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Comparison of Intramuscular, Intranasal and Combined Administration of Norovirus Virus-Like Particle Subunit Vaccine Candidate for Induction of Protective Immune Responses in Mice

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Abstract

Background and objectives: Noroviruses (NoVs) are major causative agents of non-bacterial acute gastroenteritis in people of all ages worldwide. NoV capsid VP1 derived virus-like particles (VLPs) produced in various expression systems are main vaccine candidates against NoV. The aim of this study was to investigate and compare systemic and mucosal delivery and a combination of both deliveries of NoV VLPs for induction of immune responses in BALB/c mice.

Materials and methods: BALB/c mice were immunized Intramuscularly (IM), Intranasally (IN) or sequentially (IM followed by IN) with a candidate NoV GII-4 VLP vaccine developed by our laboratory. NoV GII-4-specific serum and mucosal IgG and IgA antibodies were analyzed by ELISA. GII-4–specific T cell immune responses were investigated using an ELISPOT assay measuring production of interferon-γ (IFN-γ) at a single cell level.

Results: IM immunized mice developed a strong systemic and mucosal NoV-specific IgG antibody response but completely lacked IgA response. In contrast, mice immunized IN had strong systemic and mucosal IgG and IgA production but lacked $CD8^+$ T cell responses. Sequential immunization compensated for the deficient IgA and $CD8^+$ T cell responses induced by each delivery alone.

Conclusion: Our results show that sequential IM+IN immunization should be considered for NoV VLP vaccine delivery to activate broad immune responses.

Keywords: Noroviruses; Vaccine; Immune Response

Introduction

Noroviruses (NoV) are the leading cause of non-bacterial acute gastroenteritis (AGE) in people of all ages. NoV infections are responsible for over a million hospitalizations and up to 200,000 deaths annually in infants and children of developing countries [1]. After introduction of rotavirus vaccines in the USA, Finland, and other countries, NoVs have become the leading cause of medically attended AGE in children under five years of age [2,3]. Therefore, young children are an important target group for future NoV vaccination. NoVs are highly contagious and cause large outbreaks in community setting such as nursing homes, childcare facilities, military, and cruise ships.

NoV genogroups GI and GII are responsible for most infections in humans with GII-4 genotype being predominant for more than two decades [4,5]. After expression in vitro major NoV capsid protein self-assembles into virus-like particles (VLPs) consisting of 90 dimers of VP1 [6]. These VLPs are morphologically and functionally similar to the native virus but lack genetic material.

Human NoVs are uncultivable in vitro and, therefore, development of conventional vaccines based on live attenuated or killed NoVs is not possible at present time. Instead, NoV VLPs have been proposed as vaccine candidates against NoVs. As NoV is an enteric pathogen that uses intestinal mucosa as a port of entry, most of the immunogenicity studies in animals [7,8] as well as early phase clinical trials [9-11] have used mucosal immunization (oral or intranasal; IN) for delivery of NoV VLPs. More recently, intramuscular (IM) delivery has been considered [12-14].

Correlates of protection to NoV infection are largely unknown. Histo-blood group antigens (HBGAs) are cellular attachment factors or receptors for NoVs [15]. These complex carbohydrates are present on the surface of enteric mucosal cells as well as free antigens in body secretions. Serum antibodies, which block binding of VLPs to the HBGAs, are considered as a surrogate of neutralizing antibodies and correlates of protection to NoV infection [16-19].

Other studies have also suggested important role of cellular and mucosal immunity against NoV infection and gastroenteritis [20-22]. In this study we compared induction of potentially protective immune responses induced with NoV VLPs by systemic IM or mucosal IN immunization as well as sequential immunization (IM followed by IN) in BALB/c mice. To date, there have been no studies using combined systemic and mucosal delivery approaches with NoV VLPs. Our results show that sequential IM+IN immunization compensated for deficient NoV-specific serum IgA and T cell responses induced by IM and IN delivery alone.

