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Original article

Replication-deficient Sendai virus expressing human norovirus capsid protein elicits robust NoV-specific antibody and T-cell responses in mice

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ABSTRACT

Human norovirus (HuNoV) is a major global cause of acute gastroenteritis, with vaccine development facing several challenges. Despite years of research, there are currently no licensed vaccines available for controlling HuNoVs. Here, we describe the construction and testing of a replication-deficient Sendai virus (SeV) vector as a potential vaccine candidate against the HuNoV GII.4 genotype. SeV was chosen as the vaccine backbone due to its non-pathogenic nature in humans, its capability for long-term antigen expression in mammalian cells, and its suitability for mucosal administration. By inserting the HuNoV GII.4 capsid gene, VP1, into the SeV genome, we generated a replication-deficient SeV (SeV/dP.VP1) vector. The resultant SeV/dP.VP1 virus were observed to successfully express the inserted NoV VP1 gene upon infection. Inoculating the vaccine into wild-type mice elicited NoV-specific IgG antibodies, along with INF-γ and IL-2-producing T cells, through both intranasal (i.n.) and intramuscular (i.m.) immunization. Furthermore, a significant level of NoV-specific IgA was detected in lung homogenates after i.n. immunization, particularly using a high dose of the viral vector. Additionally, a synergistic effect was observed with heterologous prime-boost regimens using SeV/dP.VP1 and MVA.VP1 vectors, indicating the potential for more robust immune responses when the vaccine design is optimized. Our study demonstrates the potential of a SeV vaccine candidate in eliciting a broad immune response and lays the foundation for further exploration of the SeV vector platform's potential as a HuNoV vaccine. Additionally, the results emphasize the importance of vaccine dosage and administration route, highlighting the need for tailored immunization strategies.

1. Introduction

Human norovirus (HuNoV) is the main cause of acute gastroenteritis (AGE) worldwide [1]. It is a non-enveloped, positive-sense and single-stranded RNA virus [2]. Among the several genogroups of NoVs, GI, GII, and GIV give rise to human disease, especially GII.4 genotype predominantly causes the most AGE outbreaks worldwide [3]. The major challenges to HuNoV vaccine development are 1) the lack of suitable in vivo and in vitro infection models, 2) susceptibly of all age groups to multiple infections, 3) diverse strains of circulating noroviruses, 4) limited cross-reactive immunity between different genogroups, and (5) a lack of sustained immune responses against HuNoV [4,5]. The major (VP1) capsid protein, encoded by ORF2, can independently self-assemble into virus-like particles (VLPs) [6], making it the most important candidate protein in vaccine development against HuNoV. VLP-based vaccines, however, have various drawbacks, including variable/low protein yield, a lack of long-term expression in different expression systems, and the necessity of additional purification processes. Impurities and contaminants, such as host cell impurities and baculoviruses, pose challenges during purification, demanding the development of an effective procedure to remove them without compromising the immunogenicity of VLP-based

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vaccines. It has been reported that VLP-based vaccines are sensitive to changing conditions during manufacture and downstream processing resulting in a decreased immunogenicity. Overall, overcoming these technological limitations makes the development of VLP-based vaccines difficult and expensive [5].

The Sendai Virus (SeV), a murine parainfluenza virus type 1, is an enveloped virus with a nonsegmented, negative-strand RNA genome that belongs to the *respirovirus* genus of the *paramyxovirinae* subfamily [7]. SeV has received particular attention as a favorable human vaccine candidate due to several features: it is non-pathogenic in humans, and it can replicate in a wide variety of mammalian cells and chicken eggs. SeV does not need attenuation to be used as a viral vector. Thus, reversion of attenuating mutations is unlikely [8]. Being not a pathogen in humans ensures very low levels of pre-existing neutralizing antibodies if any [9]. Furthermore, SeV does not possess any risk of DNA integration into the host DNA genome. Unlike DNA viral vectors (e.g. MVA85A and AdAg85A) that use eukaryotic promoters (e.g., CMV), SeV does not use the host transcriptional machinery for antigen expression, reducing the risk of transcriptional silencing [10]. Lastly, the possibility of intranasal administration of the virus and eliciting mucosal immunity adds an extra bonus to the features of the SeV-based viral vectors [11–13].

Exploring SeV as a vaccine vector offered advantages such as non-pathogenicity in humans, broad cellular tropism, and the potential for mucosal administration. The objective of this study was to address key challenges in developing a HuNoV vaccine by leveraging the potential of SeV as a vaccine vector for the HuNoV capsid gene to pave the way for an effective and economically viable HuNoV vaccine. Our study reveals that a SeV-vector can induce robust immune responses against HuNoV and at least in mice – is a safe vaccine delivery platform.

2. Materials and methods

2.1. Cell lines and DNA plasmids

BSR-T7 cells, stably express T7 RNA polymerase, were used to rescue recombinant SeV (recSeV). The cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. Vero cell line (V3.10), expressing SeV phosphoprotein, was used as helper cells for propagation of replication deficient SeV. The cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 1× L-Glutamine, 1× Sodium pyruvate and 200 μg/ml Hygromycin B. A subgenomic SeV plasmid containing the Phosphoprotein-gene-defective SeV genome was used for the insertion of HuNoV capsid protein, VP1, in the genome of SeV. Three helper plasmids pTM-N, pTM-P/C and pTM-L encoding SeV N, P and L proteins, respectively, were used for the rescue of recombinant SeV/dP.VP1 in Vero cells.

