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Characterization of the HPV-specific memory B cell and systemic antibody responses in women receiving an unadjuvanted HPV16 L1 VLP vaccine

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Abstract

Human papillomavirus (HPV)-specific antibodies are proposed to be the correlate of protection afforded by HPV L1 virus-like particle (VLP) vaccines. Previous studies have characterized the systemic antibody response to immunization both in terms of quantity and the ability to neutralize HPV. Here, we have adapted a generalized memory B cell ELISPOT to the HPV16 system and expanded the analysis of the systemic antibody response to include an avidity measurement of HPV L1 VLP-specific antibodies. We show the results of the memory B cell ELISPOT significantly correlated with IgG and neutralizing antibody titers, but not with the avidity measurement. This is the first comprehensive study to correlate a variety of humoral aspects potentially associated with protective immunity following vaccination with a HPV16 L1 VLP vaccine.

Keywords

HPV; VLP; vaccination; humoral response

1. Introduction

Infection with human papillomavirus (HPV) is associated with the development of cervical cancer [1–2]. HPV virus-like particle (VLP)-based vaccines have been shown to induce excellent protection against infection with the HPV types contained in the vaccine and the development of precancerous lesions [3–4]. The VLP vaccines are composed solely of the L1 protein, a major component of the HPV viral capsid [5–6]. These vaccines elicit both cellular and humoral immunity after completion of the vaccine regiment [7], but correlates of long-term protection have not been identified. The humoral immune response is proposed to be the primary correlate of protection [8–10].

Several studies have relied on measuring the HPV-specific antibody response in serum [7,11] or cervical samples [12] in evaluating the immunogenicity of HPV16 L1 VLP

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vaccines. Others examined the capacity of serum samples to neutralize HPV *in vitro* as a more functional evaluation of the response [12–14]. While informative, these previous studies do not simultaneously consider all the diverse aspects of humoral immunity induced by vaccination and provide no formal evaluation of the relationship among these parameters. Two aspects of the humoral response that have received little attention in the context of HPV16 L1 VLP vaccination is the memory B cell response and the avidity of the systemic antibody response. Existing reports have looked at the ability of HPV16 L1 VLP vaccination to generate memory B cells [14–15] but the relationship between memory B cells and other aspects of the humoral response was not reported. The role of memory B cells is to provide a rapid burst of antibody upon secondary exposures [16–17]. Human memory B cells have also been proposed to play a role in maintaining serum antibody levels over time [16]. A better understanding of the memory B cell response to HPV L1 VLP vaccination may contribute to our understanding of the characteristics leading to the high clinical efficacy of these vaccines and the identification of early biomarkers that can predict long-term protection.

We chose to adapt the memory B cell ELISPOT protocol originally characterized by Crotty et al [18] to the HPV16 system. The memory B cell ELISPOT is the accepted standard for measuring the relative frequency of memory B cells. The advantage of the assay is that it can be applied to any system that has specific antigens available. The assay relies on the detection of memory B cells that have differentiated into plasma cells after stimulation with three polyclonal stimuli. This memory B cell ELISPOT protocol differs from previous ELISPOT applications in the HPV field as it incorporates the use of three polyclonal stimuli instead of a single stimulus and cytokines [14–15]. Following the stimulation, the number of antigen-specific memory B cells and total memory B cells are enumerated in an ELISPOT assay and the ratio between the number of antigen-specific spots and the total number of memory B cell spots is usually reported as a percentage.

The overall goal of this study was to broadly understand the humoral response generated against an unadjuvanted, HPV16 L1 VLP vaccine given in three doses over 6 months. We were interested in 1) describing the kinetics of the different aspects of the B cell response to vaccination against HPV and 2) defining immunological biomarkers that provide unique information and should therefore be considered in future efforts by our group and others when researching determinants of vaccine protection. To achieve our objective, we first adapted the memory B cell ELISPOT assay described above to the HPV system and investigated the kinetics and magnitude of the memory B cell response after vaccination. We expanded our evaluation of the systemic humoral response by measuring anti-HPV antibody titers by ELISA, HPV neutralizing antibody titers and antibody avidity following HPV L1 VLP vaccination, and correlated these results against the memory B cell response. Our results suggest that the frequency of memory B cells correlated with the anti-HPV16 neutralizing antibody titers induced by the vaccine at one month following third and final dose of vaccination. In contrast, the antibody avidity was not predictive of the frequency of memory B cells or the measurement of the systemic antibody response generated at months 2 and 7 following vaccination, and therefore deserves further attention in HPV vaccine efficacy studies to determine its potential role in long-term protection against infection.

