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Alphavirus-Adjuvanted Norovirus-Like Particle Vaccines: Heterologous, Humoral, and Mucosal Immune Responses Protect against Murine Norovirus Challenge▽

Anna D. LoBue,[1] Joseph M. Thompson,[1,2] Lisa Lindesmith,[3] Robert E. Johnston,[1,2] and Ralph S. Baric[1,2,3]*

Department of Microbiology and Immunology[1] and Carolina Vaccine Institute,[2] School of Medicine; and Department of Epidemiology, School of Public Health,[3] University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

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The development of an effective norovirus vaccine likely requires the capacity to protect against infection with multiple norovirus strains. Advanced recombinant genetic systems and the recent discovery of a mouse-tropic norovirus strain (MNV) provide robust model systems for vaccine efficacy studies. We coadministered multivalent norovirus-like particle (VLP) vaccines with alphavirus adjuvant particles to mice and evaluated homotypic and heterotypic humoral and protective immunity to human and murine norovirus strains. Multivalent VLP vaccines induced robust receptor-blocking antibody responses to heterologous human strains not included in the vaccine composition. Inclusion of alphavirus adjuvants in the inoculum significantly augmented VLP-induced systemic and mucosal immunity compared to the responses induced by low-dose CpG DNA, validating the utility of such adjuvants with VLP antigens. Furthermore, multivalent vaccination, either including or excluding MNV VLP, resulted in significantly reduced viral loads following MNV challenge. Passive transfer of sera from mice monovalently vaccinated with MNV VLP to immunodeficient or immunocompetent mice protected against MNV infection; however, adoptive transfer of purified $CD4^+$ or $CD8^+$ cells did not influence viral loads in murine tissues. Together, these data suggest that humoral immunity induced by multivalent norovirus vaccines may protect against heterologous norovirus challenge.

Noroviruses are annually responsible for at least 23 million infections in the United States (33) and up to 200,000 deaths in children of <5 years of age in developing countries (38). Effective vaccines are needed to control widespread norovirus outbreaks; however, immunity to noroviruses remains a controversial topic, as short-term, but not long-term, protection was observed upon reinfection of some human challenge volunteers (23, 37). Human norovirus vaccine research has also been hampered by the lack of a small animal model or an in vitro culture system for identifying key components of protective immunity. The manipulation of recombinant genetic systems, however, has allowed in vitro production of norovirus antigens from multiple strains, which may be utilized in immunogenicity studies in small animal models (5, 16, 19, 21, 34, 39, 58, 59). Furthermore, the recent discoveries of murine norovirus (MNV) strains that can replicate in vitro and in vivo provide novel tools in norovirus vaccine development (24, 56).

Previous studies with norovirus-like particle (VLP) vaccination have shown that humoral and cellular immune responses can be generated against human norovirus antigens in both humans and mice (3, 4, 34, 47, 48). Furthermore, antibody responses following infection with norovirus or immunization with VLPs can block ABH histo-blood group antigen (HBGA) binding to VLPs in a strain-specific manner (18). HBGAs are carbohydrates ubiquitously expressed on mucosal tissues and red blood cells that have been implicated as natural receptors for norovirus binding and entry, suggesting that blockade of HBGA interactions with VLPs may prevent norovirus infection (32). Additionally, $CD4^+$ T-cell responses following norovirus infection in humans or VLP vaccination in mice are also induced and have been characterized by secretion of type II interferons (gamma interferon [IFN-γ]) upon stimulation with VLPs (28, 34). Presently, however, the components of protective immunity and the impact of multiple exposures on norovirus immunity are unknown.

The norovirus family consists of more than 40 genetically diverse strains that can differ by up to 40% in capsid amino acid sequence identity between strains within a genogroup and by >50% between genogroups (15). Most studies of norovirus immunity have focused on individual strains; however, a limited number of reports have shown that antibody responses to one norovirus strain have little cross-reactivity to other strains, both within and across genogroups (17, 28, 35, 43, 53). Consequently, it is not surprising that infection with one norovirus strain fails to prevent infection with another strain in human challenge studies (57). Efficacy of norovirus vaccines, however, is dependent on protection against multiple circulating strains. Our group previously addressed this problem by showing that multivalent immunization with Venezuelan equine encephalitis (VEE) virus replicon particles (VRPs) expressing norovirus VLPs from three genetically distinct strains induced antibody responses that blocked receptor binding to a heterologous VLP not included in the vaccine composition (31). Although animal studies and in vivo protection were not evaluated, these results suggested that administration of multiple norovirus immunogens may represent a successful vaccination strategy for

* Corresponding author. Mailing address: Department of Epidemiology, School of Public Health, CB#7435, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7435. Phone: (919) 966-3895. Fax: (919) 966-0584. E-mail: rbaric@email.unc.edu.

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protection against more than one norovirus strain, including those not incorporated in the vaccine cocktail. However, the key strains necessary for eliciting a broad-based immune response to multiple noroviruses still requires more detailed studies including homologous and heterologous cross-challenge in experimental animals.

To induce robust immune responses to noroviruses, some VLP vaccines have been coadministered with several known biological adjuvants (3, 5, 16, 34, 39). Previous studies by our group have alternatively used VRPs as a delivery vaccine vector for VLP expression in vivo (19). This model is advantageous, as mammalian cells are permissive to VRP infection and VLPs are assembled in vivo in large quantities, with the caveat that different vaccine formulations are needed for each norovirus strain. However, a recent finding from Thompson et al. demonstrated that VRPs possess inherent adjuvant activity which can be uncoupled from their transgene expression function (50). In this configuration, VRPs lacking a transgene (null VRPs) demonstrate strong humoral, cellular, and mucosal adjuvant activity, even when administered from a nonmucosal site. This approach has enhanced safety features as well as the capacity to vaccinate against multiple VLPs simultaneously with a common adjuvant. However, whether this approach stimulates protective immunity against pathogenic microorganisms has not been evaluated. The concurrent discovery of murine noroviruses provides a tractable model system by which to test the efficacy of multivalent vaccine formulations as well as the mechanism(s) responsible for protection (24, 56). The murine intestine serves as an important site of replication after peroral inoculation, followed by infection of multiple secondary peripheral tissues, including the spleen. In this study, we have evaluated the immune responses elicited against human and murine norovirus strains within and across genogroups following multivalent vaccination with norovirus VLP cocktails coadministered with null VRP adjuvants. This study establishes the null VRP adjuvant platform as a robust strategy for eliciting high levels of protective humoral, cellular, and mucosal immunity against a group of significant mucosal pathogens.

MATERIALS AND METHODS

VLPs and VRPs. VRPs expressing norovirus open reading frame 2 were cloned and produced as described in reference 6. Experimental use of VLPs derived from the Southampton (SoV), Chiba, Desert Shield (DSV), Toronto (TV), and M7 virus strains and produced using the VRP system has not been described previously. Detailed characterization of each VLP will be reported in another manuscript. Null VRPs were kindly provided by the Carolina Vaccine Institute (UNC). Norovirus VLPs were produced and purified as described in reference 31 and visualized by electron microscopy to ensure appropriate particle size and structure. VLPs used in vaccination experiments were further concentrated by centrifugation at 3,000 × *g* in Centricon tubes (Millipore) overnight at 4°C.

Vaccination. Six-week-old BALB/c mice (Charles River) were vaccinated by footpad inoculation in two independent experiments with monovalent or multivalent VLP vaccines containing 2 μg of each VLP alone or in conjunction with 10^5 null VRP or 1 μg oligodeoxynucleotide 1826 CpG DNA (5′-TCCATGAC GTTCCTGACGTT-3′; Invivogen) (n = 4 per vaccination group). Mice used in VLP titration experiments received VLP doses of 0.02 μg, 0.2 μg, 2 μg, or 10 μg Norwalk virus (NV) VLP coadministered with null VRP (n = 4 per group). Other monovalent vaccination groups received NV (genogroup I.1 [GI.1]), Lordsdale-like virus (LV; GII.4), or MNV-1 (GV) VLPs. Multivalent groups received GI-specific VLPs representing SoV (GI.2), DSV (GI.3), and Chiba (GI.4) strains with or without NV VLPs; GII-specific VLPs representing Hawaii (GII.1), TV (GII.3), and M7 (GII.13) strains with or without LV VLPs; or complete VLP cocktails containing all GI and GII VLPs with or without NV and LV VLPs or all GI and GII VLPs with or without MNV VLPs (GV) (Table 1).

TABLE 1. VLP vaccination chart

VLP vaccine	Genogroup(s)	Type	VLP(s) in vaccine composition[a]
NV	GI	Monovalent	NV
GI+		Multivalent	NV, SoV, DSV, Chiba
GI−		Multivalent	SoV, DSV, Chiba (−) NV
LV	GII	Monovalent	LV
GII+		Multivalent	LV, HV, TV, M7
GII−		Multivalent	HV, TV, M7 (−) LV
GI+/GII+	GI/GII	Multivalent	All GI/GII
GI−/GII−		Multivalent	All GI/GII (−) NV/LV
MNV	GV	Monovalent	MNV
Hu+/MNV+	GI/GII/GV	Multivalent	All GI/GII, MNV
Hu+/MNV−	GI/GII	Multivalent	All GI/GII (−) MNV

[a] VLP-associated strains and years of isolation: NV, 1968; SoV, 1999; DSV, 1999; Chiba, 2000; LV, 1997; Hawaii virus (HV), 1971; TV, 1999; M7, 1999; MNV, 2003. − in parentheses indicates vaccines formulated without the following listed VLPs.

Mice were vaccinated and boosted at days 0 and 28. Donor mice for adoptive transfers (n = 8) were vaccinated a third time on day 52.

MNV infection. MNV-1 strain CW.3 was kindly provided by H. W. Virgin (Washington University School of Medicine). To generate virus stocks, murine macrophage-like raw 264.7 cells (UNC Tissue Culture Facility) cultured in complete Dulbecco's modified Eagle medium (Gibco) were infected with MNV at a multiplicity of infection of 0.1 and incubated for 36 h. Supernatant was then collected, clarified by centrifugation at 13,000 × *g* for 15 min (Beckman), and ultracentrifuged for 3 h at 100,000 × *g* over a 5% sucrose cushion to pellet purified virus. Pellets were resuspended in phosphate-buffered saline (PBS), aliquoted, and stored at −80°C until use. Titers of virus stocks were determined by plaque assay as previously described (56). Mice used in MNV challenge experiments were infected with 3×10^7 PFU MNV-1 strain CW.3 in 30 μl total volume orally on day 42 postvaccination.