2.2. Construction of recombinant Sendai virus vector encoding NoV capsid protein

To generate recombinant P-gene-defective SeV vector expressing HuNoV GII.4 VP1 protein, the NoV capsid gene, VP1, was amplified using the primer pair F: 3′CTTTCACCCCAAGCGCG CGCCACCATGAAGATGGCCTC5′ and R: 3′CTGATGCTGATAGCGC GCTAGTTATACGGCTCGTCTTC5′, and subsequently inserted into a *BssH*II site located between the P and M genes in the subgenomic plasmid, pSeV/dP. Following this, the target region in the recombinant subgenomic vector was cloned into the full-length Sendai virus vector after both vectors were cut by *Eco*RI. This process resulted in a recombinant replication-deficient Sendai Virus vector with a truncated P gene, named recSeV/dP.VP1. Throughout each step, cloning efficiency and the integrity of the inserts were assessed by digestion pattern of constructs and Sanger sequencing.

2.3. Virus rescue, propagation and purification

To rescue the recombinant viruses, BSR-T7 cells were transfected with the recombinant SeV/dP.VP1 (4 μg), and three helper plasmids: pTM-N (0.25 μg), pTM-P/C (0.15 μg) and pTM-L (0.05 μg), using Lipofectamin 2000 (Thermo Fisher Scientific, USA). Transfected cells were then incubated with the DMEM medium supplemented with 2% FCS. A second transfection was performed three days post-transfection, involving 1 μg of pTM-P/C (for SeV P gene). Viral particles were harvested from the cell culture supernatant on day 12 and used to infect the V3.10 helper cells in DMEM medium supplemented with 10% FBS, L-Glutamine, Sodium pyruvate, and 200 μg/ml Hygromycin B, for the propagation of the rescued recSeVdP.VP1 in T-125 flasks. Three days post-infection, when more than 90% of the cells were GFP positive, the cells were harvested using cell scraper, centrifuged and washed with PBS. The cell plates were resuspended in 10 mM Tris, pH 9, and subjected to three times freeze–thaw cycles to extract viruses from the infected cells. Subsequently, the virus particles were purified on a 35% sucrose cushion at 25 000 rpm for 1 h.

2.4. Virus titration and in vitro characterization

The concentration of the purified viruses was determined by calculating TCID50 in the V3.10 helper cells within 7 days post-infection with 10-fold serial dilution of the purified virus in DMEM medium supplemented with 2% fetal bovine serum. The presence of the virus particles in the purified samples was confirmed by transmission electron microscopy (TEM). Additionally, Western blot analysis was conducted to verify the capability of the recSeV/dP.VP1 viruses in expressing the transgene, HuNoV.GII4 VP1, in the infected cell. This analysis utilized VP1-specific rabbit antisera as the primary polyclonal antibody and HRP-conjugated goat anti-rabbit as secondary antibody.

2.5. Transmission electron microscopy (TEM)

For negative staining of the virus particles in TEM imaging, FCF400-Cu Formvar 400 mesh copper grids with a collodion-supported carbon film were used and glow discharged for 45s at 35 mA prior to sample application. The samples were incubated on the grids for 10 min and subsequently stained for 40s with 20 μl of 2% aqueous uranyl formate solution, including 25 mM sodium hydroxide. Imaging was performed using a FEI Tecnai T12 microscope operated at 120 kV with a Tietz TEMCAM-F416 camera at a magnification of 30 000× using the SerialEM software.

2.6. Mouse immunization

Female BALB/c mice aged 6–8 weeks were immunized either intranasally or intramuscularly with both resSeV.VP1 at both low (3×10^8/ml) and high (3.25×10^{10}/ml) virus titers. PBS-injected mice were served as controls. Each group received three times inoculations, spaced two weeks apart. In a separated group a heterologous prime-boost vaccination was employed, using recSeV.VP1 as prime vaccination (administrated twice, intramuscularly) and MVA-VP1 viral vector as a booster (3×10^7 virus particles in the third injection, intramuscularly). Mice were sacrificed, ten days after the last administration, and NoV-specific immune responses were assessed in the vaccinated mice. The recombinant MVA (recMVA) encoding norovirus GII.4 capsid protein (VP1) was previously generated in our lab, as vaccine candidates against HuNoV

GII.4. More details can be found in the original study by Kugler et al. [14].

2.7. Antibody titration by ELISA

Norovirus-specific IgG, in mice sera, and IgA, in lung and intestine homogenates, were assessed by ELISA on polystyrene 96-well microtiter plates coated with 2 μg/ml NoV capsid protein. To this end, after blocking with 5% milk powder in PBST (1× PBS containing 0.05% Tween 20) for 2 h, the wells were treated with 1:500 diluted mice sera, or 1:10 dilution of lung/intestine homogenates. After 1.5 h incubation at RT, and five washes with PBST, HRP-conjugated secondary antibodies were applied for 1 h. Thereafter, the wells were washed, and then treated with TMB substrate solution at RT at the dark. Following color development, the reaction was stopped, and the absorbance value was measured at 450 nm using a TECAN Infinite® 200 PRO microplate reader. To quantify the serum IgG concentration against NoV VP1 protein, a standard curve was generated using a serial two-fold dilution of mouse IgG in 1× PBS.

