



Research Paper

A Single Human Papillomavirus Vaccine Dose Improves B Cell Memory in Previously Infected Subjects



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ABSTRACT

Although licensed human papillomavirus (HPV) vaccines are most efficacious in persons never infected with HPV, they also reduce infection and disease in previously infected subjects, indicating natural immunity is not entirely protective against HPV re-infection. The aim of this exploratory study was to examine the B cell memory elicited by HPV infection and evaluate whether vaccination merely boosts antibody (Ab) levels in previously infected subjects or also improves the quality of B cell memory. Toward this end, the memory B cells (Bmem) of five unvaccinated, HPV-seropositive subjects were isolated and characterized, and subject recall responses to a single HPV vaccine dose were analyzed. Vaccination boosted Ab levels 24- to 930-fold (median 77-fold) and Bmem numbers 3- to 27-fold (median 6-fold). In addition, Abs cloned from naturally elicited Bmem were generally non-neutralizing, whereas all those isolated following vaccination were neutralizing. Moreover, Ab and plasmablast responses indicative of memory recall responses were only observed in two subjects. These results suggest HPV vaccination augments both the magnitude and quality of natural immunity and demonstrate that sexually active persons could also benefit from HPV vaccination. This study may have important public policy implications, especially for the older 'catch-up' group within the vaccine's target population.

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1. Introduction

Approximately 5% of the global cancer burden, including cervical, anal, oropharyngeal, vaginal, vulvar, and penile cancers, are attributable to human papilloma virus (HPV) infections (de Martel et al., 2012). There are three approved HPV vaccines that protect against those HPV types that most commonly cause cancer, HPV 16 and 18 (de Sanjose et al., 2010): the bivalent HPV (bHPV) vaccine against types 16 and 18; quadrivalent HPV (qHPV) vaccine against types 16, 18, 6, and 11; and the recently licensed nine-valent HPV (nHPV) vaccine against types 16, 18, 6, 11, 31, 33, 45, 52, and 58. In the US, a three-dose HPV vaccine schedule is recommended for adolescents but approved for a larger age range of women (9–26 years of age) and men (9–21 years of age; qHPV and nHPV vaccines only).

The HPV vaccines are most efficacious in persons who have not been infected with the vaccine types (Garland et al., 2007, FUTURE II Study Group, 2007, Paavonen et al., 2009, Joura et al., 2015). Thus, they are primarily targeted to adolescents in an effort to immunize prior to HPV exposure through sexual activity. The putative mechanism of protection is via type-restricted anti-L1 neutralizing antibodies (Abs) that block viral entry (Kwak et al., 2011). Likely as a result of these type-restricted Ab responses, the vaccines provide little cross-protection against heterologous HPV types and only limited protection against types that share high L1 nucleotide sequence homology with vaccine types, e.g., HPV 31 and 16 or HPV 45 and 18 (Bernard et al., 2010, Kwak et al., 2011).

While most sexually active adults have already been infected with some HPV types, it remains unclear whether natural immunity is sufficient to protect these persons against re-infection, i.e., autoinoculation or new infections of the same type. Natural history studies have shown that high levels of infection-elicited serum Abs are associated with protection against same-type re-infections (Ho et al., 2002, Safaeian et al., 2010, Wentzensen et al., 2011, Beachler et al., 2016). A prior study also found that both previously infected and previously

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vaccinated subjects generated anamnestic Ab responses following a single qHPV vaccine dose, indicating that both natural infection and vaccination elicit memory B cells (Bmem) (Olsson et al., 2007). At the same time, a number of independent studies have shown reduced incidence of same-type re-infection or disease in seropositive subjects that received an HPV vaccine compared to those that received placebo, suggesting that natural immunity is not entirely protective (Castellsague et al., 2011, Olsson et al., 2009, Szarewski et al., 2012). Moreover, there is no information as to what level of Abs is protective and little information as to whether qualitative differences exist between vaccine- and infection-elicited immunity. Therefore, to understand more about the immunity natural HPV infection imparts, HPV 16-specific Bmem responses were examined in subjects with serological evidence of HPV 16 infection before and after they received a single HPV vaccine dose.

2. Materials and Methods

2.1. Research Subjects and Study Design

To characterize the Bmem elicited by natural HPV 16 infection and test binding Ab, neutralizing Ab, plasmablast, and Bmem responses to a single HPV vaccine dose, ten healthy women, aged 27–45 years, were enrolled into an unblinded pilot study. At entry, the women reported \geq five heterosexual lifetime partners and were HPV 16 seropositive.

Participants of this group were randomized at day 0 by block design to either receive a single qHPV vaccine dose ($n = 5$) or serve as non-immunized controls ($n = 5$). Blood samples (~60 ml) were collected from these subjects at month -6; day 0 (± 1 month); week 1 (± 1 day); month 1 (± 1 week); and month 6 (± 1 month). On the day of vaccination, the blood draw preceded the immunization. Institutional Review Boards at both the University of Washington and Fred Hutchinson Cancer Research Center approved study protocol and the participants provided written informed consent.

2.2. Screen for HPV 16 Seropositivity

An anti-L1 IgG binding assay using glutathione-S-transferase (GST)-HPV L1 fusion proteins was performed on a BioPlex (Bio-Rad Laboratories, Inc.) with magnetic beads as previously described (Katzenellenbogen et al., 2015). Controls included the titration of serum from a high titer vaccinee that had been previously identified and the international standard HPV 16 serum (10 Units/ml, U/ml). To convert median fluorescent intensity (MFI) values to U/ml a standard curve was generated using the net MFIs (after subtracting the MFI of beads coated with GST) of the control using the sigmoidal dose response program (GraphPad Prism) with weighting ($1/Y^2$). Interpolated values were calculated from the standard curve and interpolated values were converted to U/ml using the formula: $U/ml (\text{test}) = 10 U/ml \times \text{interpolated value (standard)} / \text{interpolated value (test)}$.

To screen subjects, sera were tested at dilutions of 1:200, 1:400 and 1:800. A concentration was calculated for each dilution and the results averaged. A cutoff of 3 U/ml was selected on the basis that it was three standard deviations above the mean value obtained for a panel of sera from virgins. Following vaccination, sera were initially tested at 1:200, 1:2000 and 1:20,000. Based upon the results of that test sera were retested using 1:2 dilutions (3) starting at 1:200, 1:2000 or 1:10,000.

2.3. Flow Cytometry

To identify HPV 16-specific Bmem, Alexa Fluor 488 (AF488)-labeled HPV 16 pseudoviruses (psV) or negative control AF488-labeled bovine papillomavirus (BPV) psV were generated as previously described (Scherer et al., 2014). Samples were also enriched, stained, and sorted

the same as before, except that only 1/10th of each enriched sample was used for AF488-BPV labeling.

