KIRSI TAMMINEN

Development of a Norovirus Capsid GII-4 and GI-3 Virus-like Particle-based Candidate Vaccine

ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

KIRSI TAMMINEN

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To my family,

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Abstract

Norovirus (NoVs) is the second most common cause of pediatric viral acute gastroenteritis (AGE) after rotavirus (RV) worldwide. After the introduction of live RV vaccines onto national immunization programs, the importance of NoV as a cause of viral AGE has increased. Genetically diverse NoVs fall into six genogroups (GI–GVI), of which GI and GII contain most of the genotypes causing the human disease. The leading genotype, GII-4, accounts for 55–85% of infections worldwide. Currently there is no vaccine against NoV despite the need for one, especially for young children and for specific target groups among adults and the elderly.

In the first stage of this dissertation, we determined NoV age-related seroprevalence in 0–14-year-old Finnish children to assess the serological background for NoV vaccine development. NoV GII-4-specific immunoglubulin (Ig) G and IgA were detected in all age-groups. Maternally acquired IgG antibodies declined soon after birth. By NoV-specific IgG determination, up to 47% of children aged 6–23 months had already encountered NoV. The seroprevalence as well as the levels of serum NoV-specific antibodies increased with age. These results indicate that NoVs are encountered at a very young age and consecutive NoV infections are probably common. Thus, the NoV vaccine should be administered early to prevent the majority of NoV gastroenteritis cases in children.

There has been no success in culturing human NoVs *in vitro*, hampering the development of a live virus-based NoV vaccine. Instead, NoV subviral particles, lacking the genome but representing the whole capsid (virus-like particles, VLPs) or parts of the capsid (P-particles), have been considered as antigenically competent alternatives to a live vaccine. We demonstrated in mice that NoV VLPs induced cross-reactive humoral and cellular immune responses, whereas P-particles elicited a type-specific antibody-mediated response, suggesting that for the induction of broad NoV immunity, vaccine development should be focused on VLPs.

The high number of NoV genotypes and the resulting pre-existing antibody variability among humans has been a challenge in NoV vaccine design. There is a concern that the pre-existing immunity might have a negative impact on the immune responses generated against novel circulating genotypes or vaccine antigens. We showed in mice that pre-existing immunity against one NoV genotype did not impair

induction of humoral response against the *de novo* genotype; therefore, no proof of "original antigenic sin" among NoVs was observed.

There is strong evidence that histo-blood group antigens (HBGAs), which are expressed on gut mucosal cells, are involved in the binding and/or entry process of NoVs. The blocking assay, a surrogate neutralization assay developed for NoV, measures antibodies that are able to block the binding of NoV VLPs to HBGAs. We observed that children with a high level of blocking antibodies in their sera were less susceptible to NoV infection. As blocking antibodies correlate with protection from NoV infection, the blocking assay can be used to determine a vaccine's potential to prevent the disease. We tested the capacity of NoV VLP and P-particle subunit vaccines to elicit blocking antibodies against various NoV VLP genotypes from both major genogroups (GI and GII). Both VLPs and P-particles induced serum antibodies in mice that blocked the immunogen-specific strain efficiently. However, only VLP immunization resulted in the development of cross-blocking activity in the sera, but it was in a genogroup-restricted manner. This observation suggests that a broadly effective vaccine should contain at least one VLP from each of the two main genogroups (GI and GII).

The final part of the dissertation focuses on assessing the NoV-specific immunogenicity of a trivalent combination vaccine candidate against NoV and RV in mice. The vaccine contains two NoV VLP genotypes derived from NoV GII-4 and GI-3 and RV VP6. High levels of type-specific and cross-reactive antibodies with broad blocking activity covering genogroups I and II were detected in immunized mice sera. Anti-NoV antibodies were also present in the gut of these animals. Furthermore, the combination vaccine activated the cellular arm of the immune system, which might be important for the induction of heterologous immunity. Both humoral and cell-mediated immune responses were long-lasting, and no interference between the vaccine components was observed. In conclusion, these results suggest that a combination vaccine might be sufficient to induce protective immune responses to a vast majority of the circulating NoVs.

Tiivistelmä

Norovirus (NoV) on rotaviruksen (RV) jälkeen toiseksi yleisin lasten virusperäisen akuutin gastroenteriitin (AGE) aiheuttaja maailmanlaajuisesti. Monissa maissa NoV on kuitenkin noussut tärkeimmäksi AGE:n aiheuttajaksi sen jälkeen, kun RV-rokotteet lisättiin kansallisiin rokotusohjelmiin. Norovirukset luokitellaan geneettisesti kuuteen eri genoryhmään (GI-GVI), joista genoryhmät GI ja GII sisältävät suurimman osan ihmisessä tautia aiheuttavista noroviruksista. Maailmanlaajuisesti yleisin genotyyppi, GII-4, on taudinaiheuttajana 55–85 %:ssa NoV-tautitapauksista. Norovirusta vastaan ei ole rokotetta, mutta tarve lasten, aikuisten erityisryhmien sekä vanhusten rokottamiseen on olemassa.

Tämän väitöskirjatyön ensimmäisessä vaiheessa selvitimme NoV-vasta-aineiden ikäkohtaista esiintyvyyttä 0–14 vuotiailla lapsilla, minkä ansiosta serologinen ympäristö voitiin ottaa huomioon NoV-rokotteen kehityksessä. Kaikissa ikäryhmissä esiintyi NoV-spesifisiä immunoglobuliini (Ig) G ja IgA vasta-aineita. Vastasyntyneillä vauvoilla havaittiin äidiltä peräisin olevia NoV IgG-vasta-aineita, mutta niiden määrä väheni nopeasti syntymän jälkeen. IgG-vasta-aineiden perusteella jopa 47 % 6–23 kuukauden ikäisistä lapsista oli saanut NoV-infektion, ja sekä vasta-aineiden esiintymisen yleisyys että niiden määrä veressä kasvoivat iän myötä. Nämä tulokset osoittavat, että norovirusta esiintyy yleisesti jo pienillä lapsilla ja että toistuvat NoV-infektiot ovat tavallisia. NoV-rokote tulisikin antaa varhaisella iällä, jotta suuri osa lasten NoV-gastroenteriiteistä pystyttäisiin estämään.

Norovirusta ei ole pystytty kasvattamaan laboratoriossa, mikä on estänyt elävän NoV-rokotteen kehittämisen. Elävän rokotteen sijaan NoV-rokotekandidaateiksi on ehdotettu norovirusperäisiä proteiinirakenteita, kuten viruksen kaltaisia partikkeleita (VLP:itä) ja P-partikkeleita. Kumpikaan rakenne ei sisällä NoV-genomia ja rakenteellisesti ne eroavat toisistaan siten, että VLP:t ilmentävät NoV-kapsidin rakenteen kokonaan ja P-partikkelit vain osittain, sisältäen kuitenkin toiminnallisesti tärkeät NoV-partikkelin osat. Toisessa osatyössä vertasimme näiden kahden rakenteen immunogeenisuutta hiirissä. VLP:t aktivoivat ristiin reagoivan vasta-ainevälitteisen ja soluvälitteisen immuunivasteen, kun taas P-partikkelit indusoivat tyyppispesifisiä vasta-aineita. Kokeen tulokset osoittavat, että laajakirjoisen NoV-rokotteen tulisi pohjautua VLP:ihin.

Norovirusgenotyyppien laaja kirjo ja NoV-vasta-aineiden yleisyys vaikeuttavat NoV-rokotteen kehittämistä. Olemassa olevasta NoV-immuunivasteesta huolimatta elimistön tulisi kyetä tehokkaasti muodostamaan vasta-aineita uusia, esimerkiksi rokotteen sisältämiä genotyyppejä vastaan. Kolmannessa osatyössä testasimme hiirillä, muodostuvatko vasta-aineet spesifisesti uutta NoV-genotyyppiä vastaan, kun hiiret oli aikaisemmin immunisoitu toisella genotyypillä. Olemassa oleva immuunivaste ei heikentänyt uuden vasta-ainevälitteisen vasteen syntymistä, joten norovirusten kohdalla ei löytynyt viitteitä ”original antigenic sin” -ilmiöstä.

On olemassa vahvoja todisteita siitä, että suoliston solujen pinnalla ilmentyvät kudosveriryhmäantigeenit (histo-blood group antigens, HGBA:t) toimivat noroviruksen tarttumispintana ja/tai tekijänä sisäänmenoprosessissa. NoV-vasta-aineiden tiedetään estävän NoV-VLP:iden sitoutumista HBGA-hiilihydraatteihin *in vitro*. Osoitimme, että sitoutumista estävillä (eli ”blokkaavilla”) seerumin vasta-aineilla oli yhteys tautisuojaan lapsilla. Tämän havainnon pohjalta pystyimme arvioimaan myös testirokotteiden potentiaalia estää NoV-infektio. Sekä VLP:illä että P-partikkeleilla immunisoitujen hiirien seerumi sisälsi tyyppispesifisiä blokkaavia vasta-aineita. Ainoastaan VLP:t indusoivat ristiin reagoivia blokkaavia vasta-aineita, mutta vaste oli genoryhmäkohtainen. Tämän havainnon perusteella laajakirjoiseen NoV-rokotteeseen tulisi sisällyttää ainakin yksi VLP-genotyyppi molemmista ihmisen päägenoryhmistä (GI ja GII).

Viimeisessä osatyössä keskityimme arvioimaan NoV-RV-yhdistelmärokotteen NoV-spesifistä immunogeenisuutta hiirissä. Yhdistelmärokote sisältää noroviruksen GII-4- ja GI-3-peräisiä VLP:itä sekä rotaviruksen VP6-proteiinin. Rokote synnytti vahvan ristiinreagoivan vasta-ainevälitteisen immuunivasteen, ja seerumin vasta-aineet estivät GI- sekä GII-ryhmän eri VLP:iden sitoutumista HBGA-hiilihydraatteihin. Lisäksi NoV-vasta-aineita muodostui hiirten suolistoon. Rokotteen todettiin myös aktivoivan soluvälitteisen puolustuksen T-soluja, mikä saattaa olla tärkeää ristiin reagoivan immuunivasteen kannalta. Immuunivasteet olivat pitkäkestoisia, ja rokotekomponenttien ei havaittu häiritsevän toisiaan vasteiden synnyssä. Näiden tulosten perusteella voidaan todeta, että NoV-RV-yhdistelmärokote on lupaava ehdokas laajakirjoinen NoV-rokotteeksi.

List of original communications

This dissertation is based on the following publications which are referred in the text by their respective Roman numerals (I–IV):

I Nurminen K, Blazevic V, Huhti L, Räsänen S, Koho T, Hytönen VP, Vesikari T. (2011). Prevalence of norovirus GII-4 antibodies in Finnish children. Journal of Medical Virology 83 (3), 525–31.

II Tamminen K, Huhti L, Koho T, Lappalainen S, Hytönen VP, Vesikari T, Blazevic V. (2012). A comparison of immunogenicity of norovirus GII-4 virus-like particles and P-particles. Immunology 135 (1), 89–99.

III Tamminen K, Huhti L, Vesikari T, Blazevic V. (2013). Pre-existing immunity to norovirus GII-4 virus-like particles does not impair *de novo* immune responses to norovirus GII-12 Genotype. Viral Immunol. 2013 Apr; 26(2):167–70.

IV Tamminen K, Lappalainen S, Huhti L, Vesikari T, Blazevic V. (2013). Trivalent combination vaccine induces broad heterologous immune responses to norovirus and rotavirus in mice. PLoS One. 2013; 8(7): e70409.

Abbreviations

aa	Amino acid
AEC	3-amino-9-ethylcarbazole
AGE	Acute gastroenteritis
AP	Alkaline phosphatase
APC	Antigen-presenting cell
ASC	Antibody-secreting cell
BCIP/NBT	Nitro-blue tetrazolium chloride/ 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
B_M	B-memory
bp	Base pair
BV	Baculovirus
cDNA	Complementary DNA
CM	Cell culture media
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
FBS	Fetal bovine serum
FCV	Feline calicivirus
fuc	Fucose
FUT	Fucosyltransferase
G(I–VI)	Genogroup (I–VI)
gal	Galactose
GalNac	N-acetyl-galactosamine
GII-4 NO	GII-4 New Orleans
GlcNac	N-acetyl-glucosamine
GMT	Geometric mean titer
gn	Gnotobiotic
H	Hinge
HBGA	Histo-blood group antigen
HBSS	Hanks Balanced Salt Solution

HRP	Horseradish peroxidase
huNoV	Human norovirus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IN	Intranasal
Mab	Monoclonal antibody
MHC	Major histocompatibility complex
MNV	Murine norovirus
MPL	Monophosphoryl lipid A
NoV	Norovirus
OAS	Original antigenic sin
OD	Optical density
OPD	*o*-phenylenediamine dihydrochoride
ORF	Open reading frame
P domain	Protruding domain
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
RdRp	RNA-dependent RNA polymerase
RT-PCR	Reverse transcriptase polymerase chain reaction
RF	Replicative form
RV	Rotavirus
S domain	Shell domain
SC	Subcutaneous
SFC	Spot-forming cells
ssRNA	Single-stranded RNA
STAT1	Signal transducer and activator of transcription 1
Th	T helper
T_M	T-memory
UTR	Untranslated region
VEE	Venezuelan equine encephalitis virus
VLP	Virus-like particle
VF	Virulence factor
VP	Viral protein
VRP	Virus replicon particle

1 Introduction

Noroviruses (NoVs) are small, round-structured, single stranded RNA (ssRNA) viruses belonging to the family *Caliciviridae*. To date, NoV is associated 50% of acute gastroenteritis (AGE) outbreaks (1) and 18% of endemic AGE globally (2). Annually, NoV gastroenteritis causes up to 200,000 deaths in children under 5 years of age, mainly in developing countries (3). NoVs are typical agents in food- and waterborne outbreaks, and they cause sporadic cases of AGE in children, especially in the winter months (4,5). Heavy vomiting, diarrhea and abdominal pain are common symptoms of NoV, which can last a few days and lead to severe dehydration (1).

NoV genotypes are divided into six genogroups (GI–GVI) (6,7) and there are over 30 genotypes infecting humans, most of them belonging in genogroups GI and GII (7). Majority of the human NoV infections are caused by viruses belonging in genogroup GII (8,9), and the predominating genotype is GII-4 (10). Genotype GII-4 develops new variants that cause worldwide pandemics every 2–3 years (10-12). The most active time for NoV GII-4 epidemics is the winter months (4,13,14), hence the name "winter vomiting disease".

The prototype NoV, (GI-1, "Norwalk-virus"), was first discovered in stool filtrate in 1972 (15). Since then, attempts to cultivate human NoVs (huNoVs) have remained unsuccessful (16-18), and therefore NoV virus-like particles (VLPs) have been used as a surrogate for the native virus to investigate the structure (19,20), immunology (21-25) and biochemical properties (26,27) of NoVs. NoV VLPs are typically produced by cloning the NoV capsid viral protein (VP) 1 gene into the baculovirus (BV) genome and expressing it in insect cells to yield capsid VP1 proteins, which self-assemble to form VLPs (28). The specific receptors for NoV are not fully known, but studies utilizing VLPs have shown that histo-blood group antigens (HBGAs) expressed on the host's mucosal cells are involved in the binding and/or entry process of NoVs (26,29,30). The genetic susceptibility of humans to NoV infection is determined by the functional fucosyltransferase *(FUT) 2* gene, which encodes an enzyme responsible for the expression of HBGAs on gut mucosa and bodily secretions (31).

Almost 100% of older children and the adult population is seropositive to NoV (32-34). In children, a high level of genotype-specific serum immunoglobulin (Ig) G has been shown to correlate with protection (35-37), but in adults high antibody levels alone do not provide protection (21,38,39). However, serum antibodies that can block NoV VLP binding to HBGAs correlate with protection both in children (37) and adults (39). In addition, T cell responses have been shown to be activated upon infection and might be important in the clearance of NoV (24,40), as demonstrated in mice infected with murine norovirus (MNV) (41,42).

Currently no vaccine exists for NoV gastroenteritis. Potential target groups for the NoV vaccine include young children, the elderly, and specific target groups such as military and healthcare professionals. In children, there is increasing interest in a NoV vaccine since rotavirus (RV) vaccinations have decreased the incidence of RV gastroenteritis, making NoV the leading agent for viral AGE in children (13,43-45). Efforts on NoV vaccine development have focused on NoV subunit particles; most of the development is based on VLPs (25,46-48) but P-particles displaying only the protruding (P) domains of the NoV capsid are also under consideration (49,50). NoV VLPs have been shown to be safe, immunogenic and well-tolerated in phase I clinical studies (51-53). Furthermore, phase I/II challenge studies have shown protection from homologous NoV challenge (46) and reduced the symptoms of NoV illness upon heterovariant challenge (48). These results are encouraging for the future development of a VLP-based NoV vaccine (54).

2 Review of the literature

2.1 Structure and classification

2.1.1 NoV genomic structure and capsid morphology

NoV genome is an approximately 7.7 kilobase long positive-sense ssRNA molecule (55). The genomic RNA is organized into three open reading frames (ORFs), the 5' end is capped by the VPg protein and the 3' end is polyadenylated (56) (Fig. 1A). Short untranslated regions (UTRs) at both ends of the genome form secondary RNA structures that regulate viral translation and replication (57,58). There are overlapping regions at the junction of ORF1/ORF2 (17–20 base pairs [bp]) and ORF2/ORF3 (1 bp); the polymerase shifts to an alternative reading frame at the overlap regions. ORF1 encodes a large polyprotein, which is cleaved by viral protease to six nonstructural proteins (p48, p41 nucleotide triphosphatase [NTPase], p22, VPg, protease, and RNA-dependent RNA polymerase [RdRp]) responsible for viral replication. ORF2 and ORF3 are translated from subgenomic RNA to form major VP1 and minor VP2 structural proteins. (56)

NoV capsid (modelled by using the prototype GI-1 VLP) is made of 180 copies of single structural protein with a mass of approximately 57 kDa, namely VP1 (19). VP1 proteins form dimers which are further organized in *T=3* icosahedral symmetry to compose a NoV capsid (Fig. 1B). VP1 proteins are formed from two domains, the shell (S) and the protruding (P) domain, which are linked by a short flexible hinge (H) (Fig. 1C). The N-terminal S domains form the core of the capsid's shell from which the P domains extend outward. The P domains are only involved in the dimeric contacts with another P domain within a VP1 dimer, while S domains are responsible for the icosahedral contacts with other VP1 dimers. P domains can be further divided into two subdomains, P1 and P2 (Fig. 1C). The residues comprising the P2 domain (amino acids [aa] 279–405) locate in the middle of the P1 sequence (aa 226–278 and aa 406–520) and fold into a barrel-like structure at the outermost part of the P domain. The P1 domains are supposed to stabilize the icosahedral form of the virion (19). An HBGA-binding pocket is enclosed in the structure of P2 (Fig.

1C) and is responsible for virus attachment to host mucosal cells (59,60). The amino acid sequence of the S domain is largely conserved among NoVs (60). P1 domains show moderate conservation, while the P2 sequence is highly variable (61,62). The minor structural protein VP2 (22–29 kDa) is not required for the assembly of a capsid, but might be involved in genome encapsidation (63). Furthermore, VP2 is supposed to enhance VP1 production and to stabilize and protect it from degradation (20,57).

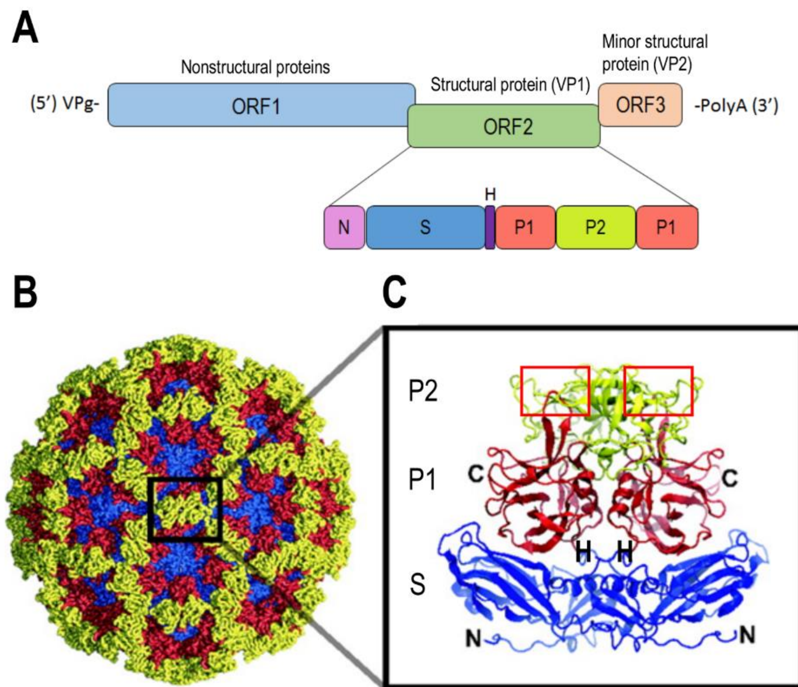

Figure 1. The genomic organization and structure of norovirus (NoV). The NoV genome is organized into three open reading frames (ORF 1–3), of which ORF2 encodes the capsid structural viral protein (VP) 1 (A). NoV VP1 proteins (n=180) are organized in *T = 3* icosahedral symmetry to form a capsid of NoV (B). The molecular structure of the VP1 dimer with carbohydrate binding sites (red rectangular boxes) is illustrated (C). The blue color represents the shell (S) domain, the red color represents the protruding domain (P) 1 and the bright green color represents the P2 domain. Abbreviations: N, N-terminus; C, C-terminus; H, hinge; VPg, viral protein g; PolyA, Poly A tail. Modified from Choi et al. (60) (B and C).

2.1.2 Classification and genotyping

NoVs are one of the five genera of small, spherical, non-enveloped ssRNA viruses in the family of *Caliciviridae* (64). In addition to NoVs, sapoviruses are infectious to both humans and animals, and the other three genera, vesiviruses, lagoviruses and neboviruses, are found solely in animals (64). NoVs are genetically divided into six genogroups, GI–GVI (6,7); the majority of the human NoVs belong to genogroups GI and GII, but a few are also classified in GIV, which mainly contains feline and canine NoVs (7). Moreover, GII contains some porcine-infecting NoVs, GIII NoVs are bovine-infecting, GV NoVs are murine-infecting and a recently discovered canine norovirus has been suggested to form the sixth genogroup, GVI (6). The genotypes can be further classified into variants (7,65) and recombinant viruses (66). Because of the error-prone nature of RNA-polymerase and the pressure of host herd immunity, new variants are developed through point mutations and by recombination, leading to great genetic diversity among NoVs (66,67).

NoVs can be detected from environmental and stool samples by a reverse transcriptase polymerase chain reaction (RT-PCR) (68-70) following molecular genotyping, which can be done by sequencing selected regions of the RNA genome, as reviewed by Stals et al. (71). NoVs have been previously genotyped based on either the polymerase (RdRp) (68,72) or the capsid (VP1) (73,74) regions. However, because of the recombinant viruses, the sheer RdRp or VP1 sequencing was found to be inadequate (75). Recombination is fairly common in NoVs and the hotspot for recombination is at the ORF1/ORF2 overlap (66). The recombination between two infecting NoVs results in a novel virus that has acquired the ORF1 (the polymerase encoding region) from one virus and ORF2–3 (the capsid encoding region) from another virus (66). Intragenotype recombinants share OFR1 and ORF2–3 sequences from two variants within a single genotype (76). Intergenotype recombinants are a combination of two different genotypes (76). There also exist "orphan genotypes" that have no corresponding VP1 sequence but are instead always detected in combination with other NoV genotype capsids (7,76). Orphan genotypes are termed with a letter instead of number (i.e. GII-e) to distinguish them from genotypes known to express both polymerase and capsid sequences.

The recently proposed NoV nomenclature and genotyping is based on the dual typing system which utilizes both polymerase (ORF1) and VP1 (ORF2) sequences (7). According to this system, there are at least nine GI and 22 GII human-infecting NoV capsid genotypes, and the polymerase sequences fall into at least 12 GII genotypes and seven orphan genotypes (7).

2.2 Infection and symptoms

2.2.1 NoV life cycle

The study of human NoV replication has been greatly impeded by the lack of cell culture (16-18), and thus there are gaps and uncertainties in the present knowledge of NoV life cycle. Valuable information has been received from the extensive study of other cultivable caliciviruses, such as MNV (77) and feline calicivirus (FCV) (78). In addition, cell-based studies with human NoV RNA, i.e. Norwalk-virus replicon system, has added to our understanding of the issue (79,80).

HuNoVs most likely use HBGAs as ligands to attach to the mucosal epithelial cells of the gastrointestinal tract (26). However, the actual entry mechanism and identity of other host membrane proteins involved in the process are currently unknown, as is the cell type that NoV uses for replication. MNV is known to be transcytosed across the intestinal epithelial cells, but it does not replicate in them (77,81). Instead, MNV replicates efficiently in the professional antigen-presenting cells (APCs), such as the dendritic cells (DCs) and macrophages, which has led to speculation that NoV possibly uses epithelial cells only for crossing the gut epithelial barrier and actually primarily infects the underlying immune cells (77,82). Recently, huNoVs were shown to infect B cells *in vitro* (83). Interestingly, the infection was promoted by the presence of HBGA-expressing enteric bacteria. This finding was not only important for the development of the cell culture system for huNoV; it also suggested that huNoV might "travel" along with enteric bacteria through the epithelial cell layer to reach the underlying permissive target cells (83).

Once NoV has entered the target cell, it releases its genomic positive-sense RNA molecule, which functions as messenger RNA, to start the initial rounds of translation of viral nonstructural and structural proteins by the host translation machinery. The VPg protein linked to the 5' end of the genomic RNA seems to be responsible for recruiting host proteins to form a translation initiation complex on the site (84). The untranslated regions (UTRs) of conserved RNA at both ends of the genome probably interact with host proteins to promote translation (57,58). The host machinery translates the large polyprotein encoded by ORF1, which is then cleaved by viral protease to release the viral nonstructural proteins. The nonstructural proteins and a few copies of the VP1 encoded in the initial translation step or from the invading virus capsid then gather to form a replication complex at the 3' end of the viral genomic RNA molecule (85). The viral polymerase (RdRp)

then initiates the transcription of viral RNA. At first RdRp synthesizes the negative-sense RNA strand by using positive-sense RNA as a template to generate a double-stranded replicative form (RF). From the RF, the positive sense genomic RNAs and the subgenomic RNAs comprising the structural genes are transcribed. The subgenomic RNAs serve as a template for viral structural protein synthesis. Replication is suggested to take place on host cellular membranes, like the endoplasmic reticulum, the Golgi apparatus and the endosomal membranes (77), which abolishes the host cell's own operations, such as the protein secretion pathway. (63)

Once there are enough VP1 proteins available, they are thought to self-assemble to form viral capsids similarly to the spontaneous formation of NoV VLPs (28) (Chapter 2.3.1). The encapsidation of the genomic RNA is supposed to occur through interactions of VP2 or VPg with RNA and capsid VP1 proteins (86,87). How newly assembled NoV virions leave the cell is mostly unknown, but apoptosis of the host cell is one of the suggested mechanisms for the release (88).

2.2.2 Transmission and symptoms of NoV gastroenteritis

NoV may be transmitted through contaminated food and water, which can lead to outbreaks in closed or semi-closed communities, such as schools, nurseries, hospitals, retirement centers, military barracks and cruise ships (1). In these settings, NoV is predominately transmitted by direct person-to-person contacts via aerosols and the vomit-oral and fecal-oral routes. In addition, sporadic cases of NoV can spread from person to person, which can eventually lead to the large pandemics that are commonly caused by the GII-4 genotype (12).

There are several reasons why NoV is highly infectious and easily transmitted. First, only a few (<10) NoV virions are needed for infection (89,90). Second, NoV is very stable and can very effectively resist disinfectants and different environmental conditions like extreme temperatures pressures and pHs (91,92). Moreover, NoV can remain virulent on surfaces from days to a few weeks (93) and in water even for months (94). Third, individuals infected with NoV can shed the virus in stools for several weeks after the acute infection and thus carry and spread it onwards (95,96). Fourth, as a typical RNA virus, NoV has a high mutation rate (97) that leads to rapid evolution of the strains, enabling escape from herd immunity and shifting between cellular ligand (HBGA) specificities (67,98,99). Finally, NoV infects and causes disease in persons of all ages (2,5).

The first symptoms of AGE typically occur about 24–48 h after the infection by NoV and last approximately 24–72 h (100). In persons with a weakened immune system, the symptoms can start more rapidly and last from weeks to years (101). During NoV illness, a person can experience one or more of the following symptoms: vomiting, diarrhea, nausea, abdominal pain, abdominal cramps and low-grade fever (1). NoV infection can also be asymptomatic (95,96). A prospective study in Tampere showed that 50% of children experienced NoV by the age of 2.5 years; most children were asymptomatic, but the infection was detected by seroconversion (36). A birth cohort study conducted in Peru showed that half of the NoV infections in children were asymptomatic during the first year of life (102). Usually, NoV illness is resolved without external care within days, but sometimes symptoms can lead to severe dehydration requiring hospitalization (103,104). Especially prone to severe illness are the elderly, the immunocompromised and small children; at its worst, NoV gastroenteritis can be fatal (103,105).

2.3 NoV subviral particles

2.3.1 Virus-like particles

In the early 1990s Jiang et al. (28) cloned the ORF2 and ORF3 sequences of NoV GI-1 in the BV genome to produce capsid proteins in insect cells; these self-assembled to form intact particles resembling morphologically and antigenically native NoV capsids. This invention led to a rapid expansion in the techniques for studying various aspects of NoV. GI-1 VLPs have served as prototype VLPs in these studies until the present times.

The three-dimensional structure of the icosahedral capsid of NoV was discovered through the use of VLPs as models in electron cryomicroscopy and computer image-processing techniques (106). Later, X-ray crystallography and mutational analyses made on VP1 completed the structural analyses and gave new, detailed information about the folding of VP1 and the domain contacts required for the assembly of the capsid (19,20). The VLPs were also a convenient material for the immunoassays that led to the development of various diagnostic assays (22,34,107).

The important discovery was that NoV VLPs bind to HBGAs, which indicated that these complex carbohydrates present on intestine mucosal cells may function as receptors or attachment factors for NoV (26,29,30). Furthermore, it was shown that

the antisera of NoV-infected humans or experimentally immunized mice blocked the binding of NoV VLPs to HGBAs (29). The monoclonal antibodies (Mabs) targeted at the P and S domains revealed that multiple sites in the P domain but not the S domain are responsible for the binding interactions between the VP1 protein and HBGAs (108,109). Based on these observations, a surrogate neutralization assay ("blocking assay") for NoV utilizing HBGAs and NoV VLPs has been developed and widely used today, e.g. in determining a vaccine's potential to prevent NoV infection (25,29,46,49,53).

The immunogenic properties of NoV VLPs were acknowledged early (110,111). As NoV VLPs are morphologically live virus surrogates, immunizations of animals and humans with NoV VLPs have given valuable information on which particular immune responses play a role in infection (41,112,113). Furthermore, the polyclonal and monoclonal antibodies raised in animals have been utilized to study the cross-reactivity of NoV genotypes (67,114,115) and to reveal the antigenically important sites of the NoV capsid (67,108,109). The ability of VLPs to bind HBGAs has been used to determine genotype specificity to certain HBGAs, and the binding patterns among NoVs (116-118).

Since the discovery of NoV VLPs, the vaccines based on this technology have been considered as alternatives for live virus-based vaccines, which remain at a standstill until the cell culture system for *in vitro* propagation of NoV is found. Preclinical studies in animals (25,111-113,119,120) and clinical phase I studies conducted in humans (40,52,110,121) using NoV VLPs have served as a base for vaccine development. Recently, the immunogenicity, safety and efficacy of two VLP-based vaccine candidates were evaluated in phase I/II clinical studies with encouraging results (46,48).

There are several recombinant expression systems developed to produce NoV VLPs. The most widely used strategy is the BV-insect cell system in which recombinant BVs carrying the NoV capsid gene are transfected in insect cells and the produced VLPs are harvested from sucrose or cesium chloride gradients (28,122-124). VLP production using the BV-expression system and sucrose gradient purification is straightforward and results in the expression of high-quality VLPs (123). Another strategy involves the use of transgenic plants, i.e. potatoes or tobaccos, in the production of NoV VLPs (111). The VLPs can be extracted and purified from plant tissue, or, alternatively, plant tissues containing VLPs could be used as an edible vaccine, which would lower costs since no expensive and time-consuming purification step would be required (125). Another approach utilizes the Venezuelan equine encephalitis virus- (VEE) based virus replicon particle (VRP)

expression system (126). In this system, the structural genes of the VEE genome have been replaced by the NoV VP1 gene, and dual transformation with these transcripts along with helper transcripts encoding VEE structural genes produces recombinant VRPs carrying the NoV gene. As the recombinant VRPs transfect the next round of cells, large amounts of NoV VP1 proteins that self-assemble into VLPs are produced. The advantage of this system is that production of VLPs occurs in mammalian cells and it enables *in vivo* vaccination with the recombinant VRPs. All of the production systems described above are able to generate VLPs that are highly stable and very resistant to various environmental factors such as temperature, pH and gamma irradiation (127,128).

2.3.2 P-particles

About a decade after the discovery of NoV VLPs, it was detected that expression of the P domain along with the hinge and without the S domain results in P domain proteins that form P-dimers able to bind HBGAs with low affinity (59,129). P-dimers tend to lose some of the HBGA binding sites when compared to the corresponding VLPs, which might affect the binding affinity (67). Later, it was shown that the expression of the P domain alone (without the H and S domains) results in the formation of about 20 nm subviral particles (130). These particles, named P-particles, are made of 24 P proteins that are organized in 12 dimers in $T=1$ icosahedral symmetry. The P-particles contain the HBGA binding sites and bind to HBGAs with severalfold stronger affinity than the corresponding P-dimers (130).

P-dimers and P-particles are usually produced in *E.coli* and genetically linked with an affinity tag (histidine), making the purification process easy (124,129,130). P-dimers and P-particles have been valuable study material in the structural analyses of the P2 domain and its involvement in carbohydrate interactions (60,131-134). The amino acids responsible for HBGA interactions in the binding interface have been resolved by co-crystallization studies of the P-dimer with the corresponding HBGA bound to it (60,131,133). Furthermore, P-particles have been used as a tool in seeking other possible ligand interactions for NoV attachment (135,136). P-particles are immunogenic in animals and induce antibodies able to block HBGA binding (49,50,137,138). P-particle-based vaccines against NoV and other viruses are under consideration (49,139,140). Small compounds can be genetically attached to the loops of P domains, which makes the P-particle a platform for antigen delivery, a feature which can be utilized in vaccine development or antibody production (49).

2.4 Carbohydrate binding and host genetic susceptibility

2.4.1 HBGA biosynthesis and expression

HBGAs are complex carbohydrates expressed on blood erythrocytes and mucosal epithelial cells, but they are also present as free compounds in secretions such as saliva and milk (141). The biosynthesis of HBGAs is controlled by several glycosyltransferases encoded by polymorphic gene families (e.g. the ABH blood group, Lewis and FUT families) and determine an individual's ABH, Lewis and secretor phenotype (142).

The biosynthesis of HBGAs in erythrocytes and on tissues such as mucosal ephitelia starts with disaccharide precursors. Of these, type 1–4 serve as a backbone for the majority of HBGA pathways, which are described below. Type 1 and type 2 (type 1/2) precursors are formed of galactose (gal) and N-acetyl-glucosamine (GlcNac) residues, and type 3/4 precursors are formed of gal and N-acetyl-galactosamine (GalNac) residues. The HBGA biosynthesis pathway in mucosal cells on type 1/2 precursors is illustrated in Figure 2. Glycosyltransferases catalyze the addition of sugar residues in a stepwise manner, resulting in the formation of HBGAs. In erythrocytes, the FUT1 enzyme is responsible for the initial step and adds a fucose (fuc) residue to a precursor to make an H antigen (O antigen), which can be further extended to A or B antigens by another set of glycosyltransferases, namely enzymes A and/or B, respectively. A similar synthesis of H antigen occurs in mucosal epithelial cells, but it is catalyzed by the FUT2 enzyme (Fig. 2, secretor pathway). H antigens serve as a backbone for more complex carbohydrates; FUT3 can act by adding a fuc residue to an H antigen to form a tetrasaccharide, namely Le^b (type 1 precursor) or Le^y (type 2 precursor). Also, other fucosyltransferases (FUT4–7 and FUT9) can catalyze the addition of a fuc residue. Enzymes A and B catalyze the addition of Gal-Nac or gal, respectively, to H antigens, resulting in the formation of A-type 1/2 or B-type 1/2. These carbohydrates can be further modified by FUT3, which adds a fuc residue, forming A $Le^{b/y}$ or B $Le^{b/y}$ pentasaccharides, respectively. FUT3 can also act directly on precursor carbohydrates (Fig. 2, nonsecretor pathway) by adding a fuc residue in a different location than FUT2, resulting in the formation of Lewis trisaccharides Le^a (type 1 precursor) and Le^x (type 2 precursor). The carbohydrate synthesized on type 1 or type 2 precursors can be also modified by additional acyl or sialyl groups catalyzed by another set of enzymes. Usually, type 1/2 precursors are used in HBGA biosynthesis in mucosal cells, but the type 3

precursor can also be extended to H-type 3, A-type 3 and B-type 3 antigens. Type 4 precursors are only used in erythrocytes. (142)

The enzymes of the biosynthesis pathway do not operate similarly in all humans, which affects the outcome of the composition of HBGAs expressed. Firstly, the activity of the enzymes described above varies from person to person, even among those of the same blood group. Secondly, mutation in both glycosyltransferase gene alleles inactivates the translation of a particular enzyme, thus affecting the possible HBGAs synthentized. The most well-known mutations involve the genes of the A and B enzymes that determine the ABH (ABO) blood group. Another mutation affects the FUT2 gene and prevents the formation of H antigens and all further structures (Fig 2, secretor pathway), being expressed on mucosal cells and secreted in body fluids. The antigens built on the H antigen core are called "secretor antigens" and the *FUT2* locus is called the "secretor locus". Persons with a functional *FUT2* gene are called "secretors" whereas individuals with a defective *FUT2* gene are called "nonsecretors". Caucasian and African nonsecretors (approx. 20% of the population) have the nonsense mutation in the *FUT2* locus and do not express ABH or $Le^{b/y}$ HBGAs on mucosal epithelial cells or in bodily fluids (143). Asian nonsecretors in turn have the missense mutation in the *FUT2* locus and express small amounts of the FUT2-encoded HBGAs on mucosal cells (143). However, most nonsecretors can express $Le^{a/x}$ (in the type 1/2 pathway) because of the action of FUT3 on disaccharide precursors. $Le^{a/x}$ carbohydrates are thus called "nonsecretor antigens". (142)

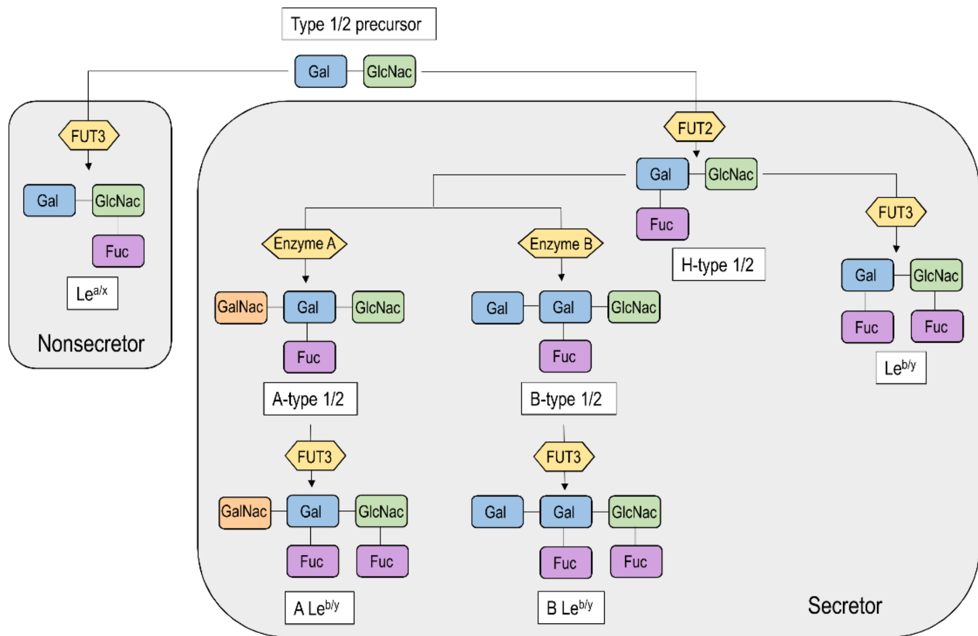

Figure 2. Biosynthesis pathway of histo-blood group antigens (HBGAs) in mucosal epithelial cells on type 1 and type 2 (type 1/2) precursors. The glycosyltransferase enzymes, namely fucosyltransferases 2 and 3 (FUT2 and FUT3), Enzyme A and Enzyme B, add carbohydrate residues on precursor carbohydrates in a stepwise manner to form ABH and Lewis (Le) HBGAs. The biosynthesis of secretor antigens, initiated by FUT2, is shown on the right-hand side of the picture and the biosynthesis of non-secretor antigens, catalyzed by FUT3, is shown on the left-hand side of the picture. The action of other glycosyltransferases have been left out for clarity. Abbreviations of the sugar residues: Gal, galactose; GlcNac, N-acetyl-glucosamine, Fuc, fucose; GalNac, N-acetyl-galactosamine. Modified from Tan and Jiang (144) and Shirato et al. (145).

