV VLPs, respectively. MNV antisera were also tested for blockade of H type 3 binding to NV (C) or LV (D) VLPs.

Figure A1.3

A.



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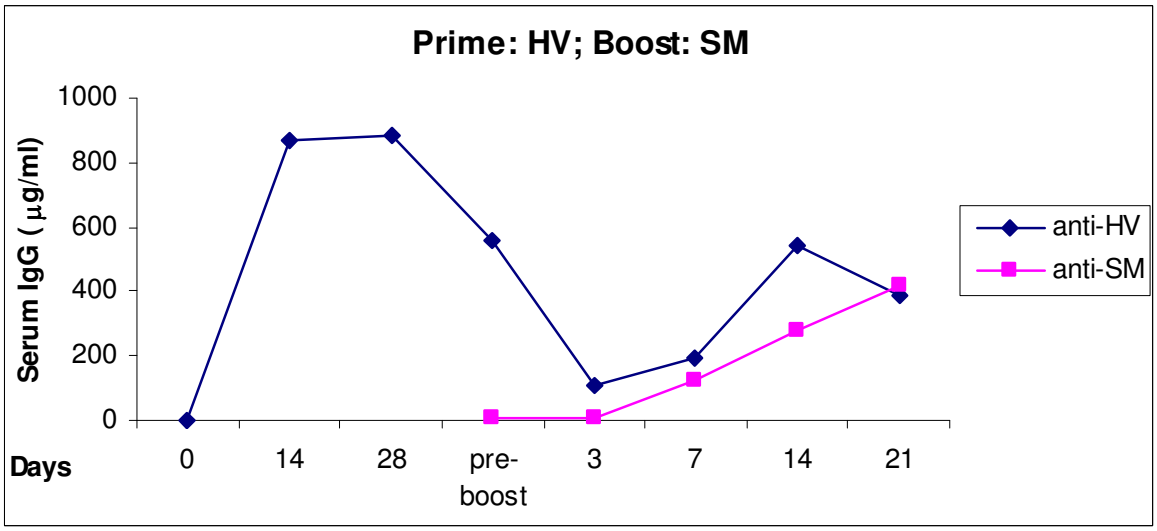