Serum samples, fecal extracts, and tissue samples. Animals were euthanized and distal ileum, spleen, mesenteric lymph node (MLN), and serum samples were harvested from mice used in MNV challenge experiments on day 45 and stored at −80°C. Tissue samples were resuspended in 1 ml complete Dulbecco's modified Eagle medium and disrupted with silica/zirconia beads (Biospec Products) using the MagnaLyser homogenizer (Roche) at 6,000 rpm for 30 s. Serum and fecal samples from all other mice were collected on day 42. Ten fecal pellets per mouse were resuspended in 1 ml PBS containing 10% goat serum and 0.01% Kathon fecal inactivator (Supelco) and homogenized by vortexing for 20 min. Solid material was then removed by centrifugation for 20 min, and fecal extracts were stored at −20°C.

ELISA and HBGA binding blockade assays. Enzyme-linked immunosorbent assays (ELISAs) for serum immunoglobulin G (IgG) antibody cross-reactivity to norovirus VLPs and binding assays for serum antibody blockade of HBGA binding were performed as previously described (31). IgG subtype ELISAs were performed as described using purified IgG1 (Sigma) or IgG2a (Sigma) as the standard control and anti-IgG1-alkaline phosphatase (Southern Biotech) and anti-IgG2a-alkaline phosphatase (Southern Biotech) as secondary antibodies. The lower limit of detection for all serum ELISAs ranged from 0.1 to 1.9 μg/ml and was assay dependent. To quantitate specific antibody in fecal extracts, 96-well high-binding plates (Costar) were coated with 2 μg VLP or serially diluted mouse IgG or IgA standard for 4 h at room temperature and blocked overnight in blocking buffer (Sigma) at 4°C. Fecal extracts diluted 1:2 in blocking buffer were twofold serially diluted and incubated in wells containing VLP for 2 h at room temperature. Wells were then incubated with antimouse IgG-horseradish peroxide or IgA-horseradish peroxide (Southern Biotech) for 2 h and developed with orthophenylene-diamine tablets (Sigma) dissolved in 1:1 0.1 M sodium citrate and 0.1 M citric acid and 0.02% hydrogen peroxide for 30 min in the dark. Reactions were stopped with 0.1 M sodium fluoride, and the optical density at 450 nm was read (Bio-Rad model 680). The limit of detection for fecal IgG and IgA ELISAs was 0.2 ng/ml. All data are representative of the results for two independent vaccination experiments.

Passive and adoptive transfers. Eight wild-type mice were immunized as described above, and unimmunized controls were treated in parallel. Serum samples and spleens from immune and control groups were harvested on day 56. Spleens from respective immunization groups were pooled, and single-cell splenocyte suspensions were obtained by manual disruption through a 100-μm

cell strainer. Splenocyte suspensions were resuspended in MACS buffer (PBS [pH 7.2], 0.5% bovine serum albumin, 2 mM EDTA) and divided in half, and $CD4^+$ or $CD8^+$ cells were purified, respectively, by magnetic bead sorting using the QuadroMACS purification system (Miltenyi) per manufacturer's protocol. For adoptive transfers, 5×10^6 $CD4^+$ or $CD8^+$ cells from immune or nonimmune mice were administered in a total volume of 500 μl intraperitoneally (i.p.) to wild-type BALB/c mice or SCID C.B.17 mice (Jackson Laboratories) ($n = 6$ per recipient group). For passive transfer of sera, immune or nonimmune serum samples were equivalently pooled, diluted 1:2 in PBS, and administered i.p. to recipient mice at 200 μl per mouse. Recipient mice were challenged with 3×10^7 PFU MNV CW.3 24 hours posttransfer, and tissues were harvested 3 days postinfection. Tissue samples were processed as described above.

FACS. Whole and purified splenocyte suspensions from adoptive transfer groups were set aside for fluorescence-activated cell sorter (FACS) analysis. A total of 5×10^5 cells per tube were blocked with anti-FcκII/III (1:500; eBioscience) in 100 μl FACS buffer (Hank's balanced salt solution plus 2% fetal bovine serum) for 20 min on ice. Cells were then pelleted, resuspended in 100 μl FACS buffer, and stained with anti-B220 conjugated to fluorescein isothiocyanate (1:400), allophycocyanin (1:400), or biotin (1:800) as single color controls for staining or cocktails containing anti-CD3–fluorescein isothiocyanate (1:200), anti-CD4–biotin (1:1,000), and anti-CD8–allophycocyanin (1:800). Cells were incubated for 45 min on ice, pelleted, and resuspended in 100 μl FACS buffer with avidin-PerCP (1:400) for 45 min on ice. Samples were then washed and resuspended in 500 μl PBS. All antibodies were obtained from eBioscience (San Diego, CA). FACS analysis was performed by the UNC Flow Cytometry Core Facility.

Statistics. All statistics comparing two groups were performed using the two-tailed *t* test; all statistics comparing multiple groups were performed using one-way analysis of variance and Tukey's posttest in GraphPad software.

RESULTS

Null VRP adjuvants induce robust systemic and mucosal antibody responses in monovalent VLP vaccines. To determine effective VLP concentrations for subsequent vaccinations, mice were immunized twice with a VLP titration series consisting of 10 μg, 2 μg, 0.2 μg, or 0.02 μg NV VLPs coadministered with 10^5 IU null VRPs. Fecal IgA, fecal IgG, serum IgG, and serum blockade of receptor binding were evaluated 3 weeks postboost (Fig. 1). Measurable IgA and IgG were detected in fecal extracts of all mice receiving 0.2 to 10 μg VLPs in the presence of VRP adjuvants (Fig. 1A). Antibody titers were increased following vaccination with increasing amounts of VLP, and fecal IgG titers were consistently higher than fecal IgA titers, in line with previous results obtained with VEE adjuvants (49, 50). Serum antibody responses were also significantly increased following vaccination with all VLP concentrations at >0.02 μg compared to vaccination with 0.02 μg VLP ($P < 0.05$) (Fig. 1B) and blocked H type 3 receptor binding increasingly effectively with increased VLP concentration (Fig. 1C). From these data, we concluded that a dose of 2 μg of VLPs elicited a robust humoral immune response in rodents, and as such, all subsequent multivalent vaccine experiments were performed with this dose. Of note, multivalent vaccines cannot accommodate all VLPs at higher concentrations (i.e., 10 μg per VLP) due to footpad volume restrictions.

To compare the effect of null VRP adjuvant activity to that of an FDA-approved adjuvant for human vaccination, we immunized mice with 2 μg NV VLPs or LV VLPs alone or in conjunction with either 10^5 IU null VRPs or 1 μg CpG DNA (25). Serum antibody responses to NV or LV VLPs were significantly increased following codelivery with null VRPs compared to with low-dose CpG adjuvants ($P < 0.01$ and $P < 0.001$, respectively), and both adjuvant groups induced significantly higher responses than did VLP alone ($P < 0.001$) (Fig. 2A). Sera from groups vaccinated with NV VLPs but not LV VLPs blocked NV VLP binding to H type 3, and adjuvanted groups blocked binding with serum concentrations lower than those of groups receiving VLPs alone (Fig. 2B). Parallel results were obtained for blockade of LV VLP binding to H type 3 following LV VLP vaccination, respectively (Fig. 2C). Percentages of sera necessary for blockade of 50% (BT50) and 90% (BT90) H type 3 binding are shown in Table 2. BT50 and BT90 values were significantly lower in adjuvanted sera than in nonadjuvanted sera ($P < 0.05$).

Multivalent vaccines induce enhanced cross-reactive and receptor-blocking antibody responses. To determine the effect of multivalent VLP vaccination with null VRP or CpG adjuvants on homotypic and heterotypic antibody responses and receptor blockade, we vaccinated mice with pools of VLPs (2 μg each VLP) alone or coadministered with null VRP or CpG adjuvants. Mice received multivalent immunizations consisting of GI VLPs, GII VLPs, or both GI and GII VLPs. GI VLPs are derived from the NV (GI.1), SoV (GI.2), DSV (GI.3), and Chiba (GI.4) strains, and the GII VLPs are derived from the LV (GII.4), Hawaii (GII.1), TV (GII.3), and M7 (GII.13) strains. VLP vaccine formulations and acronyms are summarized in Table 1. NV VLPs were excluded from GI-specific (GI−) and complete GI/GII (GI−/GII−) multivalent vaccine formulations to allow comparison of their heterotypic antibody blockade of receptor binding to NV VLPs with that elicited by vaccines containing the NV antigen. LV VLPs were likewise excluded from GII-specific (GII−) and complete (GI−/GII−) vaccine formulations. Serum IgG responses following vaccination with the complete cocktail of GI/GII VLPs (GI+/GII+) coadministered with null VRP adjuvants resulted in robust antibody responses to NV and LV VLPs, respectively, that were significantly higher than those in groups lacking adjuvant ($P < 0.001$) (Fig. 3A). Furthermore, antisera following GI−/GII− vaccination still mounted strong cross-reactive IgG responses to NV and LV VLPs, supporting our previous findings (31). GI−/GII− VLP pools coadministered with null VRPs induced significantly stronger heterotypic responses to NV and LV VLPs than did GI−/GII− VLP vaccination without adjuvant ($P < 0.05$). However, GI−/GII− heterotypic antiserum reactivity to NV and LV VLPs was significantly lower than that of homotypic GI+/GII+ antisera ($P < 0.05$). Evaluation of antiserum blockade of H type 3 binding to VLPs revealed that GI+/GII+ antisera completely blocked H type 3 binding to both NV and LV VLPs, with increased blockade in groups receiving adjuvant (Fig. 3B to C). Significantly less serum was required to attain BT90 values following GI+/GII+ vaccination with adjuvant than without adjuvant (Table 2). Furthermore, GI−/GII− antisera induced by the GI−/GII− vaccine plus null VRPs contained cross-reactive antibodies that partially ablated H type 3 binding to both NV and LV VLPs. BT50 serum concentrations were significantly higher following GI−/GII− null VRP vaccination than those following GI+/GII+ null VRP vaccination in NV VLP H type 3 blockade ($P < 0.05$); however, they were not significantly different in LV VLP H type 3 blockade. Also, BT50 concentrations were significantly lower following GI−/GII− null VRP vaccination than those following GI−/GII− VLP vaccination without adjuvant ($P < 0.001$ in NV blockade and $P < 0.05$ in LV blockade). These data suggest that multi-