Materials and Methods

Immunization of experimental animals and sample collection

To analyze NoV GII-4 VLP induced immune responses 10 female BALB/c OlaHsd mice (7 weeks old; Harlan Laboratories, The Netherlands) were immunized at day 0 and day 21 with a NoV VLP and rotavirus VP6 combination vaccine candidate developed by our laboratory [12,23] containing 10 μg GII-4 VLPs by IM or IN route. The immunogen was administered in a 50 μl volume sterile phosphate-buffered saline (PBS) into quadriceps femoris or in a 25 μl volume by gradual inoculation in each nostril. In addition, a group of mice was primed with the combination vaccine containing 10 μg NoV GII-4 VLPs by IM route at day 0 and boosted by IN route at day 21, termed sequential immunization. Naïve mice receiving carrier only (sterile PBS) either by IM or IN route were used as negative controls. Mice were terminated two weeks after the final immunization (day 35) and blood, feces and spleen were collected as described previously [24,25]. All procedures were performed in accordance with the regulations and guidelines of the Finnish Animal Experiment Board.

Serum antibody ELISA

Individual mouse sera were serially diluted starting at 1:200 and tested for NoV GII-4 specific total IgG antibodies in an ELISA assay as previously described [23]. Briefly, 96-well plates (Corning Inc. Corning, NY) were coated with 50 ng/well of GII-4 VLPs in PBS and bound antibodies detected with HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich). Groupwise pooled sera of each experimental group was 2-fold serially diluted (starting at a 1:20 dilution) and tested for GII-4 specific IgA antibodies using HRP-conjugated goat anti-mouse IgA (Sigma-Aldrich) at a dilution of 1:4000. Optical density (OD) at 490 nm was measured by Victor^2 1420 reader (Perkin Elmer) and a sample was considered positive if the OD was above the mean OD of control mice +3SD. End-point titers were expressed as the highest serum dilution giving a positive reading. Sera of each mouse at a dilution 1:200 were tested for IgG antibody avidity using ELISA as described above with an extra urea incubation step [19,26] where antibodies bound to GII-4 VLP coated plates were treated twice with 8M urea before addition of HRP-conjugated anti-mouse IgG. Avidity index was calculated as (OD with urea/OD without urea) × 100%.

Mucosal antibody ELISA

Faecal droplets from each mouse were pooled and 10% stool suspensions were made as earlier described in details [12]. Stool suspensions were serially 2-fold diluted starting at 1:5 and tested for NoV GII-4-specific IgG and IgA with the ELISA as described above.

Serum antibody blocking assay

Human type A saliva from a secretor positive individual and synthetic biotinylated H-type-3 carbohydrate were used as a source of HBGAs in blocking assays. Saliva blocking assay was performed essentially as earlier described by our laboratory [23]. 96-well plates were coated with saliva type A at a 1:3000 dilution and incubated overnight at 37°C. For synthetic HBGA blocking assay SuperBlock pretreated High Binding Capacity NeutrAvidin plates (Pierce) were incubated with 2.5 μg/ml of the synthetic biotinylated H (type 3)-PAA-Biotin (Glycotech) for 1 hour at room temperature [27]. GII-4 VLPs were pre-incubated with serially two-fold diluted (1:100–1:3200) experimental and control mouse sera for 1 h at 37°C and added to the saliva or H-type-3 coated plates. Saliva plates were further incubated for 1.5 hour at 37°C and NeutrAvidin plates for 2 hour at +4°C. The bound VLPs were detected with NoV antibody positive human serum and anti-human IgG-HRP (Invitrogen) followed by the OPD substrate. VLPs lacking the serum were used as the maximum binding control. The blocking index (%) was calculated as 100% – (OD wells with VLP serum mix/OD wells without serum; maximum binding) × 100%.

Cell mediated immune response

NoV GII-4-specific T cell responses were measured with an ELISPOT assay by quantification of interferon (IFN)-γ producing splenocytes [25].

Multiscreen 96-well HTS-IP filter plates (Millipore) were coated with anti-mouse IFN-γ antibody AN18 (2.5 μg/ml, Mabtech). Splenocytes (0.1×10^6/well) from the experimental or control mice were stimulated with a GII-4 capsid derived 15-mer synthetic peptide named NP-4 at 5 μg/ml (ProImmune Ltd., amino acids CLLPQEWVQHFYQEA) or GII-4 VLPs at 2.5 μg/ml. Cells incubated in culture media alone and cells stimulated with 10 μg/ml Concavalin A (ConA, Sigma-Aldrich) served as a background and cell viability controls.

