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Chimeric GII.4 Norovirus Virus-Like-Particle-Based Vaccines Induce Broadly Blocking Immune Responses

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ABSTRACT

There is currently no licensed vaccine for noroviruses, and development is hindered, in part, by an incomplete understanding of the host adaptive immune response to these highly heterogeneous viruses and rapid GII.4 norovirus molecular evolution. Emergence of a new predominant GII.4 norovirus strain occurs every 2 to 4 years. To address the problem of GII.4 antigenic variation, we tested the hypothesis that chimeric virus-like particle (VLP)-based vaccine platforms, which incorporate antigenic determinants from multiple strains into a single genetic background, will elicit a broader immune response against contemporary and emergent strains. Here, we compare the immune response generated by chimeric VLPs to that of parental strains and a multivalent VLP cocktail. Results demonstrate that chimeric VLPs induce a more broadly cross-blocking immune response than single parental VLPs and a similar response to a multivalent GII.4 VLP cocktail. Furthermore, we show that incorporating epitope site A alone from one strain into the background of another is sufficient to induce a blockade response against the strain donating epitope site A. This suggests a mechanism by which population-wide surveillance of mutations in a single epitope could be used to evaluate antigenic changes in order to identify potential emergent strains and quickly reformulate vaccines against future epidemic strains as they emerge in human populations.

IMPORTANCE

Noroviruses are gastrointestinal pathogens that infect an estimated 21 million people per year in the United States alone. GII.4 noroviruses account for >70% of all outbreaks, making them the most clinically important genotype. GII.4 noroviruses undergo a pattern of epochal evolution, resulting in the emergence of new strains with altered antigenicity over time, complicating vaccine design. This work is relevant to norovirus vaccine design as it demonstrates the potential for development of a chimeric VLP-based vaccine platform that may broaden the protective response against multiple GII.4 strains and proposes a potential reformulation strategy to control newly emergent strains in the human population.

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A s the primary cause of acute gastroenteritis, noroviruses annually infect more than 21 million people in the United States (1) and result in an estimated 200,000 deaths in children under 5 years of age worldwide (2). Furthermore, norovirus-associated economic losses due to health care costs and lost productivity total an estimated 5.5 billion dollars per year in the United States alone (3). Because of these staggering health and economic burdens, a norovirus vaccine is highly desirable for those who work in high-risk environments, including health care settings, schools and daycare centers, nursing homes, military barracks, and restaurants. Unfortunately, progress toward an effective vaccine has been slow due in part to the lack of a tissue culture system, the highly heterogeneous nature of different genogroups and genotypes, and lack of understanding of the mechanisms and duration of protective immunity in humans.

Noroviruses are positive-sense RNA viruses and members of the *Caliciviridae* family. They are divided into five genogroups; genogroups I and II (GI and GII, respectively) infect humans and are further divided into at least 9 and 22 genotypes, respectively (4, 5). The ~7.5-kb norovirus genome is divided into three open reading frames (ORFs). ORF1 encodes the nonstructural proteins, ORF2 encodes VP1, the major capsid protein, and ORF3 encodes VP2, the minor capsid protein (6). VP1 is divided into the shell (S) and protruding (P) domains, and upon expression of the major capsid gene, VP1 dimers self-assemble into virus-like particles (VLPs), which are antigenically and morphologically comparable

to native virions. The capsid P domain is further divided into the P1 and P2 subdomains. The capsid P2 subdomain is the most surfaced-exposed area of the virion and interacts with antibodies and cellular binding ligands, the histo-blood group antigens (HBGAs) (7–12). HBGAs are a diverse group of carbohydrates differentially expressed on mucosal surfaces and differentially targeted by the various norovirus genotypes (11–14). Importantly, in the absence of a cell culture model for human norovirus, the blockade assay (15), a correlate of human protection (16, 17), evaluates potential antibody neutralization by measuring the ability of monoclonal antibodies or serum to block VLP-ligand interactions.

Evolutionary patterns and contributions to disease outbreaks vary by genogroup and genotype. GI.1 (Norwalk virus) and other GI noroviruses exhibit a limited capacity for antigenic change over time (18), while GII.4 noroviruses demonstrate a pattern of epochal evolution, whereby a circulating strain is replaced by an

emergent strain, likely in response to herd immunity (15, 19–23). GII.3 noroviruses exhibit an evolutionary rate similar to that of GII.4 noroviruses, but the evolutionary mechanism appears to be different (24). GII.2 noroviruses, on the other hand, exhibit limited antigenic evolution over time (25). Of the remaining human norovirus genotypes, little is known about their evolutionary mechanisms. In terms of disease burden, GII.4 noroviruses consistently account for over 70% of all outbreaks (26). Other GI and GII genotypes contribute to norovirus disease outbreaks at lower levels, but relative contributions vary by year and geographic location. Since an effective norovirus vaccine should protect against the strains most commonly responsible for outbreaks, GII.4 noroviruses are the most medically relevant group to vaccinate against; however, considering the heterogeneous nature of noroviruses, a vaccine strategy that would protect against a wide array of viruses would be beneficial. In addition, due to antigenic variation in GII.4 noroviruses, the GII.4 component of a vaccine would likely need to be reformulated over time, reflecting the antigenic characteristics of newly evolved contemporary strains.

There is uncertainty in the field as to the duration of protective immunity following norovirus infection. While early human challenge studies suggested that protection lasts between 6 months and 2 years (27, 28), the acquisition of norovirus illness is dose dependent (29, 30), and the challenge dose used in these early studies was likely much higher than that of a typical human exposure, suggesting that results minimized the ability of the immune response to control a natural infection (31). Other studies showing strain replacement and extinction of early GII.4 epidemic strains suggested that many individuals may mount more-long-term protective immunity (13, 32, 33). Recent work using a community-based transmission model suggests that the duration of immune protection is much longer, from 4 to 8 years (31), meaning that an efficacious norovirus vaccine may provide some individuals protection for several years.

Several norovirus vaccine formulations are currently in human clinical trails. Results testing the GI.1 component of a commercial norovirus vaccine, the only human challenge norovirus vaccine efficacy study to date, indicate that 3 weeks after administration of an intranasal VLP-based GI.1 vaccine, gastroenteritis was reduced by 47% (16). Interestingly, 31% of the placebo group did not become ill despite administration of 10 times the 50% infectious dose (ID_{50}) (16), supporting earlier findings that some individuals may have had preexisting immunity to the virus (32). Longer-term monitoring of these participants is being conducted to determine the duration of protection provided by this vaccine.

Several different VLP-based vaccine platforms are currently being investigated, including GII.4 consensus VLP and multivalent VLP approaches. Immunization studies in mice have demonstrated that multivalent VLP vaccines induce a blockade response against VLPs representing strains included in the vaccine formulation and also broaden the blockade response against VLPs representing strains not included in the vaccine (34).