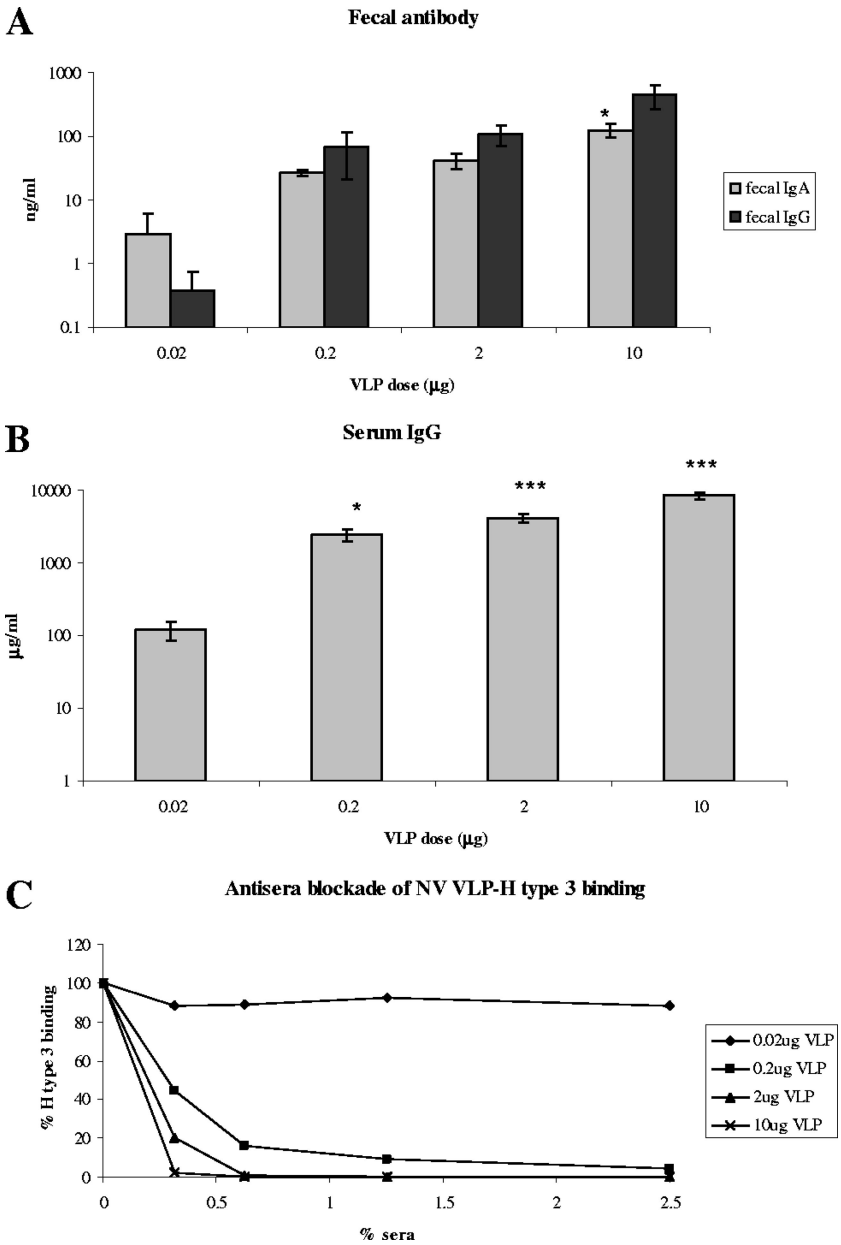


FIG. 1. Effective VLP dose for null VRP adjuvant activity. Mice were immunized with 10^5 IU of null VRPs and NV VLPs at doses of 0.02 μg, 0.2 μg, 2 μg, and 10 μg. Fecal extracts were prepared, and anti-NV IgG and IgA were quantitated by ELISA (A). Sera were also tested for anti-NV IgG and interference of H type 3 binding to NV VLPs (B and C). One asterisk (*) is representative of P values of <0.05, and three asterisks (***) are representative of P values of <0.001.

valent vaccines coadministered with null VRP adjuvants efficiently induce cross-reactive and receptor-blocking IgG responses to heterologous strains that cannot be attained following monovalent vaccination.

We performed an additional study in which mice were vaccinated with genogroup-specific VLP pools in conjunction with null VRP adjuvants. Groups of mice received immunizations of all four GI VLPs (GI+), all four GII VLPs (GII+), or three genogroup-specific VLPs lacking NV or LV VLPs (GI− and GII−, respectively) (Table 1). A comparison of serum IgG responses of genogroup-specific vaccinations to monovalent or multigenogroup VLP vaccines is shown in Fig. 4A. Cross-reactive responses of monovalent NV antisera to LV VLP and vice versa are shown as controls. All monovalent or multivalent vaccines containing NV or LV VLPs, respectively, induced highly reactive IgG responses to NV or LV VLPs that were not significantly different from one another. Genogroup-specific or multigenogroup VLP pools lacking NV and/or LV, respectively, mounted cross-reactive responses that were not significantly different from one another and were significantly lower than homotypic monovalent responses ($P < 0.01$) only, but not homotypic multivalent responses. Blockade profiles from each genogroup-specific vaccination group uphold the findings discussed above whereby multivalent genogroup-specific vaccines

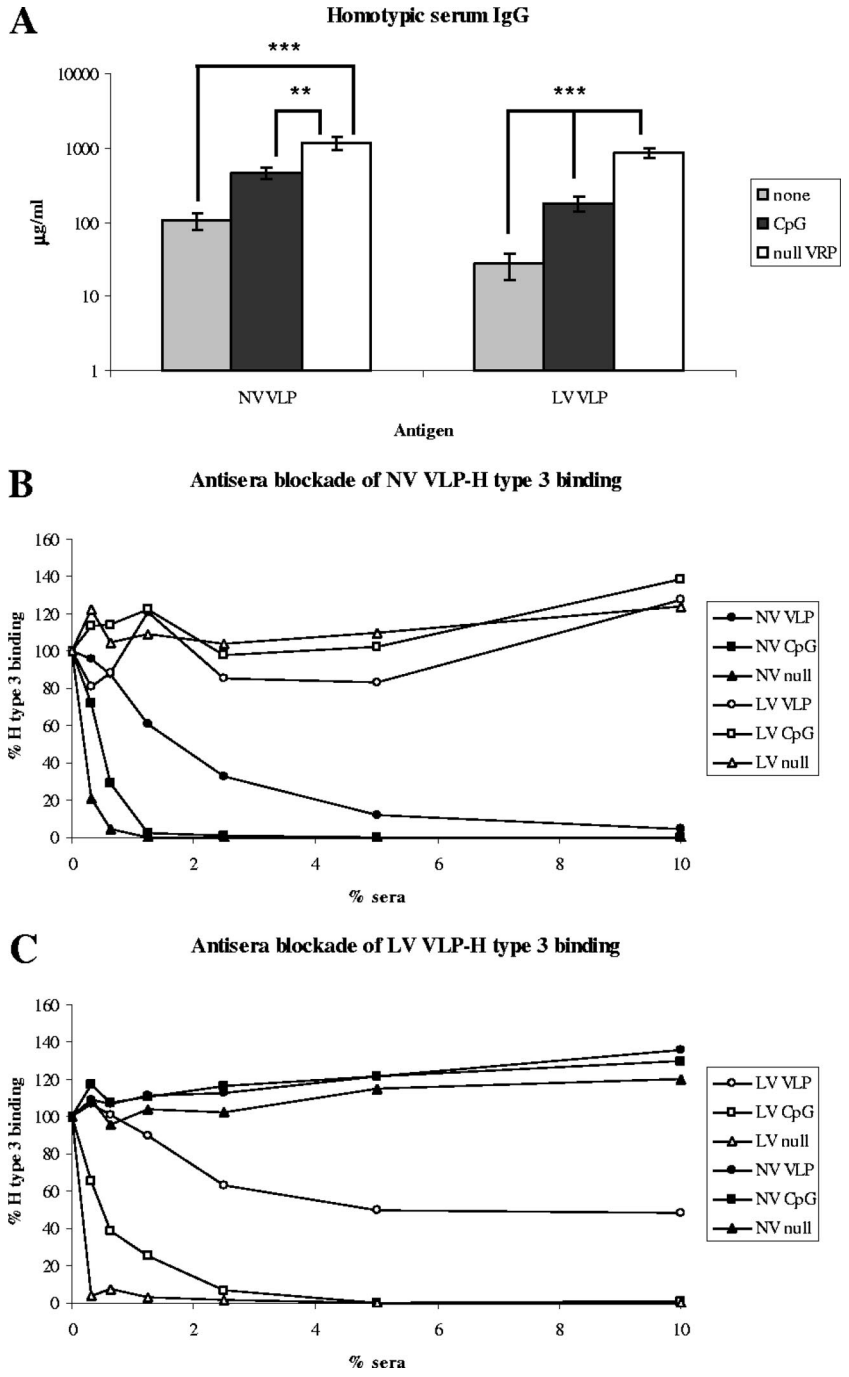


FIG. 2. Homotypic antibody responses following monovalent vaccination with and without adjuvant. Sera from mice immunized with NV or LV VLPs alone or in conjunction with CpG or null VRP adjuvants were analyzed for anti-NV or anti-LV IgG, respectively, by ELISA (A). Serially diluted antisera were also tested for blockade of H type 3 binding to NV VLPs (B) and LV VLPs (C). Two asterisks (**) are representative of P values of <0.01, and three asterisks (***) are representative of P values of <0.001.

lacking target antigens mount intermediate blockade responses (Fig. 4B and C) with BT50 values significantly higher than homotypic values ($P < 0.05$) but significantly lower than heterotypic monovalent values ($P < 0.01$) (Table 2). Furthermore, increasing the number of VLPs in the vaccine composition did not significantly change homotypic antibody titers or blockade of receptor binding. Increasing genogroup-specific VLP vaccines to include VLPs from both genogroups appeared to moderately increase cross-reactive responses to both NV and LV VLPs, respectively. Increasing the amount of null VRPs administered from 10^5 IU to 10^6 IU per vaccine did not enhance cross-reactive receptor blockade responses (data not shown).