2.4.2 Carbohydrate binding by NoV and host genetic susceptibility

Cell surface carbohydrates are known receptors for multiple pathogens (146-148). The first evidence of calicivirus binding to HBGAs was made when rabbit hemorrhagic disease virus was observed to bind to H-type 2 antigens (149). Shortly after that, Marionneau et al. (26) reported that GI-1 VLPs bind to the gastroduodenal epithelial cells of secretor individuals. More evidence on the role of HBGAs as binding ligands for NoV was found in *in vitro* VLP-binding assays that utilized synthetic or saliva-derived HBGAs (29,30,150). In addition, anti-NoV human or animal sera were found to block this interaction (29,151). The outbreak and challenge studies revealed that there might be a relationship between blood group

and susceptibility to NoV infection (152,153). This issue was defined to be related to the individual HBGA phenotype and especially to the secretor status determined by the expression of the FUT2 enzyme (31,154,155). Secretors became infected with GI-1 NoV in challenge studies, while nonsecretors seemed to be immune to the challenge virus (154). However, later nonsecretors were reported to be susceptible to infection with certain NoV genotypes, especially those from the GI genogroup (117,156,157). This can be explained by the ability of some NoV genotypes, such as GI-3, to bind Le^a and Le^x carbohydrates (117), which are expressed in the mucosal epithelia of nonsecretors with a functional FUT3 gene.

Numerous studies investigating HBGA-NoV VLP interaction have found a complex pattern of HBGA-binding specificities among NoV genotypes. It has been suggested that all NoV genotypes could be divided into specific binding groups based on the binding preference of the major H, A, B and Lewis terminal carbohydrates (30,158,159). However, some genotypes fit well into one binding group while others bind to several groups or show intermediate binding between the groups, thus complicating a strict division (159). Furthermore, the binding interfaces are known to be conserved within genogroups GI and GII but not between them (160). The GI and GII genotypes are known to bind HBGAs in different sterical positions, affecting the binding strength and possibly the dominance of GII genotypes (159). GI NoVs make fewer interactions with HBGAs and bind them in a more vertical position, whereas GII NoVs make more interactions with the horizontally positioned HBGAs. This indicates that GI and GII genotypes have evolved separately (159). As a consequence, the binding preferences of GI and GII genotypes might be the same, but the amino acids involved in the binding differ.

Taken together, binding preferences to HBGAs vary largely among genogroups, and genotypes across genogroups can have similar binding preferences (145). Some genotypes, like GII-4, can bind a large range of HBGAs, which seems to have a positive influence on the prevalence of the genotype (117,118,161). Understanding of HBGA-NoV interaction grew significantly after the crystal structures of the P domain-binding interfaces in complex with HBGA were resolved on the amino acid level (60,131,133). Several amino acids that extend outwards from the binding interface can be sterically divided in two or three regions that form hydrogen bonds and van der Waals interactions with the side chains of HBGAs. Small mutations in the amino acids involved in the interaction with HBGA or in vicinity of the binding site can change the binding preferences dramatically and eventually establish a new reservoir of individuals susceptible to that particular strain (162).

Although it is now highly plausible that NoV uses HBGAs as binding ligands on the mucosal surface, further studies are needed to investigate if other (co-)receptors are involved in the binding. For instance, it has been shown that GII genogroup NoVs bind efficiently to heparin sulfate proteoglycan (163), which is a known receptor for other viruses, bacteria and parasites (164). Recently, gangliosides were also reported to be possible ligands for NoV (136). What is the actual biochemical mechanism for NoV cell entry also remains unclear.

2.5 Epidemiology and evolution of the strains

2.5.1 Prevalence of NoV genotypes

Since the 1990s, GII-4 has been the predominant NoV genotype, and GII-4 variants have caused global gastroenteritis pandemics at intervals of a few years (10,12). There were seven pandemic GII-4 variants between 1995 and 2013: Grimsby (1995/96 US), Farmington Hills (2002), Hunter (2004), Yerseke (2006a), Den Haag (2006b), New Orleans (NO, 2009) and the most recent strain, Sydney (2012) (reviewed in (10)). Typically, a novel GII-4 variant replaces the previous variant and causes a peak in NoV-related illnesses (12,98), probably due to escape from herd immunity (67,99,161). This phenomenon was last observed with GII-4 Sydney (99), which emerged globally in 2012 and replaced the previous predominant GII-4 variant, GII-4 NO (165-168). GII-4 variants are associated both with outbreaks and sporadic NoV infections (8,169). They have a fast person-to-person transmission rate and cause a more severe form of disease than the other NoV genotypes (170,171). GII-4 strains account for 55–85% of all NoV gastroenteritis cases worldwide (10). Overall, GII-4 and other GII genotypes account for >95% of all NoV cases (8,169). Other prevalent GII genotypes (according to the ORF2 sequence) in recent years have been GII-1, GII-3, GII-6, GII-7 and GII-12 (8,172-174). Of the non-GII-4 genotypes, special interest in the 2009–2010 season was focused on the reemerging GII-12 genotype, with increasing prevalence reported all over the world; in the USA, the GII-12 genotype was responsible for 16% of all NoV infections (175).

NoV recombinants are commonly found, and recombination is one mechanism for NoV evolution, especially for the GII-4 genotype (66,161,176). In the GII-4 lineage, a common phenomenon is the recombination of a novel GII-4 variant with a former GII-4 variant, e.g. GII-4 Sydney acquired a polymerase region from GII-4

NO (177). GII-4 Sydney has also recombined with an orphan genotype GII-e, making this recombinant the current predominating NoV (167). Another prevalent orphan genotype is GII-g, which is known to recombine with GII-1 or GII-12 capsid genes (178,179). In children, GII-b/GII-3 has been the second most prevalent genotype in sporadic infections after GII-4 variants over the last decade (8). Moreover, GII7/GII6 has been a prevalent recombinant genotype in Finnish children (13,14).

GI genotypes contribute less to NoV-related illnesses than GII genotypes, but they are typical agents in environmental and waterborne outbreaks (169,180,181). Lately, the most prevalent GI genotypes (according to the ORF2 sequence) have been GI-3, GI-4, and GI-6 (172,182,183), of which GI-3 was responsible for 17% of all NoV-related outbreaks in the USA in 2013–2014 (183).

2.5.2 NoV outbreaks

NoV is responsible for 50% of all AGE outbreaks globally (1). According to the definition of the Centers of Disease Control and Prevention (CDC), a NoV outbreak occurs when two or more people develop NoV-related illness from a common source of exposure. The source of NoV can be food, drinking water, the environment or one infected person in a closed or semi-closed setting (169). In a global systematic review of NoV-related outbreaks 1993–2011 (169), GII NoVs were associated with 75% of outbreaks, GI genotypes were associated with 13% of outbreaks and mixed GI+GII genotypes were associated with 12% of outbreaks.

NoV outbreaks most commonly occur in healthcare facilities, probably because of the semi-closed nature of the facilities and the population's vulnerability to NoV infection (172,184). NoVs are introduced to these settings by incoming visitors, healthcare workers or contaminated food or water, but the main route of transmission is person-to-person (172). The outbreaks tend to be very persistent in these facilities because patients with weakened immune systems can develop chronic infection and shed the virus for a prolonged time (185). In developed countries, NoV-associated deaths occur most commonly in elderly people in long-term healthcare facilities (103). Among children, NoVs are involved in 47–96% of outbreaks that usually occur in the children's ward or day-care facilities (5).

NoV is the most common viral agent in foodborne outbreaks all over the world (186-188). Foodborne outbreaks are often local, occurring in settings where a group of people are exposed to contaminated food (189-191). Common food vehicles for

NoV are food products that are eaten uncooked like shellfish (oysters), ready-to-eat foods, leafy green vegetables, fruits and berries. NoV is usually transmitted to food products by infected food handlers, from NoV contaminated surfaces or from the fields e.g. from contaminated irrigation water (93,192,193). Outbreaks can also affect geographically distant places as certain food products commonly contaminated by NoV (e.g. frozen raspberries) are distributed internationally (194).

NoV is infectious at a very low dose and resistant to environmental conditions, making it a common agent in waterborne outbreaks (195-197). GI NoVs can withstand extreme conditions better than GII NoVs (180,198), making GI viruses more often associated with waterborne and environmental outbreaks than GII viruses (180,181,199). The vehicle for NoV can be fecally contaminated ground water, and surface, well, tap, sewage or recreational (swimming) water (181,200). Because of the diversity of NoVs in the human population, mixed GI and GII NoVs are also commonly detected in waterborne outbreaks (195,200). The largest waterborne AGE outbreak in Finland occurred in 2007 in the town of Nokia, when sewage water accidentally contaminated the drinking water system for two days (201). An estimated 8500 residents became ill and over 1200 sought medical care (201). Mixed pathogens were detected, but NoV (GII-4) was one of the main infectious agents causing AGE (195,201).

2.5.3 NoV sporadic infections

Episodes of sporadic NoV AGE especially affect children under 5 years of age, but they are detected – but less reported – in the adult population as well. Annually, NoV is estimated to cause approximately 1.5 million AGE episodes in children under 5 years of age requiring medical care in industrial countries, and results in 200,000 deaths among the same age-group in developing countries (3). NoV accounts for 15–36% of sporadic AGE in children and has been the second most prevalent viral agent in pediatric AGE after RV (1,5). In many high income countries, the proportion of NoV and RV cases has shifted in favor of NoV because RV vaccinations have reduced RV-related pediatric AGE remarkably (13,44,202,203). In Finland, before the national RV vaccinations started, NoV was found to cause about 20% of all AGE cases in children in the community (204,205). To date, NoV is the leading cause of AGE in Finnish children; in the years 2009–2011, NoV was detected in 35% of AGE cases seen in hospital and accounted for 37% of AGE hospitalizations (44). Children at risk for severe NoV illness are under 5 years of age

(3), and most the pediatric NoV infections are detected in 6–24-month age-group (44,104,206-208). Repeated symptomatic and asymptomatic infections with different NoV genotypes and GII-4 variants are common in small children (102,209).

A recent systematic review analysis assessed the role of endemic NoV in all age groups and revealed that NoV is responsible for 18% of AGE cases worldwide (2). Similar results were obtained from a large community cohort study conducted in the UK; NoV was responsible for three million of the 17 million (17.6%) AGE episodes among adults annually (210). The elderly account for most of the sporadic NoV infections in the adult population and they also have the highest NoV-associated death rate in industrialized countries (211,212).

Sporadic NoV infections follow the winter seasonality pattern, and the predominant NoV genotype causing sporadic infections in all age-groups is GII-4 (8,14,213,214).

2.5.4 Mechanisms of norovirus evolution

Virus evolution in general is based on the high mutational rate, which results in a large range of phenotypes. Phenotypes with increased epidemiological fitness are positively selected. A virus may gain fitness in several ways; e.g. by increasing its replication efficiency, infectivity or host range; by recombination; or by altering its surface epitopes allowing the virus to escape from herd immunity (215).

The general theory of "epochal evolution" is that periods of stasis (no drastic changes on the amino acid level) are followed by one or a few strategic changes favoring strain fitness (11,216). These events are suggested to drive the evolution of NoV (11,98,162,217) and other viruses, such as influenza (216). There are two main hypotheses on how alterations on the molecular level lead to the epochal evolution of NoVs. Firstly, mutations in the HBGA-binding domain may occasionally result in new HBGA-binding preferences, enabling NoVs to penetrate a previously non-susceptible population (referred as the receptor-switching theory) (162,218,219). Secondly, the antibody-driven antigenic drift in immunologically important epitopes may lead to escape from herd immunity (98,99,108).

The strategy of receptor switching has likely been the main mechanism for the evolution of the ancestral GII-4 strains (162,219). The emergence of novel GII-4 strains took place when the previous strain gained mutations in HBGA-binding epitopes that increased its host range (219). It has been suggested that the first pandemic GII-4 strain, Grimsby (1995/96), replaced the Camberwell (1987) strain

by gaining new HBGA-binding abilities (A and B antigens) in addition to the existing ones (H-type 3 and Le^y), thus expanding the reservoir of susceptible population. The next drastic change occurred when the Farmington Hills (2002a) strain emerged with additional Le^a and Le^x binding capabilities, enabling the GII-4 strain to spread into the nonsecretor population. However, the nonsecretor-binding capacity was lost in the next pandemic strain, GII-4 Hunter (2004) (219). Overall, HBGA-binding preferences and strengths are known to fluctuate to some extent between the sequential GII-4 strains, but in general the GII-4 genotype can widely recognize HBGAs from ABH- and Lewis-binding groups (99,118,219,220). The evolution of a wide-ranging HBGA-binding capacity has been proposed to be one reason for predominance of GII-4 over other genotypes with a more narrow binding range (161). GII-4 genotype also have greater mutational and evolutional rates than other less prevalent NoV genotypes (98).

The P2 domain, enclosing the HBGA-binding interface, is a target for the immune system as it lies in the outermost part of the NoV capsid and is responsible for host cell binding (19,26). Presumably, exposure to a certain NoV genotype induces neutralizing antibodies, preventing NoV attachment to the host cell (29,39,154). Neutralizing antibodies created against one specific NoV strain may not recognize the surface epitopes of the mutated strain, which allows the virus to escape from herd immunity and infect the same group of individuals repeatedly (162). Almost 100% of the adult and older child population is seropositive to NoV GII-4 (32-34,221) which has created high immunogenic pressure for this genotype. As contemporary GII-4 strains have already acquired a wide HBGA-binding range, the evolution is suggested to occur through herd immunity-driven antigenic drift in the immunologically important epitopes in the P2-domain (epitopes A–E; Chapter 2.6.3.1) (11,99,108,219). Since 2002, the pandemic GII-4 strains have been shown to be antigenically distinct (218). Furthermore, the sera collected from outbreaks caused by pandemic GII-4 strains reacted poorly with the next pandemic GII-4-derived VLPs in an HBGA-blocking assay (99,222). These observations give evidence that GII-4 lineage is evolving in response to host immunity.

Inter- and intragenotype recombination might also drive NoV evolution (176). As the recombination usually occurs in the ORF1/ORF2 region, a polymerase with a high replication efficacy might be incorporated into a capsid that is not recognized by host immune systems and result in a new strain with increased virulence (176). Interestingly, both GII-4 NO and GII-4 Sydney capsid genotypes circulated for a few years before they emerged as recombinant forms and caused pandemics, suggesting that recombination events might be important for NoV evolution (176).

2.6 Immune responses to NoV

2.6.1 Models of studying NoV-related immune responses

The lack of cell culture and the small animal model for huNoV have posed a challenge in characterizing the immune responses involved in NoV infection. Current knowledge of human immunity to natural NoV infection is mostly based on human volunteer samples collected from NoV outbreaks (22,151,223), epidemiological AGE studies (32,37) and virus challenge studies (24,38,224-226). In addition, as VLPs are an excellent virus surrogate, clinical trials conducted with VLPs give insight into human immune system activation with NoV (Chapter 2.7.2). However, in studies utilizing human samples, pre-existing immunity and genetic susceptibility to NoV must be taken into account, and the interpretation of the results can be complex (22,31,32). By contrast, immune responses in naïve animals like BALB/c mice against NoV subviral particles can be studied without concern that pre-existing immunity affects the study results (Chapter 2.7.1). Some larger animals – like gnotobiotic (gn) pigs (227) and calves (228), as well as some non-human primates (229,230) – are susceptible to huNoV, so these animals have been used as models to investigate NoV-related disease and immune responses. The gn pigs and calves develop mild diarrhea upon oral infection and the infection is short-lasting (2–3 days) (227,228). The wild-type chimpanzees can only be infected intravenously by huNoV, and they do not develop symptoms but instead shed the virus in feces for 2–6 weeks, similarly to humans (230). All of the larger animals mentioned above seroconvert upon infection with huNoV (227,228,230). The disadvantages in using large animals are the high costs and the ethical questions raised, especially concerning the use of non-human primates like chimpanzees (229).

Studies utilizing murine norovirus (MNV) have greatly added to our understanding of the immune mechanisms involved in NoV infection (41,42,77,231,232). MNV replicates efficiently in the mouse model; infection in wild-type mice is asymptomatic, but immunosuppressed mice (e.g. STAT1 and interferon (IFN)-αβγ receptor-deficient mice) develop systemic disease, which results in 100% mortality 4–9 days after infection (77,231). In addition, MNV can be grown in cell cultures (77,231). These advantages have given researchers the opportunity to study NoV infection with traditional methods *in vitro* and *in vivo* using an inexpensive and well-characterized mouse model. The neutralizing antibodies targeted on the MNV capsid have been partly identified (77,233), and genetically engineered mice have

been used to determine the innate and adaptive immune responses important in the control and clearance of MNV infection (41,77,231,232). Although the mouse immune system is not an exact representation of the human immune system, and MNV is not identical to HuNoV, these results can be applied in the study of huNoV infection, disease and pathogenicity.

2.6.2 Innate immunity

As NoV infection in immunocompetent humans is self-limiting and very short-lasting (24–48 h), the role of innate immunity in clearing the infection is probably very important, as it can respond to invading pathogens rapidly. The major agents against viral infections of innate immunity are type I IFNs (namely IFN-α and IFN-β) synthesized by most virally infected cells and by professional immune cells such as DCs. The secreted type I IFNs bind to IFN receptors (IFN-αβr) on neighboring cells, which initiates a signaling cascade leading to the expression of genes that play a role in direct antiviral defense and further activates many cell types of the innate and adaptive immune systems. Type II IFN, IFN-γ, is produced in the later stages of the infection only by professional immune cells such as natural killer (NK) cells, DCs, macrophages and T cells, and thus the action of IFN-γ contributes to both innate and adaptive immune responses (234,235).

The importance of type I and II IFNs in controlling NoV infection has been demonstrated *in vitro* (77,236,237) and *in vivo* in animal models (231,238,239). MNV replicates at higher levels in macrophages and DCs lacking IFN I/II receptors, IFN I receptors only or STAT1 (primary transcription factor in IFN pathway) than in wild-type cells (77). The pretreatment of wild-type murine cells with IFN I and II restrained the replication of MNV, which was associated with the blockage of viral nonstructural protein translation (237). A similar observation of an IFN's antiviral effect was made with HuNoV replicon-bearing cells; HuNoV replicons decreased replication activity in IFN-treated cells (236). Furthermore, depleting mice of either STAT1 or IFN I and II receptors resulted in a lethal MNV infection (231). The lack of individual IFN receptors (IFNαβr or IFNγr) did not cause mortality in mice, indicating that these receptors can compensate for each other, but the depletion of STAT1 always led to severe outcomes, underlining the importance of this particular molecule (231). Karst et al. also detected that in contrast to STAT1-deficient mice, recombination activating gene (RAG) deficient mice that lack mature B and T cells do not develop a lethal case of disease but are instead chronically infected, and the

virus RNA can be found in the intestine and also in peripheral organs (lungs, brain, spleen, liver) and blood, indicating the systemic spread of the virus (231).

In gn pigs, the level of intestinal IFN-α elevated significantly shortly after infection with HuNoV GII-4, indicating an innate immune response to the infection (240). Furthermore, IFN-α inoculation during GII-4 infection significantly reduced NoV infectivity and fecal shedding in gn pigs (239). These results suggest the important role of innate immunity in the control and clearance of NoV infection, but they do not understate the role of adaptive immunity in controlling the NoV disease and preventing sequential infections.

2.6.3 Adaptive immunity

2.6.3.1 Humoral immune responses to NoV infection

NoV infection induces systemic and mucosal antibodies

NoV infection induces typically a high serum IgG response (21,24,37,151,223,226) but IgA and IgM are also detected at lower levels (21,241-243). Serum anti-NoV IgM can be detected about 1–2 weeks after the infection (242-244) and the levels of IgM wane rapidly (21,243); therefore it can be used as a marker of recent infection (242). NoV-specific serum IgA and IgG develop on average within 2–3 weeks after the infection and are more long-lived. IgA wanes a few months after a primary infection, whereas IgG can persist for years (21,243). In adults and older children, extremely high serum IgG levels to NoV are detected even without recent infection (24,32,37). These are thought to be long-lived IgGs circulating in the blood after serial NoV infections during the lifetime. A four-fold rise in the IgG titer between acute and convalescent sera is considered to be a marker of an infection with NoV (21,38).

A method to distinguish primary from recurrent viral infections involves the avidity of antibodies to the antigen. Activated B cells undergo somatic hypermutation which, with the aid of T helper (Th2) -cells, leads to the selection of B cell clones, which have an increased affinity against their cognate antigen (246,247). Affinity maturation occurs after each infection with the same or similar epitopes bearing pathogen; thus, repeated infections define the binding sites of the secreted antibody and B cell receptors, which increases the affinity of binding significantly. After primary infection, the avidity of the virus-specific antibody is generally low and it rises after subsequent infections (248,249). Thus, antibody avidity can be used as

a tool in the seroepidemiology of some viral infections (249-251). For NoV however, repeated NoV infections have already fixed the avidity of antibodies at a high level, and the elevation of IgG might be hard to detect, at least in adults (151).

Anti-NoV IgG and IgA have also been detected in the saliva of the NoV-infected persons (31,226,245) and a saliva-based assay has also been suggested as a diagnostic tool in NoV-outbreak studies (245). NoV IgA and IgG antibodies have been detected from human stools (209). The formation of an intestinal humoral response to NoV is supported by animal studies; in gn pigs and calves, intestinal NoV IgG-, IgA- and IgM-secreting B cells and fecal NoV-specific antibodies were found after infection with NoV GII-4 (228,240).

NoV antibodies are cross-reactive

NoV antibodies are highly cross-reactive, which is demonstrated with extensive studies both in animals (25,42,119,122,252,253) and humans (24,37,119,151,223,226,241). A much higher level of antibody cross-reactivity is detected against genotypes within the same genogroup than between the genogroups GI and GII. The cross-reactivity of NoV-induced antibodies is associated with serum IgG, whereas salivary anti-NoV IgA has been shown to be only reactive with the infecting strain (226). Some genotype variants can be antigenically very different, as described for the GII-4 lineage (218,254) while others, such as GII-3 genotypes, show great intragenotype cross-reactivity (255). Interestingly, some NoV-derived VLPs are more efficient in raising inter- and intragenotype cross-reactive antibodies than others (42,122,254).

The cross-reactive antibody epitopes on the NoV capsid have been characterized, for instance, using Mabs raised against NoV VLPs in animals (256-259). Although the Mabs are usually produced for diagnostic purposes (258,259), they also provide important information on NoV cross-reactivity. Most of the intragenogroup cross-reactive antibody epitopes are conformational and locate in the P domain. The cross-reactivity increases when moving from the variable P2 domain to the moderately conserved P1 domain (109,257,260,261). Intergenogroup cross-reactive epitopes reside mostly in the highly conserved S domain (259,262,263).

Serum anti-NoV IgG has a neutralizing role

Neutralizing antibodies have been described for other caliciviruses, such as FCV (264), canine calicivirus (265) and MNV (77,233). As the traditional neutralization

assay is not available for huNoV, potential neutralizing antibodies are called "blocking antibodies", as they are shown to block NoV VLP binding to HBGAs *in vitro* (29). Based on this observation, a blocking assay, a surrogate for a neutralization assay, has been developed for NoV, and the source of HBGAs can be human saliva, synthetic HBGAs or porcine gastric mucin (29,39,260).

In mice, neutralizing antibodies against MNV have been shown to bind to conformational HBGA epitopes in the P2 domain of the MNV capsid (266), and these epitopes are also likely the targets for neutralizing antibodies in humans. Although cross-reactive antibodies can be detected at high levels between NoV genotypes and moderately between genogroups, the cross-genotype-blocking activity of these antibodies is limited (119). The cross-blocking activity of NoV-immune serum seems to be highly genogroup-restricted (24,119,151,267). NoV immune sera or Mabs have not shown cross-blocking activity over genogroups (24,119,253).

Lindesmith et al. (24) detected that GI-1-challenged individuals all had unique prechallenge blockade profiles, but after the challenge, the blocking antibody titers were increased not only to the homologous virus but also to heterologous GI-genogroup VLPs. These results suggested that potential cross-neutralization epitopes among GI-genotypes are broadly shared. GII-genotypes are known to be an antigenically more heterogenic group than GI-genotypes, and a few studies have shown weaker GII intragenogroup cross-blocking activity than that detected for GI genotypes (119,151).

Five hypervariable conformational epitopes consisting of 2–6 amino acids (epitopes A–E) on the P2 domain of GII-4 have been predicted to be important blocking antibody epitopes (67,131,217). They are located in close proximity to the HBGA-binding sites and are hot spots for amino acid variation between GII-4 variants (67,131,217). These regions can either be directly involved in the interactions with HBGAs or inhibit carbohydrate binding when antibody is bound (67). By exchanging the blocking epitopes between time-ordered GII-4 VLPs, it has been detected that just one of these epitopes can alter the blocking antibody formation significantly (67,253,268). At least one Mab for the GII-4 lineage has been identified, which was able to cross-block a panel of time-ordered GII-4 VLPs (derived from GII-4 variants from 1987-2012) and the epitope that it binds is known as epitope F (67,269). Other genotypes (such as GII-3 and GII-7) share some blocking epitopes with GII-4 indicating that some of these important epitopes might be conserved among GII genogroup (98).

The role of humoral responses in the control of and protection from NoV infection

The role of anti-NoV antibodies in protection from infection has been difficult to determine due to conflicting reports from studies investigating human serum samples pre- and post-NoV infection. Firstly, the level of serum antibody IgG has been shown to correlate with protection in children (35-37), but not in adults (21,24,38,224,226). Secondly, in challenge studies, some symptomatic volunteers who developed high antibody responses after the challenge were infected upon rechallenge, while others who did not develop antibody responses remained asymptomatic even after high doses of challenge virus (38,224). Currently, we know that susceptibility to infection is partly determined by the genes that express the HGBA ligands on the mucosa (31), which was likely the reason why some of the volunteers were not infected by NoV in the early challenge studies.

Even though there are still many uncertainties regarding immunity against NoV, some correlates of protection have been defined (31,37,39). Lindesmith et al. (31) showed that after a GI-1 challenge, an early rise (<5 days after challenge) in salivary IgA correlated with resistance to the infection. They suggested that the volunteers remained uninfected because of a memory immune response to the virus (31). However, recently it was reported that intestinal IgA and IgG antibodies did not provide protection from heterologous GII infection (209).

Simply having a high serum IgG titer against NoV does not seem to be protective; blocking assays have revealed that high levels of pre-existing anti-NoV antibodies failed to prevent the infection if these antibodies did not have a blocking ability against the infecting NoV genotype (24,37,119). The explanation for this is suggested to lie in the antigenically different blocking epitopes: The previous infecting NoV genotypes have induced cross-reactive antibodies, but not cross-blocking antibodies against the novel infecting strain. Furthermore, it was noted that most infected individuals who did not show the blocking activity in the acute serum generated one in the convalescent serum (24,37,119). A few years ago, Reeck et al. (39) showed that serum-blocking antibodies correlated with protection from clinical NoV infection in adult challenge volunteers (39), and the correlation has also been shown in children by our laboratory (37). The patterns of blocking antibody responses in humans are probably very complicated due to the high number of infections during the lifetime and the variability of exposures to different NoV genotypes.

Although the induction of humoral immune responses upon NoV infection has been extensively described, the actual mechanism of NoV-specific humoral immune responses at the cellular level is still largely unknown. Studies in MNV infected mice

have revealed some important insights into the issue. Chachu et al. (232) have demonstrated that mice lacking functional B cells failed to clear MNV infection from the mesenteric lymph nodes, and antiviral IgG was essential in clearing the infection from mucosal sites. Another study supported these findings but also demonstrated that the transfer of serum from MNV-3-genotype infected mice mediated protection not only from homotypic but partially also from heterotypic MNV-1 infection in the recipient mice (42). These studies demonstrate that B cells and antibodies play an important role in clearing a NoV infection and that cross-blocking antibodies can confer some level of protection from heterotypic infection.

2.6.3.2 Cell-mediated responses to NoV infection

NoV infection stimulates Th1-type cytokines and potentially elicits memory T cell responses

Cell-mediated immunity in NoV infection is an understudied area of NoV research. Studies conducted by Lindesmith et al. have assessed human cellular immune responses pre- and post-challenge with either GII-2 (226) or GI-1 (24) genotypes. In both studies, human peripheral blood mononuclear cells (PBMCs) were stimulated pre- and post-challenge with NoV-derived VLPs, and the cytokines produced by these cells were analyzed. In a GII-2 challenge study, serum cytokine levels were also evaluated (226). Significant elevations of serum Th1 cytokines IFN-γ and interleukin (IL)-2 against the infecting virus were detected in the post GII-2 challenge sera two days after infection. The early rise in the serum Th1 cytokine levels could reflect pre-existing T memory- (T_M) cell response activation upon the challenge. Furthermore, it was detected that both infected and uninfected volunteers' pre-challenge PBMCs were activated when stimulated with NoV VLPs, indicating the presence of an anti-NoV T_M response. Upon stimulation with homologous GII-2 VLPs, the post-challenge PBMCs secreted significant levels of Th1 cytokines IFN-γ and IL-2 and low levels of Th2 cytokine IL5. The authors identified CD4+ T cells as a primary immune cells responsible for IFN-γ production. The cross-reactive T-cell responses were investigated by stimulating PBMCs with a heterologous GII genotype, GII-1 VLPs, and a significant rise in the levels of secreted Th1 cytokines (IFN-γ and IL-2), but no Th2 cytokines were detected. However, the cross-reactive T cell response was not detected across the genogroup (against GI-1 VLPs). (226)

In the GI-1 challenge study, only IFN-γ levels were measured upon PBMC stimulation against a panel of GI VLPs (24). A subgroup of infected individuals (six out of ten) mounted an IFN-γ response either to the infecting strain or to

heterologous GI VLPs, suggesting that T cell epitopes are likely conserved among the GI genogroup. Each of the volunteers showed an individual pattern in producing homologous and cross-reactive T cell responses. The authors suggested that the complex T_M responses induced by prior NoV exposures exist and might affect the immune responses elicited against the challenge virus (24). Taken together, the results from the human challenge studies (24,226) indicate that a predominately Th1 type T cell response is activated upon NoV infection and that T cell responses show cross-reactivity inside genogroups. Also, evidence from T_M-response formation was provided.

Mouse-specific CD4+ T-cell epitopes for huNoV have been identified utilizing peptide libraries spanning across a capsid VP1 protein derived from GII-4 and GI-1 genotypes (270). A T cell epitope for GII-4 was identified in the P1 domain (aa 461–475) and it was cross-reactive among other GII-genotypes, while the GI-1 epitope was located in the S domain (aa 81-95).

The role of T cell responses in preventing, controlling and clearing NoV infection

The human challenge studies have described T cell activation, but detailed information on the mechanisms behind T cell immunity in NoV infection is still lacking. Instead, studies using an MNV-mouse model have given insight into the active and protective role of T cells in MNV infection (41,42,271). Chachu et al. (41) reported that both CD4+ and CD8+ T cells are essential in the efficient clearance of MNV infection from the intestine and intestinal lymph nodes. The depletion of CD4+ and CD8+ T cells individually led to significantly increased MNV replication, and the depletion of both T cell subgroups increased the MNV titers even more compared to either CD4+ or CD8+ cell depleted mice alone (41). Moreover, the transfer of immune CD4+ or CD8+ cells independently to chronically infected RAG-deficient mice reduced the viral titers significantly (41,271). It was detected that CD4+ cells were important in the control of acute infection in the intestine, whereas CD8+ cells acted at a later time point in clearing the virus from the intestine and intestinal lymph nodes, therefore both T cell subtypes were needed for controlling MNV infection (41). Recently, somewhat controversial results on the role of CD4+ and CD8+ cells in MNV infection have been shown (42). The authors detected that only CD4+ cells along with B cells are the primary mediators in both controlling the infection and providing protection from the infection (42). The results from the MNV studies indicate that all aspects of adaptive immunity, i.e. T

cells and B cells, are important in the effective clearance of the virus, and they can induce protective immunity against MNV (41,42,232,271).

2.6.3.3 The duration of immunity after NoV infection

The duration of immunity after natural NoV infection is largely unknown. Early challenge studies conducted in humans have given some estimates of the length of acquired immunity (38,224). These studies were conducted on healthy adults and a total of three challenges with GI-1 were given with different intervals. Collectively, the results from these challenge studies demonstrate that if the secondary challenge was given with a short interval (6 months), the majority of the susceptible volunteers (18 out of 22) were protected from NoV illness (38), but if the interval was long (2–3 years), all of the volunteers (six out of six) developed symptoms of AGE (224). However, when the volunteers who had twice become ill underwent a third challenge (2–6 months later), all but one remained healthy (38,224). These results indicate that short-term immunity to NoV lasts from six months to two years, and multiple NoV exposures might result in a higher level of protection (38,224). However, these studies were not directly comparable to natural infection, because the challenge virus doses given were several thousand-times greater than a natural infection dose (89,90); nor did these studies explain whether immunity triggered by multiple infections could be protective for time periods beyond two years.

The length of herd immunity to NoV might also provide clues on the duration of NoV immunity. Observations from epidemiological studies demonstrate that one to a few years after the pandemic GII-4 NoV strain has emerged, infections by that particular strain are less observed until a mutated strain emerges (12,99). These observations suggest that the duration of protective immunity against the homologous strain is at least two years; however, it is not known whether the immunity is even longer, because the previous GII-4 strains are largely replaced by the novel GII-4 strain and eventually become extinct (12,219). In the antigenically much more stable NoV GII-3 lineage, infections are prevalent in children but not in adults (8,272). It was hypothesized that exposures to GII-3 NoVs in childhood might be sufficient to create life-long herd immunity to this genotype, which would result in a low prevalence of GII-3 in adults (255). Presumably, the immunity is mediated by largely conserved immune epitopes on the GII-3 capsid (255).

Recently, a mathematical model was developed to give an estimate of the duration of protective immunity to NoV (273). The model was based on the published literature and took into consideration several variables (life expectancy, duration of

virus incubation period, asymptomatic infections, symptoms, relative infectiousness of the virus and genetic resistance) that would affect community NoV transmission and the duration of immunity on the individual level. The model's rough estimate of immunity against the homologous virus was 4–8 years. The authors concluded that the model indicates that immunity is created against NoV-related disease rather than infection (273).

2.7 NoV vaccine development

2.7.1 Pre-clinical studies

The creation of a NoV vaccine is still underway but the immunogenicity of multiple NoV vaccine candidates has been demonstrated in animals (Table 1).

Early preclinical studies demonstrated the potential of NoV VLPs as a NoV vaccine

Early pre-clinical studies focused on determining the immunogenicity of NoV GI-1 VLPs by oral delivery with and without an adjuvant. Mason et al. (111) used genetically engineered plants (tobacco and potatoes) to produce NoV VLPs, and the aim was to develop an edible vaccine against NoV. Ball et al. (112) in turn used a BV-insect cell system in the production of NoV VLPs and delivered the particles to mice by oral gavage. Both of these early studies resulted in the development of NoV specific serum IgG and also intestinal IgA response in a subgroup of the animals, and they provided evidence that NoV VLPs are immunogenic and have potential in vaccine development (111,112).