Our work involves the development of strategies to simultaneously investigate the capacity of rational structure-guided antigen design to expand and broaden the antigenic properties within a formulation and rapidly reformulate a preexisting GII.4 norovirus vaccine. In the manuscript, we designed and constructed a chimeric GII.4 vaccine by admixing previously identified blockade epitopes (15, 19, 22) from multiple strains into a single vaccine background to induce a more broadly blocking immune response

than a single GII.4 strain vaccine. Here, we utilize an alphavirus replicon (virus replicon particle [VRP]) system (35), which both acts as an adjuvant (36, 37) and allows for the expression of the norovirus capsid gene and the formation of VLPs in immunized mice (34, 38). As proof of principle, we demonstrate that a chimeric GII.4 capsid vaccine can induce a more broadly blocking immune response than a single GII.4 strain capsid, inducing robust blockade titers of GII.4 VLPs from strains isolated in 1987 through 2012 (representing strains GII.4-1987 through GII.4-2012, respectively), including those strains not included in the chimeric VLP. Furthermore, this broadened response is similar to a multivalent vaccine formulation, which was previously shown to broaden blockade responses (34). Our work provides a new conceptual template for the design of multivalent VLP vaccine formulations using structure-guided antigen design and represents a new and promising GII.4 norovirus vaccine strategy.

MATERIALS AND METHODS

Structure-guided antigen design. Capsid sequences for major GII.4 outbreak strains were aligned, and structural homology models based on the Research Collaboratory for Structural Bioinformatics (RCSB) PBD structure 2OBT were made for strains representing GII.4-1987 (GenBank accession number AAK50355.1), GII.4-1997 (JQ478407), GII.4-2002 (JQ478408), GII.4-2006 (JQ478409), GII.4-2009 (ADD10375), and GII.4-2012 (AFV08795.1). Changing residues in the capsid P2 subdomain were noted, and putative epitopes were defined and characterized as previously reported (15, 19, 22). Chimeric capsid sequences were created between strains whereby the GII.4-2006 A, D, and/or E epitope sites were replaced with comparable epitope site sequence(s) from one or more heterologous GII.4 strains. These sequences were synthesized (BioBasic), cloned into expression vector pVR21, and verified by sequencing.

Production of VRPs and VLPs. VRPs expressing the norovirus capsid gene were produced as previously described (35). Briefly, pVR21 3526 is an expression vector encoding the Venezuelan equine encephalomyelitis (VEE) genome yet lacks the VEE structural genes. The norovirus ORF2 gene was synthesized (BioBasic) and inserted into pVR21 3526 in place of the VEE structural genes behind the 26S promoter. RNA was made from the VEE-norovirus ORF2 construct, as well as from two additional separate plasmids expressing either the VEE 3526 E1 and E2 glycoprotein genes or the capsid gene. RNA from all three constructs was electroporated into BHK cells, and VRPs were harvested 48 h later and purified by high-speed centrifugation. Titers of VRPs were determined by counting fluorescent cells detected with fluorescein isothiocyanate (FITC)-labeled antibody. For production of VLPs, BHK cells were inoculated with VRPs as previously described (15), and VLP production was confirmed by electron microscopy, enzyme immunoassay, and carbohydrate binding.

VLP-carbohydrate ligand-binding antibody blockade assays. Pig gastric mucin type III (PGM) (Sigma Chemicals) contains HBGAs α-1,2-fucose (H antigen) and α-1,4-fucose (Lewis antigen) (13, 19, 39) and has been validated as a substrate for norovirus VLP antibody blockade assays. Blockade assays were performed as previously described (15). VLPs bound to PGM were detected by rabbit anti-GII.4 norovirus polyclonal serum. The percent control binding was defined as the VLP-ligand binding level in the presence of test antibody or serum compared to the binding level in the absence of antibody, multiplied by 100. All sera were tested for blockade potential at 2-fold serial dilutions ranging from 0.0098 to 5%. Data shown are the average of sera from five individual mice and represent two replicates that are indicative of similar data from two independent trials. Sigmoidal dose response analysis was performed as previously described (15). Fifty percent effective concentrations (EC_{50}s) among VLPs were compared using one-way analysis of variance (ANOVA) with Bonferroni's posttest. A P value of <0.05 was considered significant.
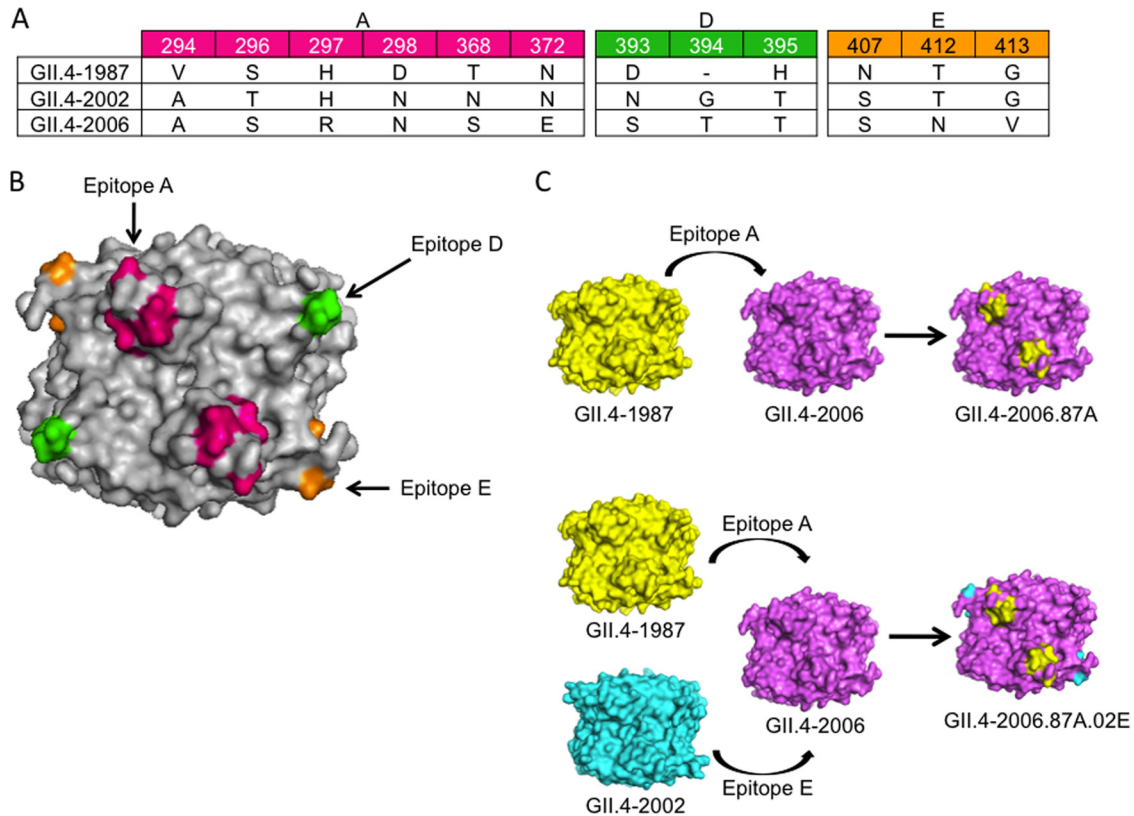
FIG 1 Chimeric VLP vaccine constructs. (A) Sequences from GII.4-1987, GII.4-2002, and GII.4-2006 were aligned, and residues in blockade epitopes A, D, and E are shown. (B) Capsid P2 dimer (gray) showing the location of blockade epitopes A (pink), D (green), and E (orange). (C) Chimeric VLP P2 dimers. Differential epitope A residues from parental strain GII.4-1987 (A294V, R297H, N298D, S368T, and E372N) are inserted into the parental GII.4-2006 strain background to create chimeric VLP GII.4-2006.87A. Chimeric VLP GII.4-2006.87A.02E contains the same GII.4-1987 residue substitutions as well as epitope E substitutions (N412T and V413G).