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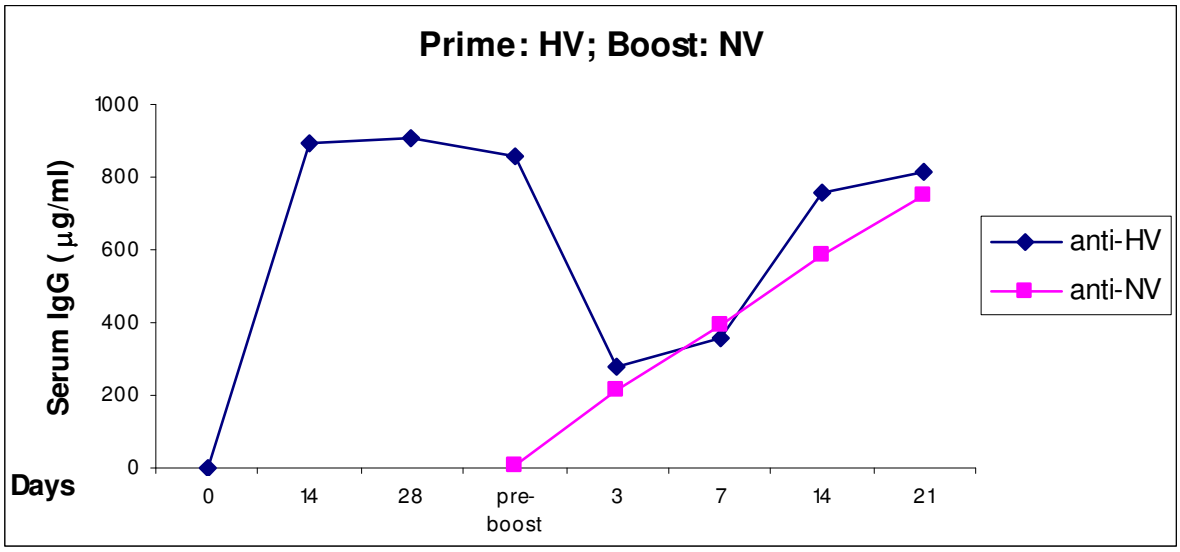
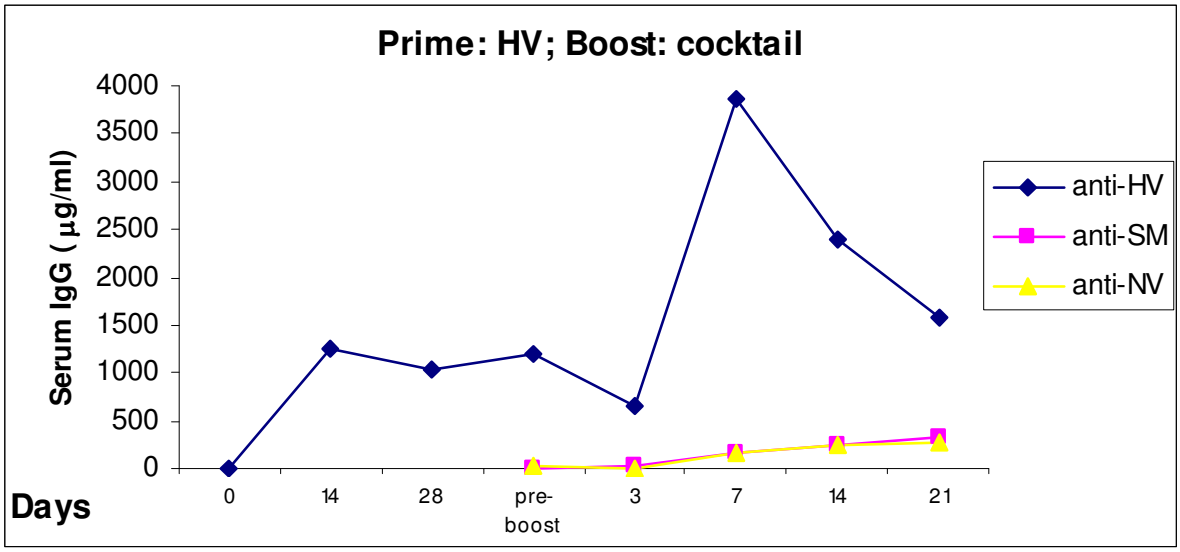


Heterologous prime-boost regimens elicit lower specific serum IgG to priming or boosting antigens than homologous prime-boost regimens. Mice were primed with HV VRP and boosted with HV VRP (A), SM VRP (B), or NV VRP (C) and antisera measured for reactivity to HV VLPs (A-C), SM VLPs (B), and/or NV VLPs (C) over a series of time points indicated on the x-axis.

Figure A1.4

A.



