Robust mucosal-homing antibody-secreting B cell responses induced by intramuscular administration of adjuvanted bivalent human norovirus-like particle vaccine

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ARTICLE INFO

ABSTRACT

Background: Two major antigenically heterogenous norovirus genogroups (GI and GII) commonly infect humans and are the leading cause of foodborne, viral gastrointestinal infections in adults.
Methods: We assessed B cell responses in participants in a double-blind, placebo-controlled, dose-escalation phase 1 study of the safety and immunogenicity of an intramuscular bivalent norovirus virus-like particle (VLP) vaccine. The vaccine contained a GI.1 VLP (Norwalk) and a consensus GII.4 VLP, representing the two major genotypes that cause human disease, and was administered on days 0 and 28 to healthy adults aged 18–49 years. Four separate cohorts received increasing doses of 5 μg, 15 μg, 50 μg, and 150 μg of each VLP adjuvanted in monophosphoryl lipid A and alum. PBMCs were analyzed for B cell activation and mucosal homing markers (flow cytometry) and VLP-specific and total IgG and IgA Ab-secreting cells (ASCs); and serum titers of VLP-specific IgG, IgA, and Pan-Ig were determined.
Results: The vaccine elicited CD27+ CD38+ plasmablasts and high frequencies of ASCs specific for both VLP antigens in the peripheral blood at 7 days after the first dose. The plasmablasts exhibited a mucosal-homing phenotype and included a high proportion of IgA ASCs. Serum antibodies increased as early as 7 days after the first immunization.
Conclusions: The data suggest that a single dose of the IM bivalent norovirus vaccine is effective in activating pre-existing B cell memory. The rapid B cell response and the mucosal homing phenotype of induced ASCs are consistent with anamnestic responses in subjects primed by prior oral norovirus infection.
  This study is registered at ClinicalTrials.gov Identifier NCT01609257.

1. Introduction

Noroviruses are estimated to cause more than 50% of viral gastroenteritis cases worldwide [1,2] and in excess of 21 million cases of acute gastroenteritis annually in the United States [3,4]. Transmission is via the fecal-oral route and outbreaks commonly result from contaminated food [5,6]. The low infectious dose and high level of environmental stability contribute to rapid viral spread in populations housed in close quarters [3,6,7].

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Table 1
Demographics and baseline characteristics by treatment group for subjects at the University of Rochester with fresh PBMC Data Cohort A, dosage escalation.

VLP Vaccine dose (prime/boost, μg)	5/5 (N = 5)	15/15 (N = 4)	50/50 (N = 4)	150/150 (N = 4)	Placebo (N = 3)	All subjects (N = 20)
Gender – n (%)						
Male	3 (60.0)	2 (50.0)	0	1 (25.0)	1 (33.3)	7 (35.0)
Female	2 (40.0)	2 (50.0)	4 (100.0)	3 (75.0)	2 (66.7)	13 (65.0)
Ethnicity – n (%)						
Non-Hispanic	3 (60.0)	4 (100.0)	4 (100.0)	4 (100.0)	3 (100.0)	18 (90.0)
Hispanic	2 (40.0)	0	0	0	0	2 (10.0)
Race – n (%)						
American Indian/Alaskan Native	0	0	0	0	0	0
Asian	0	0	1 (25.0)	1 (25.0)	0	2 (10.0)
Hawaiian/Pacific Islander	0	0	0	0	0	0
Black/African American	1 (20.0)	1 (25.0)	1 (25.0)	0	1 (33.3)	4 (20.0)
White	4 (80.0)	2 (50.0)	2 (50.0)	3 (75.0)	2 (66.7)	13 (65.0)
Multi-Racial	0	1 (25.0)	0	0	0	1 (5.0)
Other/unknown	0	0	0	0	0	0
Age						
Mean (STD)	30.2 (5.5)	33.0 (10.2)	35.8 (6.8)	36.0 (12.1)	24.0 (2.6)	32.1 (8.5)
Median	30.0	34.5	37.5	36.0	23.0	30.0
Min, Max	(23, 37)	(20, 43)	(26, 42)	(23, 49)	(22, 27)	(20, 49)
Saliva secretor status – n (%)						
Positive	3 (60.0)	2 (50.0)	1 (25.0)	4 (100.0)	3 (100.0)	13 (65.0)
Negative	2 (40.0)	2 (50.0)	3 (75.0)	0	0	7 (35.0)
Blood type – n (%)						
A	2 (40.0)	2 (50.0)	2 (50.0)	3 (75.0)	0	9 (45.0)
O	3 (60.0)	2 (50.0)	2 (50.0)	1 (25.0)	2 (66.7)	10 (50.0)
AB	0	0	0	0	1 (33.3)	1 (5.0)

Noroviruses are classified into five genogroups on the basis of phylogenetic analysis of the major viral capsid protein, VP1 [5]. Viruses in the GI and GII genogroups cause most human disease, with GII genogroup viruses predominating worldwide since the 1990s [5,8]. The genogroups are further divided into genotypes; the prototypic Norwalk virus forms the GI.1 genotype, and the GII.4 genotype represents the predominant currently circulating noroviruses.

