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Genotype Considerations for Virus-Like Particle-Based Bivalent Norovirus Vaccine Composition

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Norovirus (NoV) genogroup I (GI) and GII are responsible for most human infections with NoV. Because of the high genetic variability of NoV, natural infection does not induce sufficient protective immunity to different genotypes or to variants of the same genotype and there is little or no cross-protection against different genogroups. NoV-derived virus-like particles (VLPs) are promising vaccine candidates that induce high levels of NoV-specific humoral and cellular immune responses. It is believed that a bivalent NoV vaccine consisting of a representative VLP from GI and GII is a minimum requirement for an effective vaccine. Here, we compared the abilities of monovalent immunizations with NoV GI.1-2001, GI.3-2002, GII.4-1999, and GII.4-2010 New Orleans VLPs to induce NoV type-specific and cross-reactive immune responses and protective blocking antibody responses in BALB/c mice. All of the VLPs induced comparable levels of type-specific serum IgG antibodies, as well as blocking antibodies to the VLPs used for immunization. However, the abilities of different VLP genotypes to induce cross-reactive IgG and cross-blocking antibodies varied remarkably. Our results confirm previous findings of a lack of cross-protective immune responses between GI and GII NoVs. These data support the rationale for including NoV GI.3 and GII.4-1999 VLPs in the bivalent vaccine formulation, which could be sufficient to induce protective immune responses across NoV genotypes in the two common genogroups in humans.

Noroviruses (NoVs) are the leading cause of sporadic and epidemic nonbacterial gastroenteritis worldwide (1, 2). NoV disease is characterized by a short duration of symptoms (3), which can be severe, especially for people in high-risk groups, such as young children, the elderly, or immunocompromised patients. There is currently no vaccine available to prevent NoV infection. Cell culture models to support the propagation of human NoVs have previously failed, hampering the use of live or attenuated NoV vaccines. However, Jones et al. recently described a cell culture model to productively infect human B cells with NoV (4) that might be a step closer to successful NoV propagation. NoV capsid VP1 protein spontaneously forms virus-like particles (VLPs) morphologically and antigenically similar to NoV virions (5, 6). NoV VLPs can be efficiently produced in insect cells with baculovirus expression systems and a variety of other protein expression systems (5, 7). VLPs are promising candidates for use in a vaccine against NoV (8–10), as well as several other viruses, including influenza virus (11), parvovirus (12), and HIV-1 (13). VLP-based vaccines against hepatitis B virus (14, 15) and human papillomavirus (16) are currently licensed and used worldwide. As VLPs are highly immunogenic particulate structures, it is believed that addition of external adjuvants is not needed (17). This is very important, particularly when designing NoV vaccines for a pediatric population (9). However, clinical trials of NoV VLP vaccine conducted with adults have used adjuvants (18, 19) and proven that adjuvanted NoV VLP vaccine is safe and immunogenic.

NoVs are single-stranded, positive-sense RNA viruses in the *Caliciviridae* family and are genetically very heterogeneous, with six genogroups (GI to GVI) recognized so far (20, 21). GI and GII NoVs are responsible for most human NoV infections, comprising more than 30 genotypes that evolve rapidly to novel immune escape variants (22). GII viruses are responsible for approximately 90% of the human NoV infections that occur each year, most of which are caused by variants of a single GII.4 genotype (2, 23). New emerging strains develop approximately every 2 to 3 years, and they have been related to changes in blocking antibody epitopes in the hypervariable P2 domain of VP1 (24, 25).

Diverse putative receptors/attachment factors for NoVs, histo-blood group antigen (HBGA) carbohydrates, are found on mucosal epithelial cells and as free antigens in body secretions (22, 26). HBGA expression is associated with susceptibility or resistance to certain NoV strains (26, 27). GII.4 strain NoVs have an exceptionally broad HBGA binding repertoire and high transmissibility (2), explaining the predominance of GII.4 NoV infections worldwide (28). The quantity of genotype-specific antibodies that can block the binding of NoV VLP to the HBGA has been shown to increase remarkably after NoV infection or NoV VLP immunization in humans (18, 19, 29, 30). Prechallenge levels of blocking antibodies in human serum have been shown to positively correlate with the protection of both NoV infection and illness, and it is generally accepted that especially blocking antibodies in serum play a substantive role in protection from NoV infection (18, 31).

Natural immunity to NoV has been believed to have a short duration (32, 33); however, a more recent estimate suggests that protection could last up to 8 years (34). However, induction of

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long-term protective immunity is extremely challenging because of the rapid evolution of NoV strains that result in high genetic variability and insufficient cross-protective immunity, especially between GI and GII NoVs (30, 35, 36). It is believed that a representative of each genogroup is a minimum requirement for cross-protective NoV vaccine (10, 37, 38). Indeed, research groups working on NoV vaccine development have used VLP combinations to constitute their vaccine candidates (8, 19, 35, 38).

