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Functionality and avidity of norovirus-specific antibodies and T cells induced by GII.4 virus-like particles alone or co-administered with different genotypes

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ABSTRACT

Norovirus (NoV) is the main cause of acute gastroenteritis worldwide across all age groups. Current NoV vaccine candidates are based on non-infectious highly immunogenic virus-like particles (VLPs) produced in cell cultures in vitro. As NoVs infecting human population are highly divergent, it is proposed that the vaccine should contain at least two different NoV genotypes, potentially affecting the immunogenicity of each other. We investigated the immunogenicity of NoV GII.4 VLPs administered by intramuscular (IM) or intradermal (ID) injections to BALB/c mice either alone or co-delivered with genogroup I (GI) and other genogroup GII VLPs. Serum NoV-specific IgG binding antibody titers and antibody functionality in terms of avidity and blocking potential were assessed. Furthermore, the specificity and functional avidity of $CD4^+$ and $CD8^+$ T cell responses were analyzed using synthetic peptides previously identified to contain NoV VP1 P2 domain-specific H-2^d epitopes. The results showed that IM and ID immunization induced comparable GII.4-specific antibodies and T cell responses. Similar magnitude and functionality of antibodies and interferon-gamma producing T cells were developed using monovalent GII.4 VLPs or different genotype combinations. For the first time, degranulation assay using multicolor flow cytometry showed that NoV GII.4-specific $CD8^+$ T cells had cytotoxic T lymphocyte phenotype. To conclude, our results demonstrate that there is no immunological interference even if up to five different NoV VLP genotypes were co-administered at the same time. Furthermore, no inhibition of NoV-specific antibody functionality or the magnitude, specificity and affinity of T cell responses was observed in any of the immunized animals, observations relevant for the development of a multivalent NoV VLP vaccine.

1. Introduction

Acute gastroenteritis (GE) caused by noroviruses (NoVs) affects people of all ages worldwide with severe outcomes especially in young children and the elderly [1,2]. Highly contagious NoVs are genetically diverse with continuously emerging new variants that leads to fast spreading in human population through immune escape. NoVs are members of the *Caliciviridae* family with more than 30 recognized genotypes infecting humans that belong to genogroups (G) I, II, and IV [3]. The icosahedral viral particle of NoV, with ~38 nm diameter, is composed of 180 copies of major capsid protein, viral protein 1 (VP1). NoV virus-like particles (VLPs), formed by spontaneous self-assembly of VP1 in vitro, are morphologically and antigenically identical to the virus. VLPs has contributed significantly to the NoV research, as they are successfully used for structural and immunogenicity studies, and as promising NoV vaccine candidates. Due to the high genetic variability of NoVs and a lack of cross-protective immunity between GI and GII NoVs, it is believed that a vaccine should contain a minimum of one VLP from each genogroup. Our group has recently proposed a NoV candidate vaccine consisting of GI.3 and GII.4 VLPs in a combination with rotavirus (RV) VP6, to prevent two major causes of childhood viral GE worldwide [4,5].

Although cell culture systems for growth of NoV in vitro have recently been established, a traditional neutralization assay is still not in use [6,7]. Instead, a subset of NoV-specific antibodies that block binding of NoV VLPs to their cellular carbohydrate ligands, histo-blood group antigens (HBGA), are regarded as correlates of protection from infection [8–11]. The role of NoV-specific T cell responses in protection from NoV infection [12–16] has not yet

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been established and requires further research. Using matrix peptide pools, we have recently identified seven immunodominant H-2^d restricted NoV-specific T cell epitopes, including a highly conserved universal GII NoV-specific $CD8^+$ epitope (peptide 99-45, NNYDPTEEIPAPLGTPDF) and GII.4-1999 variant-specific $CD4^+$ T cell epitope (peptide 99-50, TRAHKATVSTGSVHFTPK) [17].

In the present study we investigated NoV GII.4-specific antibody functionality (avidity and blocking activity) and T cell responses in mice immunized with GII.4-1999 VLPs alone or co-delivered with different VLPs belonging to GI and GII NoVs, with the primary focus on mutual inhibition or immunological interference. In addition, NoV-specific cytotoxic T lymphocyte (CTL) responses were investigated for the first time.

