Antigenicity and immunogenicity of HA2 and M2e influenza virus antigens conjugated to norovirus-like, VP1 capsid-based particles by the SpyTag/SpyCatcher technology

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ABSTRACT

Virus-like particles (VLPs) modified through different molecular technologies are employed as delivery vehicles or platforms for heterologous antigen display. We have recently created a norovirus (NoV) VLP platform, where two influenza antigens, the extracellular domain of matrix protein M2 (M2e) or the stem domain of the major envelope glycoprotein hemagglutinin (HA2) are displayed on the surface of the NoV VLPs by SpyTag/SpyCatcher conjugation. To demonstrate the feasibility of the platform to deliver foreign antigens, this study examined potential interference of the conjugation with induction of antibodies against conjugated M2e peptide, HA2, and NoV VLP carrier. High antibody response was induced by HA2 but not M2e decorated VLPs. Furthermore, HA2-elicited antibodies did not neutralize the homologous influenza virus in vitro. Conjugated NoV VLPs retained intact receptor binding capacity and self-immunogenicity. The results demonstrate that NoV VLPs could be simultaneously used as a platform to deliver foreign antigens and a NoV vaccine.

1. Introduction

In the absence of an effective vaccine, new and reemerging pathogens such as influenza viruses and coronaviruses can cause rapidly spreading epidemics or pandemics with extensive morbidity and mortality rates (Jin et al., 2020). Modern vaccine technologies provide tools to develop novel vaccines containing only the essential antigenic parts of the pathogens to address the safety and production challenges encountered with traditional vaccines derived from live attenuated or inactivated whole pathogens (Karch and Burkhard, 2016). (Poly)saccharides, protein domains or even short immunodominant peptides derived from viruses or bacteria might be sufficient to induce protective immunity when displayed on the surface of larger carriers (Tan et al., 2011; Tinto et al., 2015).

Self-assembling non-infectious protein-derived nanoparticles, such as virus-like particles (VLPs), have emerged as an attractive technology in vaccine development. The intrinsic immunogenic properties of the VLPs make them promising stand-alone vaccine candidates for many diseases (Karch and Burkhard, 2016). These particles can also be exploited as delivery vehicles or platforms for heterologous antigen display (Kushnir et al., 2012), conjugating vaccine antigens to nanoparticle scaffold through different coupling approaches including genetic fusion (Neirynck et al., 1999), chemical cross-linking (Peacey et al., 2007) or isopeptide bioconjugation (e.g. the SpyTag–SpyCatcher technology) (Brune et al., 2016). Nanoparticles with repetitive structures enable expression of conjugated foreign antigens on the particle surface at high density and thus more potent antigen presentation and induction of immune responses (Fifis et al., 2004). This approach allows rapid and simple modification of the selected vaccine antigens, facilitating the development of novel vaccines in the threat of (re)emerging pandemics without a need for laborious and time-consuming pathogen cultivation and inactivation/attenuation processes. Several chimeric VLPs or other subviral particles carrying different drug substances and vaccine antigens have been created from e.g. hepatitis viruses (Neirynck et al., 1999), human papillomavirus (Murata et al., 2009), murine polyomavirus (Wibowo et al., 2013), rotavirus (Peralta et al., 2009; Tamminen et al., 2021; Philip and Patton, 2021), as well as bacteriophages (Tissot et al., 2010), and some of these have been demonstrated to be...
auspicious vaccine candidates in different stages of clinical trials.

Norovirus (NoV) is a common cause of acute gastroenteritis, affecting millions of people across all age groups worldwide. The current NoV vaccine development relies mainly on extensively studied ~30-nm VLPs (Atmar et al., 2011; Blazevic et al., 2011) formed by a shell (S) and a protruding domain (P) of a major capsid protein VP1 (Prasad et al., 1999). The feasibility of NoV VLPs as antigen carriers has been tested by non-covalent conjugation of foreign antigens to VLPs exploiting the affinity between polyhistidine-tag and tris-nitrilotriacetic acid (Koho et al., 2015). Further, two ~20-nm NoV derived subviral particles, P or S particles, consisting of only P or S domain of VP1, have been successfully implemented as platforms for presentation of short peptide epitopes and large protein antigens (Xia et al., 2018; Jiang et al., 2015). The platform technology, including NoV P particles, has been applied in the development of universal influenza vaccine candidates (Neirynck et al., 1999; Xia et al., 2011) able to induce broadly cross-protective immune responses against highly conserved regions of influenza viruses, aiming at substituting the current seasonal influenza vaccines that provide only strain-specific protection and are inefficient in protection against antigenically distinct strains (Jazayeri and Poh, 2019). Thereby, the influenza antigens employed are mostly based on the proximal stem domain of the major envelope glycoprotein hemagglutinin (HA2) or the extra-cellular domain of matrix protein M2 (M2e), HA2 inducing broadly neutralizing and cross-protective antibodies (Lee et al., 2013; Darricarrère et al., 2021; Nachbagauer et al., 2021) and M2e cross-protective antibodies and enabling activation of CD8+ T cells (Neirynck et al., 1999; Deng et al., 2015).