Complete profiles of cross-reactivity of all null VRP antiserum groups to the entire panel of VLPs are shown in Fig. 5.

TABLE 2. Average percent sera for blockade of 50% (BT50) and 90% (BT90) H type 3 binding

Vaccine	Avg % sera (range) for blockade of H type 3 binding to[a]:			
	NV VLP		LV VLP	
	BT50	BT90	BT50	BT90
NV VLP	2.2 (0.6–5)	6.9 (1.3–20)	20	20
NV CpG	0.5 (0.2–0.6)	1.4 (0.6–2.5)	20	20
NV null	0.2 (0.2–0.6)	0.4 (0.2–1.3)	20	20
LV VLP	20	20	6.3 (2.5–10)	12.5 (5–20)
LV CpG	20	20	1.0 (0.2–2.5)	2.0 (0.6–5)
LV null	20	20	0.2	0.4 (0.2–1.3)
GI+ null	0.8 (0.6–1.3)	1.7 (1.3–2.5)	20	20
GI− null	12.5 (10–20)	20	20	20
GII+ null	20	20	0.2	0.3 (0.2–0.6)
GII− null	20	20	20	20
GI+/GII+ VLP	2.9 (1.3–5)	7.7 (1.3–10)	1.5 (0.2–5)	17.5 (10–20)
GI+/GII+ CpG	0.6 (0.2–1.3)	1.8 (0.6–2.5)	0.2	0.3 (0.2–0.6)
GI+/GII+ null	0.8 (0.6–1.3)	1.7 (1.3–2.5)	0.2	0.2
GI−/GII− VLP	20	20	20	20
GI−/GII− CpG	8.0 (0.2–20)	18 (10–20)	17.5 (10–20)	20
GI−/GII− null	7.1 (2.5–10)	20	8.8 (2.5–20)	20

[a] Sera that blocked H type 3 binding at the lowest concentration tested were assigned a BT value that is half the lowest serum concentration tested (0.2%). Sera that could not block H type 3 binding at the highest concentration tested were assigned a BT value that is twice the highest serum concentration tested (20%).

Obvious trends that emerge are significantly low cross-reactivity to additional VLPs following monovalent vaccination with NV or LV ($P < 0.001$), although slightly increased cross-reactivity exists to VLPs within a genogroup; low cross-reactivity to strains in opposite genogroups following GI and GII vaccination ($P < 0.05$); enhanced cross-reactivity to heterologous NV or LV strains within a genogroup following GI− and GII− vaccination, respectively; and cumulative cross-reactivity to heterologous NV and LV strains following complete VLP vaccination. These results suggest cross-reactivity induced by multivalent vaccination is likely genogroup-specific; therefore, vaccines must contain both GI and GII strains to induce a cumulative cross-reactivity to the majority of human norovirus strains.

Because noroviruses are enteric pathogens, a likely site of neutralization is the gastrointestinal tract. We, therefore, analyzed NV-specific IgG and IgA content in fecal extracts following monovalent or multivalent VLP vaccination coadministered with no adjuvant, CpG or null VRP (Table 3). NV-reactive IgG and IgA content, as well as total IgG and IgA content, was determined, and percentages of NV-specific subtype antibody were calculated. Significantly more total IgA than IgG was present in all fecal extracts tested ($P < 0.001$); however, the percentage of IgG specific for NV VLPs was significantly higher than that of specific IgA in all samples ($P < 0.001$). Vaccines coadministered with null VRP adjuvant induced significantly more total IgG, but not total IgA, than did covaccination with CpG ($P < 0.05$) or VLP alone ($P < 0.01$). A similar trend was seen by increasing the total number of VLPs administered in the vaccine composition, although values were not significant. Monovalent NV vaccination with null VRP induced significantly higher NV-specific IgA responses than did GI+/GII+ vaccination ($P < 0.05$). Conversely, GI+/GII+ vaccination induced higher NV-specific IgG responses than did monovalent vaccination, although values were not significant. Multivalent GI−/GII− null VRP vaccination induced significantly lower NV-specific IgG ($P < 0.05$), but not NV-specific IgA, than did GI+/GII+ vaccination. Percentages of NV-specific IgG were equivalent in NV and GI+/GII+ groups receiving either CpG or null VRP adjuvant; furthermore, the presence of adjuvant resulted in a substantial increase in total measurable IgG. Percentages of NV-specific IgA, however, were miniscule. LV-specific responses following monovalent and multivalent LV VLP vaccination were lower and more variable (data not shown). These data suggest that multivalent null VRP vaccination induces a predominantly IgG subtype response in the intestinal tract, as previously described using a model antigen (49, 50).

Null VRP vaccines induce stimulation of TH1-like IgG subclass responses. Previous studies have reported the activation of $CD4^+$ T helper 1 (TH1) cells and the production of IFN-γ following norovirus infection (28). Because TH1 responses correlate with serum IgG2a subclass responses in mice, we used this alternative evaluation to determine induction of TH1 cell responses by multivalent VLP vaccination. Serum samples from mice vaccinated with monovalent or multivalent VLP vaccines alone or in conjunction with CpG or null VRP adjuvants were analyzed for IgG1 and IgG2a subclass specificity to NV and/or LV VLPs (Fig. 6). Monovalent and multivalent vaccination with NV and/or LV VLPs induced IgG2a titers that were slightly increased when coadministered with CpG and significantly increased when coadministered with null VRPs compared to when coadministered with VLPs alone ($P < 0.05$). Heterotypic IgG2a responses to NV and LV VLPs following GI−/GII− vaccination were lower than homotypic responses, and titers were not different in CpG and null VRP recipient groups. IgG1 titers were not significantly different in VLP versus adjuvant groups but maintained uniform levels of reactivity to NV and LV VLPs that were significantly lower than IgG2a titers in null VRP recipient groups ($P < 0.05$), although a spike in NV-specific IgG1 levels appeared to occur following monovalent and multivalent VLP vaccination with CpG. Increasing the number of VLPs in NV or LV null VRP vaccines from one to four to eight VLPs did not change IgG1

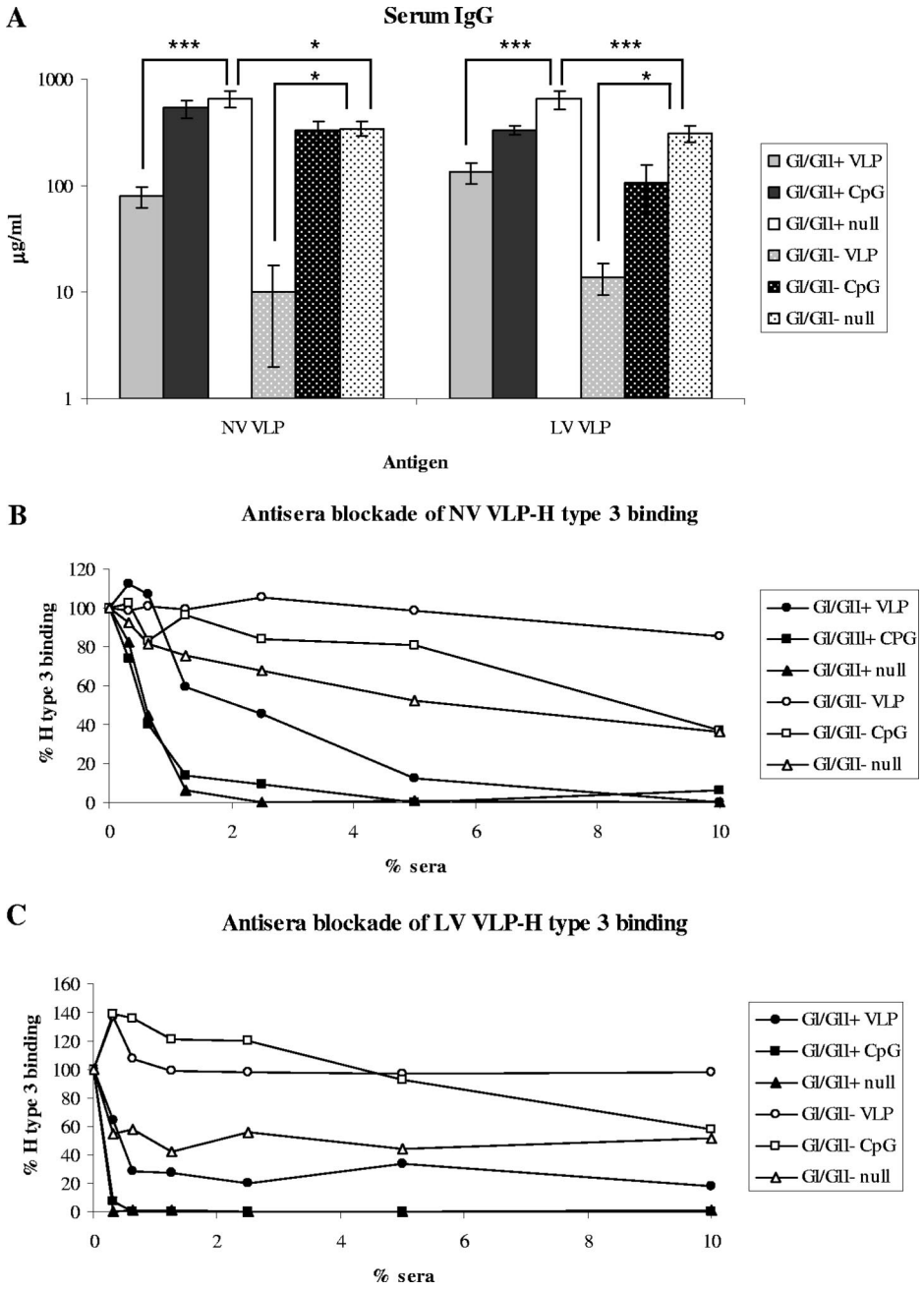


FIG. 3. Antibody responses following multivalent vaccination with or without adjuvant. Sera from animals immunized with multivalent VLP vaccines either alone or in conjunction with CpG or null VRP adjuvant were analyzed for IgG reactivity to NV or LV VLPs by ELISA (A). GI+/GII+ groups received NV and LV VLPs as a vaccine component; GI−/GII− groups did not. Serially diluted sera were also tested for interference of H type 3 binding to NV VLPs (B) and LV VLPs (C). One asterisk (*) is representative of P values of <0.05, and three asterisks (***) are representative of P values of <0.001.

or IgG2a responses specific for NV or LV VLPs, respectively. Together, these data suggest that null VRP vaccines induce IgG2a responses specific for NV and/or LV antigens, which may correlate with a TH1-type response. Furthermore, CpG and null VRP adjuvants induced cross-reactive IgG2a to NV and LV VLPs in the GI−/GII− vaccine group, implying that TH1 cross-reactivity to additional strains may also occur.