2.8. Evaluation of T cell responses by intracellular cytokine staining

Norovirus-specific T-cells were analyzed in mouse splenocytes by intracellular cytokine staining (ICS) and flow cytometry. Initially, splenocytes were isolated from the mouse spleens in RPMI medium using 100 μm cell strainers. After lysis the red blood cells and the splenocytes were incubated with NoV VP1 pooled peptides at the final concentration of 1 μg/ml. As positive controls, the cells were stimulated with PMA and Inomycin with a final concentration of 400 and 5 μg/ml, respectively. One hour later, the cells were blocked with brefeldin A (BFA), and the incubation continued for an additional 16 h. Subsequently, the overnight-stimulated cells were harvested and stained with fixable viability dye, αCD4-PE (eBiosciences, Cat #12-0041-82) and αCD8-Pacific Blue (BD Biosciences, Cat #558106) antibodies in FACs buffer (500 ml PBS + 5 ml FCS), before fixing and permeabilizing using the cytofix/cytoperm solution (BD Biosciences, Cat #554714). Intracellular cytokines were then stained using anti-IFN-γ-FITC (BD Biosciences, Cat #554411), anti-IL2-APC (eBioscience, Cat #17-7021-82), anti-TNFα-PeCy7 (BD Biosciences, Cat #557644) antibodies in 1× Perm/Wash buffer (BD Biosciences, Cat #554723). After a 25-min incubation on ice, the cells were washed with 1× Perm/Wash buffer and then with FACs buffer, before applying them to the FACs machine for analysis.

2.9. Statistical analysis

The statistical significance of the data was assessed by one-way ANOVA using the GraphPad Prism 9.5.0. The threshold for statistical significance was set at $p < 0.05$. The results are presented as means ± standard deviation (SD) of the mean.

3. Results

3.1. Construction of replication deficient Sendai virus vector encoding NoV capsid protein

A subgenomic and a full genome SeV vector were employed to construct a replication-deficient SeV vector encoding the HuNoV GII4 capsid protein, VP1. Due the unique replication cycle and transcription characteristics of the SeV, mRNA transcript abundance decreases from the 3′ end to the 5′ end of its genome (N > P > M > F > HN > L) [8]. This leads to a higher expression level of the target foreign protein when the foreign gene is positioned closer to the 3′ end of the genome. To reach a high expression level of our gene of interest, the HuNoV GII.4 VP1 was inserted immediately after the P deletion (Pdel), flanked by the P gene stop signal, an intergenic sequence, and the start signal for transgene at the 5′ end and transgene stop signal, an intergenic sequence, and the M gene start signal at the 3′ end (Fig. 1a and b). The second and final host vector was the full genome SeV vector, comprising of a 3' leader sequence, six structural genes (N, P, M, F, HN, L), an eGFP sequence inserted 3′ of the L gene, and a 5' trailer (Fig. 1c). This design allowed for the control and initiation of the expression of SeV genes and NoV GII4 VP1 by a T7 promoter located upstream of the N gene 5′. Gene expression is terminated by a T7 terminator located downstream of the eGFP 3′ end (Fig. 1d).

3.2. Virus rescue and propagation

Recombinant SeV.VP1 (recSeV.VP1) was rescued in BSRT7 cells by transfecting the cells with a plasmid mixture consisting of pTM-N (encoding SeV N gene), pTM-P (encoding SeV P gene), pTM-L (encoding SeV L gene), and a plasmid carrying the cDNA of the viral genome flanked by the T7 promoter and terminator sequences. This arrangement allows the viral genomic sequence to be transcribed by the T7 polymerase. The vector carries an eGFP gene, enabling the screening of successful recSeV.VP1 through GFP expression, observed under fluorescence microscopy, as depicted in Fig. 2. Early signs of protein expression were shown in BSRT7 cells following transfection with the respective plasmids. Notably, on day 6, we observed initial GFP expression in the transfected cells (Fig. 2b), this expression was gradually extended to neighboring cells, and by day 12, GFP expression became more substantial, with approximately 20 percent of the cells displaying positivity (Fig. 2c).

Recombinant SeV.VP1 rescued on day 12 after transfection from cell culture supernatant were subsequently propagated in the Vero V3.10 helper cells that stably express the SeV P protein (Fig. 3). It was remarkable that by day 6, nearly 100 percent of the cells exhibited GFP positivity, as shown in Fig. 3b, indicating efficient spread of recSeV.VP1. Virus propagation in the non-helper cell line (Fig. 3c) demonstrated the SeV vector's ability to propagate, although at significantly reduced levels compared to the V3.10 cells. This was evident from the lower intensity of the GFP signal observed in the transfected cells.