The finding that expressed recombinant norovirus VP1 self-assembles into virus-like particles (VLPs) has stimulated the development of norovirus vaccine candidates [5]. Each VLP consists of 90 VP1 dimers and is structurally and immunogenically similar to the intact norovirus particle [8–12]. Norovirus vaccine candidates based on VLPs have been tested in a number of clinical trials [8,9,13,14]. Oral and intranasal routes of administration have been employed with the objective of inducing mucosal immunity to optimally combat natural norovirus infection [8,9,13–16]. Norovirus VLP vaccines elicit both T and B cell immunity, as found for other VLP-based vaccines such as the licensed human papillomavirus vaccines [11,17–25]. Norovirus vaccine studies to date have mostly focused on a single genogroup, although multivalent vaccines covering more than one genogroup may generate broader protection.

Although norovirus VLP vaccines alone are immunogenic, the incorporation of appropriate adjuvants is likely to enhance immune responses and improve protection. This may be particularly important when targeting age groups that tend to respond poorly to vaccination. Monophosphoryl lipid A (MPL) and aluminum hydroxide (alum) are two adjuvants approved for use in human vaccines. MPL, a lipopolysaccharide derivative from *Salmonella minnesota* R595 [26,27], is immunostimulatory because of toll-like receptor-4 agonist activity [27] and is licensed for use in a human papillomavirus vaccine.

The norovirus vaccine used in our study consisted of VLPs from insect cell-derived, recombinant VP1 proteins representing the GI.I and GII.4 genogroups, together with the adjuvants MPL and alum. A phase I clinical trial of the adjuvanted, bivalent norovirus VLP vaccine given intramuscularly (IM) was conducted to assess safety and to measure the seroresponse to escalating doses of a two-dose regimen. Here, we present an analysis of the circulating antibody-secreting cell (ASC) response to the vaccine in a subset of trial participants.

2. Methods

2.1. Study Design and Subjects

A total of 20 healthy subjects 18 to 49 years of age were enrolled sequentially into each of four dose groups of 4–5 subjects (Table 1). Dose groups received two IM administrations (days 0 and 28) of a bivalent norovirus VLP vaccine containing 5 μg, 15 μg, 50 μg, or 150 μg of each VLP component (GI.1 and GII.4). Subjects were randomized 5:1 to receive vaccine or saline control (placebo). All subjects were tested for saliva secretor status and blood type prior to the trial. After all being informed of the nature and possible consequences of the study, subjects were enrolled under informed consent using a protocol reviewed and approved by the University of Rochester Research Subjects Review Board.

2.2. Vaccine and placebo

The norovirus bivalent VLP vaccine contained GI.1 and GII.4 VLPs, MPL and aluminum hydroxide as adjuvants, sodium chloride and L-histidine as buffer (pH 6.3–6.7), ethanol, and water for injection (Table 2).

Table 2
Composition of four IM norovirus bivalent VLP vaccine formulations per 0.5 mL.

Total VLP per dose (μg)	GI.1-VLP (μg)	GII.4 VLP (μg)	MPL (μg)	Al[a] (mg)
10	5	5	50	0.5
30	15	15	50	0.5
100	50	50	50	0.5
300	150	150	50	0.5

[a] As aluminum hydroxide. Placebo formulation consisted of sterile normal saline for injection (0.9% NaCl and preservative-free).

Table 3
Flow cytometry markers and fluorochromes used for assessing B cell phenotypes and homing potential.

Marker	Clone	Fluorochrome
Forward scatter	–	FSC
Side scatter	–	SSC
$\alpha 4\beta 7$	FIB504	FITC
CD138	MI 15	APC
Live/dead	–	Alexa 430
CCR10	6588-5	PE
CD19	J3-119	PE-Texas Red
CD27	1A4CD27	PE-Cy5
CD3	UCHT1	PE-Cy5.5
CD38	HIT2	PE-Cy7
CD62L	Dreg-56	APC-Alexa 750

2.3. Immune assays

2.3.1. B cell enrichment and phenotyping

B cells were enriched from peripheral blood mononuclear cells (PBMCs) by negative selection [28], resulting in CD19+ B cell purities from 65 to 95%. B cell phenotyping by flow cytometry involved staining aliquots of PBMCs before and after enrichment with a 10 or 11-color panel of stains (Table 3). Data on stained cells was collected on a Becton-Dickinson LSR-II flow cytometer using Diva software and analyzed using FlowJo (TreeStar).

2.3.2. Enzyme-Linked ImmunoSpot assay (ELISpot) assay for ASCs

Vaccine-specific and total IgG and IgA ASCs were enumerated by ELISpot assay (20). Briefly, 96-well IP filter plates (Millipore, Billerica, MA) were coated overnight at 4 °C with antigens or Ig capture antibodies. For detection of VLP-specific ASCs, wells were coated with 1 μg/ml × 100 μl/well GI.1 Norwalk VLP (EN544-01-10-001, Takeda Vaccines Inc., Bozeman, MT), or GII.4 VLP (EN544-01-10-001A Takeda Vaccines Inc.). For total IgG or IgA ASCs, wells were coated with 4 μg/ml × 100 μl/well goat-anti human IgM + IgA + IgG antibody (KPL, Gaithersburg, MD). Control wells were plated with PBS or with recombinant influenza H3 Wisconsin/67/05 HA protein (BEIR) diluted in PBS to 1 μg/ml × 40 μl/well. Enriched B cells were resuspended in complete medium containing either alkaline phosphatase-conjugated goat anti-human IgG (H + L) (KPL) at 0.2 μg/ml or alkaline phosphatase-conjugated goat anti-human IgA (KPL) at 0.2 μg/ml for the detection of IgG or IgA ASCs, respectively. Serial dilutions of the cells were prepared in the coated/blocked plates and the plates were incubated for at least 4 h at 37 °C in 5% CO_2. Spots representing IgG or IgA ASCs were developed with a Vector Blue alkaline phosphatase substrate kit (Vector Laboratories, Burlingame, CA) and counted using a CTL ImmunoSpot plate reader and counting software (Cellular Technology Limited, Cleveland, OH).