We have recently constructed a stable modular vaccine platform based on NoV VLPs using the isopeptide-bond-forming SpyCatcher/SpyTag pair and the two universal influenza vaccine antigens as model antigens (Lampinen et al., 2021). Both, HA2 fragment and M2e peptide, were demonstrated to be successfully presented by the NoV VLP platform, and NoV VLPs conjugated with HA2 or M2e were immunogenic in mice inducing antibodies against SpyCatcher fused influenza antigens, providing the first evidence of NoV VLP platform feasibility. The present study was aimed to further investigate induction of influenza-specific immune responses and functionality of the induced immunity as well as the effect of SpyTag/SpyCatcher conjugation on NoV-specific immunity.

2. Materials and methods

2.1. Antigenic formulations

NoV capsid VLPs derived from strains GII.4 Sydney (Syd; 2012, GenBank accession no AFV08795.1), GII.4 New Orleans (NO; 2010, accession no GU445325) and GII.12 (1998, acc. no. AJ277618) were produced in insect cells using baculovirus expression vector system as previously described (Blazevic et al., 2011). The VLPs were purified by ultracentrifugation through discontinuous sucrose gradients (10–60%) (Huhle et al., 2010) and consecutive ultrafiltration method (Tamminen et al., 2020) and confirmed for purity, antigenicity, and morphology with the procedures described in detail elsewhere (Blazevic et al., 2011; Tamminen et al., 2020). NoV VLPs were employed as antigens in immunological assays and NoV Syd VLPs were also used as control vaccine antigens in animal immunizations.

NoV VLPs were decorated with M2e or HA2 influenza antigens employing a modification (Lampinen et al., 2021) of SpyTag/SpyCatcher strategy originally described by Brune and colleagues (Brune et al., 2016). Briefly, for modular VLP formation, SpyTagged NoV GII.4 Syd VLPs (NoV VLP-SpyTag) were expressed and purified separately from SpyCatcher-fused antigens prior to conjugation of the components via spontaneous isopeptide bond formation. SpyTagged NoV VLPs were produced by expression of baculovirus recombinants in insect cells and purified by tangential flow filtration and ultracentrifugation through a 30% sucrose cushion as well as anion exchange chromatography (Lampinen et al., 2021). Instead, SpyCatcher-fused M2e (SpyCatcher-M2e) and HA2 (SpyCatcher-HA2) as well as HA2 without the SpyCatcher fusion (rHA2) were equipped with polyhistidine-tag, expressed in E. coli and purified with Ni–NTA affinity chromatography. M2e and HA2 antigens were derived from the human influenza A virus consensus sequence of the first 24 N-terminal amino acids (aa) of matrix protein M2 (Neirynck et al., 1999; Deng et al., 2015) and the influenza A virus H1N1 HA (A/Puerto Rico/8/34 subtype) stem fragment (140 aa) consisting of aa 18–41 and aa 290–323 of subunit H1A and aa 41–113 of subunit HA2 connected by GSA and GSAGSA linkers (Miallajouyula et al., 2014). Each SpyTag/SpyCatcher component was confirmed for stability, morphology, size, purity, and identity as demonstrated in our recent publication (Lampinen et al., 2021).

A 23-mer consensus peptide (SLITEVETPIRNEWGCRCNDSSD) derived from M2e protein of human H1N1, H2N2 and H3N2 influenza A viruses (Deng et al., 2015) was synthesized by Synepeptide Co. Ltd. (Shanghai, China). Recombinant HA (rHA, extracellular domain (aa 1–528) of HA, Influenza A, subtype H1N1 A/Puerto Rico/8/1934) was purchased from Sino Biological (Beijing, China). The influenza type A virus A/PR/8 (A/Puerto Rico/8/34(H1N1)), cultured in embryonated hen eggs (Blazevic et al., 2000), was used in focus reduction neutralization assay.