To identify and isolate single plasmablasts, peripheral blood mononuclear cell (PBMC) samples were rapidly thawed in pre-warmed, heat-inactivated FBS. These samples were then washed and counted in PBS and stained with Live/Dead Violet viability dye (Life Technologies) for 30 min. To stain cell surface receptors, samples were washed and re-suspended in 2% FBS-PBS and incubated with anti-CD3 V500 (clone UCHT1; RRID:AB_10612021), anti-CD19 APC-Cy7 (SJ25-C1; RRID:AB_396873), anti-CD20 PerCP-Cy5.5 (clone 2H7; RRID:AB_1727451), anti-CD27 PE-Cy7 (clone M-T271; RRID:AB_1727456), anti-CD38 APC (clone HIT2; RRID:AB_398599), and anti-IgD PE (clone IA6-2; RRID:AB_396114) for 30 min. Samples were again washed and re-suspended in 2% FBS-PBS, placed on ice, and protected from light until fluorescence-activated cell sorting (FACS). All staining was conducted with pre-optimized amounts of reagents and with samples on ice and protected from light. All Abs, including those used for Bmem staining, were purchased from BD Biosciences. Samples were analyzed with a FACSria II cell sorter (BD Biosciences), and plasmablasts were isolated in single cell sort mode. Cells were sorted into PCR plates containing 8 μ l of ice-cold lysis buffer [$0.425 \times$ RNase-free PBS (Life Technologies), 10 mM dithiothreitol, and 16 U RNasin (Promega)] and stored at -80°C . For kinetic analysis, 1×10^6 PBMC per sample were used for staining.

2.4. Ab Sequence Analysis

To analyze the number of potential nucleotide mutations and amino acid changes from germline, as well as variable gene usage of each human monoclonal Ab (mAb) cloned in this study, heavy and light chain variable region sequences obtained by Ab cloning were submitted to V-QUEST (Brochet et al., 2008). Default parameters were used except that insertions and deletions were also searched and nucleotide mutations and amino acid changes that overlapped with 'n' nucleotides or a given diversity or joining gene were not counted.

2.5. Pseudovirus Neutralization Assay

Experiments to generate HPV 16, HPV 31, and BPV psV comprised of the L1 and L2 (major and minor) capsid proteins of these respective viruses encapsidating a secreted alkaline phosphatase (SEAP) reporter gene were conducted as previously described, as was the psV neutralization assay in 293TT cells (RRID: CVCL_1D85), with the following specifications (Scherer et al., 2014, Buck et al., 2005):

To evaluate the levels of circulating neutralizing Abs in each subject over the course of the study, plasma samples collected at each time point were tested in monoplicate against HPV 16 and BPV psV, starting at a final plasma dilution of 1:100 in PBS and continuing as a four-fold dilution series in assay media. Sample controls included psV only in media and media only, and psV neutralization controls included dilution series of H16-V5 and 5B6 supernatants for HPV 16 and BPV psV, respectively. The monoplicate experiments were repeated at least twice for each subject time point. The signal obtained from the media only controls was subtracted from the signals of other controls and samples, and the percent neutralization was calculated using the following formula: $(\text{Abs } 405\text{nm}_{\text{psV only}} - \text{Abs } 405\text{nm}_{\text{Ab/plasma + psV}}) / (\text{Abs } 405\text{nm}_{\text{psV only}}) * 100$. The theoretical dilution at which each plasma sample exhibited 50% neutralization, or IC_{50} , was determined by nonlinear regression analysis of the neutralization curve using the log (inhibitor) vs. response formula (GraphPad Prism).

Each of the mAbs was screened for neutralization activity against HPV 16 psV and BPV psV in monoplicate, once, starting at a final mAb concentration of 50 $\mu\text{g/ml}$ in PBS and continuing as a three-fold dilution series in assay media. The same controls were used. If a mAb did not reach 50% neutralization at a concentration of 50 $\mu\text{g/ml}$, it was regarded as non-neutralizing. For any mAb with $\geq 50\%$ neutralization, the assay

was repeated in triplicate starting at an appropriate mAb concentration (typically 200 pM and continuing over a three-fold dilution series). For those mAbs that exhibited binding to HPV 31 L1, neutralization activity to HPV 31 psV was examined in parallel with HPV 16 psV, using H31.A6 as a positive control for HPV 31 psV neutralization.

2.6. HPV L1 Binding Assay

The test of tissue culture supernatants from Panoply was conducted in two parts. The standard HPV 16 L1 binding assay (see above, under **Screen for HPV 16 seropositivity**) was conducted using tissue culture supernatant without blocking at a final dilution of 1:3.3. Secondly, supernatants that tested positive in the first part were titrated (dilutions 1:4.4 to 1:1.06 × 10⁵) and tested against HPV 16, 31, 33, 35, 52, 56, 67 and 18 GST-L1 proteins.

Human mAbs were tested in monoplicate (no blocking was required as these Abs have extremely low non-specific reactivity) at a starting concentration of 100 nM with five 1:10 dilutions (100 nM–1 pM) versus HPV 16 GST-L1 and GST as described above. The median fluorescent intensity (MFI) values for GST binding were subtracted from the MFI values for L1 binding for each well.

2.7. Statistical Analyses

An unpaired, two-tailed student's *t*-test was used to determine if there was a significant difference between the mean increase of Bmem responses ± standard deviation (SD) in the vaccinated versus control group in Fig. 3.

2.8. Accession Numbers

All mAbs that were expressed and characterized or whose sequences contributed to data in the Figures or Supplemental data were submitted to GenBank (Table S1).

3. Results

3.1. Study Design

In order to investigate the B cell memory elicited by natural infection, ten healthy women, aged 27–45 years, were enrolled in an exploratory, unblinded pilot study on the basis of having serum Abs against HPV 16. This age group was chosen because it falls outside those currently recommended to receive the vaccine. Since natural HPV infection is assumed to mainly elicit type-restricted Ab responses (Stanley et al., 2012), women were only screened for serum Abs to HPV 16 L1. However, multiple HPV infections are possible and occur frequently in healthy, sexually active women (Clifford et al., 2005). Here, a woman was defined as being seropositive if her anti-HPV 16-L1 serum Ab levels were three standard deviations above the mean Ab level of uninfected subjects. We screened 52 women and identified 14 who were seropositive by this definition, corresponding to a HPV 16 seroprevalence rate of 27%. It should be noted that there is no clinically approved serology test for HPV 16 Abs. Moreover, natural HPV infection elicits low serum Ab responses (Stanley et al., 2012) that approach the limit of detection in current assays. Therefore, false positives cannot be ruled out.

Five women were randomized to receive a single qHPV vaccine dose to evaluate recall responses i.e., the ability of memory immune cells to elicit more rapid responses to antigen than naive cells during a primary response. The other five women served as unimmunized controls. The qHPV vaccine has been shown to be safe, efficacious, and immunogenic in women of this age compared to 18–26 year old women (Einstein et al., 2011, Munoz et al., 2009, Castellsague et al., 2011). Blood samples were collected at enrollment (six months prior to vaccination, or month -6), just prior to vaccination on the same day (day 0), at one-week post-vaccination (week 1), at one-month post-vaccination

(month 1), and at six months post-vaccination (month 6). From each blood sample, plasma and PBMC were isolated for Ab measurements and cellular analyses.