After overnight incubation at 37°C IFN-γ secretion was detected with biotinylated anti-mouse IFN-γ antibody R4-6A2 (2.5 μg/ml, Mabtech) and streptavidin-ALP (Mabtech). The spots developed with BCIP/NBT substrate (Mabtech) were counted by ImmunoSpot® automatic CTL analyzer (CTL-Europe GmbH). The results are expressed as mean spot forming cells (SFC)/10^6 cells of duplicate wells.

To determine which cell type is responsible for the IFN-γ production, splenocytes were preincubated (1 hour at 37°C) with the functional blocking antibodies rat anti-mouse CD4 or rat anti-mouse CD8 (both from eBiosciences) at a 30 μg/ml concentration prior to stimulation with the GII-4-specific peptide or GII-4 VLPs.

Statistical analyses

Fisher's exact test was used for comparison of GII-4 VLP-specific IgG endpoint titers. $P<0.05$ was considered statistically significant. All hypothesis testing was two-tailed.

Results

Serum NoV-specific antibody responses

Serum GII-4-specific IgG titers of individual mice immunized at day 0 and day 21 with a candidate vaccine containing 10 μg NoV GII-4 VLPs by IM or IN delivery route are shown in Figures 1A and 1B. Regardless of the delivery route each mouse developed a strong IgG antibody response with endpoint titers of 5log10 ($p>0.05$).

In all of the sera the GII-4 specific IgG antibodies had high avidity with an avidity index >50% (Figure 1C). As these results indicated uniform success of immunization of each mouse in the experimental group, the sera were pooled groupwise and GII-4 specific IgA titers were determined by ELISA (Figure 1D). IN immunized mice generated a remarkable serum IgA antibody response (mean OD 0.40, titer 1:20) while IM immunized mice did not (mean OD 0.07, titer 1:20).