2.2. Mouse immunizations

Immunogenicity of the NoV VLPs conjugated with influenza HA2 or M2e antigens was assessed in randomly divided pathogen-free seven-weeks-old female BALB/c mice (Envigo, Horst, the Netherlands), acclimatized under controlled specific conditions for one week prior to experiments. Native NoV VLPs, SpyTagged NoV VLPs, a mixture of SpyCatcher-fused M2e and native NoV VLPs, M2e peptide formulated with Al(OH)3 (Alhydrogel; InvivoGen, Tolouse, France) as well as carrier only (PBS; Lonza, Verviers, Belgium) served as control groups. Animals (4–5 mice/group) were immunized twice with intramuscular (im) injection into the right caudal thigh muscle at a 3-week interval (study weeks 0 and 3). Table 1 shows the employed antigenic formulations as well as the doses of immunogens in each experimental group. All immunizations were performed under isoflurane (Attane Vet®, Vet Medic Animal Health Oy, Parola, Finland) generated inhalation anesthesia. Mice were euthanized at study week 5 and whole blood was collected from each mouse. The serum was separated by centrifugation as previously described (Tamminen et al., 2012) and stored at −20 °C until further analysis.

2.3. Measurement of NoV-specific antibody responses

Antibody responses generated against NoV VLPs were determined by measuring NoV GII.4 Syd type-specific IgG antibody levels in serum samples of individual mice by enzyme-linked immunosorbent assay (ELISA) according to previously published procedures (Blazevic et al., 2011; Tamminen et al., 2012) and as outlined here. Half-area polystyrene plates (Corning Inc, Corning, NY) were coated with 50 ng of NoV Syd VLPs per well and anti-NoV IgG antibodies in serially twofold diluted serum specimens were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and FAST OPD-substrate (both purchased from Sigma-Aldrich, St Louis, MO). Optical density (OD) values at 490 nm (OD490) were measured by a microplate reader (Victor® 2, Perkin Elmer, Waltham, MA). Endpoint titers were expressed as the reciprocal of the highest serum dilution giving an OD490 value above the set cut-off value (mean OD490 of the negative control mice × 3 × SD and >0.1 OD490). Negative samples were assigned with a reciprocal titer half of the starting dilution for statistical purposes. The results are expressed as geometric mean titers (GMTs) with 95% confidence intervals (CI).
2.4. NoV VLP binding and blocking assays

The ability of SpyTagged NoV VLPs to bind to cellular binding ligands, histo-blood group antigens (HBGAs), was examined using two sources of HBGAs: pig gastric mucin (PGM) type III (Sigma Chemicals, St Louis, MO) and human type A salvia. HBGA binding assays were conducted using procedures described in detail elsewhere (Malm et al., 2017). Briefly, NoV GII.4 control VLPs as well as NoV GII.12 control VLPs (0.8–0.05 μg/mL PGM or 1:3000 diluted type A saliva from adult volunteers with previously determined ABO phenotype (Usui-Kerttula et al., 2014). Binding of serially twofold diluted NoV GII.4 Syd VLPs, SpyTagged NoV GII.4 Syd VLPs as well as NoV GII.12 control VLPs (0.8–0.05 μg/mL) was detected using human anti-NoV GII.4 or GII.12 detection serum collected from voluntary laboratory personnel (Nurminen et al., 2011) and the corresponding anti-human IgG HRP-conjugate (Novex; Thermo Fisher Scientific, Fremont, CA) followed by FAST OPD substrate. Results are expressed as the mean OD_{490} of duplicate wells. OD_{490} > 1.0 was interpreted as a strong binding (Usui-Kerttula et al., 2014). Positive reactivity was defined as a mean OD_{490} > 0.2.

To determine neutralization ability of NoV-specific antibodies, the ability of serum antibodies to inhibit the binding of NoV VLPs to HBGA, a blocking assay was conducted as a surrogate test for neutralization using PGM as the source of HBGAs (Malm et al., 2017). Briefly, NoV GII.4 Syd and GII.4 NO VLPs were preincubated with mouse sera serially diluted 2-fold from 1:100 (homologous GII.4 Syd blocking) or 1:20 (heterologous GII.4 NO blocking) and added to PGM coated plates. The PGM bound VLPs were detected with a combination of human GII.4 positive serum and anti-human HRP-conjugated secondary antibody according to the HBGA binding assays described above. The maximum binding (OD_{490}) was determined with VLPs without serum preincubation. The blocking indexes (%) were calculated as 100% – [(OD_{490} sample/OD_{490} max. binding) × 100%]. Half maximal inhibitory concentration (IC_{50}) of the blocking data was calculated from sigmoidal dose–response analysis of nonlinear data for each serum demonstrating ≥50% blockade. A serum sample failing to block ≥50% of the binding was considered negative for blocking antibodies and was assigned with the reciprocal titer of half of the starting dilution. The blocking data are expressed as the GMTs of the IC_{50} values with 95% CI.