2. Material and methods

2.1. Recombinant proteins and synthetic peptides

Five different NoV capsid VLPs derived from GII.4-1999 (reference strain accession no: AF080551), GII.4-2009 New Orleans (NO; reference strain accession no: GU445325), GII.12 (reference strain accession no:AJ277618), GI.1 (accession no: AY502016.1), and GI.3 (reference strain accession no: AF414403) VLPs were produced in baculovirus-insect cell system and purified by sucrose gradient ultracentrifugation as described in details earlier [4,18]. The purity, identity and morphology of VLPs were determined by SDS-PAGE, immunoblotting, and electron microscopy using previously described procedures [19].

Seventy-four synthetic peptides representing the entire 539 amino acid (aa) sequence of GII.4-1999 NoV VP1 were synthesized by Synpeptide Co., Ltd (Shanghai, China) as 18-mers overlapping by 11 aa. The purity was >70% as determined by high-pressure liquid chromatography. Each lyophilized peptide was dissolved in DMSO and further diluted in sterile PBS for use in the assays. In addition, all individual peptides were pooled (GII.4-99 pool) as recently described [15]. The GII.4-99 pool was used at a predetermined concentration of 2 μg/ml in all assays. Individual GII.4 peptides, 99-45 (^{309}NNYDPTEEIPAPLGTPDF^{326}) and 99-50 (^{344}TRAHKATVSTGSVHFTPK^{361}), a predicted [17] mouse H-2^d 9-mer epitope (^{318}PAPLGTPDF^{326}, included in the 99-45 sequence), and two negative control peptides, 10-mer (^{139}TMFPHIIVDV^{148}) and 17-mer (ovalbumin, OVA ^{323}ISQAVHAAHAEINEAGR^{339}), were synthetized as described above and used at a final concentration of 4 μg/ml.

2.2. Immunizations

BALB/c (*H-2^d*) mice were obtained from Envigo RMS BV (formerly Harlan Laboratories, Horst, Netherlands) and immunized at 7 weeks of age after a week of acclimatization. Forty mice were divided into 8 groups (5 mice/group) and mice were immunized either intramuscularly (IM) at the right caudal thigh muscle or intradermally (ID) at the base of the tail (Table 1). The dose of 10 μg or 50 μg of GII.4-1999 VLPs was administrated in a 50 μl volume/dose at week 0 and week 3, a standard procedure used by our laboratory [4,5]. A group of mice received a mixture of GII VLPs (GII.4-1999, GII.4 NO and GII.12, 20 μg each) by IM delivery according to the same schedule. Additional group of mice received a mixture of GII.4 VLPs (as above) and a mixture of GI VLPs (GI.1 and GI.3) simultaneously at the different sites. Two negative control groups of mice received a carrier only (phosphate-buffered saline, PBS) either IM or ID. No external adjuvants were used. Two weeks after the second immunization mice were sacrificed and serum and spleen cells were collected and prepared as previously described [4,5] for the analysis of NoV-specific antibodies and T cell responses. Mice welfare was monitored throughout the study and experiments were performed in accordance with the guidelines of the Finnish National Animal Experiment Board.

Table 1
Experimental and control groups of immunized mice. Mice were immunized intramuscularly (IM) or intradermally (ID) at day 0 and 21 with the indicated dose and terminated at day 35.

Group	Immunogen	Dose (μg)	Route
I	GII.4-1999 VLP	10	IM
II	GII.4-1999 VLP	10	ID
III	GII.4-1999 VLP	50	IM
IV	GII.4-1999 VLP	50	ID
V	GII-mix VLPs (GII.4-1999, GII.4 NO, GII.12)	60 (20 each)	IM
VI	GII-mix VLPs (GII.4-1999, GII.4 NO, GII.12)+ GI-mix VLPs (GI.1 and GI.3)	60 + 40 (20 each)	IM ID
VII	PBS	–	IM
VIII	PBS	–	ID

PBS, phosphate-buffered saline.

2.3. Serum IgG antibody titer and avidity

Serum GII.4-specific IgG antibodies were detected by enzyme-linked immunosorbent assay (ELISA) as described elsewhere in detail [4,5]. In brief, twofold diluted serum samples were analyzed on NoV VLP-coated plates. IgG antibodies were detected with HRP-conjugated anti-mouse IgG (Sigma Aldrich, Saint Louis, MO) followed by o-Phenylenediamine dihydrochloride (OPD)-substrate (Sigma-Aldrich). The optical density (OD) was measured at 490 nm in a microplate reader (Victor^2 1420, Perkin Elmer, Waltham, MA). The mean end-point titers of the individual serum were determined as the reciprocal of the highest serum dilution giving an OD above the set cut-off value (mean OD of negative control mice serum wells + 3 × SD) and at least 0.100 OD.