3.2. Ab Responses to Natural Infection and Vaccination

Each of the subjects in the vaccinated group, save one (subject 3, ◇), elicited a robust Ab response to the single qHPV vaccine dose, whether measured in terms of plasma Ab binding to HPV 16 L1 (Fig. 1a) or plasma neutralization against HPV 16 (Fig. 1b). In contrast, the unvaccinated control group showed no change in Ab levels or plasma neutralization activity. It should be noted that subject 3 elicited a similarly weak response against the other vaccine types (Fig. S1). Two of the vaccinated subjects' anti-HPV 16 Ab levels increased 45- and 92-fold at one-week post-vaccination, suggesting that they elicited anamnestic responses to vaccination (Fig. 1a; subjects 2 (○) and 4 (□), respectively). However, these subjects also elicited neutralizing Ab responses to HPV 18 at one-week and one-month post-vaccination (Fig. S2). Therefore, it is unclear whether these subjects, which were HPV 18 seronegative at day 0, simply elicited rapid vaccine responses or whether they had been previously co-infected with HPV 16 and 18 and generated recall responses to both types. The other vaccinated subjects' Ab levels to HPV 16 and 18 increased at one-month post-vaccination (Fig. 1 and Fig. S2), indicative of primary responses to vaccination.

3.3. Plasmablast Responses to Vaccination

When the magnitude of the plasmablast response was assessed for the day 0, week 1, and month 1 PBMC samples using immunophenotyping and flow cytometry (Fig. S3), subjects 2 (○) and 4 (□) showed a 6- and 41-fold increase in plasmablast frequencies and a 5- and 36-fold increase in plasmablast numbers at one-week post-vaccination, respectively (Fig. 2 a and b). Subject 3 (◇) elicited an unusual plasmablast response, in that it did not peak at one-week post-vaccination. As this subject did not elicit a strong Ab (see above) or Bmem response (see below), her plasmablast response was likely not HPV 16-specific. In fact, the plasmablast phenotype was originally based on activation status, not antigen (Ag) specificity per se (Wrammert et al., 2008). To confirm that the peak plasmablast responses were HPV 16 specific, Abs were cloned and recombinantly expressed from plasmablasts that were single cell sorted from a subject with a robust plasmablast response (subject 2, (○)). Out of 18 IgG, eight (44%), were HPV 16-specific (Fig. S4). Moreover, as the immunoglobulin sequences amplified from the plasmablasts of subjects 2 and 4 are somatically mutated and class-switched (Table S2), it suggests they derived from Bmem (Chiu et al., 2013, Priyamvada et al., 2016).

3.4. HPV 16-specific Bmem Responses to Natural Infection and Vaccination

Bmem responses were then evaluated, and HPV 16-specific Bmem isolated for further analysis. We previously found HPV 16-specific Bmem in the blood of HPV vaccinees using a combination of Ag labeling with AF488-HPV 16 psV and flow cytometry-based immunophenotyping to identify CD3⁻CD19⁺CD20⁺CD27⁺IgD⁻AF488-HPV 16-psV⁺ Bmem (Scherer et al., 2014). Similar methods were applied here to identify and isolate single HPV 16-specific Bmem from day 0 and month 1 samples by FACS (Fig. 3a). To improve the efficiency of finding such rare Ag-specific cells, PBMC were first enriched for B cells. As before, AF488-BPV psV were used as a negative control to confirm the specificity of staining and establish sorting gates. PsV are comprised of L1 and L2 proteins self-assembled around a reporter plasmid (Pastrana et al., 2004).

Statistically significant Bmem responses were noted at one-month post-vaccination in all subjects of the vaccinated group except subject 3 (◇), corresponding to a 4- to 26-fold change in the frequencies of Ag-specific Bmem (*p* = 0.04) (Fig. S5a and b) and a 3- to 27-fold change in the numbers of Ag-specific Bmem (*p* = 0.026) (Fig. 3b and c).

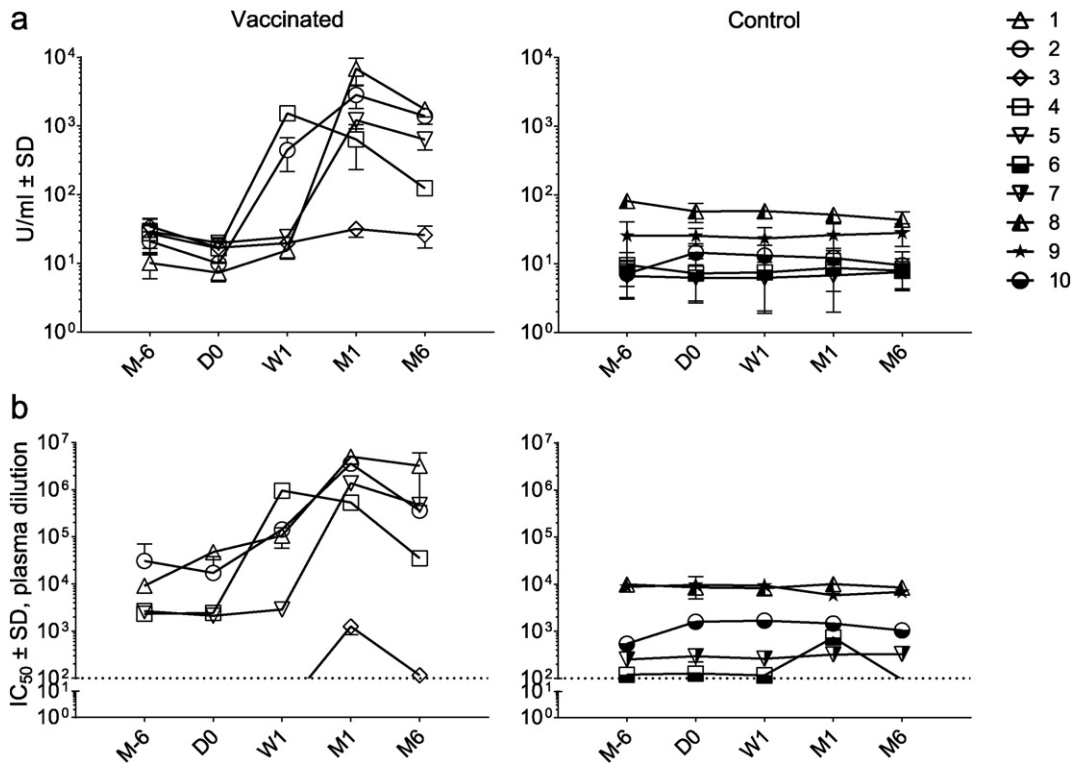


Fig. 1. A single vaccine dose boosted Ab levels 77-fold (median) in the vaccinated group. (a) Anti-HPV 16 L1 plasma Ab levels (\pm SD; $n \geq 3$ independent experiments) were measured for different time points (month -6 (M-6), day 0 (D0), week 1 (W1), month 1 (M1), and month 6 (M6)) using a binding assay and expressed in U/ml relative to an international serum standard. (b) Neutralizing HPV 16 Ab levels were measured using a 293TT psV assay and expressed as the plasma dilution at which half-maximal inhibition (IC_{50}) occurred (\pm SD; $n \geq 2$ independent experiments). Given that the dilution series started at a 100-fold plasma dilution, any neutralization curves that did not reach 50% neutralization or for which the IC_{50} was <100 are plotted below the dashed baseline. Symbols in (a) and (b) are linked to the subject ID as shown in the figure legend and remain constant between figures.