3.3. Characterization of recSeV.VP1 in vitro

The recSeV.VP1 was checked for the expression of the heterologous HuNoV GII.4 VP1 gene through Western Blot. This step ensured that the recSeV vector was capable of expressing our gene of interest. The results showed successful expression of the NoV capsid protein VP1 in the infected V3.10 cells (Fig. 4a). Notably, the Western blot showed a main band at approximately 56 KDa, corresponding to the full-length VP1 protein. Additionally, a minor band at approximately 35 KDa was observed. This minor band is likely due to a truncated form or degradation product of VP1, a phenomenon commonly reported in norovirus-related studies. This finding suggests partial proteolytic cleavage or inherent instability of the protein, which is consistent with observations in similar research. For further validation, the vector particles were concentrated using a 35% sucrose cushion and visualized by TEM. The images revealed intact Sendai virus particles in the sample, displaying the expected size and shape (Fig. 4b).

3.4. Immunogenicity of recSeV.VP1 in mice

To investigate the effectiveness of the replication-deficient recSeV.VP1 of inducing specific immunity to VoV capsid protein, VP1, we conducted a comprehensive analysis involving

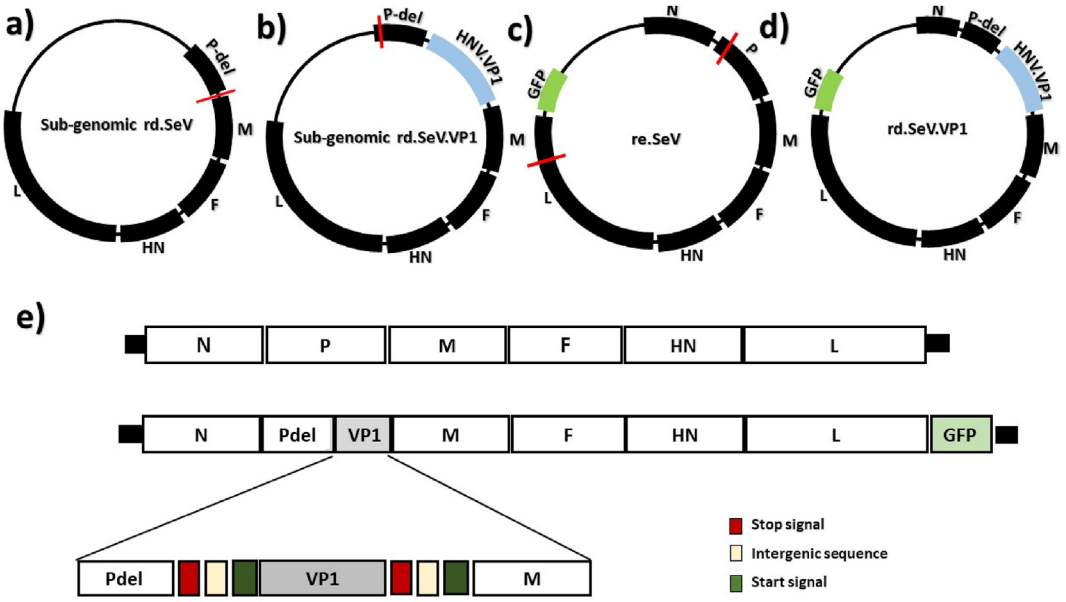

Fig. 1. Schematic representation of Sendai Vectors and cloning steps. (a) The HuNoV GII.4 VP1 gene was inserted into in *BssH*II restriction site (indicated by red line) of the sub-genomic SeV plasmid to generate (b) the recombinant sub-genomic SeV-vector plasmid. The target region was cut at *Eco*RI restriction sites (indicated by red lines (b)) and inserted into the replication-deficient SeV vector (c), resulting in a recombinant replication-deficient Sendai virus vector harboring the heterologous VP1 gene (d). The VP1 gene was inserted between the P and M genes flanked by a stop signal, intergenic sequence, and a start signal at 5′ and 3′ sites I.

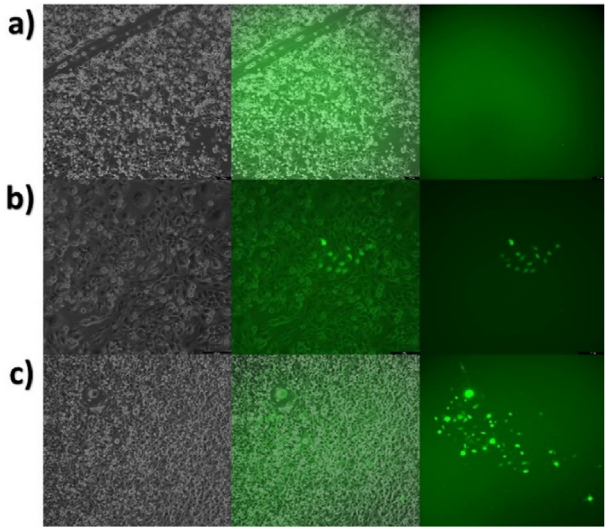

Fig. 2. Sendai Virus rescue in BSRT7 cells. The left images are phase contrast view, the middle images are fluorescent view, and the right images are overlay view. BSR-T7 cells (0.25 × 106) were transfected with the SeV plasmid and, in parallel, with pTM-N, pTM-P, and pTM-L encoding SeV N, P, and L genes, respectively. The development of eGFP-expressing cells was monitored for 12 days. Phase contrast (left column) and fluorescence microscopy (right column) on days 2 (a), 6 (b) and 12 (c). Cell supernatant containing rescued viruses was subsequently collected for further propagation in the helper Vero cells, V3.10.