Avidity assay was conducted as the IgG ELISA assay described above using 1:200 serum dilution, but additional two 8 M urea incubation steps were included to remove low avidity antibodies according to previously published method [20]. Avidity index was calculated as (OD with urea/OD without urea) × 100%.

2.4. Blocking assay

Blocking assay was used to detect antibodies preventing NoV GII.4-1999 VLP binding to the synthetic HBGAs as previously described in detail [11]. Briefly, synthetic biotinylated Le^d (H type 1)-PAA-Biotin (Glycotech, Gaithersburg, MD) was coated on NeutrAvidin plates (Pierce, Rockford, IL). After 1 h pre-incubation at 37 °C, a mixture of NoV VLPs (0.4 μg/ml) and serially twofold diluted serum samples were plated and incubated for 2 h at 4 °C. The bound VLPs were detected using NoV VLP type-specific human serum, followed by goat anti-human IgG (H + L)-HRP (Invitrogen, Carlsbad, CA) and OPD-substrate (Sigma-Aldrich). The OD was measured as described above. Maximum binding was determined by VLP lacking mouse sera. The results are expressed as the mean blocking index (%) calculated as 100% − [(OD wells with VLP − serum mix/OD maximum binding OD) × 100%]. Blocking titer 50 (BT50) was expressed as the reciprocal of the highest serum dilution blocking 50% of the maximum VLP binding.

2.5. IFN-γ ELISPOT assay

NoV-specific T cell responses were determined by the ability of short synthetic peptides to induce ex vivo IFN-γ production by splenocytes of immunized mice using interferon-gamma (IFN-γ) enzyme-linked immunospot (ELISPOT) assay [5]. Liquid nitrogen

frozen splenocytes were thawed and added to the anti-mouse IFN-γ antibody coated Multiscreen HTS-IP filter plates at 0.1×10^6 cells/well in culture medium (CM) containing 5% FBS (Sigma-Aldrich). The following peptides were used for stimulation: GII.4-1999 peptide pool, the two single peptides 99-45 and 99-50, a predicted 9-mer H-2^d epitope, and a negative 10-mer control peptide. Additionally, dose titrations (0.05–2 μg/ml) of the peptides 99-45 and 99-50 were performed to determine the functional avidity of T cells to the epitopes. CM was used as a background control and Concanavalin A (10 μg/ml, Sigma-Aldrich) as a cell viability control. After 20 h incubation at 37 °C in a humidified 5% CO_2 atmosphere, the spots were detected by biotinylated anti-mouse IFN-γ monoclonal antibody, followed by streptavidin-ALP and BCIP/NBT substrate (all from Mabtech Ab, Nacka Strand, Sweden) according to a previously described protocol [5]. The plates were analyzed by CTL-Europe GmbH (Bonn, Germany) and the results were expressed as mean spot forming cells (SFC) per 10^6 splenocytes. The experiments were performed in duplicates and each experiment was repeated at least twice.

2.6. Degranulation assay

Degranulation assay was performed to determine cytotoxic potential of the 99-45 peptide-specific $CD8^+$ T mouse splenocytes. The cells (1×10^6) were stimulated with 4 μg/ml 99-45 peptide in the presence of 3 μg/ml CD107a (clone 1D4B, BD Biosciences, San Jose, CA) FITC-conjugated antibody. As controls, unstimulated cells (spontaneous degranulation), negative control peptide (OVA) and PMA/ionomycin (Sigma-Aldrich) stimulated cells were included. After 1 h pre-incubation at 37 °C and 5% CO_2, the protein transport inhibitors brefeldin A and monensin (GolgiPlug and GolgiStop, BD Biosciences) were added and incubation was continued overnight. Treated cells were washed, blocked with rat anti-mouse CD16/CD32 (Fc Block, Clone 2.4G2, BD Biosciences) and stained with BD Horizon Fixable viability stain 780 for live cells gating. Cells were surface stained with PerCP-Cy5.5 fluorochrome-conjugated CD8-specific antibody, followed by treatment with BD Cytofix/Cytoperm Plus kit (BD Biosciences) and intracellular staining with PE-Cy7 conjugated CD3-specific antibody. The cells were acquired on BD FACSCanto II flow cytometer and analyzed using FlowJo v.10 software (ThreeStar Inc., San Carlos, CA). $CD3^+$ cells were gated from live lymphocyte gate and analyzed for CD8 and CD107a expression.