RESULTS

Design of chimeric VLP vaccine. Previous work has demonstrated both genetic and antigenic changes over time in major GII.4 norovirus strains, including changes in at least three blockade epitope sites that are linked to antigenic variation and the emergence of new epidemic strains in human populations (15, 19, 22). We aligned epitope site A, D, and E amino acid sequences from three GII.4 strains representing an ancestral strain, GII.4-1987, an early pandemic strain, GII.4-2002, and a more contemporary pandemic strain, GII.4-2006b to illustrate the changes over time in these epitopes (Fig. 1A) and mapped their locations on the surface of the capsid P2 dimer (Fig. 1B). Previous work indicates that blockade epitopes are portable among GII.4 strains, conferring blockade antibody reactivity gains and losses to the background strain (15, 19, 22). We then used structure-guided design to create synthetic genes in which broad combinations of time-

Mouse immunizations. Groups of five 5- to 7-week-old BALB/c mice were immunized by footpad injection with 5×10^4 VRPs expressing the GII.4 norovirus capsid gene from strain GII.4-1987, GII.4-2002, GII.4-2006, GII.4-2009, GII.4-2006.87A, or GII.4-2006.87A.02E or an equal multivalent mix of strains GII.4-1987, GII.4-2002, and GII.4-2006 and were boosted on day 21. Mice were euthanized and serum was harvested at 7 days postboost. Animal work performed in this study was approved by the Animal Care and Use Committee of the University of North Carolina—Chapel Hill.

ordered blockade epitopes were assembled into a single chimeric construct, which was used to produce a novel VLP-based vaccine with broadened immunogenicity. We created a single-epitope chimera, GII.4-2006.87A, which is comprised of the GII.4-2006 background with epitope site A (residues 294, 296 to 298, 368, and 372) from GII.4-1987, and a double-epitope chimera, GII.4-2006.87A.02E, which is comprised of the GII.4-2006 background with epitope site A from GII.4-1987 and epitope site E (residues 407, 412, and 413) from GII.4-2002 (Fig. 1C).

Individual GII.4 strain VLPs do not induce broad blockade responses. To determine the breadth of blockade induced by single parental GII.4 strain VLPs, we first immunized groups of five BALB/c mice with VRPs expressing the major capsid gene of GII.4 strains from 1987, 2002, 2006, or 2009 and then used sera from these mice to assess the ability of each VLP to induce a blockade response against a panel of GII.4 strain VLPs representing over 25 years of variation, including GII.4-1987, GII.4-1997, GII.4-2002, GII.4-2006, GII.4-2009, and GII.4-2012. Sera from mice immunized with GII.4-1987 VRPs were able to block carbohydrate-VLP interaction for early strains GII.4-1987, GII.4-1997, and GII.4-2002 but not for late strain GII.4-2006, GII.4-2009, or GII.4-2012 (Fig. 2A). Likewise, sera from mice immunized with GII.4-2002 were able to block only the early strains (GII.4-1987, GII.4-1997, and GII.4-2002) and not the late strains (GII.4-2006, GII.4-2009, and GII.4-2012) (Fig. 2B). Conversely, sera from mice immunized
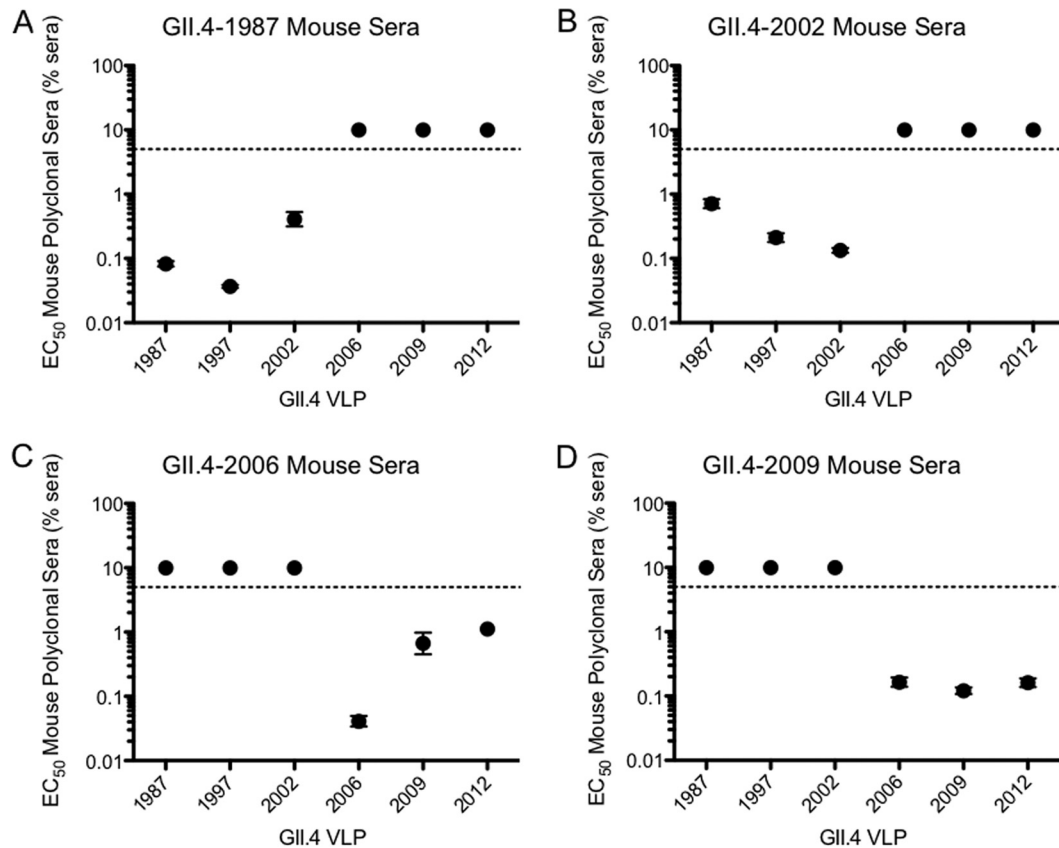
FIG 2 Single-strain VRPs do not induce a broad blockade response. BALB/c mice were immunized with VRPs expressing the norovirus capsid gene from parental strain GII.4-1987, GII.4-2002, GII.4-2006, or GII.4-2009 via footpad injection. Sera from mice immunized with GII.4-1987, GII.4-2002, GII.4-2006, and GII.4-2009 VRPs were evaluated for the ability to block VLP interaction with carbohydrate ligand pig gastric mucin type III (PGM) for VLPs representing strains GII.4-1987, GII.4-1997, GII.4-2002, GII.4-2006, GII.4-2009, and GII.4-2012. For each VLP, the mean percentage of control binding (percentage of the VLP bound to PGM in the presence of sera compared to the amount of VLP bound with no sera present) was fit to a sigmoidal curve, and the mean EC_{50} of serum was calculated (percentage of serum required to block 50% of the VLP-PGM interaction). Sera that did not block a particular VLP were assigned a value of 10% (2 times the upper limit of detection) for statistical analysis and placed as a data point above the dotted line (upper limit of detection).