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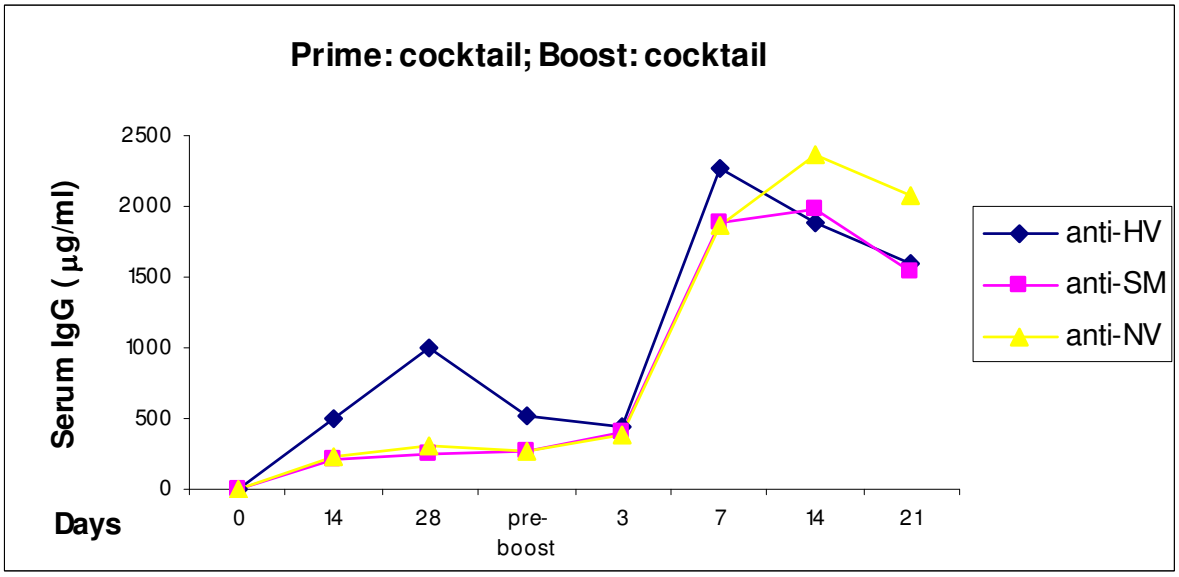