We have tested NoV GI.1, GI.3, GII.4-1999, and GII.4-2010 New Orleans (NO) VLPs representing GI and GII NoVs as vaccine candidates in BALB/c mice. GI.1 (Norwalk virus) represents a historic prototype NoV (39), and GI.3 is a commonly found GI virus that also efficiently infects children (40, 41). As NoV GII.4 has dominated worldwide for close to 2 decades (24), we have chosen an ancestor GII.4 strain (1999) and a more recent GII.4 NO (2010) strain as representatives of GII NoVs.

MATERIALS AND METHODS

VLP generation. Six NoV VLPs, GI.1-2001 (GenBank accession no. AY502016), GI.3-2002 (GenBank accession no. AF414403), GII.4-1999 (GenBank accession no. AF080551), GII.4-2010 NO (GenBank accession no. GU445325), GII.4-2012 Sydney (GenBank accession no. AFV08795.1), and GII.12-1998 (GenBank accession no. AJ277618), were expressed with a baculovirus expression system and purified from insect cells by sucrose ultracentrifugation as described previously (42, 43). VLP preparations were analyzed as described elsewhere (38, 42, 43). In brief, the total protein concentration was determined with the Pierce bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL). The purity and integrity of the VLP preparations were analyzed by SDS-PAGE and densitometric analysis. Endotoxins (<0.1 endotoxin unit/10 μg of protein) were quantified by *Limulus* amebocyte lysate assay (Lonza, Walkersville, MD). VLP morphology was confirmed by transmission electron microscopy, with all VLPs displaying particles of approximately 30 to 40 nm (38). NoV GI.1-, GI.3-, GII.4-1999-, and GII.4-2010 NO-derived VLPs were used to immunize mice as described below. In addition, GII.4-2012 Sydney and GII.12 VLPs were used in analytical methods.

Mouse immunization and sample collection. Female 7- to 8-week-old BALB/c mice obtained from Harlan Laboratories (Netherlands) were used for immunization. Two doses of 10 μg of monovalent (10 mice/group) NoV VLPs (GI.1, GI.3, GII.4-1999, and GII.4-2010 NO) in phosphate-buffered saline (PBS) were administered intramuscularly. In another set of experiments, mice were immunized with a bivalent combination of GI.3 and GII.4-1999 VLPs (five mice/group). Control mice were immunized with PBS only. Mice were immunized at weeks 0 and 3 and euthanized 2 weeks after the second immunization. Animals were anesthetized before immunization and euthanasia with a mixture of medetomidine (Dorbenevet, 1 mg/ml; Laboratorios SYVA S.A., Leon, Spain) and ketamine (Ketaminol vet, 50 mg/ml; Intervet International B.V., Boxmeer, Netherlands). Blood and spleens were collected at the time of termination for the analysis of serological and cell-mediated immune responses as previously described (44). Serum samples were stored at −20°C, and spleen cell suspensions were stored in liquid nitrogen. Experiments were performed in accordance with the guidelines of the Finnish National Animal Experiment Board.

Serum IgG, IgG1, and IgG2a ELISA. Serum of immunized mice was analyzed by enzyme-linked immunosorbent assay (ELISA) (42) to determine type-specific and cross-reactive IgG, IgG1, and IgG2a titers. Briefly, 0.4 to 1.0 μg/ml of GI.1, GI.3, GII.4-1999, GII.4-2010 NO, GII.4-2012 Sydney, or GII.12 VLPs in PBS was used to coat 96-well half-area polystyrene plates (Corning Inc., Corning, NY). Duplicates of 2-fold serial dilutions of serum samples (starting at 1:200) were incubated for 2 h at room temperature, and antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma-Aldrich, Saint Louis, MO), IgG1 (Invitrogen, Carlsbad, CA), or IgG2a (Invitrogen), followed by reaction with the SIGMAFAST *o*-phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich). Optical density (OD) at 490 nm was measured with a Victor^2 1420 Multilabel Counter (Wallac, PerkinElmer) plate reader. The background signal in wells without serum was subtracted from all of the OD readings of a plate. The wells of control mouse serum were used to count the cutoff value as the mean OD plus three standard deviations. Samples with a net OD value above the set cutoff and an OD of at least 0.100 were considered positive (45). The endpoint antibody titer was defined as the highest dilution of serum giving an OD above the set cutoff value.