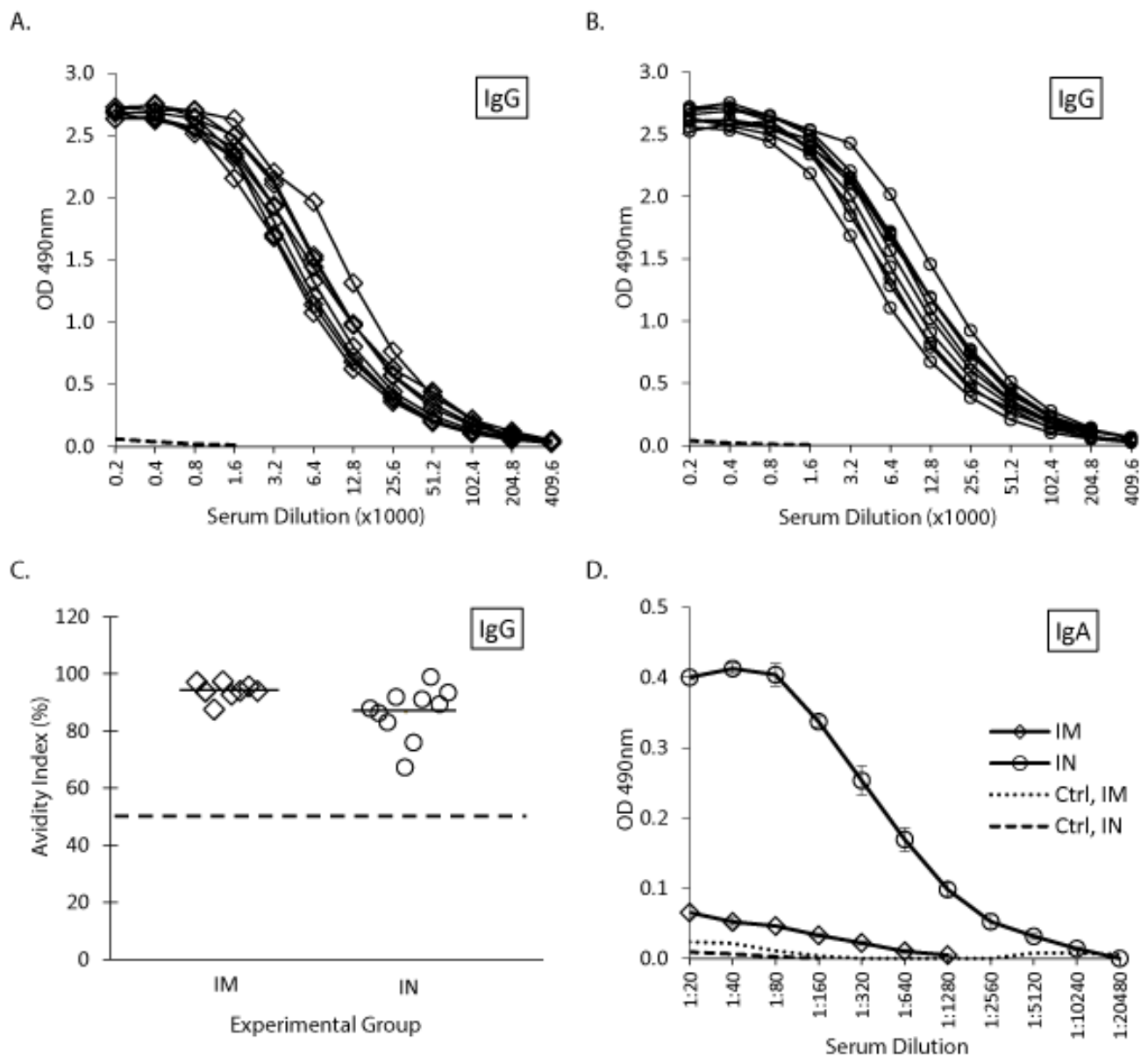

Figure 1: NoV GII-4-specific serum antibody responses in BALB/c mice. Mice (10 mice/group) were immunized twice (at day 0 and day 21) with a NoV vaccine candidate containing 10 μg GII-4 VLPs. **(A)** Termination sera of intramuscularly (IM) immunized mice was assayed for NoV GII-4-specific IgG with two-fold dilutions starting at 1:200. Shown are individual titration curves of each immune sera and titration curve of pooled control mice sera (6 mice/group) immunized with the carrier (PBS) only (dashed line). **(B)** GII-4-specific IgG titration curves of intranasally (IN) immunized mice and pooled control mice sera. **(C)** Avidity of GII-4-specific IgG antibodies of each immunized mouse was analyzed in modified ELISA assay at a dilution of 1:200 as described in the Materials and Methods. Shown is avidity index ((OD with urea/OD without urea) x 100) of each mouse sera and the mean of the group. Dashed line indicates the cut-off for high avidity antibodies (>50%). **(D)** GII-4-specific serum IgA titers were assayed with two-fold dilutions (starting at a 1:20) of group wise pooled serum. Shown are mean OD values of the IM, IN and corresponding control mouse groups with the standard errors of the mean (SEM) of the replicates.

Mucosal IgG and IgA antibodies

Groupwise pooled fecal samples of IM and IN immunized mice were tested for GII-4 specific IgG and IgA antibody content. Both delivery routes induced similar levels of IgG antibodies in the intestines (mean OD 0.57 for IM and mean OD 0.48 for IN, titer 1:50) (Figure 2A). Similar to the serum samples (Figure 1D, respectively), mice immunized with NoV GII-4 VLP by IM delivery did not develop intestinal IgA antibodies (mean OD 0.04, titer 1:5) (Figure 2B) while

mucosal IN delivery induced considerable level of intestinal IgA antibodies (mean OD 0.49, titer 1:5) (Figure 2B).

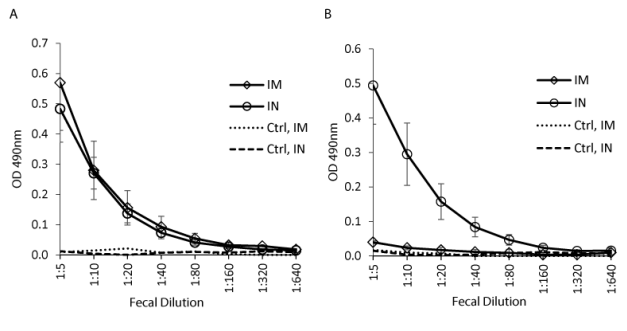

Figure 2: NoV GII-4-specific mucosal antibody responses in BALB/c mice. Mice were immunized twice with a NoV vaccine candidate containing 10 µg GII-4 VLPs. Intestinal IgG **(A)** and IgA **(B)** antibodies were analyzed from 10% fecal suspensions of IM and IN immunized mice (solid lines) or the control mice (dashed lines). Fecal samples of five mice/experimental group were pooled for analysis. Shown are mean ODs with the standard errors of the mean (SEM) of two independent experiments.