2. Methods

2.1. Patient Samples

Participants were selected from a double-blind, randomized, placebo-controlled phase II trial of a monovalent HPV16 L1 VLP vaccine without adjuvant that was conducted among 220 healthy, HIV-seronegative adult (18–25 years of age) female volunteers as described previously [19]. Briefly, subjects were enrolled at the Johns Hopkins University Center for

Immunization Research (Baltimore, MD). Pre vaccination HPV16 antibody or DNA status was not a criterion for eligibility into the trial. Subjects were determined by history to be at low risk for HPV16 exposure. Individuals were not eligible to participate if they had a history of more than four lifetime sexual partners or more than two sexual partners within the preceding 6 months. Additional exclusion criteria included history of abnormal cervical cytology, immunodeficiency, anaphylaxis to medicines or vaccines, receipt of blood products within 3 months of enrollment, current pregnancy or lactation, and any other condition that might interfere with the study objectives. Women were randomly assigned to receive three intramuscular doses of either 50 µg of HPV16 L1 VLP vaccine without adjuvant (Novavax, Rockville, MD) or placebo (0.5 ml saline) at months 0, 1 and 6. Serum samples were obtained before the initial dose (month 0), 1 month after each of the subsequent vaccinations (months 1, 2 and 7), and 6 months after the last dose was administered (month 12). PBMCs were isolated and cryopreserved before the initial dose (month 0) and 1 month after the second and third vaccine doses were administered (months 2 and 7) from blood specimens shipped to the HPV Immunology Laboratory (Frederick, MD). Twenty-five subjects (22 vaccine and 3 placebo recipients) were randomly selected for inclusion in this study. All vaccine recipients (n = 22) and placebo recipients (n = 3) had serum samples from months 0, 1, 2, 7 and 12. Nineteen vaccine recipients and all placebo recipients had PBMC samples from months 0, 2, and 7. The Johns Hopkins University Institutional Review Board approved the protocol for this study.

2.2. Media formulations

Thawing Media contained RPMI 1640 (Gibco, Carlsbad, CA) supplemented with 20% heat inactivated FBS (HyClone, Logan, UT), 2 mM Glutamax-I (Gibco), 1% Antibiotic-Antimycotic (100x, Gibco) and 10 mM HEPES Buffer (Gibco). Culture Media consisted RPMI 1640 (Gibco), 10% Heat inactivated FBS (HyClone), 2 mM Glutamax-I (Gibco) and 1% Antibiotic-Antimycotic (100x, Gibco). Thawing and culture media were prepared fresh weekly. Stimulation media was made by supplementing culture media with 50 µM 2-Mercapto-ethanol (Sigma), 6 µg per ml CpG-2006 (InVivogen, San Diego, California), 1:10⁵ *Staphylococcus aureus* Cowan (SAC, Sigma), and 100 ng per ml Pokeweed Mitogen (PWM, Sigma). Stimulation media was prepared fresh as needed. The formulation of the stimulation media was optimized to maximize production of antibody secreting cells in our hands.