Blocking of HBGA binding. ELISAs measuring antibodies able to block NoV VLP binding to HBGAs were performed by previously published methods based on the use of synthetic or human saliva HBGAs (28, 42, 44). Groupwise pooled serum was diluted 2-fold starting at 1:100 for homologous blocking and 1:10 for heterologous blocking. For a blocking assay using synthetic HBGAs, serum was preincubated for 1 h at 37°C with 0.4 μg/ml of NoV VLPs before plating on biotin-conjugated synthetic HBGA (GlycoTech Corporation, Rockville, MD)-coated NeutrAvidin plates (Pierce, Rockford, IL). Synthetic H type 1 HBGA was used for binding of all VLPs, except for GI.3 VLPs and GII.12 VLPs, which were tested on Lewis^a (Le^a) and blood type B trimer (B^{tri}) HBGAs. For a saliva blocking assay, secretor-positive human type A or O saliva (for GI.1 VLPs only) was used at a dilution of 1:3,000 in PBS to coat half-area 96-microtiter plates before adding the preincubated mixture of serum and 0.1 μg/ml of NoV VLP. Maximum VLP binding was determined in wells containing VLPs without serum. Bound VLPs were detected with human NoV antiserum (29) and an anti-human IgG-HRP secondary antibody, followed by OPD substrate as described above. OD readings were determined spectrophotometrically at 490 nm with a microplate reader. The blocking index (percent) was calculated as follows: 100% − [(OD wells with VLP and serum/OD wells without serum, maximum binding) × 100%].

IFN-γ ELISPOT assay. A gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay was used to enumerate GII.4 NoV-specific IFN-γ-producing T cells of immunized mice as previously described (38). Mouse splenocytes from either individual or groupwise pooled mice were plated on MultiScreenHTS-IP filter plates (Millipore, Billerica, MA) coated with an anti-mouse IFN-γ monoclonal antibody (Mabtech AB, Nacka Strand, Sweden) at 5 μg/ml and blocked with 10% fetal bovine serum (FBS; Sigma-Aldrich). A total of 1×10^5 splenocytes were stimulated with synthetic peptides (5 μg/ml) for 20 h in cell culture medium (CM; RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol, and 2 mM L-glutamine; Sigma-Aldrich). The following NoV capsid-derived synthetic 15-mer peptides (ProImmune Ltd., Oxford, United Kingdom) were used: NP-4, a previously published T-cell epitope of an ancestor GII.4 virus (CLLPQEWVQHFYQEA, amino acids 461 to 475) (46); NP-4 NO, a corresponding GII.4 NO-specific peptide (CLLPQEWVQYFYQ EA); and NP-12, a GII.12-specific peptide (CLLPQEWIQHLYQES). Stimulation with concanavalin A (ConA; Sigma-Aldrich) at 10 μg/ml was used as a positive control. CM alone and irrelevant rotavirus 15-mer peptide R6-3 (IFPYSASFTLNRSQP) were added as negative controls to each assay. IFN-γ was detected with a biotinylated anti-mouse IFN-γ monoclonal antibody (Mabtech; 0.5 μg/ml in PBS–0.5% FBS), followed by 1:1,000-diluted streptavidin-alkaline phosphatase (Mabtech), and spots were developed with 5-bromo-4-chloro-3-indolylphosphate (BCIP)-Nitro Blue Tetrazolium substrate (Mabtech). Plate reading and analysis were done by an ImmunoSpot automatic cytotoxic T-lymphocyte analyzer (CTL-Europe GmbH, Bonn, Germany), and the results are expressed as the mean number of spot-forming cells (SFC)/10^6 splenocytes in duplicate wells.

Statistics. A nonparametric Mann-Whitney U test was performed to assess the statistical significance of differences. Data were analyzed with IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL). A statistically significant difference was defined as a P value of ≤0.05.

