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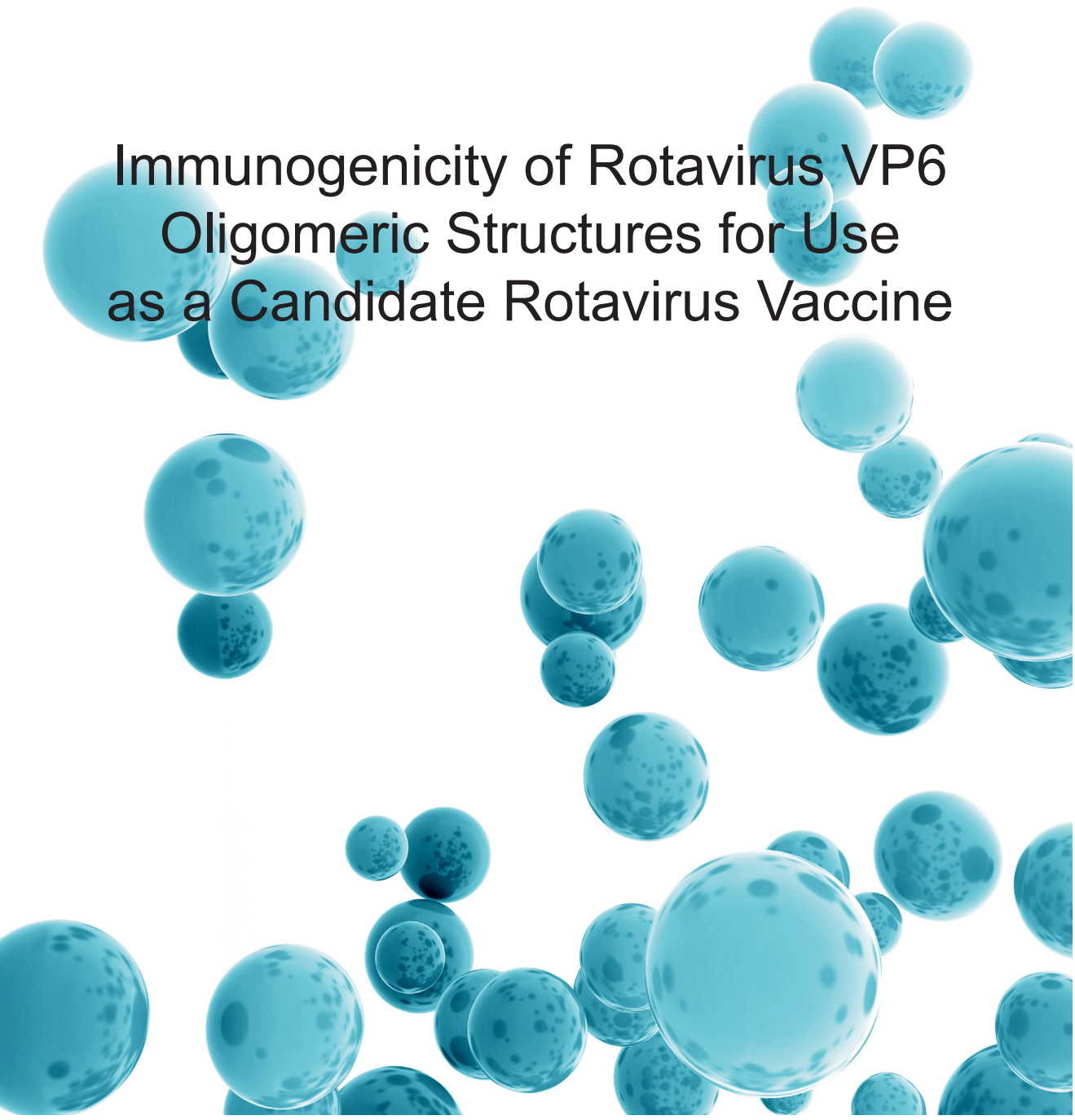

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ACADEMIC DISSERTATION
To be presented, with the permission of
the Board of the School of Medicine of the University of Tampere,
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UNIVERSITY OF TAMPERE

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To my family,

Contents

Abstract

Rotavirus (RV) is the most common cause of acute gastroenteritis (AGE) in infants and young children worldwide. An estimated 450,000 children annually, mostly in the developing world, die from diarrheal disease caused by RV. Two live attenuated oral RV vaccines, the human RV vaccine Rotarix® and the bovine-human RV vaccine RotaTeq®, are largely safe and efficacious against severe RV disease associated with various RV genotypes. Despite their high efficacy in the developed world, both oral vaccines show lower efficacy in developing countries. Moreover, safety issues, including a low risk of intussusception and the risk of reassortment between the vaccine and a wild-type strain, are concerns associated with the live-attenuated RV vaccines. Non-live subunit protein vaccines have therefore been considered for use in RV immunization.

This thesis investigates the RV inner capsid VP6 protein, a highly conserved and abundant protein of the RV particle, as a non-live RV vaccine candidate. The ability of VP6 to self-assemble into highly immunogenic oligomeric structures with repetitive multivalent expression of antigen makes it the simplest non-live subunit RV vaccine candidate. In this study, VP6 tubular structures and double-layered (dl)2/6 virus-like particles (VLPs) with core protein VP2 were produced by a baculovirus expression system utilizing VP6- and VP2-specific sequences from a Finnish patient. Both VP6 formulations were equally immunogenic in an adult mouse model by inducing strong homologous and cross-reactive IgG responses. Therefore, both structures were regarded as potential vaccine candidates against RV.

The overall objective of the studies on RV VP6 formulations is to develop a non-live subunit vaccine against RV in combination with norovirus (NoV), another leading viral cause of severe gastroenteritis, to combat AGE in children with a single vaccine. To create bivalent and trivalent combination vaccines, RV rVP6 protein was combined with NoV GII.4 VLPs or NoV GII.4 and GI.3 VLPs. Induction of similar antibody responses with rVP6 as a single antigen or in a bivalent or trivalent combination with NoV VLPs indicated that the addition of the NoV antigens to the vaccine formulation did not affect the VP6-specific responses.

The final part of the study focused on protective immunity in adult mice induced by VP6. Intramuscular and intranasal deliveries of the trivalent vaccine containing

rVP6 protein or dl2/6-VLPs induced activation of strong B and T cell immune responses. Both VP6-specific mucosal IgA antibodies and $CD4^+$ T cells have been previously implicated as mediators of protection against RV infection in mice. Comparable protection was induced with the parenteral and mucosal administrations, although serum IgA antibodies as well as higher levels of mucosal IgA were elicited by mucosal immunization. Inhibition of homologous and heterologous RV infection *in vitro* by VP6-specific mucosal IgA antibodies demonstrated the protective nature of these antibodies. Most importantly, when adult mice were immunized with the trivalent vaccines and challenged with a murine RV strain, VP6-induced immunity provided significant protection against live RV *in vivo* as measured by reduction of RV antigen shedding in feces. These results confirm that immunity induced by the conserved non-serotype-specific RV VP6 antigen can impair RV infection. Due to its highly conserved nature, VP6 protein could be expected to provide heterotypic protection against a broad range of RV genotypes. Therefore, RV VP6 protein is a promising vaccine candidate also in humans, and clinical trials are warranted in the near future.

Tiivistelmä

Rotavirukset (RV) ovat maailmanlaajuisesti yleisimpiä pienten lasten akuutin gastroenteriitin (AGE) aiheuttajia. Rotaviruksen aiheuttamaan ripulitautiin kuolee vuosittain arviolta 450 000 lasta, joista suurin osa kehitysmaissa. Tautia vastaan on olemassa kaksi suun kautta annettavaa, eläviä heikennettyjä viruksia sisältävää rokotetta: ihmisperäinen Rotarix®-rokote sekä vasikan ja ihmisen rotaviruksista johdettu RotaTeq®-rokote. Rotavirusrokotteita pidetään yleisesti turvallisina ja tehokkaina eri RV-genotyyppien aiheuttamaa vakavaa rotavirustautia vastaan. Elävät rokotteet eivät kuitenkaan ole osoittautuneet yhtä tehokkaiksi kehitysmaissa. Lisäksi elävät rokotteet on yhdistetty suolentuppeumariskiin sekä rokotekannan reassortaatioon eli yhdistymiseen villin viruskannan kanssa. Näiden syiden vuoksi elottomia proteiinirokotteita harkitaan vaihtoehdoiksi eläville RV-rokotteille.

Tämä väitöskirja tutkii RV-partikkelin sisemmän proteiinikuoren VP6-proteiinin käyttöä elottomana RV-rokotekandidaattina. VP6 valittiin rokote-ehdokkaaksi, koska VP6-kapsidiproteiinin tiedetään olevan kaikkein immunogeenisin rotavirusproteiini. Tämä proteiini on erittäin konservoitunut ja sitä on RV partikkelissa määrällisesti eniten. Lisäksi VP6 pystyy toistuvalla, monitahoisella antigeenieksпressiolla muodostamaan erittäin immunogeenisia oligomeerisia rakenteita, mikä tekee siitä yksinkertaisen ja siten sopivan elottoman rokote-ehdokkaan. VP6-proteiinit muodostavat keskenään putkimaisia rakenteita, ja yhdessä VP2-ydinproteiinin kanssa ne muodostavat kaksikerroksisia viruksen kaltaisia partikkeleita (dl2/6-VLP). Tässä työssä tuotimme bakulovirus-ekspressiosysteemillä molempia VP6-pohjaisia rakenteita RV-positiivisen potilasnäytteen VP6- ja VP2-spesifisistä sekvensseistä. Näiden rakenteiden synnyttämiä VP6-spesifisiä immuunivasteita vertailtiin aikuisissa hiirissä. Molemmat proteiinirakenteet olivat yhtä immunogeenisiä ja synnyttivät voimakkaan humoraalisen IgG-vasteen, joka reagoi useiden eri RV-kantojen kanssa. Kumpaakin VP6-rakennetta voidaan siis pitää hyvänä rokote-ehdokkaana.

Norovirus (NoV) on toiseksi yleisin lasten vakavan ripulitaudin aiheuttaja. Lopullisena tavoitteenamme onkin kehittää eloton yhdistelmärokote sekä rota- että norovirusta vastaan, jotta lasten virusperäinen AGE voitaisiin ehkäistä yhdellä ainoalla rokotteella. Bivalenttisen ja trivalenttisen yhdistelmärokotteen luomiseksi

rotaviruksen VP6-proteiini yhdistettiin joko ainoastaan noroviruksen GII.4 VLP-rakenteiden kanssa tai sekä GII.4 että GI.3 VLP-rakenteiden kanssa. Pelkällä VP6-proteiinilla saavutettiin yhtä hyvät vasta-ainereaktiot kuin bivalenttisella että trivalenttisella yhdistelmärokotteella, mikä osoitti että NoV-antigeenien lisäyksellä ei ollut vaikutusta vasteiden muodostumiseen VP6-proteiinia vastaan.

Tutkimuksen viimeinen osa keskittyi VP6-spesifisten vasteiden suojatehon arviointiin aikuisissa hiirissä. Joko VP6-putkia tai dl2/6-VLP-rakenteita sisältävä trivalenttinen rokote synnytti vahvan B- ja T-soluvasteen aktivaation niin intramuskulaarisesti kuin intranasaalisesti annosteltuna. Sekä $CD4^+$ T-soluilla että IgA-vasta-aineilla on aiemmin hiirillä todettu olevan merkitystä RV-infektiolta suojautumisessa. Toisiaan vastaavat suojatehot saavutettiin molemmilla trivalenteilla rokotteilla antotavasta riippumatta, vaikka rokotteet synnyttivätkin vahvemman limakalvo-IgA-vasteen sekä seerumin IgA-vasta-aineiden muodostumisen nenän kautta annosteltuna. VP6-spesifiset vasta-aineet estivät sekä homologisen että heterologisen RV-infektion *in vitro*. Kun trivalenttisilla rokotteilla immunisoidut hiiret altistettiin hiiren rotavirukselle, VP6-spesifinen immuniteetti suojasi elävää RV-altistusta vastaan *in vivo*, kun suojatehoa mitattiin RV-antigeenin erittymisen vähenemisenä ulosteessa. Nämä tulokset vahvistavat, että genotyyppispesifisyydestä riippumaton, konservoituneella VP6-antigeenilla indusoitu immuniteetti pystyy estämään RV-infektiot. Erittäin konservoituneen luonteensa vuoksi VP6-proteiinin voidaan odottaa suojaavan useita RV-genotyyppejä vastaan. Näiden tulosten perusteella RV VP6-proteiini on lupaava rokote-ehdokas myös ihmisille.

List of Original Communications

This thesis is based on the following publications referred to in the text by Roman numerals I–V:

I. Lappalainen S, Tamminen K, Vesikari T, Blazevic V. (2013) Comparative immunogenicity in mice of rotavirus VP6 tubular structures and virus-like particles. Hum Vaccin Immunother. 9(9):1991-2001.

II. Blazevic V, Lappalainen S, Nurminen K, Huhti L, Vesikari T. (2011) Norovirus VLPs and rotavirus VP6 protein as combined vaccine for childhood gastroenteritis. Vaccine. 29(45):8126-33.

III. 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. 8(7):e70409.

IV. Lappalainen S, Pastor AR, Tamminen K, López-Guerrero V, Esquivel-Guadarrama F, Palomares LA, Vesikari T, Blazevic V. (2014) Immune responses elicited against rotavirus middle layer protein VP6 inhibit viral replication in vitro and in vivo. Hum Vaccin Immunother. 10(7):2039-47.

V. Lappalainen S, Pastor AR, Malm M, López-Guerrero V, Esquivel-Guadarrama F, Palomares LA, Vesikari T, Blazevic V. (2015) Protection against live rotavirus challenge in mice induced by parenteral and mucosal delivery of VP6 subunit rotavirus vaccine. Arch Virol. 160(8):2075-8.

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Abbreviations

AGE	acute gastroenteritis
APC	antigen-presenting cell
ASC	antibody-secreting cell
BCR	B cell receptor
BV	baculovirus
DC	dendritic cell
DD	diarrheal dose
dl	double-layered
DLP	double-layered particle
dpi	days post infection
dsRNA	double-stranded RNA
EDIM	epizootic diarrhea of infant mice
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
EM	electron microscopy
ER	endoplasmic reticulum
FBS	fetal bovine serum
FFU	focus-forming unit
GMT	geometric mean titer
HBSS	Hanks' balanced salt solution
HPV	human papilloma virus
hsc70	heat shock cognate protein 70
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IM	intramuscular
IN	intranasal
LLR	Lanzhou lamb rotavirus
LP	lamina propria

mBc	memory B cell
MBP	maltose-binding protein
MHC	major histocompatibility complex
MOI	multiplicity of infection
NAbs	neutralizing antibodies
NELISA	ELISA-based RV antigen reduction neutralization assay
NoV	norovirus
NS	non-structural
OD	optical density
pfu	plaque-forming units
pIgA	polymeric IgA
pIgR	polymeric Ig receptor
PCV	porcine circovirus
PP	Peyer's patch
RRV	rhesus rotavirus
RT-PCR	reverse-transcription polymerase chain reaction
RV	rotavirus
RVGE	rotavirus gastroenteritis
SA	sialic acid
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFC	spot-forming cell
SC	subcutaneous
SG	subgroup
SIgA	secretory IgA
TCR	T cell receptor
Th	T helper
TLP	triple-layered particle
VLP	virus-like particle
VP	viral protein
VW	vaginal wash

1 Introduction

Human rotaviruses (RVs) have been recognized as the leading cause of severe diarrhea in infants and young children since their discovery in Melbourne, Australia in 1973 by electron microscopy (EM) in intestinal samples of children with acute gastroenteritis (AGE) (1). Each year, RV infections lead to an estimated 453,000 deaths and millions of hospitalizations and outpatient visits worldwide. The mortality is highest in developing countries because of the limited availability of healthcare (2–4).

Shortly after the identification of RV as an important enteric pathogen, development of RV vaccines became a priority. Development of RV vaccines has been primarily focused on orally administered live vaccines, which mimic protection conferred by natural infections, at least against severe RV disease (5–7). At present, two live attenuated RV vaccines, a monovalent Rotarix® (GlaxoSmithKline, Belgium) and a pentavalent RotaTeq® (Merck & Co., Inc., US), are in global use and part of routine childhood immunization programs in more than 70 countries. Both vaccines are mainly efficacious against severe RV gastroenteritis (RVGE) caused by different circulating RV genotypes, including those not part of the vaccine composition (8, 9). However, these vaccines have shown substantially lower protection and immunogenicity in some middle- or low-income countries (10–12), where the RV vaccines are needed the most. Moreover, the live attenuated RV vaccines have been associated with various safety issues such as an increased risk of intussusception (13–15), formation of reassortments between vaccine and wild-type RV strains (16, 17), and prolonged excretion of vaccine virus (18, 19).

An infectious RV particle has a complex structure consisting of three concentric protein layers. The outer layer of RV is formed by two proteins, VP7 glycoproteins with spikes of VP4, which induce protective immunity based on type-specific neutralizing antibodies (NAbs). (20) The middle layer of the virus enclosing the VP2 core consists of VP6, the most abundant and immunogenic RV protein, with the strongest antibody response generated by natural RV infection or RV vaccination being directed against VP6 (21–24). Protection levels accomplished with the current RV vaccines cannot be explained by the low levels of vaccine-induced NAbs (8, 25). Moreover, similar efficacy of monovalent and pentavalent RV vaccines against

severe RVGE associated with different RV genotypes (8, 9, 26) indicates indirectly that also mechanisms other than the type-specific NAbs directed to outer capsid VP4 and/or VP7 proteins are required for protection. Although the mechanisms or effectors of protection are not completely defined, mucosal antibodies directed to VP6, particularly IgA, have demonstrated antiviral and protective effects in mouse models (27–29).

Because of the concerns with current RV vaccines, alternative non-live vaccine candidates with the potential to alleviate safety concerns, lower costs, and improve efficacy are needed. Especially recombinant protein vaccines without the genetic material of the virus and the viral replication offer a safer approach. Since different protein assemblies are known to be powerful immunogens, the ability of VP6 to self-assemble into various oligomeric structures (30–32) makes it a potential non-live, subunit RV vaccine candidate against RVGE. Here we describe two candidate RV vaccines based on oligomeric structures of VP6 protein that can be administered parenterally or mucosally to induce immune responses protective against RV infection.

2 Review of the literature

2.1 Characterization of rotavirus

2.1.1 Architecture of the virion

Rotaviruses (RV) are non-enveloped viruses with a segmented double-stranded RNA (dsRNA) genome (20). The name *Rotavirus* (Latin *rota*, meaning "wheel") originates from the distinctive wheel-like appearance of the complete virus particles visualized under EM (33). These mature infectious RV particles possess a complex icosahedral capsid with a diameter of approximately 100 nm consisting of three concentric protein layers (20) and are therefore also termed triple-layered particles (TLPs). **Figure 1A** shows the structure of an RV particle.

The innermost capsid layer of the virion comprises 60 asymmetric dimers of a 102-kDa VP2 protein (**Fig. 1B**) in T = 1 symmetry enclosing the genome (34, 35). This thin core layer is uniform, with the exception of 12 small pores along the 5-fold axes (35). Complexes of two minor structural proteins, one copy of the viral RNA-dependent RNA polymerase VP1 (36) and the viral capping enzyme VP3 (37), are anchored to the inner surface of VP2 core shell (**Fig. 1B**) adjacent to the pores (38). An intermediate layer consisting of 260 trimers of the 45-kDa VP6 protein in T = 13 symmetry surrounds the core (**Fig. 1B**), forming a non-infectious but transcriptionally active double-layered particle (DLP) (35, 39, 40). This inner capsid protein is the most abundant RV protein, comprising over half of the mass of the RV particle (41). VP6 has a main role in the virion architecture, being in direct contact not only with the core shell but also with the external layer of TLP (**Fig. 1C**). This outer layer consists of 260 trimers of the VP7 glycoprotein on a T = 13 lattice (42), each trimer anchored to the underlying VP6 layer (39, 43), and 60 spikes of VP4 trimers projecting from the VP7 shell (**Figs. 1B and 1C**) (40). The VP7 and VP4 serve as viral attachment proteins and neutralization antigens (44–48). Cleavage of VP4 protein into VP8* and VP5* (**Fig. 1C**) by the trypsin-like proteases yields to the fully infectious TLP (49, 50). (**Table 1**)

One of the distinctive characteristics of the structure of RV virion is the presence of 132 large channels of three types penetrating through the layers of VP6 and VP7 with similar icosahedral symmetry (42). These channels include 12 type I channels located at the 5-fold axes, 60 type II channels surrounding the type I channels, and 60 type III channels around the 3-fold axes (41). The type I channels are in register with the pores in VP2 layer and serve as exit channels for the nascent viral mRNA during transcription (34).

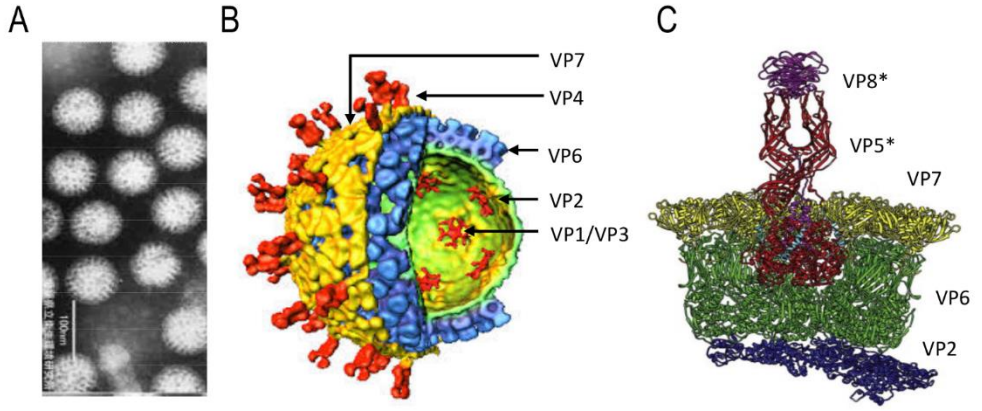

Figure 1. Structure of triple-layered RV particle consisting of a VP2 core, an inner capsid of VP6 and an outer capsid of VP7 with VP4 (VP5* + VP8*) spikes. (**A**) Electron microscopy image of TLPs. (**B**) Cutaway view of TLP demonstrating the VP7 layer with VP4 spikes, VP2 core, VP6 layer, and the transcriptional enzymes VP1 and VP3 anchored to the inner surface of VP2. Modified from (51). (**C**) Interactions of RV structural proteins. Modified from (40).

2.1.2 Genome organization

The RV genome, approximately 18.5 kb in size, consists of 11 dsRNA segments (**Fig. 2**) coding for six structural viral proteins (VP1-VP4, VP6 and VP7) and six non-structural proteins (NSP1–NSP6) (41). Each segment encodes one protein with the exception of segment 11, which is capable of encoding two proteins (NSP5/NSP6) due to overlapping open reading frames (52). The structural proteins, forming the triple-layered capsid, are named based on the descending molecular weights of the proteins, VP1 being the largest at 125 kDa and VP8*, the proteolytic fragment of VP4, the smallest at 28 kDa. Instead, the non-structural proteins are involved in replication, virus particle assembly, regulation of the host immune responses, and stimulation of viral gene expression. (20) (**Table 1**)

Table 1. Rotavirus gene segments and corresponding proteins and functions. Modified from (20).

Gene Segment	Size (bp)[1]	Protein product	Size (kDa)[1]	Location in particles	Molecules in virion	Function
1	3302	VP1	125	Core	12	RNA-dependent RNA polymerase, ssRNA binding, complex with VP3
2	2690	VP2	102.4	Core	120	RNA binding, required for replicase activity of VP1
3	2591	VP3	98.1	Core	12	Guanylyltransferase, methyltransferase, ssRNA binding, complex with VP1
4	2362	VP4 VP5* VP8*	86.8	Outer capsid	120	P-type neutralization antigen, viral attachment, protease-enhanced infectivity, virulence
5	1581	NSP1	58.7	Non-structural		Interferon antagonist, putative viral ligase, RNA binding
6	1356	VP6	44.8	Inner capsid	780	SG antigen, protection (intracellular neutralization), required for transcription
7	1104	NSP3	34.6	Non-structural		Binds 3'end of viral mRNA, surrogate of poly(A)-binding protein, inhibits host translation
8	1059	NSP2	36.7	Non-structural		NTPase, RNA binding, helix-destabilizing activities, forms viroplasms with VP1 and NSP5
9	1062	VP7	37.4	Outer capsid	780	G-type neutralization antigen, Ca^{2+}-dependent trimer
10	751	NSP4	20.3	Non-structural		RER transmembrane glycoprotein, viroporin, intracellular receptor for DLPs, role in morphogenesis of TLPs, interacts with viroplasms, modulates intracellular Ca^{2+} and RNA replication, enterotoxin, virulence
11	667	NSP5/ NSP6	21.7/ 12	Non-structural		RNA binding, forms viroplams with NSP2, interacts with VP2 and NSP6/ Interacts with NSP5, present in viroplasms, RNA binding

[1] Sizes of RNA segments and proteins according to the simian agent 11 (SA11) virus.

bp, base pair; kDa; kilodalton; RER, rough endoplasmic reticulum.

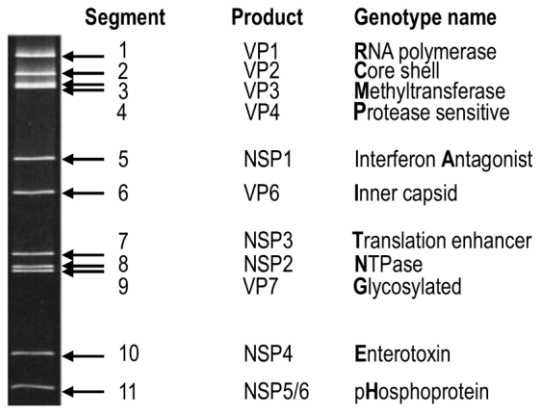

Figure 2. RNA segments of the rotavirus genome resolved by polyacrylamide gel electrophoresis, the products they encode and specific genotypes to each genome segments. The bolded letter identifies the segment in the gene constellation acronym: Gx–P[x]–Ix–Rx–Cx–Mx–Ax–Nx–Tx–Ex–Hx (x indicates the number of genotype).

2.2 Classification and strain diversity

Rotaviruses belong to the genus *Rotavirus*, one of the 15 genera of the *Reoviridae* family (53). According to the antigenic properties or, more recently, the amino acid sequences of the VP6 protein, RVs can be classified into five groups (A–E) and three tentative groups (F–H) (53, 54). (**Fig. 3**) Groups A–C and H are known to infect humans and various mammalian and avian species, whereas groups D–G have been isolated from pigs and birds (53, 54). Since viruses within the same group only are capable of genetic reassortment, each RV group is considered a unique species. The majority of human infections are caused by group A RVs (55–57), which are further divided into four different subgroups (SGs) referred to as SGI, II, I+II, and non-I/II (**Fig. 3**), based on the presence or absence of specific epitopes on VP6 (58–60). Human strains belong predominantly to either SGI or SGII (61–63). RV strains belonging to the same SG exhibit as high as 90–99% homology at the amino acid levels. Similarly, the conservation degree remains high with the homology of 87–95% among the strains belonging to the different SGs. (64)

RV strains of group A have also been designated G- and P-serotypes according to the reactivity of the antigenic epitopes of VP7 (G for Glycoprotein) and VP4 (P for Protease-sensitive) proteins (**Fig. 3**) with reference antisera (65). Nowadays, the

virus strains can also be classified into G- and P-genotypes based on the sequences of the VP7 and VP4 gene segments (66, 67). In contrast to G-serotypes, which correspond one to one with G-genotypes, part of P-serotypes comprise several P-genotypes. To date, 27 G-genotypes (G1–G27) and 37 P-genotypes (P[1]–P[37]) have been identified for RVs (68, 69). Instead, 14 P-serotypes, of which serotypes 1, 2, and 5 are further divided into subtypes with letter designations, have currently been discriminated. This has led to a double nomenclature for P-types (41) in which the P-genotype is enclosed in square brackets after the P-serotype designation together with a possible subtype letter (e.g. P1A[8]). Because of the complicated serotyping due to lack of immunological reagents and the cross-reactivity between the P-serotypes, P-types are commonly denoted by their genotypes only. Because of the segmented genome RVs have, the segments can occasionally reassort during co-infections with different serotypes, resulting in reassortant strains with gene segments originating from the two parent RV strains (70). Thus, genetic reassortment provides a mechanism for the evolution of novel strains. So far, more than 70 distinct G–P genotype combinations of human RV strains belonging to the 12 G-types and 15 P-types have been identified (71) but only five G-types (G1, G2, G3, G4, and G9) and three P-types (P[4], P[6], and P[8]) are frequently associated with human diseases (70–72).

To assess the genetic diversity and evolutionary relationships of co-circulating group A RVs, a new comprehensive nucleotide-sequence-based classification system comprising the complete genome of the virus was recently introduced as an extension to the binary G-and P-typing classification (68, 73, 74). In the full genome classification system, the abbreviations Gx–P[x]–Ix–Rx–Cx–Mx–Ax–Nx–Tx–Ex–Hx designate the gene segments respectively encoding VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 (**Figs. 2 and 3**) (73). Currently, at least 27 G, 37 P, 17 I, 9 R, 9 C, 8 M, 18 A, 10 N, 12 T, 15 E and 11 H genotypes have been differentiated according to cut-off points of nucleotide sequence identities (68, 69, 75–77). The genotype constellations of animal RVs usually differ from genotype composition of human viruses (68, 78). The majority of the human RVs appear to belong to two major genotype constellations sharing the majority of the genotypes of the internal non-G, non-P genes. Genotypes 1 (I1-R1-C1-M1-A1-N1-T1-E1-H1) are commonly encountered in combination with P[8] and various G-genotypes, and genotypes 2 (I2-R2-C2-M2-A2-N2-T2-E2-H2) with G2P[4] (79). Human RVs with internal genes predominantly of genotype 1 are referred to as genogroup 1 or Wa-like viruses and RVs with genotype 2 genes are referred to as genogroup 2 or DS-1-like viruses. Phylogenetic analysis has revealed an evolutionary relationship between

these two human genogroups and porcine and bovine strains, Wa-like RVs sharing the majority of the genotypes with porcine strains and DS-1-like RVs sharing the genotypes with bovine strains. A third, but minor, human genotype constellation, referred to as AU-1-like (I3-R3-C3-M3-A3-N3-T3-E3-H3) is believed to originate from cats or dogs. (73)

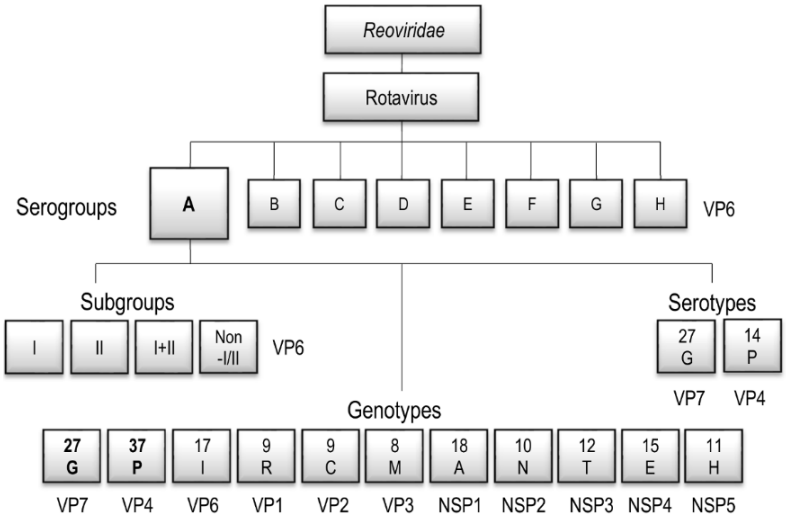

Figure 3. Classification of rotaviruses into serogroups, subgroups, serotypes and genotypes. The number of particular serotype and genotype defined for each 11 gene segments are shown. Names of genotypes are **G**lycosylated, **P**rotease sensitive, **I**nner capsid, **R**NA-dependent RNA polymerase, **C**ore protein, **M**ethyltransferase, Interferon **A**ntogonist, **N**TPase, **T**ranslation enhancer, **E**nterotoxin, P**H**osphoprotein.

2.3 Rotavirus infection

2.3.1 Life cycle of rotavirus

RVs infect the mature enterocytes at the tips of the villi of the small intestine, suggesting these cells express specific receptor(s) for the virus entry. However, extra-intestinal spread of RV (80–82) indicates a broader range of host cells and thus possible additional receptors. Because of the lack of established intestinal cell lines of small intestine origin, infection studies with this type of cells have been limited. Most commonly, the entry and replication cycle of RV have been studied in the epithelial monkey kidney cell line (MA104) or in the human epithelial colon carcinoma cell line (Caco-2), which are highly permissible to the virus. (20) Some transformed cell lines derived from breast, stomach, bone or lung are also efficiently infected by RV (83).

The efficiency of RV infection depends on activation of the external TLPs, involving cleavage of the VP4 into VP8* and VP5* (**Fig. 1C**) by trypsin-like proteases in the intestinal lumen of the host or the culture medium (49, 50, 84). Proteolytic processing of this spike precursor has been proposed to be required for the later conformational changes that mediate virus entry (84). Overall, RV entry into the cell is a complex multistep process, in which external VP4 and VP7 proteins of the virus interact with different molecules on the host cell surface (**Fig. 4**). VP8* domains located at the activated spike tips of the infectious TLPs first attach to sialic acids (SAs) in the terminal or internal position of the cellular glycans (85–88). Neuraminidase treatment of cells diminishes infection by particular RV strains (e.g. RRV) which have therefore been termed SA-dependent strains (89). Instead, RV strains capable of infecting neuraminidase-treated cells, initially called 'SA-independent' strains (e.g. Wa) (89), have been shown to bind to receptors containing SA in internal position of glycolipids insensitive to treatment by neuraminidase (87, 90). Alternatively, histo-blood group antigens, present on the surface of mucosal epithelial cells, have recently been indicated as cellular receptors for certain RV strains via binding of VP8* (91, 92). Additional cell proteins implicated in RV entry as possible co-receptors in a post-attachment step include various cellular integrins (e.g. $\alpha 2\beta 1$, $\alpha x\beta 2$, $\alpha 4\beta 1$, and $\alpha v\beta 3$) interacting with VP5* or VP7 (48, 93, 94) and heat shock cognate protein 70 (hsc70) interacting with VP5* (95, 96). Binding of hsc70

to a domain in VP6, possibly due to conformational changes in RV particles induced by binding to the host cell, has also been described (97). Following binding, the virus is internalized by receptor-mediated endocytosis (**Fig. 4**) or direct membrane penetration (98). During entry, a sequence of molecular transformations triggered by low Ca^{2+} concentrations removes the outer-layer proteins, resulting in the release of transcriptionally active DLPs into the cytoplasm (99–101).

Once the DLP reaches the cytoplasm, transcription complexes consisting of VP1 and VP3, localized at the inferior side of the VP2 core (38), start the transcription (**Fig. 4**) of the 11 gene segments from the negative strands of dsRNAs. The synthesized capped (+)ssRNAs are extruded via type I channels passing through the VP2 and VP6 layers (34), each channel possibly extruding transcripts of a specific gene segment (102). These transcripts serve as mRNAs for viral protein synthesis and later as templates for synthesis of the (–) RNAs to generate dsRNAs (103).

Once a critical amount of viral proteins is synthesized, structural and non-structural proteins (VP1, VP2, VP3, and VP6; and NSP2, NSP5, and NSP6) accumulate in cytoplasmic inclusion bodies called "viroplasms," in which replication, genome packing, and assembly of DLPs take place (**Fig. 4**) (98). The presence of the viral NSP2 and NSP5 is essential for the formation of viroplasms (104). Viroplasm-associated (+)ssRNAs interacting with core proteins are selectively packed into assembling VP2 cores and then replicated by VP1 into the dsRNAs (103). Subsequently, core particles are transcapsidated by VP6 trimers, resulting in the formation of DLPs (98) that bud into the endoplasmic reticulum (ER) via transmembrane protein NSP4 (**Fig. 4**) serving as an intracellular receptor (105). Within the ER, the particles acquire temporary envelopes of an unknown function, which are removed when the VP4 and VP7 proteins assemble (**Fig. 4**) (98). Due to NSP4 also functioning as a viroporin by releasing Ca^{2+} from intracellular stores, the intracellular levels of cytoplasmic Ca^{2+} increase (106, 107), which is required for maintaining the integrity of the mature TLPs (108). The progeny virions are released by direct cell lysis (109) or a non-classical vesicular transport mechanism (**Fig. 4**) (110).

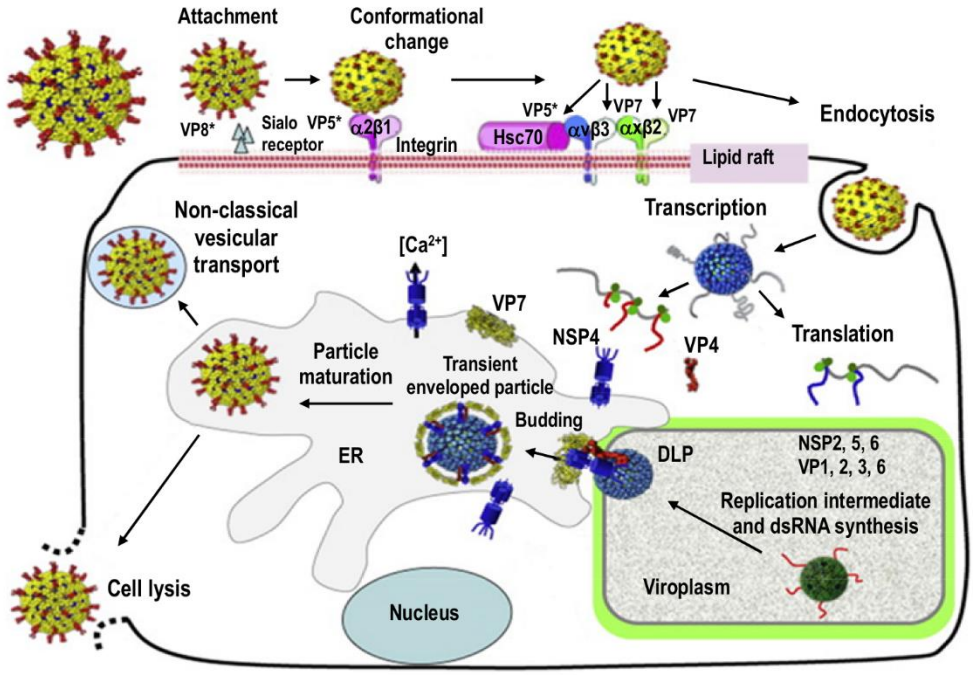

Figure 4. Main features of the replication cycle of the rotavirus. Cleavage of VP4 into VP5* and VP8* allow the TLP particle to interact efficiently with series of cellular receptors. The RV is internalized by receptor-mediated endocytosis. Low Ca^{2+} of the endosome triggers uncoating of the TLP, leading to the release of the DLP into the cytoplasm. The transcriptionally active DLP initiates transcription of mRNAs used to translate proteins. Replication and packing of the RNA genome into a newly produced DLP occur in the viroplasm. A DLP binds to NSP4, leading to the budding of transient enveloped particles into the endoplamic reticulum (ER). Subsequent removal of the transient envelope results in maturation of a TLP, following assembly of VP4 and VP7 as a consequence of the intracellular increase of Ca^{2+}. Release of the TLP occurs via cell lysis or non-classical vesicular transport. Modified from (20).

2.3.2 Transmission and pathogenesis

RVs are highly contagious; only a low number of live particles (<100) are usually required for infection of a susceptible individual (111). Moreover, they are highly resistant to ambient temperatures and a wide range of pH (pH 3.5–10) remaining viable in the environment for weeks or even months (112–114). Transmission occurs via the fecal-oral route, but spreading via respiratory droplets or aerosols has also been suggested (41).

Pathogenesis of RV infection is multifactorial, with both host and viral factors affecting the manifestation of illness. During infection, the virus may secrete a viral enterotoxin, NSP4 (115), and damage the epithelial surface, leading to blunting of the villi and defects in the fluid and electrolyte absorption of the small intestine (116). RV-induced diarrhea, the most typical outcome of RV infection, may be caused by various mechanisms, including virus-mediated destruction of enterocytes, virus-induced disruption of the absorptive enzyme expression, functional alterations in tight junctions augmenting cell permeability, epithelial damage caused by villus ischemia, effects of a virus-encoded NSP4 on Ca-dependent cell permeability and chloride secretion, and activation of the enteric nervous system by vasoactive agents from damaged cells (20, 116). Although an anti-NSP4 antibody can block or reduce RV-induced diarrhea in mice, NSP4-specific antibody levels have not been correlated with protection in children (117, 118) and therefore the mechanism of NSP4-induced diarrhea in humans remains to be determined. Recently, the pathogenesis of vomiting has been suggested to result from production of serotonin, stimulated by RV infection of enterochromaffin cells in the gut, which in turn acts through the enteric nervous system by stimulating vomiting center of the brain (119).

Although RV has a specific cell tropism for the non-dividing mature enterocytes, limited systemic replication probably occurs in different sites of the body (116, 120). Antigenemia and viremia in RV-infected individuals (80–82) implies extraintestinal spread of RV. However, evidence supporting the responsibility of this systemic spread and replication for any specific pathologic findings in normal hosts is limited (120). Susceptibility to RV infection is host-range restricted with heterologous strains replicating poorly and causing diarrhea only with larger inoculation doses compared to homologous strains (20). Despite the host-range restriction, interspecies transmission and genetic reassotment between human and some animal RVs occur, probably because of the close interactions between humans and these animals (78).

2.3.3 Clinical picture of rotavirus disease

Usually RV infection causes inflammation in the intestines that leads to a spectrum of illnesses ranging from mild diarrhea to severe and occasionally fatal dehydrating disease. However, infection can also be asymptomatic. (121) Although the clinical features of RV disease can vary and be similar to those caused by other gastrointestinal pathogens, the symptoms associated with RVGE include the sudden onset of watery diarrhea, often accompanied with vomiting and fever (122–124).

Most commonly, the clinical symptoms of infection start after a <48-hour incubation period, with symptoms resolving within three to seven days. However, the frequent watery stools and vomiting may result in to life-threatening dehydration of the infected individual unless rehydrated, malnutrition increasing the severity of disease (125).