Table 1. Summary of some preclinical studies using NoV subunit particles as immunogens

Publication (ref)	Animal	Particle	Prod. system	NoV genotype	Formul./ Adjuvant	Route	Dose per antigen	Imm. times	Challenge	Imm. responses analyzed
Mason et al. 1996 (111)	Mouse	VLP	Transgenic plants	GI-1	Solid, liquid/CT	Oral	10–80 μg	4	No	Serum IgG, Intestinal IgA
Ball et al. 1998 (112)	Mouse	VLP	BV	GI-1	Liquid/CT	Oral	5–500 μg	4	No	Serum IgG, Intestinal IgA
Guerrero et al. 2001 (113)	Mouse	VLP	BV	GI-1	Liquid/ CT, mLT	IN, Oral	10, 25, 200 μg	2	No	Serum IgG, Intestinal IgG/IgA Vaginal IgA
Harrington et al. 2002 (29,274)	Mouse	VRP/ VLP	VEE	GI-1	Liquid	SC Oral	10^7 IFU, 75, 200 μg	2	No	Serum IgG/IgM, Intestinal IgA, HBGA-blocking
LoBue et al. 2006 (119)	Mouse	VRP	VEE	GI-1 GII-1,2,4	Liquid	SC	2.5×10^7 IFU 10^7 IFU	2	No	Serum IgG, Intestinal IgG/IgA, Spleen IgG, HBGA-blocking
Souza et al. 2007 (240)	Gn pig	VLP	BV	GII-4	Liquid/mLT, ISCOM	Oral, IN	250 μg	2	GII-4	Serum IgG/IgA/IgM, serum cytokines, ASC, CMI
Chachu et al. 2008 (41)	Mouse	VRP	VEE	GII-4,GII-1 MNV	Liquid	SC	2.5×10^7 IFU 3×10^7 IFU	2	MNV	Serum IgG, CMI
LoBue et al. 2009 (23)	Mouse	VLP	VEE	GI-1,2,3,4 GII-1,3,4,13 MNV	Liquid/mock-VRPs, CpG	SC	2 μg	2	MNV	Serum IgG, Intestinal IgG/IgA, HBGA-blocking
Tan et al. 2010 (49)	Mouse	P-particle	E.-coli	GII-4	Liquid	SC	5–15 μg	3–4	No	Serum IgG, HBGA-blocking
Bok et al. 2010 (230)	Chimpanzee	VLP	BV	GI-1 GII-4	Liquid	IM	50 μg	2	GI-1	Serum IgG/IgA/IgM, Intestinal IgA, HBGA-blocking
Velasquez et al. 2011 (47)	Guinea pig	VLP	Transgenic plants	GI-1	Dry powder, Liquid/GARD	IN	10, 25 μg	2	No	Serum IgG, various mucosal IgG/IgA
Blazevic et al. 2011 (25)	Mouse	VLP	BV	GII-4	Liquid	IM, ID	10 μg	2	No	Serum IgG, AVD, Intestinal IgG, HBGA-blocking, T cells
Parra et al. 2012 (252)	Rabbit	VLP	BV	GII-4C GI-1	Liquid, powder/Multiple	IM, IN	50, 150 μg	2	No	Serum IgG
Fang et al. 2013 (50)	Mouse	P-particle	E-coli	GII-4	Liquid	IN	30 μg	3	No	Serum IgG, T cells,
Debbink et al. 2014 (253)	Mouse	VRP	VEE	GII-4C GII-4	Liquid	SC	5×10^4 IFU	2	No	Serum IgG, HBGA-blocking

VLP, virus-like particle; Ig, immunoglobulin; BV, Baculovirus; CT, cholera toxin; mLT, mutated heat-labile toxin; IN intranasal; VRP, virus replicon particle; VEE, *Venezuelan equine encephalitis* (replicon); SC subcutaneous; IFU, infectious units; HBGA, histo-blood group antigen; Gn, gnotobiotic; ISCOM, immunostimulating complex; ASC, antibody-secreting cell, CMI, cell-mediated immunity; IM, intramuscular; GARD, gardiquimod; ID, intradermal; AVD, avidity; GII-4C, GII-4 consensus

Systemic and intranasal administration routes elicited strong NoV-specific immune responses

The subsequent pre-clinical studies introduced different administration routes in order to enhance the immune responses obtained by oral route. Intranasally (IN) administered BV-derived VLPs were very immunogenic in raising especially mucosal antibody responses and also improving the serum IgG titers in comparison to oral inoculation (113). VLP immunizations resulted in a serum IgG geometric mean titer (GMT) >10,000 even without the adjuvant (113). Harrington et al. (274) in turn introduced the *in vivo* expression of VLPs in mammalian tissues by administering VRPs expressing NoV GI-1 VLPs subcutaneously (SC) to mice. The VRP-vaccination strategy without an adjuvant resulted in systemic (serum IgG and IgM) and mucosal (intestinal IgA) antibody responses that were superior compared to oral VLP administration. In addition, the cross-reactivity of serum IgG against another GI VLP was detected in VRP-immunized mice (274). For the first time, a blocking assay was used as a surrogate neutralization assay to determine the functionality of serum IgG (29). The mice immunized SC with VRPs generated serum IgG that was able to block homologous VLP binding to H-type 1 HBGAs while orally immunized mice failed to generate IgG with neutralizing activity (29).

A recent study by our lab compared the intramuscular (IM) and IN routes in generating humoral and T cell immune responses to NoV VLPs in mice (120). IM immunization induced T cell responses and high levels of systemic and mucosal IgG, but lacked an IgA response. IN immunization in turn triggered both IgG and IgA antibodies, but failed to induce T cell responses. However, when VLPs were sequentially administered by both of these routes (IM+IN) the absences in the immune responses were compensated for and a strong systemic and mucosal IgG/IgA response as well as T cell response was detected (120).

A multivalent NoV vaccine approach broadened the immune responses

The early preclinical studies were conducted with the prototype GI-1 VLPs, but as NoVs are a highly heterogenous group, a multivalent vaccine strategy against a broad array of NoV genotypes was introduced by Lobue et al. (119). The mice were immunized with monovalent or multivalent VRP-based vaccines. Immunogen-specific and cross-reactive systemic and mucosal IgG was elicited upon immunizations. Monovalent vaccines induced a 10–40 times stronger homotypic than cross-reactive antibody response and the highest level of cross-reactivity was directed against genotypes belonging to the same genogroup as the immunogen.

However, when multivalent vaccines were used, cumulative cross-reactivity against genotypes not included in the vaccine were provoked, and importantly the cross-blocking activity against these additional NoV genotypes also increased (119).

VLP immunization protects animals from homologous NoV infection

The ability of multivalent NoV vaccine to mount a protective immune response was tested in a MNV-mouse model (23). The mice were immunized with VLP-cocktails produced *in vitro* with a VEE-replicon system in several different combinations with an adjuvant. The mice were then challenged with MNV to see whether huNoV VLP vaccination with or without MNV VLPs protected them from MNV infection. Monovalent or multivalent vaccine containing MNV VLPs protected the mice from MNV infection. Although the vaccine consisting solely of huNoV VLPs did not protect the mice from MNV infection, it significantly reduced MNV replication (23).

VLP immunization has also been tested in animal models that support huNoV replication and thus can be challenged with huNoV post-vaccination (230,275). Souza et al. (275) used oral and IN routes to immunize gn pigs with BV-produced GII-4 VLPs and mucosal adjuvants. VLP vaccines were proven effective, as upon homologous challenge with GII-4 NoV, the viral burden and diarrhea were significantly reduced in immunized animals compared to the control animals (275). In another study (230), chimpanzees were immunized by the IM route with either GI-1 or GII-4 VLPs and challenged with GI-1 NoV one or 18 months later. Only the GI-1 vaccine protected the chimpanzees from homologous NoV infection conducted at both time points, and the blocking antibody activity of the serum IgG correlated with the protection (230). These results demonstrated that VLP immunization can induce protective immunity at least against the homologous virus.

Novel NoV vaccine approaches focus on broadening the cross-reactive immune responses

In recent years, some novel approaches in NoV vaccine development have been introduced and tested in animals. Nasal delivery of VLPs in a dry powder formulation (GelVac™) was demonstrated by Velasquez et al. (47). The GelVac™ inoculation without any external adjuvants showed superior systemic and mucosal immunogenicity in comparison to the liquid formulation of the same vaccine in guinea pigs.

Another novel approach by our group mixed NoV VLPs (GII-4) with RV VP6-proteins in order to generate a systemically administrable combination vaccine against NoV and RV (25). The vaccine was proven highly immunogenic against NoV

in mice, resulting in serum and intestinal IgG production. Serum IgG showed broad cross-reactivity and the immune sera also blocked homotypic and heterotypic VLPs binding to HBGAs. In addition, homologous and heterologous NoV-specific T cell responses were activated. The inclusion of RV protein in the vaccine did not impair NoV-related immune response generation (25).

Parra et al. (252) generated chimeric NoV VLPs ("consensus VLPs") containing sequences from three different GII-4 strains (2002 Houston, 2006a, 2006b) in order to respond to the rapidly developing GII-4 lineage burden in humans. The multivalent vaccine generated a higher level of cross-reactivity than any of the GII-4 variant VLPs vaccines alone. In addition, the IM route was proven to be preferable to the IN route in inducing heterologous immune responses (252).

Chimeric GII-4 VLPs were also utilized by Debbink et al. (253) who cloned an antigenically important antigenic site (blocking epitope A) from a panel of NoV GII-4 strains into a single VLP construct and tested its ability to induce blocking responses. The chimeric VLP-immunization resulted in broader serum-blocking activity in comparison to monovalent VLP immunization and a similar blocking activity in comparison to multivalent VLP immunization (253).

P-particles are another candidate for a NoV vaccine

P-particles, representing only the P domain of the native NoV, have also been suggested as a potential NoV vaccine (49,50). As antigens can be tagged to the loops of the P domain, the P-particles can also be used as a vaccine platform against other pathogens (49,139,140). P-particles have been shown to induce high homotypic serum IgG responses with a neutralizing ability when administered SC along with Freund's adjuvant (49). In another study, P-particles administered IN provoked strong humoral immune responses in mice and also elevated cellular CD4+-specific T cell responses against the homologous virus (50).

2.7.2 Clinical studies

The immunogenicity, safety and efficacy of NoV VLPs have been evaluated in clinical phase I and I/II studies (Table 2). The early clinical studies were conducted with escalating doses of BV-produced GI-1 VLPs given orally to adult volunteers in the absence of adjuvant (40,51,121). Ball et al. (51) immunized volunteers orally two times with two different doses (100 and 250 μg) of GI-1 VLPs and demonstrated that serum IgG and IgA levels and seroconversion rates rose in a dose-dependent

Table 2. Summary of some clinical studies using NoV subunit particles as immunogens

Publication (ref)	Phase	Particle	Prod. system	NoV genotype	Formul./ Adjuvant	Route	Dose per antigen	Imm. times	Chal-lenge	Imm. responses analysed
Ball et al. 1999 (51)	I	VLP	BV	GI-1	Liquid	Oral	100, 250 µg	2	No	Serum IgG, IgA
Tacket et al. 2000 (121)	I	VLP	Transgen. plants	GI-1	Solid	Oral	215-751 µg	2-3	No	Serum IgG, IgM Intestinal IgA, ASC (IgA)
Tacket et al. 2003 (40)	I	VLP	BV	GI-1	Liquid	Oral	250, 500, 2000 µg	2	No	Serum IgG, IgA, Mucosal IgA, ASC (IgG, IgA, IgM), CMI
El-Kamary et al. 2010 (52)	I	VLP	BV	GI-1	Powder/MPL, Chitosan	IN	Study 1: 5, 15, 50 µg Study 2: 50, 100 µg	2	No	Serum IgG, IgA, IgM, HAI-titers, ASC (IgG,IgA), B_M-cells (274)
Atmar et al. 2011 (46)	I/II	VLP	BV	GI-1	Powder/MPL, Chitosan	IN	100 µg	2	GI-1	Serum IgG, IgA, IgM, HBGA-blocking, protection rate
Treanor et al. 2014 (53)	I	VLP	BV	GII-4C GI-1	Liquid/MPL, alum	IM	Study 1: 5, 15, 50, 150 µg Study 2 : 50 µg	2	No	Serum IgG/IgA, HBGA-blocking
Bernstein et al. 2014 (48)	I/II	VLP	BV	GII-4C GI-1	Liquid/MPL, alum	IM	50 µg	2	GII-4	Total serum Ig, Protection rate

VLP, virus-like particle; BV, Baculovirus; Ig, immunoglobulin; ASC, antibody-secreting cell; CMI, cell-mediated immunity; Transgen., transgenic; MPL, 3-O-desacyl-4'-monophosphoryl lipid A; IN intranasal; HAI, hemagglutination inhibition assay; B_M, B-memory cells; GII-4C, GII-4 consensus; Alum, aluminum hydroxide; IM, intramuscular

manner. The vaccine was proven safe, as no side effects were reported (51). The subsequent study assessed both the humoral and the cellular immunities activated in response to oral immunization with even higher escalating doses (250, 500 and 2000 μg/dose) of GI-1 VLPs (40). The study revealed that the two highest doses (500 and 2000 μg) could not elevate the seroconversion rates nor IgG titers higher than those observed with the lowest dose (250 μg). In addition, lymphocyte proliferation and IFN-γ production from PBMCs were observed upon *in vitro* stimulation with GI-1 VLPs in the volunteers who received the 250 or 500 μg doses. The edible NoV vaccine (transgenic potatoes) was also tested for immunogenicity (121). The NoV-specific immune responses (serum IgG, IgA, IgM and intestinal IgA) induced upon immunization varied greatly among volunteers and the titers were modest overall (121).

Preclinical studies in mice suggested that the IN delivery route would significantly increase immune responses in comparison to oral inoculation (113). A GI-1 VLP vaccine adjuvanted with 3-O-desacyl-4'-monophosphoryl lipid A (MPL) and mucoadherent chitosan was tested in a phase I clinical study with a two-step protocol in which dose-dependent immunogenicity and the safety of 5, 15 and 50 μg dosages (Study 1) and 50 and 100 μg dosages (Study 2) were evaluated (52). The vaccine was shown to be well tolerated and immunogenic. IgG and IgA titers elevated in a dose-dependent manner; however, no significant difference was detected between the two highest doses. Anti-NoV IgG and IgA antibody-secreting cells (ASCs) expressing homing receptors for mucosal and peripheral tissues were detected from blood (52). B memory- (B_M) cell responses in volunteers receiving 50 and 100 μg doses were evaluated (276). A significant rise in NoV-specific IgG and IgA B_M cell frequencies circulating in the peripheral blood were observed in all of the volunteers receiving the highest dose. The ability of the B_M cells to become active ASCs upon exposure to GI-1 VLPs was also demonstrated (276).

The ability of the GI-1 VLP vaccine to prevent NoV infection and illness was further evaluated in a proof-of-concept phase I/II clinical study (46). Adult volunteers were immunized two times with 100 μg of the above-mentioned GI-1 intranasal vaccine (or placebo) and challenged with a homologous GI-1 virus three weeks after the second dose. The relative reduction of NoV-associated AGE was 47%; AGE occurred in 69% of the placebo recipients and 37% of vaccine recipients. The vaccinees were also significantly less likely to be infected by NoV (relative reduction 26%) and the vaccination also reduced the severity of the disease (relative reduction 35%). Moreover, the HBGA-blocking titers of serum IgG were

significantly increased by the vaccination and the prechallenge titer (BT50, 50% blocking of VLP-HBGA binding) of ≥200 correlated with a relative reduction of >50% in the frequency of NoV-related illness and infection (46).

The VLP-based bivalent vaccine containing GI-1 and GII-4 consensus VLPs (252) was also tested for safety, immunogenicity and efficacy in phase I (53) and phase I/II clinical studies (48). In the initial phase I clinical study, four doses (5, 15, 50 and 150 μg of each VLP) were evaluated, and after selecting the optimal dose, three age-groups were vaccinated two times with a vaccine containing 50 μg of each VLP, and the humoral immune responses were assayed (53). The vaccine was adjuvanted with MPL and 0.5 mg of aluminum hydroxide and administrated IM. The antibody GMTs increased in different age-groups after the first dose 24–118-fold for GI-1 and 9–49-fold for the GII-4-specific antibody; the second dose did not further improve the titers. The HBGA-blocking titers (BT50) rose ≥200 in the majority of the volunteers. The vaccine was proven safe and was well tolerated overall (53).

The bivalent NoV VLP vaccine was tested for efficacy in the following heterovariant GII-4 (Farmington Hill, 2002) challenge study (48). Statistically significant protection from NoV infection was not detected, as 54% of the vaccine recipients compared to 62.5% of the placebo recipients were infected. However, the vaccine reduced the symptoms (diarrhea/vomiting) of AGE and the severity of the illness significantly in comparison to the placebo group indicating that this vaccine could protect from NoV disease rather than infection (48).

3 Aims of the study

1. To define NoV seroprevalence in Finnish children.

2. To evaluate the immune responses induced in mice by two NoV subviral particles – VLPs and P-particles – considering their potential use as a candidate vaccine.

3. To examine the influence of pre-existing antibodies on generation of *de novo* immune response to a novel NoV genotype in mice.

4. To assess whether serum-blocking antibodies prevent NoV infection in children and to measure blocking antibody formation upon NoV VLP vaccine immunization in mice.

5. To study humoral and cell-mediated NoV-specific immune responses in mice induced by a trivalent NoV-RV combination vaccine containing GII-4 and GI-3 NoV VLPs and RV VP6.

4 Materials and methods

4.1 The production and purification of NoV subviral particles

4.1.1 NoV VLPs (I–IV)

For the production of NoV VLPs of genotypes GII-4 (1999; reference strain GenBank ID: AF080551), GII-4 NO (2010; reference strain GU445325), GII-12 (1998; reference strain AJ277618), GI-1 (2001; AY502016.1) and GI-3 (2002; reference strain AF414403), the complementary DNA (cDNA) of the VP1 gene of the respective genotypes were cloned in BV pFastBac1 vector (Invitrogen, Carlsbad, USA). The vectors were transfected in *Spodoptera frugiperda* (Sf)9 insect cells (Invitrogen) for the amplification of the recombinant BV stocks and production of VLPs (25,123). The VLP-containing supernatants were purified with discontinuous sucrose gradients (123) and the formation and the morphology of the purified VLPs were examined using an FEI Tecnai F12 electron microscope as described earlier (123). The purity, functionality (HBGA binding) and *in vitro* antigenicity of the VLPs were determined as described by our laboratory (118,123,277). The protein concentration was quantified with a Pierce BCA Protein Assay (Thermo Science, Rockford, USA) according to the manufacturer's instructions.

4.1.2 NoV P-particles (II)

For the expression of GII-4 1999 P-particles, the polyhistidine (his)-tagged cDNA of the VP1 P domain was cloned in pET101 directional TOPO vector (Invitrogen) and transformed in TOP10 *E.coli* cells (Invitrogen) following P-particle production in *E.coli* BL21 star cells (Invitrogen) (124). The resulting his-tagged P-particles were isolated and purified with Ni-NTA affinity chromatography as described by Koho et al. (124). The formation of P-particles was verified by electron microscope and the purity, functionality (HBGA binding) and *in vitro* antigenicity of the P-particles were confirmed (124) in prior mice immunizations.

4.2 Clinical material (I)

Human sera were collected from ≤15-year-old children hospitalized for AGE in a prospective etiological study conducted in 2006–2008 (104). A total of 492 acute-phase sera were collected and 97 of the serum donors had a NoV infection detected by RT-PCR from a stool sample, as described by Räsänen et al. (104). The convalescent-phase sera were obtained from 14 NoV-infected children. Fifteen acute-phase sera were collected from children referred to hospital due to AGE during a large waterborne outbreak (195). Acute sera were collected 0–5 days after onset of AGE and convalescent sera were collected 2–6 weeks later. Six adult sera were used as positive control sera. The study protocol was approved by the Ethics Committee of Pirkanmaa hospital district in 2006 and the children were enrolled in the study after a parent or the legal guardian had given informed consent.

4.3 Animal experiments (II–IV)

4.3.1 Animals and ethics statement

Female BALB/c OlaHsd mice obtained from Harlan Laboratories (Horst, the Netherlands) were used in all animal studies (II–IV). The mice were seven weeks old at the beginning of the experiments. Immunizations and blood sample collections were done under general anesthesia generated either with the formulation of fentanylsitrate-fluanison (Hypnorm®, VetaParma Limited, Leeds, UK) and midazolam (Dormicum®, Roche Pharma AG, Grenzach-Wyhlen, Gertmany) (II, III) or with the mixture of ketamine (Ketalar®, Pfiizer Ltd., New York, USA) and medetomidine (Dorbene®, Syva, Leon, Spain) (IV). All the procedures performed on animals were carried out according to the guidelines of the Finnish National Animal Experiment Board (permission number ESLH-2009-06698/Ym-23).

4.3.2 Vaccine antigen formulation

Monovalent vaccines (VLPs or P-particles) were formulated in sterile phosphate-buffered saline (PBS; Lonza, Basel, Switzerland) at a concentration of 200 µg/ml (II–IV). The trivalent vaccine was prepared by diluting each of the components (NoV GII-4 VLPs, GI-3 VLPs and RV VP6 protein) first to a concentration of 600

μg/ml and then mixed together 1:1:1, resulting in a final concentration of 200 μg/ml of each component (IV).

4.3.3 Immunizations and study schedules

The antigens (Chapter 4.3.2) were administered to BALB/c mice by the IM route in to tight caudal muscle with a needle injection (50 μl volume). The control mice received the carrier buffer only (PBS; Lonza). The immunogenicity studies (Fig. 3) were conducted with 10 μg of single antigens per immunization point or 10 μg of each compound in a trivalent vaccine formulation per immunization point.

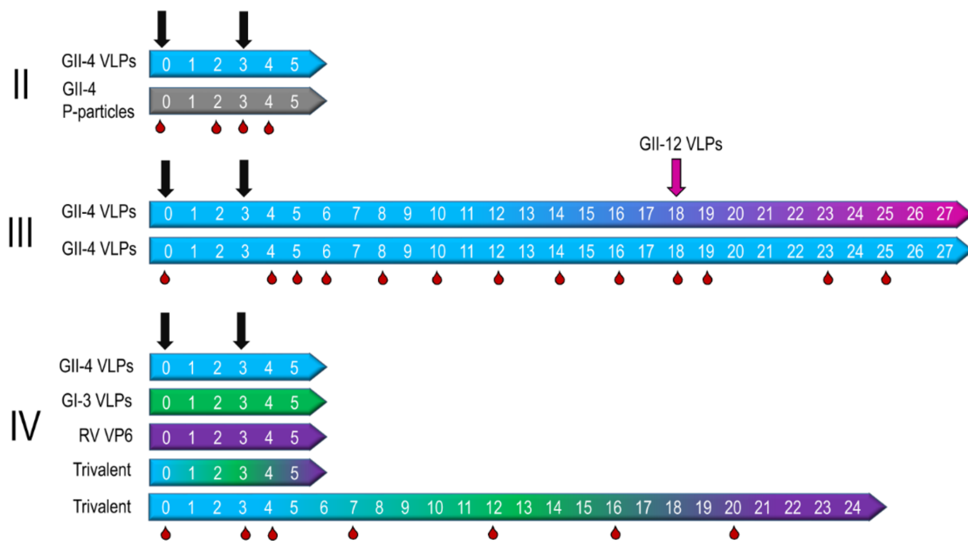

Figure 3. The mice were immunized at study weeks 0 and 3 (black arrows) with the antigen formulations stated to the right of the roman numerals (illustrating the study in which these experiments were originally performed). A group of mice primed with GII-4 VLPs (III) was boosted with GII-12 VLPs at study week 18 (pink arrow). Blood samples were collected at various time points (illustrated by red droplets). The final time points of each bar represent the termination points of the mice.

4.3.4 Sample collection and preparation

Blood samples (5 μl in 495 μl PBS) were collected from tail vein of the mice at several time points (Fig. 3) during the studies. Pre-immunization blood samples were taken

before the mice were immunized for the first time. Fresh stool samples were pooled group-wise (IV) before the mice were terminated. After termination, the whole bleed (II–IV) and spleens (II, IV) were harvested from each of the mice (278).

The blood samples were centrifuged at 3500 × g (Himac CT15RE; Hitachi, Twinsburg, USA) and the sera were separated and stored at -20°C. The stool samples were homogenized in 10 mM Tris buffer containing 100mM NaCl, 1mM CaCl2, 0.05% Tween 20, 1% aprotinin, and 10 µM leupeptine (all from Sigma-Aldrich, Saint-Louis, USA), incubated on ice for 20 min and centrifuged at 18,000 × g to obtain 10% stool suspensions, which were stored at -80°C (25). The spleens were first disrupted with a scalpel and then gently pushed through a 70 µm cell strainer (Becton Dickinson, Franklin Lakes, USA) to obtain single cell suspensions. The splenocytes were collected using Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich). The suspensions were washed by centrifugation at 300 × g (Multifuge 3SR Plus, Heraeus, Thermo Fisher Scientific, Waltham, USA) and red cells were disrupted from resuspended cell pellets by hypotonic shock with 1:10 diluted HBSS. The molarity of the suspensions was recovered with 2 × HBSS supplemented with 7.5% sodium-bicarbonate (both from Sigma-Aldrich). The splenocytes were washed and suspended in HBSS and counted in a Bürker's chamber. The cells were washed again and suspended in sterile freezing media (Roswell Park Memorial Institute medium [RPMI] supplemented with 40% fetal bovine serum [FBS] and 10% dimethyl sulfoxide [DMSO], all from Sigma-Aldrich). The vials containing 10–20×10^6 cells were frozen in freezing chambers containing 2-isopropane at -80°C and transferred to liquid nitrogen for long-term storage.

4.4 Immunoassays

4.4.1 Antibody ELISA (I–IV)

4.4.1.1 Human serum (I)

For the detection of NoV GII-4-specific IgG and IgA antibodies in human sera, an enzyme-linked immunosorbent assay (ELISA) was developed according to a published procedure (243) with modifications. Ninety-six well microtiter plates (Nunc immune Maxisorp, Thermo Fisher Scientific) were coated with 0.05 µg/well (IgG detection) or 0.2 µg/ml (IgA detection) of GII-4 VLPs. After overnight (o/n)

incubation at +4°C the plates were blocked with 5% skimmed milk. The serum samples were diluted 1:100 in sample buffer (1% milk + 0.05% Tween20 in PBS) and added to the wells. The bound antibody was detected using 1:4000 diluted anti-human IgG- or IgA- conjugated with horseradish peroxidase (HRP) (Invitrogen), reacting for 30 min with *o*-phenylenediamine dihydrochloride (OPD, Sigma-Aldrich). Between each step, the plates were washed thoroughly with PBS containing 0.05% Tween 20. All assay volumes were 100 μl/well and incubations were done at +37°C for 1 h unless otherwise stated. The optical density (OD) of the color reaction was measured at a wavelength 490 nm with a microplate reader (Victor^2 1420, Perkin Elmer, Waltham, USA). One known NoV positive and one NoV negative serum were included in each assay as controls. The background signal from blank wells (without serum) was subtracted from experimental sample OD values. A sample was defined positive if a resulting OD value was above the set cut-off value: ≥2 × negative control serum mean OD and at least 0.100 OD.

4.4.1.2 Mouse serum (II–IV) and stool suspensions (IV)

An ELISA for detecting NoV-specific IgG or IgG subtypes (II) in mouse sera or stool suspensions was conducted according to the published procedure (278). VLPs of genotypes GII-4, GII-12, GII-4 NO (II, IV), GI-3 (II, IV) and GI-1 (IV) were coated on microtiter plates at concentrations of 0.4–1.5 μg/ml. Mouse sera (diluted 1:200 or serially twofold) or 10% stool suspensions (serially diluted from 1:2) were added to the plates and the bound antibody was detected using HRP-conjugated anti-mouse IgG (1:4000; Sigma-Aldrich), IgG1, or IgG2a (both 1:6000; Invitrogen). The plates were developed and measured as described for human serum (Chapter 4.4.1.1). The end-point titer for each serum was determined as the highest dilution giving an OD value over the cut-off value (control mice: mean OD + 3 × SD and at least OD 0.100). Reciprocal titers were used to calculate GMT with ±95% confidence intervals (CI) for each experimental and control group. If the starting dilution (1:200) of a given serum sample resulted in an OD value below the above-described cut-off OD, a reciprocal titer of 100 (half of the detection limit of the assay) was used in the calculation of GMT. The Th2/Th1 ratio (II, IV) was calculated from IgG-subtype GMTs by dividing IgG1 GMT by the corresponding IgG2a GMT.

4.4.2 Avidity assay (I–IV)

The affinity of NoV GII-4-specific IgG antibodies towards their specific antigen epitopes was determined by an avidity ELISA, adopted from Rockx et al. (151). The ELISA procedure was conducted as described above for the mouse ELISA with the exception that after serum (diluted 1:200) incubation, 8 M urea in wash buffer was added for 2 × 5 minutes to dilute away the weak-affinity antibodies. The OD values from the urea treatment wells were compared to the OD values from cells lacking urea to obtain avidity indexes (OD with urea/OD without urea × 100%). An avidity index ≥50% was considered high (151).

4.4.3 Blocking assays (I–IV)

4.4.3.1 Synthetic HBGA-blocking assay (I)

To assess the ability of the serum antibody to block the binding of NoV to HBGA, a blocking assay was adopted from Harrington et al. (29). The sample and washing buffers are identical to those used in the human antibody ELISA (Chapter 4.4.1.1). GII-4 VLPs (0.2 μg/well) were coated on a microtiter plate (Nunc) in PBS (pH 7.2), in which VLPs are shown to retain their tertiary structure (127). After blocking the free antigen binding sites, human serum samples diluted 1:100 were added and incubated for 1 h at +37°C. The sera were aspirated from the plate and biotinylated synthetic H-type 3 HBGA (Lectinity Holdings Inc., Moscow, Russia) was added at a concentration of 40 μg/ml for 4 h incubation at +37°C. After washing, the bound HBGA was detected using 1:2000 diluted HRP-conjugated streptavidin (Thermo Fisher Scientific) reacting with OPD-substrate (Sigma-Aldrich), and the plate was measured as described above (Chapter 4.4.1.1). The background signal from wells lacking serum and HBGA was subtracted from the rest of the OD values on the plate and the maximum binding OD value was obtained from cells lacking serum incubation.

4.4.3.2 Saliva phenotyping and saliva-blocking assay (II–IV)

Saliva processing and phenotyping

Human saliva samples were boiled to denature naturally existing antibodies, and the supernatants extracted by centrifugation (10,000 × g, Himac CT15RE; Hitachi) (30) were stored at -20°C. Saliva samples were ABH-phenotyped by ELISA according to the published protocol (30) using anti-human A, B and H antigen-specific monoclonal antibodies (Immucor, Norcross, USA). Nonsecretors were detected as their saliva lacked A, B, and H antigens, and they were not used in further assays. Secretors' saliva samples were used to determine the binding profiles for each VLP, and a saliva-VLP pair giving a good binding signal (OD≥0.700) was selected for the saliva-blocking assays. The saliva-binding assay was conducted as described for the saliva-blocking assay below, but without the serum incubation step.

Saliva-blocking assay

Saliva of types A (for GII-4, GII-4 NO and GI-3 VLP binding), type O (for GI-1 VLP binding) and type B (for GII-12 VLP binding) were utilized in the blocking assay (25,29,137) as an alternative to synthetic HBGAs (Chapter 4.4.3.1).

Saliva samples diluted 1:3000 in PBS were coated on microtiter plates for 2 h at +37°C following o/n incubation at + 4°C. Mouse sera were diluted two-fold and pre-incubated in test tubes with VLPs (final concentrations 0.1–0.5 μg/ml) for 1 h at +37°C. The VLP-serum samples were then added to saliva-coated plates and incubated for 1.5 h at +37°C. After washing, the saliva-bound VLP was detected using NoV-positive human serum and anti-human IgG-conjugated HRP (Invitrogen) reacting with OPD substrate (Sigma) (Chapter 4.4.1.1). "Maximum binding" wells (without serum) and blank wells (without serum and VLP) were added to each plate, and the background OD value from the blank wells was subtracted from each OD value on the plate. The blocking index was calculated as 100% - (OD wells with serum/OD wells without serum, "maximum binding" × 100%).

4.4.4 ELISPOT IFN-γ (II,IV)

An enzyme-linked immunosorbent spot (ELISPOT) assay was used to quantify IFN-γ-producing T cells from mice splenocytes upon stimulation with NoV-specific peptides (Table 3). The peptides correspond to the P1-domain cross-reactive CD4+ T-cell epitope (CLLPQEWVQHFYQEA) originally published by LoBue et al. (270).

Table 3. The peptides used to stimulate splenocytes in ELISPOT IFN-γ.

NoV genotype	Amino acid sequence	Amino acids	Domain
GII-4 1999	CLLPQEWVQHFYQEA	450–464	P1
GII-4 NO 2010	CLLPQEWVQYFYQEA	451–465	P1
GII-12 1998	CLLPQEWIQHLYQES	446–460	P1

The ELISPOT-IFN-γ assay was conducted according to the published procedure (278) with some modifications. Multiscreen HTS-IP filter plates (Millipore, Billerica, USA) were coated o/n at +4°C with 0.25 µg/well (II) or 0.5 µg/well (IV) of monoclonal anti-mouse IFN-γ (Mabtech, Nacka Strand, Sweden). The plates were washed thoroughly with sterile PBS and blocked with 10% FBS (Sigma-Aldrich) diluted in cell culture medium (CM; RPMI-1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol and 2 mM L-glutamine, all from Sigma-Aldrich). The NoV peptides (0.5 µg/well) were diluted in CM and added to the plate. Concanavalin A (Sigma-Aldrich) was used as a positive stimulation control and RV VP6-specific peptide as a negative control peptide (II). Wells lacking stimulating compound (CM only) were used as a background control. The thawed and washed splenocytes were counted in a Bürker's chamber and 0.1×10^6 cells were added to each well in CM containing 5% FBS (final concentration). The plates were kept in a cell incubator (+37°C, 5% CO_2) for 20–24 h. After washing, 0.2 µg/well (II) or 0.05 µg/well (IV) of biotinylated anti-mouse IFN-γ (Mabtech) was added. The spots were developed either with streptavidin-HRP reacting with 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich) substrate (II) or with streptavidin-conjugated alkaline phosphate (AP, Mabtech) reacting with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP/NBT; Mabtech) (IV). The color reaction was stopped with water. Spot-forming cells (SFC) were counted under a light microscope by two independent

counters (II) or by automated ImmunoSpot® analyzer (CTL-Europe GmbH, Bonn, Germany) (IV). The results are expressed as the mean SFC/10^6 splenocytes of the duplicate wells. A result was considered positive if it was above the set cut-off value: $\geq$ mean SFC + 3 × SD of the negative control cells.

4.5 Statistical analyses

Pearson's chi-square test was used to assess the differences in categorical variables, i.e. antibody prevalence (I) and geometric end-point titers (GMTs) with 95% confidence intervals (II–IV) between experimental groups. The intergroup differences in antibody magnitude, avidity index, blocking index and SFC quantity were measured by Student's two tailed t-test or the Mann-Whitney U test. Statistical analyses were performed with IBM SPSS Statistics (v.22). Statistically significant difference was defined as $p<0.05$.

5 Results

5.1 Age-related NoV seroprevalence in Finnish children (I)

A total of 492 acute serum samples collected from 0–14-year-old Finnish children were examined for the presence of antibodies against the most prevalent NoV genotype in Finland, GII-4 (13,14). The results show that antibodies against NoV in childhood are very common, as 63.4% of all serum samples were positive for anti-NoV IgG and 38.9% for anti-NoV IgA. The distribution of the data into age-groups revealed that an increase in NoV seroprevalence is positively correlated with age (Fig. 4). The high IgG seroprevalence observed in 0–6 month-old-children (66.7%) likely reflects the maternal antibodies. NoV-specific IgA seroprevalence in this age-group was 20.8%. The lowest NoV-specific IgG seroprevalence was detected in the age-group of 7–23-month-old children (47.3%; $p<0.001$–0.05 when compared pairwise to other age-groups). The seroprevalence of IgA remained at the same level in the first two age-groups (20.8% for 0–6-months-old and 20.9% for 7–23-months-old). The NoV seroprevalence rose with age, reaching 91.2% for IgG and 77.9% for IgA after five years of age.

Detailed evaluation of the age-group-related OD values (reflecting to the antibody magnitude in the serum) of children positive for NoV-antibody, revealed that 7–23-month-old children had the lowest level of anti-GII-4 IgG (mean OD 0.661 ± 0.057, $p<0.05$) in the serum of all age-groups (Fig. 4). Similarly to the seroprevalence, the level of NoV-specific IgG in the serum increased in the following age-groups. NoV GII-4-specific IgA levels were relatively low (OD<0.600) in children under five years of age but rose with age in the following age-groups.

The avidity of serum IgG (I; Fig. 2) was high (avidity index $61.5\pm5.1\%$) in the under-6-months age-group, and it decreased significantly ($p<0.05$) in children aged 6–12 months (avidity index $40.4\pm3.9\%$). High avidity antibodies were again detected in the group containing older (9–14-years-old) children (avidity index $85.6\pm4.5\%$). Adult sera contained all high-avidity IgGs (data not shown).

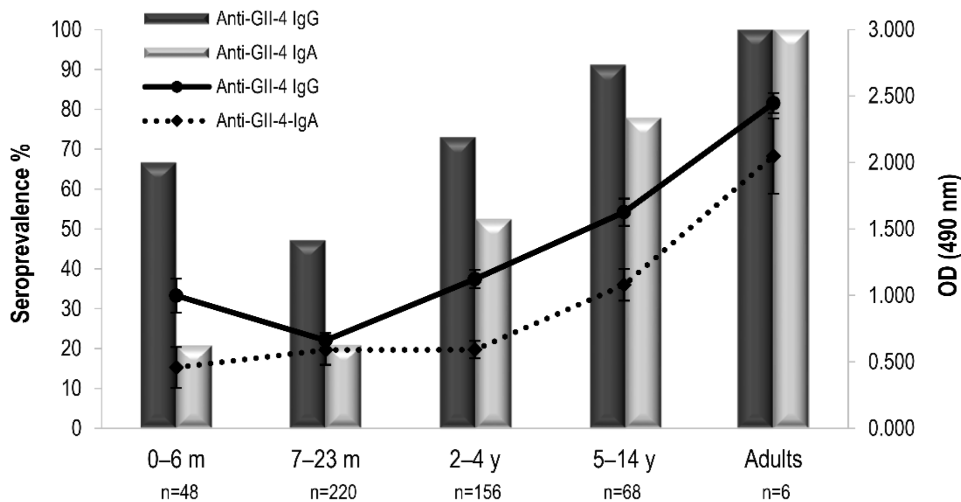

Figure 4. The age-related seroprevalence and the magnitude of NoV-specific antibody in serum. The bars illustrate the prevalence of NoV GII-4 specific IgG and IgA in the serum of Finnish children (n=492) and adults (n=6). The lines represent the mean OD-value of NoV-specific IgG and IgA in the serum of children and adults positive for NoV-antibody in ELISA.

5.2 NoV subunit particles as vaccine candidates against NoV (II, IV)

5.2.1 VLPs induce strong genogroup-specific humoral immune responses in mice (IV)

The immunogenicity of GII-4 and GI-3 VLPs were evaluated in BALB/c mice with a five-week study schedule. First, the optimal dose to induce a strong immune response was screened by administering escalating doses (3, 10 and 30 µg) of NoV VLPs and measuring the type-specific IgG response (IV: Fig. 2A & 2B). The lowest dose of VLPs induced a statistically weaker IgG response ($p<0.05$) than the two higher doses, but no difference was detected between the 10 µg and 30 µg doses ($p>0.05$); therefore the 10 µg dose was considered the optimal dose.

Single VLP immunizations with 10 µg of GII-4 VLPs or GI-3 VLPs elicited robust homotypic (immunogen-specific) IgG responses with GMTs of 77,600 (95%

CI=66,800–90,200) and 117,000 (95% CI=82,300–168,000), respectively (Fig. 5A). The antigen-specific IgGs had high avidity, as the mean avidity indexes were 82.7 ± 2.9% for GII-4 and 78.6 ± 9.9% for GI-3-immunized mice (IV; Fig. 3E & 3F). Single VLP immunizations elicited cross-reactive IgGs; higher GMTs were observed towards genotypes belonging to the same genogroup as the immunogen compared to the other genogroup ($p<0.05$, Fig 5A). IgG response kinetics after immunizations at weeks 0 and 3 showed that the first dose of GII-4 or GI-3 VLPs already induced antigen-specific IgG to a high level (both OD>2.0; data not shown). Negative control mice sera did not react with NoV-specific VLPs in the assays (OD<0.2).

The IgG subtype IgG1 and IgG2a responses were investigated after GII-4 VLP immunization (II; Fig. 3A). VLP immunization triggered both IgG subtypes at a similar magnitude indicating a balanced Th1/Th2 response.

The potential of NoV VLPs to induce a mucosal antibody response was investigated from group-wise pooled mice stool samples (10% suspension). Both GII-4 and GI-3 VLPs elicited antigen-specific fecal IgG with end-point titers of 1:4 and 1:16, respectively (Fig. 5B).

