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A human norovirus-like particle vaccine adjuvanted with ISCOM or mLT induces cytokine and antibody responses and protection to the homologous GII.4 human norovirus in a gnotobiotic pig disease model

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Abstract

We inoculated gnotobiotic pigs orally/intranasally with human norovirus GII.4 HS66 strain virus-like particles (VLP) and immunostimulating complexes (ISCOM) or mutant *E. coli* LT toxin (mLT, R192G) as mucosal adjuvants, then assessed intestinal and systemic antibody and cytokine responses and homologous protection. Both vaccines induced high rates of seroconversion (100%) and coproconversion (75–100%). The VLP+mLT vaccine induced Th1/Th2 serum cytokines and cytokine secreting cells, whereas the VLP+ISCOM vaccine induced Th2 biased responses with significantly elevated IgM, IgA and IgG antibody-secreting cells in intestine. Nevertheless, both vaccines induced increased protection rates against viral shedding and diarrhea (75–100%) compared to controls; however, only 57% of controls shed virus.

Keywords

Norovirus; immunity; VLP; calicivirus; vaccine; gnotobiotic pig model

1. Introduction

Human noroviruses (HuNoV) constitute the major cause of nonbacterial, epidemic gastroenteritis worldwide [1], causing significant disease burden associated with numerous outbreaks in settings such as nursing homes [2], hospitals [3], cruise ships [4, 5] and the military [6]. Asymptomatic infections by HuNoV are also very common and may be important for HuNoV person-to-person transmission [7]. Currently, there are no NoV vaccines available for humans or animals.

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Recombinant NoV capsids expressed as VLPs constitute a safe alternative for the development of HuNoV vaccines [8]. These particles are antigenically similar to the native virion, can be produced at high yields using the baculovirus system, and are stable at low pH [9], making them suitable for oral administration and mimicking the natural route of HuNoV infection [10].

Different strategies, have been used to produce HuNoV VLP, and their antigenicity has been evaluated in mice and in humans [11, 12]. The GI Norwalk-virus (NV) VLPs were administered intranasally (IN) to BALB/c mice in the presence or absence of the mucosal adjuvant, mutant *Escherichia coli* heat-labile toxin R192G (mLT). Low doses (10–23 µg) of NV VLPs were antigenic, and higher doses (200 µg) induced specific serum IgG, fecal and vaginal antibody responses. The use of mLT enhanced the immune responses [13]. The oral administration of 250 µg, or more of NV VLPs to human volunteers elicited 90% seroconversion and increased numbers of NV-specific IgA antibody-secreting cells (ASC) in peripheral blood mononuclear cells (PBMCs), but with only 30–40% of the volunteers developing NV-specific mucosal IgA antibodies [14].

The mLT (R192G) has a single amino acid substitution in position 192 that diminishes its toxicity while still retaining its adjuvanticity when administered orally or IN [15–17]. Immunostimulating complexes (ISCOM) also constitute an alternative as both antigen delivery system and as adjuvant. These cage-like structures are composed of subunits built from the interaction of the surfactant saponins with lipid particles (cholesterol and phospholipids) [18]. Previous studies using the gnotobiotic (Gn) pig model showed that oral priming with attenuated human rotavirus (HRV) followed by 2 IN booster doses of RV inner capsid proteins, VP2/VP6 in 2/6 VLPs in combination with ISCOM, induced protection rates against diarrhea and virus shedding similar to those of the pigs that received the three-dose attenuated human rotavirus (AttHRV) vaccine [19], and boosted antibody titers and ASC responses [16, 19, 20]. This vaccine regimen provided high protection rates against diarrhea after challenge with virulent WaHRV and induced high virus neutralization and mucosal IgA antibody titers to HRV in the intestinal contents of Gn pigs [21]. Similar strategies using ISCOM-based HuNoV VLP vaccines have not yet been tested.

Immunity to HuNoV is complex, and is largely undefined due to the absence of relevant NoV animal models of enteric disease and limitations associated with human volunteer studies. The ABO histo-blood group type and the secretor status have been identified as genetic factors that may influence susceptibility to norovirus (NoV) infection and/or disease in humans [22]. We have recently demonstrated that pigs with the A⁺ and/or H⁺ phenotype had increased diarrhea and viral shedding rates compared to Gn pigs of non- A⁺ or H⁺ phenotypes, when inoculated with a GII.4 HuNoV strain [23, 24]. However, GII.2 Snow Mountain virus (SMV) infection was not dependent on the histo-blood group or secretor status [12], and a more recent study showed that VLPs of the Bristol-like GII.4 strains, that have been implicated in various outbreaks globally for the last 10 years, did not bind to the saliva of 78% of secretor-positive individuals [25], regardless of their blood type, raising questions about the role of these antigens for certain HuNoV strains in binding to and entering into target cells.

Local immunity to HuNoV is difficult to assess and few studies, either in mice or in humans, have been done [11, 12, 25]. The Gn pig constitutes a valuable model for the study of systemic and local immunity to enteric pathogens due to its resemblance in gut physiology to humans and lack of maternal antibodies and pathogen exposure, allowing assessment of primary immune responses. We recently conducted a detailed study of the local and systemic cytokine and antibody responses to a GII.4 HuNoV (HS66) strain in the Gn pig model [26], and showed that this strain induced both antibodies and Th1/Th2 cytokine responses locally and systemically.

In this study we evaluated the systemic and local immune responses of Gn pigs to one oral followed by two IN booster doses of HuNoV GII.4 HS66 strain (HuNoV-HS66) VLPs with mLT or ISCOM adjuvant, both prior to and after viral challenge with homologous virus, and the induction of protection.