Multivalent VLP vaccines coadministered with null VRPs result in decreased viral load following MNV challenge. To determine if monovalent and multivalent vaccines can protect against norovirus challenge, we utilized the MNV infection model. Mice were immunized with monovalent MNV VLP vaccines or multivalent VLP vaccines consisting of eight human VLPs with MNV VLPs (Hu+/MNV+) or without MNV VLPs (Hu+/MNV−) (Table 1). Each was administered alone or in conjunction with CpG or null VRP adjuvants, same as the way human strain vaccines were administered, as described previously in this paper. Mice were then challenged with MNV

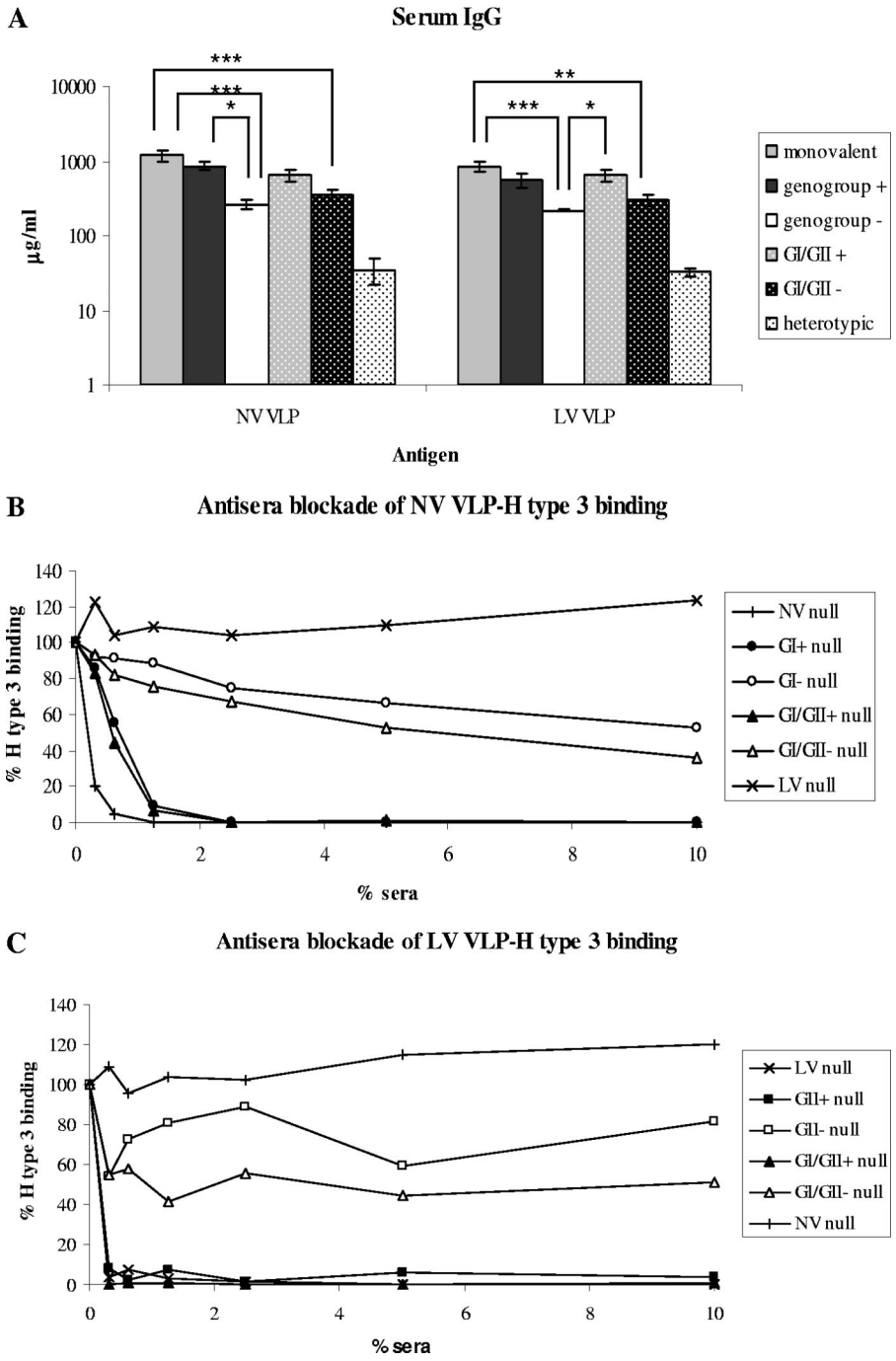


FIG. 4. Antibody responses to monovalent, genogroup-specific, and cumulative VLP cocktail vaccines coadministered with null VRP adjuvant. Sera from animals immunized with null VRP and monovalent, genogroup-specific multivalent, cumulative multivalent, or heterotypic monovalent VLP vaccines with or without NV or LV VLPs as a vaccine component were analyzed for IgG reactivity to NV or LV VLPs by ELISA (A). Serially diluted sera were also tested for interference of H type 3 binding to NV VLPs (B) and LV VLPs (C). One asterisk (*) is representative of P values of <0.05, two asterisks (**) are representative of P values of <0.01, and three asterisks (***) are representative of P values of <0.001.

3 weeks after secondary immunization, and spleens, MLNs, and distal ileums were harvested 3 days later. Viral titers of tissue homogenates were determined by plaque assay. Monovalent and MNV+/Hu+ vaccination with or without adjuvant induced complete protection from MNV infection in the spleen, with significantly lower viral titers than those induced by vaccination with null VRP alone ($P < 0.001$) (Fig. 7A). Hu+/MNV− vaccination did not completely protect against MNV infection in the spleen; however, viral loads were significantly lower in Hu+/MNV− groups coadministered with null VRP adjuvant than in those vaccinated with null VRP alone ($P < 0.05$). In contrast, viral loads in MLNs and distal ileum

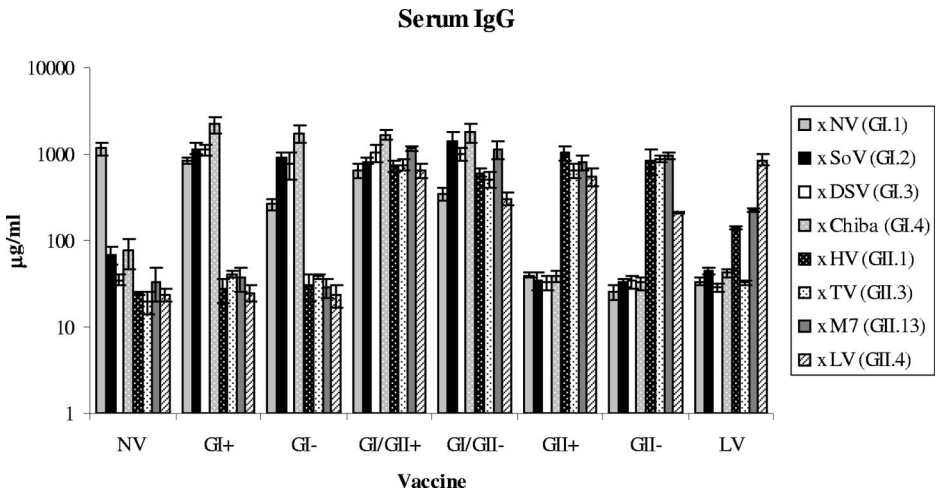


FIG. 5. Serum IgG cross-reactivity profile. Antisera from mice immunized with each monovalent or multivalent VLP vaccine coadministered with null VRP adjuvant were analyzed for IgG cross-reactivity to the VLP panel by ELISA.

were not significantly reduced following monovalent or multivalent VLP or CpG vaccination compared to those with unvaccinated controls. Null VRP administration, however, significantly reduced viral loads compared to controls following monovalent and Hu+/MNV+ vaccination in both MLNs ($P < 0.001$) and distal ileum ($P < 0.05$). Hu+/MNV− vaccination coadministered with null VRP significantly reduced viral loads in the distal ileum as well ($P < 0.05$). Vaccination and MNV challenge experiments were repeated in the null VRP adjuvant groups only (Fig. 7B) and resulted in similarly reduced viral loads in the MNV and Hu+/MNV+ vaccine groups in the spleen ($P < 0.001$), MLNs ($P < 0.05$), and distal ileum ($P < 0.01$) and reduced loads in the spleen of the Hu+/MNV− vaccine group ($P < 0.01$). MNV, Hu+/MNV+, and Hu+/MNV− antisera all contained MNV-reactive IgG antibodies following null VRP vaccination, where MNV and Hu+/MNV+ responses were equivalent and significantly higher than the cross-reactive response in Hu+/MNV− groups ($P < 0.001$) (Fig. 7C). These findings show that multivalent VRP vaccines can successfully limit the spread of norovirus infection to some peripheral tissues and can reduce viral loads in primary and additional secondary sites of replication even without the presence of homologous MNV antigen in the vaccine composition using the MNV infection model. These results lend strong support for the development of multivalent human norovirus vaccines.

Humoral immunity protects against acute MNV infection. To determine the mechanism of protection induced by null VRP vaccines, we vaccinated immunocompetent wild-type mice monovalently with MNV VLPs coadministered with null VRPs and passively transferred antisera or adoptively transferred purified $CD4^+$ or $CD8^+$ splenocytes into naïve wild-type mice or immunodeficient SCID mice. Unimmunized mice were treated in parallel as controls. $CD4^+/CD3^+$ and $CD8^+/CD3^+$ T cells from immune and nonimmune spleens were each found to be ≥90% pure by FACS analysis (data not shown).