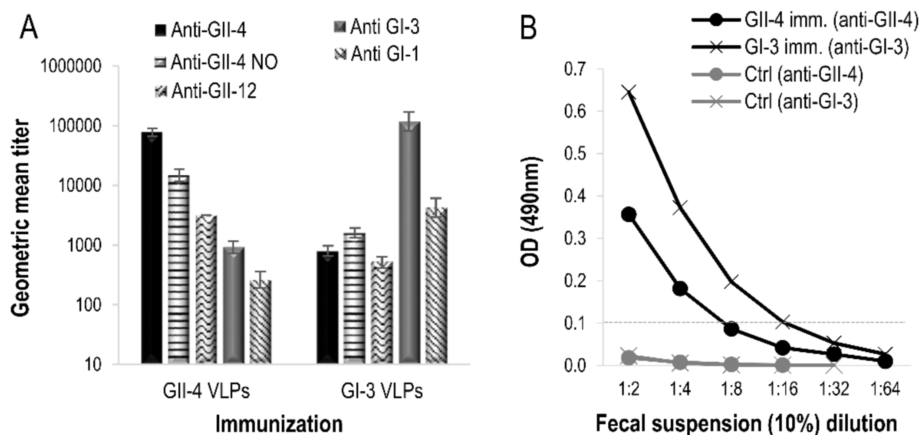

Figure 5. Humoral response induced with GII-4 and GI-3 VLP immunizations. Mice were immunized with GII-4 or GI-3 VLPs two times at study weeks 0 and 3 and terminated at study week 5. The termination serum of each mouse was titrated against NoV VLPs in ELISA, and homotypic and cross-reactive geometric mean end-point titers (GMTs) (bars) with 95% confidence intervals (error bars) were determined for each group (A). Mucosal antibody response was tested by titrating a group-wise pooled 10% fecal suspension and measuring the antigen-specific IgG content (optical density, OD) in ELISA (B). The dashed horizontal line indicates the cut-off OD-value for the assay (B).

5.2.2 VLPs induce cross-reactive cellular immune responses in mice (IV)

The capability of VLPs to induce cell-mediated immune responses was addressed by measuring IFN-γ production from mice splenocytes upon stimulation with peptides conferring the immunodominant T cell epitopes (270) of GII NoVs (GII-4, GII-4 NO and GII-12). Antigen-specific and cross-reactive cellular responses were observed as GII-4-immunized mice responded to GII-4-, GII-4 NO- and GII-12-specific peptide stimulation by producing high levels of IFN-γ in study week 5 (IV: Fig. 7A). However, splenocytes from GI-3-immunized mice did not produce detectable IFN-γ upon stimulation with any of the GII-specific peptides (IV: Fig. 7A), indicating that the cell-mediated immune response is genogroup-restricted. Unfortunately, the lack of GI genogroup-specific peptides hampered our attempts to measure GI-related cellular responses. No-antigen specific IFN-γ production was observed in the control mice or CM wells (<50 SFC/10^6 cells).

5.2.3 P-particles induce genotype-specific humoral immune response in mice (II)

BALB/c mice were immunized with NoV GII-4-derived P-particles (10 μg/dose) two times by the IM route with a five-week study schedule (Fig. 3, Chapter 4.3.3) and assayed for humoral and cellular immune responses. P-particle immunization generated type-specific (anti-GII-4) IgG with a GMT of 25,600 (95% CI = 15,300–42,800). Although the level is considerably high, it is statistically lower in magnitude than the GMT induced by the corresponding VLP immunizations (Chapter 5.2.1, Fig. 5A; $p<0.05$). In addition, the avidity of GII-4-specific IgGs was found to be very low (avidity index 5.3±0.9%) (II; Fig. 2C). P-particles failed to induce appreciable cross-reactive antibody responses (II; Fig. 6A) as the immune sera did not react with GII-12 and GI-3 VLPs in ELISA (both OD<cut-off level), and only a weak positive response against GII-4 NO VLPs with a GMT of 200 (95% CI = 143–280) (II; Fig. 6B) was observed. The kinetics of IgG generation in the serum indicated that two doses of P-particles are needed to raise the GII-4-specific IgG to a similar level as that after VLP immunizations (II; Fig. 2B). When serum IgG subtype (IgG1/IgG2a) content was examined in ELISA, a strongly Th2-type skewed response was detected; the difference in the final titers of the IgG subtypes was 32-fold in the favour of IgG1 antibody (II; Fig. 3B).

5.2.4 P-particles do not induce cellular immune responses in mice (II)

The ability of P-particles to prime T cells for IFN-γ production was analyzed in an ELISPOT assay. None of the P-particle-immunized mice responded to any of the NoV-specific peptide stimulations (II; Fig. 4), indicating a lack of Th1-type immune responses against NoV.

5.3 Pre-existing antibody responses do not hinder antibody response to a novel genotype (III)

The impact of pre-existing NoV humoral immune response on the immune response generated to novel genotype was evaluated in mice. Pre-existing NoV GII-4-specific immunity was generated by immunizing naïve BALB/c mice with GII-4 VLPs (at study weeks 0 and 3), and later (at study week 18) half of the mice were boosted with GII-12 VLPs (Chapter 4.3.3, Fig. 3). The immune responses were left to develop until study week 27, after which GII-4- and GII-12-specific humoral immunity was evaluated. The anti-GII-4 IgG level raised after the second GII-4 dose at study week 3 and persisted up until study week 27 (III; Fig. 1A), resulting in an end-point titer of 51,200 in all mice. The GII-12 boost had no impact on the pre-existing anti-GII-4 IgG level (III; Fig. 1A) observed at week 27 against GII-4 (all mice generated an anti-GII-4 IgG titer of 51,200). On the contrary, the level of anti-GII-12 IgG rose after the GII-12 boost to the same magnitude as detected for GII-4-specific IgG (III; Fig 1B). To confirm that these antibodies were GII-12-specific, the avidity of the IgGs to GII-12 VLPs was measured. The GII-12-boosted mice developed high avidity anti-GII-12 antibodies (mean avidity index 61.5±6.9%) whereas solely GII-4-primed mice had low avidity antibodies (32.6±5.6%) against GII-12, referring to the cross-reactive activity of the anti-GII-4 antibodies. In addition, the functionality of the antibodies was examined by measuring the blocking potential of the immune sera against GII-4 and GII-12 VLPs (III; Fig. 2). The GII-12-boosted mice had developed considerable GII-12-blocking activity in the serum (III; Fig 2B) while maintaining GII-4-blocking potential (III; Fig. 2A). These results indicate that pre-existing immunity to one NoV genotype does not hinder the formation of a functional humoral response to another genotype.

5.4 Natural NoV infection and NoV subunit particle immunization induce blocking antibodies (I, II, IV)

5.4.1 Blocking antibodies correlate with protection in children (I)

The acute sera of NoV-infected (n=6) and non-infected children (n=9) collected during a large waterborne outbreak were used to assess whether a serum's ability to block VLP-HBGA-binding correlates with protection (I; Fig. 5A). The acute sera of children infected with NoV (GII-4 2006b) (195) could not block VLP binding to H-type 3 HBGA, as no reduction of the mean OD value (0.715±0.176) was detected when compared to the maximum binding control (wells without serum, OD 0.712±0.005). By contrast, the acute sera from children not infected with NoV blocked the binding significantly (mean OD 0.301±0.086, $p<0.05$), indicating a correlation between blocking antibodies and protection from the disease. Six out of nine serum samples obtained from children that did not acquire a NoV infection had high blocking activity (OD≤0.200) in their acute sera.

The generation of blocking antibodies after recent NoV infection was investigated using acute and convalescent sera obtained from children with a NoV infection (I; Fig. 5B). A comparison with the maximum binding control (OD 0.910±0.066) showed that the acute sera could not block the binding of NoV VLPs to H-type 3 HBGAs (OD 0.728±0.096, $p>0.05$), whereas significant blocking activity had been developed in the convalescent serum (OD 0.372±0.084, $p<0.05$) after NoV infection.

5.4.2 NoV subunit vaccines induce blocking antibodies in mice (II, IV)

The induction of blocking antibodies in mouse sera by NoV subunit vaccines (VLPs and P-particles) was examined in saliva-blocking assays utilizing human (secretor-positive) saliva as a source of naturally occurring HBGAs. All NoV subunit particles (GII-4 VLPs, GI-3 VLPs and GII-4 P-particles) were able to induce homotypic blocking antibodies, but only VLP-immunized mice sera also had cross-blocking activity (Fig. 6). However, monovalent VLP immunizations raised blocking antibodies only against genotypes belonging to the same genogroup as the immunogen; GII-4 VLP-immunized sera blocked GII-4 NO VLPs (Fig. 6, upper panel) and GI-3 VLP-immunized mice blocked GI-1 VLPs (Fig. 6, middle panel)

binding to saliva HBGAs (IV). The low level of anti-GII-4 NO antibodies detected in P-particles-immunized mice sera (Chapter 5.2.3) failed to cross-block GII-4 NO VLPs binding (Fig. 6, lower panel) to HBGAs (II).

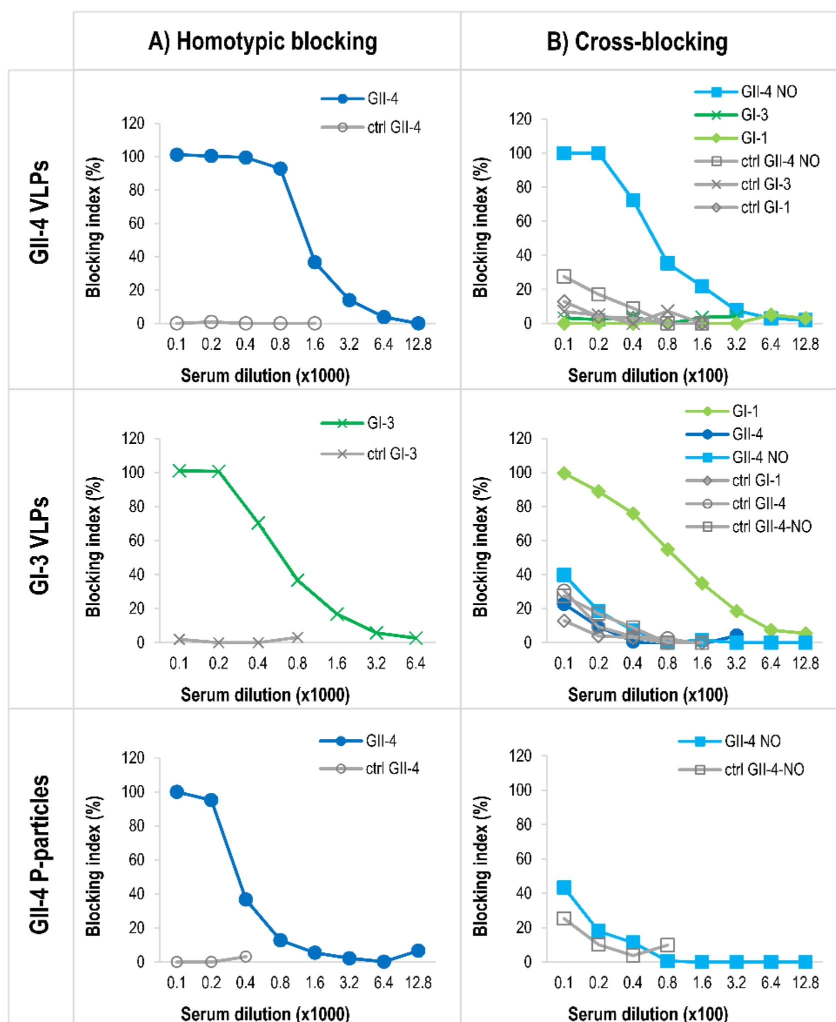

Figure 6. Blocking activity of NoV subunit particle-immunized mice sera. Group-wise pooled termination sera of mice immunized with NoV GII-4 virus-like particles (VLPs) (upper panel), GI-3 VLPs (middle panel) or GII-4 P-particles (lower panel) were used to block homotypic (column A) or heterotypic (column B) NoV VLPs binding to human saliva (type A for GII-4, GII-4 NO and GI-1 VLPs and type O for GI-1 VLPs). The blocking indexes of serially two-fold-diluted sera are shown. Negative control mice sera (unspecific) blocking activity is shown for each antigen (grey lines). The blocking indexes were calculated as 100% - (OD wells with serum/OD wells without serum) × 100%.

5.5 NoV GII-4 and GI-3 VLP combination vaccine induce broad immune responses against NoV in mice (IV)

NoV GII-4 VLPs and GI-3 VLPs were mixed with RV VP6 protein to compose a combination vaccine against NoV and RV. The immune responses elicited by the vaccine were evaluated in BALB/c mice with regular (five-week) and long-term (24-week) immunization schedules (Chapter 4.3.3, Fig. 3). A strong humoral response (Fig. 7A) with high avidity antibodies (IV: Fig. 3E & 3F) was detected against vaccine antigens at study week 5. High amounts of cross-reactive antibodies covering both genogroup VLPs were detected in the termination sera (Fig. 7A). No reduction in the homologous ($p>0.05$) or heterologous antibody magnitude was observed when antibody responses induced by the combination vaccine were compared to the antibody responses generated by the monovalent vaccines (Chapter 5.2.1, Fig 5A). On the contrary, some of the cross-reactive responses were higher in combination vaccine-immunized mice that obtained with the monovalent (GII-4 VLP) vaccine. For GII-4 NO-specific IgG, the GMT was almost twofold higher ($p<0.05$) in the combination-vaccine immunized mice (GMT 25,600, 95% CI = 21,100–31,100) than in monovalent-vaccinated mice (GMT 14,700, 95% CI = 11,700–18,500). For anti-GII-12, the difference in the GMTs between the combination and monovalent vaccination was fivefold (GMT 16,900, 95% CI 13,200–21,600 versus GMT 3200, respectively).

Both the type-specific and the cross-reactive serum IgG responses slightly decreased from study week 5 to week 24 (all $p<0.05$), but they still maintained a high magnitude (Fig. 7A), indicating the vaccine's capacity to induce long-lasting humoral response against NoV. Mucosal anti-GII-4 and anti-GI-3 IgG were detected in trivalent immunized mice stool samples at study week 5, and the responses still remained at study week 24 (Fig. 7B).

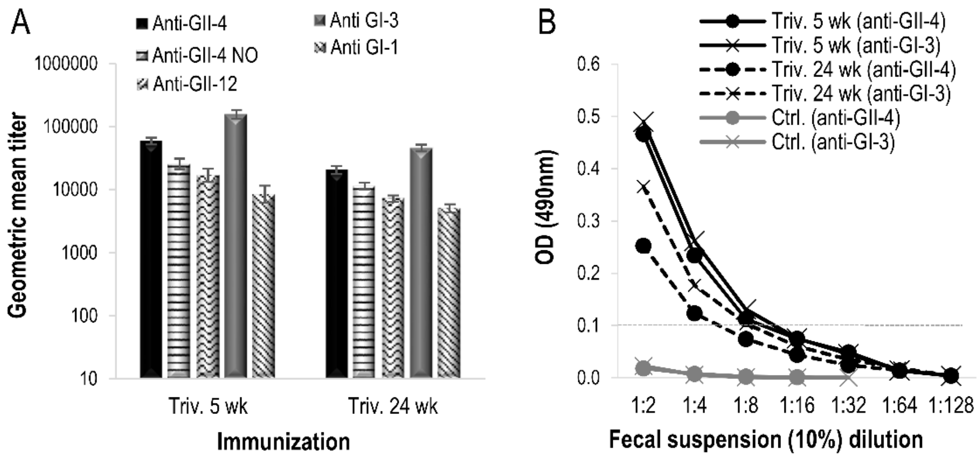

Figure 7. Humoral immune response in trivalent combination vaccine-immunized mice. Mice were immunized with the combination of NoV GII-4 virus-like particles (VLPs), GI-3 VLPs and RV VP6 two times at study weeks 0 and 3 and terminated at study week 5 (Triv. 5 wk) or 24 (Triv. 24 wk). The termination serum of each mouse was titrated against NoV VLPs in ELISA and homotypic and cross-reactive geometric mean titers (GMTs) (bars) with 95% confidence intervals (error bars) determined for each group (A). Intestinal antibody response was tested by titrating group-wise pooled 10% fecal suspension and measuring the antigen-specific IgG content in ELISA (B). The dashed horizontal line indicates the cut-off OD-value for the assay (B).

The functionality of the trivalent vaccine-immunized mice sera was examined by measuring its blocking potential against several NoV VLPs (Fig. 8). The trivalent vaccine elicited strong blocking potential not only against the vaccine genotypes GII-4 and GI-3 (Fig. 8, Column A) but also agains heterotypic VLPs GII-4 NO and GI-1 (Fig. 8, Column B). Importantly, the blocking antibodies were also long-lasting, as they were detected in mice serum at study week 24. No indication of vaccine component interference was detected, as the blocking ability of the sera was similar when compared to the monovalent vaccines (Chapter 5.4.2, Fig 6, upper and middle panels).

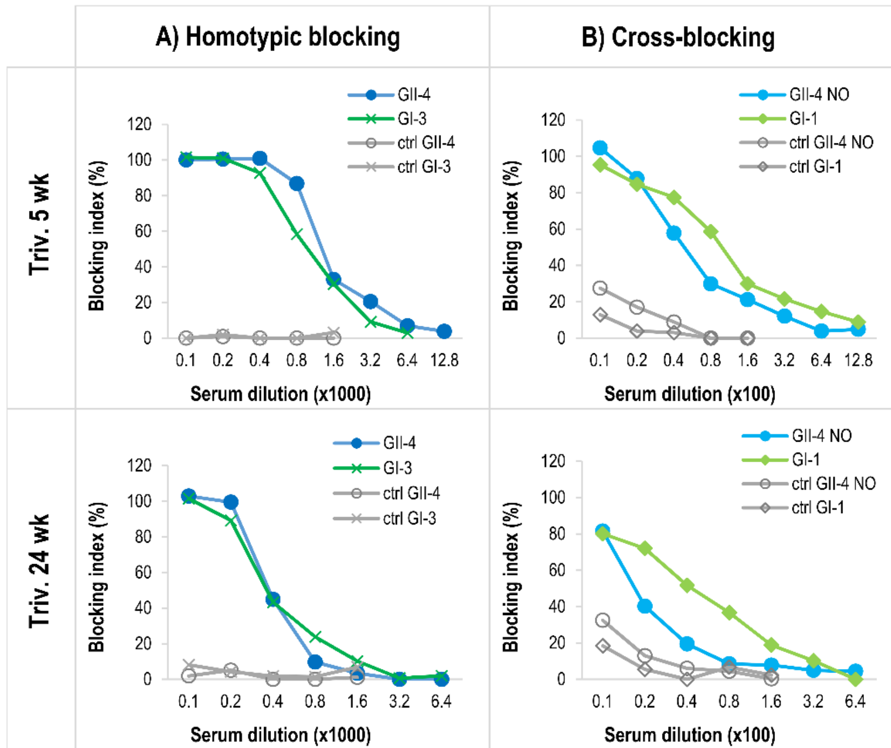

Figure 8. Blocking activity of trivalent combination vaccine- (GII-4 VLPs, GI-3 VLPs and RV VP6) immunized mice sera. Mice were immunized with a trivalent combination vaccine and terminated at study week 5 (upper panel) or 24 (lower panel), and the group-wise pooled sera were used to block homotypic (column A) or heterotypic (column B) NoV VLPs binding to human saliva (type A for GII-4, GII-4 NO and GI-1 VLPs and type O for GI-1 VLPs). The blocking indexes of serially twofold diluted sera are shown. Negative control mice sera-blocking activity (unspecific blocking) is also shown for each antigen and time point (gray lines). The blocking indexes were calculated as 100% - (OD wells with serum/OD wells without serum) × 100%.

The trivalent combination vaccine-immunized mice primed T cells that reacted strongly to NoV-peptides (GII-4-, GII-4 NO- and GII-12-specific) by secreting IFN-γ in an ELISPOT assay (IV: Fig. 7A). No reduction of the IFN-γ secreting T-cell frequencies was observed when the combination vaccine immunization was compared to monovalent GII-4 VLP immunization at study week 5 (all $p>0.05$). Furthermore, T cell response was of a long duration, as no statistical difference was detected in the IFN-γ secretion between the five-week and 24-week termination points ($p>0.05$). Overall, the trivalent NoV VLP and RV VP6 protein combination vaccine showed the potential to induce strong and non-fading humoral and cellular immune responses with no sign of component interference.

6 Discussion

6.1 Finnish children encounter NoV early in life

An early study in Finnish children had indicated a 50% seroprevalence of NoV antibodies by the age of 2.5 years (36). The present study confirmed the high overall seroprevalence (63.4% for IgG), indicating that NoV infections are common among children in Finland. The overall and age-related profile of anti-NoV antibodies in children is in line with the published seroprevalences from other developed countries (32,33,221).

Both the seroprevalence and the avidity of anti-NoV IgG in the sera of infants under six months old were high, reflecting the presence of maternally acquired IgGs, since IgG is the only Ig-class that crosses the placenta via neonatal FcRn receptors (279). Maternal IgG might confer some level of protection in the first six months. However, the prevalence of anti-NoV IgA antibodies in this age-group (20.8%) indicated that the first NoV infections can be acquired already during the first six months of life; thus, maternal antibodies are not fully protective against infection. A similar IgA seroprevalence (20.9%) was detected in the 6–23-month-old children age-group. As IgA fades within a few months after a NoV infection (21,243), we could not correlate the IgA antibody prevalence with the timing of NoV infections in children. The presence of anti-NoV IgG, however, could be used as a marker of a NoV exposure as anti-NoV IgG can persist even for years after an infection (21,243).

NoV infections occurring at 7–12 months of age are mostly primary infections, as the avidity of NoV-specific IgG was the lowest in this age-group. During the second year of life, both primary and recurrent NoV infections are likely to take place, as was shown in Peruvian children in a large birth-cohort study (102). This age-group is vulnerable because primary NoV infections may run a severe course, leading to hospital admission (3,104). This study confirmed that almost half (47.3%) of Finnish children have experienced at least one NoV infection during their first

two years of life, which is comparable to the NoV seroprevalence in Finnish children published by Lew et al. in the middle of 1990s (36).

After two years of age, IgG seroprevalence increases with age, resulting in over 90% seroprevalence in children aged 9–14 years. In this age-group, there was both a high level of NoV antibodies in the blood and a very high avidity of NoV antibodies, indicating frequent recurrent infections with NoV. The antibodies that were measured by the ELISA are not strictly GII-4-specific, but instead might be triggered by other NoV-genotype infections as well (24,37,209). The immune responses activated upon primary NoV infection are likely to be insufficient for developing cross-protection from heterologous genotypes and GII-4 variants (37), leading to multiple subsequent NoV infections in childhood. NoV infections in older children are likely to be less severe or asymptomatic, because despite the increasing NoV seroprevalence and level of NoV-antibodies in the blood, the number of children attending medical care for NoV-related AGE decreases (44,104). Presumably, the immunity acquired in the early NoV infections is sufficient in preventing the most severe NoV disease but not the infection.

As almost 50% of Finnish children experience a primary NoV infection before the age of two, children should be vaccinated early to prevent NoV infection or severe NoV-related AGE. The vaccination of young children would also reduce the disease burden in older children and adults (273), and thus lower the costs to healthcare (280).

6.2 Blocking antibodies confer protection in children

Acute sera from GII-4 NoV-infected and uninfected children were obtained during a large waterborne AGE outbreak that occurred in 2007 in the town of Nokia, Finland (195,201). These sera gave us the opportunity to examine whether children who were not infected by NoV had more blocking activity in their sera compared to the children who were infected. Our assumption was that all of these children were exposed to NoV during the waterborne outbreak, as all sought medical care for AGE due to pathogens present in the contaminated tap water (195). We found that children whose acute sera blocked the binding of GII-4 VLPs to HBGAs in a surrogate neutralization assay were significantly more protected from subsequent NoV infection than children lacking the blocking activity in the serum. A similar finding has been observed in adults (39) and later also by our laboratory using children's sera collected during the GII-4 NO pandemic season (37). Interestingly,

the VLP that we used in the blocking assays was GII-4 (1999), and we could still detect the blocking antibodies that conferred protection against the heterovariant GII-4b (2006) strain. This confirms the earlier findings that some key blocking epitopes are shared between the GII-4 strains (67,269). These observations can be applied in NoV VLP vaccine development, especially when evaluating the effectiveness of vaccines against GII-4 variants.

6.3 The effect of pre-existing immunity to NoV on *de novo* immune response generation

One theoretical concern for NoV vaccination is that pre-existing immunity to NoV might impaire immune responses to vaccine antigens or that the vaccination might skew the immune response generation in natural NoV infections later in life (24,281). This might result from the phenomenon known as "original antigenic sin" (OAS), in which immunological memory induced by the primary infection restricts the immunological response to the epitopes present in the first-encountered virus strain (282,283). As a result, the immune response against the subsequent, antigenically slightly different strains is not as effective as it would be in the primary response. The phenomenon has been detected among highly mutatious viruses, such as influenza (284) and dengue fever (285), which complicates the vaccine design against these viruses.

Lindesmith et al. observed that volunteers challenged with NoV GI-1 all generated rapid T cell response, but the responses were mostly skewed against other GI genotypes, giving reason to suspect that OAS might have influenced these responses (24). Furthermore, Parra and Green demonstrated that a child infected primarily with GII-4 generated a rapid anamnestic GII-4-specific mucosal antibody response after secondary infection with a heterologous GII genotype, GII-6 (209). Other than these two studies, OAS among NoVs is an understudied area. We studied the possible existence of OAS in naïve mice with NoV VLPs. We generated a primary immune response to GII-4 by two subsequent immunizations with GII-4 (1999) VLPs and boosted half of the mice with heterologous, genetically unrelated, GII-12 VLPs; we then examined if the immune response specific to GII-12 was induced. We found that a *de novo* antibody response was generated to the secondary immunogen, which was demonstrated by the following observations: the secondary antigen- (GII-12) specific IgG level, avidity and HBGA-blocking activity were elevated to a similar level as detected in mice immunized solely with the primary

immunogen (GII-4). The reason why we obtained contradictory results to the study by Parra and Green (209) is not clear. However, Parra and Green measured intestinal IgA (and IgG) response after natural NoV infections in one naive child while we measured systemically VLP-induced IgG response in mice. Possibly the generation of systemic IgG by IM immunization route is more efficient than the generally lower local antibody response in the gut, which might explain the different results.

Furthermore, as the genotypes that we used (GII-4 and GII-12) are genetically distinct, we cannot totally rule out the effect of OAS on the immune responses to the closely related strains, such as the GII-4 variants. With the influenza virus, the OAS is stronger among closely related strains, bearing only slightly mutated immunodominant B cell epitopes (286). In future studies, it would be interesting to examine the OAS among antigenically more closely related NoV strains and to expand the studies to also involve the T cell-related OAS.

This study adds to the general knowledge of NoV adaptive immunity and suggests that humoral OAS does not exist between heterologous NoV genotypes.

6.4 Immune responses to monovalent NoV VLP and P-particle vaccines in mice

6.4.1 Humoral immune responses

The rationale of using NoV subunit particles, rather than soluble NoV proteins, lies in the notion that vaccines that are formed from particular, repetitive structures are known to efficiently stimulate adaptive immune responses without external adjuvants; instead, the adjuvant elements are possessed in the structure of these particles (287). The repetitive, multi-display surface structures are known to efficiently cross-link B-cell receptors, which leads to robust humoral responses (288). The surface structures also often resemble the natural virus and induce neutralizing antibody formation. Unlike soluble proteins, the larger structures are effectively internalized by antigen-presenting cells (APCs), such as DCs, and thus be carried in the lymphoid organs and presented to T cells, therefore promoting adaptive immune system activation (289,290).

To assess the comparative potential of NoV VLPs and P-particles to induce adaptive immune responses, we evaluated the immunogenicity of these particles in mice. The GII-4 NoV subunit vaccines based on VLPs and P-particles are identical

to the P-domain of the GII-4 (1999) sequences, but P-particles lack the H and S domains and are smaller in size (24-mer, approx. 20 nm) than the corresponding GII-4 VLPs (180-mer, approx. 38 nm). The immunization of mice two times by the IM route with VLPs (GII-4 or GI-3) or P-particles resulted in strikingly different antibody responses against NoV. VLPs were superior to P-particles in raising both homologous and especially heterologous antibody responses with blocking activity. The conformational differences of VLPs and P-particles might explain the different immunogenicity profiles detected. The lack of the S domain in P-particles might affect the quantity and the quality of antibodies induced upon immunizations. As VLPs are structured from both P and S domains, the antibody epitopes from both domains are thus present in *in vivo* and *in vitro* assays. Presumably, some VLPs tend to partly lose their conformation upon coating on ELISA plates (291), which exposes the buried linear epitopes of the S domain to antibody binding. In addition, S domains are also partially exposed in the intact VLPs (108). VLP-immunized mice sera can recognize these S domain epitopes, which in part explains the higher overall titers.

The S domain epitopes have shown to be largely conserved among NoVs (19), although this is the case more within the genogroup than across genogroups. This could be one factor influencing the profoundly different cross-reactivity of antibodies we detected after VLP and P-particle immunizations. The linear S domain epitopes on NoV are also suggested to be important CD4+ T-cell epitopes (270). CD4+ T cells are known to stimulate the B cell activation and antibody isotype switch, and also drive antibody affinity maturation process. Indeed, high avidity antibodies were only present in the VLP-immunized mice. High avidity antibodies are shown to promote efficient virus neutralization (151,292) and have been correlated with protection in some viral infections (293,294).

The relevance of antibodies produced against the structurally occluded S domain of NoV *in vivo* is still mostly unknown, but these antibodies could, for instance, interfere with the entry process of NoV to the host cells. Presumably, the buried epitopes of the S domain become exposed for B cell recognition during the conformational change involved in the receptor attachment and entry process in the same way as is shown for HIV (295). If huNoV, like MNV (77), does in fact replicate in the cells under the mucosal intestinal cells (83), these kinds of antibodies might be important if the intestinal HBGA-blocking antibodies cannot prevent the penetration of NoVs through the mucosal layer. Another interesting finding was reported by Parra et al. (108), as they described the S domain-recognizing Mab that partially blocked the binding of GII-4 VLPs to type B HBGAs. However, the actual

relevance of S domain-specific antibodies in HBGA neutralization is probably minor; the immunodominant neutralizing B cell epitopes reside in the more exposed sites of the capsid, as the P-particles are also able to induce blocking antibodies in the serum.

In general, the conservation of NoV-immune epitopes increases when moving from the exposed sites of the virion to the inner regions (19). Mabs raised in mice against epitopes distal to the HBGA binding pocket have been shown to block the binding of VLPs to HBGAs (108,260,269) possibly by altering the conformation of the HBGA binding pockets to a more antibody-accessible form and thus inhibiting carbohydrate binding (269). Thus, it is not only the antibodies directed to HBGA binding pockets that can promote neutralization. VLPs retain extreme conformational flexibility, which allows the conserved conformational epitopes occluded in the structure to become exposed for antibody recognition (62). The flexibility could also allow the B cells to recognize the conserved buried (blocking) epitopes of the P domain and thus generate the broadly cross-reactive and possibly also cross-blocking antibodies detected in our experiments and by others (119,122). The conformational flexibility of other viruses has been shown to expose the buried epitopes, making them accessible for neutralizing antibody formation (296,297). In VLPs and native NoVs, the flexibility is mediated by a flexible hinge between the S and P domains (19), and as the hinge is missing from the P-particles, they are likely to be more rigid structures than VLPs. The lack of structural flexibility might restrict the exposition of the conserved regions of the P domain, which might in part affect the serum's lack of cross-reactivity, as we detected.

The lower immunogenic "state" of the P-particles has been discussed by Lindesmith et al. (67). They detected that the formation of P-dimers results in the loss of some blocking epitopes present in the corresponding strain VLPs, and speculated that the same possibly occurs in the formation of P-particles (67). The loss of some key blocking epitopes in the P-particles might explain why we also observed weaker homotypic blocking activity in the P-particle immunized-mice serum compared to the one generated by VLP immunizations.

Our results with P-particles are partly in conflict with the published results from Tan et al. (49,50). Tan et al. have shown that P-particles induce homotypic blocking antibody responses, which were comparable to the ones induced by the VLPs in their assay (50). They also detected overall higher responses that were observed in our assays with P-particles (49,50). However, the differences between our study designs and that of Tan et al. concern the immunization route, antigen amount and the use of adjuvant, which might explain the differences in the immune responses.

Tan et al., for instance, used the IN route for P-particle administration (without adjuvant), resulting in VLP immunization-comparable immune responses, which is something that we have not evaluated for P-particles (50). In addition, Tan et al. used P-particles or P-dimers as antigens in ELISAs and these antigens do not assess the role of S domain-specific antibodies in VLP-immunized mice (50).

6.4.2 Cell-mediated immune responses

The measurement of IgG subtypes in the immunized-mice sera suggested that VLPs also efficiently stimulate cell-mediated responses, as balanced Th1/Th2 responses, were observed after GII-4 and GI-3 VLP immunizations. We used an ELISPOT assay to further assess the amount of the Th1-specific cytokine IFN-γ induced from mice splenocytes upon stimulation with NoV-specific peptides, and we detected that GII-4 VLPs were potent inducers of cellular immune responses. The induction of INF-γ is known to drive the Th1-type responses mediated by immune cells such as CD4+ T cells, CD8+ T cells, NK cells and macrophages, which are known to be important agents in viral infections (298). Lindesmith et al. have suggested that protection against NoV could be partly mediated by T cell responses (226).

Importantly, we observed that VLP immunization triggered not only antigen-specific but also the cross-reactive T-cell responses to GII-12 and GII-4 NO. However, P-particles failed to induce cell-mediated immunity, which was observed from a strongly IgG2a-skewed IgG subtype response indicative of Th2-typic response and a total lack of IFN-γ response. As the peptides used in the immunization were derived from the P1 domain, which is present in both subunit particles, we assume that there might be some crucial difference in the *in vivo* processing of these particles by immune cells. One explanation is that the size difference between the VLPs and P-particles might result in a different level of APC uptake in tissues and thus affect the level of priming T cells in the lymphoid organs. For example, DCs, which are especially important APCs in viral infections, prefer particles that are the size of NoV VLPs (289,290). Particles sized 40–50 nm were uptaken effectively in DCs and presented to T cells in draining lymph nodes, and this particular particle size was essential for a balanced and strong immune response formation in mice (290).

VLPs have been shown to induce the activation and maturation of human DCs isolated *ex vivo* (299). A recent study showed that P-particles were also capable of activating bone marrow derived DCs of mice *in vitro* (50). However, the action of the

DCs might be different *in vivo*, as it has been demonstrated that *in vitro* cultivated bone marrow derived DCs have an unnaturally high antigen presentation capacity and do not behave similarly to DCs isolated *ex vivo* (300,301). It is also possible that P-particles are uptaken by a different subpopulation of DCs or by different APCs than VLPs, thus stimulating strongly Th2-type responses (302,303).

Contrary to our results with P-particles, Fang et al. have recently described the induction of homotypic Th1 specific cytokines from T cells after IN immunization with P-particles (50). They used a peptide that represented a predicted CD4+ T cell epitope to stimulate the mouse splenocytes; this peptide was different from the one we used. Actually, in a recent study, we have demonstrated that our peptide likely represents the CD8+ T cell receptor (120) and this might explain the difference in observations between us and Fang et al. (50). In other words, the experiments conducted by us do not exclude the possibility that P-particles could stimulate CD4+ T cell responses, especially when the immunization route is through the mucosa, which is known to be rich in APCs (298). However, based on our observations, VLPs are superior in inducing balanced immune responses through the IM route without an external adjuvant, and they should thus be primarily selected as the NoV vaccine component. With the VLP-based vaccine, a broad reactivity to the circulating NoV strains is feasible to achieve, but P-particles might be suitable for a GII-4 vaccine that is reformulated prior to every GII-4 pandemic season similarly to the seasonal influenza vaccine. P-particles with narrow cross-reactivity could also be an excellent tool in diagnostic assays for NoV.

6.5 NoV-specific immunogenicity of the candidate NoV VLP and RV VP6 combination vaccine in mice

The VLP-based vaccines in general have been shown to be safe, immunogenic and protective in preclinical and/or clinical trials (304). VLP vaccines against the hepatitis B virus and human papilloma virus (HPV) have been already registered, and promising vaccine candidates against other viruses (e.g. NoV, HIV and influenza) are under clinical evaluation (48,304-306). Similarly to NoV, HPV is a genetically versatile virus, with 30–40 genotypes known to infect humans (307). The two licensed VLP-based vaccines against HPV are multivalent (bivalent Cervarix® and quadrivalent Gardasil®), and both of these vaccines have been shown to provide cross-protection to genotypes not included in the vaccine (308). The overall good

experiences with the VLP-based vaccines for other viruses supports the development of a VLP-based vaccine for non-cultivable NoV.

The rationale for combining GII-4 VLPs with GI-3 VLPs to generate a multivalent vaccine against NoV is based on the following assumptions: Firstly, in order to stimulate immune responses – especially blocking antibodies – against both GI and GII NoVs, at least one genotype from each genogroup should be included because of the lack of cross-reactivity among the genogroups (25,119,122,252,254); secondly, the multivalent vaccine strategy likely generates higher immune responses against genotypes not included in the vaccine than any of the homotypic vaccines alone (119); and thirdly, the rationale for selection of the genotypes GII-4 and GI-3 is based on the prevalence of these genotype infections in Finnish children (13,14), and also the worldwide predominance of GII-4 both in children and adults (8,12).

We detected a comparable homotypic IgG response in mice immunized with either a combination VLP vaccine or monovalent VLP vaccines, which indicated that the two NoV components in the cocktail did not interfere the humoral immune response to each VLP. Moreover, the presence of the RV antigen (VP6 protein) did not diminish the NoV-specific responses. We observed that the antibody cross-reactivity profiles generated by the combination vaccine were at least of a similar magnitude in the monovalent vaccine-immunized mice. In fact, we found evidence that some of the cross-reactive antibody levels were elevated against non-vaccine genotypes by the combination vaccine, which is in line with the observations published by LoBue et al. (119). The reason is probably that the antigenic epitope number is a collection of all the B cell epitopes present in the VLPs included in the cocktail and thus a more heterogenic B cell population is triggered in comparison to the monovalent vaccines. As a consequence, the likelihood of the antibody pool to recognizing the epitopes from heterotypic NoV genotypes increases.

Most importantly, we observed the blocking potential of the combination vaccine-immunized mice serum extending also to heterotypic VLPs from both genogroups I and II, which is something that could not be achieved with the single VLP immunizations. Based on these experiments in mice, it seems that our vaccine components hold a high number of the cross-reactive blocking epitopes, which is important for the broad neutralization capacity of the NoV vaccine. According to recent findings by our laboratory, our GII-4 VLP component in particular seems to possess many important conserved blocking epitopes in the GII-4 lineage (254). As the GII-4 genotypes predominate, it is reasonable to make efforts for the achievement of broad protection in the GII-4 lineage. In the best scenario, a protective NoV vaccine against GII-4 genotypes would prevent approximately 80%

of NoV-related AGE and restrain worldwide pandemics, resulting in a huge health and economic saving (280). In a recent phase I/II clinical study, it was detected that GII-4 VLPs did not confer protection from the heterovariant NoV infection but instead protected against NoV disease (48,54).

As NoV infection occurs through the gastrointestinal route, it is essential that the antiviral antibodies are present at these sites. In mucosal surfases – such as the gut – secretory-IgA is the major Ig class responsible for the first-line defence against mucosal pathogens. However, as anti-NoV IgA is described to be very type-specific (226) and has not been shown to be protective against heterologous infection (209), the intestinal anti-NoV IgG might instead provide broader mucosal immunity. Chachu et al. reported that intestinal IgG is important in protection from MNV infection (41). The protective role of mucosal IgG has been shown for other mucosal pathogens as well (309,310). The combination vaccine administered parenterally (IM) triggered an intestinal NoV-specific IgG response in mice. In another pre-clinical study, a predominately IgG subtype response against NoV was induced in the gut of mice immunized parenterally (SC) by the multivalent VRP vaccine (23).