with GII.4-2006 did not block early strain GII.4-1987, GII.4-1997, or GII.4-2002 but efficiently blocked homotypic GII.4-2006 and heterotypic late strains GII.4-2009 and GII.4-2012 (Fig. 2C). Sera from mice immunized with GII.4-2009 did not block early strain GII.4-1987, GII.4-1997, or GII.4-2002 but blocked GII.4-2006, GII.4-2009, and GII.4-2012 (Fig. 2D). Overall, our data show that none of the parental-strain VLPs we tested induces a broad blockade response against ancestral and contemporary GII.4 strains, consistent with previous work demonstrating GII.4 antigenic variation over time (20, 21, 23).

Chimeric VLPs retain blockade epitopes of the component VLPs. To determine if chimeric VLPs retain the antigenic properties of multiple GII.4 strains, we tested the blockade ability of sera from mice immunized with parental-strain VRPs (GII.4-1987, GII.4-2002, GII.4-2006, or GII.4-2009) to block VLP-ligand interactions for two chimeric VLPs. Sera from mice immunized against parental strain GII.4-1987, GII.4-2002, GII.4-2006, or GII.4-2009 were all able to block both GII.4-2006.87A and GII.4-2006.87A.02E VLP-carbohydrate interactions but to differing degrees (Fig. 3). Sera from mice immunized against GII.4-1987 (Fig. 3A) blocked GII.4-2006.87A and GII.4-2006.87A.02E VLPs to similar degrees, while sera from mice immunized against GII.4-2002 (Fig. 3B) blocked GII.4-2006.87A.02E VLPs significantly

better ($P < 0.05$) than GII.4-2006.87A VLPs, likely because this chimeric VLP contained a GII.4-2002 blockade epitope. Sera from mice immunized against GII.4-2006 (Fig. 3C) and GII.4-2009 (Fig. 3D) blocked GII.4-2006.87A VLPs significantly more efficiently ($P < 0.05$) than GII.4-2006.87A.02E VLPs. These results demonstrate that chimeric VLPs can maintain antigenic blockade properties of multiple GII.4 strains.

Chimeric VLPs induce a broadly blocking immune response against homotypic- and heterotypic-strain VLPs. Since antigenic variation in GII.4 noroviruses is a major hurtle to development of an efficacious vaccine, creating a vaccine that induces neutralization against a broad panel of strains is ideal. Since chimeric VLPs retain the antigenic properties of multiple GII.4 strains, we tested their potential as a vaccine. In order to determine whether a chimeric VLP can induce a more broadly blocking immune response than parental VLP strains, we developed VRPs that express the capsid genes for chimeric sequences GII.4-2006.87A and GII.4-2006.87A.02E. We immunized groups of five BALB/c mice with VRPs expressing either the GII.4-2006.87A or GII.4-2006.87A.02E capsid gene and compared the ability of the sera from these mice to block VLP-ligand interactions against our VLP panel with previously mentioned representative GII.4 strains isolated from 1987 to 2012. Unlike the parental VLPs, which
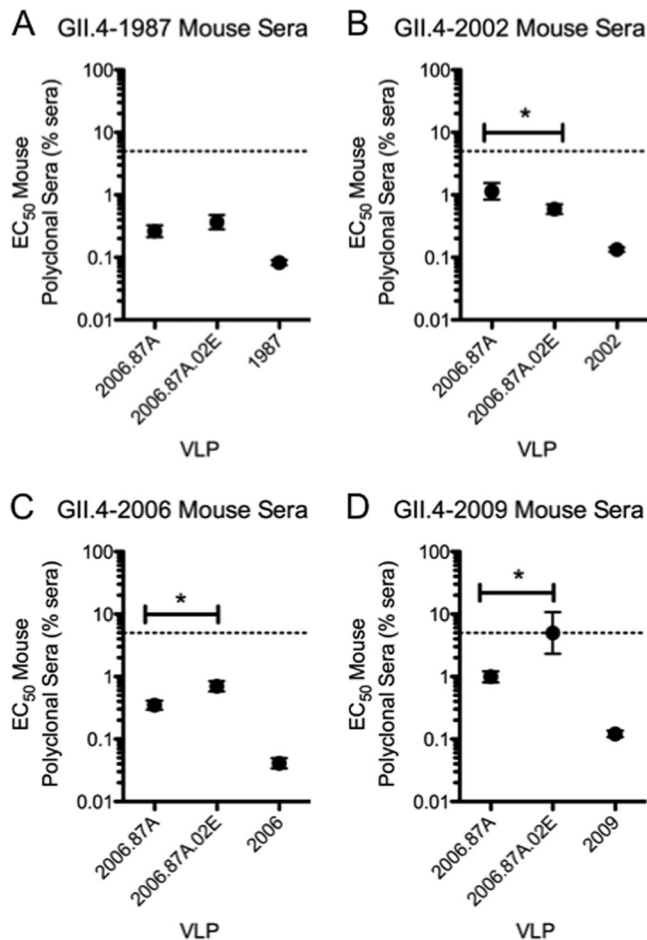
FIG 3 Single-strain VRP serum blockade response against chimeric VLPs. BALB/c mice were immunized with VRPs expressing the norovirus capsid gene from parental strain GII.4-1987, GII.4-2002, GII.4-2006, or GII.4-2009 via footpad injection. Sera from mice immunized with GII.4-1987, GII.4-2002, GII.4-2006, and GII.4-2009 VRPs were evaluated for the ability to block VLP interaction with carbohydrate ligand PGM for chimeric VLPs GII.4-2006.87A and GII.4-2006.87A.02E. For each VLP, the mean percentage of control binding (percentage of the VLP bound to PGM in the presence of sera compared to the amount of VLP bound with no sera present) was fit to a sigmoidal curve, and the mean EC_{50} of serum was calculated (percentage of serum required to block 50% of the VLP-PGM interaction). Sera that did not block a particular VLP were assigned a value of 10% (2 times the upper limit of detection) for statistical analysis and placed as a data point above the dotted line (upper limit of detection). Statistical significance was determined by one-way ANOVA with a Bonferroni posttest (*, $P < 0.05$). Blockade of parental VLPs is shown as a reference for each serum sample.