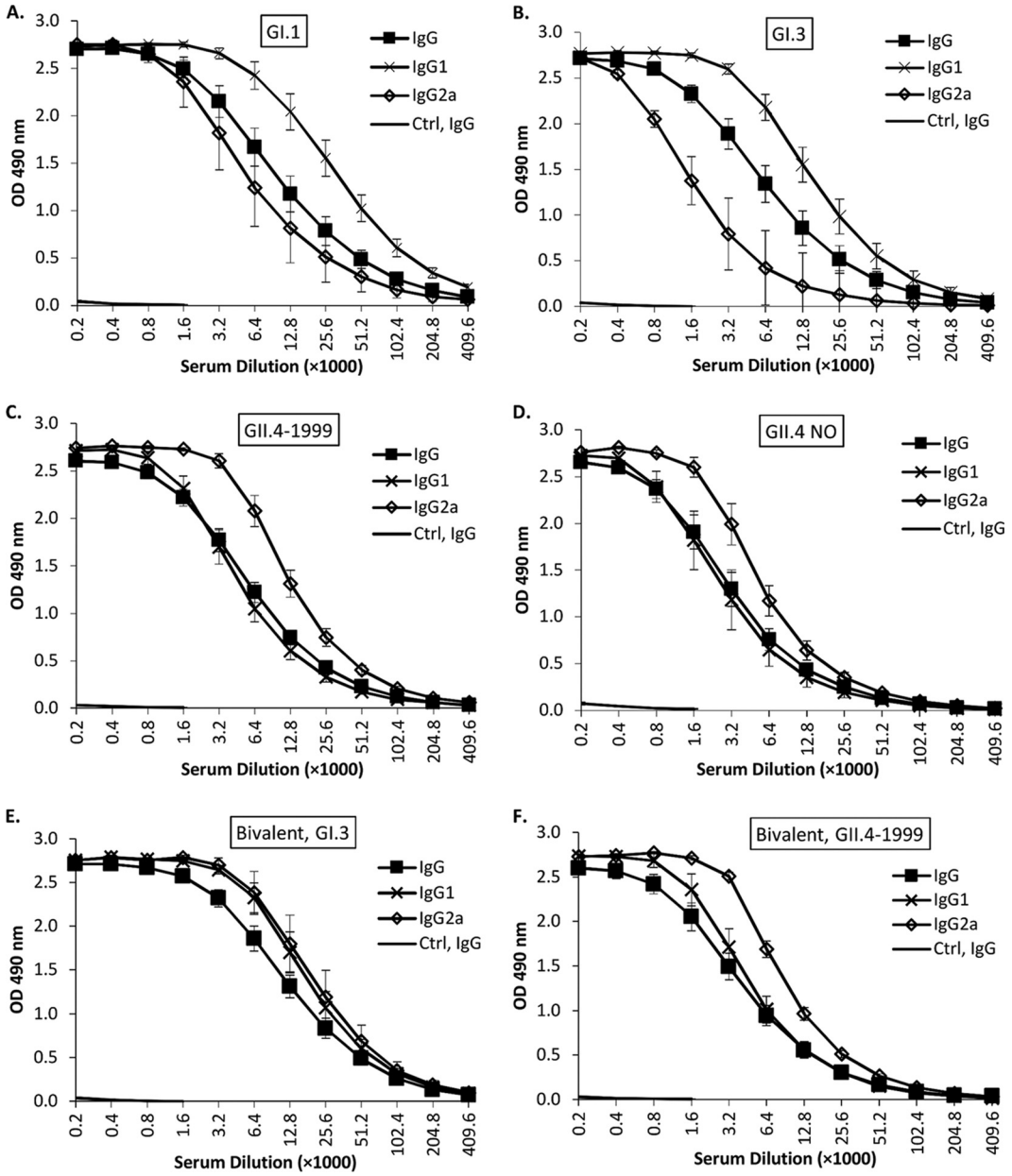

FIG 1 NoV genotype-specific serum IgG, IgG1, and IgG2a antibody titers. Termination sera of mice immunized with monovalent NoV GI.1 (A), GI.3 (B), GII.4-1999 (C), or GII.4 NO (D) VLPs were titrated against homotypic NoV VLP antigen in an ELISA. Bivalent GI.3 and GII.4-1999 VLP-immunized mouse serum was analyzed for GI.3 (E)- and GII.4-1999 (F)-specific antibodies. Serum of mice immunized with only the carrier (PBS) served as a control (Ctrl). Shown are the mean ODs of the groups with error bars representing the standard errors of the means.

RESULTS

Genotype-specific serum IgG, IgG1, and IgG2a titers. Mice were immunized with monovalent NoV VLPs or a bivalent combination of GI.3 and GII.4-1999 VLPs. The total serum IgG antibodies in mouse serum were analyzed 2 weeks after the second immunization. Robust NoV genotype-specific IgG responses against each VLP were detected (Fig. 1A to F) with endpoint titers of >51,200. In addition, IgG antibody subclass IgG2a (representing a Th1-type response) and IgG1 (representing a Th2-type response) titers in serum were measured. All VLP immunizations induced both IgG1 and IgG2a antibodies (Fig. 1A to F). Mice immunized with the bivalent combination of GI.3 and GII.4-1999 VLPs developed IgG, IgG1, and IgG2a antibody responses comparable to those of mice immunized with each of the single VLPs (all $P > 0.6$) (Fig. 1E and F). Control mouse serum was completely negative for all VLP antigens (Fig. 1A to F).

Cross-reactive IgG antibodies. In addition to type-specific antibody responses, cross-reactive serum IgG against GI.1, GI.3, GII.4-1999, GII.4 NO, GII.4 Sydney, and GII.12 NoV genotypes were analyzed. Strong intragenogroup-specific IgG responses were observed in each experimental group, but only limited, significantly lower ($P < 0.05$) intergenogroup-specific antibody re-

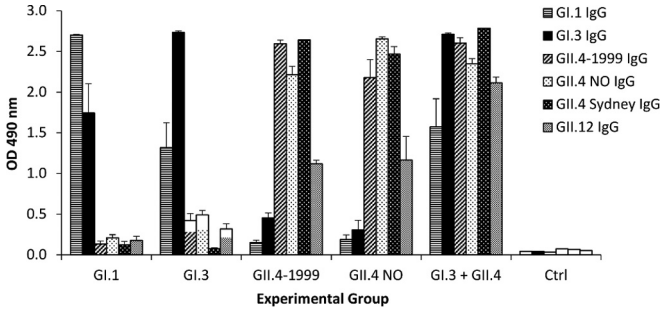

FIG 2 NoV VLP genotype-specific and cross-reactive serum IgG responses against five heterologous NoV genotypes. Serum was analyzed by ELISA at a dilution of 1:200 after monovalent GI.1, GI.3, GII.4-1999, or GII.4 NO or bivalent GI.3 and GII.4-1999 VLP immunization. Serum of PBS-immunized mice was used as a negative control (Ctrl). Shown are the mean ODs of the experimental groups with error bars representing the standard errors of the means.