Infected individuals start to shed RV particles in the feces before the onset of the symptoms and continue to do so for several days after clinical disease (126). Both asymptomatic and symptomatic patients generally shed RV for 7–10 days (127), but also prolonged viral excretion can occur, especially in immunocompromised patients (128, 129). The quantity of excreted particles may exceed 10^{10} or 10^{11} per gram of feces during infection (130). Therefore, diarrheal illness caused by RV infection is generally confirmed by detecting RV antigen or genome in feces using the enzyme-linked immunosorbent assay (ELISA) or reverse-transcription polymerase chain reaction (RT-PCR) (66, 67, 131–133). The high number of virus particles present in feces during the acute illness has also enabled the identification of RV by EM (33).

2.4 Epidemiology of rotavirus infection

Group A RVs are the most common causes of severe gastroenteritis in infants and young children globally, with millions of hospitalizations and outpatient visits associated with RV infections each year (2, 3). Annually, an estimated of 453,000 children under five years of age die from RV-related gastroenteritis, accounting for 37% of deaths attributable to diarrhea and 5% of all deaths in this age group (4). The majority of these deaths occur in the developing world due to the limited access to medical care.

RV infection can occur at any age, also in adulthood, but the incidence and severity of RV disease is usually age-dependent. Irrespective of geographic location or economic status, nearly every child in the world experiences at least one RV infection by the age of five, most before their second birthday. (121, 134) The occurrence of primary RV infection is earlier in developing countries, possibly due to year-round RV exposure and high environmental viral loads, which in turn result in RV infection shortly after the waning of transplacental maternal antibodies (135). Generally, RV causes the most severe infection in infants and young children, with the first infection at the age of 6 to 24 months causing the most severe disease (7, 124, 133, 136, 137). Instead, infections encountered in neonates and adults are most often asymptomatic or milder (5–7, 138, 139).

Despite the enormous diversity of RVs, the majority of RV infections are associated with five globally circulating genotypes: G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8]. Overall, strains of G1P[8] are most common, but fluctuations in strains with different G–P combinations vary by geography and season (56, 72, 133). In developing countries, the incidence of strains with unusual genotypes (e.g. G8 and G12) as well as genotype combinations (e.g. G1P[4], G2P[8] and G4P[6]) is often higher due to the reassortment among human RVs or reassortment between human and animal RVs. (70–72)

RV displays a seasonal distribution in countries with temperate climates, with a peak in infections in the cooler winter months (55, 140). In tropical climates, the seasonal pattern is not so distinct, with infections occurring year-round instead (140).

2.5 Rotavirus immunity

Individuals can experience multiple RV infections during their lifetime, but natural RV infection, whether symptomatic or asymptomatic, provides a protective effect against subsequent infection by reducing the frequency and severity of subsequent episodes. The severity of RV infections decreases with increasing number of RV infections, each reinfection appearing to broaden and boost natural immunity. (5–7, 137, 141) Commonly, the third infection provides complete protection against moderate-to-severe disease (7). Mechanisms responsible for protective RV immunity are not completely elucidated, but clearance of infection depends on the immunological responses of the host, comprising of combined functions of innate and adaptive immunity.

2.5.1 Humoral and cell-mediated immune responses

2.5.1.1 Generation of immune responses

The immune system is divided into unspecific innate immunity and specific adaptive immunity, innate immunity providing immediate first line of defense against invading pathogens. Functions of innate immunity include unspecific recognition of microorganisms by complement, destruction of infected cells by natural killer (NK) cells, and activation of phagocytic cells such as macrophages, dendritic cells (DCs),

and neutrophils that ingest and destroy foreign material. Cells of the innate immune system, scattered throughout the body, recognize pathogens using pattern recognition receptors (PRRs) that detect conserved pathogen-associated structures (e.g. lipopolysaccharides, lipoproteins, and DNA or RNA sequences) absent from the host. In response to recognition, these cells secrete various immunomodulators such as cytokines, which regulate the immune responses by inducing inflammatory responses, activating immune cells, and interfering with viral infection. (142)

Recognition of RV dsRNA by the infected cell initiates the host defense by stimulating the activation of interferon (IFN) response factors, which leads to the induction of different cytokines (135, 143–145), including type I IFNs (namely IFN-α and IFN-β), which are the major components of the host defense against viral infections (146). Recently, induction of type III IFNs has also been detected in RV-infected mice (147). However, RVs, like many other viruses, have evolved a mechanism to limit the early IFN response of the host. RV prevents or down-regulates the IFN induction pathway in infected cells through the action of the viral NSP1, which induces proteosomal degradation of the IFN response factors needed for the efficient expression of type I IFN (148, 149).

Adaptive immune responses

The adaptive arm of the immune system, activated at later phage of infection, cooperate closely with innate immune system. Adaptive immunity is divided into cell-mediated and humoral immune responses, mediated by T and B cells. Circulating naïve T and B cells, matured in thymus and bone marrow respectively, migrate between blood and secondary lymphoid organs including the spleen, lymph nodes, and submucosal lymphoid tissues until they encounter their cognate antigen (142). Generation of the initial immune response is influenced by the nature of the antigen as well as by the entry route of the antigen into the body. Antigen entering via the circulation evokes immune responses in the spleen, whereas entry via the skin and subcutaneous (SC) tissue generates an immune response in the regional lymph nodes. In contrast, antigen entry via mucosal surfaces induces responses in submucosal lymphoid tissues (150). Essential for adaptive immunity are the professional antigen presenting cells (APCs) such as DCs, macrophages and B cells, which can capture and engulf foreign antigens, degrade them and display antigens complexed with major histocompatibility complexes (MHCs) on their surfaces to naïve $CD4^+$ and $CD8^+$ T cells (151). Antigen-loaded APCs migrate to the T cell areas of the secondary lymphoid tissues, lymph nodes being the most important sites for

antigen presentation (150). APCs are scattered in the skin and mucosa but are also resident in lymphoid organs, where they may capture antigen directly from circulating lymph or blood (150, 152).

Cellular immune responses are mediated by the $CD8^+$ and $CD4^+$ T lymphocytes, which are primed through T cell receptors (TCRs) recognizing the processed antigens in the context of MHC molecules on APC (142, 152). $CD8^+$ T cells recognize intracellular antigens presented by MHC class I molecules, expressed on professional APCs as well as nearly all nucleated cells of the body. Instead, antigen recognition by $CD4^+$ T cells occurs via MHC class II molecules expressed predominantly by professional APCs (151). The signals derived from binding of the antigen-MHC complex by TCR and a CD4 or CD8 co-receptor as well as from interaction of co-stimulatory molecules trigger APCs to produce cytokines (142, 151), which in turn leads to proliferation and differentiation of the activated T cells. Naïve $CD8^+$ T cells differentiate into cytotoxic effector T cells capable of eliminating their target cells (153). Instead, $CD4^+$ T cells differentiate into T helper (Th) 1, Th2, Th17 or regulatory T cells with a variety of functions on the basis of the cytokines they secrete (154). Th1 cells direct the immune responses mainly towards cellular immune responses by activating macrophages and $CD8^+$ T cells through secretion of IFN-γ, and interleukin-2 (IL-2). Th2 cells stimulate humoral immune system by activating B cells and antibody class switching through secretion of IL-4, IL-5, IL-6, and IL-10 (155).

The induction of a humoral immune response requires a recognition of a soluble or membrane-bound antigen by the immunoglobulin (Ig), which serves as the B cell receptor (BCR) on the surface of B cell. The processed antigen displayed by MHC II molecules on B cells is recognized by $CD4^+$ Th cells primed by the same antigen. The specific interaction at the border of the B and T cell areas of secondary lymphoid tissue leads to the secretion of the B cell stimulatory cytokines by the T cell, which direct the proliferation of the activated B cell followed by the differentiation into antibody-secreting cells (ASCs), called plasma cells, or memory cells. (150, 156) The isotype or class of secreted antibody is influenced by the composition of antigen, entry route of antigen and the nature of T cell help via various cytokines (150). The first antibodies to be produced are always IgM, which can be expressed without class-switching. Class switching to IgG depends on the presence of IFN-γ or IL-4, whereas generation of IgE requires IL-4 (157). Instead, IgA class switching is triggered by TGF-β in combination with IL-10, IL-6, IL-5, IL-4, and IL-2 (158). The dominant class of antibody in the blood and extracellular fluid is IgG. In the mucosal tissues, the principal antibody class is IgA, secreted as a dimeric IgA molecule by

plasma cells in lamina propria (LP). However, monomeric IgA, produced in the bone marrow by plasma cells derived from B cells activated in lymph nodes and spleen, is also found in the circulation. B cells can also be activated by T cell-independent antigens in the absence of recognition by antigen-specific Th cells (142, 156). These antigens (e.g. some lipopolysaccharides, polymeric proteins and polysaccharides) activate B cells through Toll-like receptors (TLRs) or BCRs to become short-lived IgM-ASCs as well as class-switched IgG-ASCs and IgA-ASCs in a $CD4^+$ T-cell-independent manner (158).

The main induction site of RV immune response is considered to be Peyer's patches (PPs) in the intestine (159). Presentation of RV antigens by APCs leads to the activation of Th cells, followed by expansion of B and T cell responses. RV-specific B and T cells (160–162) enter the blood stream and traffic via homing receptors; ASCs traffic to the intestinal LP to secrete polymeric IgA (pIgA), memory B cells (mBc) return to PPs, and T cells return either to PPs or the LP. (163–165) Regardless of the primary site of RV infection, RV can escape from the gastrointestinal tract, resulting in antigenemia and viremia with unclear clinical significance, which in turn leads to induction of systemic and mucosal responses (80–82). According to a mouse model (159), production of mBc and ASCs stimulated by systemic RV antigen occurs in the spleen; memory cells circulate in the blood and return to the spleen, and ASCs home to the bone marrow for secretion of monomeric IgG and IgA (163–165).

2.5.1.2 Antibody responses in rotavirus-infected individuals

RV infection induces both serum and mucosal antibody responses. Humoral anti-RV responses include an early IgM response followed by the production of IgG and IgA (166–168). Although each of these antibody isotypes are generated in the serum following RV infection, especially IgA levels have been associated with protection against infection (7, 169) and more commonly against moderate-to-severe disease (169–172). To a lesser extent, also serum RV-IgG has been reported to correlate with the prevention of RVGE in children (169, 173). Furthermore, after infection, serum antibodies are accompanied by anti-RV IgA antibodies secreted at mucosal sites. Higher titers of intestinal IgA have been associated with a protective effect from infection and disease in children (174, 175) but not in adults (176, 177).

IgG and IgA antibodies induced by natural RV infection are directed against structural as well as non-structural proteins. In convalescent sera of RV-infected individuals, antibodies specific to neutralizing antigens VP7 and VP4 and to non-

neutralizing antigens VP6, VP2, NSP2, and NSP4 have been commonly detected (22, 117, 178–182).

External VP4 and VP7 proteins involved in viral attachment and entry to cells are capable of eliciting NAbs with type-specific and cross-reactive serotype responses (179, 183, 184). These NAbs have been shown to provide protection against disease caused by subsequent RV infections (183, 185, 186). The first RV exposure is believed to induce a primarily homotypic antibody response, which is broadened to heterotypic responses upon recurrent exposure (185). However, the occurrence of sequential infections even with the same serotype, although less frequently than with different serotypes, indicates that NAbs are not sufficient for protective RV immunity (7, 137, 186). In fact, the strongest antibody response to RV infection is generated against VP6, indicating the highly immunogenic nature of this RV protein (22, 178, 179). Indeed, anti-VP6 antibodies encoded by V_H1–46 immunoglobulin heavy chain variable region gene segment have been identified as the dominant B cell response following RV infection in infants and adults (162, 187).

2.5.1.3 Cellular immune responses

Cell-mediated immune responses have also been demonstrated after natural RV infection. Due to the difficulty of studying intestinal antigen-specific T cells *in situ* in humans, characterization of T cell responses has been concentrated on identification of the presence of RV-specific T cells in circulation after antigen priming (188–190). Although present in relatively low amounts, higher frequencies of RV-specific IFN-γ secreting $CD4^+$ and $CD8^+$ T cells have been detected in the circulation of RV-infected adults than in the circulation of healthy adults (191). However, the induced responses appear to be lower or even undetectable in children with RVGE (161, 191). Although the role of cell-mediated immune responses in protection against RV is poorly understood, chronic RV infection in T and/or B cell immunodeficient children (192) suggests the importance of both the cellular and humoral arms of the immune system in the clearance of RV infection.

2.5.1.4 Animal models of rotavirus immunity

Several animal species (e.g. bovine, equine, porcine, ovine, murine, canine, feline, and poultry) are susceptible to group A RV infection and suffer from RV diarrhea caused by numerous RV strains with distinct G-genotype and P-genotype

combinations (193–195). The outcome of the disease is similar in most species, and severity may range from an asymptomatic or subclinical condition to severe enteritis. As in humans, the disease is generally encountered in young animals and the susceptibility to illness decreases with age. (193) RV-associated diarrhea is a problem in livestock animals, notably in calves (<4 weeks of age), piglets (1–8 weeks of age), as well as foals (<3 months of age), being responsible for economic loss due to morbidity and mortality. Despite the diversity of RV strains, G6P[5], G6P[11] and G10P[11] are the most prevalent genotypes among cattle, G3P[12] and G14P[12] among horses, and G5P[7] among pigs. (78, 194, 195)

The contribution of different immunological components in the clearance of ongoing RV infection and in protection from subsequent infection have been investigated in various animal models, including mouse, rat, rabbit, piglet, calf, lamb, and primate models (196). However, mice and gnotobiotic piglets, especially adult mice, are most extensively employed as models to identify the immune correlates of protective immunity to RV infection and to evaluate various vaccination approaches.

First murine RV infections were described in the 1950s as "epizootic diarrhea of infant mice", EDIM (197). Thereafter, a number of other murine RVs (e.g. EMcN, EC, EB, and EHP) have been isolated, and all strains have originally been characterized as G3 or G3-like and either P[16] or P[20]. However, according to the recent phylogenetic analyses, the murine RVs could be assigned to G16 instead of G3 (73, 198). Mice of any age can be infected with different RV strains, although their susceptibility to heterologous non-murine RVs is variable and development of RV illness is age-restricted. Only infant mice are susceptible to diarrheal disease, the time period limiting merely up to two weeks of age. Therefore, an infant model provides both an infection and a disease model, whereas an adult model is an infection-only model in which mice shed RV particles without showing clinical symptoms of RV infection. (199–201)

2.5.1.5 Mechanisms of protection

Different studies have suggested the importance of humoral and cell-mediated immunity in the resolution of ongoing RV infection and in protection from subsequent infection. Because of the replication site of RVs in intestinal enterocytes, effector mechanisms are assumed to be active at the intestinal mucosa. In fact, the majority of human RV-specific B cells and $CD4^+$ T cells circulating in the blood express the intestinal homing receptor $\alpha4\beta7$, indicating trafficking of primed cells to the intestine and thus suggesting their local protective action (160–162). Although

immunological effectors preventing RV disease remain poorly understood in humans, variable mechanisms of RV immunity and viral targets of protective humoral immunity have been identified through animal models and *in vitro* studies.

The protective nature of antibodies directed to VP4 and VP7 has been demonstrated both *in vitro* and *in vivo* (45, 46, 202). These antibodies are able to neutralize RV classically by inhibiting viral attachment to the cell or decapsidation (203). More specifically, NAbs to VP5* and VP7 inhibit RV binding to integrins such as α2β1 and α4β1 (204). Moreover, passive transfer of VP4 and VP7 NAbs has been demonstrated to clear RV infection in suckling mice (46, 205).

The most apparent effector is secretory IgA (SIgA), which is produced following polymeric Ig receptor (pIgR)-mediated transepithelial transport of pIgA from basolateral side of epithelial cells to the apical surface. In consequence of proteolytic cleavage, the extracellular portion of the pIgR, called the secretory component, is attached to pIgA generating the SIgA. (206) As in humans, evidence of the role of SIgA is supported by the correlation of protection against RV infection in mice and piglets with the levels of RV-specific intestinal and serum IgA but not with serum IgG (207–211). However, it has been shown that RV-specific IgG or IgM in the gut or in sufficient amounts in serum can reach the intestine by compensatory mechanism incompletely understood and mediate protection in IgA-deficient individuals (212, 213). The predominant intestinal IgA response against RV was also demonstrated to be directed exclusively at VP6 (214).

Due to the recognition of VP4 and VP7 as neutralizing antigens, the first report describing a low level of neutralizing activity of monoclonal anti-VP6 antibodies against RV (44) was largely ignored, but evidence to the contrary has been mounting lately. Although VP6 is considered as non-neutralizing protein, certain antibodies against synthetic VP6 peptide (97) and VP6-specific llama-derived single-chain antibody fragments (215–217) have shown broad inhibitory activity against RV infection *in vitro*. Moreover, selected VP6-specific monoclonal pIgA antibodies are able to prevent and clear a chronic murine RV infection, as demonstrated in the "backpack tumor" model, in which pIgA-secreting hybridoma cells were transplanted SC into the upper back of mice (27). These antibodies appear to mediate antiviral function via intracellular neutralization (29, 218), the phenomenon previously suggested to Sendai (219), influenza (220), measles (221), and HIV (222). According to the recent publication, the neutralizing ability of human anti-VP6 antibodies results from the intracellular inhibition of viral transcription during basolateral-to-apical transcytosis of pIgA via binding of the antibodies into the transcriptional pore, the type I channel, and thus sterically blocking RNA egress

from the pore (223). The importance of anti-VP6 responses in protection has been shown in different animal models, as is described in greater detail in Chapter 2.6.2.2.

According to the knockout mouse models, RV-primed mBc and thus RV-specific antibodies seem to provide more long-term protection against subsequent RV infection, while RV-specific T cells help to limit the course of infection (164, 224). Severely immunodeficient mice have resolved RV infection after the adoptive transfer of immune $CD8^+$ T lymphocytes (225). Especially, $CD8^+$ T cells expressing $\alpha4\beta7$ gut homing marker are efficient in the resolution of RV in chronically infected mice (226). In addition to their involvement in the clearance of primary RV infection, these murine cytotoxic T lymphocytes are able to mediate short-term partial protection against re-infection (214, 227, 228). Moreover, the protective intestinal IgA response is, at least partially, T-cell-dependent. In the murine model, the $CD4^+$ T cells are important in providing help for the development of anti-RV intestinal IgA (214).

2.6 Rotavirus vaccines

Since the discovery over 40 years ago of RV as an important enteric pathogen in children, efforts have been directed at the development of RV vaccines. Because of the protection, at least against severe disease induced by natural RV infections (5–7, 137, 141), RV vaccine development has been focused mostly on oral live attenuated vaccines. Unlike live vaccines, only one non-living vaccine candidate has reached clinical trials in humans (**Table 2**).

2.6.1 Live attenuated vaccines

The first RV vaccine candidates followed the "Jennerian" approach involving immunization with an antigenically related animal RV strain considered to be naturally attenuated for humans to induce protection against human strains (229). These early vaccines based on bovine (RIT4237 and WC3) and rhesus RV (MMU18006) strains (230–232) were precursors for the first licensed oral RV vaccines.

Table 2. Currently licensed RV vaccines or vaccine candidates in clinical development.

Vaccine	Route	Strain characteristic	Developer/manufacturer	Status in development
RotaShield®	Oral	Tetravalent human-rhesus reassortant (G1–G4 + P7[5])	Wyeth (US)	Withdrawn 1999, Phase II trials of neonatal administration in Ghana
Rotarix®	Oral	Human strain (G1P1A[8])	GlaxoSmithKline (Belgium)	Licensed worldwide
RotaTeq®	Oral	Pentavalent human-bovine reassortant (G1–G4 + P7[5]; G6 +P1A[8])	Merck (US)	Licensed worldwide
LLR	Oral	Lamb strain (G10P[12])	Lanzhou Institute of Biological products (China)	Licensed and in use in China
Rotavac®	Oral	Human neonatal strain 116E (G9P8[11])	Bharat Biotech International Limited (India)	Licensed in India
Rotavin-M1®	Oral	Human strain (G1P1A[8])	Polyvac (Vietnam)	Licensed and in use in Vietnam
RV3-BB	Oral	Human neonatal strain (G3P2A[6])	Murdoch Childrens Research Institute (Australia) and Biofarma (Indonesia)	Phase II trials in Ghana
BRV-TV/ BRV-PV	Oral	Bovine-human tetravalent/ pentavalent reassortants (G1–G4 + P7[5]/G1–G4+G9 + P7[5])	Serum institute of India (India) Shantha Biotechnics Limited (India) Biotehnics Instituto Butantan (Brazil) Wuhan (China)	Phase III trial Phase II/III trials Phase I trial
P2-VP8*	IM	Non-replicating VP8 subunit (P1A[8])	Serum institute of India (India)	Phase I/II trials

2.6.1.1 Monovalent vaccines of animal origin

The first RV vaccine trials were conducted with a RIT4237 (G6P6[1]) strain of attenuated bovine RV sharing neither G or P serotypes with the predominant human types (230, 233). This vaccine was shown to be safe and highly effective in Finnish children, providing an 82–88% protection rate against severe RV disease caused by heterotypic human RVs (234). However, clinical trials conducted in developing countries revealed a considerably lower efficacy (235–237), which led to the discontinuation of the development of this vaccine.

Soon after, another vaccine candidate of bovine origin, Wistar Calf 3 (WC3) strain (G6P7[5]), was developed. Promising results were achieved in the initial studies with the WC3 vaccine in the US (232), but, due to the low-level protection observed in subsequent clinical trials (238, 239), studies of the vaccine were terminated. Nevertheless, WC3 was later reassorted with human strains to create the reassortant RotaTeq® vaccine, one of RV vaccines currently licensed worldwide.

A simian RV strain MMU18006 (G3P5B[3]), more commonly known as rhesus RV (RRV), was also investigated as a vaccine candidate during the early stage. RRV vaccination was associated with high reactogenicity with febrile responses in recipients (240), and protection rates were inconsistent, ranging from a high degree of protection to moderate or even non-existent (231, 237, 241). Later, RRV was used as a backbone for the reassortant RotaShield® vaccine.

One monovalent RV vaccine developed directly from an animal RV strain continues to be used: the G10P[12] lamb strain attenuated in cell culture. The Lanzhou lamb RV (LLR) vaccine has only been sold on the private market in China since its licensure in 2000. (242) The vaccine has not undergone controlled Phase III efficacy trials, but a retrospective case-control study demonstrated an overall vaccine effectiveness of 73% against hospitalized RV diarrhea with one dose of the vaccine (242). In a subsequent post-marketing case-control study, LLR conferred partial protection (44–53%) against RV disease (243).

2.6.1.2 Multivalent human-animal reassortant vaccines

Because of the variable protection observed in clinical trials, a “modified Jennerian" approach was developed with the effort to broaden the antigenic coverage of the vaccines (244). This strategy took advantage of the ability of RV genomes from

animal and human viruses to recombine *in vitro* to create novel reassortant viruses that consist of an animal RV genomic backbone and a selected number of immunologically important human RV genes.

The first multivalent RV vaccine, tetravalent human-rhesus reassortant vaccine (RRV-TV), is based on the simian strain RRV (G3P7[5]). The vaccine incorporates four viruses representing the most commonly circulating G types: the attenuated RRV and three mono-reassortants, each containing the gene encoding VP7 of the human G1, G2 or G4 genotype together with ten other gene segments of the parent RRV strain (244). According to the extensive RRV-TV efficacy trials conducted in Finland, the US and Venezuela, the vaccine efficacy ranged from 48% to 68% against RV diarrhea and from 69% to 91% against severe RV diarrhea (245–247). RRV-TV was licensed in 1998 under the name of RotaShield®. However, one year after its introduction, RotaShield® was withdrawn from the market because of the rare but increased risk of intussusception within the ten days after administration of the first dose (248). Subsequent studies have reported intussusception to be strongly associated with the age at the time of vaccination, especially with administration of the first dose after the three months of age (249). Therefore, RRV-TV efficacy studies have recently continued with a neonatal dosing (250).

Following the withdrawal of RotaShield®, vaccine development concentrated on reassortant strains of bovine origin and on attenuated human RV strains. The early WC3 (G6P7[5]) vaccine was made serotypically more related to human strains by introducing a VP7 or VP4 gene from different human RVs, which finally resulted in the creation of a pentavalent human-bovine reassortant vaccine (251). The developed vaccine, marketed as RotaTeq®, consists of five mono-reassortant viruses, each containing the WC3 parent strain with VP7 or VP4 protein representing human RV genotypes G1, G2, G3, G4 or P1A[8]. Because of the association of RotaShield® with intussusception, the RotaTeq® vaccine underwent an extensive multinational efficacy and safety trial in 2001–2005, with 70,000 infants in 11 countries (8). The study showed the vaccine to be safe and highly immunogenic regardless of the modest neutralizing responses to VP7 and VP4 (25). It protected against all cases of gastroenteritis (74%) and severe cases of gastroenteritis (98%) caused by G1–G4 RVs. RotaTeq® was licensed in the US and Europe in 2006, and is one of the two RV vaccines being licensed worldwide today. Lately, it was shown to protect against G9 and G12 RVs as well (252, 253).

Another multivalent reassortant vaccine, a tetravalent human-bovine reassortant vaccine (BRV-TV), was initially developed along with RRV-TV (later RotaShield®). The BRV-TV vaccine, which incorporates four reassortant viruses with a human

VP7 gene of either G1, G2, G3 or G4 genotype in the backbone of bovine RV UK strain (G6P7[5]), was evaluated in small safety and efficacy trials prior to the withdrawal of RotaShield® (254, 255). The vaccine was safe, non-reactogenic and immunogenic (254, 255), and two doses of BRV-TV induced 69% protection against any and 88% against severe RV disease, levels comparable to those observed with RotaShield® (255). Vaccine manufacturers in Brazil, China and India have recently licensed production of the BRV vaccine.

2.6.1.3 Monovalent vaccines of human origin

Another approach in the development of oral live RV vaccines has been the use of monovalent human RV strains isolated from infected children and attenuated by cell culture passage or isolated from asymptomatic neonates with a strain that appears to be naturally attenuated.

The first licensed human RV vaccine is Rotarix®, which is based on the most commonly circulating human G1P1A[8] strain. Originally, human RV strain 89-12 was isolated from a 15-month-old child with an RV infection in 1988 in Cincinnati (238). Initial trials with this vaccine strain, RIX4414, revealed that the vaccine was highly immunogenic and safe (256). The first efficacy trial of RIX4414 conducted in Finland showed 73% and 90% protection against any and severe RVGE during the first RV season and 72% protection against any and 85% against severe RVGE during two RV seasons (257). The safety and efficacy of RIX4414 was confirmed in a large-scale safety and efficacy trial involving 63,000 infants (9) conducted in Finland and 11 Latin American countries. In this sample size, the vaccine was not associated with intussusception. The protection rate was 85% against severe RVGE and 100% against most severe episodes of RVGE. The vaccine provided high efficacy against RV disease of any severity caused by homologous G1P[8] RVs (92%) and heterologous non-G1 RV strains (87%) sharing the P[8] type (G3P[8], G4P[8], and G9P[8]). The cross-protection against non-G1 and non-P[8] strain G2P[4] was considerably lower (41%). (9) In a subsequent two-year study conducted in Europe, the overall protection rate against severe RVGE was 96% in the first RV season and 86% in the second (258). Efficacy rates ranged between 58–90% and 85–96% against respectively any and severe RVGE caused by genotypes G1–G4 and G9; the lowest rates were associated with the genotype G2. The integrated analysis of several efficacy studies confirmed that the vaccine also protects against RVGE caused by non-G1 and non-P[8] RVs such as G2P[4] with a vaccine efficacy of 81% (26). RIX4414 was licensed in 2004 in Mexico under the name of Rotarix®, and the

vaccine has been included in the national immunization programs of over 60 countries.

Another vaccine based on a human G1P1A[8] RV strain, similar to Rotarix®, is Rotavin-M1®. The vaccine strain, isolated from a six-month-old child with AGE in Nha Trang, Vietnam, in 2003, was selected for vaccine development due to the global occurrence of the strain and the existence of proof of principle with Rotarix® (259). In fact, in Vietnamese Phase I/II clinical trials, Rotavin-M1® showed a safety and immunogenicity profile similar to that of the group receiving Rotarix® (260).

A human neonatal strain G3P2A[6], RV3, was isolated from a healthy newborn with an asymptomatic RV infection in Melbourne, Australia, in the 1970s (5). Asymptomatic neonates naturally infected with this RV3 strain were found to be 100% protected for their first three years of life from clinically severe RVGE caused by heterotypic RV strains (5), which led to the development of a low-titer RV3 vaccine. The vaccine was safe and well tolerated, but immunogenicity was relatively low (261, 262). Currently, the reformulated higher titer RV3-BB (Bishop-Barnes) vaccine is under development for neonatal vaccination (263).

A novel RV vaccine derived from a human neonatal strain 116E (G9P8[11]) has recently been licensed in India under the name of Rotavac®. The strain is a naturally occurring reassortant containing a VP4 gene from a bovine RV and ten other genes from a human strain, and it was originally isolated from an asymptomatically infected neonate in New Delhi, India, in the mid-1980s (264, 265). The 116E-based vaccine demonstrated a 55% protection level (56% efficacy in the first year of life and 49% in the second) against severe RVGE in an efficacy trial with Indian infants (266, 267). The vaccine is planned to be introduced into the country's national immunization program in 2015.

2.6.1.4 Correlates of protection after vaccination

Induction of broad heterotypic protective immunity has been the goal in the development of the RV vaccines. Despite the evident role of type-specific NAbs against VP4 and VP7 in the protection after natural RV infection (205), evidence of a correlation between serum NAbs and vaccine-induced protection against severe RVGE is weak (135, 268). The levels of serum type-specific antibodies induced by the pentavalent RotaTeq® have been considered too low to account for the high degree of protection provided by the vaccine (8, 25). The induced levels of NAbs have been lower in developing countries than in developed ones, but pre-existing

NAbs of maternal origin may interfere with measuring vaccine-induced NAbs (11, 12, 269). Nevertheless, similar efficacy of monovalent and pentavalent RV vaccines against severe RVGE caused by RVs with different genotypes (8, 9, 26) indirectly shows the significance of protective mechanisms other than the type-specific NAbs against VP4 or VP7. Instead, protection has been associated with IgA antibodies targeted to immunodominant VP6 (270).

Fecal RV-specific IgA has been demonstrated to correlate with protection after natural RV infection (175), but because of the interference by maternal antibodies from breast-feeding in the measurement of vaccine-induced antibodies, this parameter is not considered as useful in evaluating protection (268). Instead, serum IgA has been routinely measured in vaccine trials as a marker for vaccine immunogenicity or vaccine "take" (268). Seroconversion rates induced by Rotarix® and RotaTeq® have been lower in some developing countries in Africa and Asia (10, 12, 269, 271) than in Europe and the US (8, 25) but the efficacies of both vaccines have been correlated with serum RV-specific IgA (272, 273). Despite some disputes with serum IgA as a correlate of protection (135), a high level of serum IgA appears to be the best, although not perfect, surrogate marker for vaccine-induced protection (268, 272, 273). Studies are underway to evaluate other potential candidates, including RV-specific secretory antibodies and T cells, for correlation with protection (268).

2.6.1.5 Concerns with the current vaccines

Regardless of the high efficacy of current orally administered live RV vaccines against severe RV disease in developed world (8, 9), the vaccines have shown lower immunogenicity and protection rates in certain developing countries in Africa and Asia (10–12). According to the post-marketing studies conducted, the overall efficacy of RotaTeq® vaccine in the first year of life was 64% in Africa (Ghana, Kenya and Mali) and 51% in Asia (Bangladesh and Vietnam), with reductions in the efficacies (20% and 46% respectively) in the second year of life (11, 12). Similarly, the mean efficacy of Rotarix® in preventing severe RVGE has been lower (61%) in Malawi and South Africa (10).

The lack of protection offered by monovalent Rotarix® against RV hospitalizations during an outbreak of a heterotypic G2P[4] RV infection in Central Australia has also raised questions (274). Several possible factors have been suggested to explain the reduced efficacy, including interference of high levels of RV-specific maternal antibodies acquired either transplacentally or via breastfeeding,

compromised micro-nutritional status such as deficiency of vitamin A and zinc, enteric co-infections and other concurrent medical conditions, co-administration with oral polio vaccine, or serotype difference between the vaccine and the outbreak RV strains (275, 276). Moreover, the earlier occurrence of a primary RV infection of children in developing countries with high year-round RV exposure may have had an influence on vaccine efficacy (135).

Several other concerns have also been raised, mostly regarding the cost and safety of the RV vaccines. For instance, vaccine-derived RV illness has been described in infants with severe immunodeficiency (19). Live attenuated vaccines, including both RV vaccines, pose potential safety issues such as the risk transmission of the vaccine strain into the environment, genetic reassortment of the vaccine strain with the circulating wild-type virus, and reversion of the vaccine strain to virulence (277). Following oral administration of Rotarix® or RotaTeq®, the majority of vaccine recipients excrete RV vaccine strains in stools for an average of seven days as a result of viral replication in the intestinal tract, most commonly after the first dose of the vaccine (8, 256, 278, 279). However, prolonged shedding has been detected in immature infants and immunocompromised children (18, 19). Fecal shedding by vaccinated children has led to transmission of both vaccine viruses and vaccine-derived reassortant strains to unvaccinated contacts (280–283) and even to subsequent symptomatic vaccine-derived disease (16, 284, 285). New vaccine-derived double reassortants have also been encountered in vaccinated children with RV gastroenteritis (16, 17, 286).

As discussed above, administration of the first licensed RV vaccine, RotaShield®, was associated with an elevated risk of intussusception (approximately one excess case per 10,000 vaccinated), a potentially lethal condition with poorly understood etiology (287, 288). In the pre-licensure studies of Rotarix® and RotaTeq®, no such association was revealed (8, 9). However, according to the post-licensure surveillance data from different international settings (including the US, Australia, Mexico, and Brazil) both Rotarix® and RotaTeq® are associated with a small increase in incidence of intussusception during the seven days after the first dose with estimated risk of 1–5 excess cases per 100,000 vaccinated infants (13–15, 289–292). Although the mechanism of intussusception is unclear, a local inflammatory response in the lymphatic tissue or intestines has been implicated in the pathogenesis of intussusception. Especially viral infections have been associated with intestinal invagination resulting from hypertrophy of tissues in response to stimuli. (293, 294)

Both vaccines suffered a setback in 2010, when the unexpected presence of porcine circovirus (PCV) DNA was discovered in the vaccines, which led to

temporary suspension of the Rotarix® vaccine in the US and some European countries (295, 296). The source of PCV contamination was traced to porcine-derived trypsin used in the vaccine manufacturing process (297, 298).

2.6.2 Non-live vaccine candidates

2.6.2.1 Different approaches in the development of new rotavirus vaccines

Various disadvantages encountered with orally administered live RV vaccines have led to pursuance of next-generation non-live RV vaccines. Limitations associated with oral delivery could be overcome with parenterally administered RV vaccines. Indeed, parenteral vaccines are able to protect against mucosal pathogens, as demonstrated with the current inactivated vaccines against enteric diseases such as polio and hepatitis A (299, 300) and VLP-based human papilloma virus (HPV) vaccines against cervical cancer (301). Therefore, alternative non-living vaccine candidates designed for parenteral and mucosal administration such as inactivated RV particles (210, 302–305), recombinant RV proteins (21, 32, 306–308) or VLPs (309–313), and DNA-based vaccines (314–317) have been developed and tested in different animal models. Of inactivated RV vaccine candidates, both DLPs and TLPs, inactivated either chemically or physically, have been considered. In the veterinary field, parenteral administration of inactivated vaccines is an important approach due to the poor efficacy of live oral vaccines (318) and inactivated RV vaccines against RV disease in calves (e.g. Guardian®, Merck Animal Health; Rotavec® Corona, Schering-Plough Animal Health Limited; Lactovac®, Pfizer Animal Health) and foals (Equine Rotavirus Vaccine, Pfizer Animal Health) have been available for many years. Prevention of neonatal diarrhea in newborn animals is based on passive immunity of maternal origin and protection from disease provided by parenteral immunization of pregnant cows or mares with inactivated TLPs (194, 195, 318).

Subunit RV vaccines consisting of individual recombinant RV proteins and VLPs with different assemblies of RV capsid proteins are produced with several expression systems, the baculovirus (BV) – insect cell expression system being the most commonly employed (21, 308–313). VP6 can assemble *in vitro* into variable oligomeric structures such as tubules, spheres, and sheets composed of a different number of trimers, the type of assembly depending on pH, ionic strength, and

concentration of divalent cations (30, 31, 319). In the absence of other RV proteins, VP2 forms empty single-layered core-like particles (319). Simultaneous expression of VP2 and VP6 leads to the formation of dl2/6-VLPs mimicking the confirmation of RV DLPs (319), whereas co-expression of VP2, VP6, and VP7 with or without VP4 results in triple-layered VLPs (2/6/7-VLPs or 2/4/6/7-VLPs) resembling native infectious TLPs (320). These highly immunogenic oligomeric proteins represent a safe approach, as they are non-infectious and deprived of viral genetic material.

Moreover, soluble *E. coli*-expressed fusion proteins, most commonly VP6 as a fusion protein with maltose-binding protein (MBP), have undergone extensive preclinical testing (306, 307, 321, 322). In fact, the only non-live vaccine candidate progressed to clinical trials is a P2-VP8* subunit vaccine (**Table 2**) in which the VP8* protein from a human Wa strain (G1P1A[8]) has been expressed in *E. coli* and fused to the P2 T cell epitope of the tetanus toxin to enhance immunogenicity. This monovalent vaccine has recently been shown to be safe in a Phase I trial in US adults. Intramuscular administration of the candidate vaccine elicited a robust neutralizing antibody response to homologous P[8] strains, but less response to P[4] and P[6] strains. (323)

2.6.2.2 VP6 subunit vaccines

In addition to being highly immunogenic (21–24), VP6 proteins are highly conserved among group A RVs, with approximately 90% homology at the amino acid level (64). Therefore, in theory, immunization with VP6 from any RV strain of group A could protect against all group A RVs, making it a potential universal RV vaccine candidate. The evidence from several preclinical studies in mice and rabbits conducted with RV particles lacking the neutralizing antigens, such as inactivated DLPs (303, 304) and dl2/6-VLPs (309, 310, 312, 313, 324, 325), demonstrates that immune responses to neither VP4 nor VP7 are necessary requirements for protection against RV shedding. When the adult mice immunized intramuscularly (IM) or intranasally (IN) with the inactivated TLPs or DLPs co-administered with an adjuvant were challenged with live murine RV, protection stimulated by DLPs was not significantly different from the protection stimulated by TLPs (303, 304). Similarly, equivalent levels of protection were achieved in mice immunized IN with dl2/6-VLPs or triple-layered 2/6/7-VLPs (309). Both parenteral and mucosal immunization of mice with dl2/6-VLPs has been reported to reduce antigen shedding efficiently (70–100%), usually when an adjuvant was included in the

vaccine formulation (308, 309, 312, 313, 324, 325). In contrast to results found in mice and rabbits, IN administration of gnotobiotic piglets with dl2/6-VLPs with or without adjuvant afforded no protection against shedding or diarrheal illness (311). However, according to the subsequent studies, dl2/6-VLPs could supplement protection if given as a booster after oral priming with attenuated RV (326, 327).

That VP6 alone plays a role in RV protection is supported by the consistently high reductions in fecal shedding in mice immunized IN or orally with homologous *E. coli*-expressed MBP-VP6 (306, 328). Moreover, protection close to 100% against the homologous and heterologous challenge of two different murine RV strains, EDIM and EMcN, was elicited after IN delivery of MBP-VP6 derived from either the human CJN or the murine EDIM strains (307). Parenteral delivery of MBP-VP6 has not been extensively studied, but two studies describe partial protection (38% and 73%) following IM and SC administration (306, 329). Likewise, higher protection rates were induced with mucosal delivery of plasmids encoding VP6 (317, 330), although also parenteral DNA immunization provided significant suppression in antigen shedding (314–317). IM immunization of gnotobiotic piglets with VP6 plasmid did not provide substantial protection, but IM boosting with the plasmid after oral RV priming effectively boosted protection against infection (331). Recombinant adenovirus vector expressing VP6 did not provide complete protection (31%) in mice either when administered orally alone, but IN priming with dl2/6-VLPs conferred almost 93% protection (313). In contrast to these studies, in which inclusion of an effective adjuvant was associated with higher protection levels, induction of considerable protection against RV infection with VP6 oligomers without co-administration of an adjuvant has recently been described. In this study, both trimers and tubular structures induced >70% protection in mice against shedding after SC immunization. Only one dose of tubules was sufficient to provide protection. (308)

2.6.2.3 Protective immune responses induced with non-live VP6 vaccines

Non-live vaccine candidates without surface VP4 and VP7 antigens are able to generate heterotypic cross-protective RV immunity in mice and rabbits (304, 306, 307, 310, 312, 324, 332). Protection levels and immune responses induced by the VP6-based vaccines vary greatly depending on the amount and type of antigen, immunization route, and co-administration of different adjuvants.

The protection provided by the VP6-based vaccines has mostly been associated with induction of intestinal and/or serum IgA independently from the type of

immunogen (303, 304, 313, 314, 316, 324, 325, 330). Generally, non-replicating vaccines after mucosal immunization have elicited higher intestinal IgA in adult mice compared to parenteral routes. The protection has also been related to intestinal and serum IgG levels, especially after parenteral deliveries (308, 312). Although the mechanisms or effectors of protection are not completely defined, anti-VP6 pIgA antibodies demonstrating antiviral and protective effects in a mouse model appear to confer protection via pIgR-mediated intracellular neutralization (27–29, 218, 223).

In addition to antibody responses, the presence of $CD4^+$ T cells appears to be important in the establishment of VP6-induced protection, as memory $CD4^+$ T cells have been demonstrated to protect against RV infection in mice immunized IN with MBP-VP6, dl2/6-VLPs or DLPs (333–335). Indeed, an immunodominant $CD4^+$ T cell epitope within the VP6 protein probably responsible for protection has been identified in BALB/c mice (336). A VP6-specific $CD4^+$ T cell epitope has also been identified in a rhesus macaque infected with a human G1P[8] RV (337). These VP6-specific $CD4^+$ T cells contribute to protection either through a direct cytotoxic mechanism or by production of antiviral IFN-γ (333, 338, 339).

2.7 Norovirus – another cause of gastroenteritis

Noroviruses (NoVs) are single-stranded RNA (ssRNA) viruses of *Caliciviridae* family. They are the leading causes of viral AGE, being responsible for 50% of outbreaks of AGE and 18% of endemic AGE in all age groups (340, 341). Although NoV affects people of all ages, especially very young, elderly or immunocompromised are at risk for severe NoV disease (342). After the waning of maternal antibodies, children at the age of 6–23 months are the most susceptible to NoV infections (343–345). In fact, NoVs are the second most common causes of childhood gastroenteritis after RV, accounting for ~12% of diarrheal hospitalizations worldwide. Each year NoV infections lead to an estimated of 2 million episodes of AGE requiring medical care and 200,000 deaths in children under five years of age, the majority of hospitalizations and deaths occurring in developing countries (346).