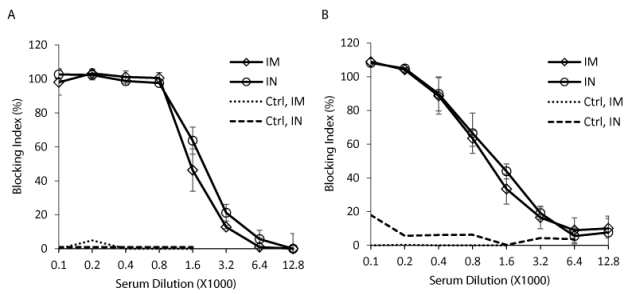

Figure 3: Blocking of NoV GII-4 VLP binding to the HBGA receptors by immune mouse sera. Group wise pooled (five mice/group), two-fold diluted sera of mice immunized by IM or IN route with a NoV vaccine candidate containing 10 µg GII-4 VLPs were assayed for blocking activity. Corresponding control group serum was used as a non-specific blocking control. **(A)** Blocking of GII-4 VLP binding to human secretor positive saliva type A. **(B)** Blocking of GII-4 VLP binding to the H-t-3 synthetic HBGA. The blocking index (%) was calculated as 100% - (OD wells with serum/OD wells without serum, a maximum binding) × 100%. Results are shown as the mean blocking index of duplicate wells with similar results from a minimum of two independent experiments.

Blocking ability of NoV-specific antibodies

Sera of GII-4 VLPs immunized and control mice were pooled groupwise and tested for blocking of GII-4 VLP binding to human type A saliva (Figure 3A) and synthetic H-type-3 carbohydrate (Figure 3B). Both IM and IN immunization induced strong blocking antibodies in the sera with 100% blocking up to a dilution 1:800 in the saliva blocking assay (Figure 3A). Blocking of the GII-4 VLPs' binding to the synthetic H-type-3 was used to confirm the results of the saliva blocking assay (Figure 3B). Both immunizations induced comparable blocking responses in the sera, which correlated to the blocking responses seen in the saliva assay. Neither of the control groups' (ctrl IM or ctrl IN, respectively) sera contained antibodies able to block GII-4 VLP binding to the HBGAs (Figure 3A and 3B).

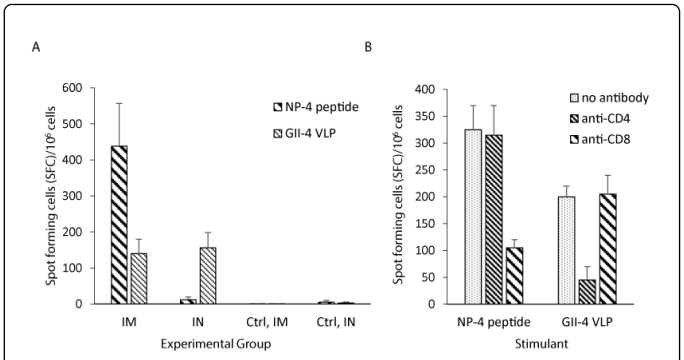

Figure 4: NoV GII-4-specific interferon-γ (IFN-γ responses. **(A)** Splenocytes of mice immunized by IM or IN route with a NoV vaccine candidate containing 10 µg GII-4 VLPs or control mice receiving PBS were stimulated in vitro with GII-4 capsid derived 15-mer peptide (NP-4) or with the homologous GII-4 VLPs. IFN-γ production at a single cell level was detected by an ELISPOT assay. **(B)** T cell restriction of the NoV GII-4-specific IFN-γ production. Cells were stimulated with the NP-4 peptide or GII-4 VLPs in the presence or absence of CD4 and CD8 specific antibodies to block the T cell activation. Results are expressed as the mean Spot Forming Cells (SFC)/10^6 cells of at least two independent experiments with standard errors.