2. Materials and Methods

2.1. Recombinant HS66 VLPs

The HuNoV-HS66 strain VLPs were produced using the recombinant baculovirus system, as previously described [24], with minor modifications. Briefly, a recombinant baculovirus that contained the capsid gene sequence of the HuNoV-HS66 strain was constructed [24] and, after a baculovirus stock was produced and plaque purified, the VLPs were produced by infecting the *Spodoptera frugiperda* (Sf9) cells with the HS66 baculovirus stock at a multiplicity of infection (moi) of 8, incubated at 27°C and harvested at post-inoculation day (PID) 10. The supernatants were collected and centrifuged at 3,000 X g for 30 min to pellet the cells and the assembled VLP were purified by CsCl density gradient ultracentrifugation, as previously described [24]. Particle integrity and reactivity were tested by immune-electron microscopy (IEM), antigen enzyme linked immunosorbent assay (ELISA) and Western blotting, and protein concentration was evaluated by the Bradford quantification method (Bio-Rad, Hercules, CA) [24]. The sterility of each VLP preparation was assessed by bacteriologic culture assays using blood agar plates and thioglycolate broth, at both room temperature (Rt) and at 37°C, for two weeks. The endotoxin levels were measured by using the *Limulus* ameocyte lysate assay (Association of Cape Cod, Woods, Hole, Mass) [19].

2.2. VLP-ISCOM vaccine

The HuNoV-HS66 VLPs were mixed with 2 M LiCl₂ for 30 min at Rt and then incubated overnight at -70°C to increase their capacity to bind to the ISCOM matrix [19]. The LiCl₂-treated VLPs (positively charged by the Li⁺⁺ ions) were mixed with ISCOM matrix (5 mg of ISCOM per 1 mg of HuNoV-HS66 VLPs) and then dialyzed in 0.09% NaCl solution for 72 hrs, and the association of the HuNoV-HS66 VLPs with ISCOM matrix was confirmed by IEM [21].

2.3. VLP-mLT vaccine

We used mLT containing a mutation (from arginine to glycine, R192G) at amino acid position 192 of the protein, kindly provided by J. Clements (Tulane University Medical Center, New Orleans, La.). The dose used in this study (5µg) was as previously determined

in Gn pig experiments in our lab using HRV VLPs [16]. The HuNoV-HS66 VLPs were mixed with the mLT (5 µg of mLT per 250 mg of VLPs), previously diluted in phosphate buffered saline (PBS) pH 7.2, immediately preceding their use.

2.4. HuNoV-HS66 virus challenge inoculum

A single aliquoted pool of the original human fecal sample identified as NoV/GII/4/**HS66**/2001/US (HS66 strain) [24], GenBank accession number: EU105469, was used for oral inoculation of Gn pigs using a dose of approximately 5.4×10^6 genomic equivalents (GE)/ml. A uniform inoculum pool was prepared by diluting the HuNoV original inoculum 1:10 in minimal essential medium (MEM) (Gibco, Invitrogen, Carlsbad, CA), further processing it by vortexing, centrifugating at $3000 \times g$ for 20 min and filtration through 0.8 µm followed by 0.2 µm filters. A subset of the pigs were challenged with one oral dose (10 ml of the 1:10 dilution) of the pooled inoculum after being administered 8 ml of 100mM sodium bicarbonate, to neutralize stomach acids.

2.5. Inoculation of the experimental pigs

Near-term pigs were derived by surgery and maintained in sterile isolator units as previously described [27]. The 30 Gn pigs used in this study were allocated into one of three groups: (1) one oral and two IN immunizations with 250 µg of HuNoV-HS66-VLP + 1,250 µg of ISCOM (n=8); (2) one oral and two IN immunizations of 250 µg of HuNoV-HS66 VLP + 5 µg of mLT (n=8); and the control group: (3) one oral and two IN immunizations with 1,250 µg of ISCOM matrix or 5 µg of mLT (n=14). The first inoculation of the Gn pigs was performed at 5 days post-derivation at post-inoculation day (PID) 0, and it was preceded by 8 ml of 100mM of sodium bicarbonate orally. The other two subsequent IN inoculations were at PID 10 and 21. Half of the Gn pigs in each group were euthanized at PID 28/post-challenge day (PCD) 0 (after receiving one oral and two IN doses of the respective vaccine) and the other half was challenged with the original HuNoV-HS66 inoculum at PID 28 and euthanized at PID 35/PCD 7.

2.6. A/H typing

The A/H phenotype of each pig, at 3 to 5 days of age, was determined by hemagglutination inhibition (HI) using buccal cells collected and tested as previously described [23].

2.7. Assessment of diarrhea

Daily rectal swabs were collected and diarrhea scores were noted and recorded (0=normal; 1=pasty; 2=semi-liquid; 3=watery) from PID 28–35 as described previously [24]. Samples with scores 2 and 3 were considered diarrheic. The diarrhea cumulative score of each pig represents the sum of daily rectal swab scores from PCD 1–6, and the mean cumulative score of each group is the sum of each pig's diarrhea cumulative score divided by the number of pigs in that group.

2.8. Viral shedding by RT-PCR

Viral shedding was determined using rectal swab fluids and 1:20 dilutions of intestinal contents (IC) by RT-PCR, using the primer pair Mon 431/433 [28] targeting the RdRp

region of HuNoV GII, using the same conditions as previously described [24]. However unlike as previously described, samples that were inhibited in RT-PCR, as revealed by the use of an internal control [24], were re-tested after being re-extracted using the RNeasy Mini kit (Qiagen Inc, Valencia, CA). Negative controls (rectal swabs from mock inoculated pigs and RNase-free water) for RNA extraction and RT-PCR were included in each assay. A microplate hybridization assay [29] was performed to confirm the product specificity using a probe specific for HuNoV-HS66 [24].