2.3.3. Serology

For the Rochester cohort, blood samples were collected before the first vaccination (day 0) and on days 7, 28, and 35 after the first dose of vaccine for evaluation of serum antibodies. Titers of norovirus-specific Ig (Pan-Ig) and class-specific (IgG and IgA) antibodies were determined by enzyme linked immunoassay (ELISA) and duplex, time-resolved, dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA), respectively [9,10].

2.4. Statistical analyses

Data was analyzed using SAS version 9.3. A regression was performed to test for serological differences between dose groups. For this analysis, the log serological response was modeled with treatment group, according to dosage (0, 5, 15, 50, or 150 μg), the log baseline serological result and their interaction as covariates.

Additionally, each subject's serological log fold rise from baseline was calculated and a student's t-test was used to evaluate any treatment effect, for all vaccine subjects collectively. Negative serological results were analyzed as half the lower limit of quantification (LLOQ), defined as the lowest amount of antibody in a sample that could be quantitatively determined. Differences between the dose groups for expression of B cell phenotype and homing markers were tested by regression analysis. Correlations between the frequencies of B cells with a plasmablast phenotype and frequencies of VLP-specific ASCs were determined by calculating Spearman's correlation coefficients and P values with a two-tailed test.

3. Results

3.1. Serology

In this report, we analyzed a local cohort of 20 subjects aged 18–49 years from a larger, multicenter cohort that included subjects aged 50–64 and 65–83 years. The older subject groups were not part of our analysis. Post hoc analysis revealed that each dose group contained 3–5 subjects and included a total of three placebo controls. Serum antibody responses to the GI.1 antigens were higher than to the GII.4 antigens, as observed in the larger cohort. When results from all dose groups were collated by time point, there were significant increases in GI.1 and GII.4 VLP-specific IgG, IgA and Pan-Ig serum antibody levels at days 7, 28, and 35 compared to the prevaccination (day 0) titers (Fig. 1). There was no evidence of a boost in serum titers after the second vaccine administration on day 28. VLP-specific antibody titers did not change in recipients of the placebo.

3.2. Antibody-secreting cell analysis

The robust serological responses indicated substantial ASC formation. To assess the magnitude and specificity of the ASC response in peripheral blood, an ELISpot assay was used to determine GI.1 and GII.4 VLP-specific and total IgG and IgA ASC frequencies. All subjects that received bivalent norovirus VLP vaccine demonstrated high ASC frequencies in the peripheral blood on day 7 after the first vaccination (Fig. 2). After the study was unblinded, the three subjects among the dose groups that had undetectable VLP-specific ASC responses were revealed to be placebo (P) recipients. The response after the first vaccination tended to be biased toward IgA ASCs (Fig. 2A, B, and E). In general, ASCs specific for GI.1 VLP antigen predominated when both IgA and IgG ASCs were viewed collectively (Fig. 2E). However, the group that received the 150 μg dose exhibited a higher proportion of GII.4 VLP-specific ASCs compared to the lower dose groups (49% vs. ∼30%; Fig. 2E). Interestingly, the VLP-specific ASC responses on day 7 were much greater in magnitude (frequency per million CD19+ cells) than we had typically observed after influenza vaccination [28,29]. The strong IgA ASC bias is consistent with a mucosal (versus systemic) immune response, yet did not lead to a similar bias in serum immunoglobulin (Fig. 1 and [30]). There were no apparent increases in ASC frequencies corresponding to higher VLP doses.