NoVs are divided into six genogroups (GI–GVI). Most of NoV infections in humans are caused by viruses belonging in two heterologous and distant genogroups, GI and GII (342, 347), that induce a limited immunological cross-protection against the viruses belonging to the different genogroup (348–350). Despite the over 30

genotypes within the genogroups GI and GII (347), the genotype GII.4 has dominated since 1990s, causing 55–85% of all NoV gastroenteritis worldwide (351).

Currently, there is no vaccine available against NoV gastroenteritis (352). Since production of live attenuated and inactivated NoV vaccines is impossible due to the lack of cell culture system for *in vitro* propagation of NoV, vaccine development has focused on vaccine candidates produced in recombinant expression systems. These vaccines are based on VLPs, the structures mimicking the native NoV structure, produced by spontaneous self-assembly of the NoV major capsid protein VP1 (353). In fact, the immunogenicity, safety and efficacy of two adjuvanted VLP-based vaccine candidates have recently been assessed in phase I/II clinical trials (354, 355). IN delivery of GI.1 VLP reduced the incidence of NoV gastroenteritis by 47% and the severity of disease following challenge with homologous NoV (354). Moreover, IM administration of GI.1/GII.4 bivalent vaccine reduced NoV-related symptoms upon heterovariant challenge (355).

The goal of our research group has been focused on development of a combined vaccine against NoV and RV gastroenteritis. The development of NoV components, GI.3 and GII.4 VLPs, and the promising results on the NoV-specific immunogenicity of the combination vaccine have been described as a part of the dissertations by Leena Huhti and Kirsi Tamminen (356, 357).

3 Aims of the study

The overall objective of the study was to develop a RV-NoV combination vaccine to combat childhood AGE with a single vaccine. The specific objectives of this thesis were the following:

I. To produce recombinant VP6 and dl2/6-VLPs for an RV candidate vaccine and assess the products for VP6-specific immunogenicity (I, II).

II. To assess VP6-specific immunogenicity induced with RV-NoV combination vaccine (II, III).

III. To investigate protective immunity against RV induced by VP6 immunization *in vitro* (III, IV) and *in vivo* (IV, V).

4 Materials and methods

4.1 Production of VP6 and dl2/6-VLPs

4.1.1 Construction of recombinant VP6 and VP2 expression systems (I, II)

In order to obtain the nucleotide sequences of gene segments of RV VP6 and VP2, dsRNA was extracted using the QIAamp® RNA viral mini kit (Qiagen, Hilden, Germany) from a 10% fecal suspension of a three-year-old RV G1P1A[8] and SGII positive child at the Tampere University Hospital in 2007 (133). The dsRNA was subjected to RT-PCR reactions with primer pairs specific for VP6 and VP2 (358), producing respective amplicons of 1362 bp and 2662 bp, followed by sequencing (ABI PRISM TM 310 Genetic Analyzer, Applied Biosystems, Carlsbad, CA) of the purified (QIAquick® gel extraction kit, Qiagen) amplicons. The DNAs coding for sequences of VP6 (accession number of reference strain GQ477131) and VP2 (accession number of reference strain HQ609556) were optimized for expression in *Spodoptera frugiperda* (Geneart AG, Regensburg, Germany) without changes in amino acids. The optimized VP6 sequence was inserted into the pFastBac1 expression vector (Invitrogen, Carlsbad, CA) at the BamHI/EcoRI restriction site and the VP2 sequence into the pFastBacDual vector (Invitrogen) at the XhoI/KpnI restriction site.

Recombinant bacmid DNAs were generated by transformation of DH10Bac™ competent *E. coli* cells with pFastBac constructs by Bac-to-Bac Baculovirus expression system (Invitrogen). Recombinant BV (rBV) stocks were produced in Gibco® *S. frugiperda* Sf9 insect cells (Invitrogen). Low-titer P1 rBV stocks were produced by transfecting the cells with 1 μg of bacmid DNAs in serum-free medium (Sf-900™ III SFM, Invitrogen), followed by culturing at 27°C for 72 hours. Similarly, high-titer P2 rBV stocks were produced by infecting Sf9 cells with the P1 stocks and culturing for six days. Titers of P2 rBV stocks expressed as the multiplicity of infection (MOI) were determined using the BacPak™ Baculovirus Rapid titer kit (Clontech Laboratories, Mountain View, CA).

4.1.2 Production and purification of the recombinant proteins (I, II)

For the optimal production of rVP6 and dl2/6-VLPs, various conditions such as cell count, MOI/cell, and days post infection (dpi) were tested. Different cell cultures were analyzed for recombinant protein contents by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with Mini-PROTEAN® TGX™ Any kD gel (Bio-Rad Laboratories, Hercules, CA) followed by PageBlue™ Protein staining (Thermo Fisher Scientific Inc., Rockford, IL). Conditions leading to the biggest yield were chosen for production, and the schematic diagram of the production and purification processes is shown in **Figure 5**. Briefly, to produce rVP6, Sf9 cells were infected with the rBV-VP6 P2 stock. To produce dl2/6-VLPs the cells were co-infected with P2 stocks of rBV-VP2 and rBV-VP6. Both cultures were harvested at 7 dpi; rVP6 proteins were purified from cell lysates while supernatants were utilized for purification of dl2/6-VLPs. Both recombinant products were concentrated by ultracentrifugation and purified on sucrose gradients. Fractions were collected by bottom puncture, and the fractions containing rVP6 or dl2/6-VLPs were pooled, dialyzed against PBS, and concentrated by ultrafiltration. The proteins were stored at +4°C.

4.1.3 Characterization of the final products (I–III)

The total protein content of the purified rVP6 and dl2/6-VLPs was quantified by the Pierce® BCA Protein Assay (Thermo Fisher Scientific Inc.). The purity of the products was defined by SDS-PAGE and PageBlue staining, followed by densitometric analysis with AlphaEase® FC Software (Alpha Innotech, San Leandro, CA). Likewise, the proportion of VP6 protein in dl2/6-VLP preparation was defined by the densitometric analysis and the concentration of VP6 was calculated from the total protein amount quantified with BCA Protein Assay. Endotoxin levels were determined using the Limulus Amebocyte Lysate (LAL) assay (Lonza, Walkersville, MD).

The integrity and morphology of the protein assemblies were determined by EM with FEI Tecnai F12 (Philips Electron Optics, Holland) following negative staining with 3% uranyl acetate pH 4.6.

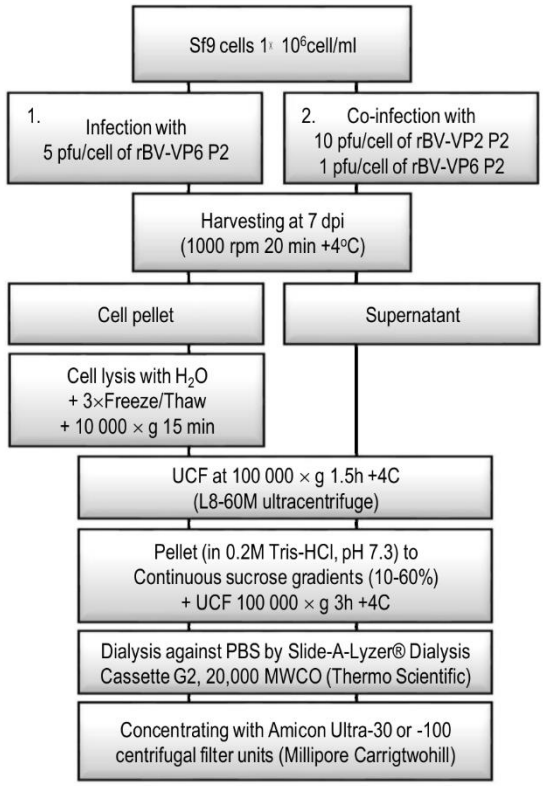

Figure 5. Schematic diagram of the main steps in the production and purification processes of rVP6 and dl2/6-VLPs. UCF, ultracentrifugation.

4.2 Preparation of virus stocks (I–V)

Fetal rhesus monkey kidney (MA104) cells were cultured in Earle's minimum essential medium supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St Louis, MO), 2 mM L-glutamine (Gibco® by Life Technologies, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco® Pen-Strep, Life Technologies) at 37°C in a humidified 5% CO_2 incubator.

Several RV strains (**Table 3**) were propagated in MA104 cells in the presence of 0.5 μg/ml of trypsin (Sigma-Aldrich). After observing the cytopathic effect at 3–4 dpi, virus stocks were prepared from infected cells by three cycles of freezing-thawing, centrifuging at 2,000 × g for 10 min for clarification, and storing at -70°C. The titer of the stocks in plaque-forming units (pfu)/ml of virus was determined by virus plaque assay (359). The amount of VP6 in the viral cultures was determined

using the Ridascreen® Rotavirus test (R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions using insect-cell-derived rVP6, expressed in ng/ml, as an internal standard. Each human, bovine, and rhesus RV culture was diluted in PBS to include equal amounts of VP6 protein before employing as antigens in ELISA or enzyme-linked immunosorbent spot (ELISPOT) assay (Chapters 4.3.5.2 and 4.3.5.3).

Murine RV strain $EDIM_{wt}$ (**Table 3**), originally received from Dr. Richard Ward (Gamble Institute of Medical Research, Cincinnati, OH), was used to challenge mice in an *in vivo* protection study (Chapter 4.3.6.2). The EDIM RV, pooled from fecal samples of infected neonatal mice, was prepared according to the method used by Ward and co-workers (201).

Table 3. Characteristics of human and animal RV strains employed in the experimental work.

Strain	Origin	Subgroup	I-type	G-type	P-type
Wa	human	SGII	I1	G1	P1A[8]
SC2	human	SGI	Ix	G2	P2A[6]
BrB	human	SGII	Ix	G4	P2A[6]
69M	human	SGI	I2	G8	P4[10]
L26	human	SGI	I2	G12	P1B[4]
WC3	bovine	SGI	I2	G6	P7[5]
RRV	rhesus	SGI	I2	G3	P5B[3]
EDIM	murine	SG non-I/II	I7	G3/16	P10[16]

x, the undetermined genotype.

4.3 Preclinical studies

4.3.1 Experimental animals (I–V)

Inbred female BALB/c OlaHsd mice (7–9 weeks old) were obtained from Harlan Laboratories (Horst, the Netherlands). Immunizations were performed under general anesthesia induced either with a mixture of Hypnorm® (VetaParma Limited,

Leeds, UK) and Dormicum® (Roche Pharma AG, Grenzach-Wyhlen, Germany) or Ketalar® (Phizer, NY) and Dorbene® (Syva, Leon, Spain). All the procedures were conducted according to the regulations and guidelines of the Finnish National Animal Experiment Board (permission numbers ESLH-2009-06698/Ym-23 and ESAVI/4106/04.10.03/2012), and all efforts were made to minimize animal suffering. The protocol for the challenge study was approved by the Bioethics Committee of the Instituto de Biotecnologia at the Universidad Nacional Autónoma de México (permission number 167-2010).

4.3.2 Antigenic formulations (I–V)

Baculovirus-produced RV rVP6 protein and dl2/6-VLPs diluted in sterile PBS (Lonza, Verviers, Belgium) were used in two different doses for animal immunizations. The doses of dl2/6-VLPs were calculated according to the proportion of VP6 protein in the preparation instead of the total protein concentration. Combination RV-NoV vaccines were prepared by mixing similarly produced RV and NoV components in equal quantities to contain similar amount of each antigen. Bivalent vaccine was prepared by combining RV rVP6 with NoV GII.4 VLPs (1:1). Trivalent candidate vaccines were produced by mixing either RV rVP6 or dl2/6-VLPs with NoV GII.4 and GI.3 VLPs in a ratio of 1:1:1. No external adjuvants were included in any vaccine formulations.

4.3.3 Animal immunizations and sample collections (I–V)

To examine immune responses induced by different immunogens, BALB/c mice (3–7 mice/experimental group) were immunized IM or IN with 50 μl volumes of different vaccine formulations containing 3 or 10 μg of VP6 protein (**Table 4**) per immunization point. Mice were immunized two times at study weeks 0 and 3. Naïve control mice received carrier only (sterile PBS). Immunizations by IM injection were administered into to the caudal thigh muscle of the hind leg. IN administration was performed in a 25-μl volume by gradual inoculation in each nostril.

Different sample types were collected at different time points. Collection of pre-immune tail blood samples (diluted 1:100 or 1:200 in PBS at the time of collection), fecal samples and vaginal washes (VWs) was conducted at week 0. Vaginal lavages were examined, since both mucosal and parenteral immunization can induce mucosal antibodies at various locations, including the genital tract (302, 360–362).

Tail blood samples were also collected at study week 3 to test the kinetics of the antibody responses in sera. The mice were euthanized at week 5, when whole blood, feces, VWs, and lymphoid tissues were collected. For long-term follow-up of the immune responses, a group of mice was sacrificed at study week 24. **Figure 6** shows the time schedule for immunizations, sample collections, and terminations.

Table 4. Antigenic formulations with VP6 doses and delivery routes employed in preclinical studies.

Immunogen	VP6 dose (μg)	Delivery route	Study
rVP6	3	IM	I, III
	10	IM	I, II, III
dl2/6-VLP	3	IM	I
	10	IM	I
VP6 bivalent	10	IM	II
VP6 trivalent	10	IM, IN	III, IV, V
dl2/6 trivalent	10	IM, IN	unpublished
None (PBS)	0	IM, IN	I-V

4.3.4 Sample preparations (I–V)

The sera of experimental mice were separated from collected tail blood and whole blood samples by centrifugation at 3500 × g for 20 min after incubating the blood samples at room temperature ~2h (363). Separated sera were stored at -20°C until analyzed.

Fresh feces collected from 3–7 animals per group were pooled group-wise, and 10% w/v stool suspensions were prepared in cold TNC buffer (10 mM Tris, 100 mM NaCl, 1mM $CaCl_2$ pH 7.4) containing 0.02% Tween, 1% aprotinin, and 10 μM leupeptine (all from Sigma-Aldrich). The suspensions were homogenized by vortexing, incubated on ice for 20 min, and centrifuged at 18,000 × g for 15 min. The supernatant was stored at -80°C.

Vaginal fluids were collected by rinsing twice with 125 μl of cold PBS (by pipetting 4–5 times up and down) followed by centrifugation at 12,000 × g for 10 min +4°C and storage of the supernatants at -20°C.

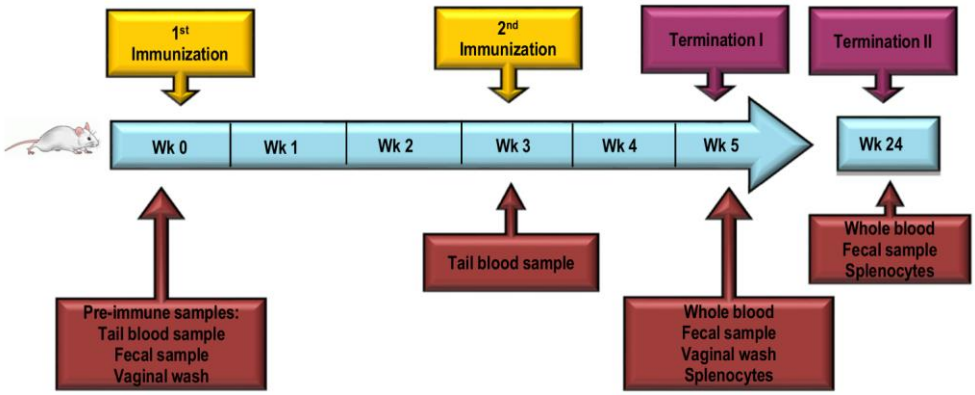

Figure 6. Preclinical study design showing the schedule for immunizations, sample collections and terminations.

At the time of euthanasia, the spleen of each mouse was harvested in Hanks' balanced salt solution (HBSS, Sigma-Aldrich), and the texture of the spleen was disrupted mechanically with a scalpel. A uniform single-cell suspension was prepared by pressing disrupted tissue through a 70-μm cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ) with the soft end of syringe plunger, followed by centrifugation at 300 × g for 10 min and resuspending of the cells in HBSS. The red blood cells were lysed by hypotonic shock with 1:10 diluted HBSS, and the molarity of the suspension was recovered with 2 × HBSS balanced with 7.5% sodium bicarbonate (both from Sigma-Aldrich). The remaining splenocytes were washed with HBSS, counted in Bürker's chamber, and resuspended in freezing medium consisting of RPMI-1640 supplemented with 40% FBS and 10% DMSO (all from Sigma-Aldrich). (363) Vials containing 10–20 × 10^6 cells/vial were stored in liquid nitrogen after cryopreserving by freezing to -80^{o}C in a freezing chamber with 2-isopropanol.

4.3.5 Immunoassays

4.3.5.1 IgG and IgA antibody ELISAs for the detection of humoral immunity (I–V)

Humoral immune responses were defined by measuring anti-VP6 IgG and IgA antibodies in collected sera, stool suspensions, and VWs. Serum samples from individual mice at 1:200 dilution and two-fold dilution series were evaluated for RV

VP6-specific total IgG and IgG subtype responses by ELISA in which Costar High Binding 96-well half area polystyrene plates (Corning Incorporated) were coated with 40 ng/well of rVP6 protein. For detection of serum (at 1:2, 1:12.5 or 1:100 and two-fold dilution series) and mucosal anti-VP6 IgA, as well as mucosal IgG (fecal samples and VWs tested at 1:2 or 1:5 dilutions and two-fold dilution series), the plates were coated with 50 ng/well of rVP6. Anti-VP6 antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma-Aldrich), IgG1 (Invitrogen), IgG2a (Invitrogen) or IgA (Sigma-Aldrich) and OPD substrate (Sigma-Aldrich). Optical density (OD) at 490 nm was measured by a Victor^2 1420 microplate reader (Perkin Elmer, Waltham, MA), and a sample was considered positive if the OD_{490} value was above the cut-off value (mean OD_{490} of control mice + 3 × SD) and at least 0.1. The end-point titers were defined as the reciprocal of the highest sample dilution with an OD_{490} above the cut-off value. One known positive sample and one known negative sample were included in each assay as controls.

Characterization of IgA forms by immunoblotting (IV)

The quality of IgA antibodies in different sample types of experimental mice was evaluated by immunodetection analysis after electrophoresis. To allow the detection of SIgA complexes, pools of sera, fecal samples and VWs were separated by SDS-PAGE with 4–15% Mini-PROTEAN® TGX™ gels (Bio-Rad) under non-reducing conditions. Each specimen was mixed equally with Laemmli sample buffer (Bio-Rad) in absence of β-mercaptoethanol and incubated at 95°C for 5 min before electrophoresis in 1 × Tris/Glycine/SDS buffer (Bio-Rad). Forms of IgA were detected after immunoblotting to the nitrocellulose membrane (Bio-Rad) with goat anti-mouse IgA-HRP (Sigma-Aldrich) and an OPTI-4CN™ Substrate kit (Bio-Rad).

4.3.5.2 Sandwich ELISA for detection of cross-reactive antibodies (I)

Cross-reactive anti-VP6 serum IgG antibodies were determined using the sandwich-ELISA. The Costar High Binding 96-well polystyrene plates were coated with polyclonal rabbit anti-RV antibody at a concentration of 1 μg/ml (DakoCytomation, Glostrup, Denmark), followed by the addition of RV cell culture (Wa, SC2, BrB, 69M, L26, WC3, and RRV) antigens at a concentration of 100 ng VP6/ml. MA104 mock cell culture was used as negative control. Antibodies in 1:200 diluted sera were detected using HRP-conjugated goat anti-mouse IgG as described in Chapter 4.3.5.1.

4.3.5.3 ELISPOT IFN-γ assay for detection of cell-mediated immunity (I, III)

VP6-specific T cell responses were analyzed by ELISPOT assay (363) by quantification of IFN-γ production from splenocytes. Multiscreen 96-well HTS-IP filter plates (Millipore, Billerica, MA) were coated with anti-mouse IFN-γ monoclonal antibody (Mabtech AB, Nacka Strand, Sweden) at 5 μg/ml. The thawed and washed splenocytes were suspended in RPMI-1640 medium supplemented with FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol, and 2mM L-glutamine (all from Sigma-Aldrich). Group-wise pooled splenocytes at 0.2 × 10^6 cells/well from the experimental mice were stimulated in duplicates with Wa RV cell culture (0.5 μg VP6 antigen/ml) or 5 μg/ml of a synthetic 18-mer VP6-derived R6–2 peptide (Proimmune Ltd., Oxford, UK), previously identified as an VP6-specific BALB/c mouse $CD4^+$ T cell epitope (DGATTWYFNPVILRPNNV, $AA_{242-259}$) (336). MA104 mock cell culture was used as negative control. Splenocytes incubated in culture medium alone and splenocytes stimulated with 10 μg/ml of concanavalin A (Sigma-Aldrich), a T cell mitogen, were included as background and viability controls. After ≥20 h incubation of the cells with the different stimuli at 37°C, IFN-γ cytokine secretion was detected with biotinylated anti-mouse IFN-γ antibody and streptavidin-ALP (both from Mabtech AB). The spots developed with BCIP/NBT substrate (Mabtech AB) were counted by ImmunoSpot® automatic CTL analyzer (CTL-Europe GmbH, Bonn, Germany), and the results were expressed as mean spot-forming cells (SFC)/10^6 splenocytes of duplicate wells.

Blocking of T cell activation with CD4- and CD8-specific antibodies (I)

To define the cell type responsible for the production of IFN-γ, splenocytes were pre-incubated for one hour at 37°C with the functional blocking antibodies, rat anti-mouse CD4 and rat anti-mouse CD8a, or rat IgG isotype control antibody (all from eBioscience) at concentration of 30 μg/ml prior to ELISPOT stimulation (364) with the VP6-specific peptide or Wa RV cell culture.

4.3.6 Evaluation of protection induced by combination vaccines

4.3.6.1 Inhibition of rotavirus infection *in vitro* (III, IV)

The neutralizing ability of VP6-specific antibodies was analyzed by measuring the reduction in RV antigen production using an ELISA-based RV antigen reduction neutralization assay (NELISA) as previously reported (365, 366). A series of two-fold dilutions (from 1:10 to 1:1280) of each group-wise pooled sera, fecal samples and VWs were mixed with equal volumes of Wa RV strain or RRV strain containing 125 pfu and pre-incubated at 37°C for 60 min. Serial dilutions of human polyclonal anti-RV serum from a patient with an RV infection and human polyclonal serum negative for RV were included as positive and negative assay controls. Confluent MA104 monolayers in 96-well plates (Nunc, Roskilde, Denmark) were overlaid with the mixture, followed by centrifugation of the plates at 1,000 × g for 60 min, replacement of the virus inoculum with culture medium supplemented with 4 μg/ml of trypsin (Sigma-Aldrich) and incubation at 37°C for 15 h. The cells were lysed with a cycle of freezing and thawing, and the plates were stored at -80°C.

The RV antigen production of each specimen, performed in duplicate, was determined by a Ridascreen® Rotavirus test using internal insect-cell-derived rVP6 standard.

Depletion of antibodies (IV)

In order to assess whether IgA antibodies mediated the neutralizing activity of mucosal specimens, IgA antibodies from VWs were depleted with magnetic bead treatment by incubating the sample prior to neutralization assay with 6.7×10^7/ml of Dynabeads M-280 Streptavidin combined with 40 μg of biotinylated goat anti-mouse IgA (both from Life Technologies). The residual neutralization activity of the IgA-depleted supernatant separated from the beads was tested by NELISA. IgA depletion of the sample was confirmed by mucosal IgA ELISA as described above (Chapter 4.3.5.1).

4.3.6.2 Virulent rotavirus challenge *in vivo* (IV, V)

For an assessment of the *in vivo* protection conferred by the trivalent combination vaccines containing 10 μg of VP6 protein, adult mice (5 mice/group) were immunized IM and IN as described above (Chapter 4.3.3). For correlation analysis, blood samples from each mouse were collected by tail bleed at study week 5. Three weeks after the last immunization, at study week 6, the immunized and control mice were challenged orally with 1×10^4 FFU (100-fold the diarrheal dose DD_{50}) of the murine RV strain $EDIM_{wt}$ (308). The fecal samples of each mouse were collected before the challenge (day 0) and daily for eight days (days 1–8) after the challenge and suspended in TNC buffer for RV antigen detection. The mice were sacrificed at day 8 post-challenge, when also the sera were collected from each mouse. The pre- and post-challenge serum samples (from study weeks 5 and 7 respectively) were tested for anti-VP6 IgG and IgA antibodies as described in Chapter 4.3.5.1. **Figure 7** shows the study design for the *in vivo* protection study.

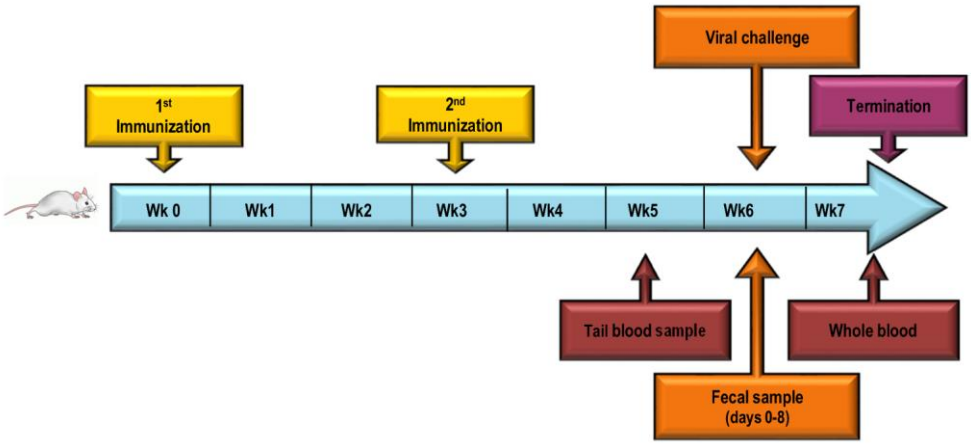

Figure 7. The *in vivo* protection study design. The schedule for immunizations, sample collections, live viral challenge and final bleeding is shown.

Detection of rotavirus antigen in fecal samples

To evaluate protective efficacy, the presence of RV antigen in stool specimens was determined with an antigen-ELISA as previously described (332). Shedding of fecal

antigen was expressed as the net OD_{405} value by subtracting the OD of pre-challenge fecal samples from the OD of the post-challenge fecal samples of each individual mouse. Viral shedding curves for each animal were plotted (OD_{405} versus day post-challenge), and the reductions in viral load were calculated by comparing the areas under the curves of the immunized animals to the mean area under the curve of the control group. A >50% reduction in antigen shedding was considered significant protection from the viral challenge (309, 313).

4.4 Statistical analyses (I–V)

The Mann-Whitney U-test and Kruskal-Wallis test were employed to determine the statistical differences between the non-parametric observations of two or more independent groups. Correlates of protection were assessed by calculating Spearman's correlation coefficients. The analysis was performed by IBM SPSS Statistics -software (SPSS Inc., Chicago, IL) version 22.0, and the statistically significant difference was defined as $p \leq 0.05$.

5 Results

5.1 Expression of rVP6 and dl2/6-VLPs (I–III)

Pure and intact RV rVP6 and dl2/6-VLPs were successfully produced by a BV expression system. **Figure 8A** shows SDS-PAGE gel with the identified VP6 (45 kDa) and VP2 (102 kDa) protein bands of the purified products. An additional cleavage product of VP2 with a molecular weight of ~90 kDa was detected in dl2/6-VLPs. Densitometric analysis detected >90% purity of the products, and the proportion of VP6 protein in dl2/6-VLP preparation was ~42% from the total protein content. Neither of the antigenic formulations were contaminated with bacterial endotoxin according to the determined endotoxin levels of <0.6 EU/20 μg protein, which is below the international standard of ≤30 EU/20 μg of protein.

The integrity and formation of high-order structures including rVP6 tubules (~0.2–1.5 μm in length) with hexagonal pattern and dl2/6-VLPs (~65 nm in diameter), were verified under EM (**Fig. 8B, panels 1 and 2** respectively). Production of rVP6 led to formation of VP6 trimers, which under neutral pH (PBS with pH 7.4) assembled into oligomeric tubular structures with occasional spheres and sheets. VP6 tubules were not detected in the dl2/6-VLP preparations.

Mixing RV components with NoV VLPs to create combination vaccines against RV and NoV did not impair the integrity or morphology of the proteins (**Fig. 8B**). The combination of rVP6 with NoV GII.4 VLPs in a ratio of 1:1 or with NoV GII.4 and GI.3 VLPs in a ratio of 1:1:1 resulted respectively in a VP6 bivalent (**Fig. 8B, panel 5**) or a VP6 trivalent formulation (**Fig. 8B, panel 6**) in which the VP6 tubules were partly filled with VLPs. Instead, the combination of dl2/6-VLPs with both NoV VLPs resulted in a dl2/6 trivalent vaccine, which was identified as a mixture of VLPs of different sizes (**Fig. 8B, panel 7**).

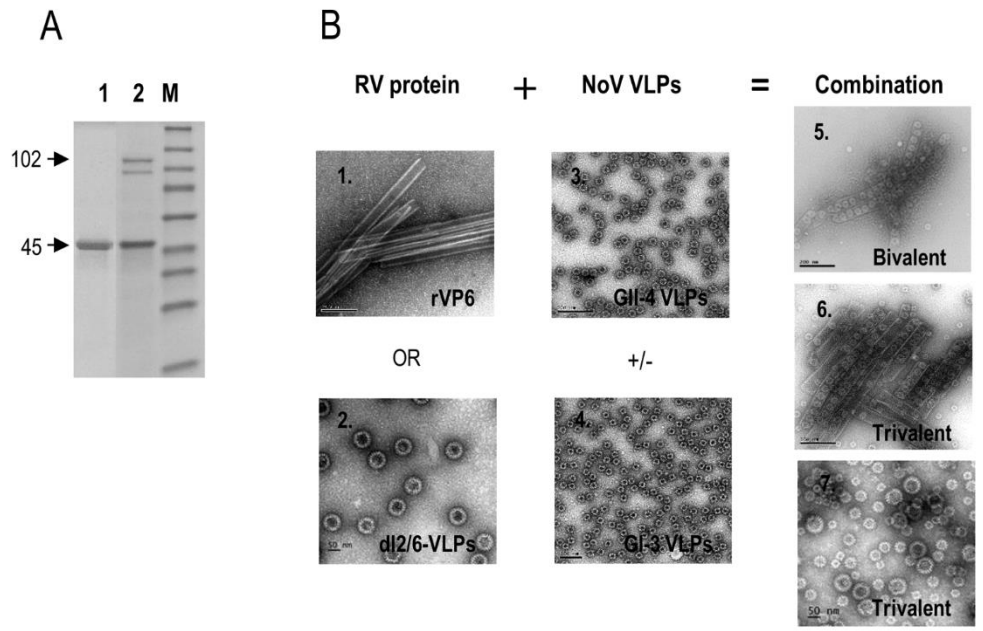

Figure 8. Characterization of RV rVP6 and dl2/6-VLPs. (**A**) Purity and integrity analysis of produced rVP6 (lane 1) and dl2/6-VLPs (lane 2) with PageBlue-stained SDS-PAGE gel. Lane M illustrates the molecular weight marker. Corresponding molecular weights are indicated with arrows on the left side of the gel. (**B**) EM images of negatively stained morphological structures assembled by RV rVP6 (panel 1) and dl2/6-VLPs containing VP2 and VP6 proteins (panel 2), respectively corresponding to SDS-PAGE lanes 1 and 2. Panels 3 and 4 respectively represent images of NoV GII.4 and GI.3 VLPs. A bivalent combination of rVP6 with GII.4 VLPs is shown in panel 5 and a trivalent combination of rVP6 or dl2/6-VLPs with GII.4 and GI.3 VLPs in panels 6, and 7 respectively.

5.2 rVP6 tubular structures and dl2/6-VLPs are equally immunogenic

5.2.1 Serum antibody responses (I)

To compare the immunogenicity of the two different RV immunogens, mice were immunized IM with two doses of rVP6 or dl2/6-VLPs at an interval of three weeks without an adjuvant (**Fig. 9A**). After the immunizations, similar levels of total IgG were observed in each immunized group ($p \geq 0.05$) at week 5 (**Fig. 9A**). Control mice remained negative for anti-VP6 IgG antibodies during the study period.

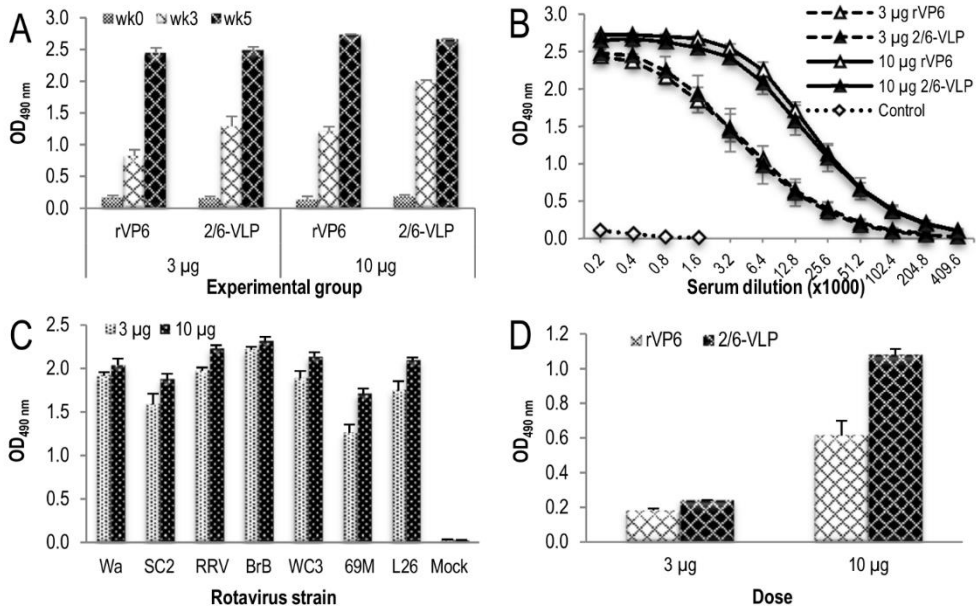

Figure 9. Humoral immune responses in mice immunized IM with 3 µg or 10 µg doses of rVP6 or dl2/6-VLPs. (**A**) Kinetics of VP6-specific IgG antibodies in 1:200 diluted serum samples of mice immunized with a 2-dose schedule of the VP6 proteins at study weeks 0 and 3. Group means with standard errors of tail blood samples from weeks 0 (pre-immune sera) and 3 (after first immunization) and termination sera from week 5 (after second immunization) are presented. (**B**) End-point titrations of serum anti-VP6 IgG antibodies of each experimental group. Control mice received PBS. Mean titration curves with standard errors of termination sera from week 5 are shown. (**C**) Cross-reactivity of VP6-induced serum IgG antibodies against various RV strains in experimental mice immunized twice with two different doses of dl2/6-VLPs. (**D**) Mucosal anti-VP6 IgG antibodies at week 5 in group-wise pooled fecal suspensions. Mean OD_{490} values with standard errors of two independent experiments are shown.

End-point titrations of termination sera indicated that 3 and 10 µg doses of rVP6 or dl2/6-VLPs were able to elicit high titers of anti-VP6 IgG in each experimental group (**Fig 9B**). Similar responses were elicited with both structures ($p \geq 0.5$). Compared with 3 µg doses, immunization with 10 µg of rVP6 or dl2/6-VLPs resulted in threefold higher increases in the titers ($p < 0.05$). Determination of VP6-specific IgG subtype titers for IgG1 and IgG2a representing Th2- and Th1-type responses (367) revealed that balanced Th1-type and Th2-type responses with similar IgG2a and IgG1 titers ($p \geq 0.2$) were induced.

To evaluate whether IgG antibodies induced with rVP6 or dl2/6-VLPs were heterotypic to RV strains, different RV cell cultures were used as antigens in ELISA.

High levels of cross-reactive antibodies against each tested RV strain, including bovine WC3 and rhesus RV, as well as several human strains, were induced, indicating broad reactivity of the VP6 protein (**Fig. 9C**).

5.2.2 Mucosal antibodies (I)

Induction of mucosal VP6-specific IgG antibody responses with two doses of rVP6 or dl2/6-VLPs was determined in the group-wise pooled 10% fecal suspensions. Comparison of IgG responses in 1:5 diluted feces indicated that only 10 μg doses of rVP6 and dl2/6-VLPs induced considerable levels of fecal IgG antibodies (**Fig. 9D**). Low levels of fecal VP6-specific IgA (OD_{490} 0.13) were induced with 10 μg doses of immunogens, but the responses were observed only in 1:2 diluted suspensions. No anti-VP6 antibodies were seen in mucosal samples of control mice ($OD_{490} \leq 0.08$).

5.3 Combination vaccine induces long-lasting humoral and cellular immunity

Due to the stronger immune responses induced at higher doses, the 10 μg dose of VP6 was selected as the optimal one and employed for all subsequent immunizations with combination vaccine formulations.

5.3.1 No interference of NoV VLPs to VP6-specific immunogenicity (I–III)

To assess the possible interference of NoV components with VP6-specific immunogenicity, mice were immunized IM on a regular two-dose schedule of rVP6 single-antigen, bivalent combination vaccine containing rVP6 and NoV GII.4 VLPs or trivalent combination vaccine containing rVP6 and NoV GII.4 and GI.3 VLPs, and VP6-specific antibody responses were compared. Moreover, long-term responses in a 24-week study schedule were evaluated in a group of seven mice receiving the VP6 trivalent vaccine. No statistical differences between the magnitudes of IgG antibody levels were detected whether rVP6 was administered alone or in the combination (**Fig. 10A**). No reduction was observed in the antibody levels of the trivalent vaccine between the study weeks 5 and 24 (**Fig. 10A**).

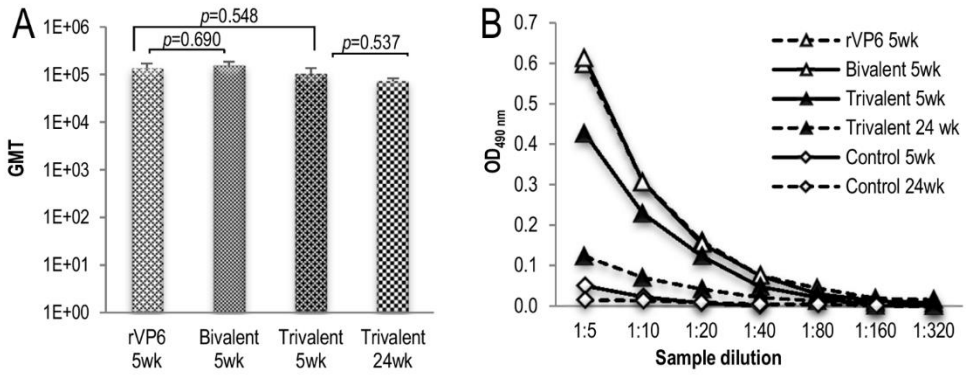

Figure 10. Humoral VP6-specific IgG responses at study weeks 5 and 24 in mice immunized IM twice with rVP6 alone or with bivalent or trivalent combination vaccines containing rVP6. (**A**) Geometric mean titers (GMTs) of anti-VP6 IgG antibodies in serum. (**B**) End-point titration of mucosal VP6-specific IgG responses in group-wise pooled fecal samples.

Moderate levels of antigen-specific fecal IgG antibodies were detected in all immunized groups at week 5 (**Fig. 10B**). Fecal antibodies were also detected at week 24, although the response decreased considerably compared to week 5 (**Fig. 10B**).

5.3.2 Intranasal delivery induces high levels of IgG and IgA (III–V)

In the following set of experiments, IM and IN delivery routes were compared for induction of VP6-specific immune responses in groups of mice receiving a two-dose immunization schedule of VP6 and dl2/6 (5 mice/group) trivalent vaccines. High levels of IgG antibodies with end-point titers >4.8log10 were induced with all immunizations. Delivery route had no effect on the IgG responses, as equal magnitudes of antibody levels between the groups immunized IM and IN with either of the trivalent vaccines (p>0.05) were detected. However, examination of VP6-specific IgA responses revealed that only IN delivery was able to induce detectable levels of serum IgA (**Fig. 11A**). Similar levels of IgA antibodies (p=1.0) were elicited with both trivalent combination vaccines (**Fig. 11A**)

Further, considerable levels of mucosal anti-VP6 IgG antibodies were detected in group-wise pooled fecal suspensions of each experimental group. Similar levels of mucosal IgG antibodies were induced IM with VP6 and dl2/6 combination vaccines (end-point titers 20 and 40), as illustrated in **Fig. 10B** with VP6 trivalent vaccine.

Mucosal immunization resulted in slightly higher fecal IgG responses (end-point titers 80). Similarly to sera, moderate levels of fecal IgA antibodies were induced with IN delivery of combination vaccines (**Fig. 11B**). Low levels of IgA could be detected in feces of IM immunized animals (**Fig. 11B**).

In addition, vaginal secretions were collected from VP6 trivalent groups for testing of mucosal antibody responses. Similarly to fecal specimens, both IM and IN immunizations induced mucosal IgG antibodies in VWs but IN delivery resulted in higher levels (end-point titers 320 and 20) of mucosal IgG (**Fig. 12A**). Similarly, higher levels of IgA antibodies were detected in VWs of IN-immunized animals in comparison to VWs of IM-immunized (end-point titers 80 and 2) mice (**Fig. 12A**). Samples of control mice remained negative for anti-VP6 mucosal antibodies.

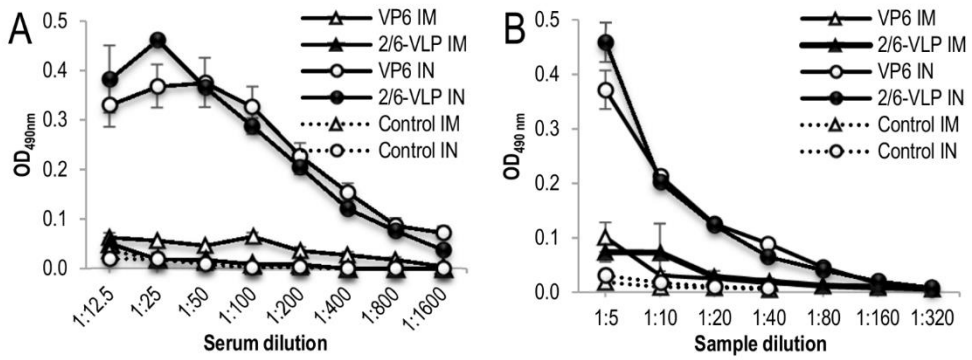

Figure 11. Anti-VP6 IgA antibodies in sera (**A**) and fecal samples (**B**) at week 5 after IM and IN administration of trivalent combination vaccines containing rVP6 or dl2/6-VLPs. Mean titers with standard errors of sera and group-wise pooled fecal samples of two independent experiments are shown.