TABLE 3. Anti-NV IgG and IgA in fecal extracts[a]

Vaccine[b]	Anti-NV IgG ± SEM (ng/ml)	Total IgG ± SEM (ng/ml)[d]	Anti-NV/total IgG (%)[e]	Anti-NV IgA ± SEM (ng/ml)	Total IgA ± SEM (mg/ml)[d]	Anti-NV/total IgA (%)[e]
VLP						
NV	0.5 ± 0.3[c]	62.3 ± 12.6	0.8	2.5 ± 1.2	47.3 ± 10	5.3E−03
GI/GII+	2.4 ± 0.7	174.6 ± 79.1	1.4	0.7 ± 0.1	25.7 ± 4.6	2.7E−03
GI/GII−	0.5 ± 0.2	110.7 ± 30.6	0.5	1.1 ± 0.7	30.6 ± 2.6	3.6E−03
CpG						
NV	12.2 ± 6.7	94.5 ± 9.7	12.9	7.2 ± 2.2	56.5 ± 2.0	1.3E−02
GI/GII+	34.9 ± 8.0	316.0 ± 203.8	11.0	3.2 ± 1.9	30.6 ± 8.1	1.0E−02
GI/GII−	10.4 ± 6.6	315.8 ± 6.0	3.3	1.8 ± 0.8	31.3 ± 1.5	5.8E−03
Null VRP						
NV	44.1 ± 9.3	254.0 ± 54.9	17.4	68.1 ± 32.2	44.0 ± 4.7	1.5E−01
GI/GII+	88.1 ± 47.9	504.6 ± 267.4	17.5	10.4 ± 8.7	27.5 ± 1.6	3.8E−02
GI/GII−	9.1 ± 4.2	318.3 ± 50.6	2.9	7.7 ± 2.4	31.5 ± 7.6	2.4E−02

[a] See text for statistical analysis.
[b] Adjuvants (if applicable) coadministered with VLP vaccines are listed, and VLP vaccine groups are listed beneath each adjuvant.
[c] Lower limit of detection for IgG and IgA assays is 0.2 ng/ml.
[d] Total Ig concentration in sample; nonspecific for antigen.
[e] The percentage of NV-specific antibody per total antibody was calculated as the anti-NV Ig concentration/total nonspecific Ig concentration × 100.

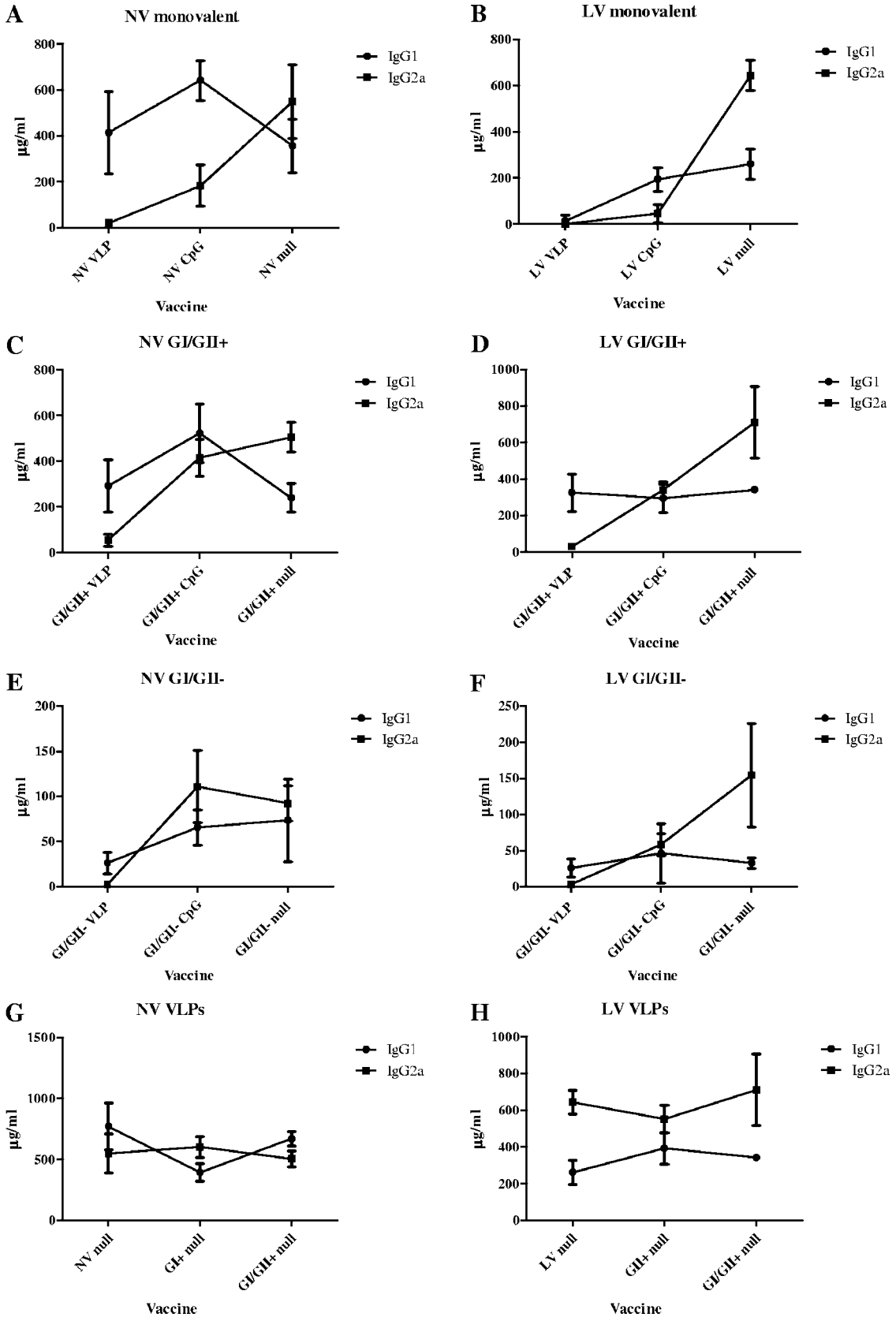
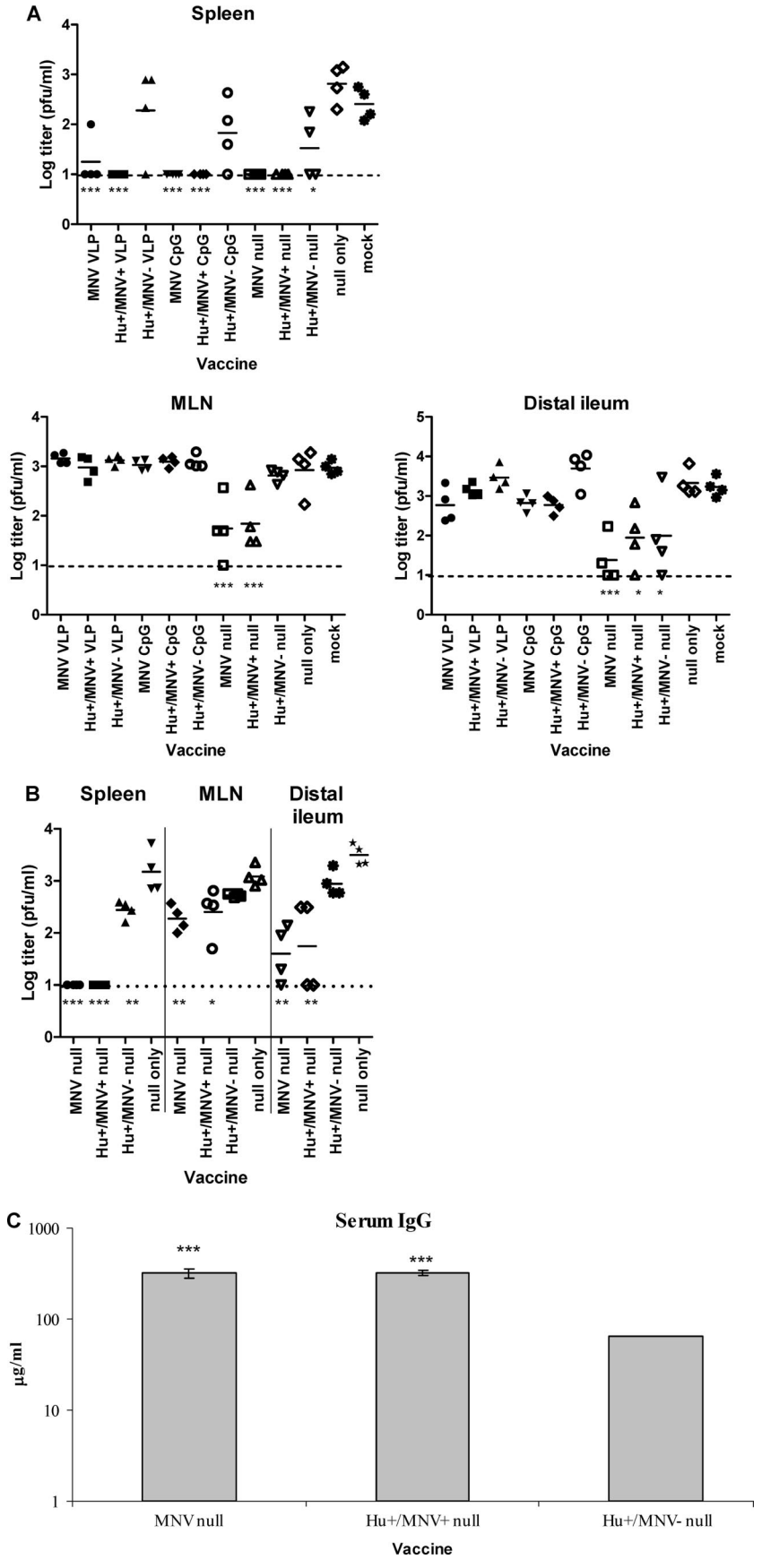


FIG. 6. IgG subtypes in serum following monovalent, multivalent, and adjuvanted vaccination. Mice immunized with monovalent (A and B) and multivalent vaccines with (C and D) or without (E and F) NV (left panels) or LV (right panels) VLPs and with or without adjuvant were analyzed for IgG1 and IgG2a serum antibody subtype responses by ELISA. Subtype responses to increasing amounts of VLPs are shown in panels G and H.