Subjects (13 out of 20) who returned on day 28 for the second vaccination were analyzed by ELISpot assay for VLP-specific and total ASC after 7 days (day 35). The frequencies of GI.1 and GII.4 VLP-specific ASCs were two orders of magnitude lower than the responses measured on day 7 after the first vaccination (Fig. 2C and D). The IgA bias was diminished and, interestingly, ASC frequencies were somewhat higher in the 5 μg (lowest) dose group than in the other groups. There was a significant ($p = 0.0111$) negative correlation between the GI.1-specific ASC frequency on day 7 with that on day 35, suggesting that a strong response to the first dose
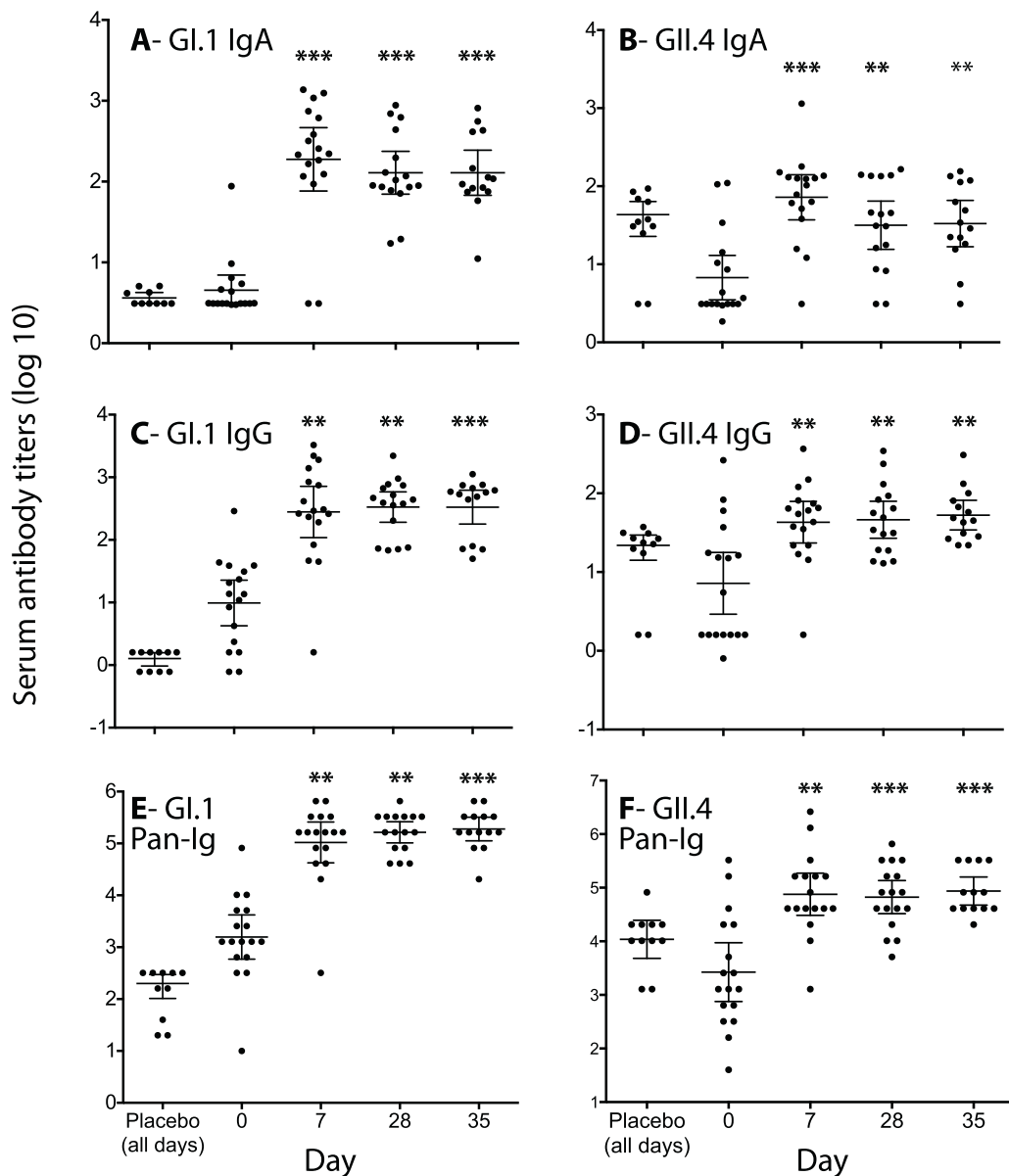
Fig. 1. Serum titers of norovirus GI.1- and GII.4-specific IgA, IgG, and Pan-Ig antibodies. (A) IgA titer against GI.1; (B) IgA titer against GII.4; (C) IgG titer against GI.1; (D) IgG titer against GII.4; (E) Pan-Ig titer against GI.1; (F) Pan-Ig titer against GII.4. Subjects were immunized IM with an adjuvanted bivalent norovirus VLP vaccine on days 0 and 28. Serum was collected on days 0, 7, 28, and 35 relative to the first immunization. Norovirus GI.1 and GII.4 VLP-specific antibodies titers were determined by DELFIA (IgA and IgG) and ELISA (Pan-Ig). Data points represent titers for individual subjects. Data from all dose groups were combined by day of blood draw before plotting and analysis. Each subject's serological log fold rise from day 0 was calculated and a student's t-test was used to evaluate any treatment effect, for all vaccine subjects collectively. Titers for placebo recipients are combined from all sampling days. Horizontal bars show the geometric mean titer and 95% confidence interval. P values relative to day 0 results are identified by asterisks ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

inhibited the response to the second dose. The day 7 versus day 35 correlations were not significant for the GII.4 VLP or total IgG and IgA. Overall, ASC measurements were consistent with the serum Ab analysis and demonstrate a rapid and vigorous B cell response to the first vaccination, but little if any boosting effect of the second vaccination, a pattern suggesting activation of pre-existing, vaccine-specific memory B cells by the first vaccination.