2.5. Measurement of influenza-specific antibody responses

Induction of influenza-specific immune responses by HA2 and M2e vaccine formulations was investigated in ELISA assays (Lampinen et al., 2021; Heinimaki et al., 2020). Anti-HA2, -HA and -M2e total IgG as well as anti-HA2 and -HA IgG subtype antibodies were measured in the sera of mice as described above for NoV-specific responses (section 2.3), but the microtiter plates were coated with rHA2 (50 ng/well), rHA (50 ng/well), or M2e peptide (500 ng/well) and detection of antigen-specific antibodies were accomplished with a combination of anti-mouse IgG, IgG1 (Invitrogen, Carlsbad, CA) or IgG2a (Invitrogen) HRP conjugate and FAST OPD or TMB (Vector Laboratories, Burlingame, CA) substrate. TMB substrate was employed in detection of responses against rHA and M2e peptide. In those cases, the OD values were measured at 450 nm (OD_{490}) instead of 490 nm used for OPD.

2.6. Influenza focus reduction neutralization assay

To evaluate the neutralizing activity of the sera against influenza virus, a focus reduction neutralization assay was established by modifying the published procedures (Okuno et al., 1990). Madin-Darby canine kidney (MDCK) cells (Sigma-Aldrich) maintained in Minimum Essential Medium (MEM, Gibco) supplemented with 1% L-Glutamin, 1% PenStrep and 10% FBS (all from Sigma-Aldrich) were seeded (30000 cells/well) in 96-well cell-culture microplates (Nunc) and cultivated at 37 °C, 5% CO2 until the cells reached confluency (2–3 d). Mouse sera diluted 1:25 were preincubated with ~100 focus forming units (ffu) per well of influenza virus stock (H1N1 A/Puerto Rico/8/1934) for 2 h at +37 °C. A repository serum originating from an adult volunteer described in NoV binding assay in the paragraph 2.4 (diluted 1:25) and monoclonal HA2 antibody (20 μg/mL, Takara Bio, Kusatsu, Japan) were used as positive controls in the assay. The virus-serum mixes were then transferred (50 μl/well) on top of MDCK monolayers and incubated for 1 h at +37 °C and 5% CO2. Virus lacking serum and blank wells containing medium only were included in each plate. After incubation, the virus inocula were removed and the MEM washed cells were covered with an overlay (0.8% carboxymethylcellulose in MEM). The plates were incubated for ~20 h at +37 °C and 5% CO2. Next day, the cells were fixed with 10% formalin and permeabilized with 1% Triton-X-100 (Sigma-Aldrich) followed by immunocolourimetric staining of the infected cells using 1:2000 diluted rabbit anti-HA IgG (Syno Biologicals) reacting with 1:3000 diluted anti-robot IgG-HP (Abcam, Cambridge, UK). The infected cells were visualized using TrueBlue substrate (Sera Care, Milford, MA) and the foci were counted visually under an inverted light microscope (Nikon, Tokyo, Japan). The results are expressed as ffu/well.

2.7. Statistical analyses

Kruskal-Wallis H-test was used to compare the non-parametric
observations (the end-point titers and the IC_{50} values) between independent groups. The Wilcoxon signed-rank test was used to compare the non-parametric measurements within a single experimental group (IgG1 and IgG2a titers). Paired samples t-test was used to assess the statistical differences of the mean OD-values (IgG1 and IgG2a levels) within a single experimental group. Statistical analyses were performed using IBM SPSS statistics (SPSS, Chicago, USA) version 25. Statistical significance was defined at p < 0.05.

3. Results

3.1. NoV-specific immune responses

3.1.1. The induction of NoV-specific antibodies

The ability of conjugated VLPs to induce NoV-specific antibodies compared with the native VLPs was evaluated in an ELISA (Fig. 1). Mice receiving either native NoV VLPs (Fig. 1A), SpyTagged VLPs (Fig. 1B) or HA2 (Fig. 1C) or M2e (Fig. 1D) -conjugated VLPs generated a robust NoV-specific IgG response. Although different doses of VLPs were employed in different immunization groups (10–40 μg, Table 1), appreciably high GMTs (reciprocal titer >4.9 log10) were detected in all groups receiving any of the VLP formulations. The conjugated VLPs induced comparable levels of antibodies to those observed with native VLPs (p = 0.102). This indicates that conjugation of VLPs with SpyTag or subsequent decoration of SpyTagged VLPs with influenza antigens did not impair induction of antibodies against NoV. Control mice receiving carrier only were negative for anti-NoV IgG antibodies (Fig. 1E).