2.3. Memory B cell ELISPOT

A published protocol by Crotty et al. [18] was adapted to the HPV16 system. Frozen PBMCs were thawed, washed, resuspended in ~1 ml of culture media, and counted using a Coulter AcTdiff 2 cell counter (Beckman Coulter, Miami, Florida). Viability was determined by trypan blue exclusion. Cells were resuspended at a concentration of 10 x 10⁶ viable cells per ml. 5 x 10⁵ viable cells were plated in 1 ml of stimulation media containing CpG, SAC and PWM (prepared as described in section 2.2) in a 24 well plate (Costar, Corning Inc.). PBMCs were cultured for 6 days at 37° C and 5% CO₂ in a humidified incubator. The wells of a Multiscreen HTS, HA filter plates (0.45 µm, Mixed Cellulose Ester; Millipore) were coated for 48 hours at 4° C with either 12.5 µg per ml of HPV16 L1 VLP (Novavax), 20 µg per ml anti-human Ig Antibody (Caltag, Carlsbad, CA) or 20 µg per ml KLH (Sigma). Coating reagents were prepared in DPBS (Gibco). On the day of the assay, coated plates were washed 2 times with DPBS (Gibco), blocked with 250 µl of culture media for 2 hours at 37° C and 5% CO₂. Stimulated PBMCs were washed in culture media, counted, resuspended and plated such that HPV16 VLP coated wells and KLH coated wells received 1 x 10⁵ and 0.5 x 10⁵ viable cells per well, respectively. Up to 12 HPV16 L1 VLP-coated wells (mean = 9 wells, median = 12 wells, range = 1 – 12 wells) were seeded with cells, depending on the availability of cells recovered from the stimulated

cultures. Anti-human Ig coated wells received 350, 700 and 1.4×10^3 viable, stimulated cells. KLH- and anti-Ig-coated wells were seeded in quadruplicate. Plates were incubated for 6 hours in a humidified incubator at 37° C and 5% CO₂. Cells were then removed and the plates were washed 4 times with DPBS and 4 times with DPBS containing 0.05% Tween 20 (Sigma). An IgG detection antibody (anti-human IgG-Horseradish Peroxidase; KPL) was added in a volume of 100 µl and allowed to incubate overnight at 4° C. The next day plates were washed 4 times with DPBS containing Tween 20 and 4 times with DPBS. IgG spots were visualized by adding 100 µl of NovaRed Substrate (Vector Laboratories, Burlingame, CA) and allowed to react for 10 minutes. Plates were extensively rinsed with water and then allowed to dry thoroughly on a biosafety hood grate. Plates were scanned and spots were enumerated with CTL Spot software (Cellular Technology Ltd., Shaker Heights, Ohio). The mean number of HPV16 VLP-specific spots from replicate wells was determined per 1×10^5 plated cells. The mean number of total memory B cells per 350, 700 and 1400 plated cells was adjusted to reflect 1×10^5 cells plated. The frequency of HPV16 VLP-specific memory B cells was calculated by dividing the number HPV-specific spots by the estimated number of total memory B cells within 1×10^5 plated cells. The 19 vaccine and 3 placebo recipients were tested over 8 batches. All samples collected from a single individual were run on the same plate. The values from month 0 and the placebos were used to establish the background response and was essentially undetectable (mean = 0.000%; range = 0.000-0.009%; background threshold set at mean + 3 SD or 0.003%). The memory B cell ELISPOT was initially assessed for reproducibility by screening three vaccinated individuals (two samples were from month 7 and one was from a collection performed at month 12) that were not part of our study cohort over 2 days in a pre-study validation. Samples were prepared each day in duplicate plates. The calculated frequencies of HPV16 VLP-specific IgG memory B cells of the three individuals were 0.065%, 0.060% and 0.20%, which fell on the lower end of the range of values observed at months 2 (0.005–0.35%) and 7 (0.026–1.6%) during the study. The mean number of spots per HPV16-coated well during the pre-study validation was low (means of 4, 5 and 12 spots) as compared to the spots per well observed at month 7 (mean = 21 spots). The coefficient of variation (CV) determined in the pre-study validation associated with the frequency of memory B cells between plates and days were 17.7% and 45.5%, respectively. The overall CV was 48.8%.