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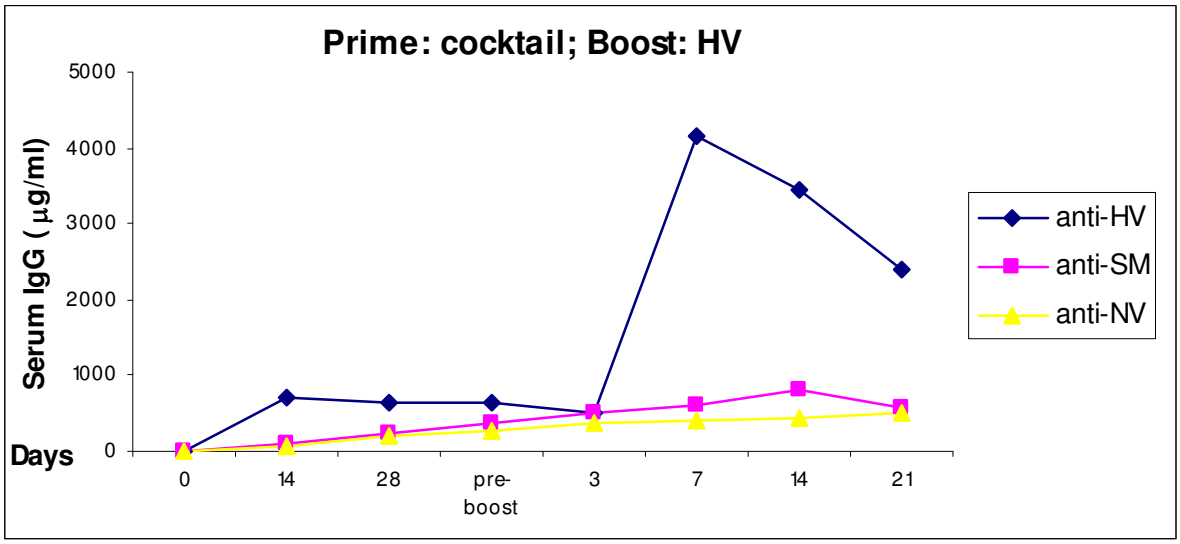
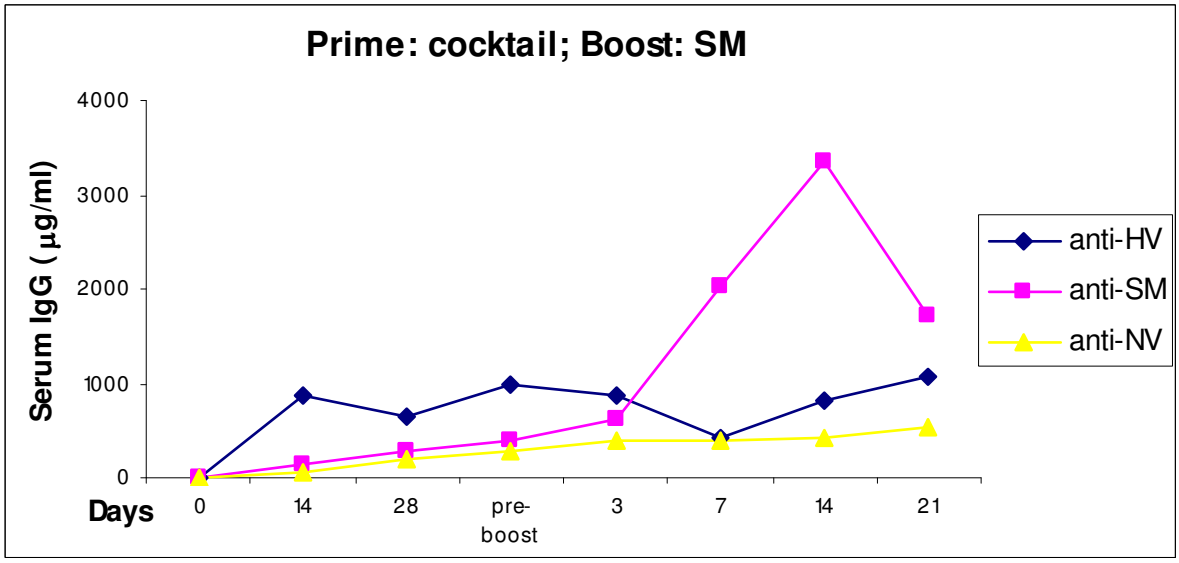
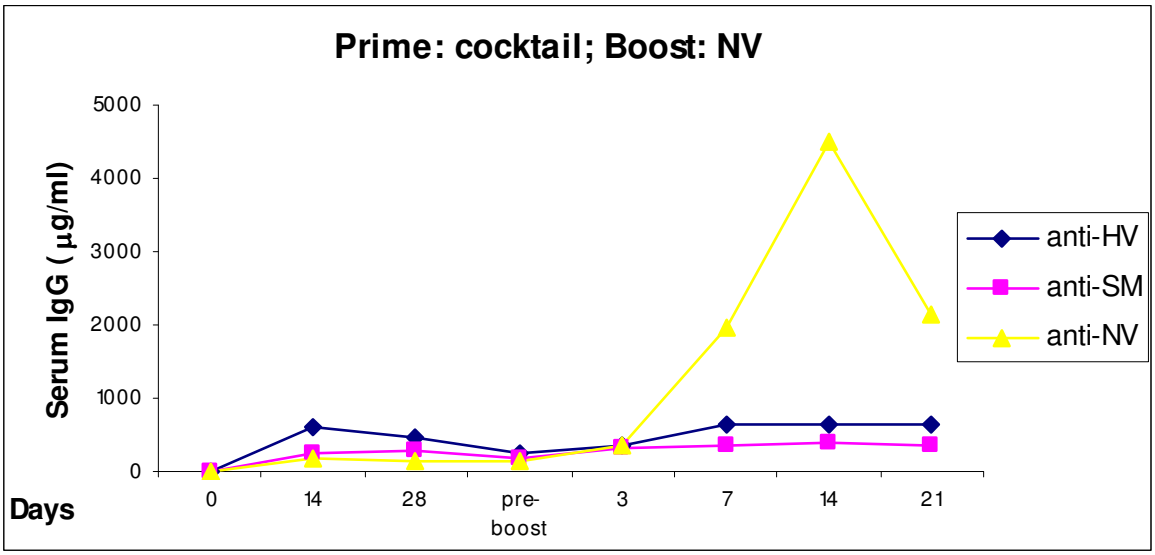

Prime: cocktail; Boost: HV
Serum IgG (µg/ml)
5000
4000
3000
2000
1000
0
Days
0
14
28
pre-boost
3
7
14
21
anti-HV
anti-SM
anti-NV

D.