blocked only a subset of the VLPs tested, sera from mice immunized with GII.4-2006.87A or GII.4-2006.87A.02E VRPs were able to block all of the VLP strains tested from GII.4-1987 through GII.4-2012 (Fig. 4). GII.4-2006.87A sera exhibited significantly more robust blockade responses ($P < 0.05$) than GII.4-2006.87A.02 sera for VLPs representing strains GII.4-1987 (Fig. 4A), GII.4-1997 (Fig. 4B), GII.4-2006 (Fig. 4D), GII.4-2009 (Fig. 4E), and GII.4-2012 (Fig. 4F), where between 1.5 to 3.9 times more GII.4-2006.87A.02E sera was needed for EC_{50} blockade. Blockade responses to GII.4-2002 VLPs were not significantly different between sera from GII.4-2006.87A- and GII.4-2006. 87A.02E-immunized mice (Fig. 4C). Importantly, this demon-

strates that the incorporation of epitope A alone is sufficient to attain a broadened blockade response against multiple GII strains.

Comparison of immune responses elicited by chimeric VLPs versus a multivalent formulation of VLPs. Previous work in our lab showed that immunizing with a multivalent VLP vaccine formulation induced a broadly blocking response against both VLPs representing strains included and those not included in the formulation (34). We wanted to compare the blockade response induced by immunizing with chimeric VRPs versus immunization with a multivalent VRP cocktail. Since GII.4-2006.87A VRPs generally induced a stronger broadened blockade response than GII.4-2006.87A.02E VRPs, we used GII.4-2006.87A to represent the chimeric immunization scheme. We immunized groups of five BALB/c mice with VRPs expressing either a chimeric (GII.4-2006.87A) capsid gene or a multivalent cocktail of parental VRPs expressing GII.4-1987, GII.4-2002, and GII.4-2006 capsid genes. Sera from mice immunized with the multivalent cocktail displayed broad blockade activity, in agreement with previous studies (34), although the blockade potential against different strains varied between the multivalent sera and the chimeric sera (Fig. 5). Sera from mice immunized with the multivalent cocktail were able to block GII.4-1987 VLP-ligand interaction at levels similar to those of chimeric sera (Fig. 5A). Multivalent serum EC_{50} blockade titers were significantly different ($P < 0.05$) for GII.4-1997 VLPs (2.2 times lower) and GII.4-2002 (6.8 times lower) VLPs from those of chimeric sera (Fig. 5B and C). Chimeric sera blocked late strains GII.4-2006, GII.4-2009, and GII.4-2012 significantly more efficiently ($P < 0.05$) than sera from the multivalent-VLP-vaccinated mice (Fig. 5D to F), with 4, 2.9, and 2.1 times more multivalent sera required, respectively, for EC_{50} blockade. When the blockade responses induced by single-strain, multivalent-strain, and chimeric-strain immunizations are compared, sera against a single parental strain blocks the homotypic VLP-ligand interaction significantly better ($P < 0.05$) than either multivalent sera or chimeric sera for GII.4-1987 (Fig. 6A), GII.4-2002 (Fig. 6B), GII.4-2006 (Fig. 6C), and GII.4-2009 (Fig. 6D) VLPs. However, for all VLPs, sera from chimeric GII.4-2006.87A or multivalent formulations blocked as well as or better than sera from the closest heterotypic strain. Chimeric GII.4-2006.87A and multivalent sera blocked GII.4-1987 VLPs significantly more efficiently ($P < 0.05$) than GII.4-2002 sera (Fig. 6A), and multivalent sera blocked GII.4-2002 VLPs significantly more efficiently ($P < 0.05$) than GII.4-1987 sera (Fig. 6B). GII.4-2006.87A sera blocked GII.4-2006 VLPs significantly more efficiently ($P < 0.05$) than GII.4-2009 sera (Fig. 6C), while blockade of GII.4-2009 VLPs was equivalent for GII.4-2006 and GII.4-2006.87A sera (Fig. 6D). Overall, our data show that chimeric and multivalent vaccine formulations can broaden the blockade response against homotypic- and heterotypic-strain VLPs compared to the response with a single-strain vaccine formulation.

DISCUSSION

GII.4 antigenic variation is a major complication in norovirus vaccine design since this genotype evolves quickly and accounts for over 70% of all norovirus outbreaks. GII.4 strains emerge every 2 to 4 years by escaping human herd immunity, suggesting that frequent vaccine reformulation may be necessary in order to continuously protect against the GII.4 genotype. An approach that uses an approved backbone gene with targeted replacements of key antigenic epitopes for eliciting protective immunity offers a
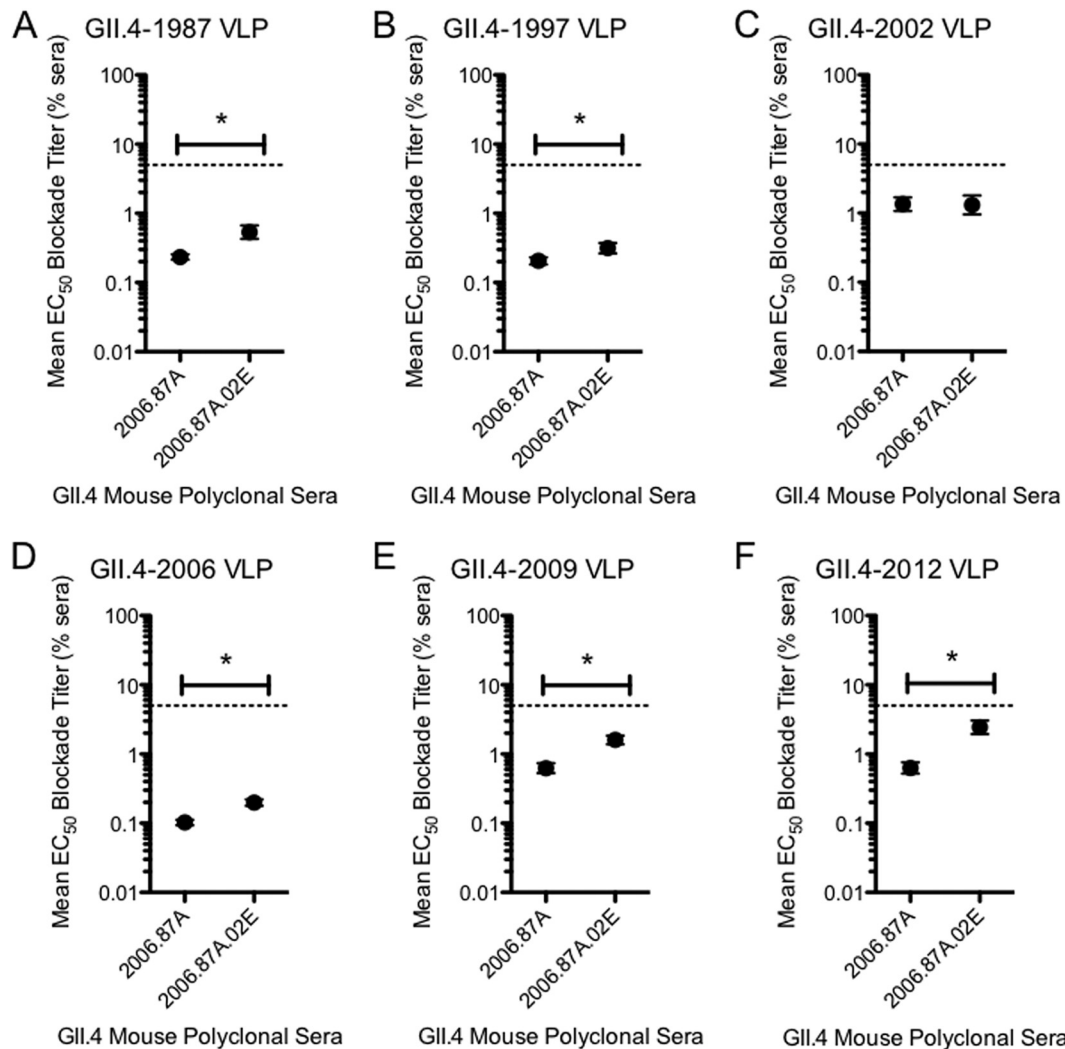
FIG 4 Comparison of chimeric VRP serum blockade response against parental GII.4 VLPs. BALB/c mice were immunized with VRPs expressing the norovirus capsid gene from chimeric sequences GII.4-2006.87A and GII.4-2006.87A.02E via footpad injection. Sera from mice immunized with GII.4-2006.87A and GII.4-2006.87A.02E VRPs were evaluated for the ability to block VLP interaction with carbohydrate ligand PGM for VLPs representing strains GII.4-1987, GII.4-1997, GII.4-2002, GII.4-2006, GII.4-2009, and GII.4-2012. For each VLP, the mean percentage of control binding (percentage of the VLP bound to PGM in the presence of sera compared to the amount of VLP bound with no sera present) was fit to a sigmoidal curve, and the mean EC_{50} of serum was calculated (percentage of serum required to block 50% of the VLP-PGM interaction). Sera that did not block a particular VLP were assigned a value of 10% (2 times the upper limit of detection) for statistical analysis and placed as a data point above the dotted line (upper limit of detection). Statistical significance was determined by one-way ANOVA with a Bonferroni posttest (*, $P < 0.05$).