3.5. Characteristics of Abs Cloned From AF488-HPV 16⁺ Bmem

To learn more about the diversity of the Bmem responding to vaccination, heavy and light chains from AF488-HPV 16⁺ Bmem that had been single cell sorted from the vaccinated group were amplified and sequenced. Bmem expressed IgA, IgG and IgM Abs with IgM⁺ Bmem enriched in the month 1 response (Fig. 4a). Collectively, the paired IgA, IgG and IgM sequences utilized 29 different immunoglobulin heavy chain variable (IGHV) genes between the vaccinated and control groups (Fig. 4a). Fourteen IGHV genes were shared among individuals; thus approximately half were unique to a given individual. Only two IGHV genes were observed in more than half of the subjects (3–23 and 3–33). Five IGHV genes were observed only at day 0, whereas nearly three-fold more IGHV genes were observed only at month 1 (Fig. 4c).

To understand if there was an increase in somatic mutations post-vaccination that would be indicative of pre-existing Bmem re-entering germinal center reactions, paired IgG sequences were examined for the number of nucleotide mutations (Fig. 4d) and amino acid changes (Fig. 4e) from germline. For two of the three subjects, we found little change in the somatic hypermutation level of IgG sequences on day 0 compared to month 1. In contrast, IgG sequences cloned from Bmem of subject 1 unexpectedly exhibited a decrease in somatic hypermutation from day 0 to month 1, which may reflect naïve cells being recruited into the response. The same results were observed when comparing the number of amino acid changes from germline.

3.6. Clonally Related Bmem

To learn more about the diversity and dynamics of the B cell clones that responded to vaccination in seropositive subjects, paired Ab sequences resulting from the original amplifying PCR reactions were

analyzed to identify those that potentially derived from the same B cell lineage and thus shared the same clonotype (Table S2). Here, sequences are defined as sharing the same clonotype if they have the same heavy and light chain variable and joining gene usages, as well as identical CDR3 lengths. The following was found: First, the response to vaccination was at least polyclonal, for there was more than one clonotype responding at one-week or one-month post-vaccination within a given subject that were HPV 16-specific based on available neutralization and binding data. Second, the sequences representing each lineage appeared to be somatic variants as most exhibited different numbers of nucleotide mutations or amino acid changes from germline and/or substitutions to CDR3 residues in the heavy and/or light chains. Third, shared clonotypes were observed between the week 1 plasmablasts and month 1 Bmem in subjects 1, 2, and 4, implying that pre-existing Bmem clones were being expanded as both plasmablasts and Bmem in response to vaccination. In only one case was a shared clonotype found between day 0 and month 1 likely due to low frequencies of HPV 16-specific Bmem at day 0. In this case, both Bmem exhibited cross reactivity to HPV 16 and 31 (discussed below). Interestingly, as for the IgG above, no overall trend toward increasing variable gene mutations with time was found for the clonotypes (Fig. S6). Instead, depending on the clonotype, increasing, decreasing, or no change in variable gene mutations was found. Thus, in addition to the aforementioned suggestion that naïve B cells were being drawn into the one-month post-vaccination response, these clonotype data imply that month 1 Bmem may also derive from less somatically mutated Bmem precursors that were not isolated as a result of their rarity or our sorting approach, which excluded IgD⁺IgM⁺ Bmem. Indeed, IgD⁺IgM⁺ Bmem have been shown to be less somatically mutated and more long-lived than class-switched Bmem (Tangye and Good, 2007, Pape et al., 2011).

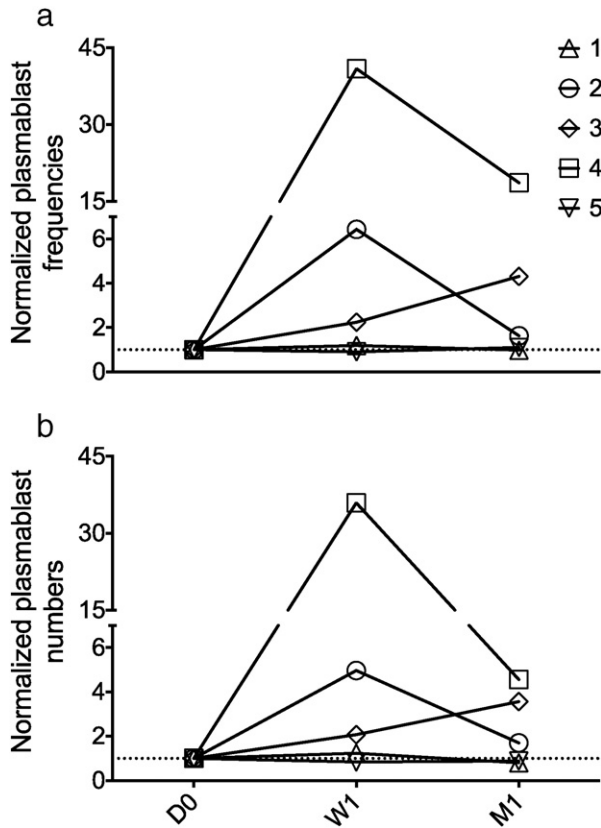


Fig. 2. Two subjects elicited rapid plasmablast responses following a single vaccine dose. Frequencies (a) and numbers (b) of plasmablasts per million PBMC at D0, W1, and M1 were normalized to D0 responses.

3.7. Binding Properties of Abs Cloned From AF488-HPV 16⁺ Bmem

In order to compare the binding affinities of Bmem elicited by natural immunity to those elicited in response to vaccination, paired heavy and light chain variable region sequences were cloned into corresponding heavy or light chain expression vectors from 21 IgG⁺ Bmem sorted at day 0 from the vaccinated and control groups and from 21 Bmem sorted at month 1 from the vaccinated group. For the day 0 samples, every IgG possible was cloned. For the month 1 samples, every IgG possible was cloned from every subject but subject 2, who had 33 paired IgG. From this subject a representative sample of IgG was chosen to bring the final count up to 21. The resulting vectors were then co-transfected in mammalian cells according to their original pairing in order to recombinantly express the IgG as mAbs.