Prime: cocktail; Boost: SM
Serum IgG (µg/ml)
4000
3000
2000
1000
0
Days
0
14
28
pre-boost
3
7
14
21
anti-HV
anti-SM
anti-NV

E.



Multivalent prime and/or boost regimens induce robust serum IgG responses to specific antigens in the vaccine composition. Mice were primed with HV VRP and boosted with multivalent VRPs (A), primed and boosted with multivalent VRPs (B), or primed with multivalent VRPs and boosted with HV VRP (C), SM VRP (D), or NV VRP (E). Antisera were tested for specific IgG reactivity to HV, SM, and/or NV VLPs.

APPENDIX 2
Chapter III.

Materials and methods.

G1.1 Westchester (WC) and GII.2 Ina and Buds capsid genes were cloned into VRP, VRPs were administered to mice to generate antisera, and VLPs were produced. Antibody blockade assays were performed as described in (149).

Results.

Heterotypic antibody responses to strains within norovirus genoclusters are robust but variable.

We cloned evolutionarily distinct capsid genes from the GI.1 WC strain in the Norwalk genocluster and the GII.2 Ina and Buds strains in the Snow Mountain genocluster into VRPs. Antisera generated in mice following VRP immunization were tested for cross-reactivity to our prototype NV and SM VLPs and vice versa. WC and NV antisera reacted strongly and equivalently to WC and NV VLPs, suggesting these strains are very closely related antigenically (Fig. A2.1a). Furthermore, NV and WC antisera both equally and completely blocked NV VLP and WC VLP binding to H type 3 (Fig. A2.1c-d). In contrast, antisera from the GII.2 strains had variable cross-reactivity to one another (Fig. A2.2). Antisera cross-reactive responses to Ina VLPs were equivalent to homotypic responses following SM or Buds immunization. However, Ina antisera did not cross-react nearly as well to heterologous VLPs. Furthermore, neither Ina or SM cross-reacted significantly to Buds VLPs. No receptor for SM VLP binding has been identified; therefore, antisera blockade assays were not done.

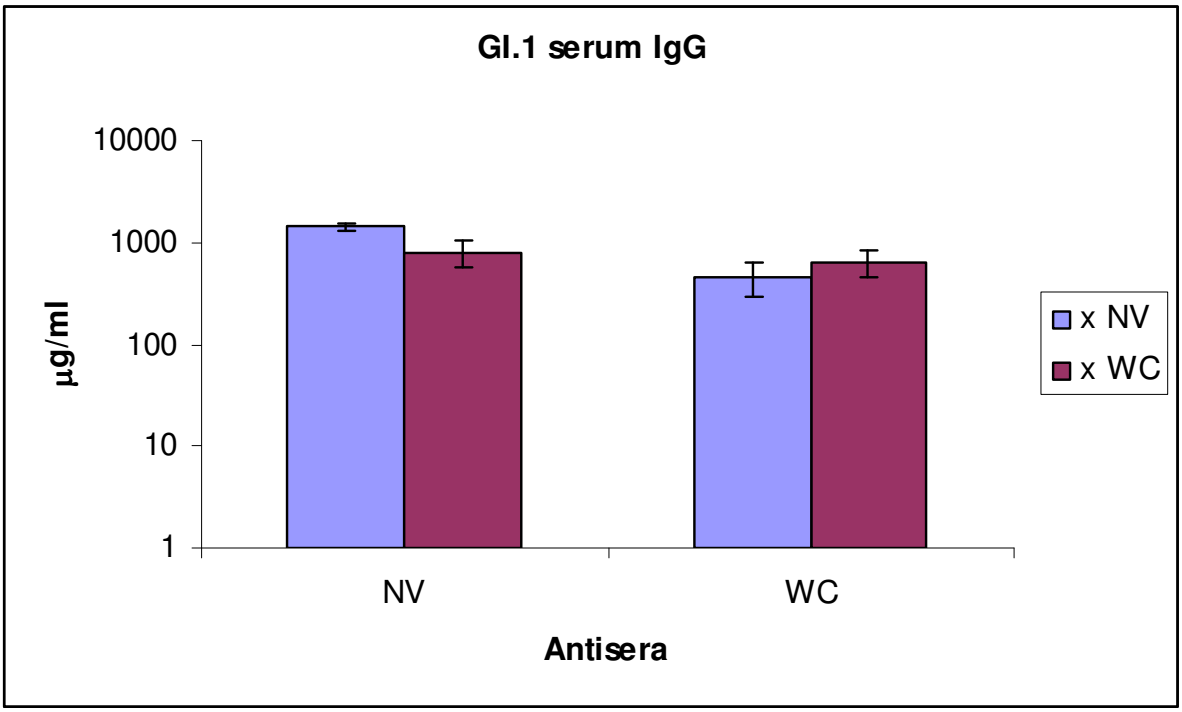
Discussion.