2.4. HPV-Specific Antibody ELISA

Anti-HPV16 IgG antibodies were detected by an enzyme-linked immunosorbent assay (ELISA), as previously described [11–12]. Polystyrene flat-bottom microtiter plates (MaxiSorp, high binding; Nunc, Thermo Fisher Scientific, Waltham, MA) were coated with 100 µl of HPV16 L1 VLP at a concentration of 2.7 µg of protein per ml. The plates were incubated at 4°C and washed with a saline-based buffer containing Tween 20. After blocking the plates with 4% Skim Milk and 0.2% Tween 20 (volume/volume) in phosphate-buffered saline, the plates were again washed. Then 100 µl of two-fold serially diluted serum (diluted in blocking buffer) was added to each well. All samples were tested in duplicate. The plates were incubated for 1 hr at room temperature. After washing four times, a solution of peroxidase-labeled goat anti-human IgG (KPL, Inc., Gaithersburg, MD) was added for 1 hr. Plates were then developed with a tetramethylbenzidine substrate solution (KPL, Inc.). After 25 min of incubation in the dark at room temperature, the reaction was stopped by the addition of 100 µl of 0.36N H₂SO₄ to each well. The absorbance at 450 nm and 620 nm were measured with a microtiter plate reader (Spectramax M5; Molecular Device, Sunnyvale, CA). To control for the background reaction, four wells were filled with blocking buffer only. To calculate the titers of the samples, eight wells were filled with 100 µl of 1:100 diluted pooled sera from healthy, unimmunized individuals, presumably negative for HPV antibodies. The mean optical density (OD) of the pooled normal serum plus 3 standard deviations was used as cutoff to determine the titer of the samples. The data were

fitted to a graph representing on the y-axis the absorbance and on the x-axis the dilutions of the samples. The reported value was the dilution interpolated from the curve for each sample that would give an OD value equal to the healthy, unimmunized control-based cut-off value. This ELISA assay was previously reported to have an overall coefficient of variation of 11.4% [12].

2.5. Avidity ELISA using elution procedure

To determine the avidity index (AI) values, chaotropic-based elution was performed as described by Polanec and coworkers with slight modifications [20]. In brief, plates were coated as described in Section 2.2. Serum samples were diluted and used in the assay such that they yielded an optical density of 1.0 ± 0.5 in the ELISA assay described in Section 2.4. Samples that did not produce a minimum OD of 0.5 at a 1:10 dilution were not included in the analysis out of concern that the change in OD induced by the chaotropic agent would not be measurable due to the small difference between the OD value and background levels at the lower range of the assay. The month 0 and 1 samples did not meet this criterion and were excluded from the study. 100 μ l of the diluted serum was added to each well for 1 hour with shaking. All incubations were done at room temperature. After antibody incubation, 100 μ l of 0.5 to 3.5 M guanidine hydrochloride (GuHCl; Sigma, St. Louis, MO) was added for 15 minutes. Buffers without GuHCl were added to control wells. The plates were washed and the remainder of the assay is the same as the ELISA assay described in Section 2.4. Absorbance readings after treatment with a range of GuHCl concentrations were converted to the appropriate percentage of the control well OD. The data were fitted to a graph representing on the y-axis the percentage of the control OD and on the x-axis the molar concentration of GuHCl. For each sample, the AI was calculated as the molar concentration of the chaotropic compound required to reduce the absorbance to 50% of the control wells. To determine potential effects of the GuHCl treatment on the integrity of HPV16 L1 VLP, the binding ability of the V5 antibody was measured after plate bound HPV16 VLP were exposure to GuHCl. The V5 antibody is neutralizing anti-HPV16 mouse monoclonal antibody [21] that recognizes a conformational dependent epitope in L1 VLP [22]. Briefly, HPV16 VLP coated wells were exposed to concentrations of GuHCl ranging from 0.5 to 3.5 M for 15 minutes as in the avidity assay. After a washing step, V5 antibody was incubated with the plate-bound HPV16 VLP for one hour. The amount of V5 bound in the wells was detected using a HPR-conjugated anti-mouse IgG antibody (Bethyl Laboratories, Inc., Montgomery, TX). The remainder of the assay is the same as described in section 2.4. The amount of V5 bound to GuHCl-treated HPV16 VLP was compared to the amount of V5 bound to untreated HPV16 VLP. Greater than 90% of V5 binding was retained when using concentrations of GuHCl ranging from 0.5 to 3.0 M as compared to V5 binding in untreated HPV16 VLP coated wells. This avidity ELISA was previously determined to have an overall coefficient of variation of 3.6% (Dauner, J.G. et al, manuscript submitted).