2.7. Statistics

The statistical differences between the experimental groups were determined by the Kruskal-Wallis test, Fisher's exact test or the Mann-Whitney *U* test, as applicable. Statistical analyses were performed using IBM SPSS Statistics for Windows (IBM Corp., Armonk, NY) version 23. Statistical significance was defined as a p value of <.05.

3. Results

3.1. Similar levels of NoV GII.4-1999 specific serum IgG antibodies induced by IM and ID delivery of GII.4 VLPs alone or co-delivered with other VLP genotypes

Immunization of the mice with GII.4 VLPs induced robust genotype-specific IgG antibodies irrespective of the dose, delivery route or co-administration of other genotype VLPs at the same time (Fig. 1A). The GII.4-specific serum IgG titers were similar in all experimental groups ($p > .05$), with end-point titers 102,400 or 204,800 for all VLP immunized mice (Fig. 1B).

3.2. NoV GII.4-1999-specific antibody blocking and avidity are not dependent on delivery route or immunogen composition

Sera of mice immunized with GII.4 VLP (10 μg groups only), GII VLP-mix, or GI VLP + GII VLP mix via IM or ID route were further analyzed for antibody avidity and blocking activity. All groups had developed high avidity antibodies (>50%) with no significant difference between the groups ($p = .161$) (Fig. 1C). Furthermore, similar blocking activity was observed for all deliveries (Fig. 1D). Sera of each experimental group reached BT50 of 800. Therefore, no immunological interference due to the competition between different genotypes was found in avidity or blocking activity of GII.4-specific antibodies.

3.3. Magnitude and specificity of NoV GII.4-specific T cell responses

Synthetic peptide pools and single peptides were used to compare T cell responses of mice immunized with 10 μg VLPs alone or co-administered with different VLP genotypes. Splenocytes of immunized and control mice were assayed for IFN-γ production against different peptide antigens (Fig. 2A). Each experimental group but not the control mice responded to a similar level ($p > .05$) to all GII.4-1999 derived peptides tested, while no response was induced by the negative 10-mer control peptide. The GII.4-99 peptide pool induced the highest IFN-γ response compared to other GII.4-specific peptides ($p < .05$) in all immunized groups. All tested NoV-specific peptides, the 18-mer peptides 99-45 and 99-50, previously identified to contain NoV GII.4-specific $CD8^+$ and $CD4^+$ T cell-specific epitopes [17], and the 9-mer predicted T cell epitope [17], induced specific SFC in immunized mice, although a response to 99-45 was somewhat higher ($p < .05$).

3.4. Functional avidity of $CD8^+$ and $CD4^+$ T cells

As we did not observe any differences in the magnitude or specificity of T cell responses of different experimental groups (Fig. 2A), we further tested epitope functional avidity of $CD4^+$ and $CD8^+$ T cells of mice immunized with different formulations, to detect possible difference. The cells were stimulated with a dose range (0.01–2 μg/ml, app. 10^{-5}–10^{-3} μM) of the single peptides 99-45 and 99-50 in the IFN-γ ELISPOT assays (Fig. 2B and C). No significant differences in the responses were observed between the groups ($p > .05$).

3.5. $CD8^+$ T cells specific for the 99-45 peptide epitope undergo degranulation

$CD8^+$ T cells specific for the peptide 99-45 were further tested for potential to lyse the NoV infected cells. Flow cytometry was used to measure surface expression of the degranulation molecule CD107a on $CD3^+CD8^+$ T cells, a marker of an effector cytotoxic population [21]. Approximately 0.8% of 99–45 peptide–specific CD8 + T cells of mice immunized with the GII VLP mix (Fig. 3A) and 0.6% of the cells of mice immunized with the GI + GII VLP mix (Fig. 3B) expressed CD107a at the surface. No 99-45 peptide-specific degranulation was seen with negative control mice cells (Fig. 3C) or when 14-mer OVA control peptide was used (data not shown).