strategy for improving vaccine performance while streamlining FDA approval processes for human use. In the manuscript, we demonstrate that detailed molecular characterization of key varying blockade epitopes, coupled with structure-guided antigen design and synthetic biology, is able to yield a VLP vaccine formulation that induces a more robust and cross-reactively broadly blocking immune response than a single-outbreak strain capsid. This and recent work from our group using a similar epitope transfer strategy in dengue virus (40) demonstrate that structure-guided antigen design provides a novel method of mapping immunogenic targets in medically important RNA viruses. The approach is also compatible with RSV mimotope vaccines based on synthetic reconstruction of key neutralizing epitopes on a portable scaffold nanoparticle (41), in our case, a norovirus VLP-based vaccine platform.

Our work demonstrates that a single-strain VLP can induce a stronger blockade response against the parental strain than either chimeric or multivalent VLPs. Whether this is due to differences in the number of epitopes from each strain presented, immune interference, or both is unknown. However, both immune interference and antigen dose are known to affect the strength of the immune response. Immune interference has been noted in other multivalent norovirus vaccines (42), multivalent VLP-based human papillomavirus (HPV) vaccines (43), live-virus vaccines (44, 45), and killed/recombinant vaccines (46). However, immune responses elicited by chimeric VLP-based vaccines should not be subject to immune interference since only one type of particle is present in the vaccine. This notion, coupled with our data showing that multivalent and chimeric sera more efficiently blocked strains that were most represented in their respective vaccines,
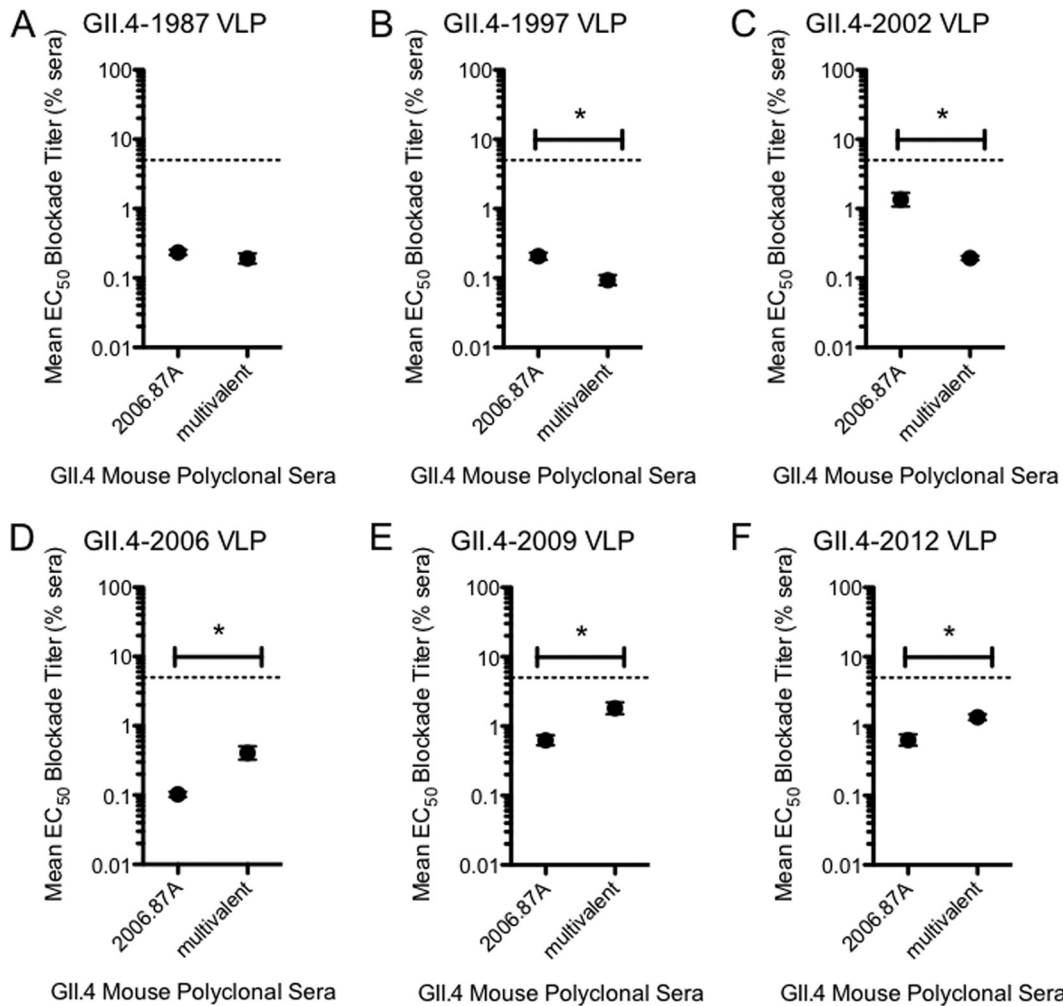
FIG 5 Comparison of chimeric versus multivalent VRP serum blockade response against parental GII.4 VLPs. BALB/c mice were immunized with VRPs expressing either the norovirus capsid gene from chimeric sequence GII.4-2006.87A or a multivalent mix of VRPs expressing the capsid gene from GII.4-1987, GII.4-2002, and GII.4-2006 via footpad injection. Sera from mice immunized with GII.4-2006.87A and multivalent VRPs were evaluated for the ability to block VLP interaction with carbohydrate ligand PGM for VLPs representing strains GII.4-1987, GII.4-1997, GII.4-2002, GII.4-2006, GII.4-2009, and GII.4-2012. For each VLP, the mean percentage of control binding (percentage of the VLP bound to PGM in the presence of serum compared to the amount of VLP bound with no serum present) was fit to a sigmoidal curve, and the mean EC_{50} of serum was calculated (percentage of serum required to block 50% of the VLP-PGM interaction). Sera that did not block a particular VLP were assigned a value of 10% (2 times the upper limit of detection) for statistical analysis and placed as a data point above the dotted line (upper limit of detection). Statistical significance was determined by one-way ANOVA with a Bonferroni posttest (*, $P < 0.05$).