2.9. Viral shedding by antigen-ELISA

The antigen ELISA was performed as previously described by Cheetham et al. [24]. Samples were considered positive when the mean absorbance (450 nm) of the positive coating wells minus the mean absorbance of the negative coating wells was higher than the mean absorbance of the negative control wells plus 3 times the standard deviation.

2.10. Antibody detection by immunocytochemistry

An immunocytochemistry assay was performed to detect HS66-specific antibodies in the serum and IC of Gn pigs, as previously described [30]. For this assay a recombinant baculovirus expressing HS66 capsid was used to infect *Spodoptera frugiperda* (Sf9) cells as the HuNoV antigen source and the recombinant baculovirus infected cells or mock cells (infected with recombinant baculovirus expressing the VP4 protein of the Ku rotavirus strain) were subsequently fixed using 10% formalin in PBS. The antibody titer was defined as the reciprocal of the highest serum dilution at which brown-stained cells representing NoV antibody complexed to HuNoV-HS66 capsid antigen could be detected.

2.11 Isolation of mononuclear cells (MNC) for ELISPOT assays

Segments of the small intestine (jejunum and ileum), spleen, and blood were aseptically collected at euthanasia and processed for the isolation of MNC populations, as previously described [31, 32]. Single MNC suspensions from each tissue and blood were prepared at concentrations of 5×10^6 MNC and 5×10^5 MNC/ml in complete medium prepared with Roswell Park Memorial Institute (RPMI)1640 (GIBCO) enriched with 8% fetal bovine serum, 20mM Hydroxyethyl-Piperazine Ethanesulfonic Acid (HEPES), 2mM L-glutamine, 1mM sodium pyruvate, 0.1mM nonessential amino acids, 100 µg of gentamicin/ml, 100 µg of ampicillin/ml, and 50 µg of 2-mercaptoethanol.

2.12. ELISPOT assay for detection of HuNoV-HS66-specific antibody-secreting cells (ASC)

An ELISPOT for detection of isotype-specific (IgM, IgA and IgG) ASC was conducted using previously published methods [33, 34]. Briefly, Sf9-cell plates infected with the HuNoV-HS66 capsid gene recombinant baculovirus and mock plates were prepared and fixed as described in the antibody detection section and washed with deionized water prior to use. Single MNC suspensions from each tissue were added to duplicate wells (5×10^5 and 5×10^4 /well). Plates were then incubated at 37°C for 12 hrs in 5% CO₂ and then washed three times with PBS buffer and incubated at 37°C for 2 hrs with 100 µl/well of HRP-labeled antibody: goat anti-pig IgM (µ) (KPL) (0.25 µg/ml); IgA (Serotec) (0.3 µg/ml); or IgG (KPL) (0.25 µg/ml). Plates were then washed three times in PBS buffer and developed

with tetramethylbenzidine (TMB) (KPL Inc.) for 2 hrs at Rt. The number of virus-specific ASC were determined by counting blue spots in the wells, using a light microscope, and were reported as the number of virus-specific ASC per 5×10^5 MNC, after any background spots (< 3), evident on the mock plates, were subtracted.

2.13. ELISPOT assay for detection of HuNoV-HS66-specific cytokine-secreting cells (CSC)

A cytokine ELISPOT for detection of pro-inflammatory (IL-6), Th1 (IL-12 and IFN- γ), Th2 (IL-4) and Th2-T-reg (IL-10) CSC was performed as previously described [35] with minor modifications. Before being added to Multiscreen-IP sterile 96-well plates (Millipore, Bedford, MA) at concentrations of 5×10^5 and 5×10^4 /well, the cells were stimulated with 50 $\mu\text{g/ml}$ of CsCl-purified HuNoV-HS66 virus-like particles (VLPs) [24] or 10 $\mu\text{g/ml}$ of phytohemagglutinin (positive control) or RPMI (negative control). Plates were then incubated at 37°C in 5% CO₂ for 48 h. The numbers of CSC were counted using an ImmunoSpot series 3A analyzer (Cellular Technology Ltd., Cleveland, OH) and expressed as CSC per 5×10^5 MNC. The HuNoV-HS66-specific CSC numbers were computed after the numbers of CSC (< 4) in the controls (RPMI-stimulated cells) were subtracted from the HS66 VLP-stimulated cells.

2.14. Cytokine concentrations in the serum and IC quantitated by cytokine ELISA

Blood was collected from pigs at PID 0, 2, 7, 10, 21, 28, 30 and 35 and intestinal contents were collected at euthanasia (PID 28/PCD 0 and PID 35/PCD 7). Serum samples were processed and stored at -20°C [35]. The IC samples were diluted 1:2 in MEM with a protease inhibitor cocktail to prevent cytokine degradation [36]. The serum and IC were immediately frozen at -20°C until further testing. An ELISA test was performed to detect IL-6, IL-12, IFN- γ , IL-4 and IL-10 as previously described [35]. In addition to those cytokines, an ELISA to detect the porcine Th2 cytokine, IL-13 was also performed (anti-Hu IL-13 kit, Biosource, Camarillo, CA). Plates were coated with a polyclonal anti-human IL-13 (1.3 $\mu\text{g/ml}$), samples were added and then a biotin-labeled monoclonal antibody (MAb) to human IL-13 (1.5 $\mu\text{g/ml}$) (Biosource) was added followed by the addition of streptavidin-HRP (0.1 $\mu\text{g/ml}$) (Biosource) and TMB (KPL). Standard curves were generated using recombinant porcine IL-6 (Biosource, Camarillo, CA), IL-12 (R & D Systems), IFN- γ , IL-4, IL-10 and IL-13 (Biosource). A computer-generated four-parameter curve-fit was used to calculate the concentration of each cytokine. The detection sensitivity limit for the reactions was 7 pg/ml for IL-6, IL-12, IFN- γ , IL-4, IL-10, and IL-13.