2.6. Neutralization Assay

The neutralization assay was performed as previously described [12]. Briefly, 293TT cells were seeded in 96-well flat bottom plates at a cell density of 3×10^4 cells per well and incubated at 37° C and 5% CO₂ for ≥ 2 h prior to the addition of controls and diluted samples. 25 μ l of serially diluted serum was incubated with 100 μ l of HPV16 pseudovirion at 4 °C for 1 h. Next, the sample and pseudovirion mixture was transferred to the 293TT cells and incubated at 37 °C for 72 h. Following incubation, supernatants were clarified by centrifugation, then transferred to 96-well plates and frozen at -70 °C until further testing. The Great EscAPe SEAP assay kit was used according to the manufacturer's protocol (BD Biosciences-Clontech Laboratories Inc., Mountain View, CA). Serum neutralizing antibody titers were calculated by linear interpolation and defined as the reciprocal of the dilution that

caused 50% reduction in SEAP activity compared to control wells. This neutralization assay was previously reported to have an overall coefficient of variation of 40% [12].

2.7. Statistical Analysis

Estimates of variability were done as previously described [12]. The correlation between assays and the relative levels between collections were based on a Spearman calculation of the correlation coefficients using Prism 4 (GraphPad Software Inc., San Diego, CA). Correlations were significant if the associated p-value was <0.05 . A Kruskal-Wallis test was performed between total IgG memory B cells with Prism 4. The Mann-Whitney test was used to calculate significance between individual collection times. P-values <0.05 were considered significant.

3. Results

3.1. Kinetics of the HPV16-specific IgG Memory B cell Response

The memory B cell ELISPOT assay results obtained from PBMC samples collected at month 0 (enrollment), month 2 (1 month post 2nd dose) and month 7 (1 month post 3rd dose) are summarized in Figure 1. PBMC samples were stimulated with optimized concentrations of CpG, SAC and PWM to drive the differentiation of memory B cells into plasma cells. At enrollment, 95% of vaccine recipients (18 of 19) and all placebo recipients were considered negative for a HPV16 VLP-specific IgG memory B cell response (frequency below background levels or $<0.003\%$). At month 2, 73.7% (14 of 19) of the vaccine recipients had a discernable IgG memory B cell response while none of the placebo recipients did. The responses present at this time point were weak in nature (median frequency at month 2 = 0.04% ; IQR = $0.008 - 0.15\%$), but were higher than the median response observed month 0 (0.009%). At month 7, 100% (19 of 19) of the vaccine recipients had a detectable HPV16 VLP-specific memory B cell response in contrast to the placebo recipients whom had no detectable response. Memory B cell frequencies observed at month 7 (median = 0.20% ; IQR = $0.14 - 0.95\%$, Figure 1) were significantly higher ($p < 0.0001$) than those observed at month 2. No correlation was observed between the frequency of memory B cells at months 2 and 7 (Spearman $\rho = -0.18$, $p = 0.45$) as well as between the total number of memory B cells and the frequency of HPV16-specific memory B cells at month 7 (Spearman $\rho = -0.032$, $p = 0.90$). As an internal control for the consistency of the stimulation protocol, we tracked the total number of IgG producing memory B cells detected at each time point because this number should not significantly change over the timeframe of the study. No significant difference was observed in the total memory B cell population over time (median spots per 1×10^5 cells: month 1 = 4851, month 2 = 3132, month 7 = 4691; $p = 0.11$).