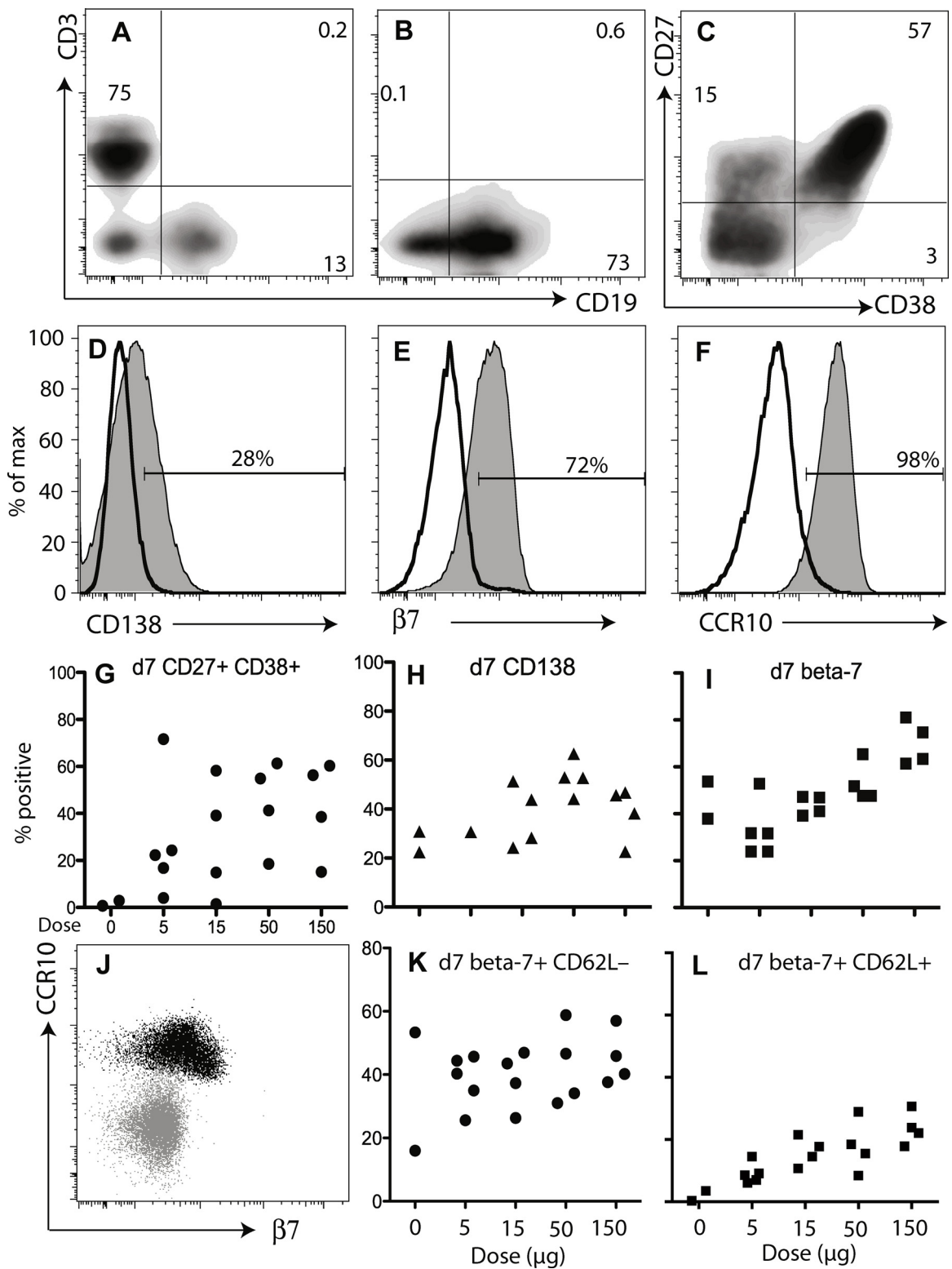### 3.3. Flow cytometric analysis of B cells in the peripheral blood

One sample collected after the first vaccination was lost during processing, leaving 19 samples for flow cytometric analysis. B cells (defined as CD3− CD19+ cells) were analyzed by flow cytometry before and after enrichment from PBMCs; dead cells and doublets