homologous and heterologous prime-boost vaccinations utilizing the recSeV.VP1 and a MVA-VP1 viral vector recently described [14]. In the homologous vaccination, mice were immunized either intranasally (i.n.) or intramuscularly (i.m.) with both low and high recSeV.VP1vector titers. In the heterologous approach after prime vaccination with recSeV.VP1 (twice), the mice were injected intramuscularly with MVA.VP1 as a booster.

To evaluate HuNoV-specific T cell responses, mouse splenocytes were stimulated with peptide pools covering the HuNoV VP1 gene.

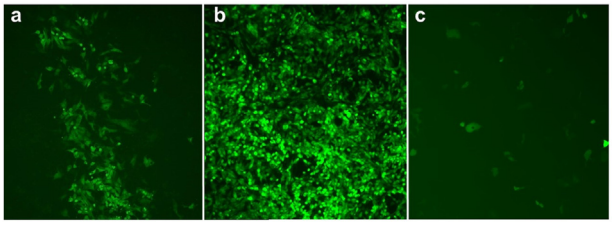

Fig. 3. SeV propagation in helper-Vero cells. Replication deficient recSeV.VP1s were propagated in Vero V3.10 helper cells. GFP expression status was depicted at day 3 (a) and day 6 (b) post-infection, with more than 90% of the cells becoming infected. Low level of GFP expression was detected in non-helper cell line (c).

Intracellular cytokine staining (ICS) and subsequent flow cytometry analysis demonstrated that both low-dose and high-dose administration of SeV.VP1 elicited $CD8^+$ and $CD4^+$ T-cell responses specific to HuNoV in mice (Fig. 5a). We observed higher NoV-specific T-cell responses with i.m. injection than using the i.n. route, regardless of the administered dose. Notably, the high-dose administration of recSeV.VP1 exhibited significantly higher HuNoV-specific T-cell responses than the low-dose, indicating a dose-dependent effect on the immune response. Using the heterologous prime-boost scheme, we detected superior T-cell immunity (Fig. 5a). We found that the booster vaccination with MVA.VP1 vector significantly enhanced both $CD8^+$ and $CD4^+$ T-cell responses specific to HuNoV-VP1 when compared to the homologous vaccination with recSeV.VP1 or MVA.VP1 viral vectors.

Both i.n. and i.m. administration of recSeV.VP1 also elicited significant levels of HuNoV-specific antibodies (Fig. 5b). Comparing the two routes of administration, the i.m. route demonstrated a stronger IgG antibody response, surpassing the levels observed with both low and high doses of the i.n. route. The heterologous prime-boost regimen also demonstrated superior efficacy in inducing HuNoV-specific antibody responses compared to individual administrations of the viral vectors alone (Fig. 5b). Anti-HuNoV IgG1 and IgG2a antibodies were detected in mouse sera.

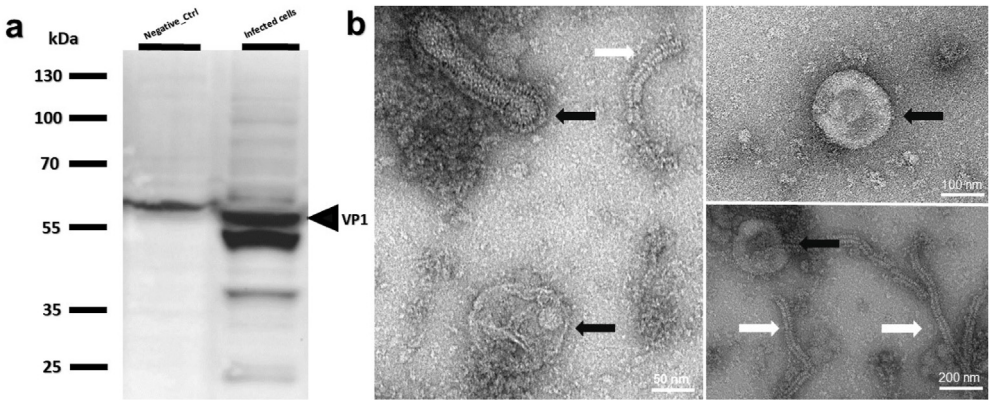

Fig. 4. *In vitro* characterization of recSeV.VP1. (a) Western Blot imaging to assess the expression of NoV capsid protein in V3.10 cells infected with recSeV.VP1. The primary band at approximately 56 KDa represents the full-length VP1 protein; the minor band at around 35 KDa is likely a cleaved or truncated form of VP1, a finding commonly reported in norovirus studies. (b) TEM imaging to validate the Sendai viral particles. Viral particles were concentrated on a 35% sucrose cushion and then visualized via TEM. Black arrows indicate the virus particles, and white arrows highlight SeV nucleocapsids released, most likely due to breakage of viral particles due to the physical pressure during centrifugation.