2.15. Statistical analysis

The cytokine concentrations, ASC (PID 28 and 35) and CSC (PID 28 and 35) numbers were compared among and within groups using Kruskal-Wallis rank sum test (nonparametric). The one-way analysis of variance (ANOVA), followed by Duncan's multiple range test was used to compare the antibody GMT among different groups. The seroconversion and coproconversion rates and the percentage of pigs with diarrhea and shedding were compared using the Fisher's exact test. Statistical significance was assessed at $P < 0.05$.

3. Results

3.1. A/H phenotype of Gn pigs

All pigs were typed for the presence of the A and/or H antigen on their buccal cells by hemagglutination inhibition (HI). Of all pigs used in the study, 16/30 (53 %) had the A⁺/H⁺ phenotype and 13/30 (43%) had A⁺/H⁻ phenotype. One of the 30 pigs (4%) was a non A/H type and belonged to the HuNoV-HS66VLP+ISCOM vaccinated group of pigs that was euthanized at PID 28. All pigs, including controls challenged with HuNoV-HS66 and euthanized at PID35/PCD7 had the A⁺/H⁺ phenotype.

3.2. The VLP vaccines protected pigs after HuNoV-HS66 challenge, with the VLP+mLT vaccine inducing complete protection

A subset of pigs in each group was euthanized at PID 28/PCD 0 and the other at PID 35/PCD 7, after receiving the HuNoV-HS66 challenge orally. Diarrhea and viral shedding were evaluated (Table 1). In the VLP+ISCOM vaccinated group, only 1/4 (25%) pigs had diarrhea for 1 day and in the VLP+mLT group none of the 4 pigs developed diarrhea after HuNoV-HS66 challenge. However, 7/7 (100%) of the pigs in the control group (ISCOM and mLT) had diarrhea after challenge. None of the pigs in either vaccinated group had detectable virus shedding by RT-PCR, microwell hybridization or antigen-ELISA; however, in the control group only 4/7 (57%) pigs shed virus. The mean days of diarrhea and the mean diarrhea score for the VLP+ISCOM and the VLP+mLT vaccinated groups were both significantly reduced when compared to the control group. The Gn pigs in the VLP+ISCOM vaccinated group had a 75% protection rate against diarrhea and those in the VLP+mLT group had 100%. Both groups had a 100% protection rate against viral shedding.

3.3. The VLPs induced systemic and intestinal antibody responses in Gn pigs regardless of the adjuvant used but the VLP+ISCOM vaccine induced significantly higher IgM and IgA antibody titers in IC and IgG antibody titers in serum and IC pre-challenge compared to the VLP+mLT vaccine or controls

The detailed antibody responses in the serum and IC are depicted in Fig 1a and the seroconversion and coproconversion rates are shown in Table 1. One-hundred-percent of the pigs that received either of the vaccine regimens (VLP+ISCOM or VLP+mLT) had seroconverted to HuNoV-HS66 at virus challenge at PID 28/PCD 0 (Table 1), compared to none of the pre-challenge controls. The IgM antibodies were first detected at PID 10 at low titers after the oral priming dose of VLP+ISCOM and VLP+mLT vaccines (GMT=12 and 9, respectively), and increased significantly at PID 21 and 28 in both vaccinated groups, when compared to controls (Fig 1a). However at PID 35/PCD 7, the IgM antibody titers only in the VLP+mLT group were significantly higher than controls.

The IgA antibodies were first detected in the serum at PID 21 in both VLP+ISCOM and VLP+mLT groups (GMT=8 and 6, respectively), and were significantly elevated at PID 28/PCD0 and at PID 35/PCD 7 in both vaccinated groups, when compared to controls.

Serum IgG antibodies were first detected at low levels at PID 21, in both vaccinated groups and were significantly higher than controls. Pre-challenge at PID 28/PCD 0, significantly

higher titers of IgG antibodies were detected in the VLP+ISCOM group (GMT=381, range 20–2,560), compared to the VLP+mLT group (GMT= 44, range 5–320). However post-challenge at PID 35/PCD 7, the IgG GMT remained the same in the VLP+ISCOM group, although in one pig, the titer increased (640 to 2,560). In the VLP+mLT group, the IgG antibody GMT increased 2-fold at PID 35/PCD 0 (GMT = 95), compared to that at PID 28 (GMT=44), and the post-challenge antibody titers were significantly higher in both groups when compared to controls (Fig. 1a).

The IgM, IgA and IgG antibody titers were also measured in the IC of the euthanized pigs at challenge (PID 28/PCD 0) and post-challenge (PID 35/PCD 7) (Fig.1b). One-hundred-percent of the pigs that received the VLP+ISCOM and 75% of those that received the VLP +mLT vaccine regimens coproconverted (PID 28/PCD 0) (Table 1) with either or both IgA and IgG antibodies in the IC. Pre-challenge antibody (IgM, IgA and IgG) titers were significantly elevated in the IC of only the VLP+ISCOM vaccine group compared to the other groups (Fig 1b.), and titers did not increase post-challenge (VLP+ISCOM), except for 1 pig that had diarrhea at PCD 2 (GMT=40 to 160; 640 to 2,560, for IgA and IgG, respectively). Titers increased for both IgA and IgG (GMT=28 to 40; 5 to 34, respectively) in the VLP+mLT group at PID 35/PCD 7 compared to those at PID 28/PCD0, and both IgA and IgG antibodies were significantly increased post-challenge compared to controls. Only 1 control pig coproconverted after viral challenge, with an IgA titer of 320 in the IC (Table 1).