sponses were detected (Fig. 2). Immunization with the bivalent GI.3 and GII.4-1999 VLPs induced a response similar to that induced by immunization with each VLP alone; thus, it generated the broadest serum IgG response. All of the ODs of the PBS-immunized control mice were <0.05.

Blocking activity of immune mouse sera. The capability of mouse immune sera to block NoV VLP binding was determined by incubating serially diluted sera with NoV VLPs before plating on microtiter plates coated with synthetic HBGAs or human type A or O saliva. On the basis of the data recently reported by our group (28), synthetic H type 1 HBGA was chosen to be used for GI.1, GII.4-1999, GII.4 NO, and GII.4 Sydney blocking assays, while Le^a was used for GI.3 VLP blocking and B^{tri} was used for GII.12 VLP blocking. Homologous blocking activity in sera from each experimental group measured by a saliva assay is shown in Fig. 3A and C, and that measured by a synthetic HBGA assay is shown in Fig. 3B and D. All immunizations with monovalent VLPs, as well as the bivalent VLP, generated strong blocking activity in serum. The serum antibody titers needed to block $\geq$90% of the VLPs from binding either to saliva or to the synthetic HBGAs ranged from 1:200 to 1:400. Control mouse sera blocked $\leq$10% of the VLP binding.

Serum blocking activity against heterologous VLPs not used for immunization was also investigated. Remarkable differences between the experimental groups were observed (Fig. 4A to E and 5A). GI.1 VLP-immunized mice did not generate any cross-blocking serum activity (Fig. 4A and 5A), while GI.3 VLP-immunized mouse serum blocked heterologous GI.1 VLP binding but no GII