3.1.2. Functionality of SpyTagged VLPs

To assess the effect of SpyTag insertion on NoV VLP functionality and integrity, ability of the conjugated antigen to bind HBGA receptors, was examined by binding of native (Fig. 2A) and conjugated VLPs (Fig. 2B) to PGM and human type A saliva. SpyTagged VLPs showed similar binding profile to native VLPs, both VLP preparations interacting equally efficiently with PGM and saliva in two repeated experiments. The results demonstrate that VLPs retained receptor binding capacity despite the SpyTag conjugation. As expected (Uusi-Kerttula et al., 2014), GII.12 VLPs used as a negative control failed to bind to the HBGAs tested in this binding assay (Fig. 2C).

3.1.3. Neutralization activity of the NoV-specific IgG

The ability of the induced antibodies to neutralize NoV was examined by measuring blocking activity of the immune sera against homologous NoV GII.4 Syd and heterologous NoV GII.4 NO VLPs with PGM-based HBGA blocking assay. All experimental groups receiving VLP formulations developed antibodies with strong homologous blocking activity with GMTs of IC_{50} values of 516 (95% CI 383–689) for native VLPs (Fig. 3A), 869 (95% CI 463–1631) for SpyTagged VLPs (Fig. 3B), 903 (95% CI 483–1688) for HA2-conjugated VLPs (Fig. 3C) and 1399 (95% CI 1268–1544) for M2e-conjugated VLPs (Fig. 3D). Although the highest blocking activity was observed in mice immunized with the M2e-conjugated VLPs, a group administered with the highest VLP dose (Table 1), the difference in the IC_{50} values between all four VLP groups tested was not statistically significant (p = 0.059). Similarly, comparable magnitudes of cross-blocking antibodies were detected in groups receiving native VLPs or any of the conjugated VLPs. The respective cross-blocking GMTs were 470 (95% CI 248–891) for native VLPs (Fig. 3A), 324 (95% CI 80–1302) for SpyTagged VLPs (Fig. 3B), 423 (95% CI 149–1203) for HA2-conjugated VLPs (Fig. 3C), and 991 (95% CI 843–1164) for M2e-conjugated VLPs (Fig. 3D). The negative control mice did not induce any blocking antibodies (Fig. 3E). Based on these results, display of SpyTag peptides on NoV VLPs or conjugation of influenza antigens on VLP via SpyTag/Catcher technology had no effect on ability of VLPs to generate NoV neutralizing antibodies.

3.2. Influenza-specific immune responses

3.2.1. Antibody responses against HA2

To examine induction of antibodies directed against HA2 displayed on NoV VLPs, the sera of mice immunized with HA2-conjugated VLPs were assayed for the presence of HA2-specific total IgG and IgG subtype antibodies. Strong IgG response against rHA2 with a GMT of 4.9 log10 was detected after administration with HA2-conjugated VLPs, the IgG titers of individual mice ranging from 4.7 to 5.0 log10 (Fig. 4A). Further, anti-HA2 IgG subtype analyses (IgG1 and IgG2a as hallmarks of respective Th2 and Th1 responses), showed induction of a mixed and balanced Th2- (Fig. 4B) and Th1-type (Fig. 4C) immune response with equally high magnitudes (p = 0.492) of IgG1 (GMT 4.9 log10, 95% CI 4.8 log10–5.1 log10) and IgG2a (GMT 4.9 log10, 95% CI 4.5 log10–5.1 log10) antibodies. Negative control mice receiving SpyTagged VLPs had no IgG (Fig. 4D), IgG1 (Fig. 4E) or IgG2a (Fig. 4F) antibodies against rHA2. Mice administered with M2e-conjugated VLPs were negative for anti-rHA2 antibodies (data not shown).

3.2.2. Antibody responses against HA

To investigate functionality of the induced HA2-specific antibodies, the immune sera were further assayed for the presence of IgG, IgG1, and IgG2a antibodies reactive with complete rHA-protein. As expected, HA2-conjugated VLPs induced responses against rHA (Fig. 5A). However, the level of the antibodies (OD 1.072 ± 0.248 at a 1:100 dilution) was considerably lower than the level detected against rHA2 (OD 2.806 ± 0.19, Fig. 4A). Further, determination of anti-HA IgG subtypes confirmed a mixed Th2- (OD 0.459 ± 0.230) and Th1-type (OD 0.715 ± 0.352) response (Fig. 5A). Although VLPs conjugated with HA2 seemed to skew the HA-response slightly towards Th1-type, the difference was not significant (p = 0.642). No HA-specific IgG, IgG1 or IgG2a antibodies were detected in the sera of negative control mice receiving SpyTagged VLPs (Fig. 5A).