4. Discussion

A broadly protective NoV VLP-based vaccine should contain at least a single representative of GI and GII viruses, as there is very little cross-protective immunity between the genogroups [5,14,16,22–24]. Concomitantly, we have recently proposed a trivalent combination of GII.4 VLPs, GI.3 VLPs and RV VP6 as a can-

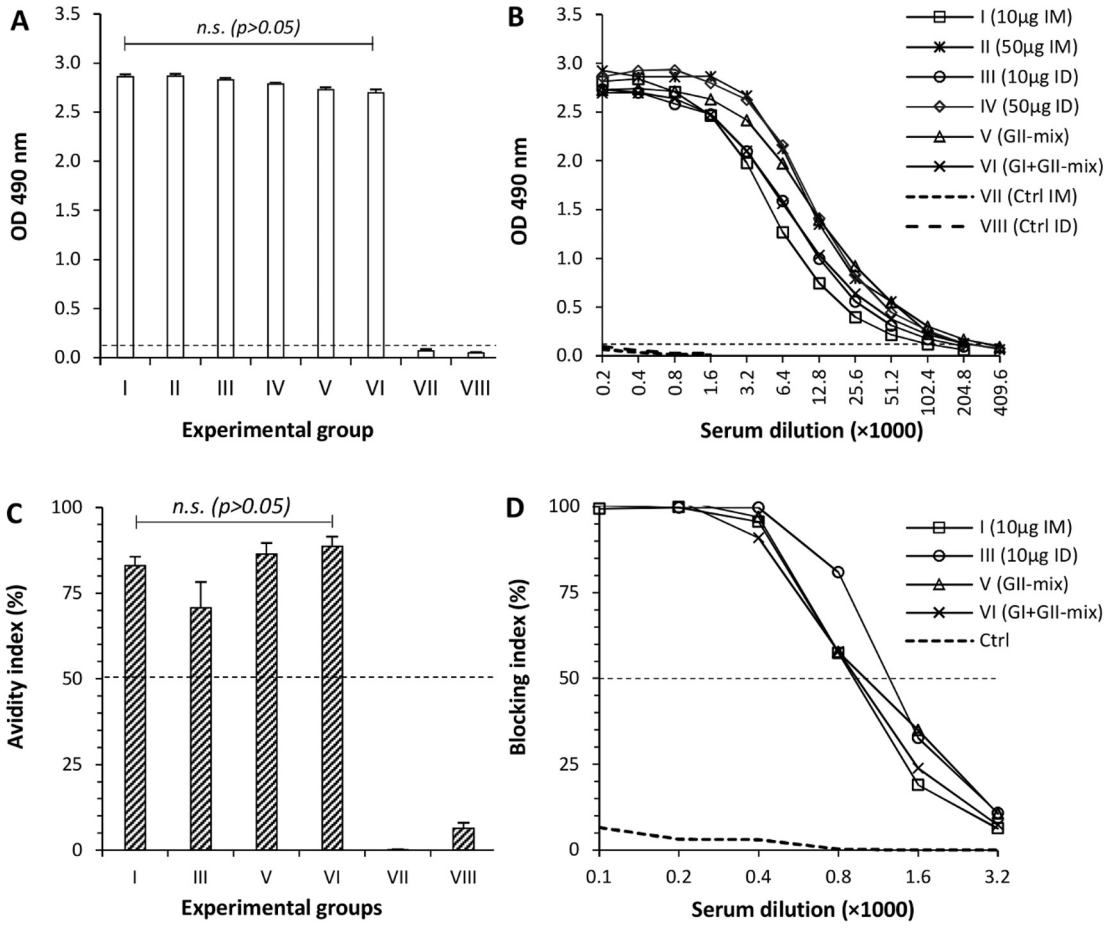

Fig. 1. Norovirus GII.4-1999-specific serum IgG responses. Individual termination serum antibodies shown as the group mean OD values with the standard errors of the mean (A) and end-point titers analyzed from the group-wise pooled sera (B). Mice were immunized IM or ID with GII.4-1999 VLPs alone (groups I to IV), GII VLP mix (group V), or GI + GII VLP mix (group VI). Mice receiving carrier (PBS) only were used as negative controls (Ctrl, groups VII and VIII). Horizontal dashed line (A and B) indicates a positivity cut-off (OD > 0.100). GII.4-specific IgG mean avidity indices (%) with standard error of the means (C) and pooled serum blocking antibody titers (D) in groups immunized with GII.4 VLPs alone (10 µg), GII mix, or GI + GII-mix. Horizontal dashed line indicates 50% avidity (C) or blocking (D) index. *n.s.*, statistically not significant ($p > .05$).