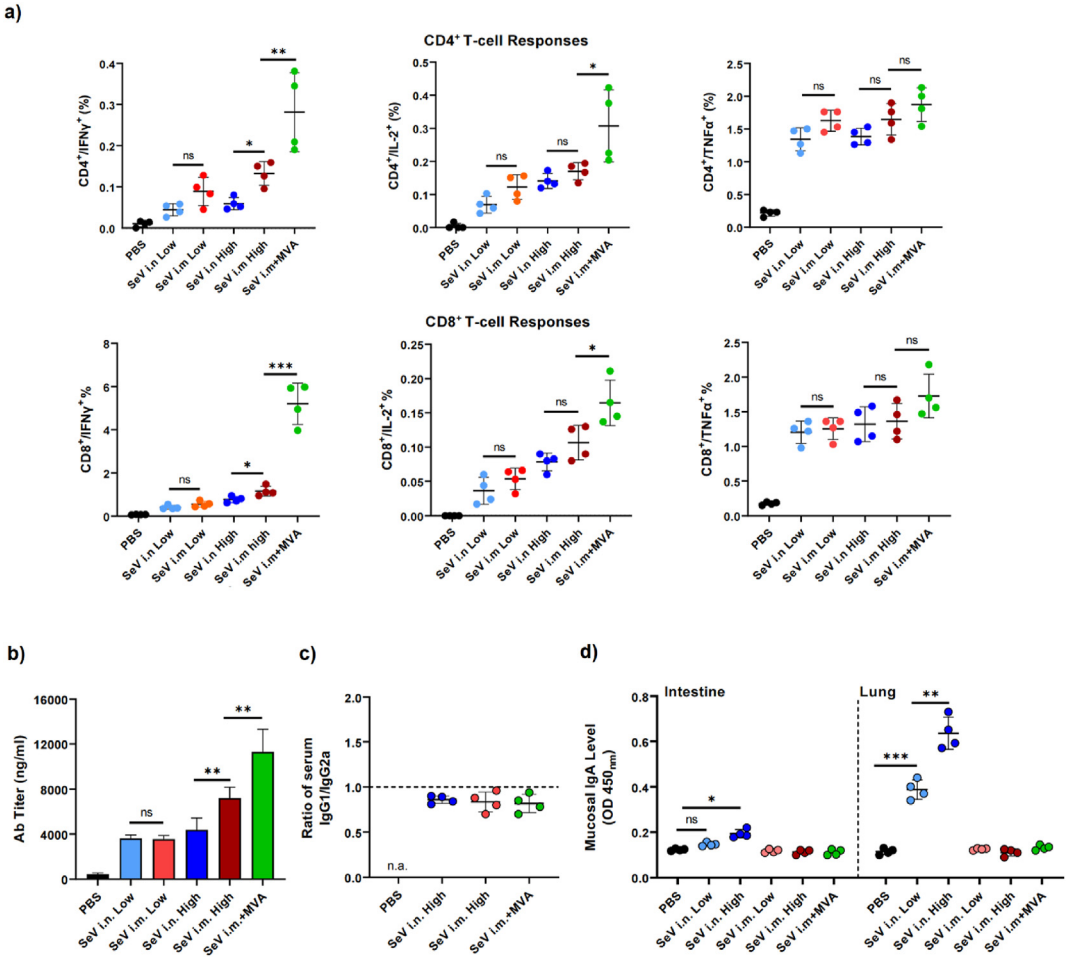

Fig. 5. Evaluation of immune responses to recombinant viral vectors, recSeV.VP1 and MVA.VP1, in mice. BALB/c mice received three times either a low dose (3×10^8/ml) or a high dose (3.25×10^{10}/ml) of recSeV.VP1 intranasal (i.n.) or by intramuscular injection (i.m.). In the heterologous vaccination scheme, mice received two doses of recSeV.VP1 i.m. and were injected with MVA.VP1 (3×10^7 IFU/ml) i.m. as a booster. After the last administration, immune responses were assessed in the mice: (a) HuNoV-specific $CD8^+$ and $CD4^+$ T-cell responses were evaluated by intracellular cytokine staining and flow-cytometry after stimulation of splenocytes with a peptide pool covering the VP1 gene. (b) VP1-specific IgG Ab response were determined by ELISA. (c) The ratio of serum IgG1 to IgG2a antibodies detected by the VP1-ELISA using IgG-type specific secondary antibodies is shown. (d) Specific IgA response intestine and lung homogenates of the mice immunized via the different routes using the indicated vaccination scheme.

All mice injected with the viral vectors showed a preference for IgG2a, indicative of a Th1-type $CD4^+$ T-cell response (Fig. 5c).

To determine whether the recSeV.VP1 could stimulate specific mucosal immune responses following i.n. administration, lung- and

intestinal homogenates were investigated for HuNoV-specific IgA level as an indicator for mucosal immunity. This revealed significantly higher levels of HuNoV-specific IgA antibodies in the lung homogenates ($p < 0.001$) after i.n. application of recSeV.VP1 than after i.m. application (Fig. 5d). In mice immunized with a high dose of the recSeV.VP1, a lower level of mucosal NoV-specific IgA was also detected in the intestinal homogenates. As expected, no boost effect on IgA responses was detected in the heterologous i.m. vaccination using recSeV.VP1 and MVA.VP1.