suggests that antigen dose and epitope dominance are more likely to account for differences in immune induction. Despite having a stronger blockade against a homotypic strain, the immune response to a single GII.4 VLP does not broadly block heterologous strains, suggesting that the ability of a single strain to induce a neutralizing response against closely related circulating GII.4 strains is limited. This is problematic for a GII.4 vaccine since emerging strains may quickly escape the herd immunity elicited by a single strain. Previous work suggests that an increase in dosage of a single-strain VLP would probably not increase blockade breadth, whereas increasing the dosage of a chimeric or multivalent vaccine could potentially compensate for the comparative weakness in blockade strength (34).

Our results also suggest that incorporating immunodominant epitope site A from one strain into another is sufficient to induce a blockade response against the donor strain. These data are con-

sistent with earlier work showing that 25 to 55% of a polyclonal blockade response targets epitope site A in multiple pandemic years (15, 20, 22, 23). Of our two chimeric VLPs, GII.4-2006.87A VLPs induce a generally stronger blockade response against all VLPs tested from GII.4-1987 through GII.4-2012, with the exception of GII.4-2002, for which there was no difference between the two chimeric VLPs. This demonstrates that epitope site A alone was sufficient to induce a blockade response against early strains GII.4-1987, GII.4-1997, and GII.4-2002 and supports earlier work demonstrating that epitope site A is likely immunodominant in GII.4 (22). The addition of GII.4-2002 epitope site E in GII.4-2006.87A.02E did not significantly increase the blockade response against GII.4-2002, suggesting that this is a minor site or that additional residues are required in this epitope to fully recapitulate the antigenic properties of the epitope.

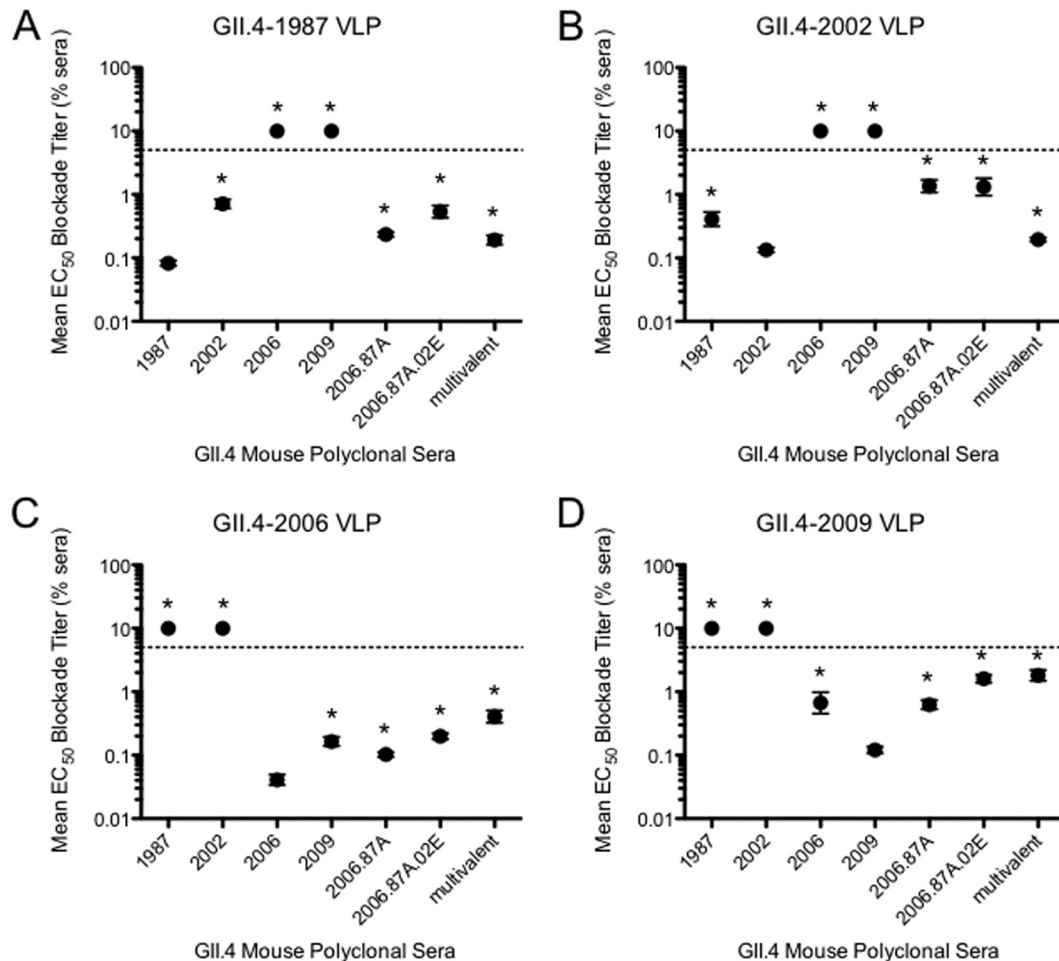Preexposure history and its effects on the immune response to