5.3.2.1 Mucosal specimens contain polymeric forms of IgA (IV)

Molecular forms of IgA antibodies were defined in IgA-positive serum and mucosal samples of mice immunized IN with VP6 trivalent vaccine by immunoblotting under non-reducing conditions. Most IgA antibodies in the fecal specimens and VWs but not in the sera appeared in polymeric forms (**Fig. 12B**).

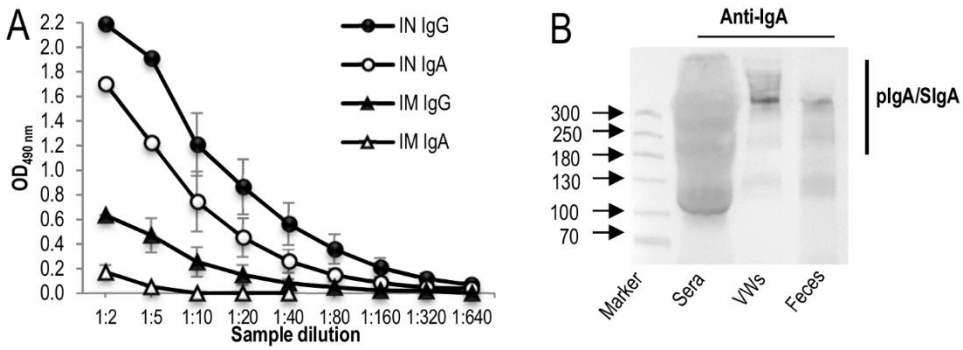

Figure 12. (**A**) Mucosal IgG and IgA antibody responses of groups of mice immunized IM or IN with VP6 trivalent vaccine. Mean titers with standard errors of group-wise pooled VWs of at least two (2–4) independent experiments are presented. (**B**) Characterization of IgA forms in sera and mucosal specimens of mice immunized IN with VP6 trivalent vaccine by immunoblot analysis. Polymeric IgA (pIgA) forms, including SIgA, were detected in mucosal samples. Molecular weight markers (in kDa) are indicated with arrows.

5.3.3 Induction of T cell responses (I, III)

T cell responses were characterized by analyzing Th1-type cytokine IFN-γ production from the splenocytes of mice immunized IM or IN with VP6 or dl2/6 combination vaccines. Regardless of the delivery route, splenocytes from the mice receiving either of the combination vaccines responded with considerable IFN-γ release to stimulation *ex vivo* with VP6-specific R6–2 peptide, representing a $CD4^+$ T cell epitope, or with RV Wa cell culture antigen (**Fig. 13A**). Similar quantities of IFN-γ secreting cells were detected in each group. MA104 mock cell culture or culture media alone stimulated no IFN-γ production (**Fig. 13A**). The T cell response was also determined to be long-lasting. At study week 24, both stimulants induced IFN-γ responses in mice immunized IM with VP6 trivalent vaccine, but the cell population producing IFN-γ was three times smaller compared to week 5.

To determine the T cell type responsible for production of IFN-γ, T cell activation was blocked with anti-CD4 and anti-CD8 antibodies. When the splenocytes of mice immunized IM with dl2/6-VLP vaccine were stimulated with a synthetic R6–2 peptide and RV Wa cell culture in the presence or absence of CD4-specific, CD8-specific or control antibodies, 50–70% of IFN-γ production by the immune cells was blocked only with anti-CD4 antibodies (**Fig. 13B**).

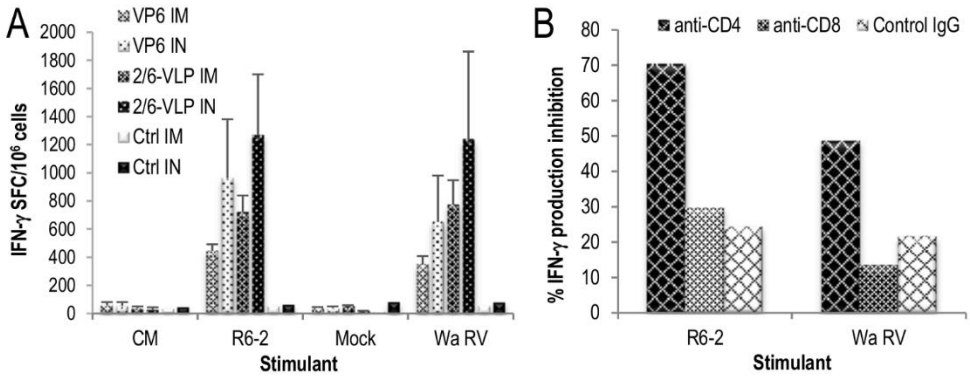

Figure 13. Detection of T cell responses by ELISPOT. (**A**) A R6-2 peptide and an RV Wa cell culture were employed to stimulate IFN-γ production from the group-wise pooled splenocytes of mice immunized IM or IN with VP6 or dl2/6 combination vaccines. A MA104 mock cell culture served as a negative control and cells incubated in culture medium (CM) as a background control. Control (Ctrl) mice received PBS. Results are expressed as mean IFN-γ spot-forming cells (SFC)/10^6 splenocytes of 2–3 independent experiments with standard errors of the means. (**B**) Determination of the T cell type responsible for IFN-γ production by stimulating the cells of mice immunized IM with dl2/6-VLP vaccine in the presence or absence of anti-CD4 and anti-CD8 or control antibodies. Results are expressed as the mean % inhibition of duplicate wells.

5.4 Protection induced by VP6 immunization

5.4.1 Inhibition of rotavirus replication *in vitro* by mucosal antibodies (III, IV)

The functionality of VP6-specific antibodies, defined as their ability to inhibit RV replication *in vitro*, was examined using an ELISA-based RV antigen reduction neutralization assay. Sera and mucosal samples of mice immunized IM or IN with VP6 trivalent vaccine were tested for neutralization against RV Wa, homologous to the VP6 protein used for immunizations, and heterologous RRV. No neutralizing effect was detected in the sera of immunized or negative control groups. Examining the neutralizing activity of fecal suspensions from experimental and control mice failed despite several attempts. Therefore, VWs containing mucosal antibodies at a level similar to that in the fecal samples were employed in neutralization experiments. As a result, samples of VWs from IM- and IN-immunized groups inhibited the infectivity of both RV strains. The cross-reactive neutralizing activity of VP6-specific

mucosal antibodies is indicated in **Fig. 14A**, which represents inhibition of RV Wa and RRV infection by VWs of the mice immunized IN with VP6 trivalent vaccine. A similar inhibition was observed with VWs of mice immunized IM with the VP6 trivalent vaccine. The VWs of control mice did not inhibit RV infection, whereas RV seropositive human control serum always neutralized both virus strains (**Fig. 14A**).

The inhibition of RV Wa and RRV replication was confirmed to be mediated by IgA antibodies with the abolished inhibitory activity after IgA depletion of VWs (**Fig. 14B**). Removal of the IgA antibodies from the VWs was verified with the reduction of the OD_{490} value of the 1:10 diluted VWs from 0.80 to 0.01. After the depletion, the VWs retained significant amounts of IgG antibodies (OD_{490} 1.4 vs. 0.8)

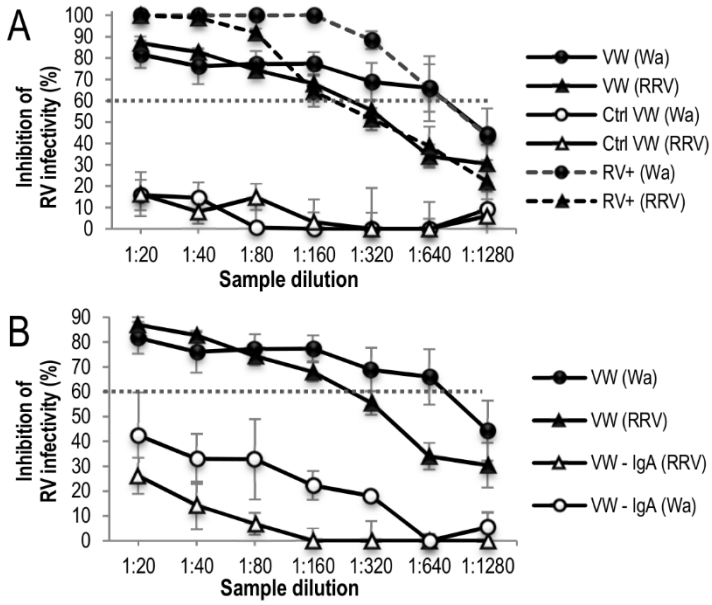

Figure 14. Inhibition of RV infection *in vitro*. (**A**) Inhibition activity of VWs of mice immunized IN with VP6 trivalent vaccine against RV Wa or RRV. VWs of control mice (Ctrl) receiving PBS were used as negative controls. Serum from a RV seropositive human donor (RV+) was used as a positive control. (**B**) IgA antibodies mediate RV inhibition. IgA depletion from VWs (VW-IgA) prior to assay reduced inhibitory activity compared with untreated VWs. Results are shown as the mean percentage of inhibition of RV infectivity in at least two independent experiments, each conducted in duplicate, with standard errors. The dashed lines indicate a 60% cut-off for reduction in virus infectivity.

5.4.2 Protection against live rotavirus challenge *in vivo* (IV, V)

The protective efficacy of VP6 and dl2/6 trivalent candidate vaccines induced by the IM and IN deliveries was determined in a new set of experiments. Mice from the experimental groups were challenged with murine RV $EDIM_{wt}$, and shedding of viral antigen in feces of vaccinated mice was compared to the mean shedding of all (IM and IN) control mice receiving only PBS. The antigen shedding was the highest at day 3 after the challenge as indicated in the viral shedding curves (**Fig. 15A**). Significant reductions in antigen shedding ($p<0.05$) were detected in all immunized groups, compared with the control group (**Fig. 15A**). Regardless of the delivery route or VP6 protein assembly, both vaccines induced significant (>50%) reduction (309, 313) in shedding (**Fig. 15B**). The total antigen shedding of mice immunized with rVP6 trivalent vaccine decreased 66% (±12%) after IM delivery and 65% (±18%) after IN delivery ($p=1.0$). The respective mean protective efficacies of mice immunized IM or IN with dl2/6-VLP trivalent vaccine were 81±3% and 92±3% ($p=0.841$) (**Fig. 15B**). Both rVP6 structures conferred similar protection rates after IM ($p=0.222$) and IN ($p=0.548$) delivery.

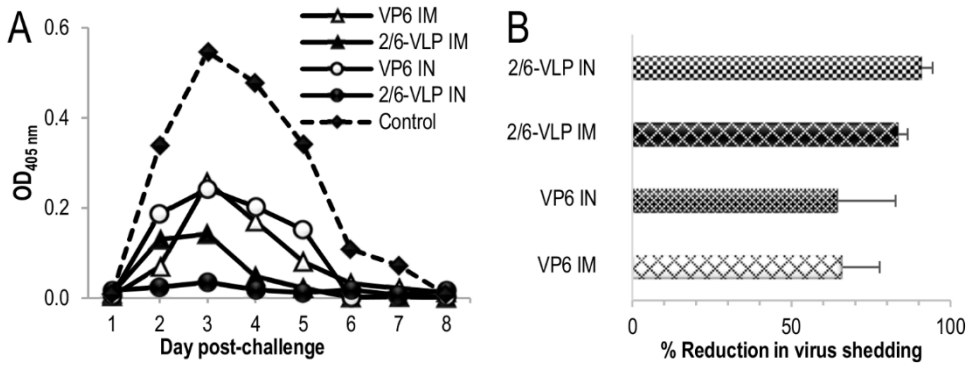

Figure 15. Protection against RV shedding in mice immunized IM and IN twice with the trivalent candidate vaccine containing rVP6 or dl2/6-VLPs. Immunized mice (5 mice/group) were challenged orally with $EDIM_{wt}$, and the quantity of RV antigen shed in stool specimens for 8 days was determined. (**A**) Viral shedding curves of experimental groups. Each point represents the daily mean of a group of mice. For the shedding curve of the control group, the results of all IM and IN control mice were combined (8 mice/group). (**B**) Reductions in virus shedding of the experimental groups after the challenge calculated by comparing the area under the curve of each mouse to the mean of the area under the curve of the control group. Mean percentage reductions of the immunized groups with standard error of the means are shown.

5.4.2.1 Association of immune responses with protection (V)

To determine whether the protection correlated with specific VP6-induced immune responses, serum anti-VP6 IgG and IgA antibody responses were examined and the correlation between the protective efficacies and the end-point titers of VP6-specific IgG and IgA antibodies of each mouse was determined. The IgG and IgA levels of tail blood samples prior to challenge from week 5 (**Figs. 16A and B**) were equivalent (p>0.05) to the previous findings with termination sera (**Figs. 10A and 11A**). There was no correlation of pre-challenge titers of IgG (r=-0.147, p=0.548) or IgA (r=0.250, p=0.289) antibodies with protection rates.

After the RV challenge, increases in VP6-specific serum IgG (≤2.6-fold increase) and IgA antibody titers (7–10-fold increase or conversion to positive) at week 7 were detected in all rVP6 or dl2/6-VLP vaccinated mice (**Figs. 16A and B**), but only the magnitudes of the post-challenge serum IgA responses (GMTs >3log10) increased significantly among all immunized groups (p<0.03). Following the challenge, the control mice also developed low levels of serum IgG and IgA (**Figs. 16A and B**) antibodies to VP6, but the titers were significantly lower than those of vaccinated mice (p<0.001).

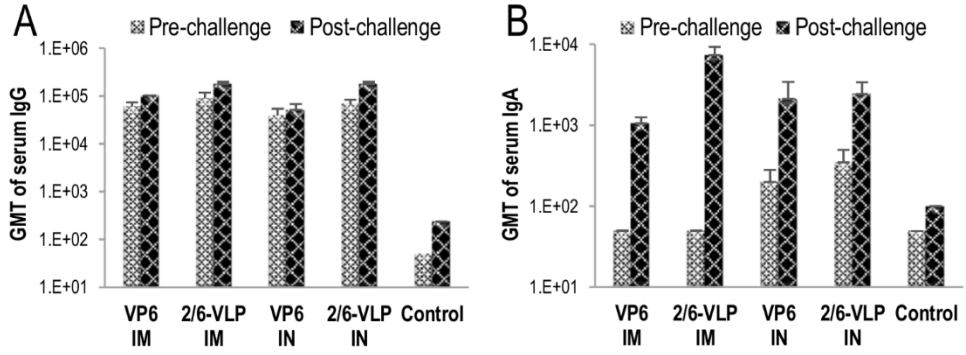

Figure 16. Pre- and post-challenge anti-VP6 IgG (**A**) and IgA (**B**) antibody titers in sera. Geometric mean titers (GMTs) of experimental groups from study weeks 5 (pre-challenge tail-blood sample) and 7 (post-challenge termination sera) are shown. Results from IM and IN control mice were combined (8 mice/group). For end-point titers of <100, a titer of 50 – a half of the starting dilution – was used to calculate the GMT.

6 Discussion

6.1 Oligomeric rVP6 tubular structures and dl2/6-VLPs are strong immunogens

Viral protein assemblies are powerful immunogens compared to soluble proteins. Therefore, the polymorphic characteristic of the RV inner capsid protein VP6 makes it a potential RV vaccine candidate. This protein has ability to form different protein assemblies *in vitro* – including trimers, tubules, spheres, sheets, and dl2/6-VLPs – depending on pH, ionic strength, concentration of divalent cations, and the presence of VP2 (30, 31, 319). An important advantage of particles or protein aggregates is their superior immunogenicity compared to soluble monomeric proteins, which usually require an adjuvant for stimulation of strong immune responses (368). Recombinant protein vaccines lacking the viral genome, thus making virus replication impossible, could represent a safe approach for RV vaccines. Currently, VLP-based vaccines against hepatitis B virus (Engerix-B® from GlaxoSmithKline and Recombivax HB® from Merck) and HPV (Cervarix® from GlaxoSmithKline and Gardasil® from Merck) have already been approved for clinical use, and several potential candidates against other viruses (e.g. NoV, influenza virus and HIV) are in clinical trials (354, 369, 370).

In the present study, rVP6 tubular structures and dl2/6-VLPs were considered VP6-based vaccine candidates. Both VP6 tubules and dl2/6-VLPs were highly immunogenic in mice by inducing robust VP6-specific B and T cell immune responses regardless of the dose or immunization route. A strong humoral immune response in different experimental animals induced by VP6 structures has previously been demonstrated by others as well (21, 32, 308, 310, 311). As demonstrated here, high levels of serum IgG were elicited even with the lowest dose or with a single dose of rVP6. Instead, induction of a strong antibody response by soluble chimeric VP6-MBP has been achieved only with co-administration of an adjuvant (306, 321). The immunogenicity of protein assemblies is strongly linked to the multivalent organization of antigen presented on the particles (371, 372). Highly repetitive structures efficiently cross-link BCRs, thus enhancing B cell activation and antibody production (371). Moreover, size of an antigen, especially within the size range of

~40–50 nm of VLPs (373), has been reported to facilitate internalization of particles by professional APCs such as DCs, which are important in activating the responses of innate and adaptive immune systems (368). Likewise, tubular structures formed from non-structural protein NS1 of the bluetongue virus, resembling rVP6 tubules in size and morphology, have been demonstrated to be efficiently internalized by macrophages and DCs (374). These tubules, when used as heterologous antigen carriers, have been shown to present B and T cell epitopes that stimulate humoral and cellular immune responses (372, 374).

Generation of a cross-reactive immune response is of high significance because of the large number of circulating RV strains. The VP6 protein is highly conserved among all group A RVs, with 87–99% homology at the amino acid level (64). Therefore, a robust serum anti-VP6 IgG response reactive with various RV strains of different subgroups (SGI or SGII) as well as G- and P-types shows the potential of rVP6 to induce cross-reactive immune response against all group A RVs. Although considerable serum IgG responses were induced even with the lower doses after IM immunization, only higher doses of rVP6 or dl2/6-VLPs were sufficient for stimulation of mucosal IgG and IgA antibody responses. Detection of mucosal response is considered to be important against RV, as the first line of defense occurs in the intestine, and intestinal IgA has been associated with protection against RV infection and disease in children (174, 175) as well as against RV infection in mice (303, 313–315, 324, 325, 330). Moreover, VP6-specific pIgA antibodies have prevented and cleared a chronic murine RV infection in a "backpack tumor" model (27).

NoV is another leading cause of viral AGE in children, with genogroups GI and GII being responsible for most cases (342). Therefore, our goal is to develop a non-live RV-NoV subunit combination vaccine for protection against childhood gastroenteritis. Since at least one representative of the two major NoV genogroups may be needed for a broadly effective NoV vaccine (342, 349), VLPs derived from genotypes GI.3 and GII.4 were chosen to be the NoV constituents of our candidate vaccine (357). Our data clearly shows that neither NoV GII.4 nor NoV GI.3 VLPs interfere VP6-specific antibody levels. Therefore, there is no impediment to the inclusion of NoV components in the vaccine formulation.

6.2 VP6-induced immunity protects against rotavirus infection

The route of vaccination influences the immune responses generated by the host. An advantage of mucosal immunization is the ability to induce immune responses not only from different mucosal surfaces of the body but also at systemic sites (360, 361). On the other hand, parenteral vaccines are also able to provide protection against enteric pathogens, as demonstrated by the current vaccines against polio and hepatitis A (299, 300). Likewise, the success of the HPV vaccines indicates the effectiveness of parenterally administered protein VLP vaccines against mucosal pathogens (301).

We compared IM and IN delivery for the induction of protective VP6-specific immune responses. Development of RV illness in mice is age-restricted, as only infant mice up to two weeks of age will develop diarrhea after the challenge (199, 200), limiting the evaluation of long-term protection in this model. Diarrhea as an end-point has mostly been used for the study of passive immunity in neonatal mice (375). Therefore, we evaluated the protective efficacy of the candidate trivalent vaccines in an adult mouse model against murine RV EDIM infection measured as decreased RV antigen shedding (201). All the experimental groups showed a significant reduction in viral antigen shedding (with mean reduction rates of 65–92%) independently of delivery route or the assembly of rVP6 used for immunizations. Despite the incomplete protection, these results demonstrate the effectiveness of the human-derived RV rVP6 protein in conferring heterologous protective immunity in mice. The importance of VP6-specific immunity is also supported by a similar protection rate (84±5%) we have observed in mice following sequential IM and IN immunizations with rVP6 alone (data not shown). The overall protection rates are in concordance with observations by others. Recently, similar protection (~70%) against murine RV challenge was accomplished in mice following SC delivery of rVP6 tubular structures or dl2/6-VLPs without an adjuvant (308). Partial protection has also been reported after IM or IN administration of inactivated DLPs or VP6 DNA vaccines (303, 304, 316). However, protection close to 100% has been achieved against homologous or heterologous RV challenge with MBP-VP6 (306, 307, 334), dl2/6-VLPs (28, 324, 325), and DLPs (304) after inclusion of an external adjuvant. It remains to be determined whether our candidate vaccines could induce complete protection when co-administered with an adjuvant. Even so, a vaccine targeted to a pediatric population should, ideally, be effective without an adjuvant (352).

Since most of the preclinical immunogenicity studies with RV VP6 or dl2/6-VLPs have been performed in adult mice (306–308, 316, 321, 325), there is no clear demonstration of reduction in RV-induced diarrhea by the VP6 protein. However, neonatal calves receiving immune colostrum from cows vaccinated IM with rVP6 protein showed a reduction in diarrhea and virus shedding (376). Despite the high protection rates against shedding in mice, dl2/6-VLPs failed to protect gnotobiotic piglets against shedding and diarrhea following IN inoculation (311, 326). Nevertheless, dl2/6-VLPs provided protection, also in a piglet model, when administered as a booster vaccine following oral priming with attenuated RV (326, 327). The potential of VP6-based candidate vaccines in protecting against RV diarrhea is further supported by experience with VP6-specific nano-antibodies. Administration of certain single-chain antibody fragments directed against VP6 have been shown to confer 60% protection against RV-induced diarrhea in a neonatal mouse model (215) as well as full protection in gnotobiotic piglets (217). Although these results suggest protection against RV-induced diarrhea in animal models, the functionality of the VP6-induced immune responses in humans will not be determined until clinical trials are conducted.

In humans, the target of RV vaccination is reduction in severe RVGE. The severity of illness has been associated with the quantity of excreted virus and duration of shedding in infected children (128, 377, 378). As RV antigen excretion can occur in the absence of clinical symptoms, protection from RV shedding might actually be a stricter measure of protection than protection against clinical illness.

6.3 Mechanisms or modulators of effectors of VP6-induced protection

The trivalent combination vaccines, containing either rVP6 or dl2/6-VLPs, stimulated similar levels of serum IgG antibodies independently of delivery route. As expected, IN delivery resulted in a higher IgA response than IM immunization, since induction of fecal IgA is more commonly detected after IN immunization with VP6-based vaccines (304, 309, 312, 313, 324, 325). Nevertheless, non-mucosal immunization can also generate an IgA response at mucosal surfaces, as demonstrated after IM inoculation of mice with live RV (302), with B cells, acting as APCs, being responsible for intestinal IgA production (379). After parenteral immunization, these APCs migrate from peripheral draining lymph nodes to

intestinal lymphoid tissues, where they induce production of RV-specific IgA secreting cells in the presence of supportive cytokines (379). Although we detected lower levels of VP6-specific IgG and IgA in the mucosal samples after IM immunization, relatively low levels of fecal IgA have been associated with protection in mice immunized with VP6 DNA vaccine (314, 330).

Antibodies directed against VP6 are generally considered as non-neutralizing, but several studies have demonstrated inhibitory activity of anti-VP6 antibodies (44, 97, 215–217) against RV infection in a traditional neutralization assay. Our results also showed that VP6-induced antibodies inhibited RV infection *in vitro*. Although neutralization experiments with fecal suspension failed, probably due to toxicity of fecal samples to the cultured cells (380), vaginal lavages inhibited replication of not only Wa RV homologous to the VP6 used for immunizations, but also heterologous RRV, which indicates a cross-reactive neutralizing ability of VP6-specific mucosal antibodies *in vitro*. The broad neutralization activity of anti-VP6 antibodies has previously been demonstrated by others as well (29, 215–217).

Certain monoclonal pIgA antibodies against VP6 have been shown to contribute to protection by impairing RV infection *in vitro* via intracellular neutralization (29, 218). Our results also suggest that antibodies responsible for the neutralizing activity were VP6-specific pIgA antibodies. Although we did not perform intracellular neutralization experiments or determine antigen-specific binding of pIgA antibodies directly by immunoprecipitation (218, 219, 223), we showed reduction in inhibition of RV infectivity by depletion of IgA antibodies. According to the results of non-reducing immunoblot analysis, IgA antibodies present in mucosal samples were mainly in polymeric form. Based on a recent publication, human VP6-specific RV6–26 IgA antibody can neutralize RV intracellularly by inhibiting RV transcription during the transcytosis of pIgA (223). The antibodies block the release of RNA from the DLP by binding to the transcriptional pore. The necessity of pIgR-mediated transport of VP6-specific antibodies for protection has also been shown in J-chain deficient mice immunized IN with dl2/6-VLPs (28). On the other hand, anti-VP6 pIgAs have not contributed impairment of RV infection when administered directly to the gastrointestinal tract of mice (27, 218). Therefore, intracellular neutralization of RV by anti-VP6 pIgA antibodies most likely contributes to human immunity to RV (223).

The intracellular mechanism could also account for the inhibition of RV replication as detected in the *in vitro* assay, since monoclonal anti-VP6 pIgA and SIgA antibodies have been shown to interact with VP6 beneath the intact TLPs (218)

through the holes in the outer capsid or via areas of partial decapsidation (381). Consequently, the anti-VP6 pIgAs could be transported inside the cells on RVs.

It has been shown that induction of protection with non-replicating RV vaccines requires $CD4^+$ T cells (333–335), but both T-cell-dependent and -independent mechanisms are needed for long-term protection in mice (335). Activated $CD4^+$ T cells constitute potential effectors of immunity by providing traditional help for B and $CD8^+$ T cell activation through the secreted cytokines. Certain cytokines may directly or indirectly inhibit RV replication, and especially IFN-γ has been demonstrated to block RV replication in cell culture (339, 382). The generation of well-balanced Th1- and Th2-type responses in mice immunized IM with VP6 tubules or dl2/6-VLPs suggested that both VP6 structures stimulate also cell-mediated immune responses. Indeed, we observed that recipients of the combination vaccines developed significant quantities of IFN-γ secreting T cells in response to stimulation with RV antigen and a VP6-specific peptide previously identified as an immunodominant $CD4^+$ T cell epitope (336). Both structures were equally potent inducers of cellular responses independently of administration route. Blocking experiments confirmed $CD4^+$ T cells as the principal lymphocyte population responsible for IFN-γ production. VP6-specific $CD4^+$ T cells, as the ones we identified, have been shown to be required for protection against RV infection in IN immunized mice (333, 334). It is possible that memory $CD4^+$ T cells directed against VP6 are generated in the immunized mice, and upon subsequent exposure to RV, they are stimulated to produce IFN-γ that directly inhibits RV replication. This does not, however, imply that B cells would not contribute to the protective immunity as demonstrated with dl2/6-VLPs or inactivated RV particles (335), but IgA production could be associated with $CD4^+$ T cells. Furthermore, $CD4^+$ T cells of VP6-primed mice have been suggested to promote cross-reactive immunity in subsequent RV infection by providing cognate help to B cells in the production of antibodies specific for neutralizing epitopes on the external VP4 and/or VP7 proteins (312, 332). On the other hand, the protective role of $CD8^+$ T cells cannot be totally excluded as adoptive transfer of $CD8^+$ T lymphocytes from BALB/c mice immunized with rVP6 eliminated a chronic RV infection in SCID mice (383). The role of $CD4^+$ or $CD8^+$ T cells in protection after rVP6 and dl2/6-VLP immunizations, can only be ascertained with genetically-modified T cell knockout mice or *in vivo* depletion of T cell populations.

Serum IgA targeted to VP6 has been considered as the best surrogate marker for vaccine-induced protection in humans (272, 273). In the present work, parenteral and mucosal delivery of rVP6 or dl2/6-VLP induced a similar clearance of RV.

Although no correlation was shown with pre- or post-challenge responses, protection against RV infection induced by the vaccines could still be IgA-dependent. This is supported by the demonstration of substantial enhancement in serum IgA titers after RV challenge in each VP6-primed mouse, regardless of delivery route. Coffin et al. showed that undetectable levels of pre-existing serum IgA expanded rapidly after replication of the challenge virus in the intestine (302). Therefore, the considerable increase in IgA titers in our study could be due to immune system priming with the VP6 tubular structures or dl2/6-VLPs, resulting in mBc that were boosted upon viral challenge.

7 Conclusions and future perspectives

This thesis investigated a novel vaccine candidate, the RV rVP6 protein, as an alternative for current live RV vaccines. Both the rVP6 tubular structures and dl2/6-VLPs were considered as vaccine candidates and were evaluated for immunogenicity and protective immunity in an adult mouse model. Since the overall objective was to develop a non-live subunit vaccine against RV in combination with NoV in order to combat AGE in children with a single vaccine, a trivalent combination vaccine of rVP6 and two NoV VLPs was designed and studied.

Both oligomeric structures of rVP6 were equally immunogenic, inducing strong homologous and cross-reactive humoral and cell-mediated immune responses. Moreover, induction of similar antibody responses with rVP6 as a single antigen or in different combinations with NoV GI.3 and GII.4 VLPs indicated the potential of the trivalent candidate vaccine to induce strong VP6-specific immune responses with no sign of interference with NoV VLP components.

The present thesis also investigated parenteral and mucosal delivery routes for the induction of VP6-specific protective immunity. Both IM and IN delivery of the trivalent vaccine containing rVP6 protein or dl2/6-VLPs induced activation of B and T cells. However, higher levels of IgA antibodies were elicited with mucosal immunization. Detection of $CD4^+$ T cells and mucosal IgA antibodies in immunized animals was of great importance because both responses have been associated with protection against RV infection in mice. Mucosal IgA antibodies induced by the rVP6 vaccines inhibited replication of homologous and heterologous RV strains *in vitro*. Most importantly, trivalent candidate vaccines induced ≥65% protection in mice against live heterologous RV challenge, regardless of administration route or rVP6 structure. These results indicate that the conserved rVP6 protein induces a heterotypic protection against RV infection.

Overall, rVP6 tubular structures and dl2/6-VLPs are non-infectious, highly immunogenic, and easy to produce, and thus potentially safer and cheaper vaccine candidates compared to live attenuated vaccines. Due to its highly conserved nature, VP6 protein could provide protection against a broad range of RV genotypes. While oral administration of live RV vaccine mimics the natural RV infection, IM or IN administration of non-live subunit vaccines could offer benefits over orally

administered live vaccines, which have been associated with an increased risk of intussusception, the formation of reassortments between vaccine and wild-type strains, prolonged shedding in immunodeficient children and lower efficacy in developing countries. Further, these recombinant protein vaccines could represent a safe approach for RV vaccines with no risk of animal-derived contaminants.

In the light of the present results, the candidate vaccines containing rVP6 protein either in the tubular form or in the form of dl2/6-VLPs can be considered as a potent non-live vaccine candidate against RVGE in humans. Whether these vaccines will be effective in humans can only be ascertained in clinical trials. If shown to be efficacious, the candidate vaccines could be used as a stand-alone vaccine in the routine childhood immunization programs. Alternatively, these vaccine candidates could be combined with routine infant immunizations as a booster vaccine in children previously vaccinated with live RV vaccines, since protective immunity induced by live RV vaccines wane over time, and live vaccines may not be given at later ages because of the risk of intussusception.

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Suvi Lappalainen

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10 Original publications

RESEARCH PAPER

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Comparative immunogenicity in mice of rotavirus VP6 tubular structures and virus-like particles

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Keywords: rotavirus, VP6, dl2/6-VLP, immunogenicity, vaccine

Abbreviations: RV, rotavirus; N-Abs, neutralizing antibodies; BV, baculovirus; dl, double-layered; VLP, virus-like particle; tl, triple-layered; Th, T-helper; IFN-γ, interferon-γ; dpi, days post infection; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; EM, electron microscopy; ELISA, enzyme-linked immunosorbent assay; IM, intramuscular; OD, optical density; VW, vaginal wash; ELISPOT, enzyme-linked immunosorbent spot assay; DC, dendritic cell; MOI, multiplicity of infection; HRP, horseradish peroxidase; SFC, spot forming cells

Rotavirus (RV) is the most important cause of severe gastroenteritis in children worldwide. Current live RV vaccines are efficacious but show lower efficacy in developing countries, as well as a low risk of intussusception. This has led to the development of parenteral non-live candidate vaccines against RV. RV capsid VP6 protein is highly conserved and the most abundant RV protein forming highly immunogenic oligomeric structures with multivalent antigen expression. Both recombinant VP6 (rVP6) or double-layered (dl) 2/6-virus-like particles (VLPs), might be considered as the simplest RV subunit vaccine candidates. Human rVP6 protein and dl2/6-VLPs were produced in Sf9 insect cells by baculovirus expression system. Formation of rVP6 tubules and VLPs were confirmed by electron microscopy. BALB/c mice were immunized intramuscularly, and immune responses were analyzed. Both rVP6 and dl2/6-VLPs induced a balanced Th1-type and Th2-type response and high levels of serum IgG antibodies with cross-reactivity against different RV strains (Wa, SC2, BrB, 69M, L26, WC3, and RRV). In addition, mucosal VP6-specific IgG and IgA antibodies were detected in feces and vaginal washes (VW) of immunized animals. Importantly, VWs of immunized mice inhibited RV Wa and RRV infection in vitro. Immunization with either protein preparation induced a similar level of VP6-specific, interferon-γ secreting $CD4^+$ T cells in response to different RVs or the 18-mer peptide ($AA_{242–259}$), a VP6-specific $CD4^+$ T cell epitope. RV rVP6 and dl2/6-VLPs induced equally strong humoral and cellular responses against RV in mice and therefore, may be considered as non-live vaccine candidates against RV.

Introduction

Rotavirus (RV) is the major cause of severe gastroenteritis in children worldwide with high rates of morbidity and mortality.[1] RVs are classified into seven distinct serogroups (A-G) with human associated infections predominantly caused by group A.[2] The capsid of RV consists of three protein layers: the core formed by 60 dimers of VP2, the intermediate layer consisting of 260 trimers of VP6 covered by the external layer of VP7 glycoprotein with 60 hemagglutinin spikes of VP4.[3,4] The outer capsid proteins VP7 and VP4 define 19 G (glycoprotein) and 27 P (protease sensitive) RV types[5] and induce protective immunity based on type-specific neutralizing antibodies (N-Abs).[6] The major capsid protein VP6 contains viral group- and subgroup-specific antigenic determinants and is highly conserved,[7] immunogenic,[2,8-10] and the most abundant RV protein.[6] When produced by recombinant baculovirus (rBV) expression system VP6 can self-assemble into different oligomeric structures, including tubules, spheres and sheets.[11] Moreover, co-expression of VP2 and VP6 results in the formation of double-layered (dl) virus-like particles (VLP)[3] while co-expression of VP2, VP6, and VP7 (with or without VP4) leads to triple-layered (tl) VLPs resembling native infectious RV particles.[12]

Two recently licensed live oral RV vaccines (pentavalent RotaTeq® and monovalent Rotarix®) are efficacious and largely safe, but they may be associated with a low risk of intussusception[13] and other issues related to live vaccines including the risk of introduction of vaccine strains into the environment, genetic reassortment between the vaccine and a wild-type strain, and reversion of the vaccine strain toward virulence.[1] Non-live vaccines against RV have therefore been considered, including inactivated RV particles,[14,15] RV VLPs of different composition,[16-18] recombinant VP6 (rVP6) protein,[8,19,20] and DNA plasmids expressing VP6.[21,22] The evidence that immune responses to the neutralizing proteins VP4 and VP7 are not necessarily required for protection against RV infection in animals is considerable.[16-18] Inactivated dl RV particles lacking VP4 and VP7 induced protection in mouse model against virus shedding.[14,15]

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When rVP6 was expressed in *Escherichia coli* as a fusion chimeric protein and administered to mice, intestinal RV antigen production was suppressed by > 93% after murine RV challenge.[19,20] Furthermore, immunization with DNA encoding VP6 induced protection in mice.[21,22] These studies suggest that VP6 alone plays an important role in RV protective immunity. Although VP6 does not induce serum N-Abs it induces heterologous cross-protective RV immunity in mice.[14,15,18,23] The mechanisms of protection are not clearly defined but it has been suggested that protection is dependent on VP6-specific $CD4^+$ T-helper (Th) cells,[18,23] which have been shown to mediate protection either by direct cytotoxic mechanism or by antiviral cytokine interferon-γ (IFN-γ) production.[24-26] In addition, mucosal VP6-specific IgG and, even more, IgA antibodies were shown to correlate with protection in a mouse RV challenge model.[22,27-29]

Therefore, the ability of VP6 to form highly immunogenic oligomeric structures (i.e., tubules and VLPs) with repetitive multivalent antigen expression[11] in vitro and to elicit protective immune response makes it the simplest non-live, subunit RV vaccine candidate relatively easy to produce. We hypothesized that both rVP6 and dl2/6-VLPs are able to induce similar VP6-specific immune responses although having different assembly conformation. However, the immunogenicity of these RV VP6 derived oligomeric subviral structures has not been compared simultaneously in animal models. Our results show that both structures are equally immunogenic in mice, supporting the use of either one as a non-live vaccine candidate against RV gastroenteritis.

Results

Expression and characterization of rVP6 and dl2/6-VLPs. Production conditions for the recombinant human rVP6 protein and dl2/6-VLPs were optimized. The best rVP6 yield (~5 mg/l) was achieved at 7 days post infection (dpi) with rBV-VP6 of 5 pfu/cell. Production of dl2/6-VLPs with 10 pfu/cell of the rBV-VP2 and 1 pfu/cell of rBV-VP6 yielded ~3–4 mg/l of the total protein concentration with the 42% proportion of VP6.

Figure 1A shows sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel with the identified RV VP6 (45 kDa) and VP2 (102 kDa) bands of purified products. In concordance with the work of others[30] we also detected an additional cleavage product of VP2 with an apparent molecular weight of ~90 kDa. Oligomeric structures including rVP6 tubules (~0.2–1.5 μm in length) with hexagonal subunit pattern and dl2/6-VLPs (~65 nm in diameter) were confirmed under electron microscopy (EM) (**Fig. 1B**). At pH 7.2 most of the rVP6 trimers formed tubular structures with occasional spheres and sheets, as shown by others as well.[11] No VP6 tubules were detected in the dl2/6-VLPs preparations.

The antigenicity of the rVP6 and dl2/6-VLPs was compared with an enzyme-linked immunosorbent assay (ELISA) assay. When equal amount of VP6 protein in both preparations was coated in the wells rVP6 and dl2/6-VLPs reacted equally with human RV antiserum (**Fig. 1C**). In addition, recombinant VP6 and dl2/6-VLP contained endotoxin at a very low level (0.57 EU and 0.21 EU per 20 μg of protein respectively).

Serum IgG and IgA antibody responses. Mice were immunized intramuscularly (IM) twice with 3 μg or 10 μg of rVP6 or dl2/6-VLPs (the doses of dl2/6-VLPs calculated according to the VP6 content, respectively). The single immunization with both doses of the two RV immunogens induced detectable VP6-specific IgG levels in the sera collected at week 3 (**Fig. 2A**). After the first immunization IgG antibodies were boosted with the second dose (**Fig. 2A**) resulting in similar levels of total IgG in each experimental group at the time of termination ($p \geq 0.05$). Although low IgG responses (optical density, $OD \leq 0.18$) of pre-immune sera at the study week 0 were seen (**Fig. 2A**), the responses were below the cut-off value (OD 0.15) in the termination sera of control mice at the study week 5 (**Fig. 2B**).

High titers of VP6 IgG antibodies were elicited with both doses of rVP6 or dl2/6-VLPs ($p \geq 0.5$) with end point titer > 5log10 in each experimental group (**Fig. 2B**). IgG1 and IgG2a serum antibodies specific for VP6 (**Fig. 2C**) were measured as indicators of Th2- and Th1-type immune responses.[31] Doses of 10 μg rVP6 or dl2/6-VLPs induced 5- and 4-fold increases in IgG1 titers compared with 3 μg doses. Similarly, compared with 3 μg doses, immunization with 10 μg of rVP6 or dl2/6-VLPs caused 3- and 6-fold greater IgG2a titers. However, both doses of rVP6 and dl2/6-VLPs induced a balanced Th1-type and Th2-type response with comparable IgG2a and IgG1 titers ($p \geq 0.2$).

IgA antibody levels were measured in groupwise pooled sera of the immunized and control mice. We did not detect any VP6-specific IgA ($OD \leq 0.07$) in the termination sera of any group even at a very low dilution (1:2, respectively).

Cross reactive serum IgG antibody responses. We further evaluated whether rVP6 or dl2/6-VLPs could induce heterotypic IgG antibodies to different RV strains using different RV cell cultures as antigens in ELISA (**Fig. 3**). Serum antibodies of mice immunized with 10 μg of antigenic formulations were cross-reactive with different RV strains including bovine WC3 and rhesus RV (RRV) as well as several human strains indicating broad reactivity as well as conserved nature of the VP6 protein. The 3 μg doses also elicited high levels of cross-reactive IgG antibodies toward each of the RV strains tested (data not shown). Significant differences were observed between all experimental groups and the control group ($p < 0.05$).

Mucosal antibodies. We also determined mucosal VP6-specific IgG antibodies in the groupwise pooled 10% fecal suspensions and vaginal washes (VW) of the experimental groups. Low levels of fecal (final dilution 1:50) IgG antibodies prior to termination at week 5 were detected in the groups immunized with 3 μg doses of rVP6 (OD 0.18) and dl2/6-VLP (OD 0.24). However, considerable levels of IgG antibodies in feces were induced in the groups immunized with 10 μg doses of rVP6 (OD 0.61) and dl2/6-VLP (OD 1.08) at a 1:50 dilution. Similar quantities of VP6-specific IgG antibodies (OD 0.61 for rVP6; OD 1.13 for dl2/6-VLP) were detected in 1:5 diluted VWs of groups immunized with 10 μg doses. No anti-VP6 IgG was detected in feces or VWs of control mice ($OD \leq 0.08$).