In Chapter III, we discussed the evolution of GII.4 norovirus strains over time. The question remained whether other GI and GII genoclusters exhibit similar evolutionary patterns. We tested the GI.1 strains NV and WC, isolated in 1968 and 2001, respectively, for antisera cross-reactivity and receptor blockade and discovered these antisera were not significantly different from one another in either category. Equivalent cross-reactivity and blockade to both strains indicate that the GI.1 genocluster did not evolve between isolation of each strain. As these evolutionary patterns were different than that observed among the time-ordered GII.4 strains, the GI.1 genocluster may be static or evolving very slowly. In contrast, the GII.2 strains exhibited differential cross-reactivity among strains. Antisera to SM, originally isolated in 1976, and to Ina and Buds, both isolated in 2002, all cross-reacted equivalently to Ina VLPs; however, neither heterologous strain cross-reacted well to Buds VLPs. In addition, Ina antisera did not cross-react to SM VLPs although SM antisera reacted to Ina VLPs. The cross-reactivity profiles of these antisera suggest that some strains within the GII.2 genocluster retain common antigenic sties over a long period of time while others have evolved rapidly. Like the GII.4 strains, some GII.2 strains appear to be more antigenically related than others, and antisera cross-reactivity to one VLP does not warrant reciprocal antisera cross-reactivity to the other VLP. This research supports our hypothesis that plasticity exists within the capsid sequence and antigenic drift may occur to avoid antibody recognition. Antibody blockade of receptor binding could not be tested as no receptor for GII.2 strains has been identified. Together, our data show that another GII genocluster may exhibit evolution over time, similar to the GII.4 cluster, while the GI genocluster tested did not change. These findings may explain the continued prevalence of GII outbreaks overall

compared to those reported for GI; however, additional studies with time-ordered VLPs from multiple GI and GII clusters must be conducted to confirm these patterns.

Figure A2.1

A.