3.2. Kinetics and function of the HPV16-specific antibodies within the serum

The quantity of HPV16 L1 VLP-specific IgG antibodies in the serum was analyzed by a standard ELISA assay and the results are shown in Figure 2. Consistent with the published literature [12], anti-HPV16 VLP IgG titers were very low in pre-immunization samples and increased with each vaccine dose (Figure 2.A). Anti-HPV16 IgG titers peaked at month 7 (Figure 2.A; median = 44,721; IQR = 19,519–113,998) and declined thereafter to month 12, 6 months after the 3rd dose (Figure 2.A; median = 10,902; IQR = 5,832–30,889). A similar trend was observed when HPV16 VLP neutralizing antibody titers were measured by a SEAP assay (Figure 2.B; month 7, median = 7,437, IQR = 4,412–41,272; month 12, median = 2,515, IQR = 1,508–6,578). For both assays, the differences between consecutive collection time points were significant (p-value ranges of <0.05 to <0.0001 ; Figure 2.A and 2.B). Consistent with work from our group and others [12–13], we observed significant correlation between HPV16-specific IgG titers and HPV16 neutralizing antibody titers

(Spearman $\rho = 0.82$, $p < 0.0001$; correlation coefficient range of 0.56–92 when individual collection time points were examined).

The avidity of HPV16 VLP-specific IgG in the serum was examined using a modified ELISA developed in our lab (Dauner, J.G. et al, manuscript submitted). The avidity assay was not performed on the month 0 and month 1 samples because HPV16 VLP-specific antibody titers were below those at which avidity can be reliably measured as described in the Section 2.5. A statistically significant increase in avidity was observed from month 2 to 7 ($p < 0.0001$) and from month 7 to 12 ($p < 0.05$; Figure 2.C). The correlation between the AI values and neutralizing titers, both measures of the antibody's functional capacity, were significant only at the month 12 collection (Spearman $\rho = 0.62$, $p = 0.0021$; Figure 3). Lastly, for each serum-based assay, we assessed if it was possible to predict the relative values from one collection to the next (month 1 to 2, month 2 to 7, and month 7 to 12). Anti-HPV16 IgG titers, neutralizing antibodies titers or AI values at month 7 were at best weakly to moderately predictive of the relative levels at month 12 (anti-HPV16 IgG titers: Spearman $\rho = 0.53$, $p = 0.011$; neutralizing antibody titers: Spearman $\rho = 0.62$; $p = 0.0022$; AI values: Spearman $\rho = 0.40$, $p = 0.067$). Comparisons between earlier collection time points showed lower degrees of predictability (data not shown).

3.3. Correlations between serum based assays and ELISPOT results

We calculated the correlation between memory B cell frequencies and each of our serum-based measures: antibody titers, neutralizing antibodies titers, and antibody avidity (Figure 4). For this analysis, we focused on HPV16 VLP-specific memory B cell frequencies observed at month 7 because this was the only collection time point at which all the participants had a detectable memory B cell response corresponding to a reasonable mean number of spots/well (21.4 spots/well at month 7 versus 1.3 at month 2). A significant correlation was observed between the frequency of memory B cells and total HPV16 antibody titers (Spearman $\rho = 0.62$, $p = 0.0047$; Figure 4.A). A similar significant correlation existed between HPV16 VLP neutralizing antibody titers and the frequency of memory B cells (Spearman $\rho = 0.76$, $p = 0.0002$; Figure 4.B). In contrast, a no significant correlation was observed between the frequency of memory B cells and avidity (Spearman $\rho = 0.35$, $p = 0.14$; Figure 4.C). Studies have suggested a link between memory B cells and the maintenance of systemic antibody levels [16]. Therefore we analyzed if the month 7 memory B cell frequency had any correlation with the month 12 antibody titers. A statistically significant correlation was observed between the memory B cell frequency at month 7 and anti-HPV16 IgG titers at month 12 (Spearman $\rho = 0.50$, $p = 0.029$).

4. Discussion

HPV L1 VLP vaccines are highly immunogenic and afforded excellent protection in clinical trials [3–4]. Results to date have demonstrated near complete protection by the vaccine against infection with HPV types included in the vaccine. The antibody response is thought to be largely responsible for this protection [8–10]. The interplay between the distinct aspects of the humoral response stimulated by vaccination has not been assessed for the HPV L1 VLP vaccine and is important in identifying markers of long-term protection against HPV infection. The objective of this study was to evaluate multiple aspects of the humoral response to better understand the relationship between the different parameters and determine which assays could provide unique information relevant to future, large-scale epidemiological studies. To this end, a previously described memory B cell ELISPOT used in evaluating immunity to smallpox and anthrax [18] was adapted to the HPV16 VLP system. Its performance was reviewed for application to a clinical trial setting using specimens from a phase II trial of an unadjuvanted HPV16 L1 VLP vaccine.