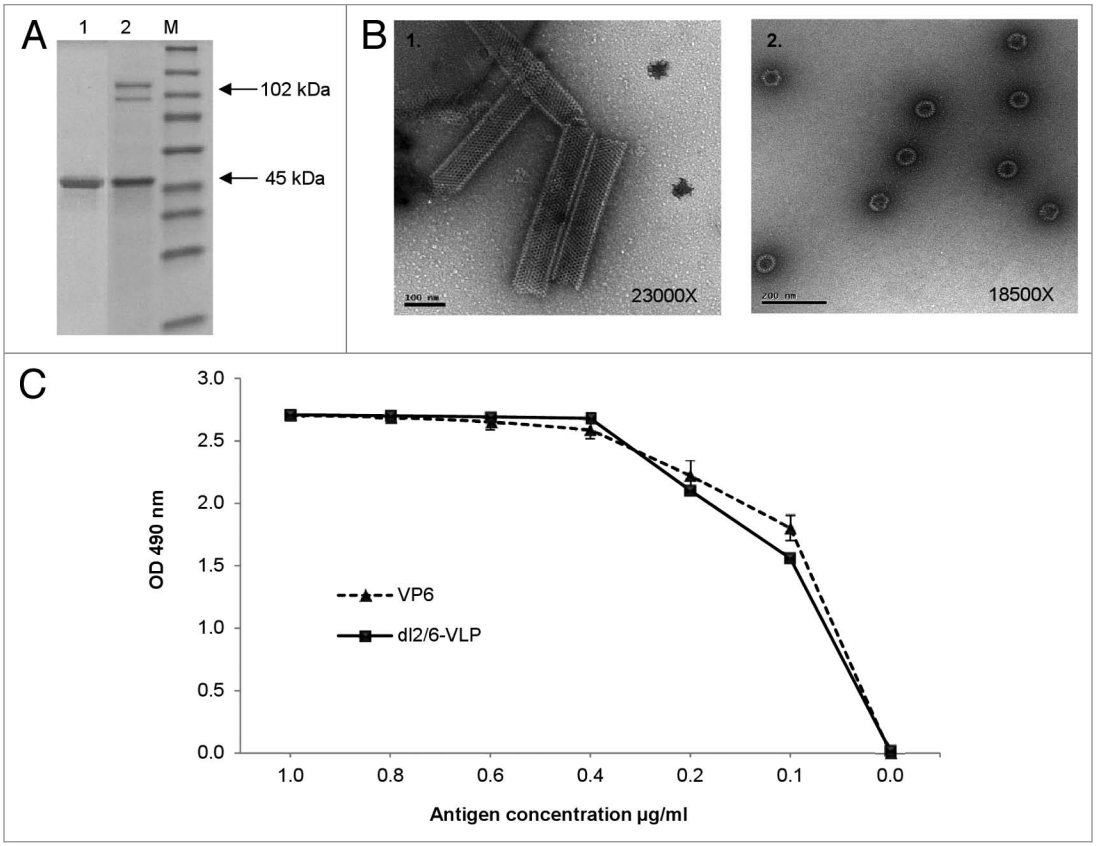

Figure 1. Characterization of the purified rVP6 and dl2/6-VLPs. (**A**) Purity and integrity analysis of RV rVP6 (lane 1) and dl2/6-VLPs (lane 2) with SDS-PAGE followed by PageBlue staining. Lane M illustrates molecular weight marker. Corresponding molecular weights are indicated with arrows on the right of the gel image. (**B**) Electron microscopy images of morphological structures assembled by RV rVP6 (panel 1) and dl2/6-VLPs (panel 2) corresponding to the SDS-PAGE lanes 1 and 2 (**A**) respectively. Protein structures were examined after negative staining with 3% uranyl acetate pH 4.6. (**C**) Evaluation of antigenicity of the purified rVP6 and dl2/6-VLPs at different concentrations with ELISA for total IgG antibodies using human polyclonal anti-rotavirus serum. Mean OD values with standard errors of duplicate wells are shown.

After two immunization doses low to moderate levels of mucosal VP6-specific IgA (OD 0.13–0.24) were detected in feces at a dilution of 1:20 and VWs at a dilution of 1:2 of groups immunized with 10 μg doses of both immunogens. Mucosal samples of control group remained negative for IgA (OD 0.01).

Cellular immune responses. The T cell immune responses from immune and naïve cells were analyzed by enzyme-linked immunosorbent spot (ELISPOT) IFN-γ assay. Splenocytes were stimulated ex vivo with different RV cell culture antigens or VP6-specific 18-mer R6–2 peptide. Immunization of mice with either rVP6 or dl2/6-VLPs revealed significant quantities of VP6-specific, IFN-γ secreting cells in response to stimulation with Wa, BrB or WC3 RV cell cultures (**Fig. 4A**). Comparisons of T cell responses indicated that levels were substantially higher in every immunized group compared with controls but were not significantly different between the experimental groups ($p > 0.05$ for all). All immunized mice developed significant T cell responses to the R6–2 peptide (**Fig. 4A**), an earlier identified VP6-specific T cell epitope.[24] MA104 mock cell culture or negative control peptide stimulated no IFN-γ production.

In order to determine the T cell type responsible for production of IFN-γ we employed functional antibodies specific for mouse CD4 and CD8 molecules to block the interaction of antigen and T cell receptor and thus T cell activation. An ELISPOT-IFN-γ assay was performed with splenocytes of mice immunized with 10 μg of dl2/6-VLPs stimulated with the R6–2 peptide and RV cell cultures in the presence or absence of anti-CD4, anti-CD8 or control antibodies (**Fig. 4B**). The results showed that only CD4 specific antibodies blocked IFN-γ production by the immune cells (50–70%, respectively) in response to the peptide epitope and the RVs indicating that $CD4^+$ T cells were the main effectors producing IFN-γ.

Inhibition of RV infection in vitro. Inhibition of RV Wa (homologous to VP6 used for immunization) and RRV infection by mucosal samples and sera of immunized and control mice was tested by ELISA-based antigen reduction neutralization assay. No neutralizing effect was detected with the serum (**Fig. 5A**) samples of immunized or control groups. Several attempts to test the neutralizing activity of fecal samples failed each time as the samples from all mice including control even at a high dilutions induced toxicity for all cell cultures, an observation seen by others as well.[32] Thus, VWs containing similar levels of mucosal antibodies were used for neutralization experiments. VWs of mice immunized with 10 μg doses of rVP6 or

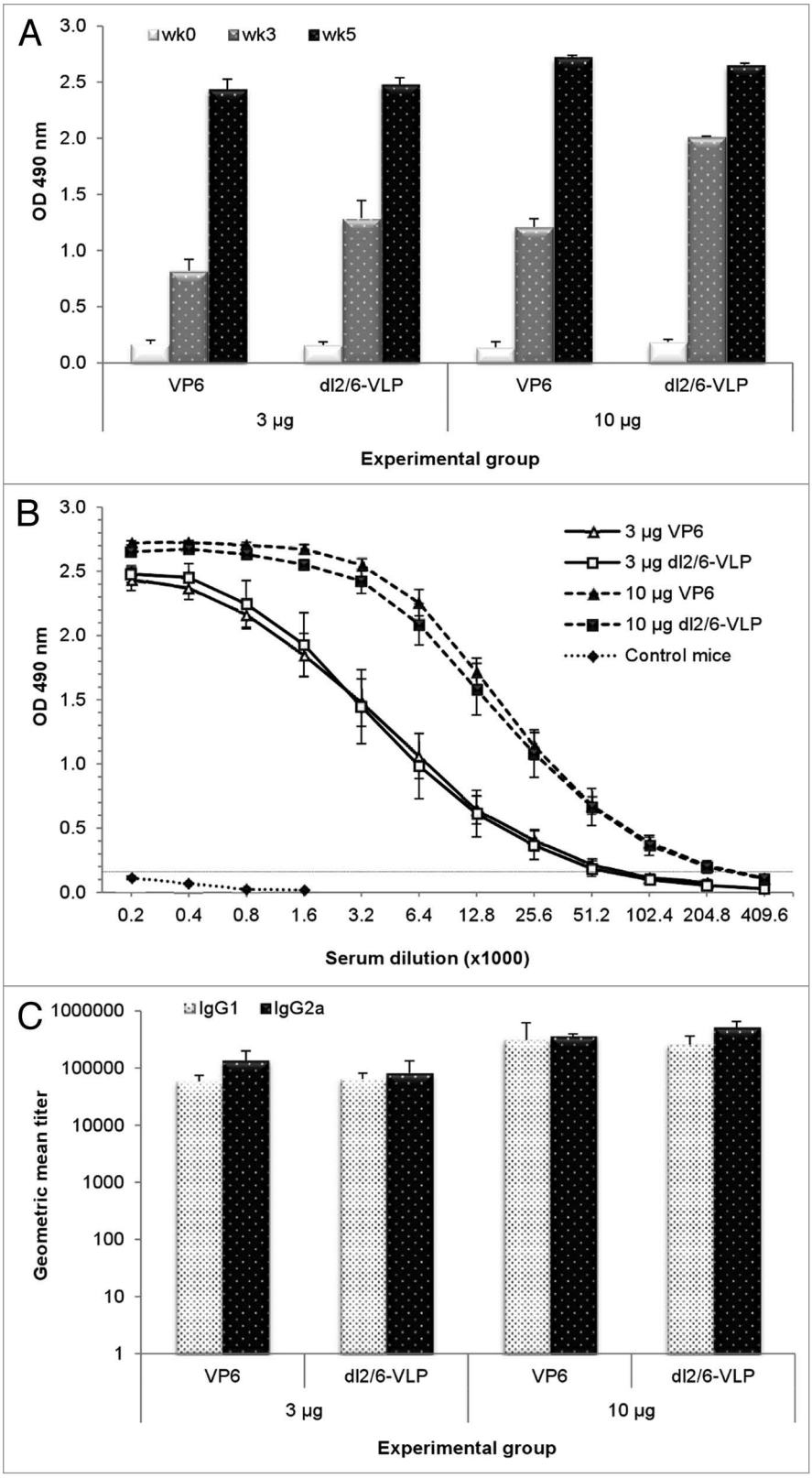

Figure 2. Humoral immune response in BALB/c mice immunized with RV VP6 proteins. (**A**) Kinetics of RV VP6-specific total IgG antibodies in sera of BALB/c mice (3–5 mice/group) immunized intramuscularly with 3 µg or 10 µg doses of rVP6 or dl2/6-VLPs at weeks 0 and 3. Group means with standard errors of tail blood samples collected at study weeks 0 (pre-immune sera) and 3 and termination sera at week 5 are shown. (**B**) End point serum titrations of RV VP6-specific IgG antibody responses of different experimental groups of mice. Control mice receiving carrier only (PBS) were used as controls. Mean titers with standard errors of termination sera at week 5 are shown. A dashed line indicates the lower cut off value (OD 0.15) calculated as follows: mean OD (termination sera of control mice) + 3 × SD (**C**) VP6-specific IgG1 and IgG2a subtype antibody responses of groups of mice immunized with rVP6 or dl2/6-VLPs. Data are expressed as the geometric mean titers (log_{10}) with standard errors of the reciprocal dilutions of specific IgG1 and IgG2a antibodies in termination sera at week 5.

dl2/6-VLPs inhibited RV infection with both strains indicating homotypic and heterotypic neutralizing activity (**Figs. 5B and 5C**). Inhibition of infectivity of RV Wa (**Fig. 5B**) and RRV (**Fig. 5C**) was detected in VWs of rVP6 immunized mice with the maximum neutralization titers of 160 and 320, respectively. The neutralizing titer of dl2/6-VLPs immunized group was 160 against Wa (**Fig. 5B**) and RRV (**Fig. 5C**). The low neutralization (titer of ≤ 10) was observed in VWs of control mice (**Figs. 5B and 5C**). All the samples were analyzed simultaneously except that the control #2 in **Figure 5B** has been run later, hence a weakness in the study. However in **Figure 5C** all VWs (including two separate pooled controls) were run simultaneously.

Discussion

We have recently proposed a combined RV VP6 – norovirus VLP vaccine to combat acute gastroenteritis in children.[33] For the RV VP6 component of the proposed vaccine candidate either rVP6 tubular structures or dl2/6-VLPs might be considered. To prove this hypothesis, RV proteins rVP6 and dl2/6-VLPs were produced by the rBV expression system and immunogenicity of these proteins was compared in a mouse model.

This comprehensive study of humoral and cellular immune responses induced by the two VP6 assembly formulations, showed that either one can be used as a simplest subunit vaccine candidate against RV. Although this study does not extend to determine in vivo RV protection with challenge experiments, it determines functionality of anti-VP6 antibodies by inhibition of RV infection in vitro.

Immunization of mice and rabbits with RV dl2/6-VLPs,[16-18] chimeric VP6 protein[19,20] or DNA encoding VP6[21,22] have been

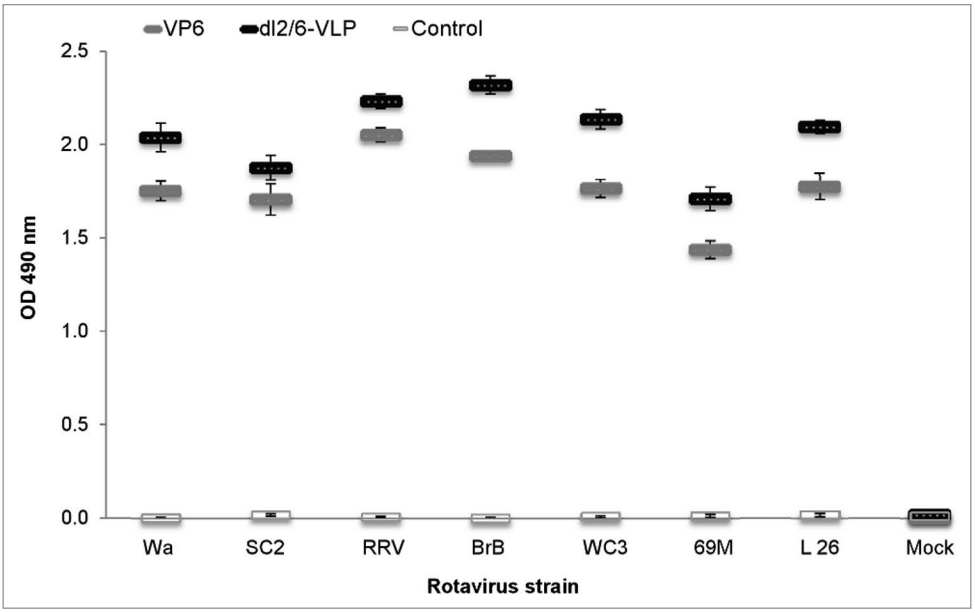

Figure 3. Cross-reactivity of rVP6 induced serum IgG antibodies to several RV strains in mice immunized twice with 10 μg of rVP6 or dl2/6-VLPs. Control mice received carrier only (PBS). Mean ODs (490 nm) of the experimental groups with standard errors are shown.

shown to efficiently protect against RV infection, suggesting that VP6 plays a notable role in RV protective immunity, and may be sufficient for protection without the surface neutralizing proteins VP4 or VP7. An indirect proof comes from the experience of live attenuated RV vaccines which are currently in use. The monovalent (G1P1A[8]) Rotarix® vaccine is as effective as the pentavalent RotaTeq® vaccine against severe RV gastroenteritis caused by different RV genotypes[34,35] indicating the importance of other types of protective mechanisms but the type-specific N-Abs to external VP4 or VP7 proteins.

We found that the repeating multivalent antigenic structures of rVP6 trimers packed into tubules or in the form of dl2/6-VLPs are both very strong immunogens in mice without the need for external adjuvants. In contrast to soluble proteins which often require an adjuvant to potentiate their function,[19,20,26] antigens displayed in a highly dense, multivalent format such as the oligomeric rVP6 structures described in this study, efficiently activate B cells leading to cross-linking of B cell receptors on the cell surface.[36] In addition, the particulate nature and the size range of around 40 nm of VLPs is optimal for the uptake of nanoparticles by dendritic cells (DC),[37] which play a key role in activating innate and adaptive immune responses.[38] Likewise, bluetongue virus nonstructural protein NS1 forms tubules similar to VP6 in morphology and size, and have been shown to be extremely immunogenic and excellent heterologous antigen carriers.[39] These tubules, internalized by macrophages and DC, are efficiently presenting T cell and B epitopes to the immune system.

In addition to the enhancing immunological features of the rVP6 protein discussed above there are other characteristics of VP6 protein which make it a promising vaccine candidate. VP6 among group A RVs is highly conserved with ~90% homology at the amino acid level.[7] Our results show that when the mice were immunized with rVP6 tubules or dl2/6-VLPs, RV-specific serum IgG antibodies and T cells were reactive to RV with VP6 type 1 (Wa), homologous to the rVP6 used for immunizations, as well as cross-reactive to heterologous RVs having VP6 type 2 (e.g., 69M, L26, WC3, and RRV) indicating broad reactivity because of the well conserved nature of VP6 among different RVs. In addition, these findings indicate the conservation of the VP6-specific B and T cell epitopes across different RV strains. When the splenocytes of mice were stimulated with a synthetic 18-mer VP6-specific peptide, the peptide previously reported to stimulate memory $CD4^+$ T cells,[24] we observed that both rVP6 and dl2/6-VLPs recipients developed significant IFN-γ producing T cells. The cells responsible for IFN-γ production in response to the RV cell culture antigens as well as to the R6–2 peptide were identified as $CD4^+$ T cells in blocking antibody experiments. VP6 specific $CD4^+$ Th cells, as the ones we detected, were shown to suffice for protection against RV infection in mice either by direct cytotoxic mechanism in mucosa or by antiviral cytokine, IFN-γ, production.[24-26] In addition, VP6-specific Th cells in mice have been shown to provide cognate help to B cells specific for neutralizing epitopes on the heterotypic VP4 or VP7 molecules of RV,[18,23] making these T cells essential for the induction of a cross-protective immune response against RV. Recently, $CD4^+$ T cells responding to conserved influenza internal proteins were shown to correlate with disease protection against influenza challenge in humans.[40] Altogether, immunization with rVP6 alone could be expected to protect against RV gastroenteritis caused by any group A RVs.

Mucosal VP6-specific IgG and even more IgA antibodies have also been implicated to account for protection against RV challenge in a mouse model[22,27] and non-neutralizing mucosal IgA against VP6 has been shown to inhibit RV replication intracellularly.[29,41] We also detected considerable levels of IgG and low levels of IgA in the intestinal lumen and VWs of mice immunized

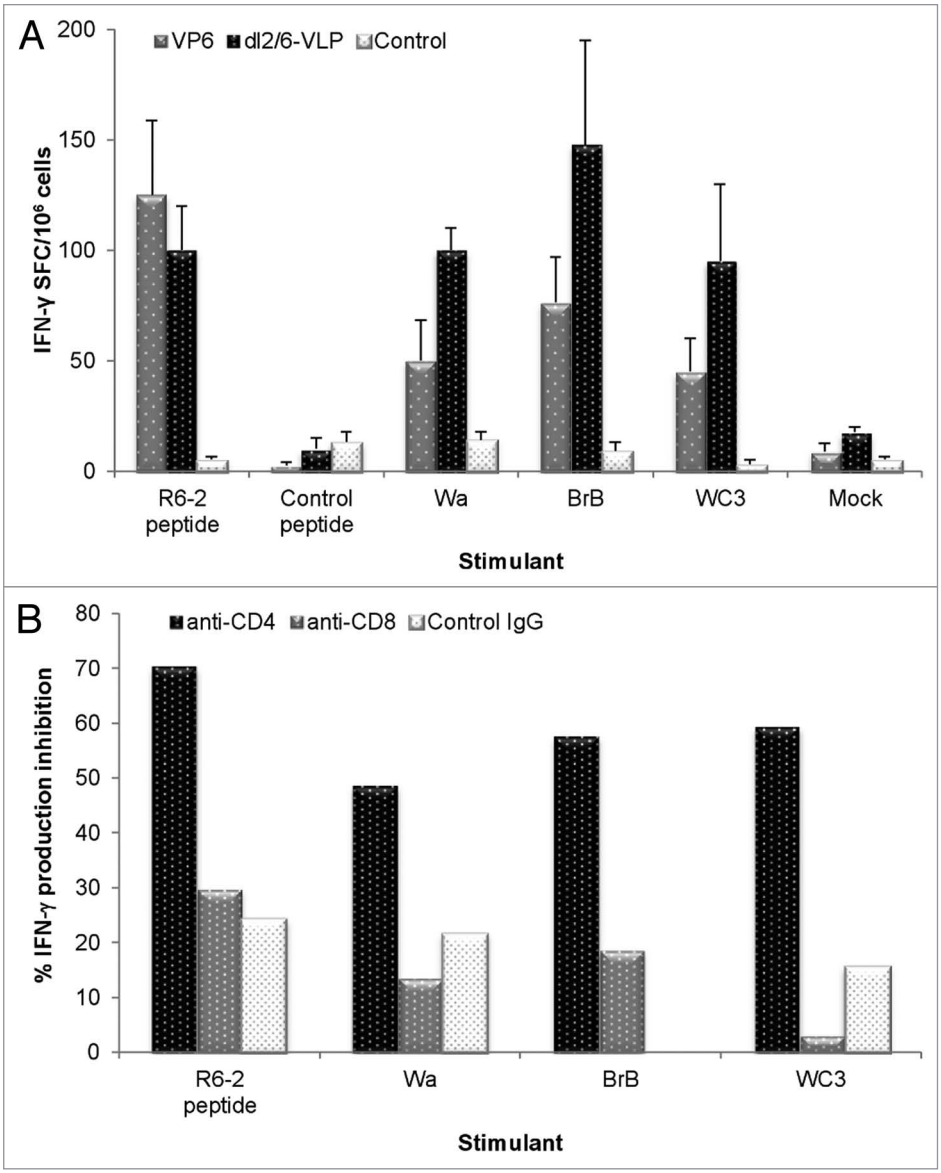

Figure 4. Rotavirus VP6-specific T-cell response detection by ELISPOT. (**A**) Different RV cell cultures and a synthetic RV R6–2 peptide were used to stimulate interferon-γ (IFN-γ) production from the splenocytes of mice immunized twice with 3 μg rVP6 or dl2/6-VLPs. Control mice received carrier only (PBS). Results are expressed as mean IFN-γ spot forming cells (SFC)/10^6 cells of duplicate wells with standard errors. (**B**) $CD4^+$ T cells are responsible for IFN-γ production of mice immunized twice with 10 μg dl2/6-VLPs. Splenocytes were stimulated with different RV cell cultures and a synthetic R6–2 peptide in the presence or absence of CD4 and CD8 specific antibodies or control antibodies to block the T cell activation. Results are expressed as the mean % inhibition of duplicate wells.

with rVP6 or dl2/6-VLPs. It may be that mucosal delivery or/and use of external adjuvants are needed to obtain higher levels of IgA antibodies. However, relatively low levels of fecal VP6-specific IgA, comparable to the levels we have detected, were induced by mucosal immunization with VP6 DNA vaccine[22] or adjuvanted dl2/6-VLPs[27] and were shown to correlate to protection against RV challenge in mice. Although it was thought that antibodies directed against VP6 have no neutralizing ability in vitro, evidence to the contrary has been mounting.[42-45] Our attempts to neutralize RV with fecal suspensions failed most likely because of toxicity of the samples to the MA104 cells.[32] Instead, in the present work we show for the first time that VWs of mice immunized with both of the VP6 preparations inhibited homologous Wa RV and heterologous RRV infection in vitro. Low neutralization background seen in VWs of control mice may be due to non-specific factors in VWs such as the estrous cycle. These results indicate the importance of VP6-specific IgA antibodies especially as the serum samples, which completely lacked IgA, also lacked RV neutralizing activity.

Although IgA antibodies of sera were not detected, the presence of serum VP6-specific IgA cannot be definitively excluded as existence of serum IgG antibodies at high titers may interfere with the detection of IgA and consequentially neutralizing activity.[46,47] Moreover, the timing of serum sample collection at the termination of mice at week 5 might be suboptimal for IgA detection. In any event, other investigators have also reported RV-specific[48] as well as VP6-specific[28] mucosal IgA antibodies without detection of serum IgA following parental[48] as well as mucosal immunization.[28]

The mechanisms by which VP6-specific antibodies exert the neutralizing effect are not yet understood but it is possible that these antibodies interfere with the binding of VP6 to the cellular receptor as has been suggested by Gualtero et al.[43] or induce conformational changes in the viral particle interfering with virus entry to the cell. Alternatively, it has been also shown that VP6-specific mucosal (secretory) IgA can impair RV infection in vitro by intracellular neutralization.[49] It is also possible that neutralizing anti-VP6 IgA we detected could be introduced to the cells on the virus and inhibit RV replication intracellularly as it was shown that VP6-specific IgA antibodies bind to intact RV particles.[49]

Recombinant VP6 and dl2/6-VLPs have been generally administered mucosally to mice in combination with different adjuvants.[17,18,20,26,27,29,50] In this study we have administered rVP6 tubuli or dl2/6-VLPs parenterally without an adjuvant. Only one study described immunogenicity of oligomeric VP6 delivered IM without the adjuvant but lacking detailed analysis of the mucosal or cellular immune responses.[8] Importantly, humoral and cellular

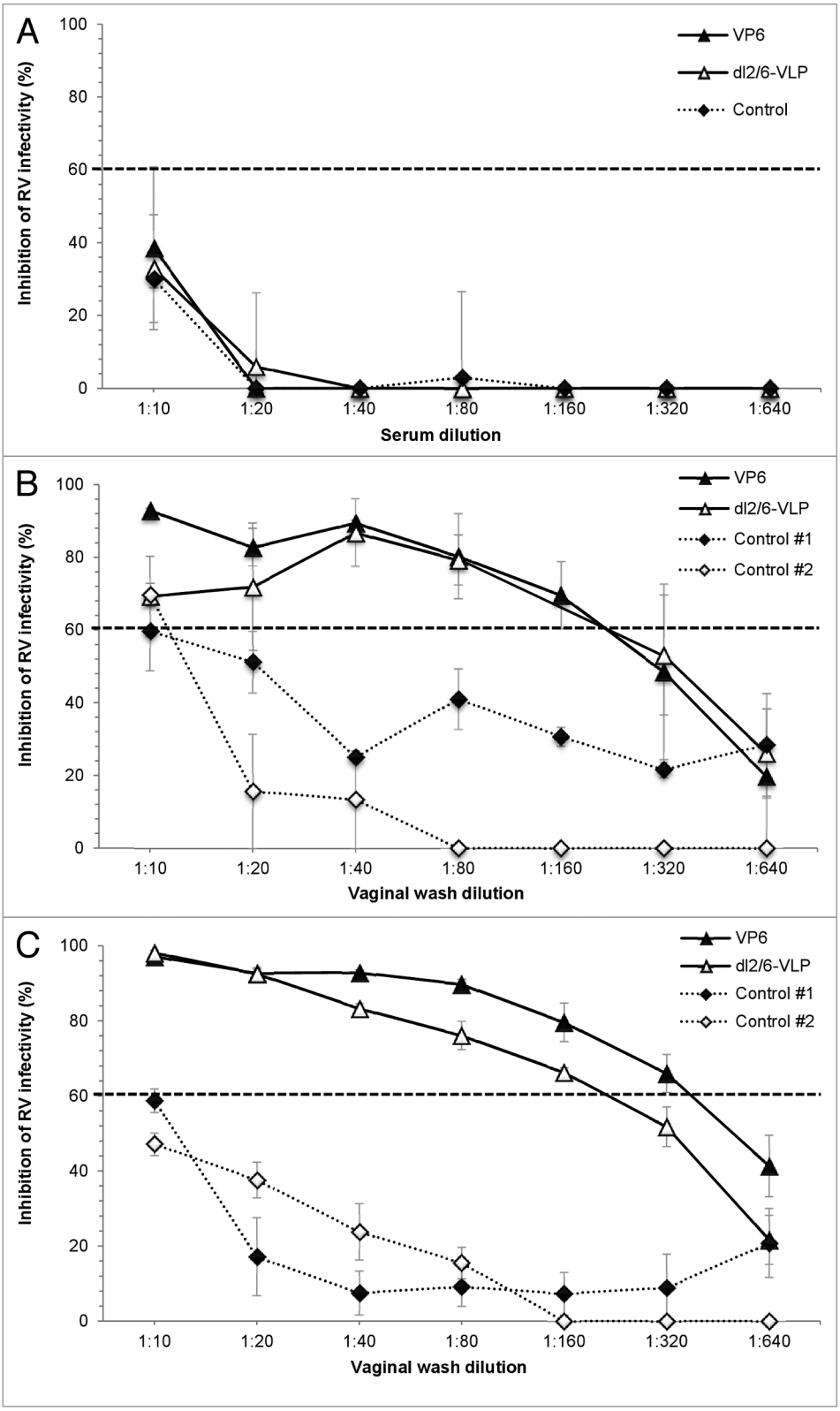

Figure 5. Inhibition of rotavirus infection in vitro. Inhibition activity of sera (**A**) and vaginal washes (**B and C**) of mice immunized with 10 μg of rVP6 or dl2/6-VLPs against homologous rotavirus Wa (**A and B**) and heterologous RRV (**C**) was analyzed by Neutralizing ELISA (NELISA). Sera and vaginal washes of mice receiving carrier only (PBS) were used as controls. Control #1 and #2 shown in panels **B and C** represent two separate pooled controls. In neutralization experiments the immunized and control samples from the same time point (week 5) were run simultaneously. Only neutralization of control #2 against Wa in panel **B** has been run later. Results are shown as the mean % inhibition of rotavirus infectivity of at least two independent experiments, each done in duplicate, with standard errors. A dashed line indicates 60% reduction in virus infectivity.

immune responses induced by the two VP6 oligomeric structures have never been compared simultaneously in an animal model. Furthermore, we showed for the first time inhibition of RV replication by VP6-specific mucosal antibodies in vitro whereas others have shown correlation of mucosal antibodies with the protection in vivo.[22,27-29]

Compared with tlVLPs containing RV external neutralizing proteins VP4 and VP7, both rVP6 and dl2/6-VLPs are simple and straightforward to produce and purify for use as a candidate vaccine. These non-replicating subunit protein vaccines are also considered safe as they are deprived of any live attenuated or inactivated viruses or their genetic material. Both VP6 derived oligomeric subviral protein structures were equally immunogenic and able to elicit strong cross-reactive B and T cell immune responses in mice, specifically IFN-γ producing $CD4^+$ T cells and mucosal IgG and IgA antibodies, which are implicated as mediators in protection against RV infection in mice.[22,24-27] We therefore propose that RV rVP6 protein either in tubular form or dl2/6-VLPs can be used to possibly induce protective RV immunity in humans. In young children, a VP6 vaccine could be used after primary immunization with live RV vaccines as protective immunity induced by live RV vaccines may wane over time, particularly in developing countries, and booster immunization may be desirable. Live RV vaccines currently in use could hardly be used for such boosting because of the risk of intussusception. Studies of VP6 containing candidate vaccines in humans are warranted and should be conducted for primary and booster immunization.

Table 1. Classification of human and animal rotaviruses according to the different genome segments

Strain	Origin	Genotype			
		VP6	VP2	VP4	VP7
Wa	human	I1	C1	P1A[8]	G1
SC2	human	Ix	Cx	P2[6]	G2
BrB	human	Ix	Cx	P2[6]	G4
69M	human	I2	C2	P4[10]	G8
L26	human	I2	C2	P1B[4]	G12
WC3	bovine	I2	C2	P7[5]	G6
RRV	rhesus	I2	C3	P5B[3]	G3

x, the genotype not determined. Classification according to Matthijnssens et al.[5]

Materials and Methods

Construction of recombinant VP6 and VP2 expression systems. To obtain the complete nucleotide sequences of VP6 and VP2 gene segments extracted dsRNA of RV from the stool of 3-y-old RV G1P1A[8] positive patient[33] was subjected to RT-PCR reactions with specific primer pairs of VP6 and VP2[51] followed by sequencing as previously described.[33] The DNAs encoding sequences of RV VP6 (Database accession number GQ477131) and VP2 (Database accession number HQ609556) were optimized for expression in *Spodoptera frugiperda* (Geneart AG, Regensburg, Germany). Nucleotide differences resulted in no amino acid changes. The VP6 and VP2 nucleotide sequences were inserted into the pFastBac1 expression vector (Invitrogen) and the pFastBacDual expression vector (Invitrogen) respectively. The generation of recombinant bacmid DNAs by a Bac-to-Bac Baculovirus expression system (Invitrogen, Cat. 10359-016), followed by the amplification of P1 and P2 rBV stocks, was performed as described earlier.[33] rBV titer of P2 stocks expressed as the multiplicity of infection (MOI) was determined by the BacPak™ Baculovirus Rapid titer kit (Clontech laboratories, Cat. 631406).

Rotavirus rVP6 and dl2/6-VLP production. To produce rVP6 and dl2/6-VLPs cell number, MOI/cell, and dpi were tested for the optimal conditions. The recombinant proteins were produced and purified essentially as previously described for rVP6.[33] rVP6 was produced in *S. frugiperda* (Sf9) insect cells (Gibco, Cat.12659-017) at a density of 1×10^6 cells/ml and MOI of 5 pfu/cell. For dl2/6-VLPs production Sf9 cells were co-infected with the rBV-VP2 P2 stock and rBV-VP6 P2 stock at a MOI of 10 and 1 pfu/cell. Cell cultures at 3–8 dpi were analyzed for recombinant protein contents by SDS-PAGE. The 7 dpi conditions were chosen as optimal for producing significant amount of the proteins for further purification steps.

Rotavirus rVP6 and dl2/6-VLP purification. Cultures were clarified at 1,000 rpm for 20 min at +4°C. Cell lysates were used for purification of rVP6 while dl2/6-VLPs were purified from the supernatant. Both proteins were concentrated by ultracentrifugation at 100,000 × g for 1.5 h at +4°C (L8–60 M ultracentrifuge, Beckman SW-32.1 Ti rotor) and purified on continuous sucrose gradients (10–60%). Fractions containing rVP6 or dl2/6VLPs were pooled, dialyzed against phosphate buffered saline (PBS) by Slide-A-Lyzer® Dialysis Cassette G2 of 20,000 MWCO (Thermo Scientific, Cat. 87738) and concentrated by ultrafiltration in Amicon Ultra-30 or -100 centrifugal filter units (Millipore Carrigtwohill, Cat. UFC903024, Cat. UFC910024). The schematic diagram of the production and purification processes resulting in the best yield of the VP6 proteins is shown in **Figure S1**. Total protein concentration of the products was quantified by Pierce® BCA Protein Assay (Thermo Fisher Scientific Inc., Cat. 23227).

Determination of purity, integrity, morphology and antigenicity of the recombinant proteins. The purity, integrity, and morphology of rVP6 and dl2/6VLPs were verified by SDS-PAGE followed by densitometric analysis with AlphaEase® FC Software (Alpha Innotech, San Leandro, CA) and EM with FEI Tecnai F12 (Philips Electron Optics, Holland) after negative staining with 3% uranyl acetate pH 4.6. The proportion of VP6 protein in dl2/6-VLPs was determined by densitometric analysis and the VP6 concentration calculated from the total protein content determined by BCA Protein Assay.

Endotoxin levels were quantified with Limulus Amebocyte Lysate assay (Lonza, Cat. N184-25) according to the manufacturer's instructions.

The antigenicity of the rVP6 and dl2/6-VLPs was evaluated by ELISA, where equal quantities of VP6 antigen (0 to 1 μg/ml PBS) in both protein preparations were coated to Costar High Binding 96-well half area polystyrene plates (Corning Inc., Cat. 3690). Human polyclonal anti RV serum originating from a patient with RV infection was added to the wells (1:200 dilution) and binding of the antibodies was detected with 1:4000 diluted horseradish peroxidase (HRP)-conjugated anti-human IgG (Invitrogen, Cat. 627120) and SIGMA FAST o-phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich, Cat. P9187-50SET). Absorbance (OD) at 490 nm was measured by Victor^2 1420 microplate reader (Perkin Elmer, Waltham, MA).

Preparation of RV cell culture antigens. In order to use RVs as antigens in ELISA and IFN-γ ELISPOT assay, MA104 cells were infected with the human RV strains Wa, SC2, BrB, 69M, L26, bovine WC3, and rhesus RV as described elsewhere.[50] **Table 1** shows the classification of the RV strains used. After observing maximum cytopathic effect (3–4 d respectively), the VP6 amount of the virus cultures was determined by Ridascreen® Rotavirus kit (R-Biopharm AG, Cat. C0901) using the internal insect cell derived rVP6 standard and expressed in ng/ml. RV cell culture antigens were diluted in PBS to contain equal quantities of VP6 protein per each culture prior to using in the ELISA or ELISPOT assays.

Immunization of mice and sample collection. Female 7–9-week-old BALB/c OlaHsd mice (3–5 mice/experimental group) obtained from Harlan Laboratories (Horst, Netherlands) were immunized IM with 3 μg or 10 μg of the VP6 or dl2/6-VLPs, at weeks 0 and 3. The doses of dl2/6-VLPs were calculated based on the amount of VP6 protein in the preparation and not the total protein concentration. Naïve mice receiving carrier only

(sterile PBS) were used as controls. No external adjuvants were used at any immunization point.

Tail blood samples (diluted 1:200 at the time of collection) and feces were collected at weeks 0 (pre-immune sample) and 3. At week 5 mice were euthanized and feces, whole blood, VWs, and lymphoid tissues were collected. Fresh feces of mice were pooled groupwise and 10% fecal suspension in 10 mM Tris (Cat. T1378) buffer containing 100 mM NaCl (Cat. 31434N), 1 mM $CaCl_2$ (Cat. C5670), 0.02% Tween (Cat. P1379), 1% aprotinin (Cat. A6279), and 10 μM leupeptine (Cat. L2884) (all from Sigma-Aldrich) were prepared as previously described.[33] Blood samples and a single-cell suspension from the spleen of each mouse were prepared according to Tamminen et al.[52] Vaginal lavages were collected by washing twice with 125 μl of cold PBS followed by a centrifugation at 12,000 × g for 10 min at +4°C. The VWs were pooled groupwise and stored at –20°C. All procedures were performed according to the regulations and guidelines of the Finnish Animal Experiment Board.

RV specific IgG and IgA detection from serum. Sera of each mouse at 1:200 dilution or 2-fold dilution series were tested for total IgG and IgG subtype antibodies by ELISA assays as previously described[33] with minor modifications. Briefly, Costar High Binding 96-well half area polystyrene plates were coated with 40 ng/well of rVP6 protein in PBS. The bound VP6-specific IgGs were detected with HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich, Cat. A4416), IgG1 (Invitrogen, Cat. A10551) or IgG2a (Invitrogen, Cat. A10685). A sample was considered positive if the OD was above the set cut-off value calculated as follows: mean OD (termination sera of control mice) + 3 × standard deviation (SD).[33] The cut-off value never exceeded OD 0.15. End-point serum titers were expressed as log10 of the reciprocal of the highest serum dilution giving a positive reading.

Sandwich ELISA[33] was used for the detection of serum VP6-specific IgA antibodies. The plates were coated with polyclonal rabbit anti-rotavirus antibody at 1 μg/ml (DakoCytomation, Cat. B0218) followed by the addition of rVP6 (50 ng/well). Antibodies in sera (at dilutions of 1:2 and 1:5) were detected with goat anti-mouse IgA-HRP (Sigma-Aldrich, Cat. A4589).

Detection of serum cross-reactive RV-specific IgG antibodies. Cross-reactive RV VP6-specific IgG serum antibodies were detected with the sandwich ELISA as described above but the plates were coated with RV cell culture antigens (homologous, Wa; and heterologous, SC2, BrB, 69M, L26, WC3, and RRV) at the VP6 antigen concentration of 100 ng/ml and serum dilution of 1:200 was utilized.

RV specific IgG and IgA detection from mucosal samples. For the detection of mucosal VP6-specific antibodies the sandwich ELISA was used as described above. Stool suspensions and VWs were used at dilutions of 1:2 and 1:5.

Cell-mediated immune response detection. VP6-specific T cell responses were measured with ELISPOT assay by quantification of IFN-γ production from splenocytes.[52] Multiscreen 96-well HTS-IP filter plates (Millipore, Cat. MSIPN4W50) were coated with anti-mouse IFN-γ monoclonal antibody AN18 (Mabtech AB, Cat. 3321-3-1000) at 2.5 μg/ml. Splenocytes (0.1×10^6 cells/well) from the immunized or control mice were stimulated in duplicates with Wa, BrB or WC3 RV cultures (500 ng VP6 antigen/ml) or 5 μg/ml of a synthetic VP6 derived 18-mer peptide (Proimmune Ltd., Oxford, United Kingdom), previously identified as an VP6-specific BALB/c mouse $CD4^+$ T cell epitope (DGATTWYFNP VILRPNNV, $AA_{242–259}$),[24] named R6–2. MA104 mock cell culture and synthetic norovirus capsid-derived peptide (CLLPQEWIQH LYQES) were used as negative controls. Cells incubated in culture media alone and cells stimulated with 10 μg/ml of concanavalin A (ConA, Sigma-Aldrich, Cat. C5275), a T cell mitogen, served as background and viability controls. After overnight incubation at 37°C cytokine secretion was detected with biotinylated anti-mouse IFN-γ monoclonal antibody R4–6A2 (Mabtech AB, Cat. 3321–6-1000) at 2 μg/ml and streptavidin-ALP (Mabtech AB, Cat. 3310–10) at 1:500. The spots developed with BCIP/NBT substrate (Mabtech, Cat. 3650–10) were counted by ImmunoSpot® automatic CTL analyzer (CTL-Europe GmbH, Bonn, Germany). The results were expressed as mean spot forming cells (SFC)/10^6 splenocytes of duplicate wells.

In order to determine cell type responsible for the IFN-γ production, splenocytes were preincubated for 1 h at 37°C with the functional blocking antibodies (rat anti mouse-CD4 (Cat. 16-0041) and rat anti-mouse CD8a (Cat. 16-0081) from eBioscience) or rat IgG isotype control antibody (eBioscience, Cat. 16-4321) at 30 μg/ml concentration prior to stimulation with the peptide or RV cell culture antigens.