Initially, each mAb was screened against HPV 16 L1 and other related (e.g., HPV 31 and 33) or relevant (e.g., HPV 18) HPV types. If a mAb exhibited reactivity to another type (e.g., HPV 31), this psV type was incorporated into our neutralization experiments. Remarkably, mAbs cloned from pre-existing naturally elicited Bmem at day 0 generally bound very poorly to HPV 16 L1 even when tested at 100 nM (except HPV16.19 and HPV16.13), whereas most of the mAbs cloned from Bmem at one-month post-vaccination exhibited high binding levels at this concentration (except HPV 16.47, HPV16.41, and HPV 16.35; Fig. 5a, note the scales).

Each mAb was then tested against psV and BPV psV in monoplicate alongside positive controls, using a high starting mAb concentration of 50 µg/ml (~333 nM). If 50% neutralization was not achieved at this concentration, the mAb was considered non-neutralizing. If a mAb exhibited ≥50% neutralization, the assay was repeated in triplicate, and neutralization curves were fitted by non-linear regression analysis to obtain IC₅₀ values (Fig. 5b). MAb clones from naturally elicited

Bmem at day 0 were found to be generally non-neutralizing (except HPV16.19), whereas all of the mAbs cloned from Bmem elicited one-month post-vaccination were neutralizing. Even if mAbs from subject 2 are excluded, which were not randomly selected, as well as mAbs from control subjects, so as to eliminate any bias that may result from differences between subjects in each group, only 10% (1/10) of the mAbs isolated at day 0 were neutralizing, whereas 100% (14/14) of subject-matched mAbs isolated one-month post-vaccination were. The finding that the naturally elicited mAbs were generally non-neutralizing was particularly surprising given that the same labeling methods, gating, and lots of reagents were used to identify and isolate the AF488-HPV 16⁺ Bmem, in addition to samples being batched together for flow cytometry experiments. Furthermore, in the case of subjects 1 and 4, the AF488-HPV 16 labeling was well separated from background (Fig. S7), making it improbable that all Bmem sorted from these samples represented noise.

3.8. Cross-reactive Antibodies Cloned From Bmem

The exception among the otherwise non-neutralizing and non-binding day 0 mAbs, HPV16.19, is also notable in that it neutralizes HPV 31 psV at a concentration approximately an order of magnitude lower than that with which it neutralizes HPV 16 (IC₅₀ HPV 31 = 1.9 pM; Fig. S8). Furthermore, a Bmem from the same subject at month 1 yielded a mAb, HPV16.47, which neutralizes HPV 31 psV 2200-fold more potently than HPV 16 psV (IC₅₀ HPV 31 0.22 pM vs. IC₅₀ HPV 16 0.48 nM; Fig. S7). Thus, this subject was suspected to have also had pre-existing HPV 31 Abs. To test this, her enrollment plasma was re-evaluated for binding to HPV 16 L1 and HPV 31 L1 in an L1 binding assay and her anti-HPV 31 plasma Ab levels were found to be 4.5 fold higher than her HPV 16 Ab levels (Fig. S9). This finding indicated that subject 1 was either previously co-infected with HPV 16 and HPV 31, or that her HPV 31 serum Abs cross-reacted with HPV 16 L1 in our assay. The fact that this subject elicited an appreciable Bmem response to vaccination at month 1 (3-fold that of her day 0 numbers), but a low plasmablast response at week 1 (1.2-fold that of her day 0 numbers) and a slower Ab response suggests the latter. Moreover, all of the remaining mAbs cloned from subject 1's Bmem at month 1 bound and neutralized HPV 16 in the absence of HPV 31 reactivity. Taken together, these data indicate that subject 1 generated a primary response to vaccination. It will be interesting to identify the cross-reactive epitopes.

Subject 5 also elicited a robust Bmem response to vaccination at month 1 (5.2-fold that of her day 0 numbers), but did not generate recall responses. However, her day 0 plasma Abs did not bind strongly to any other type in the alpha 9 papillomavirus species, i.e., the species of types with 60–70% nucleotide identity to the HPV 16 L1 open reading frame (de Villiers et al., 2004). Therefore, it is possible that she experienced a prior HPV 16 infection, but did not generate an effective Bmem response. Subject 3 who did not elicit a strong Ab response to any vaccine type or a Bmem response to HPV 16 may reflect the response of persons who require more than one vaccine dose to seroconvert.

4. Discussion

In this study we investigated whether a single HPV vaccine dose improved the B cell memory of persons with pre-existing Abs to HPV 16. We found that a single qHPV dose administered to HPV 16-seropositive women boosted Ab levels 24- to 930-fold (median 77-fold) at one-month post-vaccination; generated what appeared to be primary responses in three of the five vaccinees; and elicited HPV 16-specific Bmem that were somatically mutated, class-switched and expressed neutralizing Abs.

Therefore, pre-existing, infection-elicited Ab levels, were lower than vaccine-elicited Ab levels, which is known (Stanley et al., 2012). Moreover, we only observed potential recall responses in two of the five vaccinees. The study that first noted anamnestic Ab responses indicative of