3.4. Pre- and post-challenge, VLP+ISCOM vaccine elicited higher numbers of IgM, IgA and IgG ASC locally (intestine) compared to systemically (spleen and blood). ASC isotypes were also significantly elevated in the intestine of VLP+ISCOM versus VLP+mLT or controls groups, whereas VLP+mLT induced higher ASCs in spleen post-challenge

The results of the ELISPOT assay for detection of virus-specific ASC in HuNoV-HS66-inoculated and control Gn pigs euthanized at PID 28/PCD 0 and PID 35/PCD 7 are shown in Fig. 2. The numbers of IgM, IgA and IgG ASC elicited in VLP+ISCOM and VLP+ mLT groups were significantly higher than in the controls at both PID 28/PCD 0 and PID 35/PCD 7 for all tissues, except for IgM post- and IgA and IgG pre-challenge in blood. At both PIDs, higher numbers of IgA and IgG ASC were elicited by both vaccine regimens, locally (intestine), compared to systemically (spleen). The highest numbers of IgA and IgG ASC were detected in the intestine of the VLP+ISCOM group at PID 28/PCD 0 and PID 35/PCD 7, respectively (150 and 136; 150 and 157 ASC / 5×10^5 MNC, respectively).

At PID 28/PCD 0, significantly higher numbers of IgM ASC were detected in the intestine of the VLP+ISCOM vaccinated pigs compared to the other groups and at PID 35/PCD 7 higher numbers (2-fold) were detected after HuNoV-HS66 challenge (from 59 to 136 ASC/ 5×10^5 MNC). In the VLP+mLT vaccinated group, fewer IgM ASC (6 and 38, respectively) were detected at both PIDs. In spleen, pre- and post-challenge, the IgM ASC numbers were also significantly higher in both vaccinated groups, compared to controls, although at lower numbers compared to those observed in intestine.

The IgA and IgG ASC numbers were also significantly higher in the intestine of the VLP +ISCOM pigs compared to the other groups, both pre- and post-challenge, although no boosting in the magnitude of response to either vaccine was observed post-challenge,

consistent with the protection observed. In spleen, the IgA and IgG ASC numbers were significantly higher in both vaccinated groups, compared to controls, both pre- and post-challenge, but with all ASC isotypes significantly elevated in spleen post-challenge in the VLP+mLT versus VLP+ISCOM group. Significantly higher numbers of IgA and IgG ASC were observed in blood (PBMC) of the VLP+mLT vaccinated pigs pre-challenge compared to VLP+ISCOM and controls, and in both vaccinated groups post-challenge, compared to controls.

3.5. Both VLP vaccines induced a significant increase in serum Th1 (IFN- γ) concentrations only after homologous viral challenge

The cytokine ELISA results are summarized in Fig.3. At PID 30/PCD 2, a non-significant transient increase in the pro-inflammatory (IL-6) cytokine was detected in the control group, and at PID 35/PCD 7 increased levels were observed in the serum of both vaccinated groups. The Th1 cytokine (IL-12) was detected constitutively in the serum of pigs from all groups at birth. It was significantly elevated in the serum of only the VLP+mLT group at PID 28/PCD 0, compared to the other groups. A transient (but not significantly elevated) peak of the Th1 cytokine IFN- γ was detected in serum of the VLP+mLT group at PID 2 (after one oral dose of the vaccine), with a significant increase only post-challenge at PID 35/PCD 7. For the ISCOM+VLP vaccinated pigs, a low peak of IFN- γ was detected at PID 28/PCD 0 and increased significantly after challenge at PID 30/PCD 2 and at PID 35/PCD 7, compared to controls.

The Th2 cytokine IL-4 was significantly elevated in the serum of both vaccinated groups at PID 2 and 7 compared to controls and in the serum of the VLP+mLT pigs at PID 10 and 21, compared to the other groups. The other Th2 cytokine IL-13 was significantly elevated in the serum of only the VLP+mLT vaccinated pigs at PID 28/PCD 0 (64 pg/ml) and in the serum of both vaccinated groups post-challenge (35/PCD 7), although not significantly, compared to controls. The Th2-T-reg cytokine (IL-10) peaked (52 pg/ml) at PID 10 in the serum of the VLP+ISCOM pigs, and later (57 pg/ml) at PID 28/PCD 0 in the VLP+mLT group. Higher, but not significantly, levels of IL-4 and IL-10 were also detected in serum of the control group after viral challenge at PID 35/PCD 7.

3.6. The Th1 cytokine (IFN- γ) was significantly elevated post-challenge in IC only in the VLP+mLT vaccinated pigs

The cytokine concentrations were measured in the IC of the pigs after euthanasia (PID 28/PCD 0 and PID 35/PCD7) (data not shown). Only low (IL-6, IL-12, IL-13) to moderate (IL-4 and IL-10) cytokine concentrations were detected in the IC of both vaccinated groups at both PID/PCDs, that were not significantly higher than those in the IC of the controls, presumably due to the instability of the secreted cytokines in the IC. Significantly higher concentrations (166 pg/ml) of only the Th1 cytokine (IFN- γ) were detected in the IC of the VLP+mLT group one week post-challenge (PID 35/PCD 7), when compared to the other groups.