![Fig. 1](https://example.com/figure1.png)

**Fig. 1.** Norovirus (NoV) -specific serum IgG antibody responses induced by conjugated NoV VLP formulations. Antibodies against homologous NoV Sydney VLP antigen in sera of mice following two immunizations with NoV VLPs (A), SpyTagged VLPs (B), NoV VLPs conjugated with HA2 (C), NoV VLPs conjugated with M2e (D) or carrier only (E). Shown are individual titration curves of each mouse (dashed lines) and mean titration curves (±SEM) of the experimental groups (solid lines with symbols). Horizontal dotted lines indicate the cut-off level (OD_{90} ≥ 0.1).
Fig. 2. Effect of SpyTag conjugation on NoV GII.4 Sydney VLP binding to different histo-blood group antigens (HBGAs). Binding of native NoV VLPs (A), SpyTagged NoV VLPs (B) and NoV GII.12 control VLPs (C) to HBGAs present in pig gastric mucin (PGM) and type A saliva (Saliva) was determined at different VLP concentrations in two independent experiments. Shown are OD_{490} values of two repeated experiments (1 and 2).

Fig. 3. Induction of NoV blocking (neutralizing) antibodies by decorated NoV VLP formulations. Homologous and heterologous blockage of NoV Sydney (Syd) and NoV New Orleans (NO) VLP binding to HBGAs present in pig gastric mucin (PGM) by serum antibodies following two immunizations with NoV VLPs (A), SpyTagged NoV VLPs (B), NoV VLPs decorated with HA2 (C), NoV VLPs decorated with M2e (D) or carrier only (E). Half maximal inhibitory concentration (IC_{50}) of each mouse is shown. Each symbol represents an individual animal. Bars indicate geometric mean values of the experimental groups with 95% confidence intervals. A titer of 50 (1.7 log10) was assigned for sera with no detectable genotype-specific antibodies, being a half of the initial serum dilution in homologous blocking assay. A titer of 10 (log10) was assigned for sera with no detectable cross-reactive antibodies, being a half of the initial serum dilution in heterologous blocking assay.

Fig. 4. Induction of influenza HA2-specific IgG and IgG subtype antibody responses. The sera of mice immunized twice with NoV VLPs decorated with HA2 (A–C) or SpyTagged NoV VLPs (D–F) were tested for IgG (A, D), IgG1 (B, E) and IgG2a (C, E) antibodies against rHA2. Shown are individual titration curves of each mouse (dashed lines) and mean titration curves (±SEM) of the experimental groups (solid lines with symbols). Horizontal dotted lines indicate the cut-off level (OD_{490} ≥ 0.1).
Unable to reduce the replication of the influenza A virus H1N1 PR/8, as SpyCatcher-M2e co-delivered with native VLPs as a mixture or to those induced by M2e-conjugated VLPs (OD 0.198 ± 0.126% reduction) was observed with sera of negative control mice immunized with SpyTagged VLPs. Sera of mice immunized with HA2-conjugated VLPs were unable to reduce the replication of the influenza A virus H1N1 PR/8, as only 16.7 ± 5.3% reduction in ffu was detected. Similar result (12.1 ± 12.6% reduction) was observed with sera of negative control mice receiving SpyTagged VLPs. Instead, the known positive human serum as well as HA2 monoclonal antibody, employed as assay controls, conferred strong neutralization with 99.1 ± 0.9% and 87.9 ± 1% reductions in ffu. These results show that the antibodies induced by HA2-conjugated VLPs did not exhibit neutralizing activity against influenza A virus.

3.2.3. Induction of neutralizing antibodies against influenza A virus

Ability of antibodies induced by HA2-conjugated VLPs to neutralize influenza A virus H1N1 PR/8 (homologous to the HA2 used for VLP conjugation) was tested in focus reduction neutralization assay (Fig. 5B). Sera of mice immunized with HA2-conjugated VLPs were unable to reduce the replication of the influenza A virus H1N1 PR/8, as only 16.7 ± 5.3% reduction in ffu was detected. Similar result (12.1 ± 12.6% reduction) was observed with sera of negative control mice receiving SpyTagged VLPs. Instead, the known positive human serum as well as HA2 monoclonal antibody, employed as assay controls, conferred strong neutralization with 99.1 ± 0.9% and 87.9 ± 1% reductions in ffu. These results show that neither conjugation of M2e to VLPs nor coadministration of SpyCatcher linked M2e with VLPs (Fig. 5C). These results demonstrate that the conjugation of M2e to VLPs did not exhibit neutralizing activity against influenza A virus.