didate vaccine against childhood GE [4,25]. In the present study, we determined antibodies and T cell immune responses to GII.4-1999 VLPs administered either alone or co-administered with different VLP genotypes in mice, to determine possible immunological interference or inhibition of both humoral and cellular immune responses. As the representatives of GII NoVs, three different VLPs were used, an ancestor GII.4-1999, the more recent GII.4 NO (2009) genotype and antigenically distant GII.12 genotype. GII.4 NoVs have been dominant in causing sporadic cases and GE outbreaks for more than 20 years, with newly emerging variants every 2–4 years [26]. NoV GI.1 and GI.3 VLPs were chosen as the representatives of GI NoVs.

High serum IgG antibody titers generated against NoV GII.4 were detected in mice immunized with GII.4-1999 VLPs either alone or in any combination. Furthermore, the functionality of these antibodies, in terms of virus neutralizing potential and avidity, determining the strength of molecular interaction between polyclonal serum antibodies and antigens, was very good. This is important as blocking antibodies are considered the most significant correlate of protection from NoV infection and disease identified so far [8,22,24,27]. High avidity antibodies, produced as a result of B cell avidity maturation, better tolerate variations within the target epitopes [17,23,28,29] and are important for protection from viral infections [30–32].

NoV-specific blocking antibodies in humans and mice are highly type-specific [23,28,33] and therefore the biological significance of other arms of protective immunity, such as cross-reactive T cell responses, should be considered besides blocking of ligand interaction. It has been suggested that protective immunity to NoV might be partially dependent on the activation of T cell immunity, [14,34–37], however, very little is known about the role of NoV-specific T cells in natural infection [14] and even less on the vaccine induced T cell responses in humans or experimental animal models [35,36]. T cell immunity has a significant role in protection from other viruses such as influenza [38], measles [39], and human immunodeficiency virus [40,41]. To the best of our knowledge, our group is the only one who has identified NoV-specific T cell epitopes in naturally exposed humans [15] and BALB/c mice immunized with the NoV VLPs [17]. Limited number of GII.4-specific T cell epitopes was detected in a heterogeneous human population, in contrast to seven cross-reactive T cell epitopes identified in a single inbred mouse strain, suggesting that T cell immunity might significantly improve after NoV VLP vaccination [15,17].

In contrast to naïve mice, in humans the vaccination is influenced by the complex prior NoV exposure history, however, the effect is yet unknown [10,42]. Vaccination may improve the pre-existing immunity in humans by eliciting strong recall immune responses towards conserved epitopes, despite the wide genetic inter- and intragenogroup variability of NoVs [10]. Clinical trials in adults have suggested that vaccination induces cross-protective memory immune responses, such as cross-blocking antibodies, directed towards strains encountered in the past, in