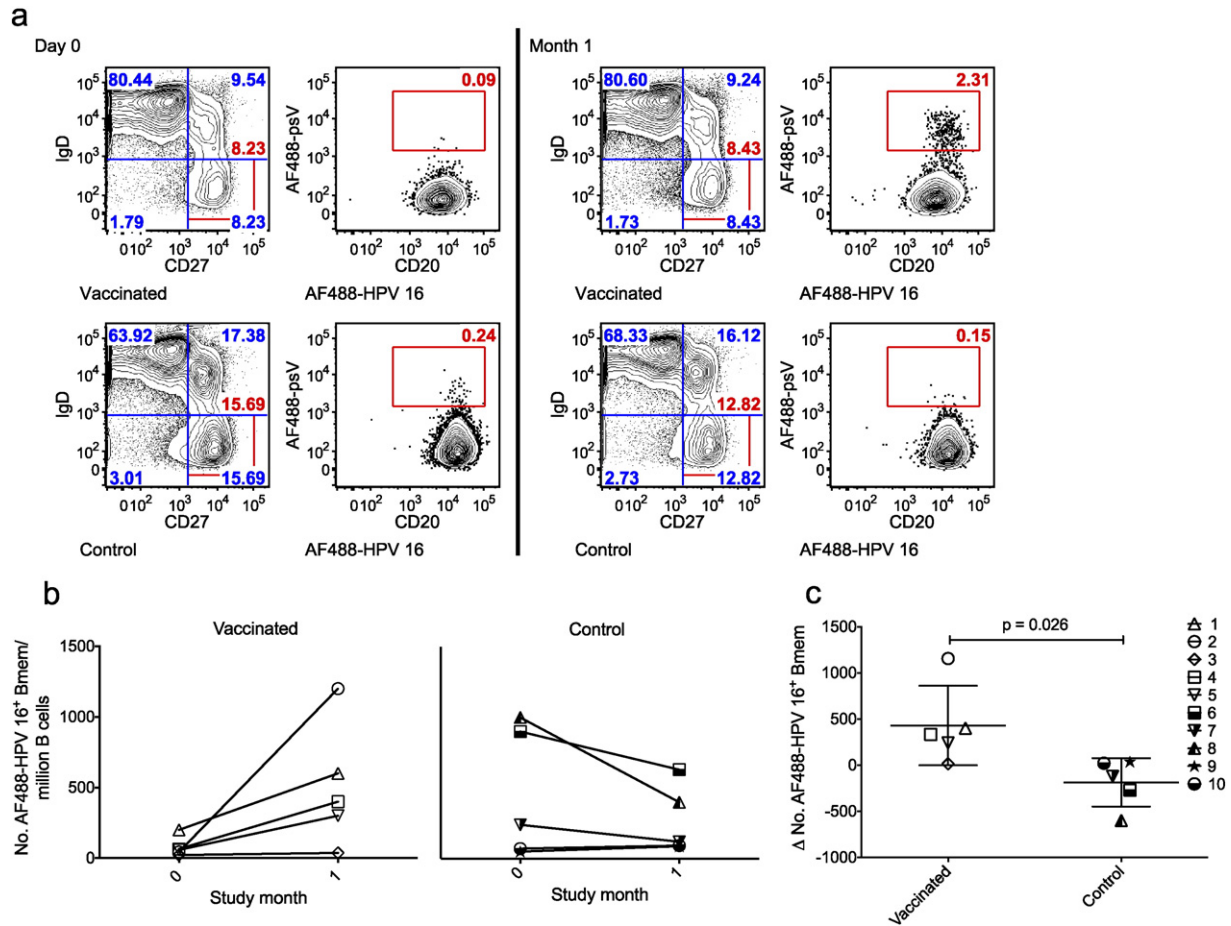


Fig. 3. A single vaccine dose elicits a robust Bmem response in HPV 16-Exposed subjects. (a) Dot plots show the gating strategy used to identify HPV 16-specific Bmem at day 0 and month 1 in a representative subject from the vaccinated and control groups (subjects 2 and 7, respectively). Flow cytometry data were first gated to include lymphocytes (FSC vs. SSC), to exclude doublets and dead cells, and to include B cells (CD3⁻CD19⁺ cells). The dot plots in the first and third columns show the frequency of bulk Bmem (CD27⁺IgD⁻, red gate) in each sample, whereas dot plots in the second and fourth columns show the frequency of those Bmem that bound to AF488-labeled HPV 16 psV. Numbers associated with the quadrants and insets indicate the percentage of cells. (b and c) Graphs illustrate the change in HPV 16-specific Bmem numbers between day 0 and month 1 for subjects in each of the indicated groups (b), as well as the increase in AF488-HPV 16⁺ Bmem numbers between day 0 and month 1 (\pm SD, unpaired, two-tailed student's *t*-test) (c).

Bmem following a single vaccine dose in previously infected subjects also reported a high degree of variability in subject responses (Olsson et al., 2007). The quality of B cell responses generated by natural infection must thus vary from person-to-person.

We also found that most infection-elicited Bmem expressed non-neutralizing Abs despite being class-switched and somatically mutated. Given that vaccinated subjects possessed low levels of neutralizing activity in their plasma at day 0 (Fig. 5b) and were included in the study on the basis of having low levels of HPV 16 Abs in their serum, we know they elicited HPV 16-specific plasma cells. We assume that these subjects also elicited HPV 16-specific Bmem. If this assumption is true, there are two possible reasons for the finding that the AF488-HPV 16⁺ Bmem expressed non-neutralizing mAbs: The non-neutralizing mAbs were cloned from Bmem that were not truly HPV 16-specific, but resulted from noise in the method. Therefore, we did not efficiently find their rare HPV 16-specific Bmem. The discovery of one day 0 Bmem that expresses a potent neutralizing HPV 16/31 mAb supports this interpretation. However, the explanation that the remaining AF488-HPV 16⁺ Bmem at day 0 are all non-specific seems improbable based upon the frequency of neutralizing mAbs cloned from Bmem at month 1 post-vaccination using the same methods and reagents, as well as the frequency of neutralizing mAbs isolated from AF488-HPV 16⁺ Bmem at one-month post-vaccination in our previous study (7/11 mAbs or 64%) (Scherer et al., 2014). Furthermore, we find that some non-

neutralizing mAbs do exhibit weak binding to L1 (e.g., HPV16.13, subject 2_C7, and potentially HPV16.14, HPV16.15, HPV16.25, and HPV16.30).

An alternative explanation is that the B cell receptor avidities of the naturally elicited Bmem were sufficient to bind fluorescently labeled HPV 16 psV, but their corresponding affinities as soluble Abs were insufficient to bind appreciably to HPV 16 L1 or to neutralize HPV 16 psV. In this scenario, the polyclonal response may have resulted from plasma cells producing an aggregate of similarly weak Abs. If this were true, it would indicate a fundamental difference in the quality of Bmem elicited by natural infection versus those elicited by vaccination. The finding that the avidities of naturally elicited HPV-specific serum Abs are significantly lower than those of bHPV vaccine-elicited serum Abs further supports this interpretation (Scherpenisse et al., 2013).

It is unclear how much overlap exists between Bmem, plasmablast and serum Ab repertoires, as studies examining this subject are scarce in the literature (Purtha et al., 2011; Lavinder et al., 2014). Purtha and colleagues found that Bmem repertoires responding to West Nile virus infection in mice possessed additional specificities beyond the dominant clonotype expressed by plasma cell and serum Ab repertoires, suggesting that the Bmem repertoire is better adapted to respond to viral variants (Purtha et al., 2011). Lavinder et al. found little overlap between the Bmem and serum Ab repertoires or plasmablast and serum Ab repertoires elicited in response to tetanus toxoid boosting, as only