The source of intestinal IgG might be the active receptor-mediated transport of systemic IgG via FcRn receptors present in polarized mammalian epithelial cells (311-313). Thus, robust serum IgG might be a direct correlate to the presence of intestinal IgG. For influenza, it has been shown that IM immunization triggers IgG-mediated mucosal immunity, and the high antigen-specific IgG content in serum and mucosa correlates with protection in mice (309). The action of anti-NoV IgG in the intestine is likely neutralizing, thus preventing the attachment of NoV on the HBGAs present on the intestinal cells. Furthermore, one possible mechanism could involve the bi-directional recycling of anti-viral IgG across mucosal cells via FcRn-receptors. The IgGs can opsonize the pathogen in the gut lumen following the FcRn receptor-mediated transport of the immune complexes back into the lamina propria where they are further processed by APCs and presented to T cells in draining lymph nodes (311-313). This mechanism for FcRn-mediated transport has been observed e.g. for HIV-1 in the genital tract (314). Although this mechanism has not been shown for NoV, the systemically-induced IgG transport in the gut might be important in strengthening broad T cell responses upon natural NoV encounters.

Although the neutralizing IgG is likely very important in protective immunity from NoV, one should not forget the possibly crucial role of cellular immunity in the control of NoV infection, especially in cross-protection. Cell-mediated immunity could be important in controlling the progress of NoV infection, as demonstrated in the MNV-mouse model (41,271). We showed that the combination vaccine

efficiently stimulated homologous and heterologous T cell responses in mice. T cell responses are likely more cross-reactive than neutralizing B cell responses, as T cell epitopes are derived from more conserved inner parts of the capsid (270). Indeed, a high IFN-γ response to peptides derived from heterologous genotypes were observed in both monovalent and combination VLP vaccine-immunized mice. Presumably, the cell-mediated immunity could compensate for the lack of cross-reactivity of blocking antibodies and direct the protection especially towards the heterologous strains.

We demonstrated that both humoral and cell-mediated immune responses induced by the combination VLP vaccine were long-lasting in mice. We could detect high levels of anti-NoV antibodies for six months after the immunizations, and although the magnitude of the response was slightly decreased from the one observed in the short-term study, the sera still possessed neutralizing activity to homologous and heterologous genotypes. In future studies, it should be evaluated whether the B_M cells can be activated to rapid (neutralizing) antibody production if encountered by the NoV antigens. For T cell immunity, we showed that there was no statistical difference in the production of IFN-γ after six months in comparison to the five-week immunization schedule, which suggests that at least T_M cells were efficiently elicited.

Taken together, the blocking antibodies are likely important in protecting against NoV infection, but cell-mediated immunity might be important in cross-reactive immunity and in controlling the progress of NoV disease. Because we still do not know precisely which of the immune mechanisms leads to protective immunity to NoV, it would be wise to proceed with a vaccine candidate that is able to stimulate both the humoral and cellular arms of adaptive immunity and has the potential to promote long-term immune responses. According to our findings from preclinical studies in mice, we have shown that our NoV-RV trivalent combination vaccine has this potential, and it should next be evaluated in clinical studies.

7 Conclusions

The aims of this dissertation can be divided in two parts; first, we conducted a serological study on NoV to assess the need and serological background for NoV vaccinations in Finnish children. The major part of this dissertation, however, evaluated the various aspects of the immunogenicity of non-live NoV antigens (VLPs and P-particles) in mice as monovalent vaccines and VLPs in a trivalent combination vaccine with a RV VP6 protein. Moreover, the importance of blocking antibodies in preventing NoV infection was addressed in children, and the ability of the NoV subunit particle-based vaccines to raise blocking antibodies was evaluated in mice. In addition, the importance of OAS in *de novo* immune response generation was briefly investigated. The main conclusions of this dissertation are the following:

- Finnish children acquire NoV antibodies early in life and seroprevalence increases rapidly with age. Maternal IgG antibodies are present in the sera of young infants and they might be of a short-term protective nature. After the maternal antibodies wane, children 6–23 months seem most vulnerable to NoV infections. Early infections do not generate strong protective immunity; instead, recurrent NoV infections are likely to be common in childhood as the levels of NoV-specific antibodies and the avidity increase with age. Children should be vaccinated early to enhance the generation of protective immunity against NoV.
- Based on preclinical immunogenicity studies in mice, NoV VLPs are superior in comparison to P-particles in inducing broad humoral and cellular immune responses, and they should be selected as the base for a non-live NoV subunit vaccine.
- OAS in the generation of humoral immune responses against NoV was not detected in mice. Thus, a possible NoV vaccine should be able to stimulate strong immune responses without concern of an immune response skewing to the originally encountered NoV strain or the vaccination impairing future immune responses to heterologous NoV genotypes.

- The presence of antibodies in the serum that block the binding of NoVs to the putative HBGA receptors correlates with protection in children. The homotypic and cross-blocking antibodies were efficiently triggered in mice by NoV VLPs.
- A trivalent NoV-RV vaccine consisting of GII-4, GI-3 VLPs and RV VP6 antigens is a potent vaccine against NoV. In mice, it generated both major NoV genogroups (GI and GII) extending humoral immunity with a neutralizing ability. In addition, the combination vaccine stimulated strong cellular immunity, which is possibly important in cross-reactive immune responses. In addition, two administrations of the vaccine generated long-term immunity. All of the points listed meet the requirements for a potentially successful NoV vaccine and support the testing of a trivalent NoV-RV combination vaccine in clinical trials in the future.

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Prevalence of Norovirus GII-4 Antibodies in Finnish Children

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Noroviruses (NoVs) are the second most common cause of viral gastroenteritis after rotavirus in children. NoV genotype GII-4 has emerged as the major type not only in outbreaks of NoV gastroenteritis but also endemic gastroenteritis among infants and young children worldwide. Using baculovirus-insect cell system virus-like particles (VLPs) of NoV genotype GII-4 and an uncommon genotype GII-12 were produced. These VLPs were used in enzyme-linked immunosorbent assays (ELISA) for detection of NoV-specific immunoglobulin G (IgG) and IgA antibodies in 492 serum specimens from Finnish children 0–14 years of age collected between 2006 and 2008. NoV IgG antibody prevalence was 47.3% in the age group 7–23 months and increased up to 91.2% after the age of 5 years. Avidity of NoV IgG antibodies was low in the primary infections while high avidity antibodies were detected in the recurrent infections of the older children. In GII-4 infections, the homologous antibody response to GII-4 VLPs was stronger than to GII-12 VLPs but cross-reactivity between GII-4 and GII-12 was observed. Binding of GII-4 VLPs to a putative carbohydrate antigen receptor H-type 3 could be blocked by sera from children not infected with NoV during a waterborne outbreak of acute gastroenteritis. Therefore, protection against NoV infection correlated with strong blocking activity. ***J. Med. Virol. 83:525–531, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: NoV; antibody responses; ELISA; antibody avidity; blocking antibodies

INTRODUCTION

Noroviruses (NoVs) are common causative agents of acute non-bacterial gastroenteritis in children and adults worldwide [Green, 2007]. After rotavirus, NoVs are the major cause of pediatric gastroenteritis, often requiring hospitalization [Pang et al., 1999; Moreno-Espinosa et al., 2004]. NoVs belong to a genetically diverse family of *Caliciviridae* and there are currently at least 25 NoV genotypes infectious to humans, of which eight belong to genogroup GI and 17 to genogroup GII [Zheng et al., 2006]. The prevalence of genotypes fluctuates annually [Gallimore et al., 2007] but during the last decade variants of the GII-4 genotype have become the dominant genotypes in NoV epidemics worldwide [Siebenga et al., 2009].

The inability to grow NoVs in in vitro cell cultures [Duizer et al., 2004] has impeded the development of serotype-specific antibody assays. Thus, NoV capsid antigens used in serological diagnostic assays have to be produced by recombinant protein production techniques including baculovirus-insect cell expression system [Jiang et al., 1992]. NoV capsid genes are cloned into the baculovirus genome and expressed in insect cells to yield capsid proteins which self-assemble to form virus-like particles (VLPs), mimicking the structural, antigenic and immunogenic properties of the native virus [Jiang et al., 1992].

NoV seroprevalence studies using NoV VLPs have been conducted in several countries worldwide [Gray et al., 1993; Lew et al., 1994; Nakata et al., 1998; Jing et al., 2000; Talal et al., 2000; Kobayashi et al., 2009]. The prevalence of NoV-specific antibodies has been observed to follow an age-related pattern [Gray et al., 1993; Jing et al., 2000; Talal et al., 2000]; children acquire antibodies against NoV at an early age and almost 100% of adults have NoV-specific antibodies [Jiang et al., 2000].

To distinguish primary from recurrent infections, the avidity of the immunoglobulin G (IgG) antibodies may be determined [Gutierrez and Maroto, 1996; Kanno and

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Kazuyama, 2002]. The significance of antibody level or avidity as a correlate for protection from NoV infection is not clear but some studies have shown that high pre-existing IgG levels may be protective in children but not in adults [Ryder et al., 1985; Gray et al., 1994; Lew et al., 1994]. In the absence of a cell culture model, a surrogate NoV neutralization antibody assay is used to demonstrate the ability of antibodies to block the binding of NoV VLPs to ABH histo-blood group antigens (HBGAs) [Rockx et al., 2005; Cannon et al., 2009]. HBGAs likely play an important role in virus entry to the gut mucosal cells being the docking site or receptor for NoVs [Harrington et al., 2002]. High blocking ability of the antibodies has been suggested to correlate with protection against infection [Harrington et al., 2002; Lindesmith et al., 2003; LoBue et al., 2006].

The purpose of this study was to determine the seroprevalence of NoV GII-4 IgG and IgA antibodies in Finnish children, and to study if the magnitude and the avidity of the antibodies correlate with susceptibility to infection. In addition, cross-reactivity of NoV antibodies between a common (GII-4) and a rare (GII-12) genotype was investigated.

MATERIALS AND METHODS

Study Material

A total of 492 acute stage sera were collected from 0 to 14 year-old children hospitalized for acute gastroenteritis (AGE) in Tampere University Hopital and Kuopio University Hospital from August 2006 to August 2008. The study protocol and consent forms had been approved by the ethics committee in the Pirkanmaa hospital district in 2006 and the patients or the legal guardians volunteered for the study after having given an informed consent. NoV infection was detected in the stool samples of 97 children by reverse transcription PCR which is described in details elsewhere. Paired serum samples were obtained from 14 of the children with NoV infection. Acute sera were collected within 5 days and convalescent sera 2–6 weeks after onset of the disease. In addition, acute sera from six adults were collected as a control. The sera were kept at -20°C before testing.

Antigens

Recombinant capsid proteins in the format of VLPs of the two NoV genotypes, GII-4 [004/95M-14/1995/AU] and GII-12 [HU/NLV/Wortley/90/UK] were used as coating antigens in enzyme-linked immunosorbent assays (ELISA). The VLPs were produced using baculovirus-insect cell expression system as previously described [Jiang et al., 1992]. VLP production and purification are described in details elsewhere [T. Koho et al., manuscript in preparation]. The morphology and the antigenicity of the VLPs produced were verified with electron microscopy and Western blot (data not shown).

Serum Antibody ELISA and Avidity Assay

Sera were tested for anti-GII-4 and anti-GII-12 IgG and IgA by ELISA as previously described [Iritani et al., 2007] with modifications. VLPs were used to coat (4°C overnight) 96-well microtiter plates (Nunc Immuno Maxisorp, Thermo Fisher Scientific Inc., Waltham, MA) in 0.01 M carbonate–bicarbonate buffer (pH 9.6) at a concentration 0.5 μg/ml for IgG and 2 μg/ml for IgA detection (100 μl/well). The optimal antigen concentration for coating was determined in preliminary experiments by testing serial dilutions of the antigens with positive and negative sera and plotting saturation curves. After washing three times with 10 mM phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) the plates were blocked at room temperature (RT) for 1 hr with PBS containing 5% skimmed milk (Sigma–Aldrich, St. Louis, MO). The wells were then washed three times with PBS-T and incubated 1 hr at 37°C with 100 μl of serum diluted 1:100 or two-fold dilution series in PBS-T containing 1% skimmed milk. All serum samples were tested in duplicate wells. After washing six times, horseradish peroxidase (HRP) conjugated anti-human IgG or IgA (Zymed, Invitrogen, Carlsbad, CA) diluted 1:4,000 in 1% milk in PBS-T was added to the wells. After incubation (1 hr, 37°C) the plates were washed and *o*-phenylenediamine dihydrochloride (SIGMA*FAST* OPD, Sigma–Aldrich) substrate was added at a concentration of 0.4 mg/ml. The plates were incubated at RT in the dark for 30 min and the reaction was stopped with 2 M sulfuric acid (H_2SO_4). Absorbance (optical density, OD) at a wavelength of 490 nm was measured in a microplate reader (Victor^2 1420, Perkin Elmer, Waltham, MA). One known positive and one known negative serum sample was added to all plates as controls. Background signal from the blank wells (wells without serum) was subtracted from all of the OD readings at a plate. A sample was considered positive if the net absorbance value was above the set cut-off value, calculated as follows: $\geq 2\times$ mean OD readings from the negative control serum wells at the same dilution and at least 0.100 OD. Negative antigen control wells included Sf-9 mock baculovirus infected insect cell lysate and were routinely <0.100 OD. A seroconversion was considered as a $\geq$4-fold rise in the titer between acute and convalescent sera.

To determine the IgG avidity of NoV antibodies a urea elution was used to remove the low avidity antibodies [Kanno and Kazuyama, 2002]. After incubation of sera on VLP-coated plates the sera were aspirated from the plate and 8 M urea in PBS-T was added. After 5 min of incubation the treatment was repeated. Plates were washed four times prior to the addition of HRP-conjugated anti-human IgG as described above. The avidity index was calculated as [OD with urea/OD without urea] $\times$ 100% and the index value $>50\%$ was considered as high avidity.

Carbohydrate Blocking Assay

The assay for blocking the binding of HBGAs to NoV VLPs with human serum was performed as previously described [Harrington et al., 2002]. Briefly, microtiter plates were coated with GII-4 VLPs in PBS (pH 7.2) at a concentration of 2 μg/ml and incubated 4 hr at RT. After washing, the plates were incubated with 5% milk in PBS overnight at 4°C for blocking. Sera diluted 1:100 were added to the wells and the plates were incubated 1 hr at 37°C and then aspirated from the plate. Biotinylated H-type-3 or Lewis B (100 μl, for negative control; Lectinity Holdings, Inc., Moscow, Russia) was added at a concentration of 40 μg/ml in 1% milk in PBS-T. After 4 hr at 37°C the wells were washed and a 1:2,000 dilution of streptavidin-conjugated HRP (Thermo Fisher Scientific Inc.) was added and incubated 1 hr at 37°C. The development of the color reaction and the measurement of absorbance at a wavelength 490 nm were conducted as described above. OD reading from the wells incubated without serum was considered as a maximum signal for the binding of H-type 3 to GII-4 VLP. No binding of VLPs to the negative control HBGA (Lewis B) was detected. Background signal from the blank wells (wells lacking HBGA) was subtracted from all of the OD values of tested samples.

Statistical Analyses

Pearson's chi-square test was performed to examine the differences in antibody prevalence between age groups. Student's *t*-test was used to assess the intergroup differences in antibody magnitude and avidity. All hypothesis testing was two-tailed. Pearson's correlation coefficient was used to determine the correlation between GII-4 and GII-12-specific antibody levels. Mann–Whitney *U*-test was used to compare the intergroup differences in the blocking ability of the sera. Statistical significance was defined as $P < 0.05$.

RESULTS

Overall and Age-Related Serum IgG and IgA Antibodies Against GII-4

Of the 492 serum specimens 63.4% and 38.9% sera were positive for GII-4-specific IgG and IgA, respectively. The age-related seroprevalence is shown in Figure 1. The prevalence of IgG antibodies to GII-4 was 66.7% in the age group 0–6 months and 47.3% in the age group 7–23 months. After the age of 2 years the prevalence increased to 73.1% and continued to increase up to 91.2% in children 5–14 years of age. The NoV IgG antibody prevalence in the age group 7–23 months was significantly lower compared to the other age groups ($P < 0.001$–0.05). The corresponding seroprevalence for GII-4-specific IgA antibodies was low (20.8%) in infants 0–6 months of age and rose with increasing age. Overall the prevalence for IgG was significantly higher in each age group compared to IgA seroprevalence ($P < 0.001$–0.05). Samples positive for both IgG and IgA were detected in 16.8% of 0–6 months old

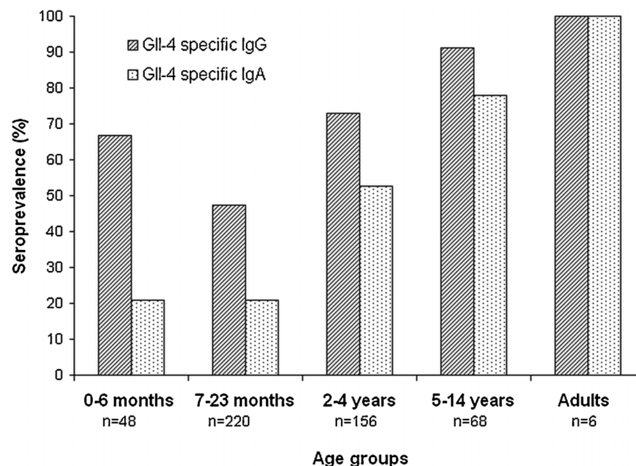

Fig. 1. Age-related prevalence of IgG and IgA serum antibodies to NoV GII-4 in Finnish children and adults.

infants, 20.5% of 7–23 months old, 51.3% of 2–4 years, and 79.4% of 5–14 years old children (data not shown). IgG and IgA GII-4-specific antibodies were detected in 100% of the adult sera (Fig. 1).

Antibody Avidity

The avidity indexes of NoV IgG antibodies in the three age groups representing newborns (0–6 months of age), presumable primary/recent infections (7–12 months of age), and recurrent infections (9–14 years of age) are shown on Figure 2. High avidity IgG antibodies (mean avidity index 61.5 ± 5.1%) were detected in infants ≤6

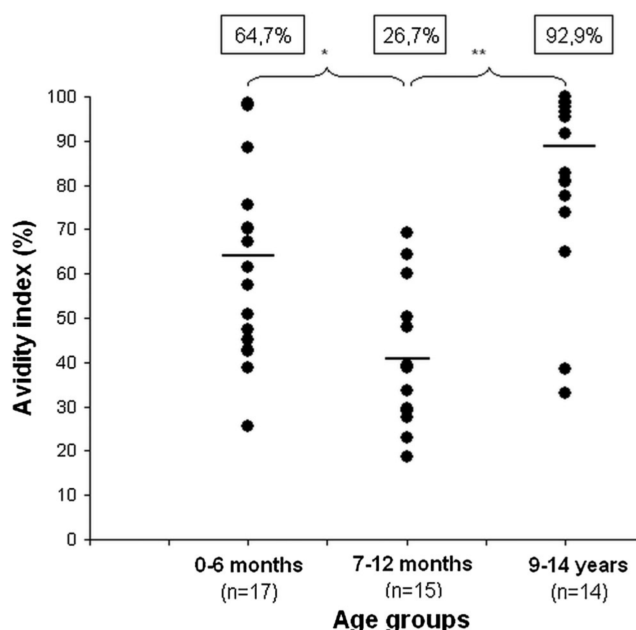

Fig. 2. Avidity indexes of IgG antibodies to NoV GII-4 in three age groups. Bars represent the mean avidity index of the group. Boxed values indicate the frequency (%) of high avidity antibodies in the age group. $^{*}P < 0.05$, $^{**}P < 0.0001$ (Student's *t*-test).

months of age. The mean avidity index was significantly lower in children aged 7–12 months (40.4 ± 3.9%) than in children aged 0–6 months ($P < 0.05$). The highest of all mean avidity indexes (85.6 ± 4.5%) were detected in older children, who had most probably encountered several NoV infections during their lifetime. The lowest frequency of the high avidity antibodies was detected in the primary infections whereas the highest frequency was observed in the recurrent infections ($P < 0.001$). All the adult control sera (n = 6) contained high avidity antibodies (data not shown).

Cross-Reactivity of NoV Antibodies

To determine the immunological cross-reactivity of the sera the seroprevalence was assayed against GII-12 which so far has not been reported in Finland [Maunula and Von Bonsdorff, 2005]. Of the GII-4 positive sera 70.8% were also IgG positive against GII-12 VLPs. A subset of GII-4 IgA positive samples (96 out of 197) was tested and found that 47.6% of these were IgA cross-reactive for GII-12. When the GII-12 antibody responses were compared to the responses obtained with GII-4 VLPs a positive correlation was detected overall (r = 0.64) although the mean ODs were generally lower for GII-12-specific responses (data not shown). Sera of the adult individuals were all positive for GII-12 (data not shown). Cross-reactivity between the two genotypes was further confirmed using paired sera of three patients infected with GII-4 NoV (Fig. 3). All the paired sera showed IgG seroconversion not only to GII-4 (Fig. 3A) but also to GII-12 antigen, although with lower magnitude of the response (Fig. 3B).

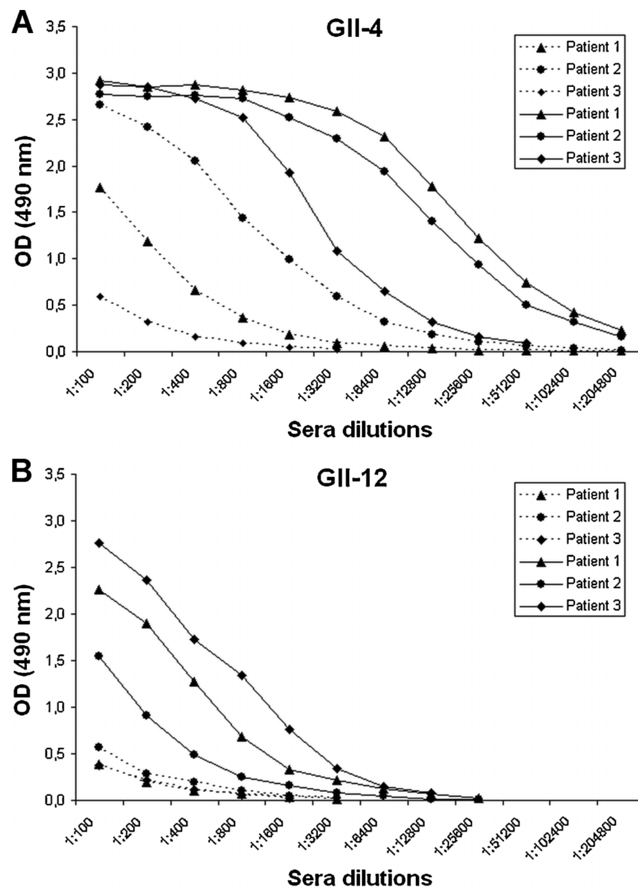

Fig. 3. Serum titration of three NoV GII-4-infected pediatric patients against NoV VLPs. Sera were diluted two-fold starting from 1:100 and tested in an ELISA using GII-4 VLPs (**A**) or GII-12 VLPs (**B**) as antigens. Acute sera titrations are shown in dotted lines and convalescent sera titrations in full lines.

Correlation of NoV Infections to Antibody Prevalence and Levels

Of the 492 children encountered in hospital because of AGE, 97 (19.7%) were diagnosed with NoV infection by reverse transcription-PCR. The incidence of NoV gastroenteritis was highest in children aged 7–23 months (53 out of 97 cases; 54.6%). Of these cases 60.4% were primary infections as judged by the absence of NoV antibodies at the acute stage while 39.6% had pre-existing antibodies to NoV. To determine the possible protective effect of the pre-existing NoV-specific antibodies against a subsequent NoV infection the OD values in the different age groups obtained in ELISA IgG assay were compared. The lowest pre-existing antibody level (mean OD 0.66 ± 0.06) as well as antibody prevalence (47.3%, Fig. 1) were detected in the age group 7–23 months (Fig. 4). In the age group 2–4 years mean OD level increased (1.11 ± 0.07) and by the age of 14 years children had high levels of pre-existing antibodies to NoV (mean OD 1.62 ± 0.10) in the majority of individuals (91.2%, Fig. 1) and the least infections (14.7%; Fig. 4). Therefore, a trend to a negative association between the incidence of infection and the level of pre-existing antibodies was observed.

Antibody Blocking

Acute sera obtained from children infected during a waterborne outbreak of AGE in the town of Nokia, Finland [Räsänen et al., 2010] with NoV GII-4 or other agent causing acute gastroenteritis were tested for blocking of the GII-4 VLPs binding to H-type-3 HBGA. Higher blocking activity was seen in the sera of children not infected with NoV than in the sera of children with NoV infection ($P < 0.05$) (Fig. 5A). In addition, blocking of the VLPs binding to H-type-3 antigen was performed with 1:100 dilutions of paired sera from eight children with NoV gastroenteritis (Fig. 5B). The blocking activity increased after a recent NoV infection. The acute sera could block on average 24.8% of the maximum GII-4 binding to H-type 3 whereas 61.6% of the binding was blocked by the convalescent sera ($P < 0.05$).

DISCUSSION

A study conducted 20 years ago to determine the Norwalk virus (GI-1) seroprevalence in young Finnish children [Lew et al., 1994] showed that 49% of children 4–23 months of age had NoV antibodies. At that time

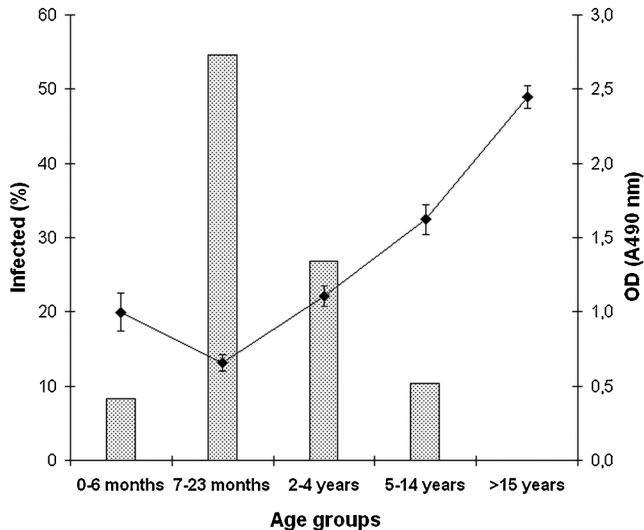

Fig. 4. Age distribution of frequency of cases of NoV infections encountered in hospital and the levels of NoV GII-4-specific IgG antibodies by age group. The bars represent the percentages of NoV-infected children in each age group of 97 total infection cases. The mean OD values of each group sera (1:100 dilution) tested by ELISA are shown (◆) on the secondary y-axis.

NoV GII-4 was not common. Nearly identical seroprevalence for GII-4 in the similar age group was found in the present study. The data therefore confirm that Finnish children acquire NoV infection at an early age and with increasing age the number of natural encounters with the virus accumulates, as judged by the increasing seroprevalence and the prevalence of high avidity antibodies. Prospective studies in Finland have shown that clinically manifest NoV gastroenteritis is seen in 20–30% of Finnish children by the age of 2 years [Pang et al., 1999; Zeng et al., 2010]. As the seroprevalence increases even more rapidly, it is likely that many NoV infections are subclinical. The prevalence of NoV antibodies in children has been reported in several European countries, Asia, Africa, and Latin America [Gray et al., 1993; Nakata et al., 1998; Jing et al., 2000; Talal et al., 2000; Farkas et al., 2006; Kobayashi et al., 2009] and the acquisition of NoV GII-4-specific antibodies, especially in the developed countries, is consistent with the results of the present study [Pelosi et al., 1999; Nicollier-Jamot et al., 2003]. Infants under 6 months of age typically have high antibody levels, which are explained by the presence of maternal antibodies [Gray et al., 1993; Numata et al., 1994]. After 6 months of age, when the maternal antibodies decline, the seroprevalence decreases and starts rising again after 12–24 months of age reaching 90–100% in older children and adult population [Matsui and Greenberg, 2000].

All the adult sera and the majority of children's sera tested in this study recognized both GII-4 and GII-12 VLPs in ELISA, although the level of reactivity was higher against GII-4. The likely reason may be cross-reactive epitopes rather than previous exposure to NoV GII-12 as GII-12 genotype has not been reported in Finland [Maunula and Von Bonsdorff, 2005]. In addition, these results imply the existence of highly conserved antibody epitopes between the genotypes within a genogroup. We also showed that a seroconversion caused by GII-4 infection could be detected with a heterotypic antigen (GII-12) although the response to homotypic VLPs was stronger. Other reports also found that seroconversion can be observed in convalescent sera using VLPs from genetically distinct virus from the one causing the primary infection [Belliot et al., 2001]. Despite the cross-reactivity, greater seroresponses are detected against homologous strain, and strains belonging to the same genogroup react more strongly than the strains across the genogroups [Treanor et al., 1993; Farkas et al., 2003; Lindesmith et al., 2010]. Because of the cross-reactive immunity and the presence of the pre-existing antibodies detected, ELISA method as the one described in this study and routinely used by others [Rockx et al., 2005; Iritani et al., 2007] does not give precise serotype-specific determination of the immune response.

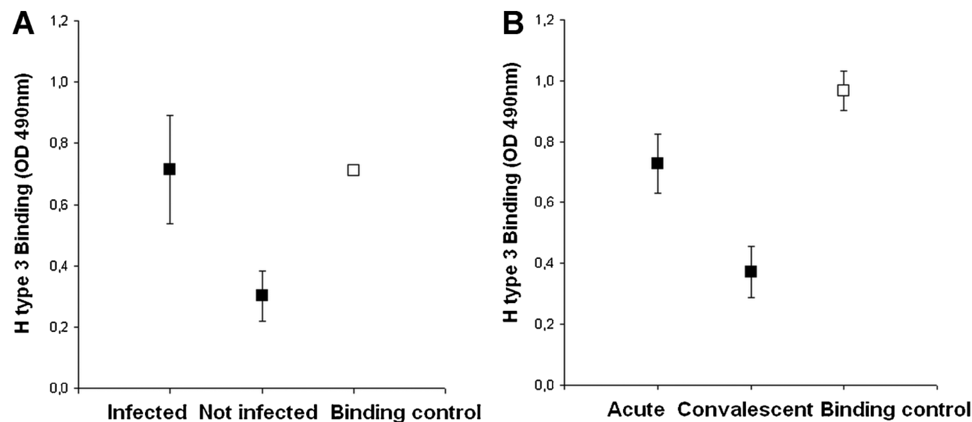

Fig. 5. Blocking of NoV GII-4 VLPs binding to H-type-3 HBGA by the acute sera of NoV GII-4 infected (n = 9) or not infected (n = 6) children (**A**), and by the paired sera from children (n = 8) infected with NoV GII-4 (**B**). Shown are mean OD values of the tested sera (1:100 dilutions). The mean OD of the binding control indicates the maximum binding of the GII-4 VLPs to H-type-3. Error bars represent the SEM.

The avidity and the level of NoV-specific IgG antibodies are low after primary infections in young children. The avidity increases with time and particularly as a result of repeated infections when molecular evolution specifies the antigen binding domains of the antibodies to recognize the target epitopes more precisely [Murphy et al., 2008]. Indeed, high avidity antibodies were detected in older children and adults who are likely to have experienced NoV infections repeatedly throughout life. The high avidity antibodies detected in infants <6 months of age presumably represent transplancentally acquired maternal IgG antibodies [Gray et al., 1993; Numata et al., 1994]. The results show that 20% of infants under 6 months of age already had NoV infection as judged by the presence of NoV IgA antibodies. Such incidence of infection suggests that transplacentally acquired IgG antibodies do not offer full protection.

The present study showed that children who were exposed to NoV in a waterborne outbreak of AGE [Räsänen et al., 2010] but were not infected by NoV, had higher blocking activity of GII-4 VLPs binding to H type 3 HBGA compared to those who were infected. Interestingly, the acute sera from not-infected children had similar blocking ability as the convalescent sera from children recently suffering from a NoV infection. The increase in the blocking ability of the serum after the NoV infection has been hypothesized to be associated with a short-term immunity to NoV infection [Rockx et al., 2005]. The results shown in the current study also suggest that high blocking activity of VLPs binding to HBGA in the acute serum may confer protection against reinfection. Blocking activity of the antibodies may be a relevant surrogate marker of NoV protection when considering different vaccine approaches.

In this study, the majority of sporadic NoV cases treated in hospital occurred in children 7–23 months of age. This age group also had the lowest pre-existing antibody levels. Therefore, a trend to a negative correlation between the susceptibility to NoV infection and pre-existing IgG antibodies was observed. A previous study conducted in Finland [Lew et al., 1994] suggested that children with low NoV-specific IgG titers (<1:50) are more susceptible to NoV infection in the next 10 months than children with higher titers (>1:50). In the current study it was shown that older children with higher NoV-specific antibody levels and avidity had also fewer infections than children <2 years old. It may be speculated that NoV infections before the age of 2 years induce protection for later ages as the children develop protective immune response to NoV. This is in support of the concept of NoV vaccination in young infants in order to decrease the burden NoV of infection as well as the hospitalization cases due to NoV gastroenteritis.

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A comparison of immunogenicity of norovirus GII-4 virus-like particles and P-particles

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Summary

Norovirus (NoV) -derived virus-like particles (VLPs) resemble empty shells of the virus and NoV P-particles contain only protruding domains of the NoV capsid. Both NoV-derived subviral particles show similar functionality and antigenicity *in vitro* and are considered to be potential vaccine candidates against NoV gastroenteritis. BALB/c mice were immunized with baculovirus-produced GII-4 VLPs or the corresponding *Escherichia coli*-produced P-particles by the intramuscular or intradermal route and the NoV-specific antibody and T-cell immune responses were compared. Elevated antibody levels were induced with a single VLP immunization, whereas P-particle immunization required a boost. High avidity antibodies were raised only by VLP immunization. VLP immunization resulted in a balanced T helper type 1/type 2 immune response whereas P-particles induced a T helper type 2-biased response. Only VLP immunization primed T cells for interferon-γ production. Most importantly, cross-reactive B and T cells were induced solely by VLP immunization. In addition, VLP antiserum blocked the binding of heterotypic VLPs to human histo-blood group antigen receptor and saliva. The findings in this study are relevant for the development of NoV vaccines.

Keywords: histo-blood group antigen blocking; immune response; norovirus; P-particles; virus-like particles

Introduction

Noroviruses (NoVs) are the leading cause of non-bacterial acute gastroenteritis worldwide. Most of the NoVs that infect humans belong to the genogroups GI and GII[1] and genetic variation among NoV genotypes is wide.[2] At present most NoV outbreaks and sporadic cases are caused by genogroup GII viruses, especially by genotype GII-4.[3] The NoVs are non-enveloped viruses and have an outer capsid consisting of a single structural protein, the capsid protein (VP1) organized into 90 dimers, which exhibit $T = 3$ icosahedral symmetry.[4] The capsid proteins have two major domains, a shell (S) domain and protrusion domains (P), which are linked together by a short hinge (H) region. The S-domain is responsible for the formation of the interior shell from which arch-like P-dimers extend outwards.[5] The P-domain consists of P1 and P2 subdomains located on the surface of the capsid. The majority of the genetic variation is identified in the P2 domain.[4,5] Mutagenesis[6] and X-ray crystallography[7] studies have shown that P2-domain binds to histo-blood group antigen (HBGA), which is the putative NoV docking site and receptor for entry into the host cell.[8]

Norovirus VP1 proteins have the ability to self-assemble to form virus-like particles (VLPs) deprived of viral genetic material, which morphologically and antigenically resemble the native virus.[9] Different expression systems have been developed to produce the capsid in the form of VLPs. Most commonly, recombinant baculoviruses are used to express NoV capsid proteins in insect cells.[9] P-domains alone have also been expressed *in vitro*, typically in a bacterial *Escherichia coli*-based cloning and expression system.[10–12] The P-domain monomers form dimers *in vitro* which can further self-assemble into larger complexes, P-particles, consisting of 12 P-dimers having the total molecular weight of 830 000.[13] The relevance of the *E. coli* system is that large quantities of a recombinant protein can be produced at low cost.[11] Furthermore, linking the P-domain genetically with an affinity tag makes the purification process reasonably straightforward.

Morphological and biological characterization of NoVs has been challenging because of the lack of a cell culture

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system.[14] Use of the two subviral particles, VLPs and P-particles, has added greatly to the understanding of the NoV structure and biology. Several studies, including our own, showed similar functionality and antigenic properties of recombinant NoV VLPs produced by the baculovirus expression system and recombinant P-particles produced in *E. coli*.[10,11,13,15] In particular, HBGA binding studies revealed that P-particles are able to bind to the HBGA receptor with intensity similar to that of the corresponding VLPs.[15] In addition, a hyperimmune animal serum raised against the P-domain is able to recognize NoV VLPs and can be used, for example, in various enzyme immunoassays.[10,15] The P-particles have also been proposed as an alternative to VLP-based vaccine development against NoV gastroenteritis because they have been shown to induce antibody responses in mice able to block binding of NoV VLPs to the HBGA receptor.[15,16] In general, VLP are very immunogenic and various VLP-based vaccine candidates to other viruses (i.e. hepatitis B virus, human papillomavirus, influenza virus, parvovirus, HIV etc.) have been found to be safe, immunogenic and effective in pre-clinical and clinical trials and some of them have already been commercialized.[17]

The present study compares the *in vivo* immunogenicity of the two potential NoV subunit vaccine candidates, GII-4 VLPs and GII-4 P-particles in BALB/c mice. Despite earlier findings of similar antigenic and receptor-binding properties described above, our results demonstrate the superiority of the VLPs in the induction of a T helper type 1 (Th1) and Th2 balanced cross-reactive immune response compared with the P-particles.

Materials and methods

Production and purification of baculovirus-expressed NoV VLPs and E. coli-expressed P-particles

The NoV GII-4 (1999, GenBank ID: AF080551), GII-4 New Orleans (GII-4 NO, 2010, GenBank ID: GU445325), GII-12 (1998, GenBank ID: AJ277618) and GI-3 (2002, GenBank ID: AF414403) VLPs used in immunizations and as antigens in ELISAs were expressed in a BV–insect cell system and purified by sucrose gradients as described earlier.[10,18] Polyhistidine-tagged P-proteins were produced in *E. coli* and the protein was isolated by Ni-NTA affinity chromatography as described in detail elsewhere.[10] The purity of the VLPs and P-proteins was verified by SDS–PAGE.[10,18] The morphology and the integrity of the VLPs and the P-protein formation in P-particles were verified by electron microscopy (Fig. 1). The double-stranded DNA (dsDNA) content of the VLP preparation was determined by the Quant-it dsDNA Broad-Range Assay kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and found to be 10 ng/dose. The functional and antigenic properties of both products were tested in an HBGA binding assay, Western blot and ELISA methods as published earlier.[10,18,19]

Study animals, immunization and sample collection

Female BALB/c OlaHsd mice were obtained from Harlan Laboratories (Horst, the Netherlands). The mice were 7 weeks old at the time of the first immunization. All procedures were authorized and performed according to the guidelines by the Finnish National Animal Experiment Board. The mice were anaesthetized before immunization with a formulation of Hypnorm (VetaPharma Limited, Leeds, UK) and Dormicum (Roche Pharma AG, Grenzach-Wyhlen, Germany). The mice were immunized (four to five mice/experimental group) twice, at week 0 and week 3 with 10 μg GII-4 VLPs or P-particles by a needle injection administered intramuscularly (IM) or intradermally (ID). Blood samples were collected from the tail vein at study weeks 0 (pre-immunization bleed), 2, 3 and 4. Sera from mice receiving no antigen (naive mice) were used as a negative control. Mice were killed at study week 5 and whole blood and lymphoid tissue were collected.