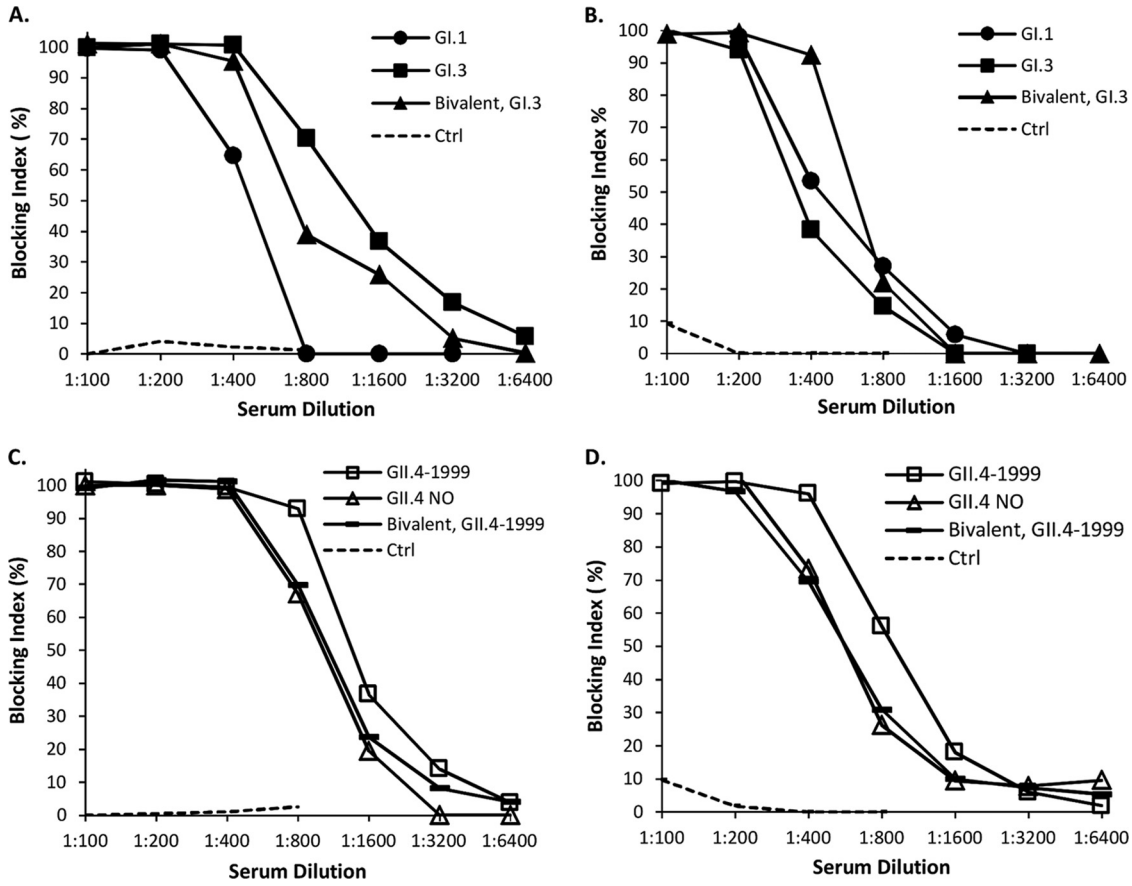

FIG 3 NoV genotype-specific blocking activities of monovalent and bivalent VLP-immunized mouse sera. Serum samples were diluted 2-fold and assayed for the blocking of homologous NoV VLP binding to human saliva (A and C) and synthetic (B and D) HBGA-coated plates. Type A saliva was used, except for GI.1 VLP binding, where type O saliva was used. H type 1 HBGA was used in GI.1, GII.4-1999, and GII.4 NO synthetic blocking assays, while Le^a HBGA was used for GI.3 VLP blocking. GI-specific blocking is shown in the upper panels, and GII-specific blocking is shown in the lower panels. Nonspecific blocking by control (Ctrl) mouse serum is shown as dashed lines. The blocking index (percent) was calculated as follows: 100% − [(OD wells with serum/OD wells without serum, maximum binding) × 100%].

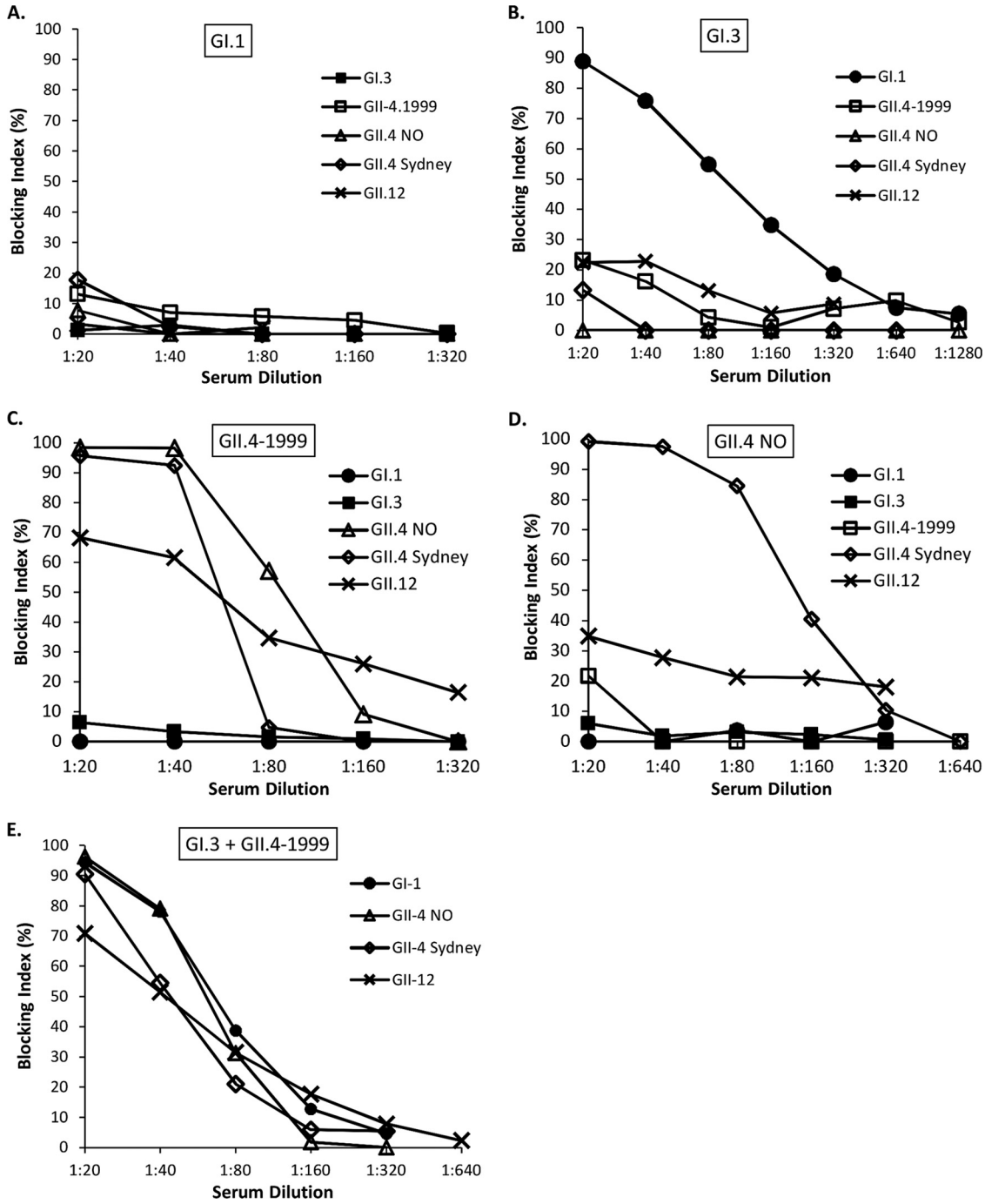

FIG 4 Serum blocking of heterologous VLP binding. The serum of mice immunized with GI.1 (A), GI.3 (B), GII.4-1999 (C), GII.4 NO (D), or bivalent GI.3 and GII.4 (E) VLPs was serially 2-fold diluted starting at a 1:20 dilution and analyzed in a blocking assay against heterologous GI and GII VLPs.