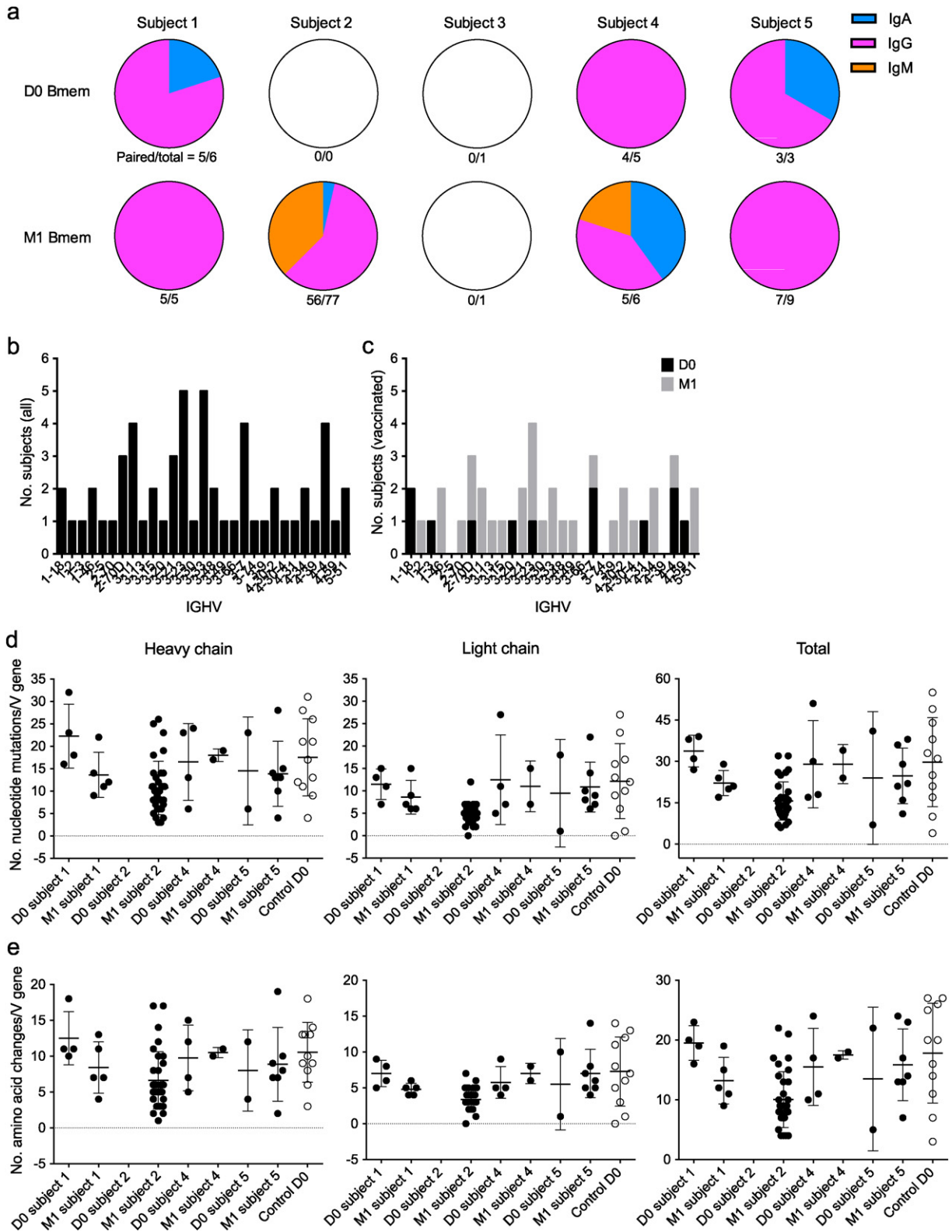
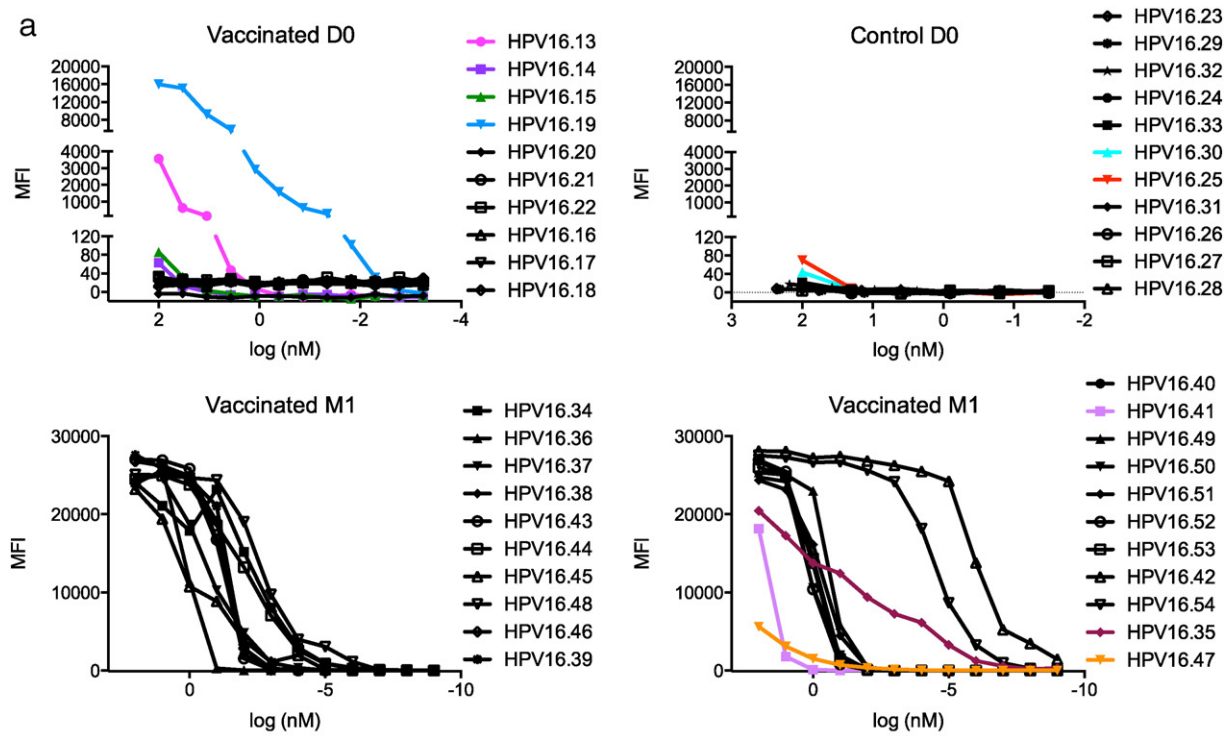


Fig. 4. Abs cloned from both infection- and vaccine-elicited Bmem include class-switched Abs, utilize diverse variable genes, and are somatically mutated. (a) The isotype distribution of paired Ab sequences amplified from AF488-HPV 16⁺ Bmem is shown. These Bmem were single cell sorted from D0 and M1 samples of the vaccinated group. The ratio below each graph indicates the number of paired Ab sequences obtained out of the total heavy chain sequences (both paired and unpaired). Only those heavy chains with high quality sequences that are predicted to be productive and do not contain large deletions (>20 nucleotides) were included. The two unpaired heavy chains amplified from subject 2's D0 Bmem with IgA and IgM reverse primers yielded either unproductive or indiscernible sequences and thus were not included in the total for this subject at this time point. (b and c) Immunoglobulin heavy chain variable (IGHV) gene usage of paired Ab sequences isolated from IgA/IgG/IgM Bmem of all subjects (b) or vaccinated subjects only (c) were determined by sequence analysis. Stacked bar graphs show the number of subjects whose D0 and/or M1 Bmem utilized that particular IGHV gene [e.g., the IGHV gene 2-70D was utilized by D0 Bmem in one subject and M1 Bmem in two subjects]. (d and e) The number of nucleotide mutations (d) and amino acid changes (e) observed in the heavy chain variable gene, light chain variable gene, or in both chains (total) of paired IgG Bmem (SD) at D0 and M1 are shown for each responding subject in the Vaccinated group and at D0 for the Control group as a point of comparison.