A
Spleen
Log titer (pfu/ml)
MNV VLP
Hu+/MNV+ VLP
Hu+/MNV- VLP
MNV CpG
Hu+/MNV+ CpG
Hu+/MNV- CpG
MNV null
Hu+/MNV+ null
Hu+/MNV- null
null only
mock
Vaccine
MLN
Distal ileum
B
Spleen
MLN
Distal ileum
C
Serum IgG
µg/ml
MNV null
Hu+/MNV+ null
Hu+/MNV- null

After 24 h, transfer recipient mice were infected with MNV, and tissues were harvested 3 days later. Adoptive transfers of immune or nonimmune $CD4^+$ or $CD8^+$ splenocytes did not prevent establishment of MNV infection in the spleens of wild-type or SCID mice, as determined by plaque assay (Fig. 8A). Passive transfer of antisera, however, was able to protect SCID mice from MNV infection in the spleen in all mice tested, whereas transfer of nonimmune sera had no effect on viral titers ($P < 0.001$) (Fig. 8A). Wild-type mice also exhibited reduced viral loads in the spleen following passive transfer of immune sera compared to those exhibited following passive transfer of nonimmune sera, with a number of animals cleared of detectable virus. Significant MNV-specific antibodies were found to be circulating in both donor wild-type mice and recipient mice but not in nonimmune controls ($P < 0.001$) (Fig. 8B). Viral titers in the MLNs and distal ileum of wild-type recipient mice were not reduced following transfer of immune sera, $CD4^+$ T cells, or $CD8^+$ T cells compared to those following nonimmune transfers (data not shown). Because SCID mice do not maintain a competent adaptive immune system and have underdeveloped immune organs, MLNs were not analyzed in this group. Furthermore, SCID mice did not support measurable viral titers in the distal ileum in either transfer group (data not shown). Together, however, these data clearly indicate that humoral immunity induced by monovalent null VRP vaccination can prevent establishment of acute MNV infection and provide further support for the development of null VRP vaccines in humans.

DISCUSSION

Multivalent vaccination has become a popular tool for generating cross-reactive immunity to heterologous strains of bacterial and viral pathogens. Cattle immunized with bivalent adjuvanted vaccines containing two viral subtypes of killed bovine viral diarrheal virus-produced neutralizing antibody and IFN-γ responses to both strains (40). Mice vaccinated simultaneously with VRPs expressing three different cowpox proteins survived infection and were protected from clinical signs better than monovalently vaccinated mice (52). In humans, adjuvanted multivalent streptococcal peptide vaccines from six serotypes are in phase I clinical trials (26), and multivalent vaccines containing 26 serotypes are in development (20). Of particular significance, effective adjuvanted quadrivalent human papillomavirus VLP vaccines are currently available (reviewed in reference 45), which are as effective as monovalent vaccines at inducing seroconversion (13). Multivalent vaccination is not without its drawbacks, however. Immune interference was reported in one study in which diphtheria and tetanus toxoids had reduced immunogenicity when coadministered with pertussis toxoid (54). Furthermore, multivalent vaccines do not necessarily elicit cross-reactive immunity to additional heterologous strains (10). This is the first study to address the efficacy of multivalent norovirus VLP vaccines using a codelivered adjuvant as well as the first study to address the efficacy of null VRP adjuvants as vaccine components in a small animal challenge model. In this study, null VRPs represent a novel vaccine adjuvant that should not only be safe for use in human vaccine trials (12) but also reproducibly generate higher immune induction to coadministered antigens than a low dose CpG DNA adjuvant, which has been approved for use in some human vaccines. While previous vaccine studies in BALB/c mice have administered up to 100 μg CpG (oligodeoxynucleotide 1826), much lower concentrations are required to induce sufficiently strong immune responses (1, 11). Given the low dose of CpG used in these studies, we would anticipate that increased concentrations of CpG may result in more-robust antibody responses against norovirus VLPs.

We have systematically designed and tested the efficacy of monovalent and multivalent norovirus VLP vaccines coadministered with null VRP adjuvants in generating cross-reactive and receptor-blocking antibody responses and protection against heterologous MNV challenge. These findings are supported by evidence showing that (i) immunodeficient mice were completely protected against MNV infection following transfer of antisera from wild-type mice following monovalent MNV VLP vaccination coadministered with null VRP adjuvant, most likely by antibody-mediated neutralization; (ii) increasing the number of antigens in the vaccine composition did not significantly blunt the immune response to the original antigens; (iii) VLP vaccines lacking target antigens induced strong cross-reactive antibody responses to heterologous strains that partially blocked receptor binding to these strains; and (iv) VRP-adjuvanted VLP vaccines lacking target antigens significantly reduced viral loads in murine tissues following heterologous viral challenge. Although multivalent vaccination did not provide protection from heterologous MNV infection, the significant reduction in observed viral load may be tightly correlated with reduction of clinical disease, as seen with human immunodeficiency virus (HIV), respiratory syncytial virus, or human papillomavirus infections (7, 14, 55), or may alter transmission rates following infection. However, one study recently showed no differences in viral load in symptomatic and unsymptomatic norovirus-infected individuals (36). Overall, our data encourage the development of multivalent VLP/null VRP vaccines against highly heterogeneous noroviruses.

Alphavirus VRPs are single-hit vectors which traditionally express high concentrations of transgene in infected tissues, and the dogma has argued that coexpression is essential for vaccine efficacy (41). Interestingly, the adjuvant activity of VRPs lacking a transgene has been clearly documented using model antigens. Our data clearly show that coadministration of VLPs with null VRP adjuvants induces not only significant

FIG. 7. Viral titers and antibody responses following MNV challenge in monovalent, multivalent, and adjuvant-vaccinated mice. Mice immunized with adjuvanted or unadjuvanted monovalent MNV VLP or multivalent VLPs ± MNV VLPs were challenged with MNV, and tissues were harvested 3 days postinfection. Plaque assays were performed on homogenized spleen, MLNs, and distal ileum to determine viral titers (A). Vaccination and challenge in all null VRP recipient groups were repeated, and MNV titers were determined in corresponding tissues (B). Serum IgG reactivity to MNV VLPs was determined by ELISA (C). One asterisk (*) is representative of P values of <0.05, two asterisks (**) are representative of P values of <0.01, and three asterisks (***) are representative of P values of <0.001.

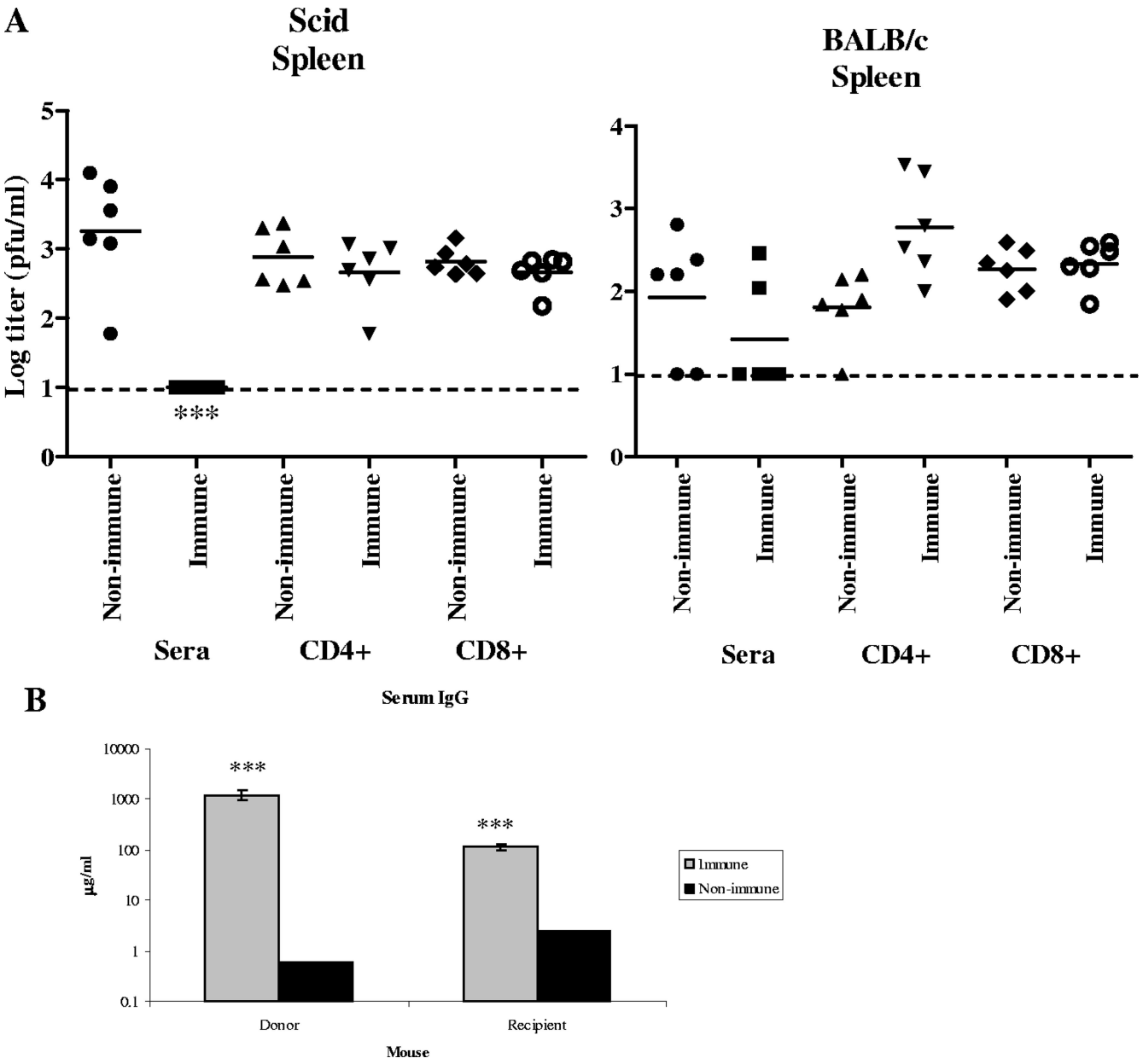


FIG. 8. MNV infection of naïve mice following transfer of immune T-cell subsets or sera. Wild-type mice were immunized three times with MNV VLPs coadministered with null VRPs. Unimmunized controls were treated in parallel. Two weeks after the final boost, sera and $CD4^+$ and $CD8^+$ splenocytes were harvested and purified. Sera, $CD4^+$ splenocytes, or $CD8^+$ splenocytes were passively or adoptively transferred to naïve SCID knockout mice or wild-type mice. Twenty-four hours posttransfer, mice were infected with MNV-1, and tissues were harvested 3 days postinfection. Spleens were evaluated for MNV titers by plaque assay (A). MNV-specific serum IgG in serum donor and recipient mice was measured by ELISA (B). Nonimmune mice had no detectable MNV antibody and were assigned values that were half the lower limit of detection per assay. ***, $P < 0.001$.