3.7. Th1 (IL-12 and IFN- γ), pro-inflammatory (IL-6) and Th2/T-reg (IL-10) CSC were elicited at higher numbers systemically only in the VLP+mLT vaccinated Gn pigs pre-challenge

and significantly higher Th2 (IL-4) CSC numbers were elicited locally and systemically post-challenge in the VLP+ISCOM vaccinated pigs

Generally, higher numbers of CSC were elicited in both vaccinated groups compared to controls post-challenge (Fig 4a and 4b). However, only in systemic tissues (spleen and PBMC) and only in the VLP+mLT pigs were IL-6 and IFN- γ CSC numbers significantly elevated (28 and 23 CSC/ 5×10^5 MNC; 58 and 25 CSC/ 5×10^5 MNC, respectively) pre-challenge. The IL-12 CSC numbers were also significantly elevated in spleen and IL-10 CSC numbers in PBMC of the VLP+mLT pigs pre-challenge.

Post-challenge, the highest numbers of the pro-inflammatory IL-6 CSC were detected in blood (PBMC) of both vaccinated groups, although not significantly higher compared to controls. In spleen, significantly higher numbers of Th1 (IL-12 and IFN- γ) CSC were observed in the VLP+ISCOM (140 and 98 CSC/ 5×10^5 MNC, respectively) and in the VLP+mLT (328 and 39 CSC/ 5×10^5 MNC, respectively) vaccinated pigs, compared to controls. Significantly higher numbers of Th2 (IL-4) CSC were detected only in the VLP+ISCOM pigs in all tissues and in blood (34–48 CSC/ 5×10^5 MNC), compared to the other groups and only IL-4 CSC were significantly elevated in the intestine. Significantly higher numbers of Th2/T-reg (IL-10) CSC were observed in blood (PBMC) of both vaccinated groups (320 CSC/ 5×10^5 MNC in mLT+VLP and 663 CSC/ 5×10^5 MNC in ISCOM+VLP), compared to controls.

4. Discussion

The HuNoV infections are usually self-limiting and transient; however, the increasing numbers of NoV outbreaks worldwide, particularly ones associated with GII.4 strains, and their impacts in settings such as nursing homes, hospitals, and the military indicate that the development of effective vaccines would be beneficiary [5, 37]. In this study, we evaluated the immunogenicity of HuNoV-HS66 VLPs administered in three doses (one oral and 2 IN) in conjunction with each of two adjuvants (ISCOM or mLT), and evaluated protection elicited by both vaccine regimens against HuNoV-HS66 oral challenge.

None of the vaccinated pigs in either the VLP+ISCOM or VLP+mLT groups shed virus and the protection rate against diarrhea was 75% and 100%, respectively. However, post-challenge, only 57% of control pigs shed detectable virus and 43% coproconverted with IgA antibodies by PCD 7. A previous study in our lab [23] showed that the pigs with the A⁺ and/or H⁺ phenotype had increased diarrhea and viral shedding rates compared to non- A⁺ and H⁺ Gn pigs after infection with the GII.4 HuNoV-HS66 strain. Because all the pigs in the study were typed for the A/H phenotype and all pigs that were challenged with HuNoV had A⁺/H⁺ phenotype, we cannot conclude that the A/H phenotype influenced the outcome of the study. However, the older age at which the Gn pigs were challenged could have played a role in the lack of virus shedding detected in the control group. In our previous studies we inoculated the pigs at an earlier age (3-to-7-day-old compared to 40-day-old) and maybe Gn pigs are more resistant to infection at an older age. However, because 7/7 (100%) of pigs in both the ISCOM and mLT control groups had diarrhea compared to only 1 pig in the VLP+ISCOM group, we assume that viral replication, undetectable at very low levels, may have occurred in the control pigs.

The immunogenicity of HuNoV VLPs, administered alone or with mLT, has been evaluated in mice, in which the VLPs induced both systemic and mucosal (fecal and vaginal) immune responses [13]. The immune responses to NV VLPs have also been determined in human volunteers [14]. However, previous exposures to HuNoV can be confounding variables in interpreting the magnitude of the immune responses elicited by these VLPs. Therefore, Gn animals, such as pigs and calves constitute valuable models for the study of both local and systemic immune responses to animal NoV VLPs [17] and HuNoV VLPs.

The high seroconversion rates observed in our study (100% in both vaccinated groups pre-challenge) are very similar to those previously observed in humans (90%) [14] and in mice [11] orally immunized with 2 or 3 doses of NV VLPs. Serum antibody responses were also induced in Gn calves immunized with bovine norovirus (BoNoV) VLPs and various adjuvants, including mLT and ISCOM, and routes of inoculation [17]. Low to moderate serum IgG antibody titers (GMT= 60 to 100) were detected pre-challenge in calves immunized IN with BoNoV VLP+mLT. Post-challenge, the highest increases in serum IgG antibody titers were also detected in this group, followed by the VLP+mLT/orally vaccinated calves [17].

In IC, we detected significantly elevated IgA and IgG antibodies in both vaccinated groups post-challenge (PID 35/PCD7), but only in the VLP+ISCOM group were IgM, IgA (highest) and IgG antibody titers significantly elevated pre-challenge, compared to the other groups, likely the local presence of these antibodies as also reflected by the significantly elevated numbers of ASC, with also IgA highest in the gut at challenge played a role in the protection induced by the VLP+ISCOM vaccine. Mucosal (from saliva, feces, vaginal and semen washes) anti-NV IgA was also observed in only 35% of volunteers orally immunized with the same dose of NV VLP (without adjuvant) as used in this study [14]. Moreover in our study, the coproconversion rate (IgA or IgG antibodies in IC) pre-challenge was higher (75% in the VLP+mLT group and 100% in the VLP+ISCOM group) than for the non-adjuvanted NV VLPs in humans, likely reflecting the enhanced intestinal antibody responses induced by mucosal adjuvants. The high IgA antibody titers observed in IC in the VLP+ISCOM group differed from results of the BoNoV VLP study where fecal IgA antibodies were detected only in 2/4 calves that received either 2 or 3 doses of VLP+mLT/IN [17]. Also in contrast to our pig studies, no fecal IgG antibodies were detected pre-challenge in any of the calves in the VLP+ISCOM or VLP+mLT (oral or IN) groups. However, after BoNoV challenge, fecal IgG antibodies were elicited in calves of all vaccination regimens [17].