Inhibition of RV infection in vitro. Neutralizing activity of VP6-specific sera, fecal samples, and VWs was determined by reduction in RV antigen production using an ELISA-based antigen reduction neutralization assay (NELISA) as described previously by others.[53,54] In each assay the samples of immunized and control mice from the same time point (week 5) were always run simultaneously. 2-fold dilutions (from 1:10 to 1:640) of each sample were equally mixed with Wa RV and RRV containing 250 focus-forming units and incubated at 37°C for 60 min. The mixture was overlaid to confluent MA104 cell monolayers in 96-well plates and the plates were spun (1000 × g) for 60 min. The virus inoculum was replaced with medium containing trypsin (Sigma-Aldrich, Cat. T4799) at 4 μg/ml followed by incubation at 37°C for 15 h. The cells were lysed by one cycle of freeze and thaw and the plates were stored at –80°C. RV VP6 antigen production in each sample performed in duplicates was measured by a Ridascreen® Rotavirus test as described above. A reduction in 450 nm OD value greater than 60% compared with the positive control wells (trypsin activated RV Wa or RRV without the test sample) was considered to indicate neutralization. Neutralizing titers were expressed as the reciprocal of the highest sample dilution yielding neutralization.

Statistical analyses. To compare the intergroup differences in the VP6 responses Mann-Whitney U-test was used. Statistical analyses were conducted using IBM SPSS Statistics (SPSS Inc., Chicago, IL) version 19.0, where $p < 0.05$ was considered to be statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
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Norovirus VLPs and rotavirus VP6 protein as combined vaccine for childhood gastroenteritis

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ABSTRACT

Noroviruses (NoVs) and rotaviruses (RVs) are the two most important viral causes of severe gastroenteritis in young children worldwide. Live oral RV vaccines are already part of routine childhood immunization in many countries, but may be associated with low risk of intussusception and other potential risks associated with live vaccines. NoV capsid-derived virus-like particles (VLPs) are in early phase clinical trials, but there is no vaccine available yet. We suggest that there is a need for non-live vaccines against both enteric pathogens. We have combined NoV GII-4 VLPs and human RV recombinant VP6 (rVP6) protein produced by recombinant baculovirus (BV) expression system in insect cells and used this combination vaccine to immunize BALB/c mice parenterally. Strong systemic cross-reactive and cross-blocking antibody responses towards NoV and RV were induced, and there was no interference of the immune response to either antigen given in combination. Rather, we observed an adjuvant effect of rVP6 on the NoV-specific homologous and heterologous immune responses to genotypes not included in a vaccine formulation.

1. Introduction

Noroviruses (NoVs) cause sporadic acute gastroenteritis (AGE) in children and food- and water-borne outbreaks of gastroenteritis worldwide. In young children, NoVs are the most important viral cause of AGE after rotaviruses (RVs) [1], and annually cause approximately 1 million hospitalizations and more than 200,000 deaths [2]. With the emergence and global distribution of GII-4 genotype there has been an increase in severe NoV gastroenteritis in children worldwide [3,4]. A unique feature of the capsid protein VP1 of NoV is its ability to self-assemble into the empty virus-like particles (VLPs) morphologically and antigenically similar to the native NoV [5]. These VLPs have been used for development of candidate vaccines against NoV [6,7]. Because of their repetitive, multivalent structures, VLPs are extremely immunogenic and effective in cross-linking B cell receptors [8]. The particulate nature of VLPs facilitates the uptake by professional antigen-presenting cells (APC), namely, dendritic cells (DC), which play a central role in activating innate and adaptive immune responses [9].

RV is the most important cause of severe gastroenteritis in young children worldwide, with high morbidity and mortality (500,000 deaths/year) [10]. The RV genome is enclosed in a capsid formed by three protein layers: a core protein VP2, an inner capsid protein VP6, and an outer capsid glycoprotein VP7 with a hemagglutinin spike protein VP4. The proteins VP7 and VP4 contain neutralizing epitopes and induce protective immunity based on neutralizing antibodies (N-Ab) [11]. The major capsid protein VP6 determines viral group specificity and is the most conserved [12], immunogenic [13–16], and abundant RV protein [11]. It forms oligomeric structures including tubules, spheres and sheets in vitro, composed of a variable number of trimers [17]. Although VP6 does not induce N-Abs, it induces heterotypic cross-protective RV immunity in mice by eliciting strong T helper (Th) cell responses, which promote cross-reactive immunity [18,19]. VP6-specific CD4+ T cells have been shown to protect mice from murine RV infection [20,21]. Correlates of protection in RV infection in humans are not clearly defined, but a generally accepted correlate of protection against RV gastroenteritis is a high titer of IgA (and IgG) antibodies targeted to the non-neutralizing VP6 protein [15,16]. Most of the immunogenicity and vaccine studies in animal models performed by today have been accomplished using a non-human bacterially produced recombinant VP6 (rVP6) protein with an adjuvant [21–23] or different RV VLPs [18,24,25]. Human clinical trials with non-live subunit RV protein vaccines have not been accomplished so far.

Administration of live oral RV vaccine (RotaShield®, Wyeth) was associated with an increased risk for intestinal intussusception in infants [26]. The currently licensed vaccines RotaTeq® (Merck) and Rotarix® (GSK) do not carry a similar risk, but a rare association

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cannot be excluded [27]. Recently, porcine circovirus DNA was discovered in both licensed live vaccines [28,29]. Both of these issues are inherent to live vaccines, and emphasize the need to develop non-live alternatives for RV vaccine.

The unique properties attributed to the VLPs, including NoV VLPs, and to the VP6 protein suggest that a vaccine consisting of these two components may represent a viable strategy to immunize against NoV and RV. We have developed such a combination vaccine and used it to immunize BALB/c mice. The results show not only that the two antigens are highly immunogenic in a mixture, but also that RV VP6 may have the potential to act as an adjuvant for a NoV VLP vaccine.

2. Materials and methods

2.1. Norovirus GII-4, GII-12 and GI-3 VLPs production and purification

RNA of NoV GII-4 was extracted from the stool of a 20-month-old girl with gastroenteritis in Lahti in 1999 by the QIAamp RNA viral mini kit (Qiagen, Hilden, Germany). The amplification and cloning of the GII-4 VP1 capsid gene are described in detail elsewhere [30]. NoV GII-4 New Orleans (GII-4 NO, 2010), GII-12 (1998), and GI-3 (2002) VP1 genes, originated from the stools of NoV-infected patients in Finland, were amplified by the RT-PCR with the following primers: GII-4 NO and GII-12 fwd (5′-GTGAATGAAGATGGCGTCGA-3′) [31], GII-4 NO rev (5′-TTATAATGCACGTCTACGCCC-3′), GII-12 rev (5′TTACTGTACTCTTCTGCGCCC-3′), GI-3 fwd (5′-GTAAATGATGATGGCGTCTAA-3′), and GI-3 rev (5′-TGGGCCATTATGATCTCCTAAT-3′). The amplicons (1.6 kb) were sequenced and strains were classified according to EMBL/Genbank and FBVE (database accession numbers, OB2009166 (GII-4 NO), AJ277618 (GII-12), and AF414403 (GI-3)). GII-12 VP1 was cloned into the pFastBacDual transfer vector, GII-4 NO and GI-3 into the pFastBac1 (Invitrogen, Carlsbad, CA). The GII-12 and GII-4 New Orleans were chosen as candidate NoV strains along with GII-4 to represent genogroup II NoVs. The first one is the least common in Finnish children while variant New Orleans is becoming the predominant GII-4 subtype [32]. The GI-3 is a representative of the genogroup I of NoVs and is the most prevalent of all genogroup I types in Finnish infants and children [33].

To generate a recombinant bacmid DNA, pFastBac constructs were transformed into DH10Bac™ competent *E. coli* cells by a Bac-to-Bac Baculovirus expression system (Invitrogen) according to the manufacturer's instructions. Recombinant BV (rBV) stocks were produced in *Spodoptera frugiperda* (Sf9) insect cells. The cells were transfected with 1 μg of bacmid DNA in serum-free medium (Sf 900 SFM III), cultured at 27 °C, and P1 rBV1 stock was collected after 3 days. P2 rBV stock was produced by infecting Sf9 cells with the P1 stock. BV titers expressed as the multiplicity of infection (MOI) of the P2 stocks were determined by the BacPak Rapid Titer kit (Clontech laboratories, USA).

For the production of the NoV VLPs, Sf9 cell cultures were set up at a density of 1×10^6 cells/ml and the cells were infected with BV P2 stock at a MOI of 1. The VLPs were purified twice with discontinuous sucrose gradients as described previously [34]. Total protein concentration was quantified using the Pierce® BCA Protein Assay (Thermo Scientific, Rockford, USA). Purity and integrity was verified by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by densitometric analysis with AlphaEase® FC Software (Alpha Innotech, San Leandro, CA) and electron microscopy (EM). Endotoxin levels were quantified with Limulus Amebocyte Lysate (LAL) assay (Lonza, Walkersville, MD) according to manufacturer's instructions.

2.2. Rotavirus rVP6 production and purification

To obtain the sequence of VP6 gene, RNA of 10% stool suspension originating from a 3-year-old RV G1(P8(RT-PCR positive boy with AGE at the Tampere University Hospital in 2007 [35] was extracted by the QIAamp RNA viral mini kit. The extracted dsRNA was subjected to RT-PCR reactions with a specific primer pair of VP6 [36], producing amplicon of 1362 bp, followed by sequencing (ABI PRISM™ 310 Genetic Analyzer, Applied Biosystems, Carlsbad, CA) of the purified (QIAquick gel extraction kit, Qiagen) amplicon. The sequence of VP6 was cloned into pFastBac1 vector. The generation of recombinant bacmid DNA, followed by the amplification of P1 and P2 rBV stocks, were performed as described above for NoV.

For the production of rVP6, Sf9 cells were infected with the rBV P2 stock at a MOI of 5 pfu/cell at a cell concentration of 1×10^6 cells/ml. Culture supernatants were collected at 6 dpi and clarified at 1000 rpm for 20 min at +4 °C. Recombinant protein was pelleted by ultracentrifugation at 100,000 × *g* for 1.5 h at +4 °C, suspended in 0.2 M Tris–HCl pH 7.3, and purified on sucrose gradients (10–60%) at 100,000 × *g* for 3 h at +4 °C. Fractions of sucrose containing VP6 protein were pooled, dialyzed against PBS and concentrated by centrifugation in Amicon Ultra-30 filter units (Millipore Corporation). The concentration, purity, and integrity of the VP6 were determined as described for NV VLPs.

2.3. Rotavirus propagation and preparation of ELISA antigen

Fetal rhesus monkey kidney (MA104) cells were infected with the human RV strains Wa (G1P1A [8]), SC2 (G2P2 [6]), BrB (G4P2 [6]), 69 M (G8P4 [10]), L26 (G12P1B [4]), bovine WC3 (G6P7 [5]), and rhesus rotavirus (RRV, G3P5B [3]). The infected cultures were grown in minimum essential medium supplemented with 0.5 μg/ml of trypsin (Gibco) [37]. Maximum cytopathic effect was observed and the viruses were collected for use in capture ELISA.

2.4. Immunizations

Female 7–9-week-old BALB/c mice (4–5 mice/group) were immunized intradermally (ID) or intramuscularly (IM) with 50 μl of different antigen formulations twice, at weeks 0 and 3. The antigenic formulations used were as follows: GII-4 capsid VLPs, rVP6 protein and a mixture of these two, referred to as a cocktail or combined antigenic formulation. The antigen doses were 50 μg, 10 μg, 1 μg, and 0.1 μg per immunization time. Tail blood samples and feces were collected at weeks 0 (pre-bleed, non-immune sera), 2, 3, and 4. The mice were euthanized at week 5 or at week 27 (long-term follow-up), when feces, whole blood, and lymphoid tissue were collected. Naïve mice receiving no immunogen were used as a control. All procedures were performed according to the guidelines by the national Animal Experiment Board. Prior to immunization the mice were anaesthetized with a formulation of Hypnorm (VetaPharma Limited, Leeds, England) and Dormicum (Roche Pharma AG, Grenzach-Wyhlen, Germany).

2.5. Fecal suspension

At certain study weeks feces of 3–5 animals/group, depending on their need to defecate, were collected and pooled. To resume 10% fecal suspension, fresh feces were suspended in 10 mM Tris buffer containing 100 mM NaCl, 1 mM $CaCl_2$, 0.05% Tween, 1% aprotinin, and 10 μM leupeptine (all from Sigma–Aldrich) and homogenized by vortexing. The suspensions were incubated on ice for 20 min, centrifuged for 15 min at 18,000 × *g* and collected for storage at −80 °C.

2.6. NoV and RV serum IgG, IgG1 and IgG2a ELISA

Sera from immunized and control mice were tested for NoV- and RV-specific immunoglobulin G (IgG), IgG1, and IgG2a antibodies by enzyme-linked immunosorbent assay (ELISA) as previously described [38] with some modifications. GII-4, GII-4 NO, GII-12 or GI-3 VLPs were coated at 0.2 μg/ml, 0.4 μg/ml, 0.4 μg/ml or 1 μg/ml (100 μl/well), respectively. VP6 protein was used at 1 μg/ml in bicarbonate/carbonate buffer (pH 9.55). Serum samples at 1:200 dilution or two-fold dilution series were utilized. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma-Aldrich), IgG1 or IgG2a (both from Invitrogen) were employed for the detection of total IgG and IgG subtype responses. Absorbance (optical density, OD) at 490 nm was measured in a microplate reader (Victor^2 1420, Perkin Elmer, Waltham, MA). The background signal from the blank wells (wells without serum) was subtracted from all OD readings at a plate. A sample was considered positive if the net absorbance value was above the set cut-off value (at least 0.100 OD), calculated as follows: mean OD (naïve mice) + 3 × standard deviation (SD).

2.7. RV-specific capture ELISA

For the detection of cross-reactive RV-specific serum IgG antibodies, 100 μl (1 μg/ml) Polyclonal Rabbit Anti-Rotavirus (DakoCytomation, Glostrup, Denmark) was used for coating (+4 °C overnight) microtiter plates. After washing, the plates were coated with 100 μl/well of 1:10 diluted RVs, cultured as described above, or 1 μg/ml rVP6 (+4 °C overnight). The plates were blocked with PBS containing 5% skimmed milk (Sigma–Aldrich), and ELISA was continued with 1:200 diluted sera as described for the serum IgG ELISA above. RV-capture ELISA was also used for detection of fecal RV-specific IgG antibodies in 1:5 diluted fecal suspensions.

2.8. Avidity assay

To determine the avidity of NoV and RV IgG antibodies, urea elution was used to remove the low-avidity antibodies. The ELISA assay was performed as previously described [38]. Avidity index was calculated as [OD with urea/OD without urea] × 100%, and index value ≥50% was considered as high avidity.

2.9. Blocking assays

The blocking of binding to synthetic biotin-conjugated H-type 3 and Lewis b (Le^b) histo-blood group antigen (HBGA) (GlycoTech Corporation, Rockville, MD) of NoV GII-4 and GI-3 VLPs was performed with the sera from immunized mice as described earlier [38], with slight modifications. Microtiter plates were coated with the VLPs in PBS at a concentration of 2 μg/ml and sera were serially diluted for the detection of homologous and heterologous blocking (cross-blocking). A saliva-based blocking assay was used to detect duration of blocking activity in the long-term followed termination sera (27 weeks) as well as to detect cross-blocking of GII-4 NO VLPs binding to HBGA receptors as described elsewhere [39]. Briefly, microtiter plates were coated with type A saliva diluted 3000 fold (2 h +37 °C, +4 °C overnight). The blocking effect of the mouse sera on the GII-4 NO VLP-saliva binding was measured by pre-incubation of 0.1 μg/ml of the VLPs with serially diluted sera for 1 h at 37 °C before the mixture was added to the saliva coated wells. Blocking indices were calculated as follows: 100% − (OD [wells with serum]/OD [wells without serum] × 100%). OD reading from the wells incubated without serum was considered as a maximum signal for binding of VLPs to HBGA.

2.10. Statistical analyses

Mann–Whitney *U*-test was used to compare the intergroup differences in the NoV- and RV-specific antibody responses, avidity indexes, and blocking ability of the sera. Statistical significance was defined as $P \leq 0.05$.

3. Results

3.1. Protein expression and morphology

Purity and integrity of the produced GII-4 VLPs, rVP6 protein and a mixture of the two were verified. Fig. 1 A shows SDS-PAGE gels with the identified NoV (rVP1 doublet of 58 and 64 kDa, [34]) and RV (rVP6, 44 kDa) proteins. High-order structures including GII-4 VLPs (~38 nm) and rVP6 tubules were confirmed under EM (Fig. 1B). Under pH 7.2 most of the rVP6 trimers were associated into tubules [17] with occasional spheres and sheets. Mixing the GII-4 VLPs and rVP6 in a cocktail at a ratio of 1:1 did not impair protein integrity or the morphology of either part in the cocktail, and EM identified rVP6 tubules filled with the GII-4 VLPs.

3.2. Dose response

Groups of mice were immunized with 10, 1 or 0.1 μg of a single antigen (Fig. 2). Tail blood collected at weeks 0, 2, 3, and 4 and termination sera collected at week 5 were tested for NoV- (Fig. 2A) and RV-specific IgG (Fig. 2B). A similar level of NoV-specific antibody response was induced by the 10 μg and 1 μg doses ($P \geq 0.05$), but a lower response was observed with the 0.1 μg dose ($P = 0.02$)

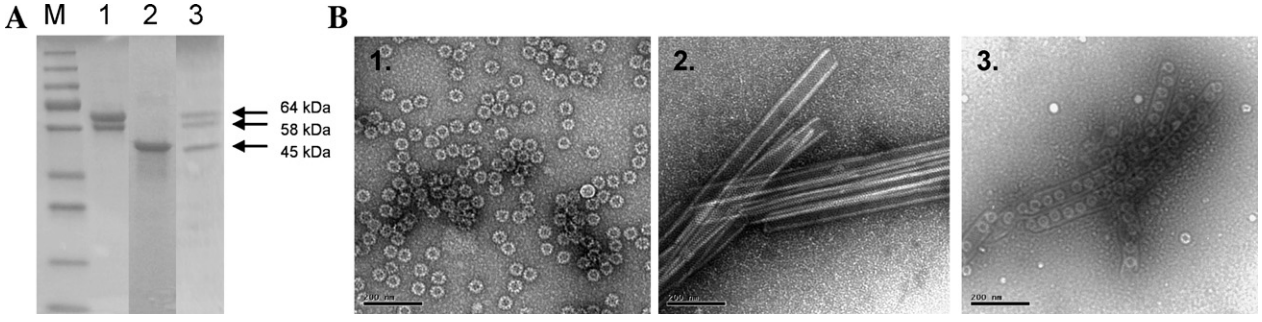

Fig. 1. (A) Purity and integrity analysis of NoV GII-4 VLPs (lane 1), RV rVP6 (lane 2), and cocktail vaccine formulation (lane 3) with SDS-PAGE followed by PageBlue staining. Lane M illustrates molecular weight marker. Corresponding molecular weights are indicated by arrowheads on the right of the gel. (B) Electron microscopy images of morphological structures assembled by NoV GII-4 VLPs (panel 1), RV rVP6 (panel 2), and cocktail (panel 3) corresponding to SDS-PAGE lanes 1–3, respectively. Structures of purified proteins were examined by FEI Tecnai F12 electron microscope (Philips Electron Optics, Holland) with 18,500× magnification following negative staining with 3% uranyl acetate (UA), pH 4.6.

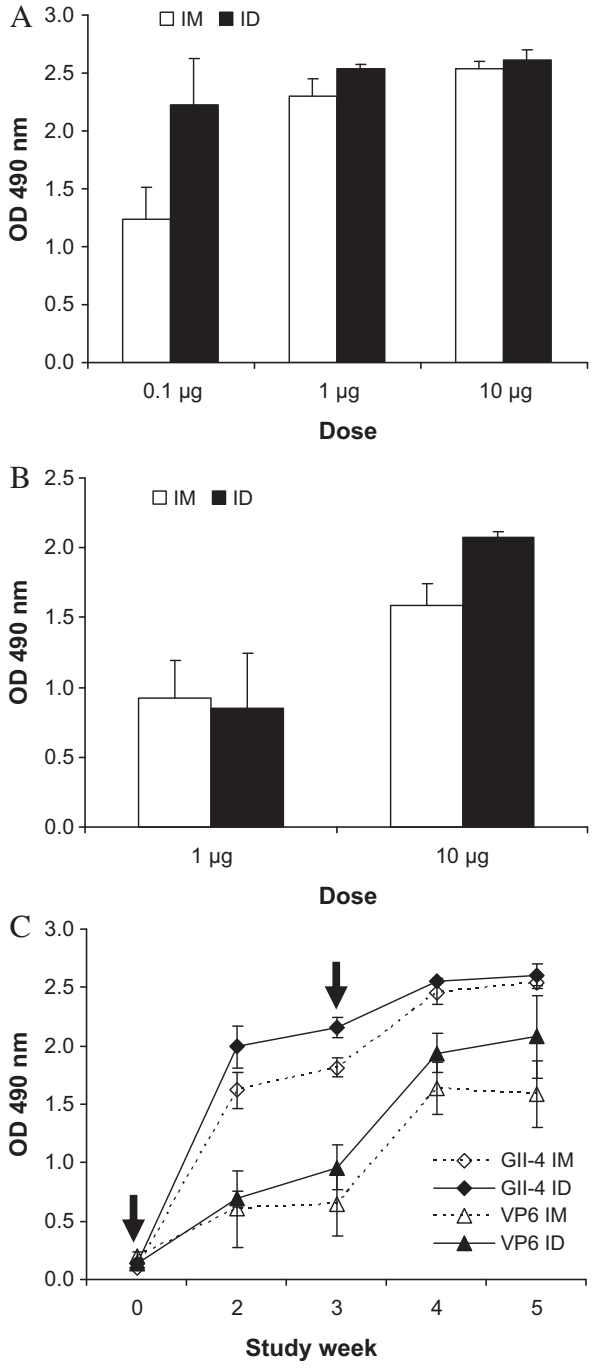

Fig. 2. (A) NoV-specific IgG dose responses of BALB/c mice (4 mice/group) immunized intramuscularly (IM) or intradermally (ID) with 0.1 μg, 1 μg or 10 μg of GII-4 VLPs. (B) RV-specific IgG responses of BALB/c mice immunized IM or ID with 1 μg or 10 μg (4–5 mice/group) of rVP6. Group means with standard errors of termination sera are shown. (C) Kinetics of NoV- and RV-specific IgG immune responses in mice immunized IM or ID twice with 10 μg of GII-4 VLPs or rVP6. Group means with standard errors of tail blood samples collected at indicated study weeks and termination sera at 5 weeks are shown. Immunizations at weeks 0 and 3 are shown with arrows.

(Fig. 2A). Significantly lower antibody responses were measured in termination sera of mice immunized with 1 μg compared to 10 μg of rVP6 ($P = 0.03$). There was no increase in the responses when using a 50 μg-dose for immunization (data not shown). Therefore, the 10 μg dose was chosen as the optimal one and used for all subsequent immunizations with NoV and RV antigen formulations. Fig. 2C shows the kinetics of the IgG antibody response measured in blood specimens collected weekly. Immunization with one dose already induced strong NoV- and RV-specific immune responses that were enhanced by the second dose. The antigenic formulations (VLPs and rVP6, respectively) were not contaminated with bacterial endotoxin (<0.1 EU/20 μg of protein).

3.3. NoV- and RV-specific serum and fecal ELISA antibody responses

Groups of mice were immunized IM or ID with 10 μg of NoV or RV single antigen formulation or 20 μg of the cocktail containing 10 μg of each single antigen, and antibody responses induced by the single versus cocktail vaccines were compared (Fig. 3). All antigen formulations induced high (reciprocal titer ≥5log 10) levels of total IgG antibodies with no difference in the magnitude of the responses induced with the single vaccine or the combination (data not shown). All formulations generated both IgG1- and IgG2a-subtype antibodies, indicating a mixed Th1-type and Th2-type immune response (Fig. 3). Immunization with both single and cocktail vaccine induced NoV- ($81.6 \pm 8.5\%$ and $82.7 \pm 7.8\%$ respectively; $P = 1.0$) and RV-specific ($61.7 \pm 2.0\%$ and $50.4 \pm 6.1\%$ respectively; $P = 0.121$) IgG antibodies of high avidity (Fig. 4A). In addition, significant levels of NoV- and RV-specific fecal IgG antibodies presumably transferred from the serum to the gut lumen were detected in immunized mice, but not in control mice (Fig. 4B).

3.4. Cross-reactive antibody responses

Cross-reactive immune responses to NoVs were detected using GII-4 NO, GII-12, and GI-3 VLPs as heterologous antigens for the GII-4 VLPs used in immunizations. Mice immunized with GII-4 VLPs either as single vaccine or the GII-4 VLP + RV VP6 combined vaccine generated antibodies that were reactive with the heterologous antigens, both within the same genogroup (GII-4 NO and GII-12) and with a GI antigen (GI-3) (Fig. 5A). The cross-reactive antibody response to GI-3 was 50% higher in mice immunized with the combination vaccine (OD 1.121) compared to the single GII-4 VLPs vaccine (OD 0.558), suggesting an adjuvant effect of the RV VP6 protein on NoV antibody response. However, because of the small group size no significant difference ($P = 0.12$) was noticed. The cross-reactive anti-RV responses are shown in Fig. 5B. The serum antibodies of mice immunized with the single rVP6 or combined antigenic formulations were cross-reactive with a broad spectrum of human (G1P [8], G2P [6], G4P [6], G8P [10], G12P [4]), bovine and simian RV strains. No significant difference was observed between rVP6 and the cocktail vaccine ($P \geq 0.05$ for all).

3.5. Blocking activity of sera

Sera from immunized and control mice were serially diluted and tested for blocking of the GII-4 and GI-3 VLPs binding to HBGA H-type 3 (Fig. 6A and data not shown). As expected, no binding of GII-4 VLPs to negative control Le^b HBGA was detected [34,40]. High blocking ability (blocking index 83%) at serum dilutions 1:3200 (ID route) and 1:1600 (IM route) was detected for the sera of mice immunized with the GII-4 VLPs as a single vaccine (Fig. 6A). A two-fold greater titer (1:3200 vs. 1:6400) was needed to maximally block the binding of GII-4 VLPs to H-type 3 antigen of mice immunized with the single compared to the combined antigenic formulation, showing that the rVP6 in the combination formulation did not suppress the blocking activity of the GII-4-specific sera. Similarly, blocking of the GI-3 VLPs binding to H-type 3 was best with the sera of mice immunized with the cocktail vaccine (data not shown). Sera of mice immunized with GII-4 VLPs cross-blocked over 50% of the GI-3 VLPs binding to H-type 3 at a 1:400 dilution, while control mouse sera did not block any VLPs binding

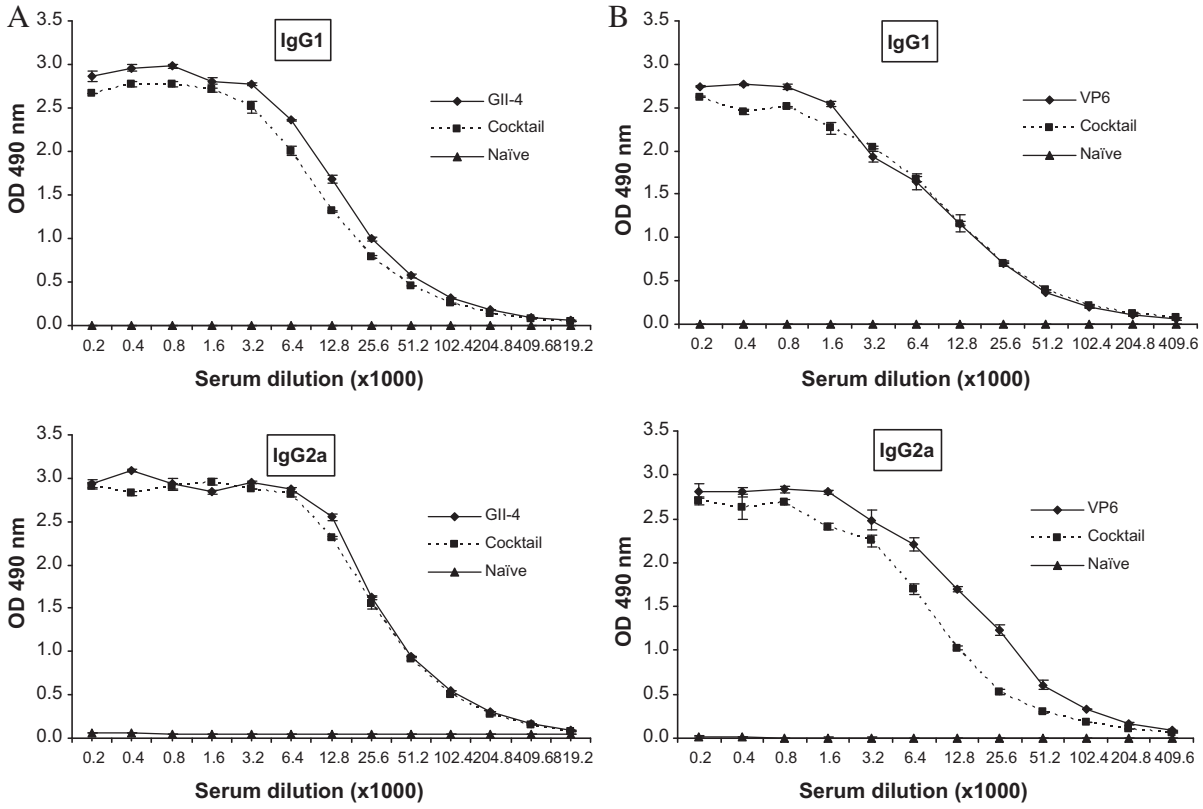

Fig. 3. End point serum titrations of (A) NoV-specific and (B) RV-specific IgG1 and IgG2a subtype antibody responses of groups of mice immunized intradermally twice either with 10 μg of GII-4 VLPs alone (4 mice/group), 10 μg of rVP6 alone (4 mice/group) or 20 μg of combination of both in cocktail (5 mice/group). Naïve mice receiving no immunogen were used as controls. Mean titers with standard errors of pooled sera from a group are shown. Upper panels show IgG1 responses and lower panels show IgG2a responses.

(maximum blocking index 1.2% at a 1:200 dilution). Long-term effect of the vaccination on blocking activity of the sera was tested using termination sera at 27 weeks following the initial immunization (Fig. 6B). As shown in Fig. 6B the blocking activity remained for 27 weeks. Maximum blocking of GII-4 binding to HBGA in saliva decreased 4-fold compared to 5 weeks termination sera (1:200 vs. 1:800) but still remained at a considerable level. Blocking of heterologous GII-4 NO VLPs binding to HBGA receptors in saliva is shown in Fig. 6C. The mouse sera after ID immunization with the single or the cocktail vaccine cross-blocked binding of GII-4 NO VLPs (maximum blocking index 80%) while sera of control mouse did not show such blockade (Fig. 6C). Approximately two-fold higher serum titer (1:20 vs. 1:40) of mice immunized with the single compared to the cocktail formulation was needed to maximally cross-block the binding of GII-4 NO VLPs. In addition, a significant difference ($P = 0.03$) in blocking activity of the cocktail immunized sera compared to single vaccine formulation was reached at a 1:40 dilution. Overall, the above blocking results suggest an additional adjuvant effect of the rVP6, similar to the one detected in the cross-reactivity experiments described above.

4. Discussion

NoV VLPs and RV VP6 protein are excellent antigen candidates for a combined NoV and RV vaccine for several reasons. Because of their repetitive, multivalent structures, VLPs are extremely immunogenic. The presentation of an antigen in a highly organized array on the surface of VLPs cross-links B cell receptors and provokes strong antibody responses [8]. The particulate nature of VLPs, especially in the size range of around 40 nm, is optimal for uptake by DC [41], which play a central role in activating innate and adaptive immune responses and are involved in long-lived memory IgG production. The RV capsid protein VP6 determines viral group specificity and is the most conserved [12] and immunogenic RV protein [13–16]. VP6 does not induce N-Abs, but has been shown to induce heterotypic cross-protective RV immunity in mice by eliciting strong Th cell responses [18–21]. The work mentioned above describes protection in a mouse model where protection is against virus shedding and not against diarrheal disease. Although these results are encouraging the protection against RV induced by cross-reactive, non-neutralizing VP6 antigen in humans remains to be determined.

The results in this study show that combined antigen formulations, consisting of a mixture of GII-4 VLPs and rVP6, induce strong systemic cross-reactive NoV- and RV-antibody responses in mice. There is no mutual inhibition of the immunogenicity of either antigen in a combination. Most importantly, the results indicate a viable strategy to combat two different enteric pathogens with a single vaccine shot. Recent work by Tan and collaborators [39] describes the use of NoV p-particles and RV VP8 protein as a combined vaccine but, to the best of our knowledge, this is the first report describing the use of NoV VLPs and RV rVP6 as a mixture to immunize against these viruses. The NoV VLPs and RV VP6 combination vaccine was produced by in vitro mixing of the two antigens. However, a similar combination vaccine can also be produced by co-infection of Sf9 cells with recombinant BVs expressing NoV capsid and RV VP6 simultaneously, resulting in a mixture of NoV VLPs and RV rVP6 with a similar EM appearance and immunogenicity to the in vitro mixed combination (unpublished observation).

The strong systemic immune response in mice induced by parenteral immunization with NoV VLPs described here contrasts with the type-specific response generated after NoV infection

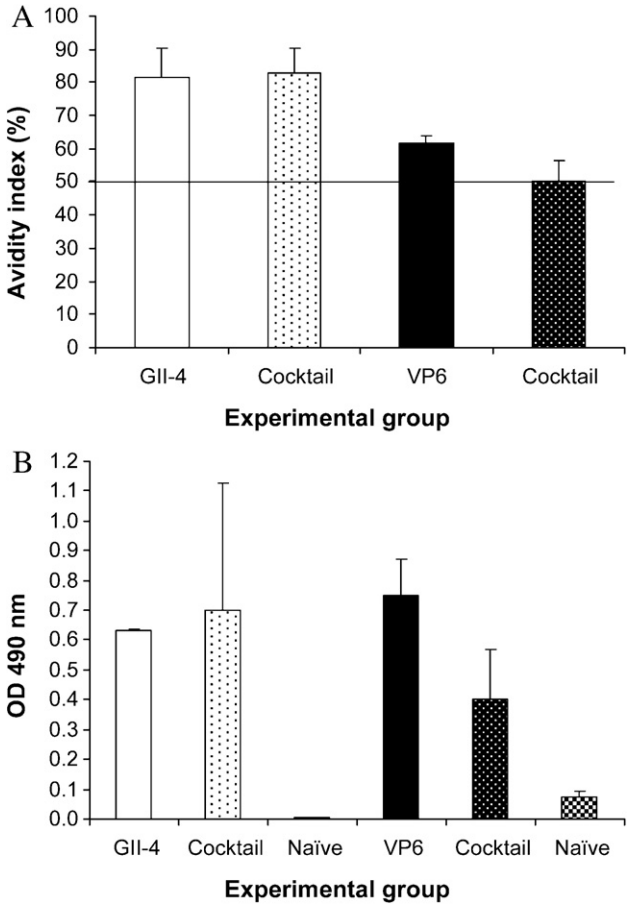

Fig. 4. (A) Mean avidity indices (%) with standard errors of GII-4-specific (white columns) and rVP6-specific (black columns) serum IgG antibodies following intradermal immunizations with 10 μg of GII-4 VLPs alone (4 mice/group), 10 μg of rVP6 alone (4 mice/group) or 20 μg of cocktail formulation (5 mice/group). Avidity index was calculated as [OD with urea/OD without urea] × 100%. (B) Levels of NoV-specific (white columns) and RV-specific (black columns) fecal IgG antibodies induced by intradermal immunizations twice with 10 μg of GII-4 VLPs alone, 10 μg of rVP6 alone or 20 μg of cocktail formulation. Naïve mice received no immunogen. Fecal suspensions were used at 1:5 dilutions. Mean OD values with standard errors of three independent experiments are shown.

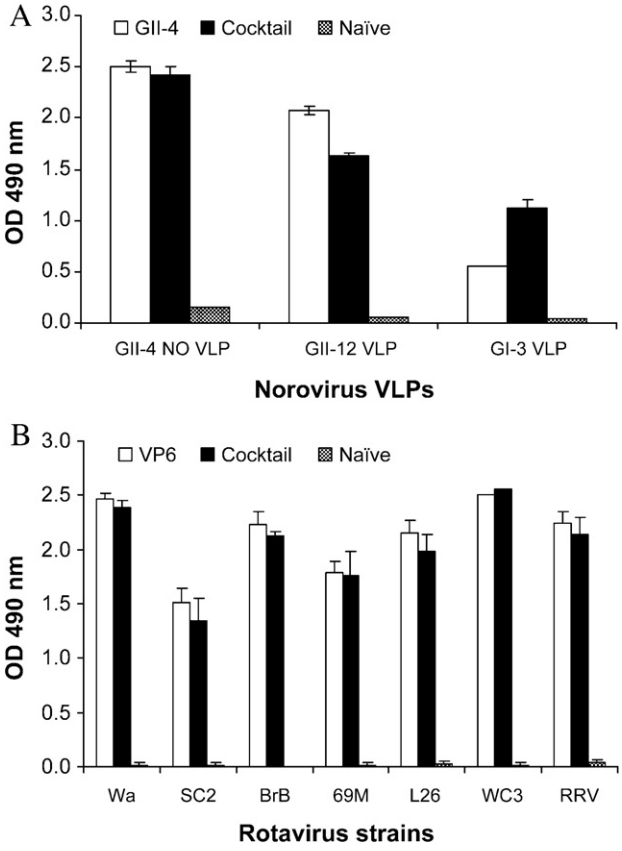

Fig. 5. (A) Levels of cross-reactive IgG antibodies to GII-4 NO, GII-12, and GI-3 VLPs. Mice were immunized intradermally with 10 μg of GII-4 VLPs alone (4 mice/group) or 20 μg of cocktail formulation (5 mice/group). (B) Cross-reactivity of rVP6 induced antibodies to several RV strains. Mice were immunized intradermally with 10 μg of rVP6 alone (4 mice/group) or 20 μg of cocktail (5 mice/group). Naïve mice received no immunogen. Results are presented as mean ODs at 490 nm with standard errors.

in humans [40,42]. The induction of a strong systemic immune response achieved by vaccination, in contrast to the local mucosal response induced by natural infection, might be necessary for a longer-lasting protection against NoV. Using a mouse model for evaluation of the immunogenicity of candidate human vaccines has limitations. Use of inbred animals restricts the immune response diversity due to the simplified genetic background. On the other hand, using inbred mice has the advantage of more reproducible results as the variation in heterogeneous human population is bypassed. In addition, naïve mice lack pre-existing antibodies to NoV and RV which might interfere with the vaccine induced responses as is the case with live RV vaccines [16]. We have performed studies in which mice were immunized twice with the GII-4 NoV VLPs (week 0 and 3) as described in the present paper followed by GII-12 VLPs immunization at week 18. The results showed that there was no impairment of de novo response to GII-12 by the pre-existing antibodies to GII-4 (manuscript in preparation). Also, vaccination with RV VP6 in piglet model boosted protection rates when administered as a booster vaccine after priming with live human RV vaccine [15]. In any case, the final evaluation of the functionality of the immune responses in humans can only be assessed in clinical trials.

Immunization with a mixture of NoV GII-4 VLPs and RV rVP6 induced a transfer of serum IgG antibodies into the gut lumen of BALB/c mice. It can be anticipated that the higher the systemic immune response elicited by the administered vaccine, the higher the level of antibodies transferred to the gut and the better the protection. Although each vaccinated group had measurable fecal antibodies high variation between the groups was noted, which contrasts to the serum antibody levels. The difference is probably due to the fact that body excretions contain different levels of immunoglobulins at a different time points which reflexes the individual need of mice to defecate. In addition, the combined antigenic formulation induces high-avidity antibodies specific for both NoV and RV. High-avidity antibodies have been shown to correlate with the protective efficacy of the vaccines [43]. Recent data by our group showed that children with high-avidity NoV-specific IgG serum antibodies had fewer NoV infections than children with low-avidity antibodies [38]. Furthermore, the combined antigen formulation also induced a mixed and balanced Th1-type and Th2-type immune response. The IgG1 isotype is representative of the Th2-type response, and IgG2a of the Th1-type response. This is an important observation, considering that both types of immune responses are likely to mediate protection against NoV and RV.

The induction of a cross-reactive immune response is of high significance when considering viruses such as NoV and RV, which have large numbers of different genotype/serotypes circulating. Our results show that cross-blocking between NoV genotypes and genogroups is feasible with monovalent VLP vaccination which contrasts to primarily type-specific responses observed by others

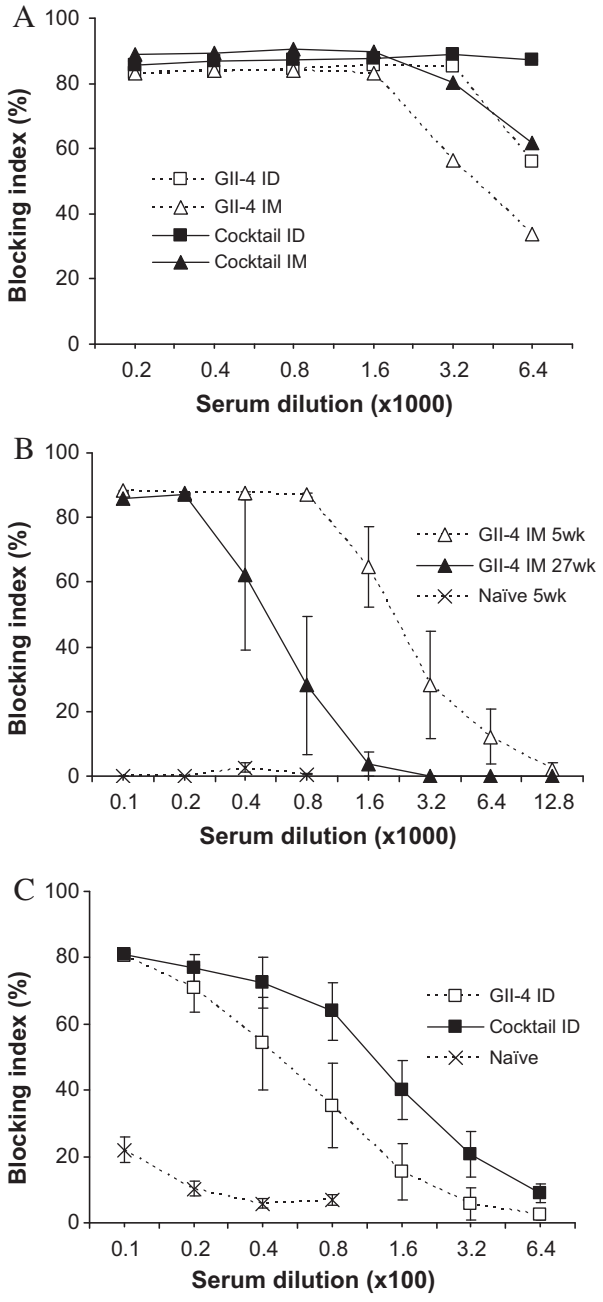

Fig. 6. (A) Homologous blockage of GII-4 VLPs binding to synthetic human histo-blood group antigen (HBGA) H-type-3 by NoV GII-4-specific serum antibodies (5 week termination sera) of mice immunized twice intradermally (ID) or intramuscularly (IM) with 10 μg of GII-4 VLPs alone (4 mice/group) or 20 μg of cocktail (5 mice/group). Blocking index was calculated as follows: 100% – (OD [wells with serum]/OD [wells without serum] × 100%). OD reading from wells incubated without serum was considered as maximum signal for binding of VLPs to H-type 3. Mean titers of pooled sera from a group are shown. (B) Long-term duration of the blocking activity of NoV GII-4-specific serum antibodies of mice immunized twice IM with 10 μg of GII-4 VLPs alone. Data represent homologous blockage of GII-4 VLPs binding to HBGA receptor (type A saliva) by sera of mice terminated at 5 or 27 weeks following initial immunization. Naïve mice received no immunogen. Mean blocking indices with standard errors are shown. (C) Cross-blockage of GII-4 NO VLPs binding to HBGA receptor (type A saliva) by NoV GII-4-specific 5 week termination serum antibodies of mice immunized twice ID with 10 μg of GII-4 VLPs alone or 20 μg of cocktail. Group means with standard errors are shown.