Blood and lymphoid tissue preparation

Tail blood and whole blood samples were centrifuged (Himac CT15RE; Hitachi, Twinsburg, OH) at 3500 ***g*** 20 min and the serum was separated and stored at −20°. Spleens from killed mice were collected in Hanks' balanced salt solution (HBSS) (Sigma-Aldrich, St Louis, MO). The structure of the spleen was disrupted with a scalpel and a single-cell suspension was prepared using a 70-μm cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ). Cell suspensions were centrifuged 300 × ***g*** for 10 min (Multifuge 3SR Plus; Heraeus, Wehrheim, Germany) and the cells were resuspensed in HBSS. The HBSS diluted 1 : 10 and pH balanced with 7·5% sodium bicarbonate (Sigma-Aldrich) was added to lyse the red blood cells and the molarity of the suspension was recovered with 2 × HBSS. The cells were washed in HBSS, counted in Bürker's chamber and stored in liquid nitrogen in sterile freezing medium (RPMI-1640 supplemented with 40% fetal bovine serum (FBS) and 10% DMSO, all from Sigma-Aldrich) for further use.

Synthetic peptides

Peptides that were 15 amino acids long covering a region of the P1 capsid domain of NoV GII-4 (CLLPQEWVQHFYQEA), GII-4 NO (CLLPQEWVQYFYQEA) and GII-12 (CLLPQEWIQHLYQES) corresponding to a T-cell epitope (amino acids 461–475) published earlier by LoBue *et al.*[20] and a rotavirus 14-mer VP6 peptide (amino acids 289–302, RLSFQLMRPPNMTP[21]) were synthesized by Proimmune Ltd. (Oxford, UK). The lyophilized peptides

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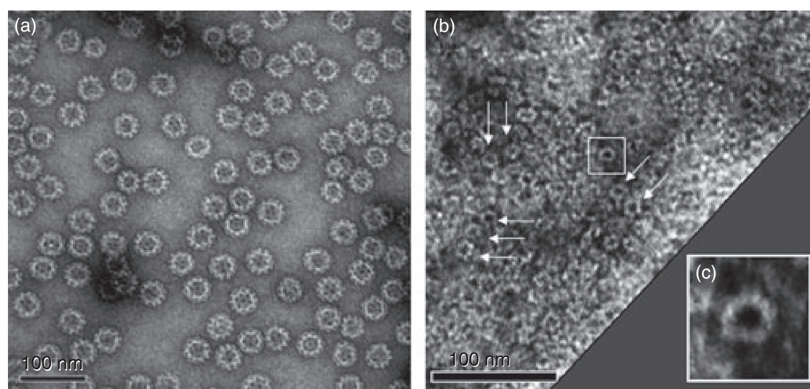

Figure 1. Electron microscopy images of purified norovirus (NoV) capsid GII-4 virus-like particles (VLPs) (a) and P-particles (b). Typical ring-shaped structures of P-particles are indicated with arrows. An enlarged image of a single P-particle (squared in panel b) is shown (c). VLPs and P-particles were negatively stained with 3% uranyl acetate (pH 4.5) and the preparations were examined using FEI Tecnai F12 electron microscope operating at 120 kV.

were dissolved in DMSO (Sigma-Aldrich) and diluted in working stock aliquots (0·1 mg/ml) in sterile PBS (Sigma-Aldrich) and stored at −80° for further use.

Serum IgG and IgG subtype ELISA

Sera from immunized and naive mice were tested for total IgG, IgG1 and IgG2a by ELISA. NoV GII-4, GII-4 NO, GII-12 and GI-3 VLPs in PBS were used to coat 96-well Nunc Immuno Maxisorp plates (Thermo Fisher Scientific Inc., Waltham, MA) (0·4–1 μg/ml). After blocking with 5% milk (Sigma-Aldrich) in PBS the serum samples diluted 1 : 200 or serial twofold dilutions were added to the plates and incubated for 1 hr at 37°. All the serum and secondary antibody dilutions were prepared in 1% milk + 0·05% Tween-20 in PBS and added to the wells at a volume of 100 μl. Between each step the plates were washed six times with 0·05% Tween-20 in PBS. For the detection of antigen specific IgGs, horseradish peroxidase (HRP) -conjugated anti-mouse IgG (Sigma-Aldrich) was used at a dilution of 1 : 4000. Anti-GII-4 IgG subtype responses were determined using goat anti-mouse IgG1 or IgG2a HRP conjugate (Invitrogen) at a dilution of 1 : 6000. The secondary antibody was incubated for 1 hr at 37° and *O*-phenylenediamine dihydrochloride (SIGMA-*FAST* OPD, Sigma-Aldrich) at a concentration of 0·4 mg/ml was used as a substrate for HRP. After 30 min the colour reaction was stopped with 2 M sulphuric acid. Absorbance (optical density, OD) at a wavelength of 490 nm was measured in a microplate reader (Victor2 1420; Perkin Elmer, Waltham, MA). Each plate, in addition to test sera, contained blank wells, a NoV GII-4/GII-4 NO/GII-12/GI-3-positive mouse serum and naive mouse serum in duplicate. Background signal (mean OD value from blank wells) was subtracted from all readings on the plate. Cut-off value was calculated from the OD values of the naive mice sera as follows: mean OD + 3 × SD. A sample was considered positive if the net OD value was above the set cut-off and at least 0·100 OD. End-point antibody titres were defined as the highest dilution of serum giving an OD above the set cut-off value. A Th2/Th1 response ratio was calculated by dividing the end-point titre of IgG1 response with the corresponding IgG2a titre.

Antibody avidity

An avidity assay to detect high avidity NoV antibodies was adopted from Rockx *et al.*[22] Briefly, the IgG ELISA was conducted as described in the previous section except that after the incubation of serum samples (1 : 200 dilution) on NoV GII-4 VLP-coated microtitre plates the samples were aspirated and 8 M urea (Sigma-Aldrich) was added (250 μl/well). Two 5-min incubation steps with urea were used to remove the low avidity antibodies. The avidity index was calculated as (OD with urea/OD without urea) × 100% and an index value of ≥ 50% was considered to be high avidity.

Denaturing and native Western blot analysis

Norovirus capsids (VLPs) were separated by 12% denaturing SDS–PAGE or non-denaturing (native) PAGE and transferred under similar conditions onto the nitrocellulose paper. Native PAGE was run similarly to the SDS–PAGE with the exception that no *β*-mercaptoethanol and SDS were added and the protein samples were not boiled. In non-denaturing (native) Western blot the proteins were transferred on nitrocellulose paper at 4° to avoid protein denaturing. The staining of proteins on the nitrocellulose paper was performed as described elsewhere.[10] Briefly, transferred proteins were detected using sera from mice immunized with NoV GII-4 VLPs or P-particles

(dilution 1 : 500), bound IgGs were detected with HRP-conjugated goat anti-mouse IgG (Sigma Aldrich).

Antibody blocking assays

The blocking assays using synthetic carbohydrate HBGAs and human saliva were conducted as described by others[8,15] with some modifications. Binding of NoV GII-4 and GI-3 VLPs to synthetic carbohydrate HBGA receptors H-type-3 and Le^b (Lectinity Holdings, Inc., Moscow, Russia) was tested before conducting blocking assays. Both VLPs bound to H-type-3 but not to the control carbohydrate Le^b (ref. 18 and data not shown). In the blocking assay all the dilution buffers, incubation conditions and washing steps were identical to those described for ELISA unless otherwise specified. Microtitre plates were coated overnight at 4° with either GII-4 (200 ng/well) or GI-3 (50 ng/well) VLPs. The sera were serially diluted starting from 1 : 200 for the detection of homotypic blocking or heterotypic cross-blocking. After the incubation of sera, the biotinylated H-type-3 was added at a concentration of 40 μg/ml (100 μl/well) and incubated at 37° for 4 hr. The bound carbohydrate was detected using 1 : 2000 diluted streptavidin-conjugated HRP (Thermo Fisher Scientific Inc.) and OPD substrate. In the saliva blocking assays human type A saliva was diluted 1 : 3000 in PBS and coated on a microtitre plate. For the type-specific blocking assays GII-4 VLPs (final concentration 0·2 μg/ml) were pre-incubated with two-fold titrated serum samples (1 : 100 to 1 : 12 800 dilutions) at 37° for 1 hr before adding to the plates. For the cross-blocking, GII-4 NO VLPs (0·1 μg/ml) were pre-incubated with the sera diluted 1 : 10 to 1 : 640. The plates were incubated for 1 hr and developed and measured as described above. An OD reading from the wells lacking serum was considered as a maximum signal for the binding of VLPs to the synthetic HBGAs or saliva. Blocking index was calculated as the percentage of the maximum binding blocked by a certain serum dilution and was calculated as 100% – ([OD wells with serum/OD wells without serum] × 100%).

ELISPOT interferon-γ assay

Enzyme-linked immunosorbent spot (ELISPOT) assay was used to measure NoV-specific T-cell response by quantification of interferon-γ (IFN-γ) production from the splenocytes. Multiscreen HTS-IP filter plates (Millipore, Billerica, MA) were coated with 100 μl anti-mouse IFN-γ monoclonal antibody (Nacka Strand; Mabtech AB, Sweden) at 2·5 μg/ml. After washing with cell culture grade PBS (Bio-Whittaker; Lonza, Basel, Switzerland) the plates were blocked with cell culture medium (CM) (RPMI-1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol and 2 mM L-glutamine, all purchased from Sigma-Aldrich) containing 10% FBS (Sigma-Aldrich) for 3 hr at room temperature. The NoV-capsid-derived peptides (GII-4, GII-4 NO and GII-12 specific) and a negative control peptide (rotavirus VP6 specific), were added to the plates at a final concentration of 5 μg/ml. Liquid nitrogen frozen splenocytes were thawed, washed, suspended in CM with 10% FBS and added to the wells ($0{\cdot}1 \times 10^6$ cells/well). Cells without the peptides (CM alone) and cells stimulated with concanavalin A (Sigma-Aldrich) at 10 μg/ml were used as controls. After 24 hr of incubation at 37° and 5% CO_2 the cells were discarded and the plates were washed first with PBS and then with PBS containing 0·05% Tween and 1% FBS. Biotinylated Anti-Mouse IFN-γ monoclonal antibody (Mabtech) was added in PBS containing 10% FBS at 2 μg/ml and incubated for 16–18 hr at 4°. After incubation the plates were washed and streptavidin-HRP in PBS/10% FBS diluted 1 : 500 was added (50 μl/well). After 1 hr of incubation the plates were washed and the spots were developed with a 3-amino-9-ethylcarbazole substrate staining kit (Sigma-Aldrich). The reaction was stopped after 8 min with tap water. The spots were counted under a dissection microscope by two independent counters and the results are expressed as mean spot-forming cells (SFC) per 10^6 splenocytes of duplicate wells. The result was considered positive if the mean SFC with the specific peptide was above the mean SFC of the negative control peptide + 3 × SD.

Statistical analyses

Mann–Whitney *U*-test was used to assess the intergroup differences in antibody magnitude and blocking ability. All hypothesis testing was two-tailed. Statistical significance was defined as $P < 0{\cdot}05$.

Results

High levels of NoV-specific IgG antibodies were induced by a single VLP immunization

Genogroup II (GII)-4 VLP immunization induced a strong GII-4-specific IgG response in all the study animals by both immunization routes (Fig. 2a). Although GII-4 P-particle immunization also resulted in a relatively high level of IgG, the antibody responses of the groups immunized with the P-particles by either of the routes were significantly lower than in the groups immunized with the VLPs (all $P < 0{\cdot}05$) (Fig. 2a). No GII-4-specific antibodies were detected in the naive mice. To determine the kinetics of the GII-4-specific IgG antibody response development tail blood samples were taken at several time-points during the study and the level of antibodies was determined by ELISA (Fig. 2b). A sharp increase of IgGs was

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detected 2 weeks after the first immunization with the GII-4 VLPs and the levels rose slightly after the second immunization. The first immunization with the GII-4 P-particles induced low levels of GII-4-specific IgGs at study week 3. The antibody levels were on average sixfold lower ($P = 0{\cdot}01$) than what was observed with the VLP immunization at the corresponding time-point. At the time of terminations and after receiving the second dose of the P-particles, the IgG levels had risen to levels similar to those induced by the VLPs after the first immunization, indicating that a booster immunization was needed with the P-particles.

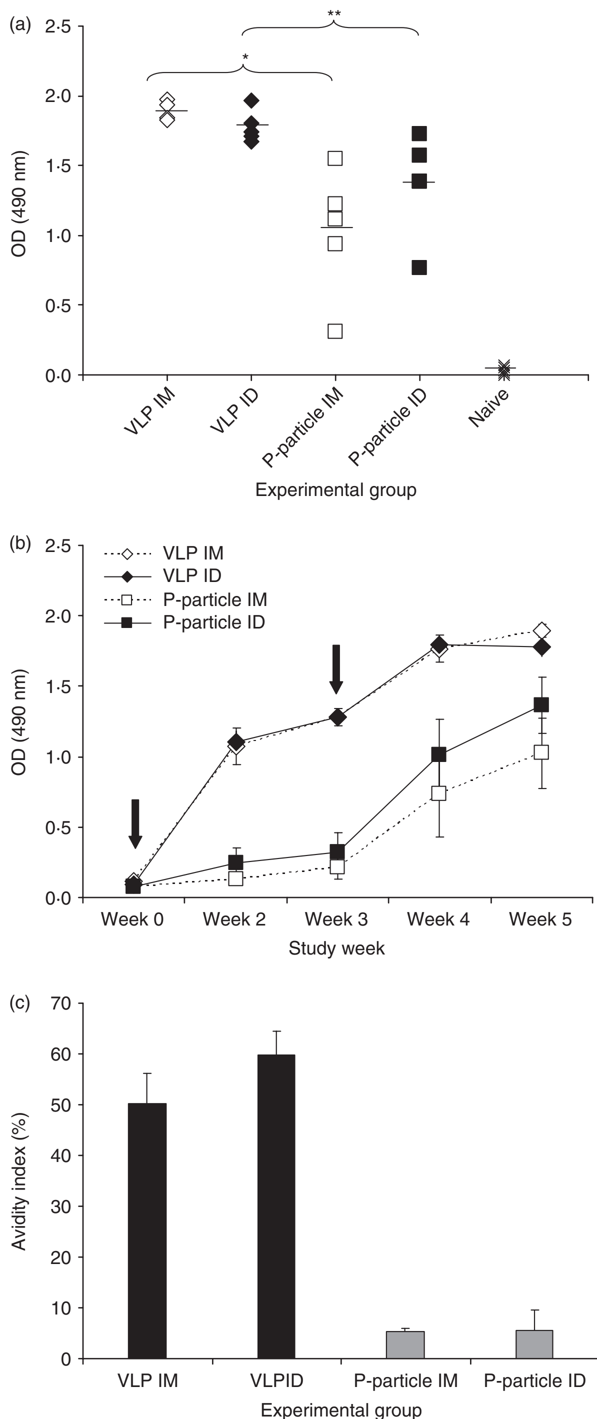

Figure 2. Anti-GII-4 IgG response. BALB/c mice were immunized intradermally (ID) or intramuscularly (IM) twice with 10 μg of norovirus (NoV) GII-4 virus-like particles (VLPs), GII-4 P-particles, or no immunogen (naive). (a) Termination sera were tested in ELISA at 1 : 200 dilutions. The values shown are the optical density (OD) values of each individual serum and the mean OD of the group (horizontal bar). Groups were compared by Mann–Whitney *U*-test; $^{*}P = 0.016$, $^{**}P = 0.009$. (b) Kinetics of norovirus GII-4-specific serum IgG response. Serum samples collected at study weeks 0, 2, 3, 4 and 5 were tested for GII-4-specific IgGs in ELISA at 1 : 200 dilutions. Mean OD values of the groups with standard errors are shown. Immunizations at week 0 and week 3 are indicated with arrows. (c) Mean avidity indices (%) of norovirus GII-4-specific serum IgG antibodies. Termination sera are tested at the dilution of 1 : 200. Bars show the mean avidity index of an experimental group.

High avidity IgGs were induced by the VLPs

The avidity of GII-4-specific antibodies in the termination serum was assayed from all immunized mice and the mean avidity indices of each group are presented in Fig. 2(c). The VLP immunization by both IM and ID routes resulted in IgG antibodies with high avidity (mean avidity indices 50·2 ± 5·9% and 59·7 ± 4·7%, respectively). Instead, extremely low avidity of IgGs for the specific antigen was obtained in groups immunized with the P-particles (mean avidity indices 5·3 ± 0·7% and 5·6 ± 4·0%). It is noteworthy that, in both groups immunized with the P-particles four of the five mouse serum IgGs were stripped down completely by the urea treatment (OD value 0·003–0·036).

Th1/Th2 balanced immune response induced by the VLPs

The systemic NoV GII-4-specific IgG response was further characterized into Th1 and Th2 responses by measuring IgG antibody subtypes IgG2a and IgG1,[23] respectively. Figure 3 shows the GII-4-specific IgG1 and IgG2a antibody levels of immunized mice measured in an end-point serum titration ELISA. The VLPs induced high levels of both IgG subtypes resulting in identical end-point titres (1 : 204 800) of IgG1 and IgG2a and therefore in a Th2/Th1 ratio of 1 (Fig. 3a) as described in the Materials and methods section. In contrast, P-particle immunization elicited relatively high levels of IgG1 (end-point titre 1 : 51 200) but low levels of IgG2a (end-point titre 1 : 1600) resulting in a Th2/Th1 ratio of 32 (Fig. 3b). Mice immunized with the VLPs produced on average

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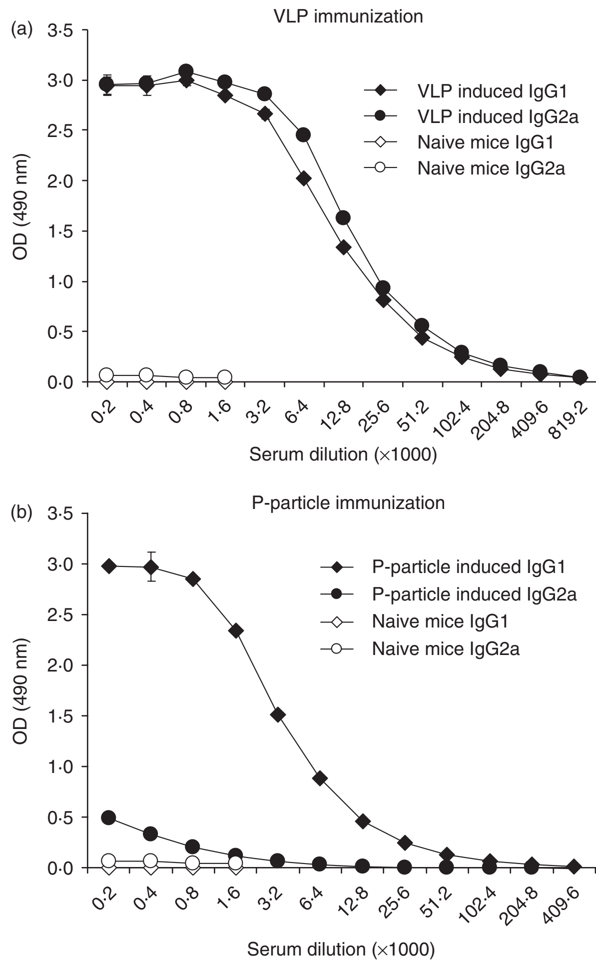

Figure 3. End-point serum titrations of GII-4 specific IgG1 and IgG2a subtype antibody responses of groups of mice immunized intramuscularly with GII-4 virus-like particles (VLPs) (a) or P-particles (b). Mean (± SEM) titres of the sera from each group are shown.

128-fold higher levels of IgG2a than mice immunized with the P-particles as judged from the end-point titres.

The difference in the induction of Th1 responses by VLPs and P-particles was further studied by measuring IFN-γ production from mice splenocytes in an ELISPOT assay. Cells of mice immunized by the VLPs produced IFN-γ when stimulated *in vitro* with synthetic 15-mer peptides representing a T-cell epitope[20] of the P1 capsid domain of GII-4 (mean 551 ± 73 SFC/10^6 cells), and a corresponding sequence in GII-4 NO capsid (mean 318 ± 41 SFC/10^6 cells) and GII-12 capsid (mean 372 ± 47 SFC/10^6 cells) (Fig. 4). In contrast, P-particle immunization did not induce IFN-γ production by T cells when stimulated with any of the NoV peptides (Fig. 4). Cell viability was similar in all groups controlled by concanavalin A stimulation. No response to the negative control peptide (rotavirus VP6 derived) was detected in any of the groups.

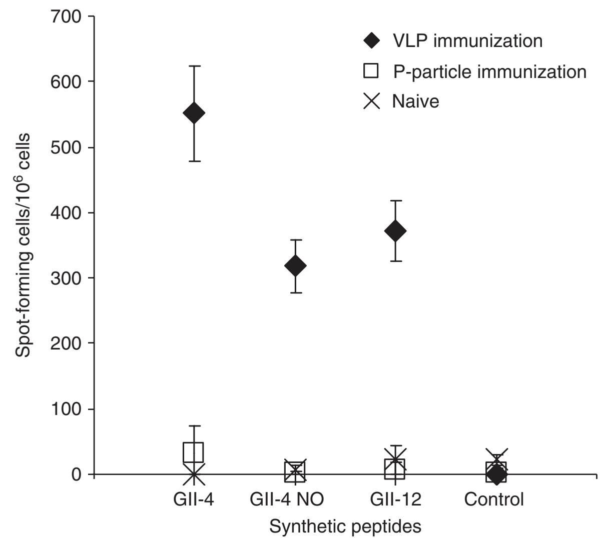

Figure 4. Norovirus (NoV) -specific interferon-γ (IFN-γ) response after immunization with GII-4 virus-like particles (VLPs) or P-particles. Splenocytes from the immunized or naive mice were stimulated with the synthetic NoV-specific peptides from the three different NoV genotypes (GII-4, GII-4 New Orleans and GII-12) and a negative control peptide (rotavirus VP6-specific) and analysed for IFN-γ production with the ELISPOT. The mean spot-forming cells (SFC)/10^6 cells with the error bars are shown.

P-particle antisera did not detect the unfolded structure of the GII-4 capsid proteins in Western blot analysis

The immunological properties of the serum antibodies were further characterized by Western blot analysis. Sera from the mice immunized with the GII-4 VLPs or the P-particles were used to detect the denatured or non-denatured NoV capsid proteins. As shown in Fig. 5(a) under denaturing conditions, VLP antisera detected the unfolded capsid proteins with a great intensity but no binding was detected with the P-particle immunized mouse serum. However, when the proteins retained their native conformation (Fig. 5b) sera from both immunized groups were able to detect NoV capsids with equal intensity. No binding of naive serum to capsid proteins was detected (data not shown).

Heterotypic immune response was generated by the VLP but not the P-protein immunization

To examine the cross-reactivity of the antibodies induced by the GII-4 VLPs or P-particles the termination sera were assayed against heterologous GII-4 NO, GII-12 and GI-3 VLPs in an ELISA (Fig. 6a). VLP immunization induced cross-reactive antibodies to all of the heterologous VLPs. In contrast, P-particle immunization did not stimulate a cross-reactive antibody response to any of the

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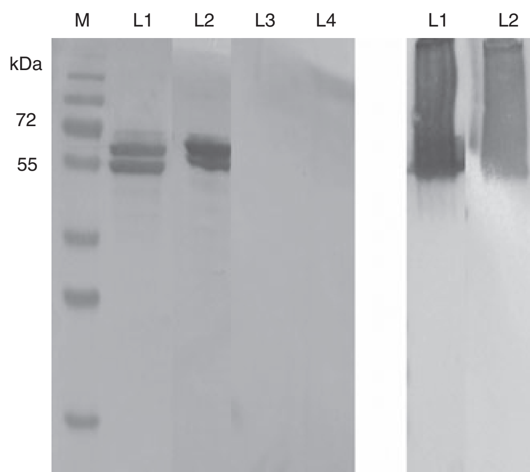

Figure 5. Detection of norovirus GII-4 capsid protein in a Western blot. (a) Sera from mice immunized with GII-4 virus-like particles (VLPs) intramuscularly (IM; L1) or intradermally (ID; L2) or immunized with P-particles IM (L3) or ID (L4) were used to detect denatured GII-4 capsid proteins. M; protein weight marker. (b) Sera from mice immunized ID with GII-4 VLPs (L1) or with P-particles (L2) were used to detect GII-4 capsid proteins in their native (non-denatured) conformation. Native PAGE in which the protein samples were not denaturized was run in a similar manner to the denaturing SDS–PAGE with the exception that no β-mercaptoethanol and SDS were added, the protein samples were not boiled and the transfer of the proteins on nitrocellulose paper was conducted at 4° to avoid protein denaturation.

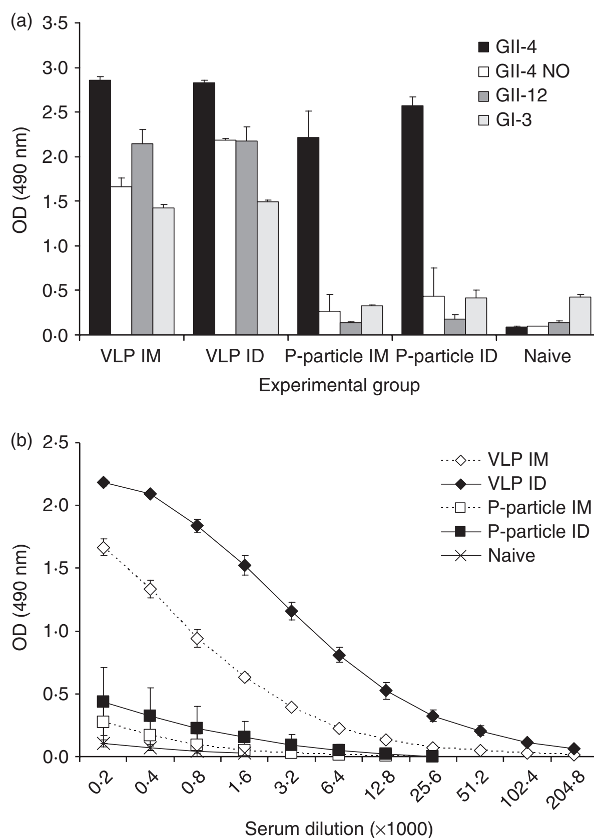

Figure 6. Levels of cross-reactive serum IgG antibodies to norovirus (NoV) GII-4 NO, GII-12 and GI-3-derived virus-like particles (VLPs). Mice were immunized intramuscularly (IM) or intradermally (ID) with GII-4 VLPs or P-particles and termination sera were tested in ELISA with homologous and heterologous VLPs. Naive mice were used as negative controls. (a) The mean IgG levels (± SEM) for different VLPs used are shown for each experimental group. (b) GII-4 NO-specific IgG responses were analysed in serum end-point titration ELISA. The mean optical density (OD) values of each serum dilution and the SEMs for each experimental group are shown.

heterologous antigens and the level of cross-reactive antibodies in the sera was comparable to that in the sera of naive mice (all $P > 0{\cdot}05$). Slightly elevated NoV GII-4 NO-specific IgG levels were detected in both P-particle-immunized groups (Fig. 6a) as the mean ODs of the groups exceeded the cut-off value and therefore the magnitude of the NO-specific response was further analysed by an end-point titration assay (Fig. 6b). The mean end-point titres of GII-4 NO-specific antibodies were 1 : 400 (IM immunization) and 1 : 1600 (ID immunization). The corresponding mean end-point titres of the NO-specific sera for VLP immunized mice were 1 : 12 800 and 1 : 102 400. Interestingly, the cross-reactivity of the IgG antibodies observed with the VLP immunization was directed at NoV capsid antigens within the genogroup and between the genogroups. Moreover, only the VLP immunized mice showed T-cell responses to GII-4 NO and GII-12-derived synthetic peptides (Fig. 4) as described in detail in the section on the Th1/Th2 balanced immune response induced by the VLPs.

VLP antiserum showed greater blocking activity than P-particle antiserum

Histo-blood group antigen blocking assays were conducted to test GII-4 VLP and P-particle antiserum blocking of GII-4 VLPs binding to the synthetic H-type-3 receptor (Fig. 7a,b) and to human type A saliva (Fig. 7c,d). In the synthetic H-type-3 blocking assay high blocking ability (maximum blocking, 83%) at serum dilutions 1 : 1600 (IM route) and 1 : 3200 (ID route) was detected for the VLP antiserum. To obtain maximum blocking with the P-particle antiserum eightfold and fourfold (Fig. 7a,b, respectively) more concentrated serum dilutions were needed compared with the VLP antiserum. Maximum blocking of the naive mouse serum was 1·2% at a 1 : 200 dilution (data not shown). A similar difference in the blocking activities of GII-4 VLP antisera and P-particle antisera was observed in saliva blocking assays for IM-immunized groups (Fig. 7c) and ID-immunized groups (Fig. 7d). In addition to the type-specific blocking described above, the blocking activity of the cross-reactive antibodies to

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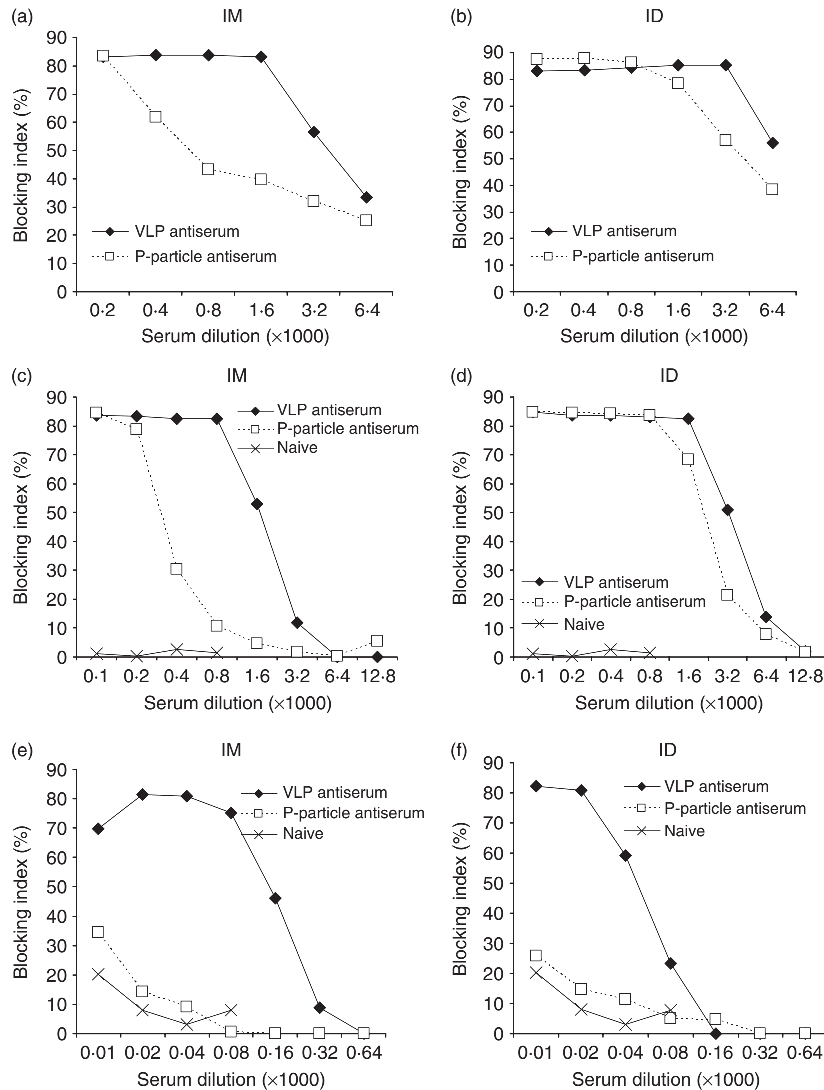

Figure 7. Blockage of norovirus (NoV) GII-4 virus-like particles (VLPs) binding to histo-blood group antigen (HBGA) H-type-3 receptor (a and b) or to human type A saliva (c and d) by sera of mice immunized with the GII-4 VLPs or P-particles. Blockage of NoV GII-4 New Orleans (NO) VLPs binding to human type A saliva (e and f) by sera of mice immunized with the GII-4 VLPs or P-particles. The panels on the left show the results of intramuscular (IM) immunizations and those on the right show the results of intradermal (ID) immunizations. The blocking indices (%) from group-wise pooled and twofold titrated sera are shown.

GII-4 NO was tested in a saliva blocking assay. As shown in Fig. 7(e,f) only VLP immunization resulted in cross-blocking antibodies against GII-4 NO. Furthermore, the blocking activity of GI-3-specific antibodies detected after VLP immunization was tested in an assay in which GII-4 VLP antiserum was used to block the GI-3 VLPs binding to the synthetic H-type-3 receptor. A blocking index of 53% was obtained with a serum dilution of 1 : 400 and a serum dilution of 1 : 800 still blocked 27% of the specific binding (data not shown). The results from these assays indicate that NoV VLP but not the P-particle immunization induces cross-blocking antibodies.

Discussion

The functional and antigenic properties of NoV VLPs and P-particles have previously been characterized.[10,15,16,18] Although P-particles contain only the P-domains of the NoV capsid and lack the conserved shell domain, several studies have demonstrated the similar antigenic and receptor-binding properties of the two NoV subunit particulate structures.[13,15] NoV VLPs induce systemic and mucosal immune responses in small animal models[24,25] and in humans.[26–29] P-particle immunogenicity studies have been conducted in mice.[15,16] Both NoV VLPs and P-particles possess unique immunogenicity-enhancing

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features including particulate nature and repetitive, high density display of antigens and both are attractive candidates for NoV vaccine development.[30–32] However, no studies have been conducted to compare the antibody-mediated and cellular immune responses induced by the VLPs compared with P-particles. Our data demonstrate the superiority of the NoV GII-4 VLPs in the induction of a high quality heterotypic immune response compared with the GII-4 P-particles.

Consistent with the results of others[15,16,24,25] high levels of type-specific IgG antibodies were obtained by both NoV GII-4 VLP and P-particle immunizations of BALB/c mice, but the quality of the immune response was different. A strong GII-4-specific IgG response was achieved only after two doses of the P-particles, whereas a similar response was obtained after one dose of the VLPs. Furthermore, IgG subtypes obtained with the P-particles were predominantly of IgG1 isotype, indicative of a Th2 immune response, whereas VLPs were strong inducers of both subtypes, IgG1 and IgG2a, indicative of a mixed and balanced Th1/Th2 response. As Th1 cytokine IFN-γ production was induced only in the cells of mice immunized with the VLP the result confirmed that only VLPs were able to activate T cells and stimulate cell-mediated immunity even in the heterologous strains. LoBue *et al.*[20] also showed that IFN-γ secretion was achieved when the cells were stimulated with the peptides corresponding to homologous and heterologous T-cell epitopes. Here we showed that IFN-γ production from the T cells can be induced when stimulated by a genetically related genotype (GII-4 NO) and a distant genotype (GII-12) derived peptides having up to three amino acids different in a single T-cell epitope (GII-12), which in part supports the hypothesis of GII NoV T-cell epitope conservation.[20] In a NoV challenge study[33] conducted in humans the authors discussed that activation of Th1 responses may have been associated with protection against NoV infection in some volunteers. Moreover, a murine norovirus challenge study revealed that the clearance of the virus from the tissues was achieved only when $CD4^+$ and $CD8^+$ T cells were activated along with the B cells.[34] The results described above[20,33,34] strongly suggest that T-cell responses are an important factor in protective NoV immunity.

The dramatic difference observed between the Th1 responses induced by the VLPs and P-particles indicates that there may be different immunological mechanisms involved in antigen processing. Possibly the macromolecular structure of VLPs is preferred in the endocytic process of the antigen-presenting cells, especially dendritic cells and macrophages, thereby activating particulate T-cell responses. Possibly another set of antigen-presenting cells, other than dendritic cells, or another dendritic cell subset is responsible for the antigen presentation of P-particle, which leads to a strongly Th2-biased response.[35,36] However, it is not excluded that the trace amount of DNA found in the VLP preparations (10 ng/dose, respectively) could have influenced the induction of IgG2a as earlier described by Jegerlehner *et al.*[37]

The immunological properties of serum antibodies were also characterized by Western blot analysis. All antibodies induced by the P-particle immunization were presumably formed against conformational epitopes of NoV P-domain as they did not react in Western blot with denatured NoV capsid proteins. On the contrary, antibodies induced by VLPs reacted against both conformational and linear epitopes of the NoV capsid. The reason for this observation may be that immunodominant linear epitopes are formed by the shell domain of the capsid, which is lacking in the P-particles. The role of these antibodies in NoV infection is not known but linear epitopes of feline calicivirus capsid protein have been shown to induce neutralizing antibodies.[38] This leads to the conclusion that conserved as well as variable domains of the capsid protein should be included in NoV subunit vaccines.

Avidity or affinity maturation of antibodies is the indicator for how morphologically precise the antigen-binding epitope is and usually the avidity increases in time and as a function of antigen exposures.[39] High avidity antibodies appear to correlate with protection of the disease in the case of several viral infections[40–43] and lower titres of antibodies are needed to neutralize the virus when the avidity of antibodies is high.[44,45] The induction of high avidity IgGs by NoV GII-4 VLPs shown here and poor avidity IgGs by P-particles towards NoV indicates that there probably are some critical elements in the repetitive structure of the VLPs affecting the antibody binding site formation. Possibly the antibodies formed toward linear epitopes of an antigen possess more avidity or high avidity antibody formation may be NoV capsid shell domain dependent.

Different genotypes of NoVs fluctuate in a year-by-year manner and an effective NoV vaccine should provide protection against a number of existing and future genotypes, within and between genogroups.[30,31] We studied the cross-reactivity of antibodies induced by the monovalent GII-4 VLPs and P-particle immunizations using GII-4 NO as a representative genotype in the GII-4 genocluster, GII-12 VLPs as a representative genotype in the GII genogroup distinct from the GII-4[1] and GI-3 VLPs as a representative genotype in the GI genogroup as antigens in ELISA. The VLP immunization induced a heterotypic response with cross-reactive antibodies towards all the heterotypic VLPs used in this study. In contrast, no statistically significant cross-reactive antibody response was induced by the P-particles, indicating a type-specific immune response only. Furthermore, only VLPs but not P-particles could stimulate the cell-mediated immune response towards heterotypic NoV genotypes (GII-4 NO and GII-12). We suggest that a Th1/Th2 balanced

immune response has a role in the observed cross-reactivity. Stimulation of cell-mediated immunity reflected by the production of IFN-γ as well as the induction of high IgG2a antibody levels by VLP immunization may be important for the induction of heterotypic immunity in a fashion similar to that of the proteins of influenza virus and rotavirus.[46,47]

As NoV has been shown to attach to HBGA carbohydrates displayed on the surface of mucosal cells or as free antigens in biological fluids[48] these are considered to serve as a receptor for NoV entry in host cells.[8,49,50] The neutralizing antibodies raised against NoV after a natural infection or in an animal model have been shown to block the binding of NoV VLP to the synthetic HBGA receptor[8,15,51] and to human saliva[16] containing a number of different HBGAs. Moreover, recent studies have suggested that HBGA blocking ability is an important factor in achieving protective immunity against NoV[19,52] and the present vaccine approaches are focused on stimulating heterotypic HBGA blocking antibodies.[28,30,31] Our results demonstrated that both the VLP and P-particle antisera were able to block the GII-4 VLPs binding to HBGA receptor and A-type human saliva. However, in contrast to studies by others,[15] our data showed that depending on the immunization route and blocking assay used, a twofold to eightfold greater titre of P-particle antiserum was needed to achieve maximum type-specific blocking compared with the VLP antiserum. Furthermore, the important finding was the ability of GII-4 VLP antiserum to cross-block GII-4 NO and GI-3 VLPs binding to human saliva or to the synthetic H-type-3 receptor, a desirable feature in NoV vaccines. The difference of the blocking activity between VLP and P-particle antisera was somewhat smaller for ID immunized animals compared with IM immunized animals. This difference might be partly explained by the abundance of professional antigen-presenting cells, dendritic cells, present in the epidermis compared with the muscle and therefore more efficient antigen presentation.