4. Discussion

This study presents the development of a replication-deficient Sendai virus vector expressing the HuNoV GII.4 capsid gene, VP1, as a promising vaccine candidate against norovirus. Through detailed experimentation, we demonstrated the successful expression of the NoV VP1 gene by the engineered SeV vector, leading to the elicitation of robust immune responses in mice. The selection of SeV vector was driven by its unique attributes, which hold significant promise for human vaccination. SeV has not been associated with any known human diseases. Unlike some other viral vectors, SeV is not derived from an attenuated human virus, eliminating concerns about its potential to revert to a harmful state [15,16]. The primary focus of this research was to generate recombinant SeV encoding HuNoV GII.4 capsid protein for vaccination against HuNoV GII.4 genotype, responsible for a significant number of acute gastroenteritis cases globally. To achieve this, a replication-deficient SeV viral vector was generated by employing two key vector candidates: a subgenomic SeV vector and a full genome SeV vector. This robust expression of the VP1 protein in helper cells underscores the vector's potential for expressing the proteins of interest efficiently. Moreover, purified virus particles exhibited the anticipated size and shape of the virus. The vector's capability to propagate in non-helper cell lines was also examined before testing in vivo. Although the expression persisted, it was evident that non-helper cells yielded lower levels of expression and intensity, signifying the vector's ability to function in a diminished capacity in these conditions. It is worth mentioning that although the replication-deficient platform addresses the improved safety of the Sendai viral vector, it results in much lower protein expression levels compared to the replication-efficient platforms. This specifically can impact the efficiency of the vaccine in optimally inducing specific immune responses.

The principal objective of the second phase of the study was to assess the vaccine's capacity to instigate targeted immune responses to the HuNoV capsid protein. The results reveal several key insights into the immune responses triggered by recSeV.VP1, offering important implications for vaccine development and immunization strategies. The study encompassed both homologous and heterologous prime-boost vaccinations, which allowed for a thorough evaluation of the replication-deficient SeV vector's immunogenicity. The findings indicate that recSeV-VP1 can stimulate robust $CD8^+$ and $CD4^+$ T-cell responses specific to the NoV capsid protein in mice. These responses were characterized by the induction of IFN-γ and IL2 cytokines, highlighting the activation of T cell-mediated immunity. The dose-dependent effect observed in both intranasal and muscular administrations, with the high-dose regimen resulting in an enhanced B- and T-cell responses, emphasizes the importance of vaccine dosage in influencing the magnitude of the immune response. Comparing different routes of administration, the study revealed that intramuscular injection of the vaccine candidates led to significantly higher antibody and T-cell responses specific to the NoV capsid protein compared to the intranasal route, regardless of the dose administered. This observation suggests that the choice of administration route plays a crucial role in shaping the magnitude and nature of the immune responses. Furthermore, it underscores the importance of optimizing the delivery method for maximizing the vaccine's immunogenicity. A similar study [17] aimed to assess the vaccine's capacity to evoke PIV3- and RSV-specific T cell responses. The results demonstrated a substantial level of IFN-γ, signifying a robust Th1 response. Remarkably, mice immunized with rcPIRV exhibited higher IFN-γ production than their rdPIRV counterparts, underscoring the vaccine's impact on Th1 responses, suggesting the capability of PIV3 antigens within the SeV envelope to induce cell-mediated immune responses. Conversely, inactivated rdPIRV-immunized individuals did not develop a CTL response against RSV due to the absence of antigen expression. A clear stimulation of IFN-γ expression in splenocytes by a replication-deficient vaccine candidate against RSV has also been demonstrated in another study [18]. They observed low-level IFN-γ values in SeV -GFP-immunized mice, indicating a possible contribution of the RSV antigen in the vaccine to IFN-γ expression. These collective results reinforce the significance of the replication-deficient Sendai vector as a promising vaccine platform to effectively stimulate immune responses, offering promising insights into its immunogenic potential and underscore the critical roles of dosage, administration route, and the synergistic effects of viral vectors in shaping immune responses.

To further assess the vaccine's immunogenicity, we analyzed NoV-specific antibody response in mouse sera. The results demonstrated the effective induction of NoV-specific antibodies through both i.n. and i.m. administration routes. Notably, the i.m. route demonstrated a more potent IgG antibody response, surpassing the levels observed with both low and high doses of the i.n. route. Consistent with the results for the T-cell responses to the viral constructs, the i.m. administration route holds greater potential for inducing robust IgG antibody production to the target capsid protein. We also delved into the balance between two different types of immune responses, namely Th1- (IgG2a) and Th2-like (IgG1). Our investigation found a tendency to IgG2a, in the vaccinated mice, which is a representative of Th1 mediated response. In a related study, Li Guo et al. utilized a different vaccine platform targeting HuNoV VP1 [19]. Similarly, they research revealed strong humoral immune responses in mice subsequent to intranasal administration of a recombinant adenovirus targeting NoV capsid protein. Consistent with our findings, they reported significant increases in specific antibody titers, indicating effective stimulation of the humoral immune response to the target antigen. Despite the differences in vector types, both studies achieved potent B and T-cell immune responses against their respective target antigens.