In comparing intestinal and systemic ASC responses the highest numbers of ASC of all three isotypes were detected locally (intestine) in the VLP+ISCOM vaccinated pigs. These findings reinforce that the administration of VLP by the oral plus IN combined routes constitutes an efficient vaccine strategy likely mimicking the natural routes of HuNoV infection and also reflect the particulate and immunogenic nature of the VLPs and the efficacy of the ISCOM adjuvant to induce mucosal antibodies, especially IgA. The magnitude of the ASC responses in the intestine however, was not boosted by the HuNoV-HS66 challenge, likely reflecting the high degree of protection observed (no virus shedding). Because 10-fold higher pre-challenge ASC numbers were elicited in the intestine of the 3-

dose vaccinated pigs in this study, compared to those detected at PID 28 in naïve Gn pigs orally inoculated with the same HuNoV-HS66 challenge inoculum [26], multiple doses of HuNoV non-replicating VLP vaccines may be needed to elicit strong immune responses and possibly protection [14].

Low to moderate, but significantly elevated, IgM, IgA and IgG ASC responses were detected post-challenge systemically (spleen and blood) in both vaccinated groups compared to controls, but were induced at a higher magnitude in the VLP+mLT group compared to the VLP+ISCOM group (except for IgM and IgG in PBMC). Systemic IgA ASC responses were also detected from the PBMCs of volunteers who were orally vaccinated with NV VLPs and the numbers of ASC elicited did not differ significantly among different VLP doses (250 µg to 2000 µg). However, unlike in the pig model, the intestinal ASC responses could not be evaluated in the human volunteers [14].

The relatively higher concentrations (166 pg/ml) of IFN-γ, compared to the other cytokines, in the IC of the VLP+mLT pigs corroborates those concentrations found in serum and likely represents the boosted memory Th1 responses previously elicited by the HuNoV VLPs [14]. Increased concentrations of IFN-γ were also detected in IC of the VLP+mLT vaccinated pigs post-challenge. In a human volunteer study, the IFN-γ levels were measured in the PBMC culture supernatants of NV-VLP vaccinees after in vitro NV-VLP stimulation, and significant increases in IFN-γ production were detected in PBMC collected 21 days after the first oral NV VLP immunization, in comparison to pre-immunization levels; however, no IFN-γ responses were detected in the PBMC supernatants of volunteers that received a high dose of VLP (2000 µg), and the authors suggested that the lack of response could be due to tolerance induced by the high VLP dose [14].

The Th2 (IL-4) concentrations were significantly higher in the serum of both vaccinated groups at PID 2, 7 and 21, and in the serum of the VLP+mLT group at PID 10, compared to the other groups. In the adult volunteer study in which IFN-γ was detected in the PBMC culture supernatant, the IL-4 concentrations were also evaluated. Because no IL-4 was detected, the authors concluded that a dominant Th1 response was induced by the NV VLPs [14]. However, this must be interpreted carefully because other Th2 cytokines exist that were not evaluated; therefore, a more complete panel of cytokines should be evaluated before this conclusion is reached. We also evaluated the concentrations of another Th2 cytokine (IL-13) in the serum of the Gn pigs and significantly higher levels were detected at PID 28 and PID 30/PCD2 in the VLP+mLT group, when compared to the other groups. The IL-13 plays an important role in the modulation of the immune responses, especially to intracellular parasites [38]. Interestingly, the increased levels of IL-13 post-challenge (PID 35/ PCD 7) in the serum of both vaccinated groups paralleled that of the pro-inflammatory IL-6 cytokine, perhaps reflecting the effect of IL-6 in inducing the anti-inflammatory IL-13.

The pro-inflammatory (IL-6), Th1 (IL-12 and IFN-γ), Th2 (IL-4) and Th2-T-reg (IL-10) CSC responses were also evaluated both locally (intestine) and systemically (spleen and blood). To our knowledge, intestinal CSC responses have not been examined previously for NoV infection in any species. The VLP+mLT group had higher pro-inflammatory, Th1 and Th2/T-reg CSC numbers in systemic tissues pre-challenge, compared to the VLP+ISCOM

group. This balanced Th1/Th2 CSC profile pre-challenge coincided with complete protection against virus shedding and diarrhea observed in this group.

The Th2 (IL-4) CSC numbers were significantly elevated post-challenge only in the VLP +ISCOM group, both locally (intestine) and systemically (spleen and blood), when compared to the other groups. In comparison, no IL-4 secretion by CSC isolated from the blood of human volunteers previously immunized with one or two doses of NV VLPs was detected [14]. Because the IL-4 CSC responses were enhanced only in the VLP+ISCOM group, this suggests that ISCOM may have influenced this Th2 response compared to the lack of adjuvant with NV VLPs in human trials.

In conclusion, both VLP+ISCOM and VLP+mLT vaccine regimens induced high rates of seroconversion, coproconversion and protection against viral shedding and diarrhea when compared to the controls. Although not all control pigs shed virus, all had diarrhea with significantly higher diarrhea scores compared to the vaccinated pigs. Pre-challenge, the VLP +ISCOM vaccine induced higher IgA and IgG ASC both systemically and locally and IgA and IgG antibodies in IC, with significantly elevated Th2 (IL-4) intestinal CSC post-challenge. In contrast, the VLP+mLT induced higher systemic Th1 and Th2/T-reg CSC numbers pre-challenge and the highest Th1 cytokine responses in IC, post-challenge. Collectively, our results indicate that VLP+mLT induced a more balanced Th1/Th2 response in serum pre-challenge, whereas the VLP+ISCOM induced a more Th2 biased response pre-challenge, but with significantly higher systemic Th1 (IFN- γ) and local and systemic Th2 (IL-4) CSC responses post-challenge. Both vaccines induced protection upon challenge with HuNoV, although protection against diarrhea was less efficient in the latter group. Based on these data, use of adjuvants with VLPs induce balanced Th1/Th2 immune responses but with significantly elevated intestinal ASCs (mLT) or more Th2 biased responses with significantly higher intestinal IgA and IgG ASCs (ISCOM), but can both induce adequate protection.