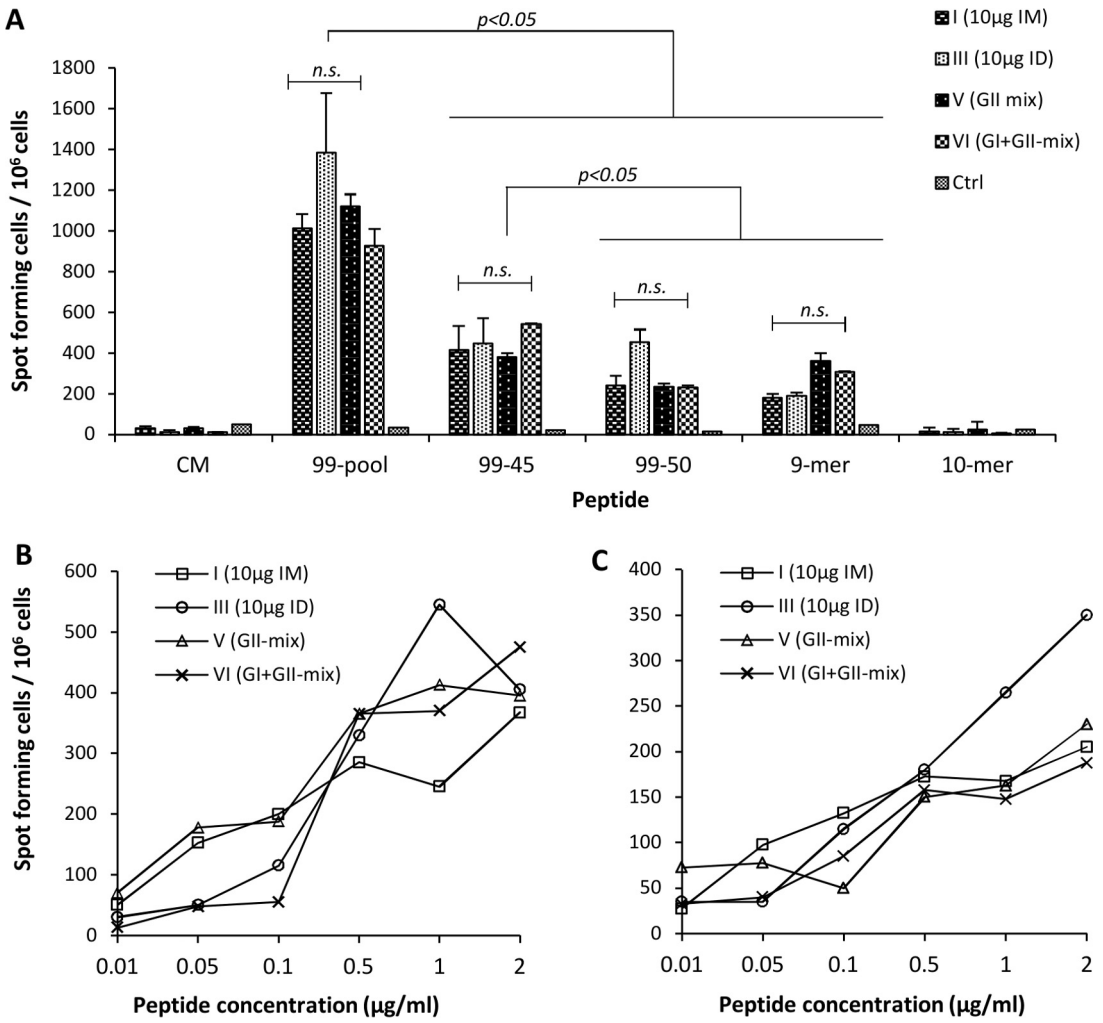

Fig. 2. NoV GII.4-specific T cell responses. Splenocytes of mice immunized IM or ID with 10 µg GII.4 VLP alone, GII VLP mix or GI + GII VLP mix were analyzed for IFN-γ production by ELISPOT assay. The control mice (Ctrl) received PBS only. Complete GII.4-99 peptide pool, 18-mer single peptides 99-45 and 99-50, and a predicted 9-mer T cell epitope were used to stimulate the cells. An irrelevant 10-mer peptide served as a negative control and culture media (CM) as a background control (A). Stimulation of the cells with increasing concentrations of the peptides 99-45 (B) and 99-50 (C) was used to determine the functional avidity of T cells. Mean IFN-γ spot-forming cells per 10^6 splenocytes of repeated experiments with standard errors of the mean are shown. P value < .05 was considered statistically significant. *n.s.*, statistically not significant (p > .05).