This is the first study to correlate an assortment of humoral response measurements against one another over the first year after vaccination with HPV16 L1 VLP and to include a description of the changes in the avidity of anti-HPV16 VLP antibodies over time. The B cell memory ELISPOT data significantly correlated with the neutralization assay (Spearman $\rho = 0.76$; $p = 0.0002$) and with the ELISA assay (Spearman $\rho = 0.62$; $p = 0.0047$). Although the correlation between the ELISA and memory B cell ELISPOT results reported here is similar to levels of correlation previously reported in other settings, the reported values cover a wide range of correlations [23–28]. The discrepancies observed between reports could be due to differences in the timing of specimen collection (steady state measurements versus peak responses), vaccination strategy (formulations of live attenuated, recombinant subunit or inactivated immunogens), vaccine regimens (number and timing of doses), antigens studied or age groups. The level of correlation observed may be impacted by the notion that long-lived plasma cells are the predominant source of systemic antibodies in the steady state, not memory B cells [29]. Therefore, a total agreement between the circulating antibody levels and memory B cells may not be necessarily expected.

While the HPV16 VLP-specific antibody and neutralizing antibody titers in response to HPV L1 VLP vaccination have been previously described [7,11–13], the addition of an avidity measurement of these antibodies provides novelty to this study. Modified ELISA are routinely used to measure the avidity of serum antibody samples in the context of vaccination [30–34] and infection [35–36]. The results of modified ELISA have been shown to correlate with results obtained using Biospecific interaction analysis [7,11–13] and equilibrium dialysis [37]. Nevertheless, the relationship between Biospecific interaction analysis and the modified ELISA in the HPV16 system is to be investigated in future studies. The level of HPV16-specific antibody avidity over time warrants further studies. The avidity of HPV16 VLP-specific IgG only significantly correlated with other assays 6 months after the completion of the vaccination schedule. The avidity assay is low cost and can easily be adapted for high throughput justifying its continued evaluation to better understand its potential role in long-term protection afforded by HPV vaccination.

The AI value analysis indicated that affinity maturation within the IgG fraction continued through month 12. This increase in avidity occurs during a time when antibody titers and neutralizing antibody titers are decreasing suggesting that the quality of the response may be increasing while the quantity contracts. Published data sets agree with this observation as it has been shown to occur after vaccination in humans [38–39] and monkeys [40]. One explanation for this interesting observation is that as the neutralizing titers decrease, those antibodies remaining continue to undergo affinity maturation. Alternatively, the antibodies with the highest avidity may be selectively maintained as the antibody levels recede from the peak levels. Without the ability to monitor individual clones or responses to specific epitopes over time, these interpretations are highly speculative in nature. While the difference in avidity between months 7 and 12 was significant ($p = 0.021$), the magnitude of increase was small (1.2-fold increase) and should be confirmed in a larger population. Further, it is not known if this increase results in any biologically relevant change. It will be interesting to analyze additional collection time points to see if affinity maturation peaked before month 12 or if it is continuing to increase beyond this period. At collection times before the completion of the vaccination schedule, the AI values did not significantly correlate with either the neutralizing assay ($\rho = -0.21$ to 0.27 , $p = 0.22$ to 0.24) or the ELISA ($\rho = 0.084$ to 0.10), suggesting that it captures a unique aspect of the immune response to HPV L1 VLP vaccination not reflected in the traditional measurements of the humoral response.

The memory B cell frequency observed here at month 7 based on an ELISPOT assay was slightly higher than that reported previously by Einstein et al [14], but this difference most

likely reflects the nuances of the protocols employed, including the stimulation cocktails (multiple polyclonal stimuli or a single polyclonal stimulus and cytokines) and absence or presence of adjuvant. The HPV16 L1 VLP-specific memory B cell ELISPOT displayed high variability. The variability was largely influenced by the rarity of HPV16-specific cells. With mean spot counts in the range of 1–10 spots, any fluctuation is going to lead a relatively large CV. Considering the amount of cellular manipulation (robust stimulation conditions, multiple cell counts and long incubation periods), the variability is on par with other biological assay, like the neutralization assay [13].