systemic and mucosal immune responses but also protective homotypic and heterotypic immunity at mucosal sites. The mechanism by which null VRPs function as adjuvants is not completely known, but stimulation of immune cells by a single round of viral RNA replication in mammalian cells, as proposed by Thompson et al. (49–51), is likely responsible for VRP immunogenicity. Importantly, the safety of VRPs as vaccine vectors has been questioned due to the presence of functional VEE genes, the possible production of replication-competent viruses during vaccine manufacture, and the biosafety level 3 requirements for safety testing (42). Despite this, the safety record for VRPs and other alphavirus replicon vectors is robust, and VRPs have proven safe in human phase I clinical trials for HIV (12), influenza, and human cytomegalovirus.

Previous work from our lab has shown that antibodies from both infected humans and VRP-vaccinated mice can block HBGA binding to homologous norovirus strains (18, 31). We also published an original study showing that VRP vaccination

can generate intermediate cross-reactive receptor-blocking antibodies to heterologous norovirus strains when multiple vectors are administered simultaneously (31). While this study laid the groundwork for the research presented here, only four VLPs were available to us at that time, and it was not possible to assess protection from infection. This work utilizes a representative panel of eight human VLPs that together account for ≥95% of all norovirus infections, including the predominantly circulating GII.4 strains. Furthermore, we are now able to address intergenogroup versus intragenogroup cross-reactivity and cross protection following multivalent vaccination, which was not possible with the single GI VLP or lack of the MNV infection model previously available to us. Our findings obviate a clear discrepancy in cross-reactivity between genogroups. Monovalent vaccines generated very low cross-reactive antibody responses to all heterologous strains, although strains within a genogroup elicited a slightly higher cross-reactive response to additional intragenogroup strains. Multivalent genogroup-specific vaccination, however, elicited strong cross-reactive and intermediate receptor-blocking antibody responses to other genogroup-specific strains but in no way enhanced cross-reactivity to strains between genogroups. This result is most likely caused by a cross-reactive epitope repertoire that is greater when multiple closely related but distinct antigens are included in the vaccine composition than when a single antigen is included. Alternatively, we may be stimulating the synthesis of antibodies with greater affinity and avidity to more-variant but cross-reactive sites. The addition of strains from both genogroups in our cumulative VLP vaccines did not detract from either genogroup-specific response but rather accentuated cross-reactivity and receptor blockade to intergenogroup strains. Antisera from multivalent vaccination groups could also block receptor binding of evolutionarily distinct GI.1 (NV genocluster) and GII.4 (LV genocluster) VLPs (30; also data not shown), suggesting that blockade responses may be genocluster rather than strain specific and may be of particular importance when applied to the GII.4 norovirus vaccine design. Previous studies of multivalent vaccination to *Neisseria* and *Streptococcus* species induced cross-reactive antibodies that could neutralize heterologous serotypes (2, 22); however, multivalent HIV envelope vaccines failed to induce cross-reactive neutralizing antibody responses (10). Together, these data support the rationale for including multiple norovirus strains from different genogroups in a comprehensive norovirus vaccine.

Multivalent human VLP vaccines coadministered with null VRPs and lacking the NV and LV components nevertheless elicited intermediate receptor blockade responses to NV and LV VLPs in vitro in our surrogate neutralization assay. No receptor for MNV has been identified to date; however, multivalent mouse VLP vaccines coadministered with null VRPs lacking the MNV VLP component lent intermediate protection against MNV challenge in vivo. We can speculate that these two findings are related and intermediate protection is conferred to heterologous strains following multivalent vaccination. However, several additional factors must be considered when evaluating the vaccine design of this study that may impact protective outcomes. VLP vaccination lacking adjuvant imparted specific but significantly lower receptor-blocking responses in vitro and elicited no protection against MNV challenge in the ileum and MLNs of infected mice. This can be explained by poor immune responses against unadjuvanted VLP vaccines (3, 5). In contrast, CpG-adjuvanted vaccines resulted in significant antibody induction to homologous and heterologous antigen; however, null VRP adjuvants imparted significantly more protection following MNV challenge at these doses. While serum antibody reactivity and blockade were not significantly different following CpG- and null VRP-adjuvanted vaccination, fecal IgG and IgA as well as the serum IgG2a subtype were significantly higher in null VRP vaccinated mice, which may explain this discrepancy. Unmethylated CpG DNA adjuvant activity is the result of innate immune activation through Toll-like receptor 9, which promotes antigen-specific adaptive immune responses (27, 44). The mechanism of null VRP adjuvant activity is not fully known but is likely linked to induction of the innate response by replicon RNA replication (50). The protective effects of null VRP adjuvant vaccination may, therefore, be linked to differential mechanisms of immune induction or a higher activation of antibody and T-cell responses in selective tissues that may not be represented by serum IgG responses.

Preparation of VLP reagents may represent a limitation in our vaccine design. We surmise that concentration by centrifugation causes some VLPs to lose particle structure. A previous study by Harrington et al. showed that antisera increased following vaccination with norovirus capsid proteins containing a mutation that does not allow particle assembly only partially blocked receptor binding (18). Because we achieve complete blockade in our receptor binding assays, we infer that the VLP immunogens retain proper structure. However, complete protection versus partial protection in the MNV infection model may reflect the degree of structural integrity in the VLP preparation. Because we observed only partial protection in some tissues in monovalent and multivalent MNV vaccination experiments adjuvanted with null VRPs (Fig. 7), we boosted wild-type donor mice a third time with unconcentrated VLPs prior to performing passive transfer experiments. The use of unconcentrated VLP, an additional immune boost by a third immunization, or both likely resulted in significant protection against challenge following passive transfer of antisera (Fig. 8). We also showed in our original VLP titration experiments that vaccination with 10 μg of VLPs induces better receptor blockade than that with the 2 μg administered in the experiments presented here. We were unable to increase this concentration in multivalent vaccines due to the finite volume that can be administered to a mouse footpad and the concentrations we can achieve with individual VLP preparations; however, higher concentrations of VLP may be required to induce an entirely protective state.

Naïve wild-type mice had reduced viral loads in the spleen but not the MLNs and distal ileum following passive transfer of immune sera, although values were not significant. These data may be the result of additional adaptive immune interference in these immunocompetent animals, more efficient virus replication in various compartments like the MLNs which may be compromised in SCID mice, or more efficient replication overall. SCID mice, however, exhibited significant protection from MNV infection in the spleen following passive transfer of immune sera. Both donor and recipient mice had robust circulating anti-MNV IgG titers. Because adoptive transfer of im-

mune $CD4^+$ and $CD8^+$ splenocytes did not affect viral loads in any mice tested, whereas passive transfer of immune sera decreased viral loads in both wild-type and immunodeficient mice, we can conclude that humoral immunity is the likely mechanism of protection following MNV VLP vaccination (8). Clearly, additional experiments with swine inoculated with multivalent VLP-adjuvanted vaccines and/or passive transfer experiments would provide important support for these findings.

The MNV model has some limitations as a model for human norovirus infection. Virus loads are reduced compared to titers seen following human infection, and it is likely that different tropisms exist for mouse and human strains. Moreover, the MNV model does not capture the strain heterogeneity noted among human strains and does not produce notable diarrheal disease. Fortunately, several human challenge inocula exist for NV and related norovirus, allowing for vaccine testing following human challenge (28, 29). However, prior to these essential human studies, it will also be important to determine if null VRP norovirus vaccines protect swine from HS66 infection (9). In the swine model, gnotobiotic pigs support symptomatic infection and shedding of the HS66 human GII.4 norovirus, develop clinical diarrheal disease, and are protected from infection following homologous VLP vaccination (46). Using this robust model, we can test homologous monovalent vaccines as well as heterologous multivalent vaccines in conjunction with null VRP adjuvants to determine efficacy in a symptomatic infection model. Together, mice and swine provide substantive, complementary models for rigorous, preclinical vaccine evaluation. Our data support the argument that similar studies in swine should be conducted prior to progressing to human experimentation.

Overall, our data suggest that increased antibody cross-reactivity to heterologous norovirus strains following multivalent VLP vaccination coadministered with null VRP adjuvant may significantly protect against subsequent norovirus infection. Homologous vaccination induced antibodies that completely blocked receptor binding and was able to completely protect against infection in transfer experiments. Multivalent vaccines also induced robust cross-reactive antibody blockade responses, concentrated mucosal IgG, and limited viral loads following MNV challenge. Unfortunately, mice do not develop clinical disease after MNV challenge making it impossible to determine if reduced viral loads in vaccinated mice correspond to reduced morbidity. Similar experiments with swine would address this issue directly. However, the efficacy of norovirus vaccine formulations containing multiple distinct VLP antigens is supported by our findings that incorporation of up to nine different norovirus strains did not detract from the overall specific immune response generated to each individual antigen; thus, more significant protection might be afforded against the vaccine strains included in the cocktail. Currently, human VLP vaccines containing GII.4 components are widely needed to prevent frequent norovirus outbreaks; however, multivalent vaccines containing additional GI and GII components may be crucial in preventing further isolated outbreaks and emergence of new predominant strains. The data presented in this study support our conclusion that multivalent norovirus VLP vaccines supplemented with VRP adjuvant will likely provide a safe and effective platform for controlling norovirus infections in humans.

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