VLP binding (Fig. 4B and 5A). Serum of GII.4-1999 VLP-immunized mice blocked the binding of all of the GII VLPs tested, i.e., GII.4 NO, GII.4 Sydney, and GII.12 (Fig. 4C and 5A). On the contrary, GII.4 NO VLP-immunized mouse serum was able to cross-block only GII.4 Sydney VLP binding (Fig. 4D and 5A). Mice immunized with the bivalent VLP combination blocked the binding of all of the heterologous VLPs tested (Fig. 4E and 5A). The control mouse serum blocking index against each VLP at a dilution of 1:20 was <20% in every assay (data not shown).

IFN-γ ELISPOT assay. We further tested the T cell responses of mice immunized with GII.4-1999 and GII.4 NO VLPs to 15-mer synthetic peptides specific for GII.4 (NP-4 and NP-4 NO, respectively) and GII.12 (NP-12) capsid proteins in an IFN-γ ELISPOT assay (44). Immunization with GII.4-1999 VLPs induced robust self NP-4 peptide-specific (304 SFC/10^6 cells) and cross-reactive NP-4 NO-specific (206 SFC/10^6 cells) and NP-12-specific (269 SFC/10^6 cells) IFN-γ responses (Fig. 6). On the contrary, GII.4 NO VLP-immunized mice did not respond to any of

A

Exp. group \ VLP	GI.1	GI.3	GII.4-1999	GII.4 NO	GII.4 Sydney	GII.12
GI.1						
GI.3						
GII.4-1999						
GII.4 NO						
GI.3 + GII.4-1999						

B

GII.4 Variants	Blockade epitopes														
	A						B		C		D			E		
	294	296	297	298	368	372	333	382	340	376	393	394	395	407	412	413
GII.4-1999	A	**S**	H	D	T	N	L	**K**	E	Q	N	-	N	N	T	G
GII.4-2010 NO	P	**S**	**R**	**N**	A	**D**	**V**	**K**	**T**	**E**	S	**T**	**T**	**S**	**N**	I
GII.4-2012 Sydney	T	**S**	**R**	**N**	E	**D**	**V**	**K**	**T**	**E**	G	**T**	**T**	**S**	**N**	T

FIG 5 Serum blocking activity and blockade epitopes. (A) Schematic representation of homologous and heterologous blocking activities of immune mouse serum. Dark gray shading indicates homologous blocking, and light gray shading indicates heterologous blocking. Absence of shading indicates absence of blocking activity. (B) Variation in the amino acid sequences of blockade epitopes A to E (22, 51) in subdomain P2 of the three GII.4 variants used in this study. Identical amino acids are in boldface.

the peptides (Fig. 6). Negative-control 15-mer peptide R6-3 did not induce IFN-γ responses in any of the mice (<50 SFC/10^6 cells). ConA stimulation indicated good cell viability (all >5,000 SFC/10^6 cells, data not shown). Control mice did not respond to any peptide antigen stimulation (<50 SFC/10^6 splenocytes).

DISCUSSION

NoV GI.1, GI.3, GII.4-1999, and GII.4-2010 NO VLPs representing GI and GII NoVs were used as monovalent vaccine candidates to immunize BALB/c mice. NoV VLP immunization of mice induced humoral and cell-mediated immune responses, which is in concordance with previously published results (38, 42, 44). Immunizations with each monovalent VLP resulted in high genotype-specific and cross-reactive intragenogroup-specific serum IgG titers. Low levels of cross-reactive intergenogroup-specific IgG antibodies detected were probably directed to the conserved N-terminal region and S domain of NoV capsid VP1 (47). Importantly, strong blocking of NoV VLP binding by homologous immune sera was observed in each experimental group.