3.2.4. IgG responses against M2e

To examine the effect of conjugation on induction of antibodies directed against M2e displayed on NoV VLPs, the sera of mice immunized with different M2e vaccine formulations were assayed for the presence of M2e-specific IgG. The IgG responses of mice receiving M2e-conjugated VLPs were compared to the responses induced by SpyCatcher-M2e co-delivered with native VLPs as a mixture or to those obtained with alum-adjuvanted M2e peptide. Conjugation of HA2 to M2e peptide indicated that extremely low IgG responses were induced by M2e-conjugated VLPs (OD 0.198 ± 0.111) as well as SpyCatcher-M2e in combination with native VLPs (OD 0.180 ± 0.126), antibody levels being significantly lower (p = 0.016) than the levels induced by M2e peptide formulated with Al(OH)₃ (OD 1.742 ± 0.097) (Fig. 5C). These results demonstrate that neither conjugation of M2e to VLPs nor coadministration of SpyCatcher linked M2e with VLPs improved the development of antibody response against M2e peptide. Sera of negative control mice immunized with SpyTagged VLPs did not react with M2e peptide (Fig. 5C).

4. Discussion

Particulate delivery platforms for presentation of heterologous antigens are considered as next generation subunit vaccines and the first vaccine based on Hepatitis B surface antigen (HBsAg) nanoparticles carrying a T cell epitope of the Plasmodium falciparum malaria parasite (Mosquirix®) has been recently approved for clinical use (Tinto et al., 2015). Despite extensive research on the potential of various nanoparticles acting as vaccine platforms, the applicability of the VLPs formed by a VP1 capsid protein of NoV as antigen carriers has not been thoroughly studied. However, the successful presentation of several foreign antigens inserted into the surface loops of NoV VP1 capsid-derived subviral P domain (Tan et al., 2011; Jiang et al., 2015; Xia et al., 2011) or the hinge of S domain (Xia et al., 2018) supports the potential of NoV VLPs to act as a vaccine platform. These VLPs can be easily produced in baculovirus-insect cell expression system at high quantities. In addition, NoV VLPs have been considered as vaccine candidates against NoV infection and disease (Atmar et al., 2011; Blazevic et al., 2011). We have previously described a nanocarrier platform based on NoV VLPs and non-covalent chemical conjugation of a C-terminal polyhistidine-tag projecting out of the VLP surface (Koho et al., 2015). Subsequently, we created a NoV VLP platform, where the M2e or HA2 of influenza A virus were successfully displayed on the surface of NoV VLPs as model antigens by exploiting SpyTag/SpyCatcher conjugation technology (Lampinen et al., 2021). This versatile technology allows decoration of different particles irreversibly with virtually any protein or peptide antigen that can be produced separately in standard expression systems. To demonstrate further the feasibility of this vaccine platform to deliver foreign antigens as well as to serve as a NoV vaccine, we examined potential interference of SpyTag/SpyCatcher conjugation with induction of antibodies directed to conjugated universal influenza antigens, M2e peptide and HA2, as well as NoV VLP carrier.

We have recently demonstrated that C-terminally SpyTagged NoV VLPs exhibit uniform size, good thermal stability, and indistinguishable morphology to native VLPs as determined by dynamic light scattering, differential scanning fluorimetry and electron microscopy (Lampinen et al., 2021). In here, we have further demonstrated a strong and unaltered binding profile of SpyTagged VLPs to HBGAs, putative cellular receptors for NoV (Harrington et al., 2002; Marionneau et al., 2002). Furthermore, the conjugated NoV VLPs retained comparable antigenicity and immunogenicity to their native counterpart, as addressed by induction of equal amounts of NoV-specific antibodies in vivo in mice, including antibodies with the potential to prevent binding of VLPs to HBGAs. These blocking antibodies are considered an indirect indication of neutralization and the best correlate of protection against NoV infection (Reeck et al., 2010). This data indicates that insertion of SpyTag to the C-terminus of VLPs did not mask important antigenic and immunologic determinants important for virus neutralization.
receptor binding sites on the P domain of NoV VLPs. Importantly, conjugation of SpyTagged VLPs with SpyCatcher fused influenza M2e or HA2 antigens did not alter significantly immune responses against NoV either. Congruent with our observations, the conjugation of polyhistidine-tag or other antigens to the C-terminus of NoV VLPs (Koho et al., 2015) or to surface loops of NoV P particles (Tan et al., 2011; Xia et al., 2011) have not affected the particle assembly, receptor binding or antigenic capacity, thus supporting the notion of NoV VLPs as a potent vaccine platform.