[44]. Immunization with GII-4 derived VLPs (so-called monovalent vaccine) induced a cross-reactive or heterotypic immune response against other genotypes (GII-4 NO, GII-12, and GI-3, respectively) not contained in the formulations, including the newest variant of GII-4 detected for the first time in October 2009 [45]. Most importantly, these antibodies were able to block homologous (GII-4) and heterologous (GII-4 NO and GI-3) VLP binding to a putative receptor and potentially neutralize the virus. In addition, the blocking potential of the sera stayed at a high level for at least 27 weeks following the initial immunization. The blocking assay is considered a surrogate neutralization assay for NoV, as the virus is unable to grow in cell cultures, and therefore a classical neutralization assay cannot be performed [40,46]. Although the receptor/s for NoV is/are not completely elucidated, it is known that NoVs recognize HBGAs [40,46,47], found on red blood cells and mucosal epithelial cells or as secreted free antigens in biological fluids. The binding of NoV VLPs to HBGAs is expected to be blocked with the antibodies that have neutralization properties. Indeed, the binding of GII-4 VLPs to H-type 3 was blocked by preexisting antibodies from children not infected with NoV during a waterborne outbreak of AGE [38], and protection against NoV infection correlated with the strong blocking activity of the sera. Therefore, the blocking activity of the antibodies may be considered a relevant correlate of NoV protection in humans [48].

RV rVP6 protein appeared to act as an adjuvant in terms of broadening the immune response induced by the NoV GII-4 VLPs and by increasing blocking activity of the sera of mice immunized by the cocktail, in comparison to the GII-4 VLPs alone (Figs. 5 and 6, respectively). Although this is an interesting observation it requires further study. We are currently investigating adjuvant effect of rVP6 on NoV-specific B and T cell responses in greater detail. It has previously been shown that RV VP6-specific Th cells in mice provide cognate help to B cells specific for neutralizing epitopes on the heterotypic VP4 or VP7 molecules of RV [19], making these T cells important for induction of a cross-protective immune response against RV.

The immunogenicity and the true potential of a human vaccine can completely be evaluated only in humans. Therefore we plan to proceed to phase I clinical trials with a combined NoV VLP + RV VP6 vaccine in the near future. We propose that there is a need for such non-live combined NoV and RV vaccine, especially for the prevention of AGE in children. Both RV and NoV gastroenteritis have a peak incidence between 6 months and 3 years of age [35,38,49]. A combined, non-adjuvanted, injectable NoV + RV vaccine could be targeted in children in two ways: (1) as part of the routine immunization schedule of young infants before the age of 6 months, followed by a booster at 12 months, or (2) used as a booster vaccination for RV following a series of live vaccine, and primary vaccination for NoV at about 12 months of age. Protection against RV will wane gradually after primary vaccination, and a booster vaccination would be desirable. Current live RV vaccines cannot be used for booster vaccination because of the risk for intussusception at an older age [50]. While a proportion of NoV gastroenteritis occurs in infants under 12 months of age, the majority of the cases occur after this age. Therefore, a vaccine starting at 12 months of age would still have the potential to prevent most cases of NoV gastroenteritis in childhood.

Conflict of interest

None of the authors have conflict of interest.

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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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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 ($0.1x10^6$ 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 $p \leq 0.05$.

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

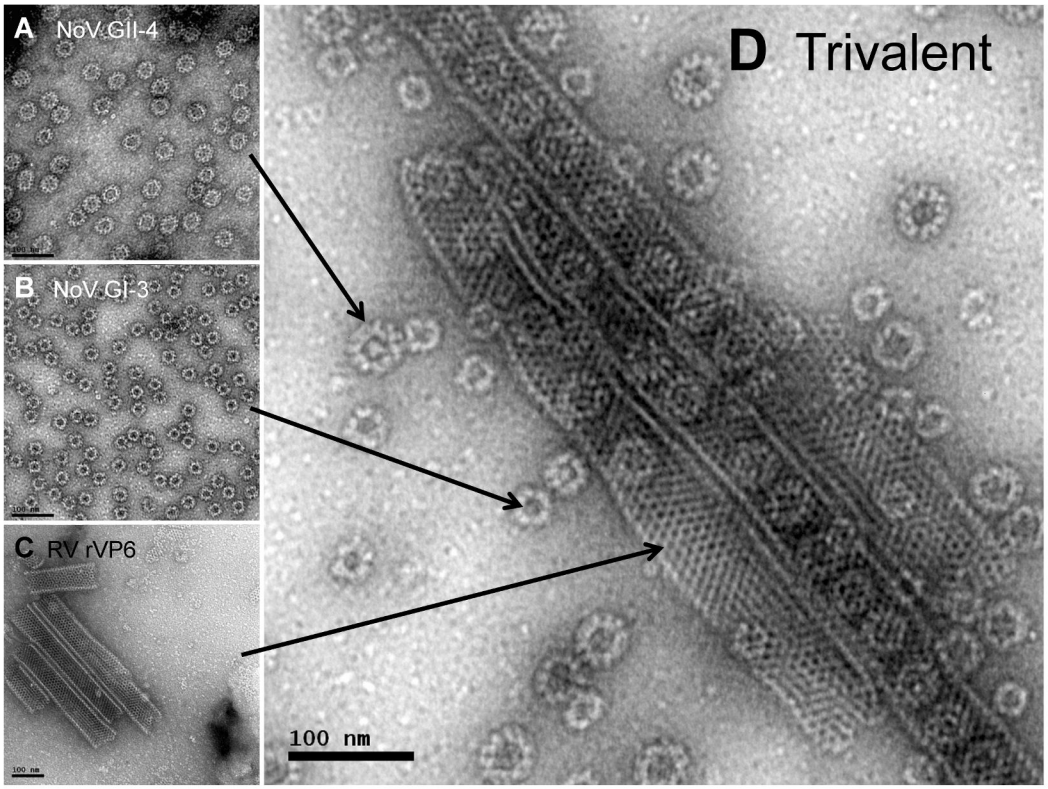

Figure 1. Electron microscopy images of the single antigens and the trivalent combination used to immunize BALB/c mice. Morphological assemblies of NoV GII-4 capsid (A), GI-3 capsid (B) and RV rVP6 (C) proteins, and the trivalent combination (1:1:1 of each antigen) of the structures depicted in panels 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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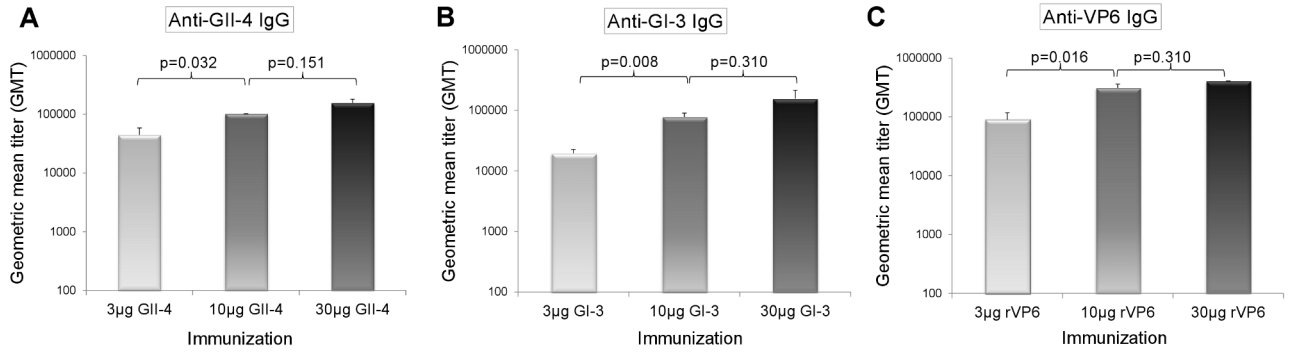

Figure 2. Antigen-specific serum IgG dose response. Mice were immunized twice at study weeks 0 and 3 with 3, 10 and 30 μg of single antigens and the geometric mean titers (GMTs) induced by GII-4 VLPs (A), GI-3 VLPs (B) and RV rVP6 (C) were measured in an ELISA. The error bars represent the standard error 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

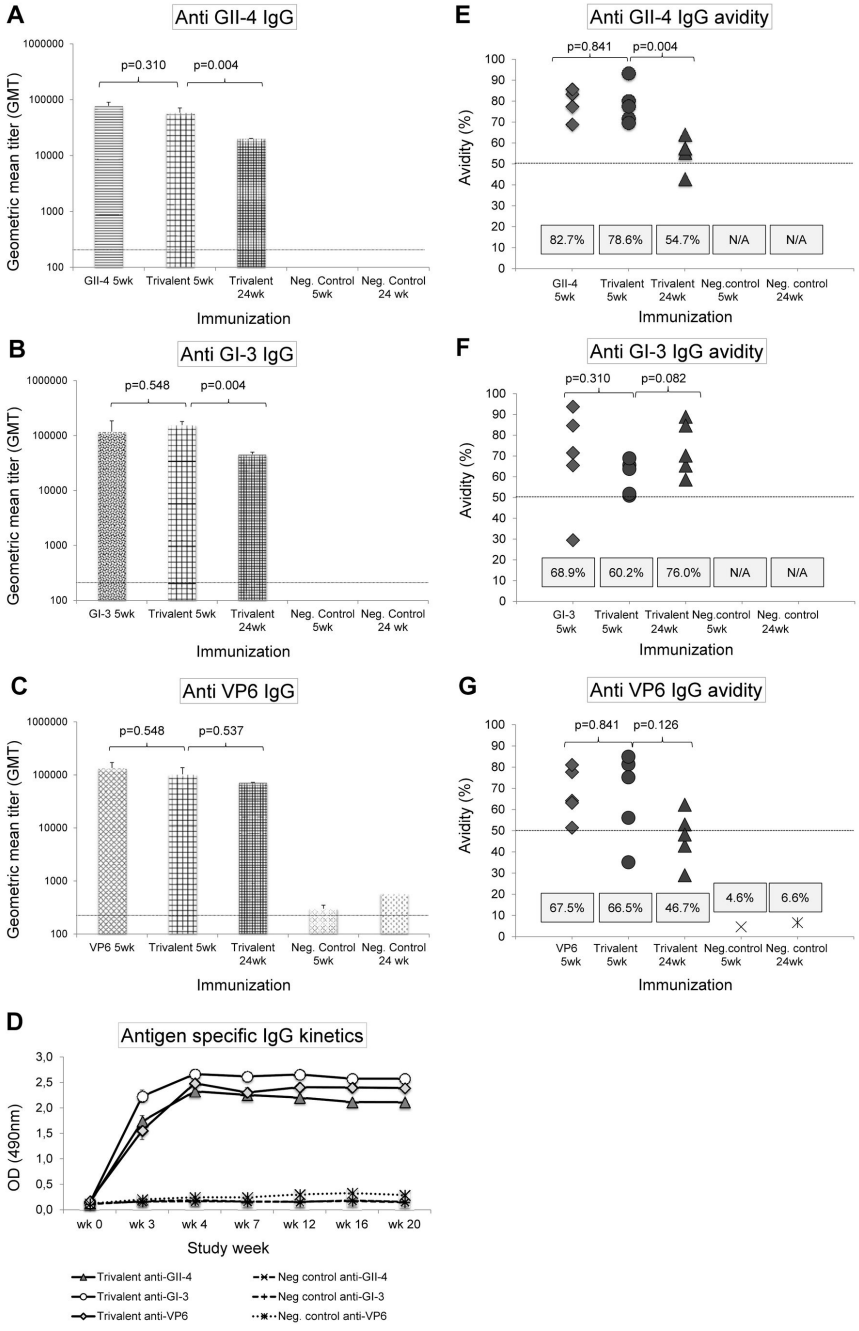

Figure 3. Serum IgG responses induced by the single antigens versus trivalent combination. Mice were immunized two times with 10 μg of the single antigen (GII-4 VLPs, GI-3 VLPs or rVP6) or the trivalent combination (each antigen at a 10 μg dose), and the sera at study week 5 and 24 were tested against 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

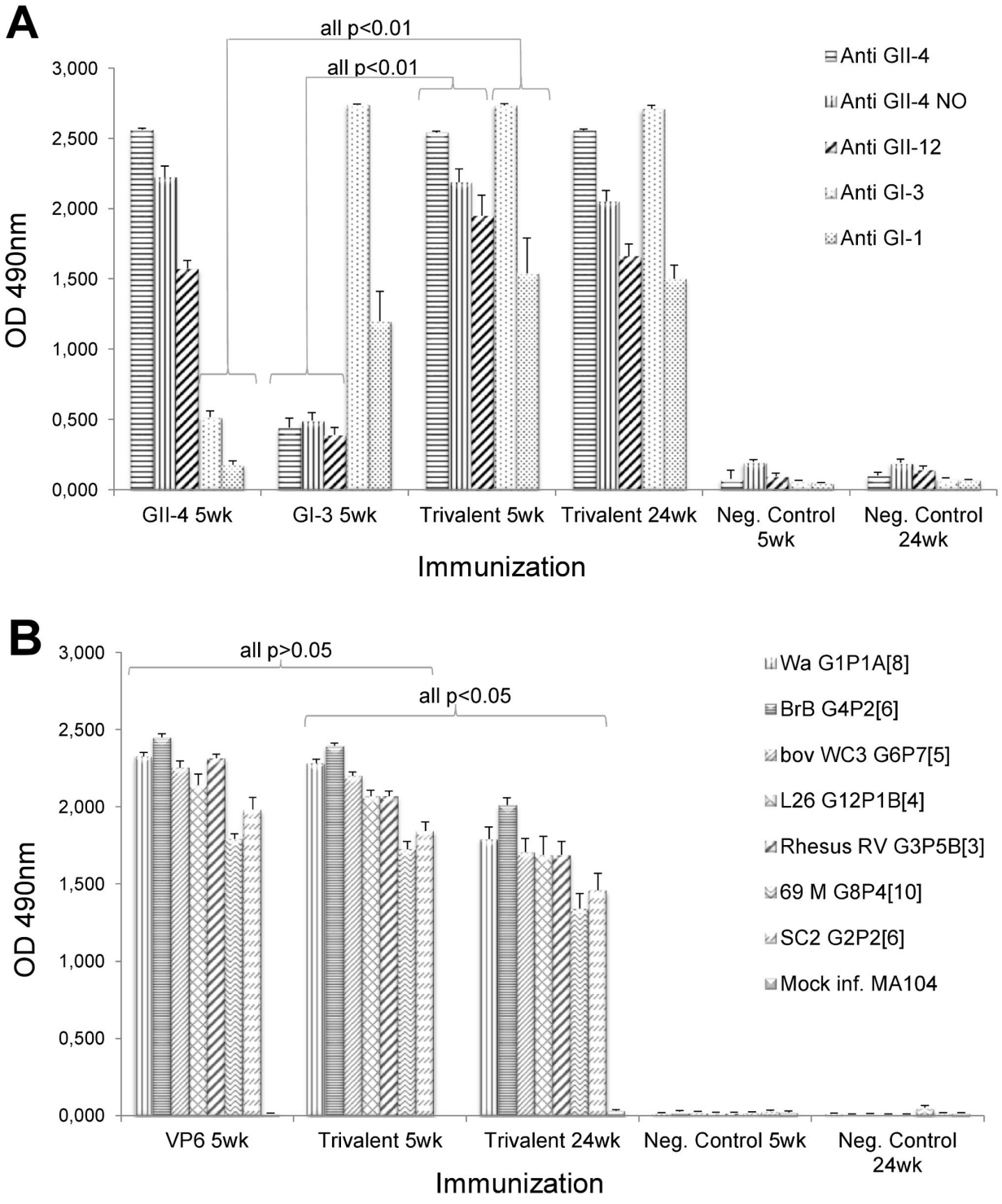

Figure 4. Cross-reactive serum IgG antibodies. Mice were immunized two times with 10 µg of the single antigen (GII-4 VLPs, GI-3 VLPs or rVP6) or the trivalent combination (each antigen at a 10 µg dose) and the sera were tested against heterologous NoV VLPs (A) and RV cell culture antigens (B) in ELISA. 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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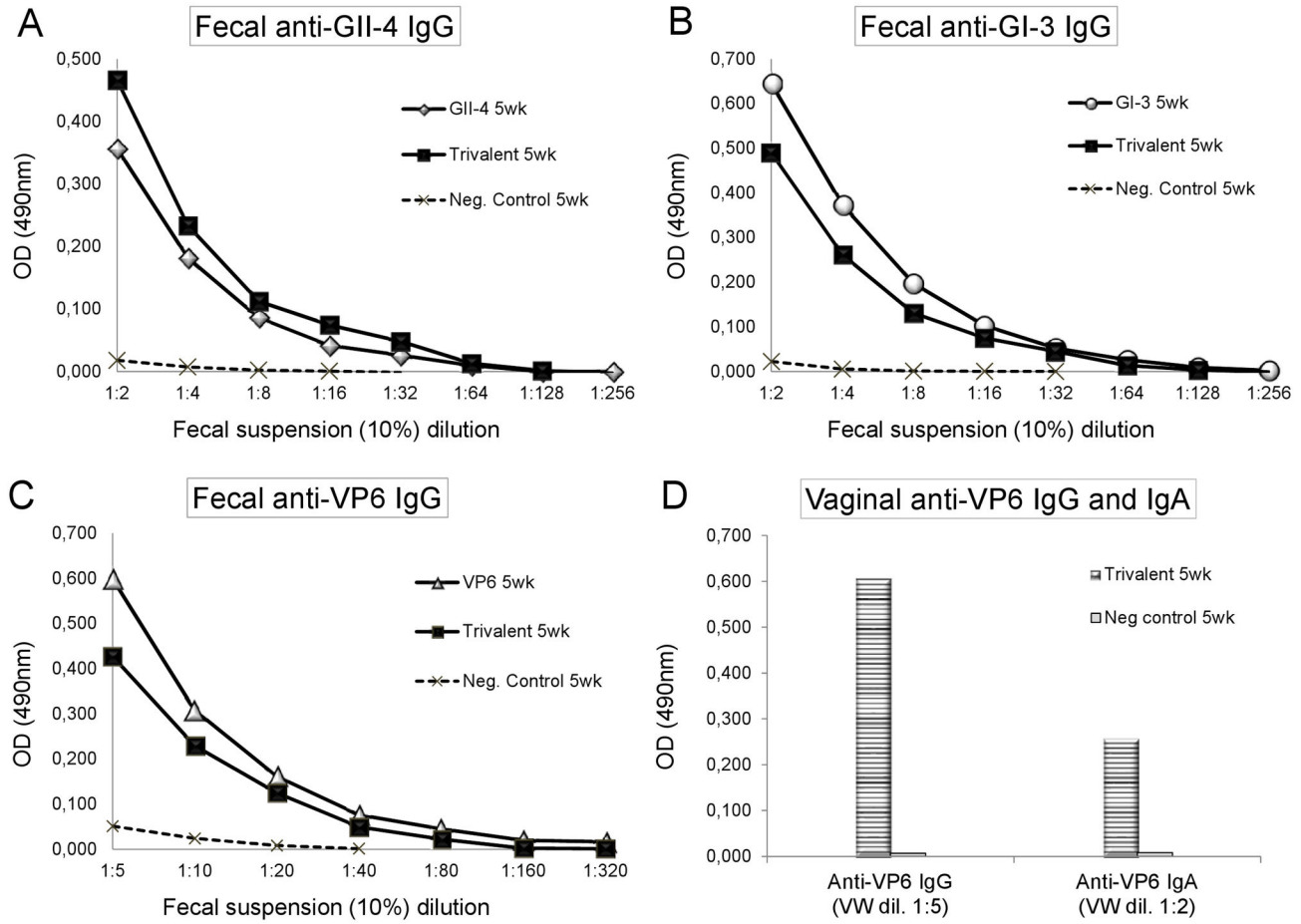

Figure 5. Mucosal antibody response. Group-wise pooled stool samples (10% suspension) of mice immunized with the single NoV GII-4 VLPs, GI-3 VLPs or RV rVP6 antigens or the trivalent combination vaccine were titrated two-fold and anti-GII-4 (A), anti-GI-3 (B) and anti-VP6 (C) IgG content was measured 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

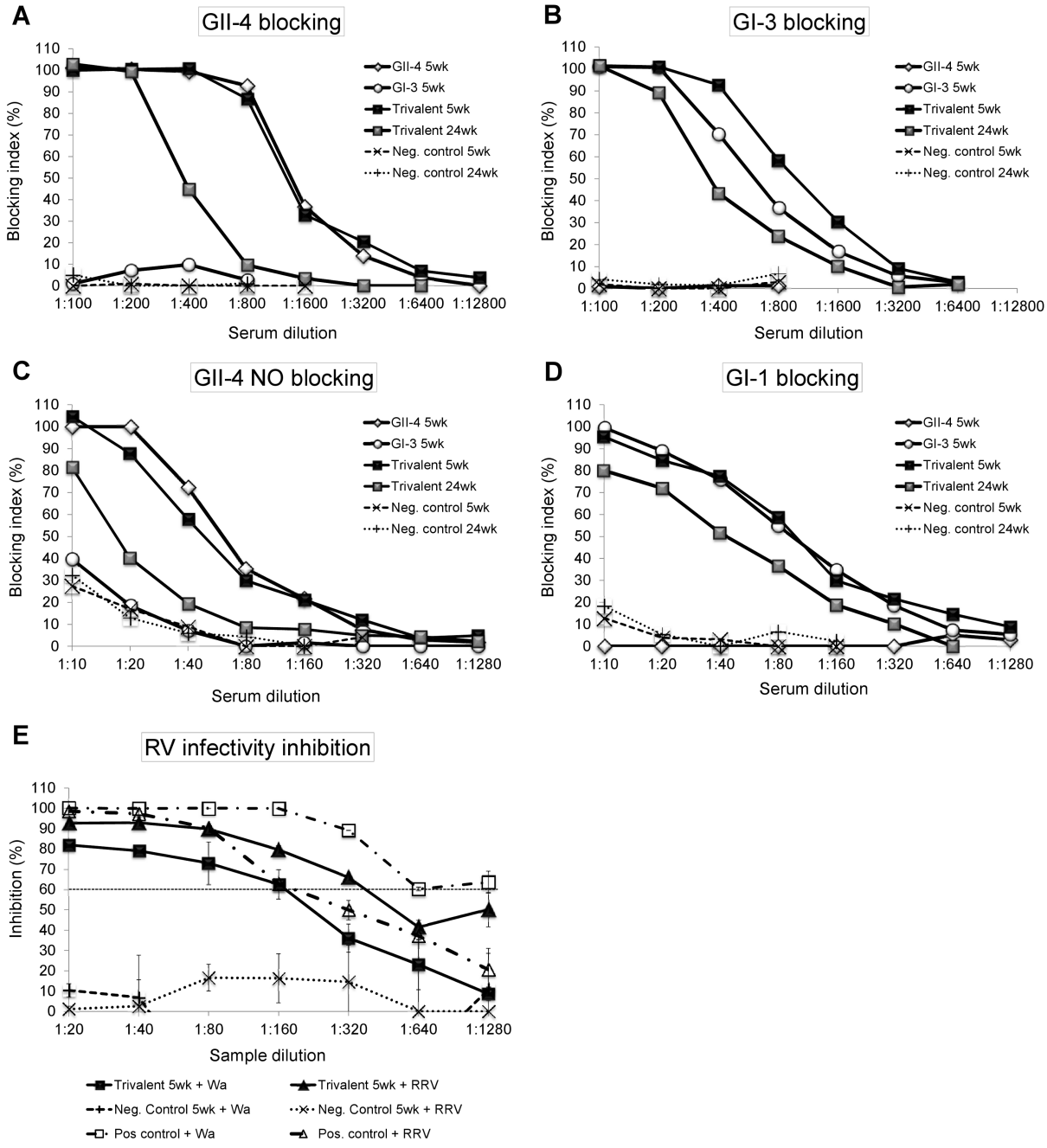

Figure 6. Functionality of NoV and RV-specific antibodies. Termination sera of mice immunized with the single NoV GII-4 or GI-3 VLPs antigens or the trivalent combination vaccine were pooled group-wise, titrated two-fold and used for blocking the binding of homologous GII-4 and GI-3 VLPs (A, B) or heterologous 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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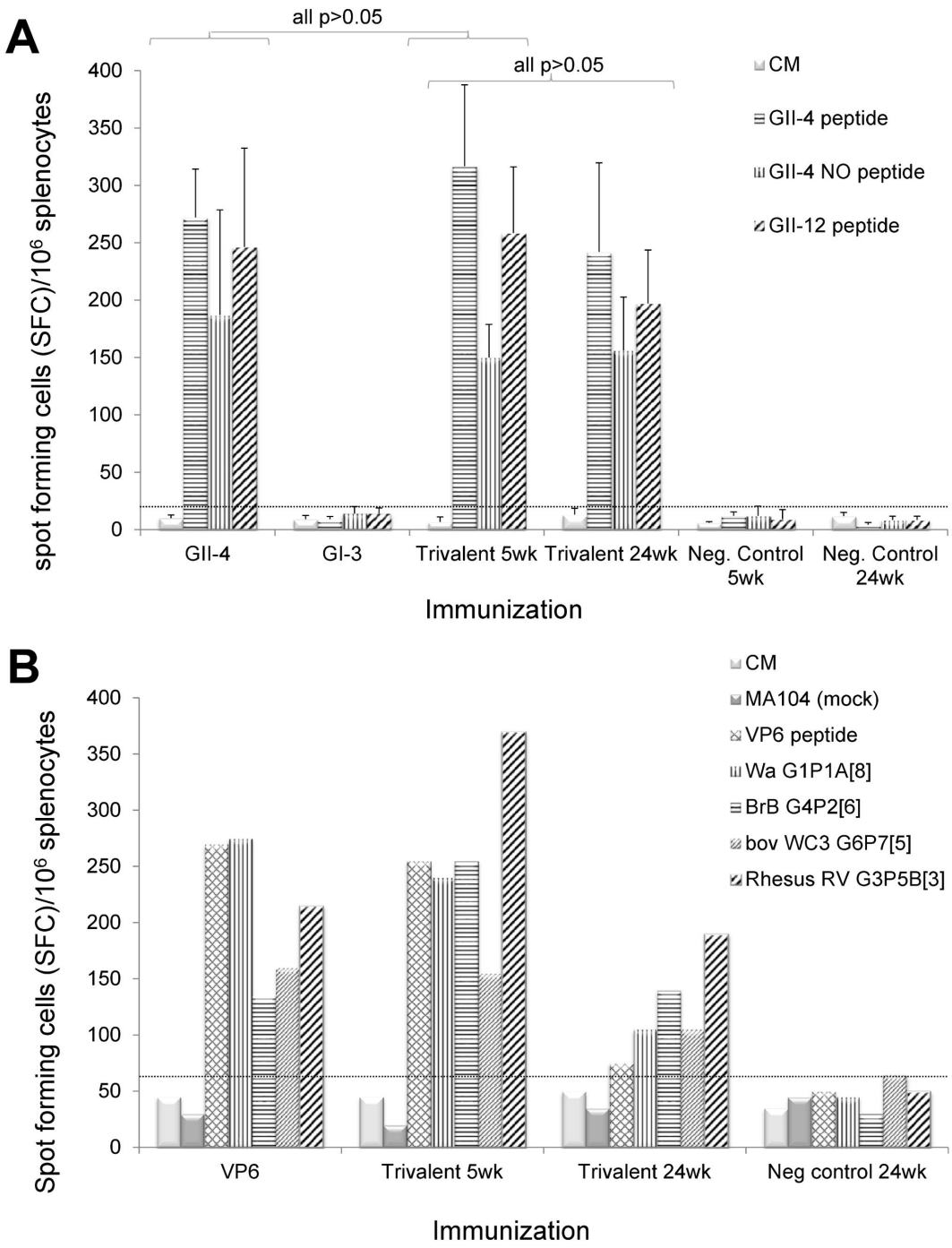

Figure 7. NoV and RV-specific IFN-γ responses. Splenocytes of mice immunized with the single NoV GII-4 or GI-3 VLPs or the trivalent combination vaccine were stimulated with synthetic NoV capsid-derived 15-mer peptides from different NoV genotypes and analyzed for IFN-γ production by an ELISPOT assay (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.

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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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RESEARCH PAPER

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Immune responses elicited against rotavirus middle layer protein VP6 inhibit viral replication in vitro and in vivo

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Keywords: rotavirus, VP6, neutralization, mucosal IgA, intranasal immunization

Abbreviations: dl, double-layered; ELISA, enzyme-linked immunosorbent assay; FFU, focus forming units; GE, gastroenteritis; HRP, horseradish peroxidase; IM, intramuscular; IN, intranasal; NAbs, neutralizing antibodies; NELISA, ELISA-based antigen reduction neutralization assay; NoV, norovirus; OD, optical density; OPD, *o*-phenylenediamine dihydrochloride; PFU, plaque-forming units; pIgA, polymeric IgA; RV, rotavirus; rVP6, recombinant VP6; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SG, subgroup; SIgA, secretory IgA; VLP, virus-like particle; VW, vaginal wash

Rotavirus (RV) is a common cause of severe gastroenteritis (GE) in children worldwide. Live oral RV vaccines protect against severe RVGE, but the immune correlates of protection are not yet clearly defined. Inner capsid VP6 protein is a highly conserved, abundant, and immunogenic RV protein, and VP6-specific mucosal antibodies, especially IgA, have been implicated to protect against viral challenge in mice. In the present study systemic and mucosal IgG and IgA responses were induced by immunizing BALB/c mice intranasally with a combination of recombinant RV VP6 protein (subgroup II [SGII]) and norovirus (NoV) virus-like particles (VLPs) used in a candidate vaccine. Following immunization mice were challenged orally with murine RV strain $EDIM_{wt}$ (SG non-I-non-II, G3P10[16]). In order to determine neutralizing activity of fecal samples, sera, and vaginal washes (VW) against human Wa RV (SGII, G1P1A[8]) and rhesus RV (SGI, G3P5B[3]), the RV antigen production was measured with an ELISA-based antigen reduction neutralization assay. Only VWs of immunized mice inhibited replication of both RVs, indicating heterotypic protection of induced antibodies. IgA antibody depletion and blocking experiments using recombinant VP6 confirmed that neutralization was mediated by anti-VP6 IgA antibodies. Most importantly, after the RV challenge significant reduction in viral shedding was observed in feces of immunized mice. These results suggest a significant role for mucosal RV VP6-specific IgA for the inhibition of RV replication in vitro and in vivo. In addition, these results underline the importance of non-serotype-specific immunity induced by the conserved subgroup-specific RV antigen VP6 in clearance of RV infection.

Introduction

Group A rotavirus (RV) is common etiological agent of severe gastroenteritis (GE) in infants and young children worldwide with great mortality in the developing world.[1] RV particles possess a triple-layered capsid enclosing a genome of 11 segments of double-stranded RNA.[2] The external layer of infectious RV particles is formed by 2 proteins, the glycoprotein VP7 and VP4 (forming spikes with hemagglutinating activity in some RV strains), which define the G (glycoprotein) and P (protease-sensitive) genotypes, respectively, of the virus.[2] Both of these proteins are essential for virus attachment and entry to the host cells[3,4] and contain major antigenic epitopes which induce type-specific RV neutralizing antibodies (NAbs).[2] The intermediate layer of the RV surrounding the VP2 core consists of VP6, which contains viral group (A-G/H) and subgroup (SGI, II, I+II, non-I-non-II for group A) specific antigenic determinants.[2,5] The inner capsid protein VP6 is highly conserved with approximately 90% homology at the amino acid level among group A RVs.[6] It is also the most abundant[2] and highly immunogenic RV protein.[7–10] Development of serum VP6-specific antibodies, especially IgA, has been regarded as an indicator of protection after natural RV infection or vaccination.[11]

Two live attenuated oral RV vaccines, the pentavalent human bovine (WC3) reassortant rotavirus vaccine (RotaTeq®, Merck) and the monovalent G1P1A[8] human rotavirus vaccine (Rotarix®, GlaxoSmithKline) were licensed in 2006 and are now used extensively, but the mechanisms or effectors of protection

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against RVGE are not clearly defined.[1,11] A role of type-specific NAbs to external VP4 and VP7 proteins in the induction of protective immunity after natural RV infection and oral immunization with live RVs is evident,[12] but other mechanisms are also important in protection.[1,11] This is indirectly indicated by the finding that monovalent Rotarix® vaccine and the pentavalent RotaTeq® vaccine show similar levels of clinical protection against severe RVGE caused by different RV genotypes.[13,14] Moreover, the levels of NAbs induced by the vaccines are low and therefore cannot account for the high level of protection of these vaccines.[15,16] The evidence that the immune response to VP4 and VP7 is not absolutely required for protection is also supported by the induction of protection against RV infection in mice and rabbits by inactivated double-layered (dl) RV particles,[17,18] dl2/6-virus-like particles (VLPs),[19–21] chimeric VP6 protein[22,23] or DNA encoding VP6.[24,25] The above studies suggest a significant role of VP6 in RV protective immunity, although dl2/6-VLPs have failed to induce protection against disease in gnotobiotic piglets.[26]

VP6-specific mucosal (intestinal) antibodies, especially IgA antibodies in mice immunized with recombinant VP6 (rVP6) or dl2/6-VLPs have been implicated as correlates of protection against RV challenge.[25,27,28] Moreover, it has been reported that anti-VP6 polymeric IgA (pIgA) impairs RV infection by intracellular inhibition of RV replication.[29–33] While it is generally accepted that antibodies directed against the internal RV protein VP6 have no neutralizing activity in vitro, there are a few reports to the contrary,[34–37] mainly relating to the inhibitory activity of llama-derived single-chain antibody fragments.[35–37] To add to the evidence, in the present study we show that mucosal VP6-specific IgA antibodies inhibit RV infection in vitro. Furthermore, VP6-specific immune response induced in vivo protection in BALB/c mice challenged with murine RV strain EDIM.

Results

Intranasal immunization induced high systemic and mucosal antibody responses

High systemic and mucosal IgG and IgA responses were induced by intranasal (IN) immunization of mice with the candidate combination vaccine containing equal quantities of RV rVP6 protein and norovirus (NoV) VLPs as determined by enzyme-linked immunosorbent assay (ELISA). IN immunization route was used to obtain maximum amount of mucosal antibodies, specifically IgA, as IgA antibodies have been implicated in protection against RV infection in vivo[25,27–30] and in inhibition of RV replication in vitro.[31–33]

Two immunization doses resulted in high levels of anti-VP6 IgG and moderate levels of anti-VP6 IgA antibodies in the serum of the experimental group with end point titers of >4.4log10 (OD_{490} 2.4 at a 1:200 dilution) and >2.5log10 (OD_{490} 0.4 at a 1:10 dilution), respectively (**Fig. 1A and B**). Further, considerably high levels of IgG antibodies were detected in groupwise pooled 10% fecal suspensions (OD_{490} 1.4 at a 1:2 dilution) and vaginal washes (VWs, OD_{490} 2.2 at 1:2 dilution) of immunized animals (**Fig. 1C**). Similarly, IgA antibodies were detected in the mucosal secretions, with OD_{490} values of 0.9 and 1.7 (ratio 0.5) for 1:2 diluted fecal samples and VWs, respectively (**Fig. 1C**). The ratios of serum IgA to VW and fecal IgA were 0.5 and 1.8, respectively. All samples from control mice were negative for both RV-specific IgG and RV-specific IgA antibodies (**Fig. 1A–C**).

Mucosal samples contain polymeric forms of IgA

Molecular forms of IgA were determined in sera, feces, and VWs of immunized mice by immunoblotting under non-reducing conditions. Most IgA antibodies in mucosal samples but not in the serum samples of the experimental group were found in the polymeric forms (**Fig. 1D**).

In vitro inhibition of RV infection

The ability of VP6-specific antibodies to inhibit RV infection was studied in vitro by an ELISA-based RV antigen reduction neutralization assay.[38,39] Functionality of the neutralization assay against RV Wa and RRV was confirmed using human RV-positive and RV-negative sera (data not shown). Mouse samples after 2 immunizations were tested for neutralization against these 2 RV strains, namely Wa (SGII, G1P1A[8]) homologous to the rVP6 used for immunization, and RRV (SGI, G3P5B[3]) (**Fig. 2**). No neutralizing effect was detected in the sera (**Fig. 2**) of immunized or control groups. Several attempts to test the neutralizing activity of fecal samples failed each time, as the samples from the mice, including control, and even at high dilutions, were toxic for cell cultures—an observation made by others as well.[40] Instead, VWs containing levels of mucosal antibodies similar to those of the fecal suspensions (**Fig. 1C**) were used in neutralization assays. VWs from immunized group neutralized both RV strains belonging to different SGs. These results indicate cross-reactive neutralizing activity of VP6-specific mucosal antibodies in vitro. More precisely, inhibition of infectivity of RV Wa and RRV was detected with neutralizing titers of 569 and 213, respectively (**Fig. 2**). The VW samples from control mice did not inhibit RV infection, whereas positive human control serum always neutralized both viruses.

VP6-specific mucosal IgA mediates RV inhibition

The neutralizing activity of the VWs was confirmed to be associated with the VP6 binding by blocking experiments. The neutralization ability against Wa was completely blocked by preincubation of the VW samples with rVP6 protein compared with the untreated VW samples ($P = 0.001$) (**Fig. 3A**). In contrast, preincubation of serial VW dilutions with unrelated recombinant NoV GII-4 VLPs had no effect ($P = 0.073$) on the neutralizing activity (**Fig. 3A**). Similar results were observed with RRV, when preincubating the samples with rVP6 reduced the neutralization ($P = 0.001$) by immune VWs (**Fig. 3A**). Furthermore, IgA antibodies were shown to mediate the RV Wa and RRV inhibition. When IgA of VWs was depleted with magnetic beads prior to use in a neutralization assay, the neutralizing activity for RV Wa as well as RRV was abolished (**Fig. 3B**). An IgA ELISA assay confirmed removal of the IgA antibodies from the VWs of the experimental group with the finding of OD_{490} values of 0.80 and 0.01 for the 1:10 dilutions of VWs before and after the depletion,

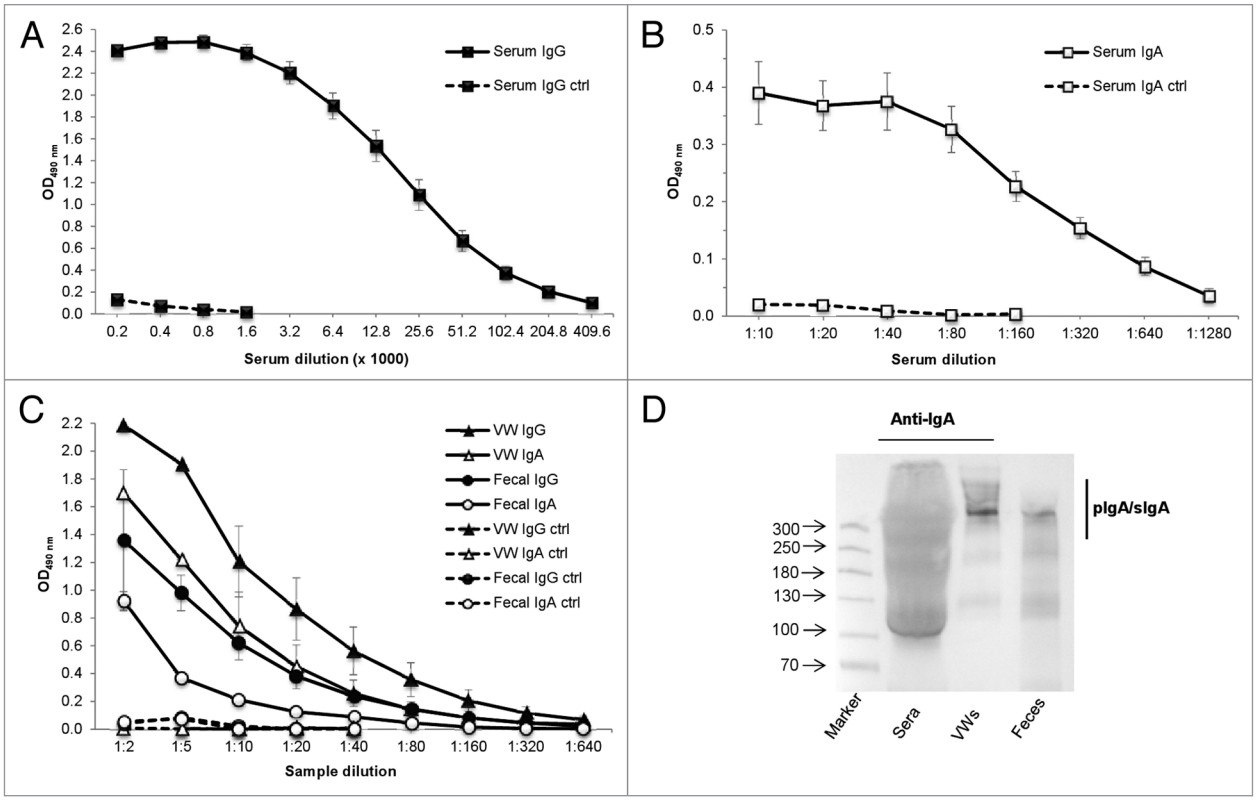

Figure 1. Detection of serum and mucosal VP6-specific antibody responses following intranasal immunization. Endpoint titration of VP6-specific IgG (**A**) and IgA (**B**) antibodies in serum of individual mice (5 mice/group) after 2 intranasal immunizations with the combination vaccine containing rotavirus rVP6 and norovirus VLPs, each at a 10 µg dose. Control (ctrl) mice received only PBS. Mean titers of sera at study week 5 are shown. Error bars represent standard error of the means. (**C**) Endpoint titration of VP6-specific IgG and IgA antibodies in mucosal samples of experimental groups. Mean titers of groupwise pooled (5 mice/group) vaginal washes (VW) and fecal suspensions of at least 2 (2–4) independent experiments at week 5 are shown. (**D**) Characterization of different IgA forms in mucosal samples from immunized mice by immunoblot analysis under non-reducing conditions. Polymeric IgA (pIgA) forms including secretory IgA (SIgA) were confirmed upon immunodetection with goat anti-mouse IgA. Molecular weight markers (in kDa) are indicated with arrows.

respectively. IgA depletion did not drastically alter the IgG content of the samples, which retained significant amount of IgG (OD_{490} 1.4 vs. OD_{490} 0.8).