The results of the present study show that the quality of immune response should be comprehensively studied when searching for vaccine candidates against NoV disease. We believe that an efficient NoV vaccine should induce cross-reactive antibody responses with neutralizing activity as well as T-cell immunity. We showed that these features are conferred by the VLPs but not the P-particles.

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Disclosures

None of the authors have conflict of interest.

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Pre-existing Immunity to Norovirus GII-4 Virus-Like Particles Does Not Impair *de Novo* Immune Responses to Norovirus GII-12 Genotype

Kirsi Tamminen, Leena Huhti, Timo Vesikari, and Vesna Blazevic

Abstract

Noroviruses (NoVs) are one of the leading causes of acute nonbacterial gastroenteritis in humans of all ages. In the 1990s, NoV genotype GII-4 became responsible for the majority of NoV sporadic gastroenteritis cases and outbreaks worldwide. Vaccine development against NoV GII-4 is underway. At the same time, there is concern of new emerging NoV genotypes, such as GII-12, which has been recently associated with increasing numbers of NoV outbreaks worldwide. The specific question is whether type-specific pre-existing immunity to NoV GII-4 might impair cognate immune response induced by new viral infections or vaccines. Using GII-4 and GII-12 virus-like particles, we tested the impact of the immunity generated against NoV GII-4 on *de novo* antibody responses to GII-12 in mice. We found that pre-existing immunity to NoV GII-4 did not impair *de novo* immune response to the novel antigen, therefore suggesting lack of original antigenic sin (OAS).

Introduction

Noroviruses (NoVs) are genetically highly diverse viruses that are causing the majority of outbreaks and sporadic cases of viral gastroenteritis in humans worldwide (6). Among almost 30 genotypes infectious to humans, genotype GII-4 has dominated since the 1990s (19). NoVs recognize human histo-blood group antigens (HBGAs) as entry enhancement factors (7,14) found on red blood cells but also on mucosal cells in the gastrointestinal track and in biological fluids (14). Serum HBGA blocking ability has been shown to correlate with protection against NoV infection (15,17).

The NoV genome encodes a capsid protein (VP1) that self-assembles into virus-like particles (VLPs) when expressed in a recombinant baculovirus-insect cell system (10). These VLPs are morphologically and antigenically similar to the native virus (10). Considerable effort has been put into the development of NoV vaccines based on VLP's (1,2,25). However, challenges to the vaccine development are high genetic diversity and the antigenic variation of NoV strains (6) and high level of NoV pre-existing antibodies (11,15), which might impair the immune response to the vaccine. We determined the impact of pre-existing immunity to GII-4 on the generation of *de novo* immune response to a novel NoV genotype GII-12 in a mouse model.

Materials and Methods

VLP production

The RNA (ORF-2 gene) of GII-4-1999 and GII-12-1998, extracted from NoV infected human stool samples were amplified by RT-PCR (2) and cloned in baculovirus vector (Invitrogen, Carlsbad, CA) for the production of NoV VLPs in a SF9-insect cell system (Invitrogen) (12). Purification of the VLPs was done by sucrose gradients as described previously (9). Protein purity, morphology, *in vitro* antigenicity, and functionality are described elsewhere (9,15).

Mice immunization

Seven-week-old female BALB/c mice (Harlan Laboratories, Horst, the Netherlands) were immunized twice (at study weeks 0 and 3) with 1 μg of GII-4 or GII-12 VLPs by intradermal (ID) route; mice were sacrificed at week 5. In other sets of experiments, groups of mice received two doses of 10 μg GII-4 by intramuscular (IM) route at the above schedule. Half of the mice receiving 10 μg GII-4 doses were boosted with 10 μg of GII-12 VLPs at study week 18, and all mice were sacrificed at week 27. No adjuvants were incorporated in immunizations. Blood samples were collected from each mouse at study weeks 0 (pre-bleed), 4, 5, 6, 8, 10, 12, 14, 16, 18, 19, 23, 25, and 27. All procedures were

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authorized and performed according to the guidelines of the Finnish National Animal Experiment Board.

Enzyme-linked immunosorbent assay (ELISA) and avidity assay

ELISA was used to measure antibody levels in mouse serum as described earlier (21). Briefly, GII-4 or GII-12 VLPs were coated (0.2–1 μg/mL) on a 96-well plate (Nunc-Immuno Maxisorp, Thermo Fisher Scientific Inc., Waltham, MA) prior to addition of serum dilutions. Antibodies were detected by goat anti-mouse IgG HRP and OPD-substrate (both from Sigma-Aldrich, St. Louis, MO). Termination sera were serially diluted two-fold and tested for GII-4- and GII-12-specific IgG serum titers. Serum dilution with an OD greater than or equal to 0.1 and three times the mean OD of the negative control serum was regarded as positive. Serum from mice receiving carrier only (PBS) was used as a negative control.

Antibody avidity was determined by testing individual mouse serum samples (diluted 1:200) in a modified ELISA in which 8 M urea was included in two washing steps after incubation of the serum samples in order to elute low-avidity antibodies (15,18). The avidity index was calculated as (OD with urea/OD without urea) $\times$ 100% and avidity index $\geq$50% was considered as high avidity.

Saliva phenotyping and HBGA blocking assays

Saliva ABO phenotyping was conducted using monoclonal antibodies (Immucor, Houston, TX) against A, B, and H antigens, as described by others (8). Saliva from secretor-positive individuals used in the blocking assays were selected by predetermined saliva-VLP binding profiles (data not shown). A saliva blocking assay (7) was conducted as described earlier (2). Briefly, GII-4 and GII-12 VLPs (0.2 μg/mL and 0.5 μg/mL, respectively) were pre-incubated with serially diluted mice antisera (week 27) and added to saliva type A (for GII-4 binding) or type B (for GII-12 binding) coated plates. The bound VLPs were detected using human NoV antiserum (15) and anti-human-HRP conjugate (Invitrogen) reacting with OPD-substrate (Sigma Aldrich).

Results

BALB/c mice were immunized twice with NoV GII-4 or GII-12 VLPs alone with a short-term immunization schedule and low dose of antigen (1 μg per dose). Immunizations with both VLPs induced strong antigen-specific IgG response with mean serum reciprocal titers 5 logs10 each (data not shown). After confirming good immunogenicity of the VLPs, mice were immunized twice with 10 μg dose of GII-4 VLPs, and the immune response was followed up until week 27 either with or without GII-12 VLP boost at study week 18. After the two doses of GII-4 VLPs, the homologous and cross-reactive IgG responses to GII-12 were stable until study week 27 (Fig. 1A and B, dashed lines). The boost with GII-12 VLPs did not impair the immune response to the primary antigen as seen from the unaltered level of anti-GII-4 IgG during the study (Fig. 1A, solid line). In addition, the final antibody titer of GII-4-specific antibodies was identical between the groups receiving the GII-12 VLP boost or not (1:51200 titer for both). Rather, GII-12-specific IgG level rose two-fold from the mean prior-boost level (OD = 1.065 ± 0.140) to the post-boost level (OD = 2.180 ± 0.151) and remained steady for the rest of the study (Fig. 1B, solid line). Anti-GII-12 mean IgG titer increased four-fold after the GII-12 VLP boost (mean antibody titer 1:51200) compared to the group receiving no boost (mean antibody titer 1:12800).

Antibody avidity of both experimental groups was high to the primary antigen GII-4 (mean avidity index 69.6 ± 3% for GII-4 immunized group and 64.8 ± 7.9% for the GII-12 boosted group), but only the GII-12 boosted group had anti-GII-12 IgGs with high avidity (mean avidity index 61.5 ± 6.9%).

To show the potential neutralization of the serum antibodies, blocking assays were used in which the antisera from mice terminated at week 27 were used to block the binding of NoV VLPs to HBGA carbohydrates present in human saliva (14). Blocking of GII-4 VLP binding to HBGAs

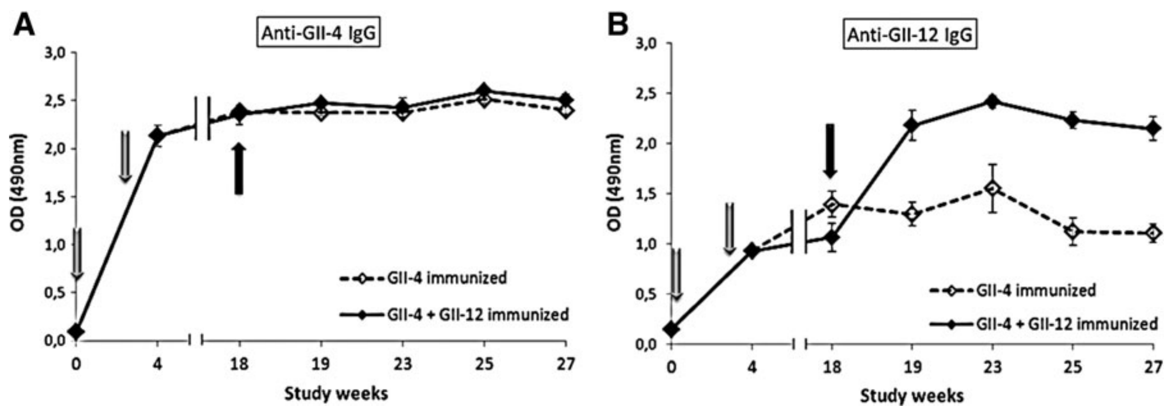

FIG. 1. Antibody-mediated immune response induced by NoV VLPs. BALB/c mice were immunized twice with 10 μg GII-4 VLPs (*light gray arrows*) and terminated at study week 27. A group of mice received a 10 μg boost with GII-12 VLPs (*dark gray arrows*) at week 18. *Dashed lines* represent the mean IgG antibody levels of solely GII-4 immunized group, while *solid lines* represent the mean IgG levels of the GII-12-boosted group. GII-4-specific IgG **(A)** and GII-12-specific IgG **(B)** were measured from blood samples taken at various time points of the study. Individual mouse sera were tested in each assay and means of the groups with standard errors are shown.

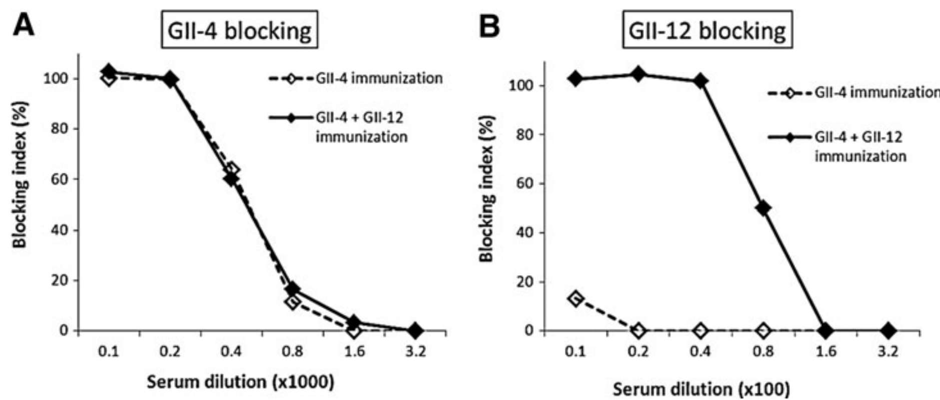

FIG. 2. Blockage of VLPs binding to human saliva. Sera of mice immunized twice with 10 μg GII-4 VLPs without (*dashed lines*) or with (*solid lines*) GII-12 boost (10 μg) were used to block GII-4 **(A)** and GII-12 **(B)** VLP binding to human secretor positive saliva type A (GII-4 binding) or type B (GII-12 binding). The blocking index was determined as the percentage of maximum binding blocked by a certain serum dilution and was calculated as 100% – ([OD VLP pre-incubation with serum/ OD VLPs pre-incubated without serum] × 100%). The blocking indices (%) from group-wise pooled and two-fold titrated sera are shown.

was achieved with the sera of GII-4 VLP-primed mice with or without GII-12 VLP boost at a similar level (Fig. 2A). However, GII-12 VLP binding to HBGAs was completely blocked only by antisera from the GII-12-boosted mice (Fig. 2B).

Discussion

The NoV genotype GII-4 has been circulating and evolving for at least 4 decades, as the earliest finding of the ancestral NoV GII-4 dates back to 1974 (3). Since the early 1990s, it has become the most abundant NoV genotype, accounting for 80% of current NoV outbreaks worldwide (19). In 2009, a novel GII-12 NoV strain emerged in the United States, causing 16% of NoV outbreaks (24), and its emergence has been reported worldwide (5,16,20). Nearly all adults and the majority of children over 5 years of age are seropositive to NoV GII-4 (11,15). There is a concern (13) that pre-existing immunity may impair immune response to new emerging strains or to vaccination with NoV VLPs in similar fashion as with influenza virus and HIV infections (23). This phenomenon is known as "original antigenic sin" (OAS) in which the immune response to new infections or vaccination is impaired by the host's infection history (22). Immune responses generated upon NoV challenge were skewed to strains other than the infecting strain, giving reason to suspect OAS involvement in the NoV immunity (13). Studying OAS in NoV infections is extremely challenging because of the multiple strains of NoVs a volunteer is exposed to during the lifetime, and the variable genetic susceptibility of individuals to NoV infection (4). The mouse immunogenicity model is a useful tool to study OAS in NoV-induced immunity because the "exposure history" can be generated and controlled by the researchers.

We used two genetically distant NoV capsid-derived VLPs, GII-4 and GII-12 genotypes, to immunize naïve BALB/c mice. Both VLPs were able to induce a similar level of antibodies at a very low antigen dose (1 μg/dose). Two immunizations with 10 μg GII-4 VLPs were further used to generate long lasting immunity to primary antigen (2) prior to boosting with GII-12 VLPs. In this way we hoped to induce immunological memory specific to the primary immunogen. Our results show that a strong (reciprocal IgG titer 5 log10) GII-4-specific antibody response lasting up to 27 weeks was induced. A heterologous boost with GII-12 VLPs at 18 weeks did not reduce the IgG antibody level or the HBGA blocking activity of GII-4 antisera. Furthermore, *de novo* immune response to the new secondary antigen was induced as confirmed by the following: 1) GII-12-specific serum IgG content after the boost reached the same level as for the primary antigen GII-4; 2) a four-fold rise observed in the GII-12 specific geometric mean serum titer compared to the nonboosted group sera; and 3) blocking potential (100%) of GII-12 antisera to block GII-12 VLPs' binding to HBGA compared to the group not receiving the boost (<20% blocking). To further confirm that the rise in anti-GII-12 antibody response did not merely reflect the cross-reactive antibodies induced by the GII-4 immunization (2), we used an avidity assay to show the increase in the affinity of GII-12-specific antibodies. The results show that GII-4 immunization alone was not able to induce high avidity anti-GII-12 IgGs, whereas GII-12 VLP boost did increase the avidity of GII-12 specific antibodies, indicating formation of specific antibody response and functional antibody maturation to *de novo* antigen.

Our study in a mouse immunogenicity model shows that immunity to NoV induced by VLPs is long-lasting and protective as measured by blocking activity of the antisera. Furthermore, the pre-existing immunity to GII-4 did not impair *de novo* immune response generation to the novel antigen GII-12. Therefore, our results encourage the use of VLPs in NoV vaccine development as no evidence of OAS was detected; however, further studies are needed to confirm these results. Vaccination with VLPs could boost the pre-existing immunity to extend to a wide range of genotypes and likewise the circulating strains could boost the vaccine-induced immunity.

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Author Disclosure Statement

No competing financial interests exist.

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Trivalent Combination Vaccine Induces Broad Heterologous Immune Responses to Norovirus and Rotavirus in Mice

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Abstract

Rotavirus (RV) and norovirus (NoV) are the two major causes of viral gastroenteritis (GE) in children worldwide. We have developed an injectable vaccine design to prevent infection or GE induced with these enteric viruses. The trivalent combination vaccine consists of NoV capsid (VP1) derived virus-like particles (VLPs) of GI-3 and GII-4 representing the two major NoV genogroups and tubular RV recombinant VP6 (rVP6), the most conserved and abundant RV protein. Each component was produced in insect cells by a recombinant baculovirus expression system and combined *in vitro*. The vaccine components were administered intramuscularly to BALB/c mice either separately or in the trivalent combination. High levels of NoV and RV type specific serum IgGs with high avidity (>50%) as well as intestinal IgGs were detected in the immunized mice. Cross-reactive IgG antibodies were also elicited against heterologous NoV VLPs not used for immunization (GII-4 NO, GII-12 and GI-1 VLPs) and to different RVs from cell cultures. NoV-specific serum antibodies blocked binding of homologous and heterologous VLPs to the putative receptors, histo-blood group antigens, suggesting broad NoV neutralizing activity of the sera. Mucosal antibodies of mice immunized with the trivalent combination vaccine inhibited RV infection *in vitro*. In addition, cross-reactive T cell immune responses to NoV and RV-specific antigens were detected. All the responses were sustained for up to six months. No mutual inhibition of the components in the trivalent vaccine combination was observed. In conclusion, the NoV GI and GII VLPs combination induced broader cross-reactive and potentially neutralizing immune responses than either of the VLPs alone. Therefore, trivalent vaccine might induce protective immune responses to the vast majority of circulating NoV and RV genotypes.

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Competing interests: The authors have a patent on the use of norovirus virus-like particles and rotavirus rVP6 as a combination vaccine (patent no 122520). This study was in part supported by UMN Pharma Inc., Japan (www.umnpharma.com/en/) as compensation for licensing the patent (patent no 122520). This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Acute gastroenteritis (AGE) is a leading cause of morbidity and mortality in children all over the world [1]. Viruses are responsible for a significant number of AGE cases and two leading agents for viral gastroenteritis are rotavirus (RV) and norovirus (NoV) [1]. Following the introduction of live RV vaccines Rotarix® (GlaxoSmithKline plc, UK) and RotaTeq® (Merck & Co., Inc., USA) into national immunization programs, NoV's epidemiological importance is rising and in some countries NoV has already overtaken RV as the most important cause of pediatric AGE [2–5].

Development of a NoV vaccine is underway [6–8]. Since the cultivation of NoVs has not been successful [9], the main direction in vaccine development has been the use of non-live NoV virus-like particles (VLPs), which mimic the structure and the antigenic properties of the native NoVs [10]. These VLPs are constructed of the core protein VP1, which self-assembles into VLPs when produced *in vitro* [10]. An additional challenge in the NoV vaccine development is the high genetic variation of NoVs [11]. The major NoV genogroups infecting human beings are genogroup I (GI) and genogroup II (GII) with at least 25 different genotypes belonging in these genogroups [11]. There is a great molecular variation inside the genotypes themselves and the driving force in the evolution seems to be herd immunity [12]. For over two decades the most prevalent NoV genotype has been GII-4, currently accounting for over 80% of all NoV cases [13,14]. There is some immunological cross-reactivity between GI and GII genogroups [15] but no protective immune responses across genogroups in humans have been

observed [16]. It has been suggested that a broadly effective NoV vaccine should be a combination of at least two genotypes; one from each of the major genogroups [17–19].

RV annually accounts for ~450 000 deaths in children under 5 years of age, the majority of the deaths taking place in developing countries [20]. Since the introduction of the two live-attenuated RV vaccines, the cases of RV-caused AGE have decreased dramatically [5,21,22]. Despite the efficacy of RV vaccines, there are still certain limitations associated with both of these vaccines. The introduction of the vaccines into developing countries has been challenging [23] and safety issues like increased risk of intussusception [24,25] and the reassortment of vaccine viruses in higher virulence [26,27] are concerns involved in the currently available live-attenuated RV vaccines.

RV has a double stranded RNA genome enclosed in the triple layered capsid [28]. VP7 forms a virion surface from which spike-like structures (VP4) extend outwards and are responsible for cell attachment [28]. The inner capsid consists of VP6, which is highly antigenic and the most conserved RV protein [28]. Although neutralizing antibodies targeted against VP4 and VP7 are most strongly associated with RV immunity [29], anti-VP6 antibodies and CD4+ T cells have also been suggested to play a role in the protection [30–33]. RV recombinant VP6 (rVP6) has the ability to form various assemblies *in vitro* [34] and these structures are considered the second-generation vaccine candidates for non-live RV vaccine development [35].

We have previously shown that a dual combination of NoV GII-4 VLPs and RV rVP6 tubules induced strong humoral immune responses without mutual inhibition when delivered parenterally into BALB/c mice [7]. In the present study we have included GI-3 VLPs as a representative of GI NoVs into the above combination in an attempt to broaden NoV-specific immune responses. Induction and long-term duration of NoV and RV-specific cell mediated immunity in addition to humoral immune responses was investigated. Our data indicates that the trivalent combination vaccine containing GII-4 VLPs, GI-3 VLPs, and rVP6 induces robust, long-lasting and broadly cross-reactive NoV and RV-specific cellular immune responses and antibodies with neutralizing abilities against both viruses.

Materials and Methods

Ethics Statement

The protocol for the study was approved by the Finnish National Animal Experiment Board (permission number ESLH-2009-06698/Ym-23). All the procedures performed on the animals were conducted according to the guidelines of the Finnish National Animal Experiment Board and all efforts were made to minimize animal suffering.

Production and purification of NoV VLPs and rVP6

NoV GII-4 VLPs, GI-3 VLPs, GII-4 New Orleans (NO) VLPs, GII-12 VLPs, GI-1 VLPs, and RV rVP6 used for immunizations and/or as antigens in immunological assays were produced by a baculovirus-insect cell expression system and purified by sucrose gradients as previously described [7,36]. The reference strains for each genotype were determined according to the EMBL/Genbank classification and FBVE as the following: AF080551 (GII-4-1999), AF414403 (GI-3-2001), GU445325 (GII-4 New Orleans, GII-4 NO-2010), AJ277618 (GII-12-1998), AY502016.1 (GI-1-2001) and GQ477131 (RV G1P1A [8]-2007 derived VP6). The morphology, integrity, purity, *in vitro* antigenicity and protein concentration were determined for each protein as described previously [7,36].

Cultivation of RVs in cell culture

The RV cultures used as antigens in the enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunosorbent spot (ELISPOT)-interferon-γ (IFN-γ) assays were propagated in an MA104 cell line (ATCC CRL-2378, LGC Standards, UK) as described by others [37]. In short, MA104 cells were infected with the human RV strains Wa (G1P1A [8]), SC2 (G2P2 [6]), BrB (G4P2 [6]), 69M (G8P4 [10]), L26 (G12P1B [4]), bovine WC3 (G6P7 [5]), and rhesus rotavirus (RRV, G3P5B [3]) and after observing the maximum cytopathic effect (3-4 days respectively), the viruses were collected and the VP6 protein amount in each culture was determined by capture ELISA using insect cell-derived rVP6 as an internal standard. The RV cell culture antigens were diluted in phosphate-buffered saline (PBS) to contain equal quantities of VP6 protein per each culture.

Mice immunizations and sample collections

To determine the optimal amount of each antigen, three doses (3, 10 or 30 µg) of NoV GII-4 VLPs, GI-3 VLPs or RV rVP6 were administered intramuscularly (IM) to 7-week-old female BALB/c mice (Harlan laboratories, Horst, the Netherlands). The mice were immunized (5 mice/group) at study weeks 0 and 3 and euthanized at study week 5. After the optimal dose selection, naïve BALB/c mice (5 mice/group) were immunized in another set of experiments according to the above schedule with a single NoV GII-4 VLPs, GI-3 VLPs or RV rVP6 antigen (each at a 10 µg dose) or the trivalent combination (10 µg GII-4 VLPs + 10 µg GI-3 VLPs + 10 µg rVP6) and euthanized at study week 5. A group of mice receiving the trivalent combination vaccine (7 mice/group) were euthanized at study week 27 for the long-term follow-up of the immune responses. Negative control groups of mice (5-7 mice/group) received carrier only (PBS) and were terminated at week 5 or week 27. Blood samples were collected at study weeks 0 (pre-immune serum), 3, 4, 7, 12, 16, and 20 as previously described [38]. Whole blood, feces, lymphoid tissues and vaginal washes (VW) were collected at the time of euthanization. Serum was separated from the blood of each mouse and 10% (w/v) stool suspensions were prepared from group-wise pooled stools according to the published procedures [7]. Preparation of the cell suspensions and freezing of the splenocytes were conducted as described earlier [38]. VWs were collected by pipetting 2 × 125 µl of cold PBS into the vagina 4-5 times up-and-down, after which the VW were centrifuged at 12,000 × g for 10 minutes at +4˚C and the supernatant stored at -20˚C.

NoV and RV-specific immunoglobulin (Ig) detection from serum and IgG avidity assay

ELISA used to measure antigen-specific IgG, IgG1, and IgG2a from serum is described in details elsewhere [7,38]. Briefly, 96-well half-area polystyrene plates (Corning Inc., Corning, NY) were coated with GII-4, GI-3, GII-4 NO, GII-12 or GI-1 VLPs (0.4-1.5 µg/ml) or rVP6 (0.8 µg/ml). For the detection of antibodies against various RV culture antigens (described above) the plates were precoated with rabbit anti-rotavirus polyclonal antibody (DakoCytomation, Glostrup, Denmark) at 1 µg/ml in PBS followed by the addition of RV cell culture antigens at VP6 antigen concentration of 0.1 µg/ml. The serum samples (at 1:200 dilution or 2-fold dilution series) from immunized and control mice were added to the plates and the bound antibody was detected with HRP conjugated goat anti-mouse IgG (Sigma-Aldrich, Saint Louis, MO), IgG1 (Invitrogen, Carsbad, CA) or IgG2a (Invitrogen) followed by the reaction with the OPD substrate (Sigma-Aldrich). The optical density (OD) was measured at 490 nm (Victor2 1420; PerkinElmer, Waltham, MA). The background signal from the blank wells (wells without serum) was subtracted from all of the OD readings at a plate. The cutoff value was calculated from the wells of negative control mice serum as mean OD + 3 × SD. A sample was considered positive if the net OD value was above the set cut-off and at least 0.100 OD. End-point antibody titers were defined as the highest dilution of serum giving an OD above the set cut-off value. A Th2/Th1 response ratio was calculated by dividing the end-point titer of IgG1 response with the corresponding IgG2a titer.

Serum IgG avidity was measured by ELISA as described above with an extra urea incubation step to remove the low avidity antibodies [39,40]. The avidity index was calculated as (OD with urea/OD without urea) × 100% and avidity index ≥ 50% was considered high avidity.

NoV and RV-specific immunoglobulin (Ig) detection from mucosal samples and RV-specific IgA detection from serum

NoV-specific IgG content was tested from stool suspensions (10% suspension) with the ELISA as described above. RV rVP6-specific IgG and IgA in the stool suspensions and VWs and rVP6-specific IgA in serum were detected by sandwich ELISA as follows. The 96-well plate was first coated with rabbit anti-rotavirus polyclonal antibody (DakoCytomation, Glostrup, Denmark) at 1 µg/ml in PBS followed by the addition of rVP6 (1 µg/ml in PBS). After washing the unbound rVP6, 10% fecal suspensions (serially diluted from 1:5), VW samples (diluted 1:5 for IgG detection and 1:2 for IgA detection) or serum (diluted 1:2) were added and the plate was developed with 1:4000 diluted HRP conjugated goat anti-mouse IgG or IgA (both from Sigma-Aldrich) and OPD substrate.

NoV VLP blocking assays

Saliva-based blocking assays were used as a surrogate neutralization assay for NoV [41] and the procedure is described in details elsewhere [38]. In brief, serum dilutions from immunized and control mice were pre-incubated with NoV VLPs (at concentrations 0.1-0.2 µg/ml) for 1 h at 37˚C and added to secretor positive human saliva type A (for GII-4, GII-4 NO and GI-3 VLPs binding) or type O (for GI-1 VLP binding) coated 96-wells plates. VLPs lacking the serum were used for maximum binding of VLPs to the saliva. The VLPs bound to histo-blood group antigens (HBGAs) present in saliva were detected with NoV antibody positive human serum [40] and anti-human IgG-HRP (Invitrogen) following the OPD substrate development. The blocking index (%) was calculated as 100% – (OD wells with serum/OD wells without serum, maximum binding) × 100%.

Inhibition of RV infectivity in vitro

The ability of mucosal and serum antibodies to abolish RV infectivity *in vitro* was determined by an ELISA-based RV antigen reduction neutralization assay (NELISA) as described by others [42,43] with slight modifications. Two-fold dilution series of group wise pooled and 1:10 diluted fecal samples, VWs and sera from immunized and control mice were mixed with Wa (G1P1A [8]) RV strain homologous to the immunizing rVP6 protein or RRV (G3P5B [3]) containing 125 focus-forming units (ffu). RV antibody positive human serum diluted from 1:10 was used as a positive assay control. After 1 hour incubation at +37˚C the mixtures were overlaid to confluent MA104 cell monolayers in 96-well cell culture plates (Nunc, Roskilde, Denmark) following centrifugation for 60 min at 1000 × g. The virus inoculum was replaced with a culture medium containing trypsin (Sigma-Aldrich) at 4 µg/ml and the plates were incubated for 15 h at +37˚C. After lysing the cells with a thaw freeze cycle the RV detection in duplicate samples was performed by a Ridascreen® kit (R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions. A reduction in OD value greater than 60% compared with the positive control wells (trypsin activated RV without the test sample) was considered to indicate neutralization. Neutralizing titers were expressed as the highest sample dilution yielding neutralization.

Detection of interferon-γ (IFN-γ) producing T cells

NoV and RV-specific T cell responses were analyzed by quantification of IFN-γ production from splenocytes by ELISPOT [38] with slight modifications. Ninety-six well MultiScreenHTS-IP filter plates (Millipore, Billerica, MA) were coated with monoclonal anti-mouse IFN-γ (Mabtech Ab, Nacka Strand, Sweden) at 5 µg/ml. After blocking the plates with 10% fetal bovine serum (FBS, Sigma-Aldrich) the antigens and the cells in the culture media (CM, RPMI-1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol and 2 mM L-glutamine, all purchased from Sigma-Aldrich) and 5% FBS were added. NoV capsid-derived synthetic 15-mer peptides (Proimmune Ltd., Oxford, UK) identical to a published T-cell epitope of GII-4 (CLLPQEWVQHFYQEA, amino acids 461–475) [44] and corresponding peptides of GII-4 NO (CLLPQEWVQYFYQEA) and GII-12 (CLLPQEWIQHLYQES) were used at 5 µg/ml to stimulate individual mouse splenocytes (0.1×10^6 cells/well) for NoV-specific INF-γ production. For detection of RV-specific IFN-γ producing cells, group-wise pooled splenocytes were stimulated with VP6 derived 18-mer peptide previously

identified as a VP6-specific CD4+ T cell epitope (DGATTWYFNPVILRPNNV, amino acids 242-259) [45] at 5 µg/ml or RV cell culture antigens (Wa G1P1A [8], BrB G4P2 [6], WC3 G6P7 [5] and RRV G3P5B [3]) at a VP6 concentration of 0.5 µg/ml. Mock infected MA104 cell cultures were used as a negative control. Background control (cells with CM only) and cell viability control (cells stimulated with 10 µg/ml of Conacavalin A, Sigma-Aldrich) were added to each assay. The plates were incubated for 20 h at +37˚C and 5% CO^2 after which the cells were discarded and the plates were thoroughly washed with PBS. Biotinylated anti-mouse IFN-γ monoclonal antibody (Mabtech, 0.5 µg/ml in PBS / 0.5% FBS) was added and the plates incubated for 2 h at RT. After washing, 1:1000 diluted streptavidin-ALP (Mabtech) was added and the plates were incubated for 1h. The spots were developed with BCIP/NBT substrate (Mabtech) and the formation of color reaction stopped with tap water. The spots were counted by an ImmunoSpot® automatic CTL analyzer (CTL-Europe GmbH, Bonn, Germany) and the results are expressed as mean spot-forming cells (SFC) per 10^6 splenocytes of duplicate wells.

Statistical analyzes

A Mann–Whitney *U*-test was used to assess the statistical difference between non-parametric observations of two independent groups. Statistical analyses were done by IBM SPSS Statistics -software (SPSS Inc., Chicago, IL) version 19.0 and the statistical significant difference was defined as p ≤ 0.05.

Results

Morphology of NoV VLPs and RV rVP6 and formulation of the trivalent vaccine

The assembly conformations of NoV GII-4 VLPs, GI-3 VLPs and RV rVP6 were verified by transmission electron microscopy (TEM) as described previously [36]. As illustrated in Figure 1, recombinant BV-produced NoV VP1capsid proteins self-assembled into the GII-4 VLPs of ~38 nm (Figure 1A) and GI-3 VLPs (Figure 1B) of ~30 nm in diameter. RV rVP6 production resulted in conformation of VP6 trimers, which under neutral pH conditions (PBS, pH 7.4) assembled into tubular structures but also to a minor number of sheets (Figure 1C) [34]. The combination of both NoV VLPs and rVP6 in the ratio of 1:1:1 resulted in the trivalent formulation where the VP6 tubules were partly filled with the VLPs (Figure 1D).

Dose response of single antigen immunizations

The optimal amount of antigens to be used in the trivalent vaccine was pre-determined by a dose response study in BALB/c mice immunized with 3, 10 and 30 µg of NoV GII-4 VLPs, GI-3 VLPs or RV rVP6 as single antigens. The dose responses to each antigen were screened by measuring antigen-specific serum IgG antibody titers in ELISA. All three antigens induced robust systemic IgG responses in mice (Figure 2A–C). No significant difference (p>0.05) in the levels of IgG in the termination sera was detected between the groups immunized with 10 and 30 µg of the antigens, whereas a 3 µg dose raised the significantly lower IgG response to each of the antigens (p<0.05). Additional immunological assays including antigen-specific IgG avidity, IgG subtype ratio (IgG1/IgG2a), IgG cross reactivity, NoV VLPs blocking activity and intestinal antibody content confirmed the same result (data not shown). Therefore, the 10 µg dose for each antigen was used in the further immunogenicity studies described below.

Magnitude and avidity of antigen-specific IgG responses and IgG subtype balance

Groups of BALB/c mice were immunized two times with 10 µg of NoV GII-4 VLPs, GI-3 VLPs or RV rVP6 as single antigens or with the combination of all three proteins (10+10+10 µg) and the immune responses induced in each group were compared at study week 5. The duration of the immune response induced by the trivalent formulation was followed in another group of mice terminated at study week 24. All antigens induced a robust homologous IgG response (Figure 3A–C) and there were no statistical differences between the immune responses induced by single antigens versus the trivalent formulation (all p>0.05). Although approximately one-fold decrease in the NoV-specific titers occurred from week 5 to 24 (Figure 3A-B), the magnitude of the response still remained high with GII-4 and GI-3-specific titers of 4log10. Kinetics of GII-4, GI-3 and rVP6-specific IgG measured from tail blood samples showed that after the second immunization (at week 3) there were no variations in the levels of antigen-specific IgGs up to study week 20 (Figure 3D). The antigen-specific IgGs were of high avidity (mean avidity index >50%) and no statistically significant differences (p>0.05) were observed between the single versus trivalent combination immunizations (Figure 3E–G) at study week 5. The avidity was long-lasting as high avidity IgGs against all three antigens were still observed 24 weeks after the last immunization in the majority of mice sera receiving trivalent formulation (Figure 3E–G). Antigen-specific IgG subtype titers for IgG1 (representing a Th2 response) and IgG2a (representing a Th1 response) were also measured (data not shown) and Th2/Th1 ratios determined. Trivalent immunization resulted in GII-4, GI-3, and rVP6-specific Th2/Th1 ratios of 0.5, 0.6 and 0.8 respectively, indicating that the combination vaccine triggers a well-balanced Th2/Th1 response.