NoV GII-4-specific CD8$^+$ T cell responses

NoV GII-4-specific T cell responses measured by IFN-γ production at a single cell level in ELISPOT assay were different in mice immunized by IM or IN route. IM immunized mice had high frequency of IFN-γ producing cells in spleen in response to the GII-4 capsid derived 15-mer peptide NP-4 (438 ± 119 SFC/10^6 cells) while IN immunized mice completely lacked these responses, as did the the control mice (Figure 4A). The T cell responses were also tested using NoV GII-4 VLPs as an in vitro stimuli and comparable responses were detected in mice immunized by IM (140 ± 40 SFC/10^6 cells) and IN delivery route (156 ± 42 SFC/10^6 cells) (Figure 4A). In order to determine which T cells responded to the peptide and GII-4 VLPs, blocking antibodies to CD4 and CD8 cell surface antigens were used to show the restriction of the NoV GII-4-specific IFN-γ responses (Figure 4B). The T cell responses to the NP-4 peptide were only blocked by anti-CD8 antibody (mean 68 % inhibition), while GII-4 VLP responses were blocked by anti-CD4 antibody only (mean 77% inhibition). These results indicate impaired functionality of CD8$^+$ T cells in mice immunized by IN delivery in contrast to IM immunized mice. Furthermore, the results also show that both delivery routes induced functional CD4$^+$ T cells by responding to GII-4 VLPs.

Immune responses by sequential immunization

As IM and IN immunizations alone induced deficient immune responses we next combined the two to determine combined effect of the delivery routes. Immunization of mice with NoV GII-4 VLPs first IM (at day 0) and sequentially IN (at day 21) induced serum and mucosal GII-4 specific IgA antibodies (Figure 5A) and NP-4 peptide

specific CD8+ T cell IFN-γ responses (Figure 5B) comparable to the optimal responses induced by each route separately (Figure 1D, 2B and 4A respectively). Serum and fecal GII-4 specific IgG antibodies as well as blocking antibody activity were comparable to each route separately as well. Serum GII-4 IgG endpoint titer was 102400 and the mean avidity index was 94.5 ± 0.2 %. Serum dilution of 1:800 blocked 92% of GII-4 VLP binding in the saliva blocking assay.

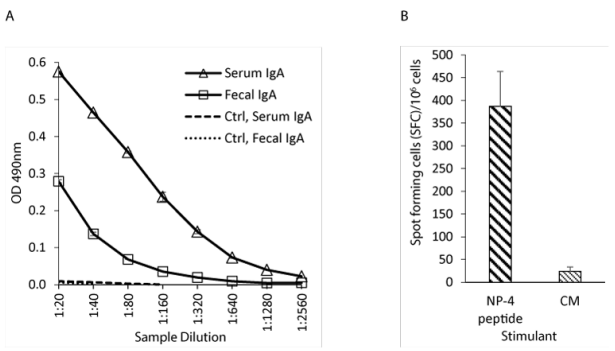

Figure 5: NoV GII-4-specific humoral and cell-mediated immune responses induced by sequential immunization. Group of mice were immunized with a NoV vaccine candidate containing 10 µg GII-4 VLPs at day 0 by IM delivery and day 21 by IN delivery. **(A)** NoV GII-4-specific IgA antibodies were analyzed from serum and fecal suspensions. Serum samples were two-fold diluted starting at a 1:20 dilution and 10% fecal suspension were analyzed starting at a 1:2 dilution. Shown are mean ODs with standard errors of the replicates. **(B)** NoV GII-4-specific IFN-γ responses after stimulating the splenocytes with GII-4 capsid derived 15-mer peptide (NP-4) or culture media (CM) only. Shown are the mean spot forming cells (SFC)/10^6 cells of the individually tested mice with the standard errors.

Discussion

NoV VLPs are excellent vaccine candidates as they resemble native virions morphologically and antigenically and are highly immunogenic [6], in addition to being safe. NoV VLPs can be given by mucosal delivery orally or IN, or parenterally (IM or intradermally, ID). As NoV is an orally transmitted enteric pathogen and natural immunity to NoV is of a short duration [28-30] it remains to be determined if systemic immunization with NoV VLP vaccine might be a better choice than mucosal one to induce long lasting protective immunity. In here we studied different delivery of NoV VLP vaccine by comparing immune responses induced by IN and IM immunization. Our results indicate that there are inherent differences in immune system activation by NoV VLPs administered systemically or mucosally.