Protective efficacy against RV challenge

To determine the protection conferred by the combination vaccine, immunizations were repeated and mice from immunized and control groups were challenged with murine RV $EDIM_{wt}$ at study week 6. Viral shedding curves show that the viral antigen shedding in feces of VP6 immunized mice was decreased significantly (P = 0.021) compared with the control mice (**Fig. 4**). High shedding was observed from day 2 to day 5 in control group, whereas the immunized group shed virus only at day 3. Total reduction in shedding of the vaccinated group was 62.0% (± 18.3%) compared with the control group. For undetermined reasons in one mouse antigen shedding was higher than in any of control mice. More consistent reduction of virus shedding (77.5 ± 12.6%) was detected in remaining 4 of the 5 animals immunized (**Fig. 4**). Since all experimental mice had similar levels of serum VP6-specific IgG and IgA (data not shown) before the challenge, the failure in protection of the particular mouse cannot be explained with the pre-existing antibody levels. In addition, intestinal IgA antibodies were detected prior to the challenge as well (OD_{490} 0.3 ± 0.08 at 1:10 dilution) and the level was similar to the level shown in **Figure 1C**.

Discussion

We have previously shown that a candidate combination vaccine against NoV and RV containing a mixture of NoV VLPs and RV VP6 protein delivered parenterally to BALB/c mice induced high levels of systemic VP6-specific cross-reactive serum IgG antibodies as well as T cells.[41–43] Further, mucosal antibodies induced by intramuscular (IM) application of the combination vaccine inhibited RV infection in vitro.[43] Since mucosal VP6-specific IgA antibodies have been associated with protection against RV in vivo[25,27–30] and inhibition of RV replication in vitro,[31–33] in the present work we have used IN delivery of the combined vaccine in order to induce high levels of mucosal VP6-specific antibody responses. Our results show that mucosal IgA antibodies inhibited RV infection in vitro. Importantly, in vivo protection from RV challenge was induced by immunization with VP6 protein.

Although in vivo protection from RV challenge correlating with VP6-specific mucosal (intestinal) IgG and IgA antibodies

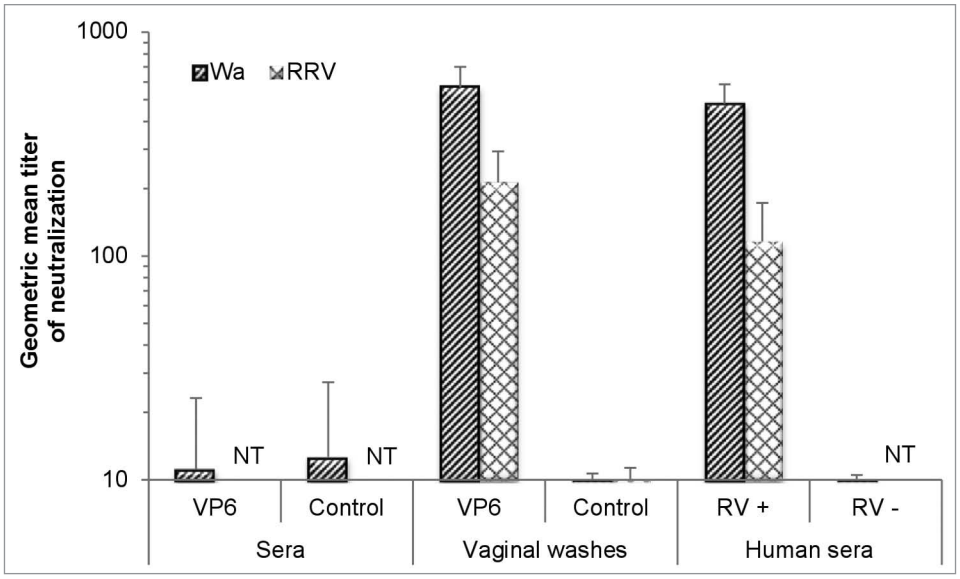

Figure 2. Inhibition activity of sera and vaginal washes of intranasally immunized mice against human rotavirus (RV) Wa (homologous VP6 to the immunizing rVP6 protein) or rhesus RV (RRV) using an ELISA-based antigen reduction neutralization assay. Mice were immunized with the combination vaccine containing RV rVP6 and norovirus virus-like particles. Control mice received no immunogen. Sera of RV-seropositive (RV+) and RV-seronegative (RV–) human donors were used as assay controls. The reciprocal of the sample dilution that generated >60% reduction in virus infectivity was considered its titer. If the highest dilution (1:10) did not yield neutralization of >60%, a titer of 5 was assigned as the neutralization titer of the sample. Results of study week 5 are represented as the geometric mean neutralizing titer of at least 2 independent experiments, each done in duplicate, with standard errors. NT, not tested.

in mice immunized with rVP6 has been documented,[25,27,28] antibodies against VP6 have been thought to have no neutralizing activity in vitro. However, few studies have described neutralizing activity of VP6-specific serum antibodies,[34–37] mainly relating to small-sized llama-derived antibody fragments against VP6,[35–37] in a traditional in vitro assay. Nevertheless, anti-VP6 IgA mucosal antibodies have been indicated to impair RV infection in vivo and in vitro by intracellular neutralization.[29–33]

Intranasal immunization with different antigens has been shown to be effective in inducing antigen-specific secretory IgA (SIgA) in intestinal surfaces but also in other mucosal sites including vagina.[44,45] Similarly, in the present study mucosal VP6-specific IgG and IgA were detected in considerable quantities in intestine and VWs of immunized mice. However, neutralization experiments of RVs by fecal suspensions failed in spite of several attempts. Failures to detect VP6-specific in vitro neutralization activity can most likely be explained by toxicity of the fecal suspensions to the MA104 cells, as seen previously by others.[40] For this reason, the VWs containing comparable levels of mucosal IgG and IgA antibodies as fecal suspensions were used for the neutralization experiments, where antibody content of these VWs served as a proxy indicator for fecal antibodies. Samples of VWs were able to inhibit infection of MA104 cells with human Wa RV strain homologous to the immunization protein rVP6, as well as with a heterologous rhesus RV strain. A similar inhibition was also observed previously with VWs from mice immunized IM with the candidate vaccine, rVP6 or dl2/6-VLPs[43,46] although the neutralization titer to Wa RV was increased by a factor of 3.6 with IN administration, possible due to the higher levels of mucosal antibodies in VWs of IN immunized mice. Neutralization of both RV strains irrespective of subgroup or G- and P-type indicates heterotypic protection of these antibodies in vitro, which is in concordance with the work of others.[30,35]

Preincubation of VWs with rVP6 protein efficiently reduced the inhibition of RV infectivity by blocking antibody binding sites and consequently neutralization activity. By contrast, an unrelated protein, GII-4 VLPs derived from NoV capsid VP1, did not reduce this activity. These results strongly suggest that heterotypic neutralization activity was conferred by VP6-specific antibodies. In addition, depletion experiments using magnetic beads indicated that IgA antibodies were mediating the RV inhibition. However, the role of mucosal IgG antibodies as mediators of the inhibition warrants further investigation. Although neutralizing activity of sera containing considerable levels of VP6 specific IgA was not detected, potential neutralizing capacity of serum VP6-specific IgA cannot be definitively excluded. Since no inhibition of RV infectivity by sera could be seen, preferably, VP6-specific IgA in the polymeric form present in mucosal washes may be responsible for prevention of RV infectivity. Generally, the reliability of using anti-RV serum IgA antibody titers as a correlate of protection or vaccine efficacy is controversial.[47–50] Recently, studies in developing countries have indicated that RV-specific IgA levels in serum are not an optimal correlate of protection following vaccination.[47]

The mechanisms by which VP6-specific antibodies exert the neutralizing effect are not yet understood, but it has been shown that VP6-specific pIgA neutralizes RV by inhibiting virus replication intracellularly.[31,32] Indeed, recent data by Aiyegbo and coworkers[33] indicate that the neutralizing activity of human VP6-specific IgA antibody results from the inhibition of viral

transcription inside the cells during polymeric Ig receptor-mediated transcytosis of pIgA, which in turn suggests that intracellular neutralization by naturally-occurring RV VP6-specific pIgAs likely contributes to human protective immunity to RV. This mechanism could also partly account for the RV inhibition we have observed, although our studies have not specifically examined intracellular mechanisms with polarized cells.[32,33,51] This assumption is based on non-reducing immunoblot analysis, where mostly polymeric forms of IgA were detected in the mucosal samples. These pIgAs as well as SIgA have been shown to bind to intact RV particles[32] through the holes in the capsid or via areas of partial decapsidation[52] and could therefore interact with dl particles inside the cell. In support of the above, we have also detected direct binding of VP6-specific mucosal but not serum IgA antibodies to the non-denatured RVs coated onto ELISA plates (data not shown). Alternatively to the post-entry infection events, RV infection could be inhibited during the virus entry, as shown by Gualtero and colleagues.[34] They suggested that VP6-specific antibodies blocked binding of VP6 to heat shock cognate protein 70, a co-receptor for RV entry, into the susceptible cells.

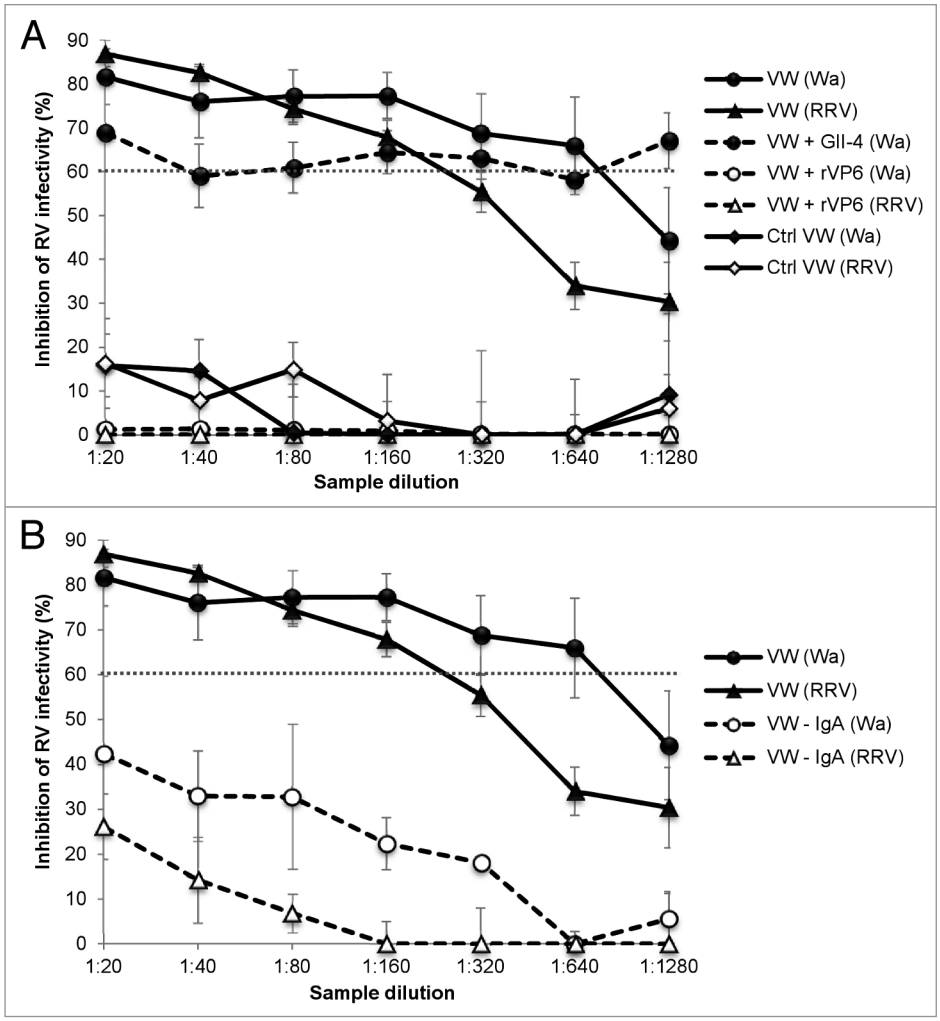

Figure 3. VP6-specific mucosal IgA mediates RV inhibition in vitro. (**A**) Blocking of the inhibition of human rotavirus (RV) Wa and rhesus RV (RRV) infection in vitro by VP6-specific antibodies of mice immunized intranasally with the combination vaccine containing RV rVP6 and norovirus (NoV) virus-like particles (VLPs). Control (Ctrl) mice received no immunogen. Vaginal wash (VW) samples from immunized mice were preincubated with rVP6 protein or NoV GII-4 VLPs prior the neutralization assay or left untreated. (**B**) IgA antibodies mediate RV Wa and RRV inhibition in vitro. IgA was depleted (–IgA) from VWs of immunized mice prior to neutralization assay, resulting in reduced inhibitory activity compared with untreated VWs. The dashed line indicates the 60% cut-off for reduction in virus infectivity. Results of at least 2 independent experiments, each done in duplicate, with standard errors are shown.

In this study, in vivo protection against RV challenge induced by human-derived RV VP6 protein was also assessed. Protective efficacy was measured in an adult mouse model based on decreased RV antigen shedding originally described by Ward et al.[53] Consequently, the candidate vaccine administered IN induced 77.5% reduction in RV replication in mice challenged with murine RV strain EDIM suggesting that the candidate vaccine is effective in conferring immunoprotection against RV challenge. Although 1 of 5 mice was not protected for unknown reason, very recent publication describing similar reduction in RV shedding in mice immunized with VP6 tubular structures[54] support our observation. These results indicate the importance of VP6-specific immune response in heterotypic protection, which is in concordance with the previously published results where protection against 2 different murine RV strains, EDIM and EMcN, was demonstrated after mucosal immunizations with *E. coli*–expressed MBP-VP6 derived from human CJN strain.[23] Although VP6-specific mucosal IgA antibodies have been implicated as correlates of protection against RV challenge in mice immunized with VP6 or dl2/6-VLPs,[25,27,28] other studies imply no protective role for VP6 specific mucosal antibodies.[22,23,55] These differences may be explained with the induction of different effectors or mediators of protective immune responses depending on nature of RV VP6 protein used for immunization, e.g., being a monomer[55] or oligomeric structures.[28] In addition, the difference may come from an adjuvant being used or not.[28,55] Nonetheless, the protection we observed in adult mouse shedding model not necessarily assure protection in RV animal disease models. For example dl2/6-VLPs have not conferred protection against RV disease in RV-infected gnotobiotic piglets.[26] Since the vaccine formulation necessary to induce protection from RV disease or virus shedding

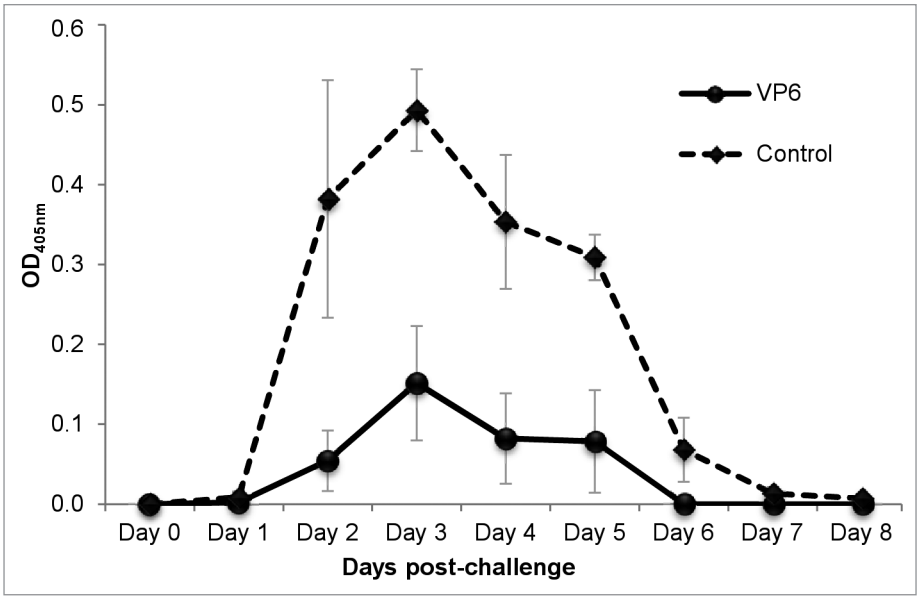

Figure 4. Effect of intranasal immunization with candidate vaccine on rotavirus shedding. Groups of mice were immunized intranasally twice with the candidate vaccine containing rotavirus (RV) rVP6 and norovirus virus-like particles, each at a 10 μg dose. Three weeks after the second dose mice were challenged orally with $EDIM_{wt}$ (100-fold DD_{50}) and the quantity of RV antigen shed in fecal samples was determined up to 8 d post-challenge by ELISA. Each point represents the daily average of a group of mice (4 mice/group) with standard error of the means.

may differ, the protective efficacy of vaccine formulation will need to be tested in different animal models and eventually in humans.

In conclusion, in the present study we show that VP6-specific mucosal IgA antibodies induced by IN immunization inhibited RV infection in vitro. Most importantly, the mice immunized with the candidate vaccine containing RV VP6 protein were protected against RV challenge in vivo. Although the mechanisms of protection were not explored directly, our results give important insights on the non-serotype-specific protective immunity to RV induced by the VP6 protein immunization.

Materials and Methods

Rotavirus rVP6 production and characterization

Human RV rVP6 protein originating from a fecal sample from a RV G1P1A[8]-positive patient was produced in Sf9 insect cells by the Bac-to-Bac baculovirus expression system (Invitrogen, Cat. 10359-016) and purified by ultracentrifugation and ultrafiltration as previously described.[41,42,46] The rVP6 protein and NoV VLPs were mixed in equal quantities to produce a combination for a candidate vaccine[41] used for immunization in a series of preclinical studies including the present study. In addition, the purified rVP6 was used as an antigen in ELISA.

Immunization of mice and sample collection

Female 7-wk-old BALB/c OlaHsd mice (Harlan Laboratories) were immunized IN twice (at weeks 0 and 3) with the candidate vaccine containing a mixture of recombinant RV VP6 and NoV VLPs,[41,42] each at a dose of 10 μg per immunization point, without an external adjuvant. The immunogen was administered in a 25-μL volume by gradual inoculation in each nostril. Naïve mice receiving carrier only (sterile PBS) were used as controls. The immunization procedures were performed 3 separate times in different groups of mice (4–5 mice/group).

Blood samples, feces, and VWs were collected at week 0 (pre-immune sample) and at the time of termination at week 5. Fresh feces of mice were suspended to 10% w/v in cold TNC buffer (10 mM Tris, Cat. T1378; 100 mM NaCl, Cat. 31434N; 1 mM $CaCl_2$, pH 7.4, Cat. C5670) supplemented with 1% aprotinin (Cat. A6279) and 10 μM leupeptin (Cat. L2884) (all from Sigma–Aldrich). Serum samples were prepared according to Tamminen et al.[56] Since IN delivery is effective in inducing SIgA antibodies in different locations including the vagina,[44,45] vaginal secretions were collected by washing twice with 125 μl of cold PBS (4–5 times up and down) followed by centrifugation at 12 000 × *g* for 10 min at +4 °C. Antibody content of these VWs served as a surrogate for mucosal immune responses. All procedures were performed in accordance with the regulations and guidelines of the Finnish Animal Experiment Board.

Humoral immune response detection

Induction of VP6-specific systemic and mucosal antibody responses was determined by measuring levels of RV VP6-specific IgG and IgA in sera, fecal suspensions, and VWs. Sera of each mouse at 1:200 dilution and 2-fold dilution series were tested for total RV VP6-specific IgG antibodies by ELISA assay,[41,46] where Costar High Binding 96-well half area polystyrene plates (Corning Inc., Cat. 3690) were coated with 40 ng/well of rVP6 protein in PBS. For detection of serum and mucosal VP6-specific IgA, as well as mucosal IgG antibodies (sera, stool suspensions, and VWs tested at 1:2 or 1:5 dilutions and 2-fold dilution series), the plates were coated with 50 ng/well of rVP6. VP6-specific antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma-Aldrich, Cat. A4416)

or anti-mouse IgA (Sigma-Aldrich, Cat. A4789) at a dilution of 1:4000 and SIGMA FAST *o*-phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich, Cat. P9187-50SET). Optical density at 490 nm (OD_{490}) was measured by Victor^2 1420 microplate reader (Perkin Elmer) and a sample was considered positive if the OD_{490} was above the set cut-off value (mean OD_{490} of control mice + 3 × SD) and at least 0.1. The titers were defined as the reciprocal of the highest sample dilution with a mean OD_{490} above the cut-off value.

Immunoblotting for characterization of IgA forms

The quality of serum and mucosal samples of immunized mice was assessed by electrophoresis followed by immunodetection analysis. Pools of sera, stool samples and VWs were prepared and separated by sodium dodecyl sulfate PAGE (SDS-PAGE) with 4–15% Mini-PROTEAN® TGX™ (Bio-Rad Laboratories, Cat. 456-1083) gels under non-reducing conditions. Each sample pool was mixed with an equal volume of Laemmli sample buffer (Bio-Rad, Cat. 161-0737) without β-mercaptoethanol and incubated for 5 min at 95 °C before being electrophoresed in 1 × Tris/Glycine/SDS buffer (Bio-Rad, Cat. 161-0732) for ~45 min at 200 V. This allowed the detection of SIgA complexes followed by immunoblotting to the nitrocellulose membrane (Bio-Rad, Cat. 162-0115) with 1:1000 diluted goat anti-mouse IgA-HRP antibody (Sigma-Aldrich, Cat. A4789). Bound antibodies were detected with OPTI-4CN™ Substrate kit (Bio-Rad, Cat. 170-8235) according to the manufacturer's instructions.

Preparation of viral stocks

Fetal rhesus monkey kidney (MA104) cells were maintained in Earle's minimum essential medium (Gibco, Cat. 21090-022) supplemented with 10% fetal bovine serum (Sigma, Cat. F9665), 2 mM L-glutamine (Gibco, Cat. 25030-024), 100 U/mL penicillin (Gibco, Cat. 15140-122), and 100 μg/mL streptomycin (Gibco, Cat. 15140-122) at 37 °C in a 5% humidified CO_2 incubator. Human RV strain Wa (SGII, G1P1A[8]) and rhesus rotavirus (RRV, SGI, G3P5B[3]) were propagated in MA104 cells in the presence of 0.5 μg/mL of trypsin from porcine pancreas (Sigma-Aldrich, Cat. T4799). After observing the cytopathic effect, viral stocks were prepared from infected cells by 3 cycles of freezing–thawing, centrifuging at 2000 × *g* for 10 min for clarification, and storing at –70 °C. The viral titer of the stocks in plaque-forming units per milliliter (PFU/mL) of virus was determined by virus plaque assay.[57]

The murine RV strain $EDIM_{wt}$ (SG non-I-non-II, G3P10[16]) used in challenge study was originally obtained from Dr Richard Ward (Gamble Institute of Medical Research, Cincinnati, Ohio). The EDIM virus, pooled from fecal specimens of infected neonatal mice, was prepared as previously described.[53]

Determination of neutralizing anti-RV antibodies in vitro

Neutralizing activity of VP6-specific antibodies in sera, fecal samples, and VWs of immunized mice was determined by measuring the reduction in RV antigen production with an ELISA-based antigen reduction neutralization assay (NELISA) as described previously by others.[38,39] The functionality of the assay was first confirmed against RV Wa by using human polyclonal anti-RV serum obtained from a patient with RV infection, later always included as a positive assay control. A human polyclonal serum negative for RV was used as a negative assay control. The stool samples from human serum donors were first analyzed for RV detection by RT-PCR.[58] Both sera were then characterized for the presence or absence of RV-specific immunoglobulins by an ELISA.[59] A series of 2-fold dilutions (1:10 to 1:1280) of each groupwise pooled specimen from immunized and control mice were mixed with equal volumes of Wa RV or RRV containing 125 PFU and preincubated at +37 °C for 60 min to ensure that anti-VP6 antibodies would bind to any exposed VP6 in the virion. Confluent MA104 monolayers in 96-well plates (Nunc, Cat. 167008) were overlaid with the mixture and the plates were centrifuged at 1000 × *g* for 60 min. The virus inoculum was replaced with medium supplemented with 4 μg/mL of trypsin followed by incubation at 37 °C for 15 h, lysing of the cells by a cycle of freezing–thawing, and storage at –80 °C. RV antigen production of each sample in duplicate was measured by an ELISA using insect-cell-derived rVP6 as an internal standard. A reduction of OD_{450} value in the duplicate wells by >60% compared with the untreated virus control wells (trypsin activated RV Wa or RRV without the test sample) was considered to indicate neutralizing ability of the sample. Neutralizing titers were expressed as the reciprocal of the highest sample dilution yielding neutralization. If the highest sample dilution (1:10) failed to neutralize, a titer of 5 was assigned as the neutralization titer of the sample.

To determine whether VP6-specific antibodies were responsible for the neutralizing activity, the antibody binding sites were blocked by preincubation of VW serial dilutions with 10 μg of the rVP6 protein or with recombinant baculovirus produced unrelated GII-4 capsid VP1 protein derived from NoV[41] at +37 °C for 1 h prior to neutralization assay.

VP6-specific antibody depletion

To assess whether IgA antibodies mediated the RV inhibition, IgA of VW was depleted with magnetic bead treatment, where 6.7 × 10^7/mL of Dynabeads M-280 Streptavidin (Life Technologies, Cat. 11205D) combined with 40 μg of biotinylated goat anti-mouse IgA (Life Technologies, Cat. M31115) were incubated with the sample prior to neutralization assay. The IgA-depleted supernatant separated from the beads by the magnet (DynaMag™-Spin, Life Technologies, Cat. 12320D) was employed in the NELISA to test the residual neutralization activity. The level of IgA depletion of the VW was verified by the ELISA as described above.

Virulent murine RV challenge and detection of RV antigen in fecal samples

For the protection study 8–10 mice/group were immunized as described above. Three weeks after the last immunization half of the immunized mice were challenged orally with 1 × 10^4 FFU (100-fold the diarrheal dose DD_{50}) of the murine RV strain $EDIM_{wt}$. The feces of challenged mice were collected prior to challenge (day 0) and daily for 8 d after the challenge, and the presence of RV antigen in fecal samples suspended in TNC buffer was determined with ELISA as previously described.[60] Fecal antigen shedding was expressed as the net OD value (OD of the post-challenge fecal sample minus the OD of pre-challenge

sample from the same mouse). A sample was considered positive if the net OD_{405} was ≥0.1. Viral shedding curves for each animal were plotted and the reduction in viral load was calculated by comparing the mean area under the curve of the immunized mice to the mean area under the curve of the control group. A >50% reduction in virus shedding was considered significant protection from virus challenge. Mice were sacrificed at day 8 after the challenge. To confirm the success of immunizations, pre-challenge sera from study week 5 were tested for VP6-specific IgG and IgA antibodies as described above.

Statistical analyses

Mann–Whitney U-test was employed to assess the statistical difference between 2 independent groups. Analyses were conducted by IBM SPSS Statistics -software (SPSS Inc.) version 20.0, where $P \leq 0.05$ was considered to be statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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BRIEF REPORT

Protection against live rotavirus challenge in mice induced by parenteral and mucosal delivery of VP6 subunit rotavirus vaccine

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Abstract Live oral rotavirus (RV) vaccines are part of routine childhood immunization but are associated with adverse effects, particularly intussusception. We have developed a non-live combined RV – norovirus (NoV) vaccine candidate consisting of human RV inner-capsid rVP6 protein and NoV virus-like particles. To determine the effect of delivery route on induction of VP6-specific protective immunity, BALB/c mice were administered a vaccine containing RV rVP6 intramuscularly, intranasally or a combination of both, and challenged with murine RV. At least 65 % protection against RV shedding was observed regardless of delivery route. The levels of post-challenge serum VP6-specific IgA titers correlated with protection.

Keywords Rotavirus · VP6 · IgA · Intranasal · Intramuscular · Protection

Rotavirus (RV) causes severe gastroenteritis in infants and children under 5 years of age with high mortality and morbidity rates [1]. Currently, two live oral RV vaccines, the monovalent Rotarix® (GlaxoSmithKline) and the pentavalent Rotateq® (Merck), are licensed and used extensively [2, 3]. However, these oral vaccines are less efficacious in developing countries [4, 5] and are associated with safety concerns such as a risk of intussusception [6]. Non-live subunit RV vaccines are therefore considered as alternatives for RV immunization.

Correlates of protection against RV infection are not fully understood. Type-specific neutralizing antibodies against the external proteins VP4 and VP7 have a role in protective immunity after natural RV infection [7, 8], but their role in vaccine-induced protective immunity against severe RV gastroenteritis has not been shown. Although serum anti-RV antibody IgA titers as a correlate of protection have been disputed [9], the best surrogate marker for RV vaccine-induced protection appears to be a high level of serum RV IgA antibody targeted to the inner capsid protein VP6 [10, 11], which determines viral group (A-H) and subgroup (SGI, II, I+II, non-I/II for group A) specificity [12] and is highly conserved [13], immunogenic [14, 15] and the most abundant RV protein [12]. VP6 does not induce classical neutralizing antibodies, but it induces heterotypic cross-reactive protection in mice [16–18].

Norovirus (NoV) is another leading cause of acute gastroenteritis in children, with genogroups GI and GII being responsible for the majority of NoV cases [19]. For protection against childhood gastroenteritis, we have introduced a concept of vaccination against RV and NoV with a combined trivalent vaccine consisting of RV rVP6 protein and NoV GI.3 and GII.4 virus-like particles (VLPs) [20]. We have previously shown that a candidate combination vaccine delivered intramuscularly (IM) to mice was highly immunogenic [20], and intranasal (IN) immunization protected mice against murine RV challenge [21]. Delivery requirements for the NoV components in the induction of protective NoV immune response were published recently [22]. In this work, we compared IM and IN delivery and the combination of both for induction of VP6-specific protective immunity against RV challenge, and we examined humoral immune responses for correlation with protection.

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Human RV rVP6 protein (SGII) used for immunization and as antigen in ELISA was produced using a baculovirus expression system in Sf9 insect cells [23]. The trivalent RV-NoV combination vaccine was prepared by mixing the rVP6 tubules and NoV GI.3 and GII.4 VLPs in equal amounts [20].

Female 7-week-old BALB/c OlaHsd mice (5 mice/group) (Harlan, Horst, The Netherlands) were immunized IM or IN twice (at study weeks 0 and 3) with the trivalent vaccine containing 10 μg or RV rVP6 per immunization point. Moreover, sequential IM and IN immunizations (4 mice/group) with 10 μg of rVP6 alone were performed to determine whether administration at two distinct sites would enhance protection. No external adjuvants were used. Naïve mice receiving PBS served as controls. Pre-immune (week 0) and pre-challenge (week 5) tail blood samples of individual mice were collected, processed to obtain sera and diluted 1:100 in PBS. At week 6, mice were challenged orally with 1×10^4 focus-forming units (FFU) (100 times the diarrheal dose DD_{50}) of the murine RV strain $EDIM_{wt}$ (SG non-I/II, G3P10[16]), originally obtained from Dr. Ward (Gamble Institute of Medical Research, Cincinnati, OH). Fecal samples were collected prior to challenge (day 0) and daily for 8 days (days 1-8) after the challenge. Mice were euthanized at day 8, when whole blood samples were also collected. The protocol for the study (permission number 167-2010) was approved by the Bioethics Committee of the Instituto de Biotecnologia (Universidad Nacional Autónoma de México).

RV VP6-specific pre- and post-challenge antibody responses were determined by measuring levels of anti-VP6 IgG and IgA in individual sera at 1:100 and two-fold dilution series by ELISA according to previously published procedures [20, 21].

The presence of RV antigen in fecal samples was determined using an antigen ELISA [16]. Fecal antigen shedding was expressed as the net OD_{405} value (the OD of the pre-challenge fecal sample subtracted from the OD of the post-challenge samples of the individual mouse).

The pre-immune sera of all mice were negative for anti-VP6 IgG and IgA (data not shown). Robust systemic IgG responses were induced by each immunization route (Fig. 1a). Geometric mean titers (GMTs) of serum IgG achieved by the IM, IN and IM+IN routes were equivalent ($p = 0.663$). IN and IM+IN delivery elicited detectable IgA antibodies ($p = 0.556$), while IM immunization did not (Fig. 1b). No anti-VP6 antibodies were detected in sera of control mice prior to the challenge (Fig. 1a and b).

The quantity of RV antigen shed in fecal samples was determined up to 8 days post-challenge (Fig. 2a). A significant difference in viral shedding was detected between the mice immunized IM, IN and IM+IN and the control

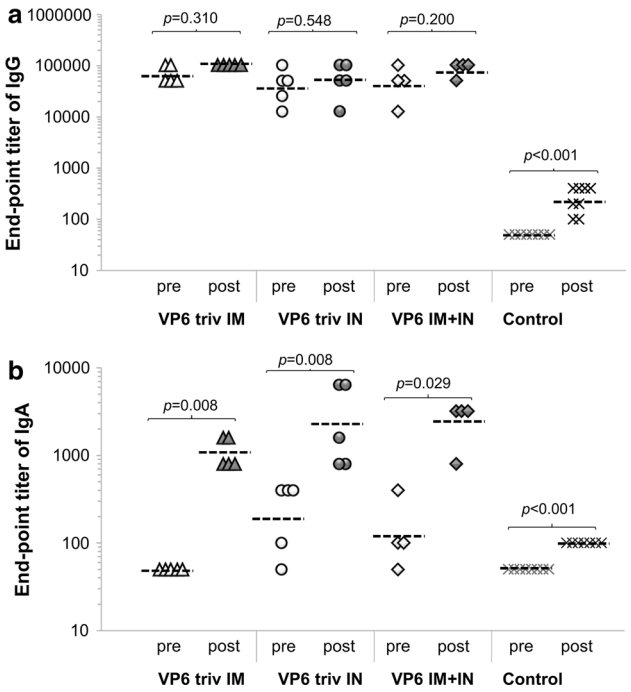

Fig. 1 Pre- and post-challenge VP6-specific IgG (**a**) and IgA (**b**) antibodies in sera of individual mice immunized IM and IN with the trivalent vaccine containing rVP6 (5 mice/group) or sequentially IM+IN with rVP6 (4 mice/group). A sample was considered ELISA positive if the optical density at 490 nm (OD_{490}) was above the set cutoff value (mean OD_{490} of control mice + 3 × SD) and ≥0.1. All control mice were combined (8 mice/group). Endpoint titers of individual mice, expressed as $\log_{10}$ of the reciprocal of the highest sample dilution giving a positive reading, as well as geometric mean titers of the groups (———) at study weeks 5 (pre-challenge tail-blood sample) and 7 (post-challenge termination sera) are shown. A titer of 50 was assigned for all negative samples, being a half of the starting serum dilution. The statistical differences between non-parametric observations of independent groups were assessed by Mann-Whitney U-test (SPSS Inc, Chicago, IL); $p \leq 0.05$ was considered to indicate a statistically significant difference

mice ($p = 0.011$), whereas the shedding between the immunized groups was not different ($p = 0.514$). The total antigen shedding of mice immunized IM and IN decreased 66 % (±12 %) and 65 % (±18 %) compared to the controls (Fig. 2a and b). Although sequential IM+IN immunization conferred a numerically higher protection rate (84 ± 5 %) (Fig. 2b), it was not statistically different from the groups immunized IM or IN.

No correlation of pre-challenge titers of IgG ($r = -0.455$, $p = 0.127$) or IgA ($r = -0.198$, $p = 0.497$) antibodies with protection rates was detected. After the RV challenge, VP6-specific serum IgG and IgA antibody titers increased in all VP6-immunized mice (Fig. 1a and b), but only the levels of the post-challenge IgA increased significantly compared to the pre-challenge levels ($p < 0.03$). Protection levels correlated with the levels of serum IgA after the challenge ($r = 0.607$, $p = 0.006$). Following the challenge, control mice also developed low levels of IgG

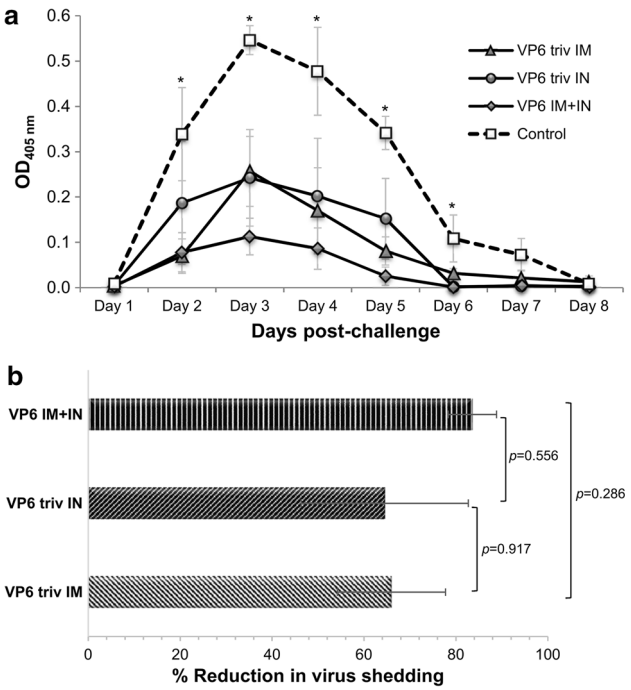

Fig. 2 Protection against RV shedding in immunized mice. Viral shedding curves (OD_{405} versus day post-challenge) for each animal were plotted and the reduction in viral load was calculated by comparing the mean area under the shedding curve of the immunized mice to the mean area under the curve of the controls. **a.** Viral shedding curves of experimental groups. Each point represents the daily average of antigen shed per group with standard error of the mean. Asterisks (*) indicate a significant difference ($p \leq 0.05$; Mann-Whitney U-test) in daily shedding between the immunized and control mice. **b.** Reductions in virus shedding of VP6-immunized mice following challenge. Mean percent reductions of the experimental groups with standard error of the means are shown. A >50 % reduction in virus shedding was considered significant protection from virus challenge, as reported previously

(GMT $\leq$ 2.5 $\log_{10}$) and IgA (GMT 2 $\log_{10}$), but the titers were significantly lower than those of the vaccinated mice ($p < 0.001$).

RV VP6 has been proposed as a subunit vaccine candidate against RV by us [14, 20, 21, 23] and others [17]. It forms different oligomeric structures *in vitro* [24], which are highly immunogenic in mice without the need for external adjuvants [14, 20, 21, 25]. Due to the repetitive multivalent antigenic structures, these oligomers are able to cross-link B-cell receptors very efficiently [26], whereas soluble VP6 generally requires an adjuvant for induction of an immune response [17]. Although the role of VP6 in protective immunity is still unclear, VP6 may be sufficient for protective immunity, as induction of protection against RV infection in mice and rabbits has been achieved with inactivated double-layered (dl) RV particles [27], dl2/6-VLPs [28] and VP6 protein [17, 21, 25] without the surface VP4 and VP7 antigens. Unlike the surface proteins, antibodies to the inner capsid VP6 are non-neutralizing. However, anti-VP6 IgA, but not IgG, is able to inhibit RV replication intracellularly [18, 29].

Human RV-derived rVP6 protein given parenterally or mucosally induced similar levels of protection against RV $EDIM_{wt}$ infection. Protection was evaluated in an adult mouse model, which is an infection model but not a disease model, by measuring reduction in fecal RV antigen shedding after viral challenge [30]. Immunized mice showed significant reduction (>65 %) in virus shedding when compared to the controls. The protection was incomplete but of the order of magnitude that is achieved against any RV disease in humans after live RV vaccination. These results indicate efficacy of the rVP6-based vaccine in conferring protective immunity against live RV challenge independently of the delivery route. Similar reduction rates were previously published for mice immunized subcutaneously with rVP6 tubules [25]. Partial protection was also achieved with inactivated dl RV particles [27] and VP6 DNA vaccines after IM administration [31]. Protection close to 100 % against shedding of two murine RV strains has been elicited after IN immunization with MBP-VP6 only after inclusion of an external adjuvant [17].

Although intestinal IgA was shown to be critical for RV clearance and protection in the mouse model [32], serum RV IgA targeted to VP6 has been considered the best surrogate marker for vaccine-induced protection in humans [10, 11]. We detected a positive correlation between post-challenge VP6-specific serum IgA levels and the RV protection rate in mice. Both parenteral and mucosal delivery induced similar clearance of RV, even though only the IN and IM+IN routes led to detectable pre-challenge serum IgA antibodies. IM immunized mice may have had undetectable pre-existing serum IgA level, which expanded rapidly after viral replication in the gut [33]. Viral replication possibly led to a significant increase in serum IgA titers in VP6-primed mice, which correlated with reduction in RV antigen shedding and therefore protection. However, evidence of a correlation of serum IgA with protection has been contradictory in animal models [34]. By contrast, correlation of protection with serum IgA has been presented in mice following IN immunization with dl2/6-VLPs and cholera toxin [28].

In conclusion, the human RV rVP6 protein induced considerable protection in mice against live heterologous RV challenge, independently of the immunization route. These results highlight the importance of non-serotype-specific antibody responses induced using the highly conserved VP6 protein in heterotypic protection.

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Conflict of interest The authors declare that they have no conflict of interest.

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