FIG 6 Parental strain blockade comparisons among homotypic, heterotypic, chimeric, and multivalent sera. BALB/c mice were immunized with VRPs expressing either the norovirus capsid gene from GII.4-1987, GII.4-2002, GII.4-2006, GII.4-2009, chimeric sequences GII.4-2006.87A or GII.4-2006.87A.02E, or a multivalent mix of VRPs expressing the capsid gene from GII.4-1987, GII.4-2002, and GII.4-2006 via footpad injection. Sera from these mice were evaluated for the ability to block VLP interaction with carbohydrate ligand PGM for VLPs representing strains GII.4-1987, GII.4-2002, GII.4-2006, and GII.4-2009. For each VLP, the mean percentage of control binding (percentage of the VLP bound to PGM in the presence of sera compared to the amount of VLP bound with no sera present) was fit to a sigmoidal curve, and the mean EC_{50} of serum was calculated (percentage of serum required to block 50% of the VLP-PGM interaction). Sera that did not block a particular VLP were assigned a value of 10% (2 times the upper limit of detection) for statistical analysis and placed as a data point above the dotted line (upper limit of detection). Statistical significance was determined by one-way ANOVA with a Bonferroni posttest, and statistical differences compared to the homotypic VLP are shown (*, $P < 0.05$).

subsequent norovirus infections are not well understood. Blockade data from outbreak serum samples collected over time suggest that exposure to new GII.4 strains does not boost cross-blocking antibodies to previous strains, but this may be due to several factors besides a lack of conserved neutralization sites, including limitations in methods capable of identifying cross-neutralizing epitopes, the long duration of time between infections in an individual, or a quickly waning antibody response. Whether a successful norovirus vaccine containing vaccine components to multiple strains would boost previous immune responses, providing a more broadly cross-reactive response over time, is a topic of future investigation and will need to be resolved over time.

Public health preparedness requires a platform strategy to survey disease prevalence in human populations, categorize sequence variation, identify antigenic variants that may seed future outbreaks, and inform vaccine design. A similar strategy to the one used for reformulation of the annual influenza virus vaccine could

be implemented to address reformulation of future norovirus vaccines. The current influenza paradigm involves a synchronized effort by public health agencies and centers around the world to conduct ongoing epidemiological antigenic testing and genetic characterization of influenza virus isolates and make predictions about which may cause the greatest threat to human health worldwide (47). Norovirus surveillance systems, like the CDC Calicinet (48) and the European NoroNet, while relatively new, represent key emerging platforms for developing a similar, widespread, coordinated effort to track norovirus epidemiology and emergent strain identification for improved vaccine design (49). Epidemiological work on GII.4-2012 Sydney has shown that this strain was present at low levels in the population for at least a year before its emergence as a major circulating strain (50), showing that new strains are likely present early enough before emergence to allow detection, antigenic testing, and targeted surveillance. As is done for influenza virus, panels of defined antibodies could be used to
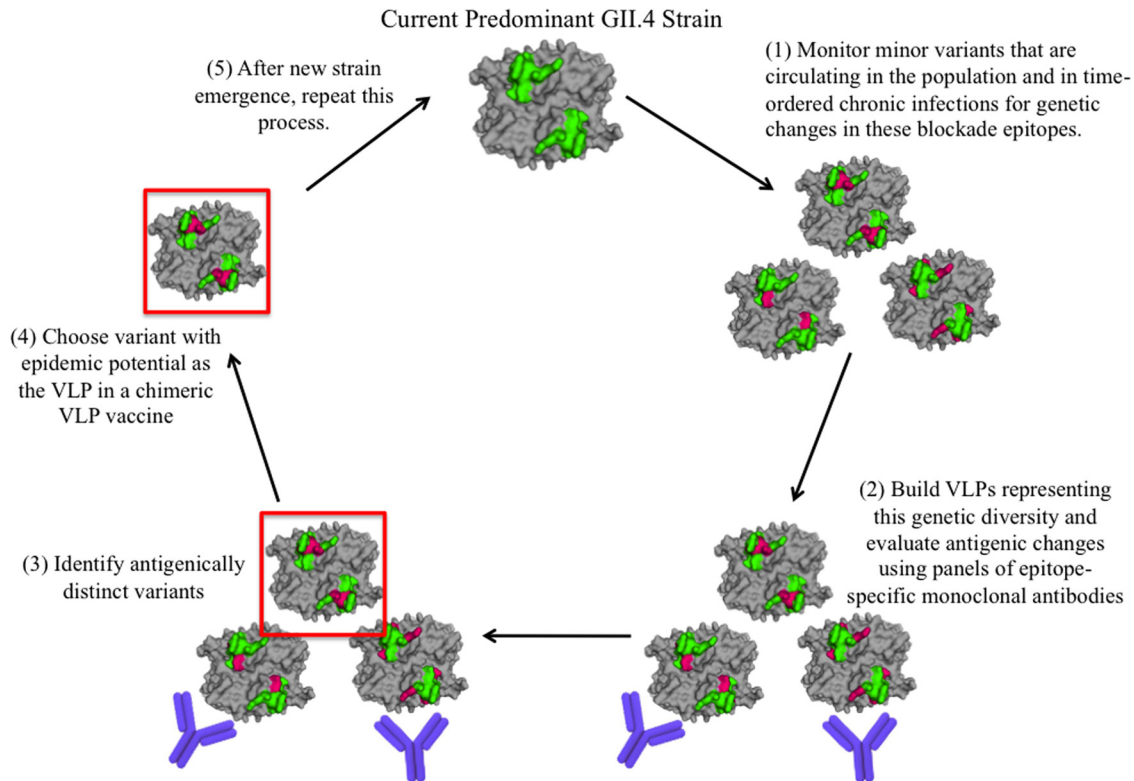
FIG 7 Model of chimeric GII.4 VLP-based vaccine design and reformulation strategy. Starting with the current predominant circulating GII.4 strain, (1) epidemiological studies identify sequence changes in epitope A in minor variants that arise in the population (2). Using the sequences of these epitope A variants, a panel of VLPs representing this genetic diversity is built, and monoclonal antibodies and polyclonal sera can be used to evaluate changes in antigenicity for each variant (3). From these data, specific variants that are antigenically distinct from the predominant strain are identified and can be more closely monitored in the population (4). If one of these variants seems likely to emerge as a new predominant strain, the chimeric VLP is already made and can quickly be introduced into a new vaccine formulation (5). When a new predominant strain emerges, this process can be repeated in order to consistently address GII.4 antigenic variation.

probe the antigenic properties of epitope site A in preemergent strains. When coupled with epidemiologic surveillance data, this system could provide a mechanism for predicting future emergent strains and for vaccine reformulation prior to epidemic seasons (Fig. 7), providing a key public health preparedness platform and vaccine reformulation strategy for noroviruses.

These studies, coupled with studies reported by other groups, highlight the power of antibody-based identification of key neutralizing epitopes in viral pathogens. This information provides an opportunity to map the conformational epitopes and identify key residues which influence immunogenicity and protective immunity. When coupled with structure-guided antigen and synthetic gene design, chimeric VLPs can be constructed with novel properties that broaden neutralization antibody responses and protective immunity even against strains not included in the original design. This fledgling strategy promises to revolutionize the design of the next generation of vaccines, with the ultimate goal of improving the global health of populations.

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