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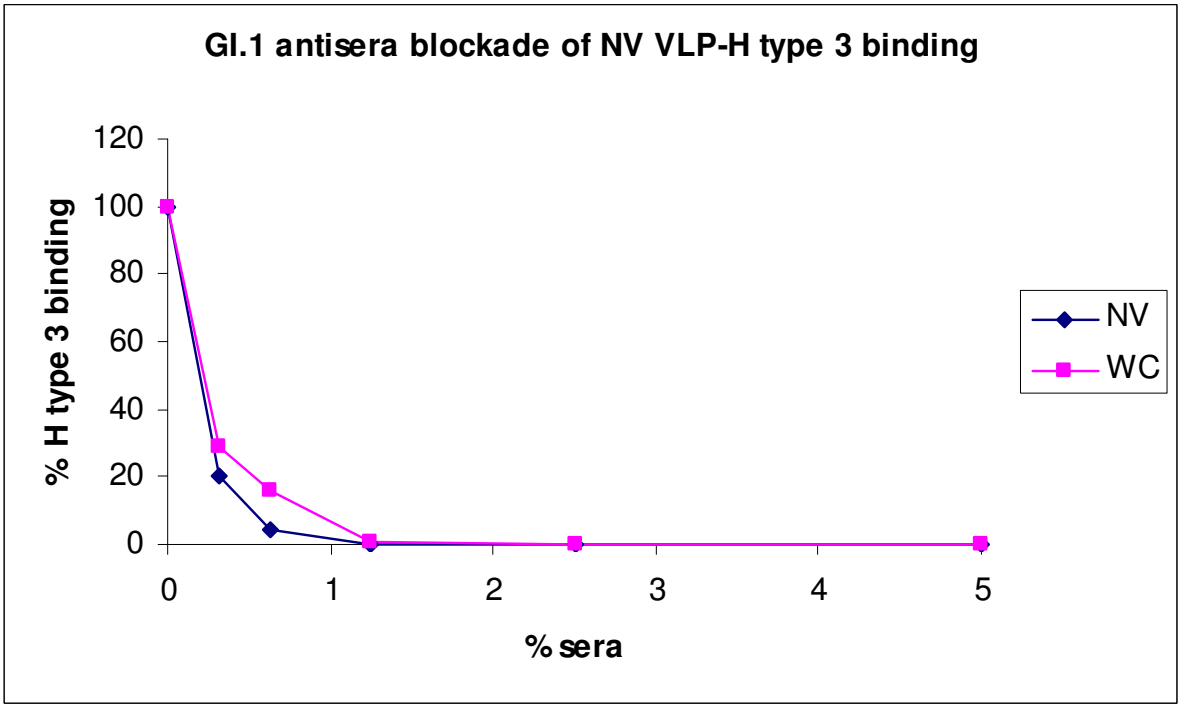
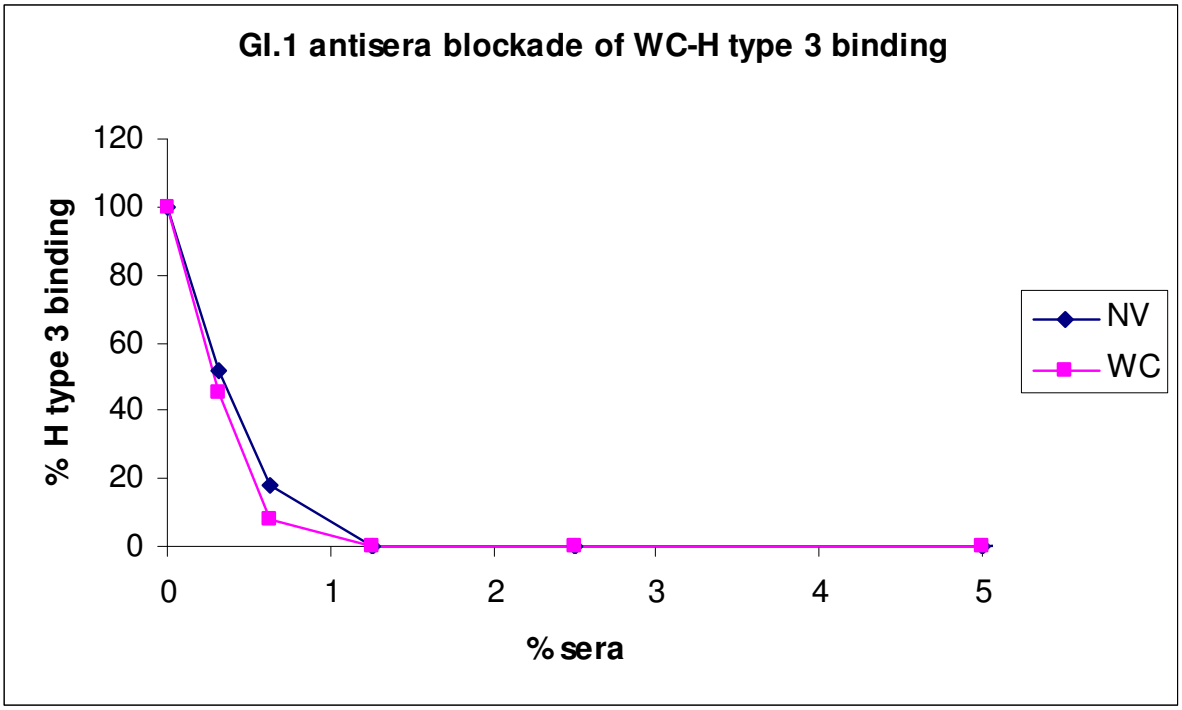