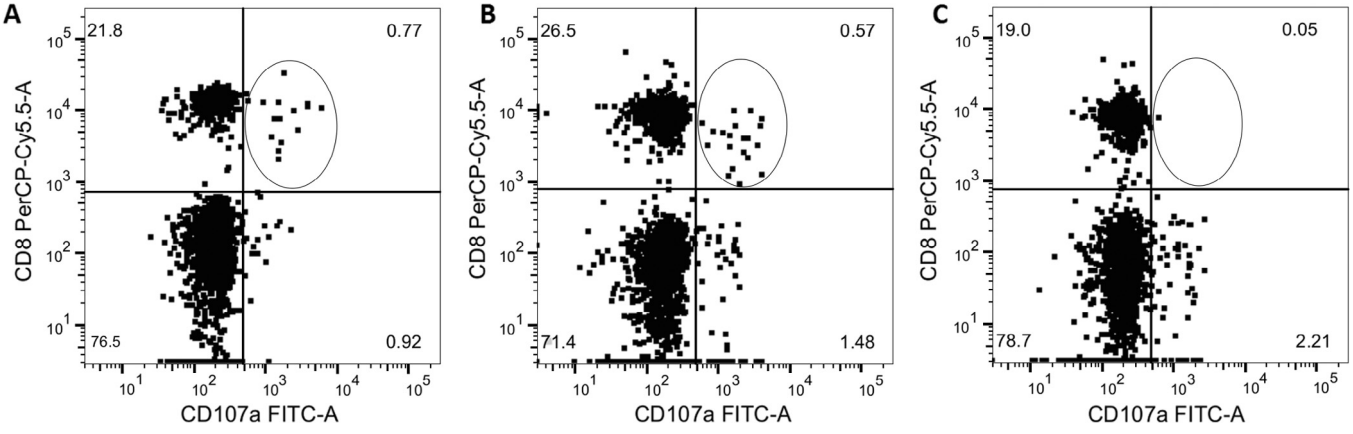

Fig. 3. Expression of CD107a in NoV-specific CD8 T cells. The group-wise pooled splenocytes of mice immunized with GII VLP mix (A) or GI + GII VLP mix (B), or splenocytes of the control mice (C), were stimulated with the single peptide 99-45, in the presence of CD107a FITC and protein transport inhibitors, before flow cytometry analysis on FACS Canto II. Events (%) shown are gated for live $CD3^+$ $CD8^+$ T cells and circled are $CD8^+CD107a^+$ cells.

addition to eliciting immune responses against strains included in the vaccine [10,43].

In this study, the T cell responses were comparable in all experimental groups tested to the $CD4^+$ T cell epitope 99-50 (^{344}-TRAHKATVSTGSVHFTPK^{361}), $CD8^+$ T cell epitope 99-45 (^{309}NNYDPTEEIPAPLGTPDF^{326}), and, for the first time, to a minimal epitope (^{318}PAPLGTPDF^{326}) conforming the predicted H-2^d binding motif [17]. The functional avidity is the activation threshold of the T cells, describing how well the cells respond to a given concentration of an antigen, as reviewed extensively by Vigano et al. [44]. Considering the major role of the high functional avidity of T cell receptor to a given peptide epitope in driving T cell antiviral activity [45–48], it is of importance that T cell magnitude, specificity and avidity were similar in all GII.4 VLP immunized mice, regardless of the administered formulation.

Importantly, the results of this study are the first to suggest the true CTL potential of the $CD8^+$ T cells specific for the peptide 99-45, which contains the highly conserved epitope across GII NoV genotypes. Increased cell surface CD107a expression in immunized mice cells is associated with loss of intracellular perforin, and indicates the ability of these cells to lyse NoV infected cells [21]. CTLs are considered as the main effectors against intracellular pathogens, conferring protection from the illness in the case that not all free virus is neutralized at the port of entry, owing to their ability to lyse the infected cells prior virus replication and release of new virions [49]. Unlike in NoV VLP immunized inbred mice as described above, highly conserved $CD8^+$ T cell epitopes in humans remain yet to be identified. Although human population is heterogeneous, it is possible that some protein domains in NoV VLPs are immunoprevalent, i.e. contain epitopes restricted by multiple MHC alleles [50,51].

IM delivery is the most commonly used for vaccinating adults, infants and small children. Even though ID immunization, with the abundance of antigen presenting cells in the close proximity, might be the most optimal route for delivering VLPs [52–54], the results of this study showed that IM and ID deliveries induced similar quantities and qualities of NoV GII.4-specific antibodies and $CD4^+$ and $CD8^+$ T cells. This finding is of a significant importance considering the easiness of IM delivery to small children, compared to ID delivery. Even though the importance of IgA and mucosal immunity in protection from NoV infection and disease has not been definitely established, they are likely to play protective role in NoV infection transmitted via mucosal route [55–57]. In GII.4 challenge studies, pre-existing NoV-specific serum IgA and mucosal IgA were associated with decreased frequency of infection and severe illness [58]. However, in the present study using parenteral routes of immunization, no IgA responses were evaluated.

We believe that protection against NoV reflects the sum of various immune responses, including antibody and cell-mediated, especially T cell, immunity. Our results show the potential for a multivalent NoV VLP vaccine to induce humoral and cell-mediated immune responses without immunological interference.

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Conflict of interest

We declare no conflicts of interest.

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