b

Study subject day 0	plasma IC ₅₀ ± SD	mAb	mAb IC ₅₀	Vaccinated subject month 1	plasma IC ₅₀ ± SD	mAb	mAb IC ₅₀	
5	2,120 ± 307	HPV16.13	17 pM	5	1,370,000 ± 293,000	HPV16.36	5.5 pM	
		HPV16.14				HPV16.37	8.4 pM	
		HPV16.15				HPV16.38	0.46 pM	
HPV16.16	HPV16.39	1.4 pM						
HPV16.17	HPV16.40	0.83 pM						
HPV16.18	HPV16.41	0.26 nM						
HPV16.19	HPV16.42	2.6 pM						
HPV16.20	HPV16.34	1.6 pM						
HPV16.21	HPV16.35	65 pM						
HPV16.22	HPV16.43	1.4 pM						
1*	47,600 ± 10,400	HPV16.23	HPV16.44	5.0 pM	1*	5,030,000 ± 1,580,000	HPV16.45	18 pM
		HPV16.24	HPV16.46	0.49 pM				
		HPV16.25	HPV16.47	0.48 nM				
6	127 ± 96.9	HPV16.26	HPV16.48	2.1 pM	2*	3,570,000 ± 273,000	HPV16.49	0.47 pM
		HPV16.27	HPV16.50	1.1 pM			0.1–0.99 pM	
		HPV16.28	HPV16.51	0.68 pM			1–99 pM	
10	1,600 ± 331	HPV16.29	HPV16.52	0.18 pM	2*	3,570,000 ± 273,000	HPV16.53	79 pM
		HPV16.30	HPV16.54	1.4 pM			>333 nM	
		HPV16.31						
7	296 ± 12.8	HPV16.32						
9	9,650 ± 4,750	HPV16.33						

* plasma exhibited non-specific neutralization activity

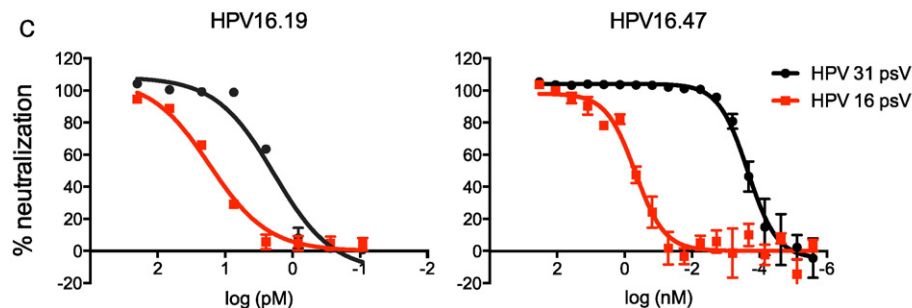


Fig. 5. MAbs isolated from Bmem one-month post-vaccination exhibit markedly improved binding and neutralization activities compared to those from pre-existing naturally elicited Bmem. (a) MAbs cloned from naturally elicited Bmem in the vaccinated group at D0 (HPV16.13–HPV16.22); naturally elicited Bmem in the control group at D0 (HPV16.23–HPV16.33); or from Bmem elicited one-month post-vaccination (HPV16.34–HPV16.54) exhibit differing levels of binding to HPV 16 L1 coated beads in a binding assay. The amount of mAb bound is represented as the median fluorescence intensity (MFI) of a fluorescently conjugated secondary Ab. (b) Neutralization potencies of the subjects' plasmas at the time the mAbs were cloned, as well as the potencies of the mAbs themselves, are shown. The subject from whom each mAb was isolated is also noted. Warm colored boxes represent increasing neutralization potency and gray boxes indicate no neutralization (IC₅₀ > 50 µg/ml or ~333 nM).

a small fraction of Bmem and plasmablast clonotypes identified through next generation sequencing overlapped with Ab clonotypes found in serum by proteomics analysis (Lavinder et al., 2014). Here, we noted highly expanded clonotypes shared between Bmem and plasmablast repertoires using single-cell approaches, which Franz et al. also observed in response to tetanus toxoid vaccination (Franz et al., 2011). It would be interesting to see if the highly expanded clonotypes in our study were among the predominant serum Ab clonotypes, given that highly expanded plasmablast clonotypes have been previously found at elevated levels in serum and in the long-lived plasma cell repertoire of human bone marrow (Lavinder et al., 2014, Halliley et al., 2015).

Collectively, the results from this study indicate that vaccination augments natural HPV immunity by boosting Ab levels and Bmem numbers, as well as by potentially improving the quality of Bmem that are elicited. Thus, persons previously infected with HPV vaccine types could also benefit from vaccination. Whether vaccination will lessen the recurrence of HPV infections in persons who have previously been treated for neoplastic lesions is unknown. Analysis of both Bmem and serum Abs in future vaccine trials in previously infected subjects would be valuable for identifying correlates of protection against HPV re-infection. The contribution of vaccine-elicited T cells to efficacy is likely negligible, given that HPV infection of basal epithelial cells is temporally and spatially separated from L1 protein expression in the highly differentiated epithelia at/near the surface (Kwak et al., 2011).

The major strengths of this study are that we have described previously unknown characteristics of naturally elicited Bmem and shown that vaccination improves the quality of B cell memory in previously infected persons. In addition, we have sampled B cell populations responding to vaccination longitudinally and interrogated the functional properties of the Abs expressed by these cells. The limitations of our study include small cohort sizes, the omission of AF488-HPV 16⁺ IgD⁺-IgM⁺ Bmem from our sorting approach, and the restriction to only a single HPV type. Our study would also benefit from a parallel next generation sequencing analysis to improve our sampling depth for finding clonotypes over time and in different B cell populations.

The high titers of neutralizing plasma Abs following a single dose of qHPV vaccine in this study and finding that all mAbs isolated from HPV 16-specific Bmem were neutralizing speak to the extraordinary immunogenicity of the HPV vaccines. It is perhaps not surprising then that the WHO has approved a two-dose qHPV vaccine schedule for 9–13 year old girls based on immunogenicity studies, or that recent evidence has been presented to suggest that a single dose may be sufficient for protection (Kreimer et al., 2015). If the serum Ab levels elicited by such reduced dose schedules do not remain stable at levels near the minimum Ab level associated with protection in clinical trials, it will be important to investigate the durability, magnitude, and quality of the B cell memory elicited by these schedules.

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Conflict of Interest Statement

D.A.G. is a member of Merck's Global Advisory Board for HPV, and A.W. serves on a Merck DSMB for a clinical trial not related to HPV.

Author Contributions

E.M.S., A.W., and D.A.G. conceived the project. E.M.S. and D.A.G. supervised the project. A.W. supervised the clinic where subjects were recruited and enrolled. E.M.S., J.J.C., N.D.T., A.W., and D.A.G. wrote and edited the paper. E.M.S., R.A.S., D.F.G., J.J.C., G.C.W., and T.T. performed experiments. E.M.S., R.A.S., D.F.G., J.J.C., G.C.W., T.T., N.D.T. and D.A.G. analyzed data. M.S. enrolled and collected samples from study subjects.

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