Samples from Month 7 were the only ones evaluated that produced a consistent and appreciable response in the vaccinated cohort. The minimal response prior to vaccination (95% of recipients with no memory B cell response) is expected since individuals in this trial were selected to have a reduced risk of exposure to HPV and are therefore likely to be immunologically naïve to HPV16 prior to vaccination. It is also possible that natural infection with HPV induces a low systemic level of HPV16 specific memory B cells. The lower rate of responders and smaller scale of response observed at month 2, when compared to month 7 is in agreement with a previous report [15] and suggests the need for the full course of vaccination before levels of specific memory B cells can be quantified by our assay. In other systems, such as influenza, the memory B cell ELISPOT may reach higher response levels because individuals are routinely exposed to the virus leading to strong, robust immune responses that are then further boosted through vaccination. While the memory B cell ELISPOT is not ideal for those interested in identifying an early indicator of long-term protection, it could be of value to better understand mechanisms involved in the long-term maintenance of the response elicited after completion of the vaccination schedule because it has been suggested that memory B cell contribute to the maintenance of circulating antibody levels [16].

In future, large-scale epidemiological studies, it would be more efficient, both in terms of time and resources, to rely on either the ELISA or the neutralization SEAP assay instead of the memory B cell ELISPOT to measure the responsiveness of vaccine recipients. The significant correlation at month 7 (Spearman $\rho = 0.62$ – 0.76 , $p = 0.0047$ – 0.0002) between the assays supports this conclusion. Therefore, further studies using the ELISPOT may be of interest in better characterizing the B cell memory response to the HPV VLP vaccine and its role in long term protection, but unless the assay can be further optimized, it would be best to restrict its use to after the vaccination series are completed when the HPV16-specific response is more readily measurable. This reinforces the need to develop reliable flow cytometric reagents that are able to detect antigen-specific memory B cells, which will eliminate the need of extensive cell culture periods and potentially reduce the amount of sample required. Additionally, it would allow for the flow cytometric determination of other parameters of interest, such as activation and cellular subset markers.

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Fig. 1.

Summary of the HPV16-specific IgG memory B cell response kinetics. Participants were vaccinated with an unadjuvanted, HPV16 L1 VLP vaccine at months 0, 1, and 6. HPV16-specific memory B cells secreting IgG after polyclonal stimulation were tracked at months 0, 2, and 7. The open circles represent each individual with a positive memory B cell response. The values in parentheses are the number of positives within the total number of individuals tested. Background frequency was determined as the mean of the placebo and month 0 responses plus 3 SD and set at 0.003%. The bars depict the median values and the interquartile ranges.

Fig. 2.

Summary of the serum antibody response kinetics. Participants were vaccinated with an unadjuvanted, HPV16 L1 VLP vaccine at months 0, 1, and 6. The HPV16 L1 VLP-specific IgG titers (A) and neutralizing antibody titers (B) were tracked over months 0, 1, 2, 7, and 12. The avidity index (C) was tracked over months 2, 7 and 12. The open circles represent each individual. The bars depict the median values and the interquartile ranges. Statistical values calculated using a Mann-Whitney test between consecutive time points (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Fig. 3. Correlation between neutralizing antibody titers and avidity at months 2 (A), 7 (B) and 12 (C). Within each graph the total number of samples used in the analysis is represented by “N”. The Spearman correlation coefficient and p-value are also indicated on each graph.

Fig. 4.

Correlation between memory B cell frequencies and measurements of the systemic antibody response in HPV-16 L1 VLP recipients at month 7. Correlations between IgG memory B cell frequencies against HPV16-specific IgG titers (A), neutralizing antibody titers (B) or avidity indices (C). Within each graph the total number of samples used in the analysis is represented by “N”. The Spearman correlation coefficient and p-value are also indicated on each graph.