were excluded (Fig. 3A and B). B cells transitioning into antibody-secreting plasmablasts express CD27 and CD38 [28,31] and those actively secreting antibodies are enriched among the CD138+ subset [32]. On day 7 after the first vaccination, a robust population of CD27+ CD38+ B cells was detected in most vaccine recipients, but not in placebo (0 μg dose) controls (Fig. 3C and G), indicating a strong B cell response to the vaccine. The potential to home to peripheral lymphoid tissues or to mucosal tissues such as the gut was assessed by measuring expression of the integrin chain β7, the chemokine receptor CCR10+, and the lymph node homing receptor CD62L (L-selectin). A comparison of differentiation and mucosal homing markers on CD27+ CD38+ and CD27− CD38− B cells demonstrated upregulation of CD138 (Fig. 3D and H) and particularly β7 (Fig. 3E and I) and CCR10 (Fig. 3F) on the CD27+ CD38+
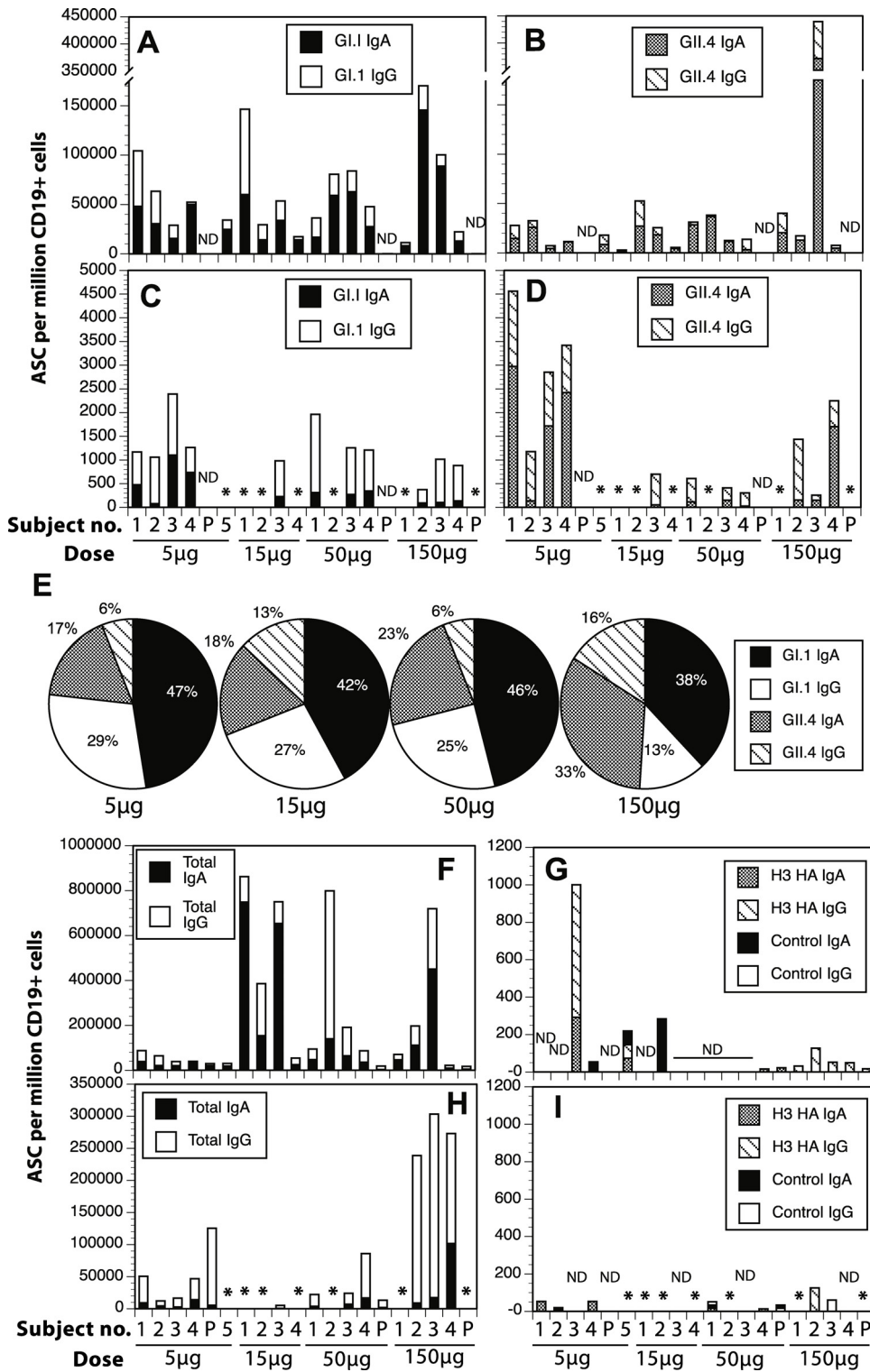
Fig. 2. Analysis of circulating GI.1 and GII.4 VLP-specific and total ASCs. Subjects were immunized twice (day 0 and day 28) with 5 µg, 15 µg, 50 µg, or 150 µg doses of an IM adjuvanted bivalent norovirus vaccine. B cells enriched from PBMCs collected on day 7 and day 35 (day 7 after the second vaccination) were analyzed by ELISpot assay for VLP-specific and total ASCs. (A and C) GI.1 VLP-specific IgA (black columns) and IgG (white columns) ASCs on day 7 (A) and day 35 (C). (B and D) GII.4 VLP-specific IgA (gray columns) and IgG (striped columns) ASCs on day 7 (B) and day 35 (D). Only placebo ("P" on the x-axis) recipients (distributed among dose groups) had no VLP-specific ASCs detected (shown as ND, none detected). Asterisks indicate that no PBMC sample was available for analysis because subjects did not return for the day 35 sampling. (E) The proportions of VLP-specific IgA and IgG ASCs in each dose group. For each dose group, ASCs of each VLP specificity and Ig class were summed and are shown as a percentage of the sum of all VLP-specific IgA and IgG ASCs. Pie chart shading corresponds to the shading in (A–D). (F and H) Total IgA (black columns) and IgG (white columns) on day 7 (F) and day 35 (H) in each dose group. As negative controls, ASCs were measured in wells coated with influenza H3 rHA or PBS (for non-specific binding). Results on day 7 (G) and day 35 (I) are shown for each dose group (gray columns = H3-specific IgA ASCs; striped columns = H3-specific IgG ASCs; black columns = non-specific IgA ASCs; white columns = non-specific IgG ASCs).