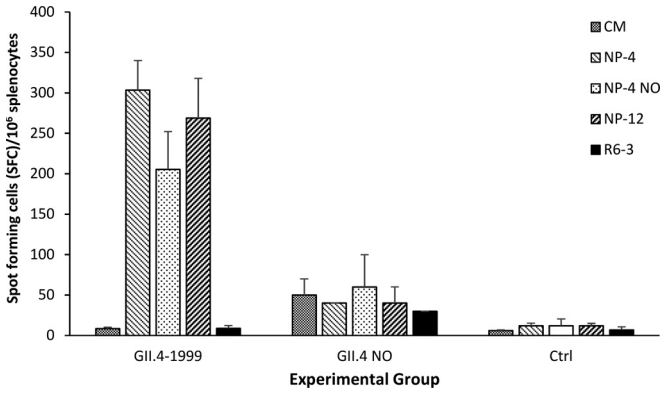

FIG 6 NoV-specific IFN-γ responses. Splenocytes of mice immunized with the monovalent GII.4-1999 and GII.4 NO VLPs or control (Ctrl) mice immunized with the carrier (PBS) only were stimulated *in vitro* with 15-mer NoV capsid-derived synthetic peptides specific for GII.4-1999 (NP-4), GII.4 NO (NP-4 NO), and GII.12 (NP-12). An irrelevant peptide (R6-3) was used as a negative control, and CM served as a background control. The results shown are the mean numbers of SFC/10^6 cells and the standard errors of the means.

Comparison of monovalent immunizations with several NoV VLPs revealed different potentials of the proteins to induce cross-reactive blocking antibodies. As GI NoVs are very well conserved (48), we expected to see similar results induced by the GI.1 and GI.3 VLPs. However, GI.3 VLPs induced serum antibodies able to block GI.1 VLP binding to the HBGAs but not vice versa. Also, although NoV VLPs GII.4-1999 and GII.4-2010 NO belong to the same genotype, having approximately 95% homology in the VP1 capsid protein amino acid sequences (21, 49), there is remarkable difference in the putative protective immune responses they induce. We demonstrated in this study that GII.4-1999 ancestor VLPs induced cross-blocking antibodies against distant and rare but recently reemerging GII NoV genotype GII.12 (23, 50), as well as to GII.4 NO and to the most recent GII.4-2012 Sydney VLPs. On the contrary, GII.4 NO VLP immunization induced cross-blocking antibodies only to closely related GII.4 Sydney. Figure 5B shows the variation in the blockade epitopes (22, 51) of GII.4-1999, GII.4-2010 NO, and GII.4-2012 Sydney variants. Although 14/16 amino acid residues differ between the 1999 and 2012 variants and only 4/16 amino acid residues differ between the 2010 and 2012 variants, it is remarkable that GII.4-1999 VLP immunization induced maximum blocking against GII.4 Sydney at a level similar to that of more closely related GII.4 NO VLPs. An explanation for the different cross-protective responses seen in this study might be that GII.4-1999 and GI.3 VLPs induce serum antibodies with much stronger affinity that better tolerate the variations within the target epitopes (8, 22, 35). In addition, there might exist as-yet-undetermined cross-reactive blockade epitopes

across the NoV capsid protein. Furthermore, Zhu et al. recently showed that two genetically highly related intracluster murine NoV strains displayed very different abilities to induce protective immune responses (52). We also showed that GII.4-1999 VLPs had a T cell immune response-inducing ability superior to that of GII.4-2010 NO VLPs, which is congruent with the cross-blocking antibody responses detected. It is possible that the stronger the T cell responses generated are, the better the cross-reactive blocking antibodies induced are.

Altogether, the results of this study indicate that measurement of the NoV strain-specific IgG level or the cross-reactive binding antibodies induced by immunization with different NoV VLPs alone is not adequate to determine the quality of immune responses (31, 51). This is in line with our results obtained with human serum (30), where genogroup-specific cross-reactive IgG titers were raised after natural NoV infection but no equivalent blocking antibody titer increase was induced.

The challenge for the development of a NoV vaccine is related to multiple circulating NoV strains that readily escape the protective immunity induced by previous infections (53, 54). To the contrary, there are indications that immunization of mice with a cocktail of different NoV VLP genotypes may induce heterotypic antibodies that can bind novel variants not included in the cocktail (35). Therefore, it is important to generate as broad a response as possible by vaccination, particularly when immunizing young children with no or a less extensive NoV exposure history. It has been suggested that although the NoV genome accumulates mutations over time, they tend to revert to the amino acid composition of the older strains (40, 55). This would mean that updating the vaccine composition on a regular basis according to the prevalent circulating strain might not be necessary, but instead, cross-reactive immunity should be induced at a sufficient level.

Our results indicate that a bivalent NoV vaccine containing GI.3 and GII.4-1999 VLPs might be sufficient to induce protective immune responses across NoV genotypes and genogroups. The bivalent VLP combination showed an additive effect of the VLPs without mutual inhibition. Moreover, as no cross-protective immunity between the two NoV genogroups was induced, our results confirm the previous findings (38) and support the idea that a NoV vaccine should consist of at least two NoV VLPs belonging to GI and GII (10, 37, 38). However, the genotypes chosen for vaccine design should be carefully considered, as even variants of a single genotype, as shown here, have different abilities to induce cross-protective immune responses. Eventually, only field trials with humans with complex pre-existing NoV immunity will give a definitive picture of the protection induced by VLP-based vaccines.

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