Cross reactive antibody responses

The cross-reactivity of the serum antibodies induced by the single versus trivalent immunizations were measured in ELISA against heterologous NoV VLPs derived from genogroup II (GII-4 NO and GII-12) and genogroup I (GI-1) not included in the immunization. GII-4 and GI-3 VLP immunizations induced high levels (mean OD>1.5) of cross-reactive antibodies against VLPs belonging to the same genogroup and significantly lower levels (mean OD<0.6, p<0.01) of antibodies against the VLPs belonging to the other genogroup (Figure 4A). The trivalent vaccine immunization triggered high levels of cross-reactive IgGs to all NoV VLPs tested, therefore indicative of a strong humoral response generation against both genogroups of NoVs. In addition, similar levels of intra genogroup antibodies (all p>0.05) were observed in the trivalent combination

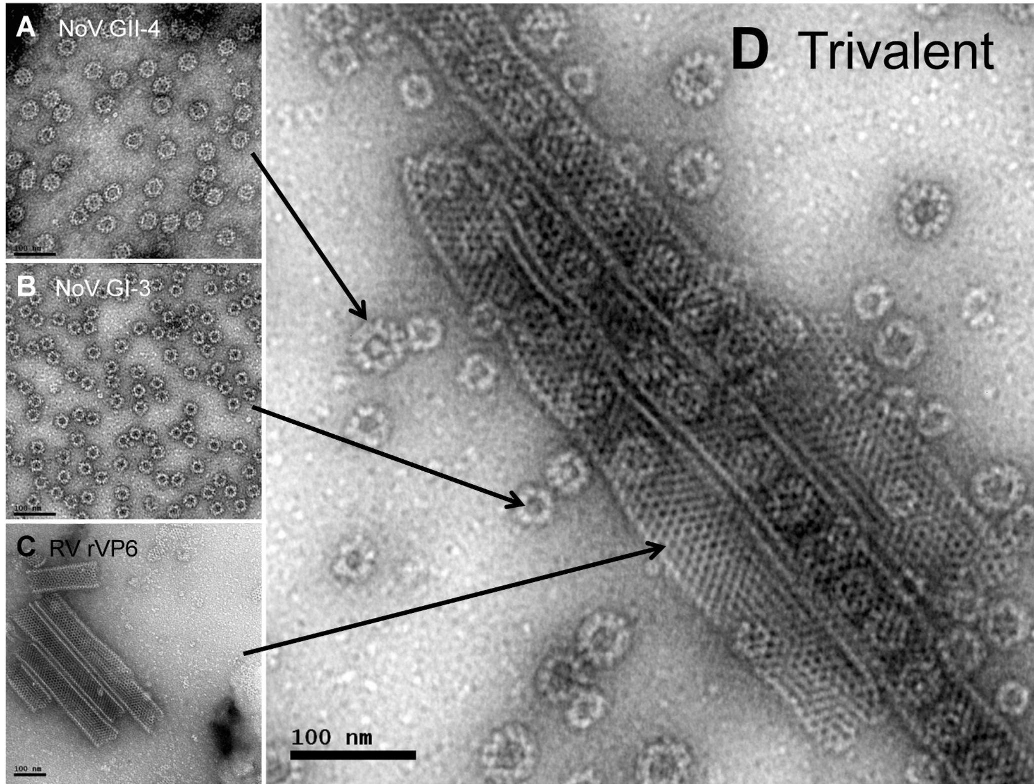

Figure 1. Electron microscopy images of the single antigens and the trivalent combination used to immunize BALB/c mice. Morphological assemblies of NoV GII-4 capsid (A), GI-3 capsid (B) and RV rVP6 (C) proteins, and the trivalent combination (1:1:1 of each antigen) of the structures depicted in panels A-C (D) were examined by transmission electron microscopy (TEM) using a FEI Tecnai F12 electron microscope (Philips Electron Optics, Holland) with 18,500 × magnification following negative staining with 3% uranyl acetate (UA), pH 4.6. The arrows represent each structure (A–C) in the trivalent assembly (D). Bar 100 nm.
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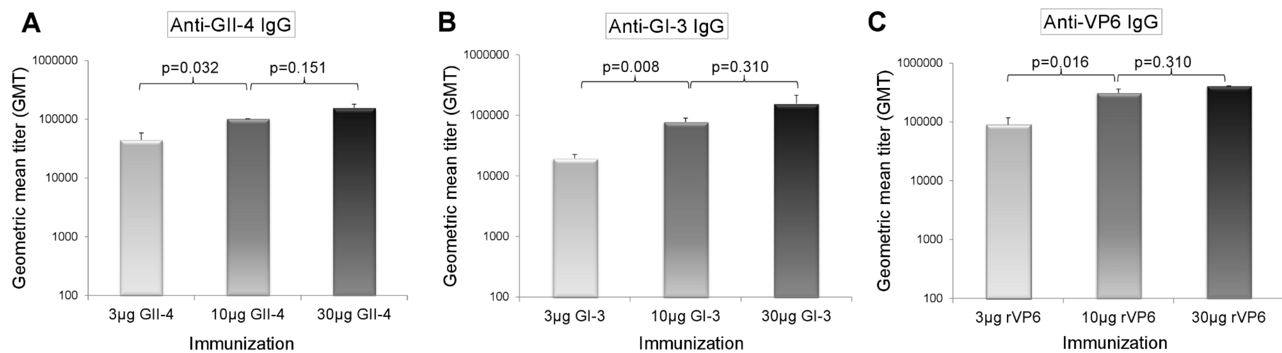

Figure 2. Antigen-specific serum IgG dose response. Mice were immunized twice at study weeks 0 and 3 with 3, 10 and 30 µg of single antigens and the geometric mean titers (GMTs) induced by GII-4 VLPs (A), GI-3 VLPs (B) and RV rVP6 (C) were measured in an ELISA. The error bars represent the standard error of the means. Statistical differences between any two experimental groups were determined by a Mann–Whitney *U*-test and the p-value ≤ 0.05 was considered a statistically significant difference.
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immunized group compared with the group of VLPs immunized separately, indicating that there was no mutual inhibition of the antigens in the combination (Figure 4A). These cross-reactive NoV-specific IgGs were also of long duration (Figure 4A). In

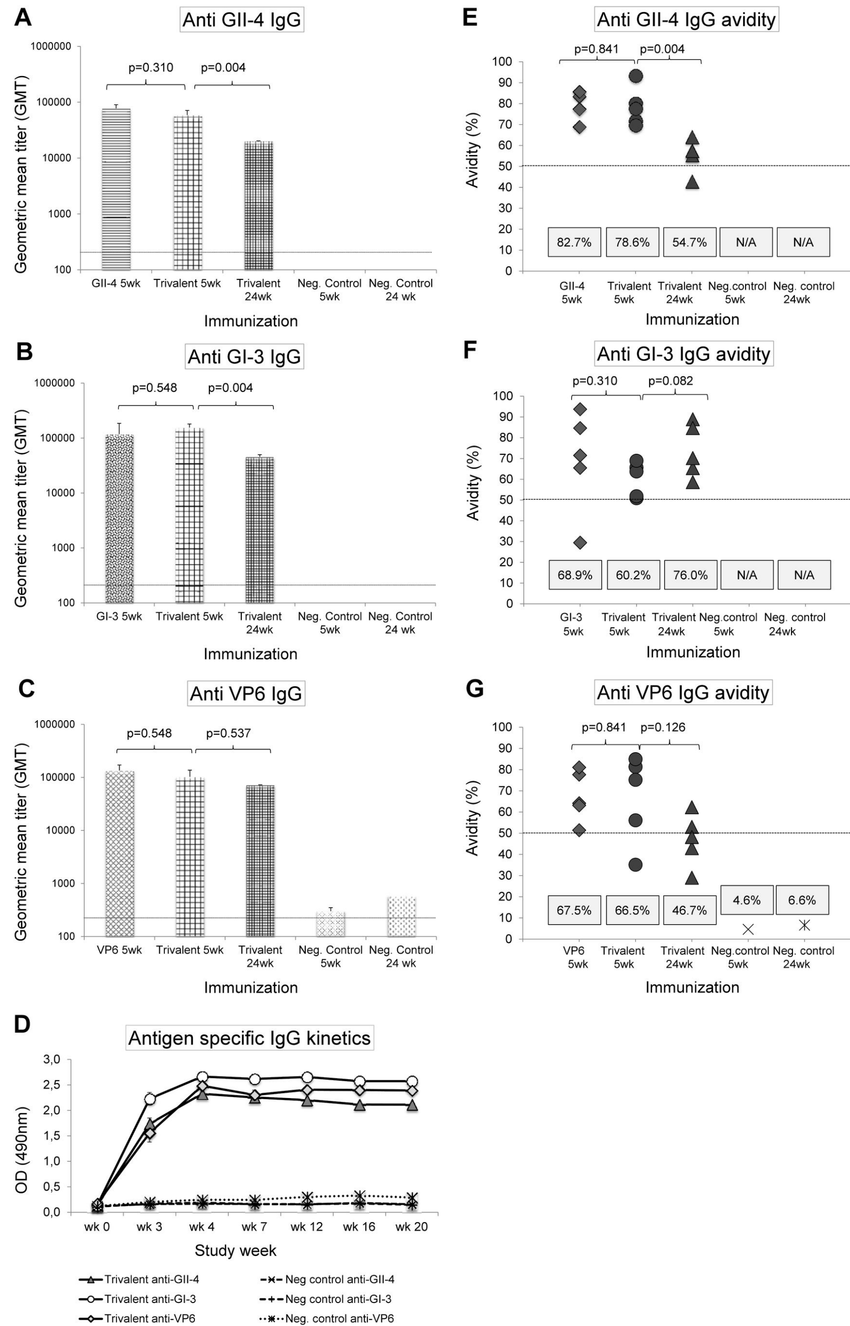

Figure 3. Serum IgG responses induced by the single antigens versus trivalent combination. Mice were immunized two times with 10 µg of the single antigen (GII-4 VLPs, GI-3 VLPs or rVP6) or the trivalent combination (each antigen at a 10 µg dose), and the sera at study week 5 and 24 were tested against GII-4 VLPs (A), GI-3 VLPs (B) and rVP6 (C) in ELISA. Shown are the geometric mean titers (GMTs) of the sera with standard errors of the means. The horizontal lines on panels A-C indicate the limit of detection for the assay. Kinetics of GII-4, GI-3 and VP6-specific IgG responses induced by the trivalent vaccine were measured from tail blood samples of immunized and control mice, and the OD values representing the quantity of antigen-specific IgG at any given time point are shown (D). The avidity of GII-4 (E), GI-3 (F) and rVP6-specific (G) serum IgG antibodies was tested from individual mouse termination sera (at 1:200 dilution) in a modified ELISA in which urea was used to strip off the low avidity antibodies. Shown are the individual mice antigen-specific avidity indexes (%) and the boxed values at the bottom of each figure indicate the group mean avidity indexes. The avidity index was calculated as (OD with urea/OD without urea) × 100%. Avidity index ≥ 50% was considered high avidity. Statistical differences between any two experimental groups were determined by a Mann–Whitney *U*-test and the p-value ≤ 0.05 was considered a statistically significant difference.

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addition to the serum IgG levels represented by the OD value, the GMTs were determined for each study group to confirm the results of the magnitude of cross-reactive IgG response. Similarly to the OD values, the GMTs of cross-reactive antibodies were higher (16 to 32-fold higher, $p<0.05$) in the trivalent than single immunized mice groups when considering inter genogroup responses (data not shown).

Cross-reactive antibodies against seven RV cell culture antigens belonging to human (G1PA [8], G4P2 [6], G2P2 [6], G8P4 [10] and G12P [4]), bovine (G6P7 [5]), and rhesus RV strains (G3P5B [3]) were detected in mice sera after rVP6 immunization (Figure 4B). No difference in the antibody levels ($p>0.05$) were noted whether rVP6 was administered alone or in the trivalent combination with NoV VLPs. The magnitude of the response was somewhat lower at the week 24 than at week 5 but still high levels of cross-reactive antibodies (mean OD 1.3-2.0) were detected (Figure 4B).

Mucosal antibodies and serum VP6 specific IgA

Intestinal NoV and RV-specific IgG were measured from group-wise pooled 10% fecal suspensions in ELISA. Moderate levels of antigen-specific intestinal anti-GII-4 IgG (Figure 5A), anti-GI-3 IgG (Figure 5B), and anti-VP6 IgG (Figure 5C) were detected after each antigen immunizations alone or in the trivalent combination. The stool suspensions from the negative control mice were all IgG negative (Figure 5A–5C). VW samples at study week 5 from the mice immunized with the trivalent combination were tested in ELISA for the detection of RV-specific IgG and IgA antibodies. A moderate level of rVP6-specific IgG and a low level of rVP6-specific IgA were detected from VW samples (Figure 5D). A low level of VP6 specific IgA (OD 0.176, at a 1:2 dilution) was detected from the trivalent combination immunized mice serum (data not shown).

NoV blocking assays and RV inhibition assay

Saliva blocking assays were conducted to study blocking of homologous (immunogen-specific) and heterologous (non-immunogen-specific) NoV VLPs binding to the saliva HBGAs with mice antiserum (Figure 6). Group-wise pooled sera of mice immunized with the single antigen or the trivalent combination blocked homologous GII-4 (Figure 6A) and GI-3 (Figure 6B) VLP binding to saliva HBGAs with a similar intensity. The serum titers for total (100%) blocking of the homologous VLPs binding to the saliva were at maximum 1:400 for GII-4 and 1:200 for GI-3 VLPs. However, mice sera immunized with the GI-3 VLPs alone did not cross-block binding of GII-4 to the saliva (Figure 6A). Likewise, sera of mice immunized with the GII-4 VLPs alone did not cross-block GI-3 VLP binding (Figure 6B). These results indicate that NoV cross-genogroup blocking activity cannot be induced with a single NoV VLP immunization, although cross-reactive binding antibodies were detected in ELISA (Figure 4A). The trivalent combination immunized mice sera were able to block both of the VLPs binding with a similar intensity as the single VLPs immunized mice, and these activities were preserved for the whole 24-week study period (Figure 6A and 6B). Serum blocking of non-immunogen GII-4 NO VLPs (Figure 6C) and GI-1 VLPs (Figure 6D) binding to the saliva was also obtained genogroup-wise; GII-4 immunization induced GII-4 NO and GI-3 immunization GI-1 blocking antibodies. The heterologous blocking activity against VLPs inside the genogroup was similar whether the antigen was administered alone or in the trivalent combination vaccine (Figure 6C and 6D) and lasted until study week 24.

To detect the functionality of VP6-specific antibodies fecal suspensions, VWs and sera were used to inhibit RV infectivity *in vitro* by ELISA-based antigen-reduction neutralization assay [42,43]. Our attempts to use fecal suspensions in the assay failed, probably because of the toxicity of the suspensions for MA104 cells, as previously shown by others [46]. Therefore, we used VWs instead, which likewise to fecal suspensions, contain mucosal antibodies as described above. Inhibition of the infectivity of RVs Wa (G1P1A [8]), homologous to the immunizing protein and RRV (G3P5B [3]), was detected with the VW of the trivalent combination immunized mice with maximum neutralizing titers of 1:160 and 1:320, respectively (Figure 6E). The VW samples from negative control mice did not inhibit RV infection, whereas the positive human control serum neutralized both viruses (Figure 6E). In addition, mouse immune sera did not inhibit RV infectivity *in vitro* (data not shown). The experiments were repeated several times with consistent results.

Cell mediated immune responses

NoV and RV-specific IFN-γ producing cells were quantified from mice splenocytes by an ELISPOT assay (Figure 7). Mice immunized with the GII-4 VLPs or the trivalent combination vaccine elicited a robust IFN-γ response when stimulated with the 15-mer peptides representing capsid P-domain T-cell epitopes [44] derived from homotypic GII-4 or heterotypic GII-4 NO and GII-12 genotypes as described in Materials and Methods. No statistically significant difference was observed in any responses between these experimental groups ($p>0.05$) at study week 5 (Figure 7A). The IFN-γ response induced by the trivalent vaccine did not diminish over time as IFN-γ producing cell frequency was similar ($p>0.05$) at study week 5 and 24. GI-3 VLP immunization did not induce any cross-reactive IFN-γ responses to any of the GII peptides. No IFN-γ responses were detected to any peptides by the cells of negative control mice. Immunization with rVP6 either as a single antigen or in the trivalent combination resulted in considerable IFN-γ production when the cells were stimulated with the synthetic peptide representing CD4+ T cell epitope [45] or RV cell culture antigens Wa G1P1A [8], BrB G4P2 [6], bov WC3 G6P7 [5] and rhesus RV G3P5B [3] (Figure 7B). IFN-γ responses were detected against all stimulants at study week 24 but the magnitude of IFN-γ response was up to 3-fold lower in some instances compared with study week 5. No response to mock-infected MA104 cells was detected in any immunized group (Figure 7B) while cell viability was similar in all groups controlled by Con A stimulation (data not shown).

Discussion

In our previous work we introduced the concept of vaccinating against NoV and RV by parenteral injection of a

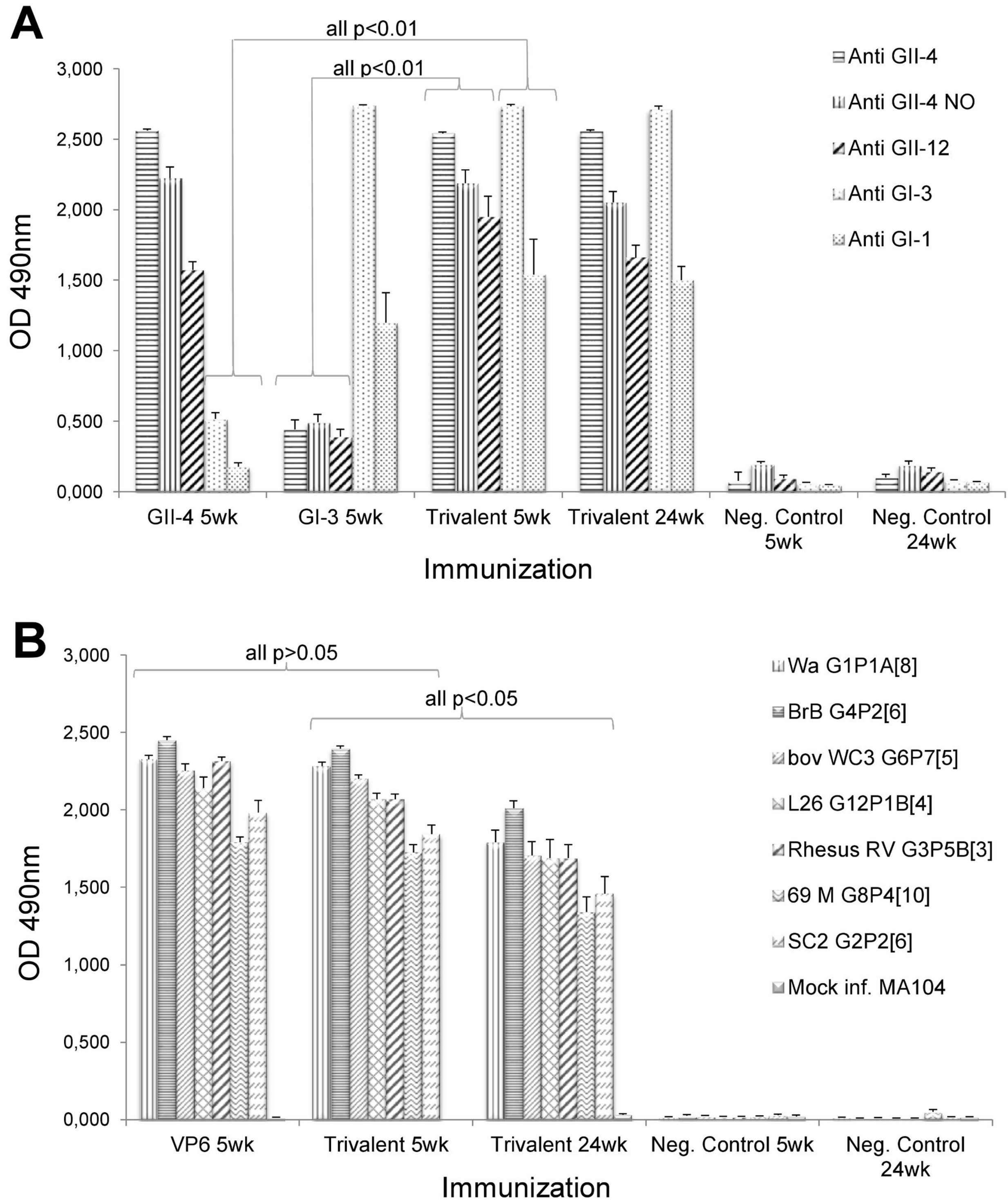

Figure 4. Cross-reactive serum IgG antibodies. Mice were immunized two times with 10 µg of the single antigen (GII-4 VLPs, GI-3 VLPs or rVP6) or the trivalent combination (each antigen at a 10 µg dose) and the sera were tested against heterologous NoV VLPs (A) and RV cell culture antigens (B) in ELISA. Shown are experimental and control groups' mean OD values representing the quantity of antigen-specific IgG. The error bars represent standard errors of the mean. A Mann–Whitney *U*-test was used to determine statistical differences between single antigen-induced IgG quantities compared with trivalent vaccine induced IgG quantities at study week 5 and IgG quantities induced by the trivalent vaccine at study weeks 5 and 24. The p-value ≤ 0.05 is considered a statistically significant difference.
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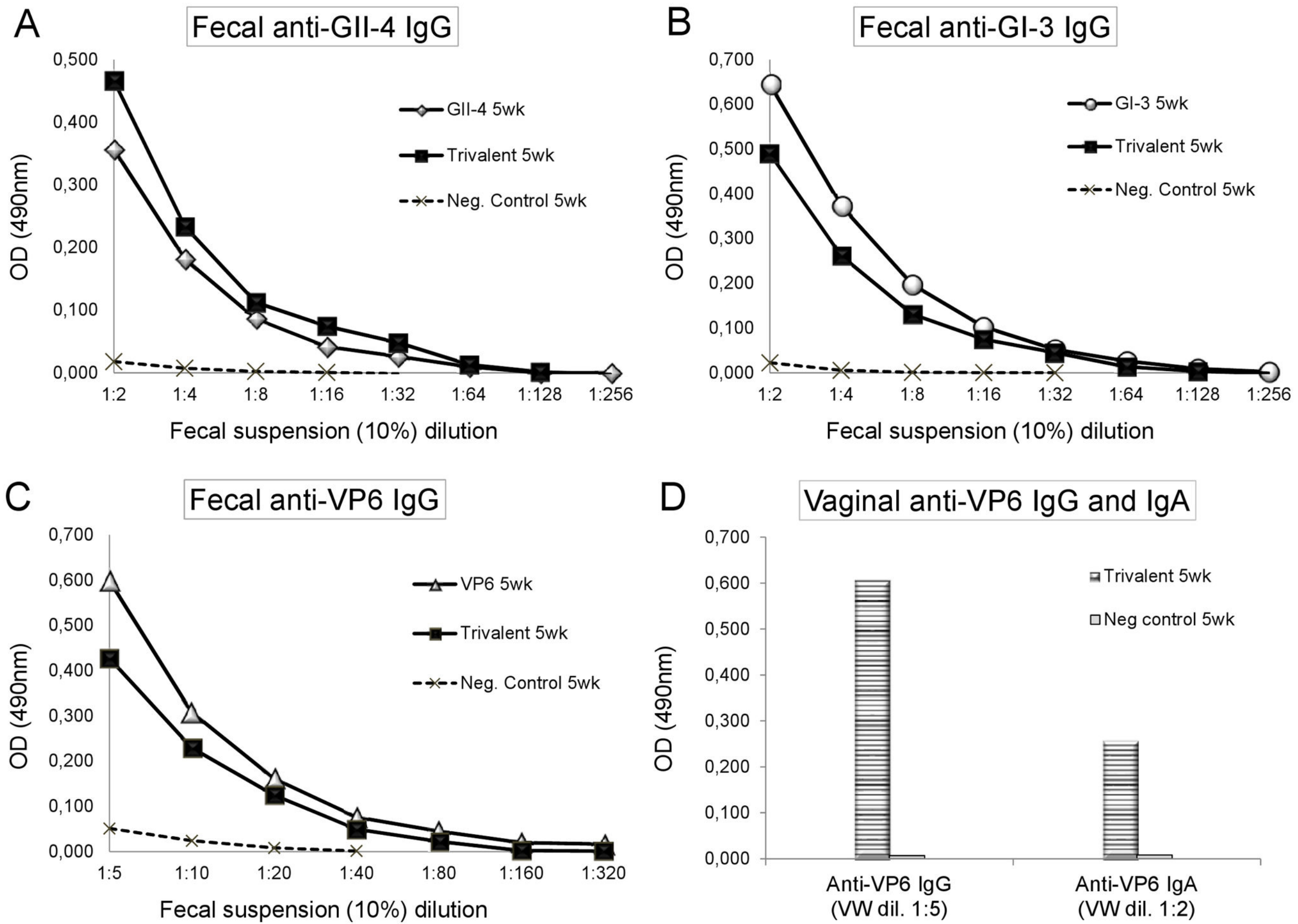

Figure 5. Mucosal antibody response. Group-wise pooled stool samples (10% suspension) of mice immunized with the single NoV GII-4 VLPs, GI-3 VLPs or RV rVP6 antigens or the trivalent combination vaccine were titrated two-fold and anti-GII-4 (A), anti-GI-3 (B) and anti-VP6 (C) IgG content was measured in ELISA. Anti-VP6 IgG and IgA antibodies were measured from the trivalent combination vaccine immunized and control mice vaginal wash samples diluted 1:5 for IgG detection and 1:2 for IgA detection (D). Shown are experimental and control groups' mean OD values representing the quantity of antigen-specific antibody.

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dual combination vaccine consisting of NoV GII-4 VLPs and RV rVP6 [7]. In the present study we have included GI-3 VLPs as a representative of GI NoVs in the dual vaccine candidate and generated a trivalent combination in an attempt to develop potentially neutralizing cross-reactive antibody responses against GI and GII of NoVs. We also investigated induction of NoV and RV-specific cell mediated immunity as well as RV inhibition by VP6-specific antibodies.

Genogroup I NoVs are antigenically very well conserved [47] and we have chosen GI-3 genotype in the trivalent vaccine combination as it is an important agent in NoV outbreaks and has been the most prevalent GI genotype in pediatric NoV gastroenteritis in Finland in recent years [3]. We hypothesized that by combining VLPs derived from GII-4, the most prevalent NoV genotype worldwide [14], and GI-3 in a single vaccine would give the substantial amount of cross-reactivity needed from a broadly effective NoV vaccine. Recombinant VP6 protein was selected as a part of the trivalent combination vaccine as numerous studies in animal models have documented the protective role of VP6-specific antibodies and T cells in RV infection [30–33]. Both NoV VLPs and rVP6 tubular structures are optimal for dentritic cells uptake [48,49]. The size difference observed between GII-4 and GI-3 VLPs (~38 nm and ~30 nm, respectively) did not affect the immunogenicity of the VLPs as similar immune responses were induced with both of these particles. Although we did not attempt to identify the reason/s for the VLPs size difference, it may be that different number of VP1 monomers are assembled in a single VLP similarly to observations made by White et al. [50]. In addition, natural amino acid differences in the VP1 proteins may drive different size VLP formation [51].

The results from the present study show that two IM immunizations with NoV GII-4 or GI-3 VLPs, either alone or in the trivalent combination with RV rVP6 without an external adjuvant, induced a strong, long-lasting antigen-specific IgG response in mice. In addition the presence of NoV IgG in the

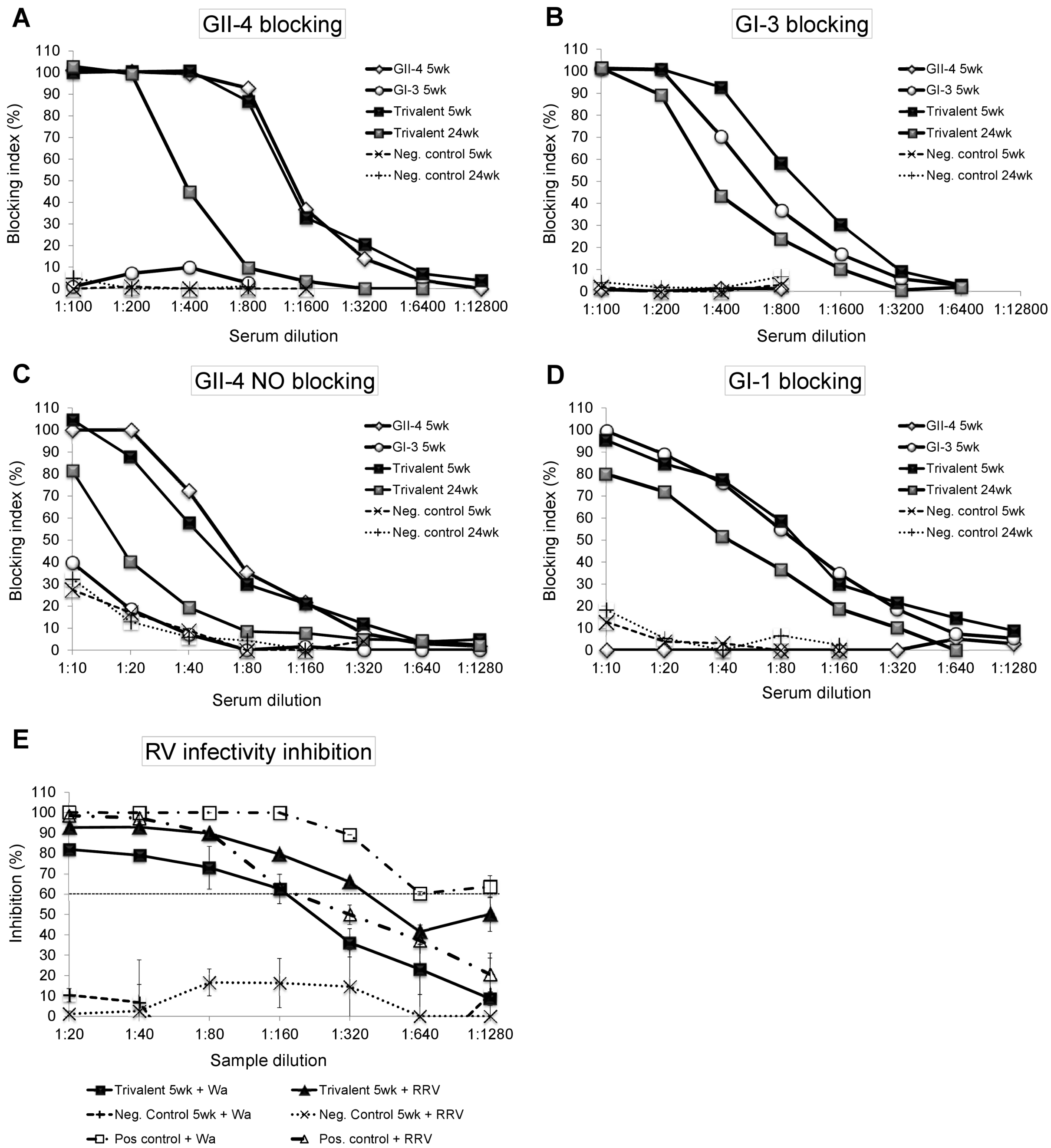

Figure 6. Functionality of NoV and RV-specific antibodies. Termination sera of mice immunized with the single NoV GII-4 or GI-3 VLPs antigens or the trivalent combination vaccine were pooled group-wise, titrated two-fold and used for blocking the binding of homologous GII-4 and GI-3 VLPs (A, B) or heterologous GII-4 NO and GI-1 VLPs (C, D) to human secretor positive saliva (type A for GII-4, GII-4 NO and GI-3 binding and type O for GI-1 binding). Serum from mice receiving the carrier only (PBS) was used as a negative control. The blocking index (%) was calculated as 100% – (OD wells with serum/OD wells without serum, maximum binding) × 100%. Vaginal washes of mice immunized with the trivalent combination vaccine were tested for inhibition of human RV Wa (G1P1A [8]) strain homologous to the immunizing rVP6 protein, or rhesus RV (G3P5B [3]) infectivity by neutralizing ELISA (NELISA). Vaginal washes of mice receiving the carrier only (PBS) and serum from a RV seropositive human donor were used as negative and positive controls. Results are shown as the mean percentage (%) inhibition of rotavirus infectivity of duplicate wells with standard errors. A dashed horizontal line indicates 60% reduction in virus infectivity.
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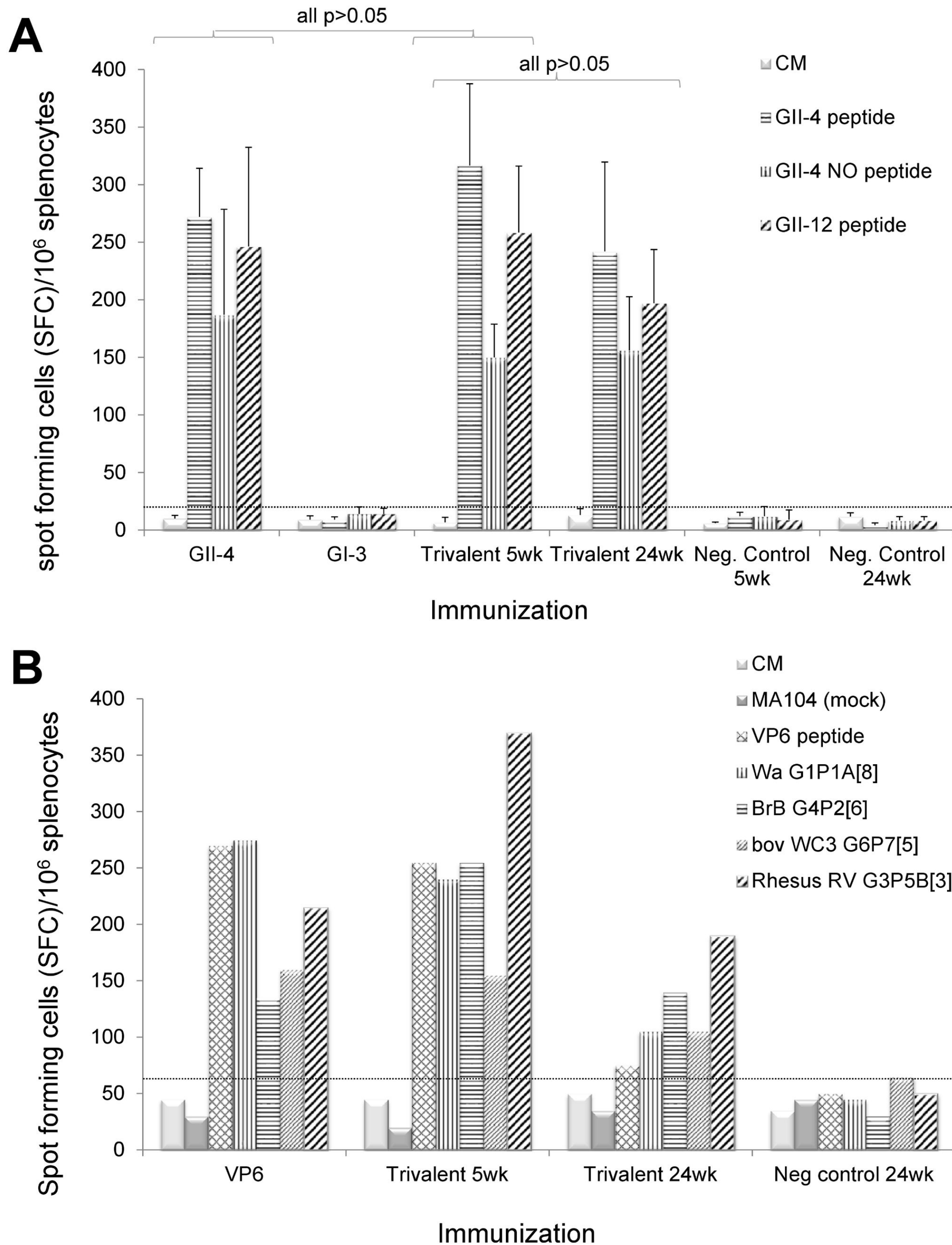

Figure 7. NoV and RV-specific IFN-γ responses. Splenocytes of mice immunized with the single NoV GII-4 or GI-3 VLPs or the trivalent combination vaccine were stimulated with synthetic NoV capsid-derived 15-mer peptides from different NoV genotypes and analyzed for IFN-γ production by an ELISPOT assay (A). The mean spot forming cells (SFC)/10^6 cells are shown. The error bars represent the standard errors of the mean. The statistical differences between any two experimental groups' response to a given peptide were determined by a Mann–Whitney *U*-test and the p-value ≤ 0.05 was considered a statistically significant difference. Splenocytes of rVP6 or the trivalent vaccine immunized mice were pooled group-wise and stimulated with synthetic VP6-derived 18-mer peptide or RV cell culture antigens and analyzed for IFN-γ production by the ELISPOT (B). Splenocytes from mice receiving the carrier only (PBS) were used as negative control cells. The mean spot forming cells (SFC)/10^6 cells of the replicate wells are shown. The dashed line in each figure indicates the maximum background level (cut-off limit) obtained from cells incubated in a culture media (CM) only (mean SFC/10^6 + 3× SD).

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gut lumen as detected in here is considered to be an important mechanism in protection against gut infection [52]. As NoVs have great antigenic diversity and are fast evolving viruses, the antibody response elicited by NoV vaccine should be cross-reactive across GI and GII genogroups [19,53]. Our results show that a robust cross-reactive NoV antibody response against both genogroups was solely achieved by the trivalent vaccine, whereas single vaccinations induced a much stronger intra than inter genogroup antibody response. Virus neutralizing potential of the antibodies is an important correlate of protection [6,40,54]. As the traditional neutralization assay is not an option for NoVs that are not able to grow in cell cultures [9], a surrogate neutralization assay named blocking assay using NoV VLPs and HBGAs has been developed instead [41,55]. We have detected high titer of type-specific blocking antibodies in the sera of immunized mice and each antisera was able to block binding of the heterologous VLPs not included in the immunizing formulation but belonging to the same NoV genogroup, namely GII-4 NO and GI-1 VLPs. However, neither GII-4 nor GI-3 VLP immunization alone could induce blocking antibodies towards the VLPs from the other genogroup although cross-reactive binding antibodies were induced (Figure 4A). These observations are in line with the previous findings showing that blocking antibodies are genogroup specific and there is very little inter genogroup blocking activity [47,53,54]. When GII-4 and GI-3 VLPs were combined in the trivalent vaccine, the mice antiserum could block binding of the immunizing and non-immunizing VLPs from both genogroups. The data obtained herein further supports the hypothesis that only multivalent NoV vaccination will induce broadly protective NoV immunity [17–19,53].

The research involving NoV immunity has been largely focused on the antibodies however, cell-mediated immune responses might be important in the clearance of NoV, as has been shown for other viruses [56,57]. We have detected that T cells in the immunized mice produce high levels of IFN-γ in response to synthetic peptides representing T cell epitopes derived from the immunizing (GII-4) [44] and heterotypic (GII-12 and GII-4 NO) NoV genotypes. Lindesmith and co-workers [58] have shown that T cell responses (specifically IFN-γ and IL-2 production) might have been associated with protection in NoV challenge study.

Due to the highly conserved nature [28] RV VP6 protein could provide protection against a broad range of RV serotypes. Although VP6 does not induce serum neutralizing antibodies it has been suggested that VP6 confers protection in mice by inducing a strong CD4+ T-cell response [59] and/or by stimulating mucosal antibodies, especially IgA [60–62]. Our results show that rVP6 assembled in tubular forms is very immunogenic in mice, stimulating a robust, long lasting, high avidity IgG response in serum reactive with various RV strains. Anti-rVP6-specific IgG and IgA were also found in the mucosal samples indicating that an anti-VP6 antibody was being transferred to the gut, the location where the first line of defense is taking place. These mucosal VP6-specific antibodies in contrast to the serum antibodies, inhibited human and rhesus RV infectivity *in vitro*, indicative of the heterotypic protective antibody induction against RVs. Although the mechanism of inhibition remains to be determined, we believe that VP6-specific mucosal IgG and especially IgA are responsible for the inhibition. To support of this, although high level of VP6-specific IgG and low level of VP6-specific IgA were present in serum as well, it did not inhibit RV infectivity. Others have shown that RV VP6 protection from RV infection in vitro and in vivo was mediated by the VP6-specific mucosal IgA and not the VP6-specific serum antibodies [60–64]. Although IM immunization usually elicits systemic immune responses without decent mucosal immunity, it has been shown [65] that naïve B cells acting as antigen presenting cells (APC) are responsible for RV-specific IgA production in the gut after parental immunization in mice. After IM inoculation these APC migrate from draining lymph nodes to mucosal lymphoid tissue, where they induce the production of virus-specific IgA secreting cells. Indeed, Parez and co-workers [66] have shown that specifically RV VP6 protein interacts with a large fraction of naïve B cells via surface immunoglubulins.

We also observed that the cellular immune responses were activated upon rVP6 immunization as the cells of immunized mice produced IFN-γ when stimulated with the VP6-derived peptide representing CD4+ T cell epitope [45] or with various RV cell culture antigens. In our earlier work we have identified CD4+ T cells as being the principal lymphocyte population accountable for IFN-γ production [67]. McNeal and co-workers have shown that CD4+ T cells as the ones we describe here, are the only lymphocyte population responsible for the protective immunity against murine RV [31].

Our results show that the trivalent vaccine consisting of NoV GII-4 and GI-3 VLPs and RV rVP6 1) stimulates strong systemic cross-reactive antibody responses to both viruses with inter NoV genogroup neutralizing ability; 2) induces mucosal antibodies able to inhibit RVs infectivity; and 3) activates the cellular arm of the immune responses to both viruses. Importantly, all the immune responses induced by the trivalent vaccine were long-lasting and no mutual interference and/or inhibition of the vaccine components in the formulation was observed. The results obtained here are encouraging and support the development of a non-live subunit combination vaccine against NoV and RV for humans.

Acknowledgements

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Author Contributions

Conceived and designed the experiments: VB KT SL TV. Performed the experiments: KT SL. Analyzed the data: KT SL VB. Contributed reagents/materials/analysis tools: SL KT VB

LH. Wrote the manuscript: KT VB. Project and manuscript supervision: VB TV.

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