Fig. 3. Flow cytometric analysis of circulating B cells for plasmablast and homing markers. B cells were enriched from PBMCs collected on day 7 after IM vaccination with an adjuvanted bivalent norovirus vaccine and analyzed by flow cytometry. Representative staining is shown for CD3 and CD19 before (A) and after (B) B cell (CD19+) enrichment, and for CD27 and CD38 (C) after gating on the CD3− CD19+ population in the lower right quadrant in (B). The CD27+ CD38+ population (shaded histograms) was compared with the CD27− CD38− population (open histograms) to determine the expression of CD138 (D), β7 (E), and CCR10 (F) (panels show representative results). The proportion of CD19+ cells that were CD27+ CD38+ (G), CD27+ CD38+ CD138+ (H), and CD27+ CD38+ β7+ (I) are plotted for individual subjects in each dose group (dose = 0 μg for placebo recipients). (J) Representative staining of CCR10 and β7 among the CD27+ CD38+ (black dots) and CD27− CD38− (gray dots) cells. The proportions of CD19+ CD27+ CD38+ β7+ cells that were CD62L− (K) or CD62L+ (L) are plotted for individual subjects in each dose group (dose = 0 μg for placebo recipients).

cells. The mucosal homing phenotype of the CD27+ CD38+ compared to CD27− CD38− B cells was most obvious when looking at dual expression of β7 and CCR10 (Fig. 3J). There was a significant ($p < 0.0001$) trend toward higher proportions of CD27+ CD38+ cells that expressed β7 cells as the VLP dose increased (Fig. 3I). In addition, there was a significant difference ($p = 0.0013$) between the 5 μg and 150 μg dose groups in the proportion of CD27+ CD38+ β7+ cells. The proportion of CD27+ CD38+ cells that expressed CCR10 was consistently 97–99% (Fig. 3F).

Expression of CD62L is associated with preferential homing to lymphoid tissues rather than peripheral tissues [33]. A higher proportion of CD27+ CD38+ β7+ cells were CD62L− (Fig. 3K and L). Overall, the expression profiles of CCR10, β7 integrin, and CD62L on circulating CD27+ CD38+ cells on day 7 after vaccination are consistent with a bias toward mucosal homing. Flow cytometric analysis of B cells on days 28 and 35 (day 7 after the second vaccination) showed no difference between pre and post immunization in vaccine recipients, or from placebo controls (not shown), consistent with our other data indicating a minimal B cell response to the second vaccination. For comparison, in a separate study of adults receiving trivalent inactivated seasonal influenza vaccine, the proportion of CD27+ CD38+ B cells (7.5%) was lower after the influenza vaccine than after the norovirus vaccine in the current study, but the proportions of CD27+ CD38+ cells that expressed CD138 (22.6%) were similar. The proportion of CD27+ CD38+ cells expressing β7 was lower after the influenza vaccine (29.5%), with more CD62L− cells among the β7+ cells (79.5%), suggesting a peripheral homing pattern. Thus, compared to unadjuvanted influenza vaccine, the adjuvanted bivalent norovirus vaccine elicited a more robust plasmablast response and a mucosal homing phenotype among plasmablasts.

4. Discussion

This study provides the first analysis of cellular aspects of the humoral response to an experimental bivalent norovirus vaccine to be tested in healthy adults. As reported [30], the vaccine elicited strong serological responses to both VLP antigens and our analysis of circulating ASCs on day 7 after the first vaccination demonstrated rapid and vigorous formation of GI.1- and GII.4-specific ASCs. There was a marked predominance of IgA over IgG ASCs in both the GI.1- and GII.4-specific responses. Notably, the serum antibody response [30] and the ASC response were stronger than in previous studies of norovirus VLP vaccines [8,9,13,14]. Similar adjuvants were used in the different studies, so the key difference is the route of immunization. The IM route ensures that the entire dose of antigen has the potential to stimulate the immune system, whereas only a small proportion of antigen given intranasally or orally is likely to be taken up and delivered to immune inductive sites. Similarly, the IM route may be more effective because the depot antigen prolongs stimulation of the immune system.

The rapid and vigorous ASC response to the first vaccination indicates a recall response by memory B cells (MBCs), rather than a response resulting from the activation of naïve B cells. Approximately 90% of adults carry norovirus-specific antibodies, indicating past exposure to circulating noroviruses. Most subjects in our study probably generated a strongly IgA-biased norovirus-specific MBC response following mucosal norovirus infection. This is likely to explain the large IgA ASC component in the response to IM vaccination. Group I noroviruses caused the majority of human infections prior to 2001, when the GII serotypes began to circulate more widely [5,34]. Currently circulating noroviruses continue to be of the GII lineage, leading to the prediction that more recent exposures to circulating virus would drive a dominant GII response. This is not what we observed, and there is no reason to believe the GI.1 VLP is

more or less immunogenic or antigenic than the GII.4 VLP. Instead the B cell responses recalled by the vaccine may reflect early priming by the GI viruses that were more common prior to 2001 when most of our 18–49 year-old subjects were likely to have been first exposed to the virus. We saw evidence for more equal frequencies of GI.1- and GII.4-specific ASCs at a high vaccine dose (150 μg), suggesting that a greater amount of antigen may in part counter differences in the frequencies of specific MBCs.

B and T cells activated by mucosal antigen exposure express sets of homing molecules that promote localization in mucosal tissues. In a study of an adjuvanted norovirus VLP vaccine given intranasally, El-Kamary and colleagues [13] investigated homing molecule expression by VLP-specific ASCs. CD19+ CD27+ ASCs were sorted based on expression of CD62L and β7 prior to VLP-specific ELISpot assay. Essentially, all VLP-specific IgG and IgA ASCs expressed β7; all of the IgG ASCs and a proportion of the IgA ASCs also expressed CD62L. Interestingly, in the present study the expression of CD62L was mixed among the CD27+ CD38+ and CD27−CD38− B cell (CD19+) subsets, though we did not distinguish Ig isotype. In our analysis on day 7 after the first vaccination, there was a positive correlation between dose and the percent of CD19+ CD27+ CD38+ ASCs that expressed β7. Greater than 98% of ASCs also expressed CCR10, a chemokine receptor that has a broad role in mucosal homing. Collectively, our analysis indicates that IM norovirus vaccination of adults generates ASCs with mucosal homing characteristics. This fits with the concept that these ASCs were derived from preexisting, mucosally generated MBCs re-activated by IM administered antigen [35].