We have previously shown that systemic delivery (IM and ID) of NoV VLPs either alone or in a combination with rotavirus VP6 protein induces robust humoral and cell mediated immune responses specific for NoV [12,23,25]. The results in this study confirm the earlier findings that IM immunization with GII-4 VLPs induces both IgG antibody and T cell responses ($CD4^+$ and $CD8^+$, respectively) but in addition show the absence of serum and mucosal IgA although significant levels of IgG were detected in the gut mucosa. Transfer of serum IgG into the gut lumen is likely an important protective mechanism against gut infection [31]. On the contrary, IN delivery induced similar IgG antibody responses to NoV in the serum and the gut as observed with IM delivery, but the T cell responses, specifically $CD8^+$ T cells, were deficient. Velasquez et al. [8] have shown low levels of IgG2a antibodies (a marker of a Th1 type immune response) by IN delivery of NoV VLPs which is in a support of our findings. The importance of T cells in NoV infection is not well known and there is a limited number of studies addressing cellular immunity [21,22,32]. It is believed that in contrast to live viral vaccines subunit protein vaccines require T cells to induce protection [33]. In general, $CD8^+$ T cells, namely cytotoxic T lymphocytes (CTL), are critical for clearance of virally infected cells [34,35]. A direct evidence for the role of T cells in NoV infection comes from a study of murine norovirus (MNV) showing that $CD8^+$ and $CD4^+$ T cells cleared infection in mice [36].

IN delivery of NoV GII-4 VLPs in this study induced high systemic and mucosal GII-4 specific IgA antibodies. Contradictory findings are related to the role of NoV-specific mucosal immunity. Lindesmith et al. [20] reported a significant role for mucosal immunity, especially salivary IgA in a protection from infection. On the contrary, other researchers have reported insignificant role of serum IgA and intestinal IgA in a chimpanzee model of a NoV infection [37] and natural infection in young children [30]. Comparable level of blocking/neutralizing activity of serum derived from IM and IN immunized mice that were detected in this study (Figure 3, respectively) would also suggest a negligible role for serum IgA antibodies, as IgA was detected only in sera of IN immunized animals.

Two human challenge studies have been published recently in subjects immunized with candidate NoV VLP vaccines administered IN [16] and IM [14]. Interestingly, IN delivery of GI.1 VLP vaccine (with an adjuvant) resulted in significant protection against homologous NoV infection [16], whereas IM delivery of GII.4 vaccine did not [14]. However, IM immunized subjects had significant protection against severe NoV gastroenteritis [14]. These results suggest different mechanism of protection against NoV infection, induced by IN and IM immunization, respectively. Protection against NoV infection requires immunity at the mucosal surfaces whereas protection against the severe disease may require different mediators for protection. Our findings in mice may shed light on the interpretation of the results of the human challenge studies.

Our results show that sequential IM+IN immunization of mice with NoV GII-4 VLPs compensate for deficiencies resulting from IM and IN deliveries alone. This schedule was chosen as immunogenicity studies of hepatitis B surface antigen in mice [38] showed that the strongest systemic immune response associated with a strong mucosal response was induced by IM prime followed by IN boost and not vice versa. Sequential immunization induced GII-4 specific T cells of both phenotypes ($CD4^+$ and $CD8^+$) and IgA antibodies in serum and mucosa. It is possible that external adjuvant in combination with NoV VLP vaccine might direct immune response in a desirable pathway. However, in vaccine development in general, NoV VLP vaccine without an adjuvant would be preferred particularly for use in children. It remains to be determined why there is no induction of effector CD8+ T cells producing IFN-γ after IN delivery of NoV VLPs. It may be that these cells are activated at the mucosal delivery site but do not disseminate to secondary lymphoid tissue, as has recently been suggested [39,40].

Our laboratory has worked on the development of NoV and rotavirus combination vaccine against the two most devastating

enteric pathogens in children [12,23]. As natural immunity to NoV is of a short duration, we postulated that IM immunization might induce stronger and more durable immune responses than mucosal delivery [13]. The results in this study show remarkable differences in the immune responses induced by IM and IN delivery of NoV VLPs in mice; absence of mucosal immunity, specifically intestinal IgA in IM immunized mice and lack of NoV-specific CD8+ T cell immune responses by IN delivery. It remains to be determined which responses are relevant to protection against NoV infection and/or disease and therefore for choosing of the route of NoV VLP vaccine delivery. Before the exact correlates of protection are identified, sequential immunization might be a good choice to ensure that different effectors/mediators of the immune response are activated.

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