Generation of strong HA2-specific antibody response by NoV VLPs conjugated with HA2, the headless HA stem antigen consisting mainly of HA2 subunit (Mallajosyula et al., 2014), demonstrated high immunogenicity of the antigen. In addition, a moderate response to the complete recombinant HA protein, containing also the highly variable and prominent head domain HA1, was detected. While the NoV-HA2 construct was immunogenic, the induced antibodies were unable to neutralize homologous H1N1 PR/8 influenza A virus in vitro. This may suggest that HA2 as presented by SpyTag/SpyCatcher conjugation on NoV VLPs was structurally different from the HA stem structure in the native influenza virus particle. Some reports have shown induction of neutralizing antibodies by HA2 stalk (Mallajosyula et al., 2014; Deng et al., 2018), while others have demonstrated a lack of neutralization in vitro (Bommakanti et al., 2010, 2012), the latter of which corroborating our observations. This does not necessarily imply an unprotective nature of the induced antibodies, as HA2 immunization has been demonstrated to protect mice in vivo from lethal virus challenge in the absence of in vitro neutralization (Bommakanti et al., 2010, 2012).

Although neutralizing antibodies are generally considered a correlate of vaccine-induced protective immunity against influenza virus, also non-neutralizing antibodies could confer protection or recovery from disease. HA2-induced protection can be mediated by antibody-dependent effector functions such as an antibody-dependent cell-mediated cytotoxicity or complement-dependent cellular cytotoxicity (Bommakanti et al., 2010, 2012). Immunization of mice with viral replicon particle vectors expressing influenza virus HA has failed to induce significant neutralizing and IgG1 antibodies while stimulating IgG2a antibodies, which correlate with clearance of virus and increased protection against lethal influenza challenge (Huber et al., 2006). In the present study, HA2 decorated VLPs elicited robust IgG1 and IgG2a responses, demonstrating a mixed Th2/Th1 response against HA2. The IgG2a antibody subtype has been demonstrated to be important in host defense against different viral infections in mice (Nimmerjahn and Ravetch, 2006) due to the ability of the antibody to activate the complement system (Klaus et al., 1979) and stimulate antibody-dependent cell-mediated cytotoxicity (Kipps et al., 1985).

Antibodies directed to M2e are involved in viral clearance by binding to infected cells expressing M2e on their surface (Deng et al., 2015). Surprisingly, our results show that C-terminal conjugation of a short M2e peptide to NoV VLPs as a SpyCatcher fusion did not result in significant induction of anti-M2e IgG response in immunized mice, which is inconsistent with the strong immunogenicity of M2e following conjugation to various other nanocarriers (Neiryvck et al., 1999; Tissot et al., 2010; Xia et al., 2011). The discrepancies in our results compared with the previously published ones could be due to the conjugation technique employed, as usually genetic fusion was used to display the M2e epitope (Neiryvck et al., 1999; Tissot et al., 2010; Xia et al., 2011). It may be that SpyTag/SpyCatcher conjugation did not enable correct presentation of the M2e antigen on the surface of NoV VLPs. Further, M2e representing the N-terminus of the influenza M2 protein, may require a free N-terminus for proper presentation and induction of immunity, as demonstrated with some short peptides being unable to generate antibodies if directly fused to the C-terminus of a carrier (Tähti et al., 2010). Indeed, high levels of M2e antibodies were detected, when M2e was coupled to N-terminus of Hepatitis B virus core antigen or bacteriophage AP205 (Neiryvck et al., 1999; Tissot et al., 2010). Finally, the large SpyCatcher conjugation protein, derived from a common human pathogen, Streptococcus pyogenes (Zakeri et al., 2012), may exhibit immunodominant nature, overwhelming or hindering the responses against the smaller and poorly immunogenic M2e epitope. Very high immunogenicity of SpyCatcher was noticed in our recent study, where antibodies induced by M2e-decorated VLPs or SpyCatcher-fused M2e cross-reacted with SpyCatcher fused HA2 and vice versa (Lampinen et al., 2021). Short peptides with sizes of ~20 aa will likely require a different conjugation methodology. Therefore, experiments with genetic fusion of the short peptides to NoV VLPs are currently in progress.

In conclusion, our data shows that SpyTagged NoV VLPs can be exploited as a platform to deliver foreign antigens in the form of longer polypeptides while preserving receptor binding capacity and excellent self-immunogenicity. Thus, NoV VLPs may not only serve as a carrier but also as a NoV vaccine offering new insights in the design of combination vaccines. However, the SpyTag/SpyCatcher conjugation technology, while not significantly affecting the autologous antigenicity or immunogenicity of NoV VLPs, will require further research to ensure presentation of heterologous antigens on the NoV VLPs in the correct native structure.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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