The frequencies of IgG + IgA ASCs specific for the GI.1 antigen and for the GI.1 + GII.4 antigens correlated ($p = 0.0040$ and $p = 0.0306$, respectively) with the frequencies of CD27+ CD38+ (CD19+ CD3−) B cells (Fig. 4A and B). IgA ASCs specific for GI.1 ($p = 0.0017$) and for GII.4 ($p = 0.0114$) also correlated with plasmablast frequencies assessed by flow cytometry. The stronger correlations of the flow cytometry data with the IgA secreting and GI.1-specific B cell responses is very much in line with the overall bias to IgA and GI.1 depicted in Fig. 2E. ASC frequencies on day 7 have been correlated with serum Ig titers on day 28 for influenza vaccines [28,36,37]. However norovirus VLP-specific serum antibody titers, measured at any time point, and the ASC frequencies did not significantly correlate in the present study. The coincident rises in circulating antibodies and ASC frequencies may obscure a cause and effect relationship, even though changes in serum antibodies must certainly follow activation of B cells to become ASCs.

Circulating frequencies of VLP-specific ASCs measured 7 days after the second vaccination were substantially lower than after the first vaccination and serum titers of VLP-specific IgG, and IgA were unchanged [30], consistent with a very low B cell response. This result is somewhat surprising, since an expansion of VLP-specific MBCs would be expected to accompany the vigorous ASC response to the first vaccination. However, the outcome of non-mucosal activation of IgA-expressing MBCs is unclear and it is possible that the MBC pool available to respond to antigen administered via the same route could be transiently diminished. This could result from newly formed IgA+ MBCs exiting the site of formation and migrating through mucosa-associated tissues before homeostasis is regained. The greater frequency of GI.1-specific IgG ASCs than IgA ASCs after the second vaccination, in marked contrast to the situation after the first vaccination, may also reflect a change in the systemic availability of IgA+ MBCs. Another factor limiting the response to the second vaccination may have been the high levels of circulating VLP-specific Abs induced by the first vaccination [30]. Preexisting specific Abs are thought to increase the rate of clearance of administered antigens and down-regulate immune responses.

In summary, we demonstrate that an adjuvanted, bivalent norovirus VLP vaccine elicits a vigorous, IgA-dominated ASC
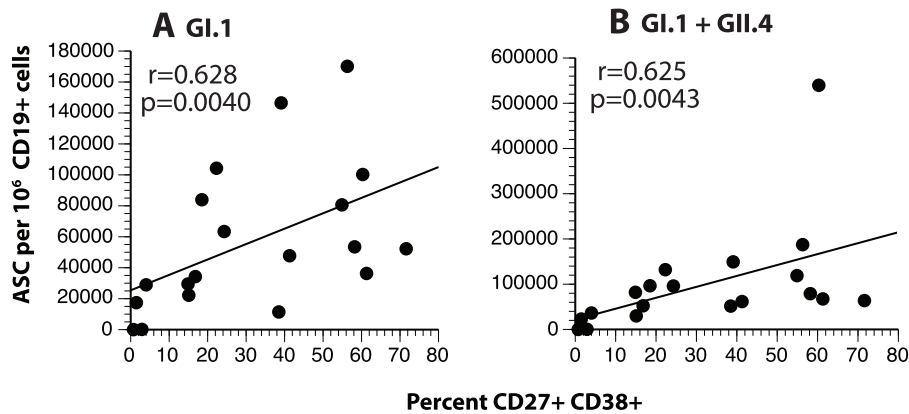
Fig. 4. Correlation between plasmablast and VLP-specific ASC frequencies. The percentage of CD27+ CD38+ cells among CD3− CD19+ lymphocytes measured by flow cytometry (*X*-axis) is plotted against the corresponding frequencies of GI.1-specific IgG + IgA ASCs (A) and GI.1- and GII.4-specific IgG + IgA ASCs (B) measured by ELISpot assay. The two-tailed Spearman correlation coefficient (*r*) was calculated for each *X*–*Y* comparison.

response against both vaccine antigens. Importantly, our findings indicate that a non-mucosal route of vaccination not only has advantages in terms of the magnitude of the response generated, but also elicits ASCs with mucosal homing characteristics. Thus, the vaccine is likely to enhance Ab-mediated protection at the site of norovirus encounter in individuals that have been previously primed by oral infection. Future studies are required to determine the immunogenicity of the IM bivalent vaccine in naïve individuals.

Funding

Authors' contribution

AS performed cellular immune assays and data analysis, MS participated in data analysis and manuscript preparation, SF, RA, WC, RB, PM, JT and DT contributed to study design, JF performed statistical data analysis and quality control, JT conducted the clinical trial, and DT supervised the immune assays, prepared the manuscript, and performed data analysis.

Conflict of interest

RB and PM are employed by the sponsor Takeda Vaccines, Inc. No other conflicts of interest are declared.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.09.073.

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