To our knowledge this is the first study to evaluate the immunogenicity and protective potential of GII HuNoV VLPs and also to assess, in detail, both local and systemic immune responses to HuNoV VLP vaccines prior and subsequent to homologous viral challenge in an experimental animal model.

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Fig. 1.

a. Isotype-specific (IgM, IgA, and IgG) antibody responses in the serum of gnotobiotic pigs vaccinated with 1 oral and 2 IN doses of each vaccination regimen (HuNoV-HS66 VLP +mLT or ISCOM) or controls. Symbols: $\blacklozenge$ HuNoV-HS66 VLP+ISCOM, $\blacksquare$ HuNoV-HS66 VLP+mLT, $\triangleleft$ controls (ISCOM and mLT). Different letters denote statistical difference ($P < 0.05$).

b. Isotype-specific (IgM, IgA and IgG) antibody responses in the IC of Gn pigs vaccinated with 1 oral and 2 IN doses of each vaccination regimen (HuNoV-HS66 VLP+mLT or ISCOM) or controls. Symbols: $\square$ HuNoV-HS66 VLP+ISCOM, $\blacksquare$ HuNoV-HS66 VLP+mLT, $\square$ or controls (ISCOM and mLT). Different letters denote statistical difference ($P < 0.05$).

Fig. 2.

Mean isotype-specific (IgM, IgA and IgG) antibody-secreting cells (ASC) in the intestine, spleen and PBMC of Gn pigs vaccinated with 1 oral and 2 IN doses of each vaccination regimen (HuNoV-HS66 VLP+mLT or ISCOM) or controls. Symbols: ▨ HuNoV-HS66VLP+ ISCOM, ■ HS66VLP VLP+mLT, □, controls (ISCOM and mLT). Different letters denote statistical difference ($P < 0.05$).

Fig. 3.

Th1 (IL-12 and IFN- γ), proinflammatory (IL-6), Th2 (IL-4 and IL-13) and Th2/T-reg (IL-10) cytokine concentrations in the serum of Gn pigs vaccinated with 1 oral and 2 IN doses of either vaccination regimen (HuNoV-HS66VLP +ISCOM or mLT) or controls. Symbols: $\blacklozenge$ HuNoV-HS66 VLP+ISCOM, $\blacksquare$ HuNoV-HS66 VLP+mLT, $\triangle$ controls (ISCOM and mLT). Different letters denote statistical difference ($P < 0.05$).

Th1

Pro-inflammatory

a

Th2/T-reg

b

Fig. 4.

a and b. Mean numbers of Th1 (IL-12 and IFN- γ), proinflammatory (IL-6), Th2 (IL-4) and Th2/T-reg (IL-10) cytokine-secreting cells (CSC) in the intestine, spleen and PBMC of Gn pigs vaccinated with 1 oral and 2 IN doses of each vaccination regimen (HuNoV-HS66VLP +ISCOM or mLT or controls. Symbols: HuNoV-HS66 VLP+ISCOM, HuNoV-HS66 VLP+mLT, , controls (ISCOM and mLT). Different letters denote statistical difference ($P < 0.05$).

Table 1

Fecal virus shedding, diarrhea, seroconversion, protection rates and coproconversion in Gn pigs vaccinated with either HuNoV-HS66 VLP+ISCOM, HuNoV-HS66 VLP+mLT or controls (ISCOM and mLT)

Vaccine # pigs	Virus shedding # pigs ^a /total % prot ^b	Diarrhea # pigs ^c /total % prot ^b	Diarrhea (Mean days) (Mean score) ^c	% IgA or IgG seroconversion ^d PID28-PCD0/ PID35-PCD7 (Ab titer range)	% IgA or IgG coproconversion ^{de} PID28-PCD0/ PID35-PCD7 (Ab titer range)
VLP+ISCOM (n= 4)	0/4 (100)	1/4 (75)	0.3 ^{bf}	100/100 ^A (20–2,560)	100/100 ^A (20–2,560)
VLP + mLT (n= 4)	0/4 (100)	0/4 (100)	0 ^B	100/100 ^A (40–2,560)	75/100 ^A (5–2,560)
Controls (n=7)	4/7 (NA)	7/7 (NA)	2 ^A	0/0 ^B (5)	0/43 ^B (5–320)

^a Virus shedding was detected by RT-PCR and hybridization assay after HuNoV-HS66 challenge (PID28–35)

^b Protection rate was based on the control group (adjuvants alone) lack of protection against diarrhea. However, only 57% of the pigs in the control groups shed virus after HuNoV-HS66 challenge, although 100% had diarrhea

^c Diarrhea present if fecal swab scores were 2 after HuNoV-HS66 challenge. Represents the sum of daily rectal swab scores (PCD 1–6) of each pig divided by the number of pigs in that group

^d Seroconversion and antibody titers in intestinal contents determined by immunocytochemistry at PID 28/PID 35, values in parentheses reflect the Ab titer range detected.

^e coproconversion with either IgA or IgG antibodies at PID 28/PID 35.

^f Values in columns with different superscript letters (A or B) differ significantly (p<0.05)