To assess the potential for mucosal immune responses, we examined NoV-specific IgA levels in lung and intestinal homogenates from vaccinated mice. Remarkably, a significant level of NoV-specific IgA was observed in lung homogenates of the mice intranasally administered with recSeV.VP1, particularly with high doses of the viral vector. In contrast, only a minor level of secreted NoV-specific IgA was detected in intestinal homogenates of these mice. As expected, the i.m. route of administration had no effect on inducing mucosal IgA responses compared to the i.n. route. The lack of enhanced IgA responses following the heterologous i.m. vaccination scheme in our study aligns with this understanding.

These findings indicate that intranasal administration of recSeV.VP1 is also effective in eliciting mucosal immune responses, including in the intestinal tract. This observation aligns with previous research by Li Guo and colleagues, who also reported strong mucosal immune responses against NoV capsid protein following intranasal administration of the recombinant adenovirus [19]. According to the concept of mucosal immune system cross-talk

[24,25], immune responses in one mucosal site can influence and induce responses in other mucosal sites, albeit at lower levels. Although our study shows predominant IgA production in the lungs, some degree of cross-talk may facilitate IgA production in the intestine as well. However, detecting IgA in the intestinal mucosa is challenging due to the complexity of intestinal tissue and potential interference in assays. Our methods may not have been fully optimized, possibly leading to an underestimation of intestinal IgA levels. Additionally, the lack of suitable animal models that accurately replicate HuNoV infection restricts our ability to fully assess the protective efficacy of the intestinal IgA response. Future studies should focus on developing more representative models to evaluate the vaccine's effectiveness in inducing mucosal immunity in the gastrointestinal tract more comprehensively.

MVA is a highly attenuated form of the vaccinia virus, which has been used in vaccine development due to its safety profile and ability to induce strong immune responses. In the context of heterologous vaccination, MVA is often used as a boost following an initial priming with a different type of vaccine. Several studies have demonstrated that heterologous prime-boost strategies using MVA vaccine can result in superior immune responses compared to homologous vaccination [22,26,27]. To provide a more comprehensive assessment, the study incorporated a heterologous prime-boost strategy involving the previously developed MVA.VP1 vaccine vector (16). This approach served to explore the potential synergistic effects of different viral vectors in enhancing the immune responses to the target protein. The results demonstrated that the combination of recSeV.VP1 and MVA.VP1 in a prime-boost regimen significantly augmented both the $CD8^+$ and $CD4^+$ T-cell responses specific to NoV capsid protein. The prime-boost regimen, following a similar pattern to the indication of T-cell responses, exhibited superior efficacy in inducing a higher HuNoV-specific antibody response than the homologous administrations of the SeV construct alone. This highlights the synergistic effect achieved by combining the two viral vectors, fostering a more potent and targeted antibody and T-cell responses against HuNoV. This effect has also been noted in other investigations [20–23], highlighting the potential of heterologous prime-boost strategies in enhancing vaccine-induced immune responses. Such findings suggest promising advancements in future vaccine strategies, where combination of different vaccine platforms may enhance overall vaccine efficacy.

5. Conclusion

The detailed analysis presented in this study provides strong evidence that the replication-deficient SeV possesses significant immunostimulatory properties. It effectively induces robust antibody and T-cell immune responses specific to HuNoV VP1, underscoring its potential as a promising candidate for the development of effective vaccines against HuNoVs. Our results also highlight the critical role of dosage and administration route in vaccine design, emphasizing the need for tailored immunization strategies. While intranasal administration has shown efficacy in eliciting systemic and respiratory mucosal immune responses, enhancing the magnitude of intestinal IgA responses remains a key objective. To improve intestinal IgA production, future research will focus on exploring alternative mucosal delivery routes, optimizing adjuvant formulations, employing advanced delivery systems, and refining immunization regimens. Strategies such as oral or rectal administration, incorporation of mucosal adjuvants, and use of nanoparticle-based delivery systems will be evaluated to enhance direct stimulation of the gastrointestinal mucosa and improve overall vaccine efficacy. Additionally, the demonstration of synergistic effects through heterologous prime-boost regimens using SeV.VP1 and MVA.VP1 viral vectors opens new possibilities for designing more potent vaccines. These findings not only contribute to our understanding of immune responses to novel vaccine candidates but also highlight their potential in combating infectious diseases like HuNoV.

Ethics statement

This study was conducted according to protocols approval by legal issues health, consumer protection and pharmacy, Government of Upper Bavaria (ROB-55.2-2532.Vet-02-18-77).

CRediT authorship contribution statement

Yazdan Samieipour: Writing – original draft, Methodology, Formal analysis, Data curation. **Marian Wiegand:** Validation, Methodology. **Elena M. Willner:** Methodology. **Dieter Hoffmann:** Writing – review & editing, Funding acquisition. **Kamyar Shameli:** Writing – review & editing, Software. **Ulrike Protzer:** Writing – review & editing, Resources. **Hassan Moeini:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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