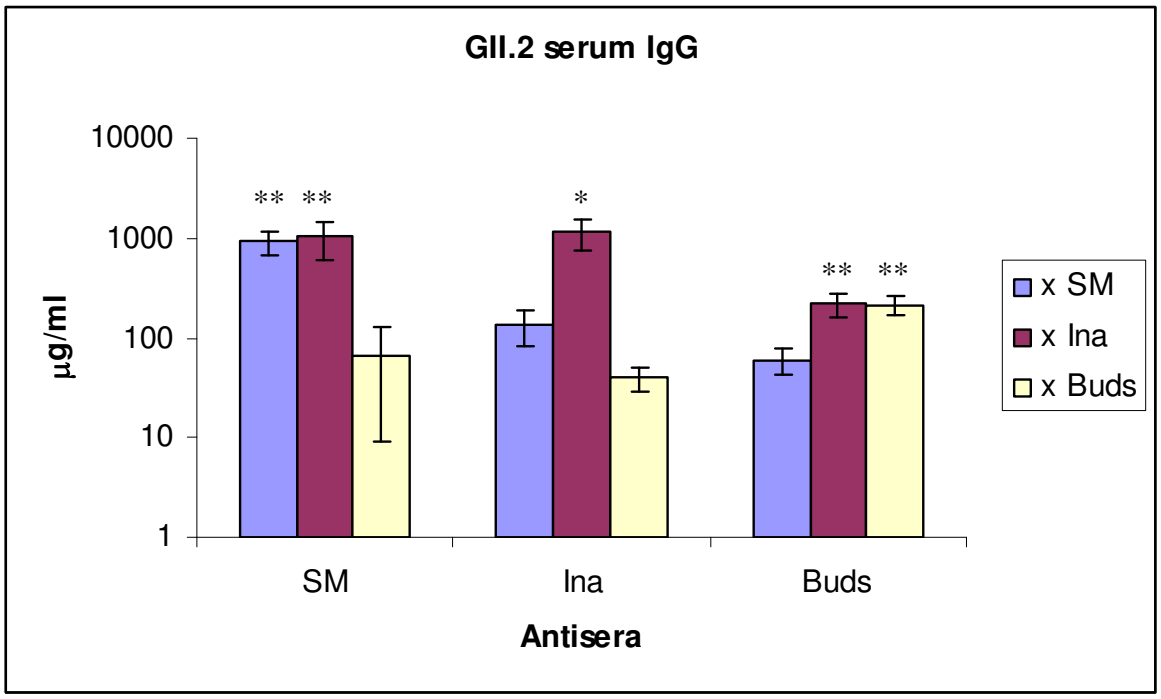
C.



Strains within the GI.1 genocluster are antigenically equivalent. Antisera from mice immunized with the NV and WC strains were analyzed for specific IgG cross-reactivity to NV and WC VLPs (A), blockade of NV-H type 3 binding (B), and blockade of WC-H type 3 binding (C).

Figure A2.2

A.



Strains within the GII.2 genocluster are antigenically distinct. Antisera from mice immunized with GII.2 strains SM, Ina, and Buds were tested for specific IgG cross-reactivity to SM, Ina, and Buds VLPs.

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