



Cellular immune responses to HPV-18, -31, and -53 in healthy volunteers immunized with recombinant HPV-16 L1 virus-like particles

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Abstract

Human papillomavirus-like particles (HPV VLP) are candidate vaccines that have shown to be efficacious in reducing infection and inducing robust antiviral immunity. Neutralizing antibodies generated by vaccination are largely type-specific, but little is known about the type-specificity of cellular immune responses to VLP vaccination. To determine whether vaccination with HPV-16 L1VLP induces cellular immunity to heterologous HPV types (HPV-18, HPV-31, and HPV-53), we examined proliferative and cytokine responses in vaccine ($n=11$) and placebo ($n=5$) recipients. Increased proliferative and cytokine responses to heterologous types were observed postvaccination in some individuals. The proportion of women responding to heterologous types postvaccination (36%–55%) was lower than that observed in response to HPV-16 (73%). Response to HPV-16 VLP predicted response to other types. The strongest correlations in response were observed between HPV-16 and HPV-31, consistent with their phylogenetic relatedness. In summary, PBMC from HPV-16 VLP vaccine recipients can respond to L1VLP from heterologous HPV types, suggesting the presence of conserved T cell epitopes.

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Introduction

Infection with one of approximately 15 HPV types is now recognized as the necessary cause of cervical cancer (Bosch et al., 1995; Walboomers et al., 1999). Two of these HPV types, HPV-16 and HPV-18, account for 60–70% of all cervical cancer cases worldwide (Ho et al., 1998; Munoz et al., 2003). Vaccines containing virus-like particles (VLP) from these two HPV types

have been shown to be well tolerated and provide strong short-term protection against transient and persistent HPV-16 and HPV-18 infections (Harper et al., 2004; Harro et al., 2001; Koutsky et al., 2002; Villa et al., 2005). These findings support the notion that cervical cancer might be preventable through vaccination.

High levels of neutralizing antibodies generated after vaccination are believed to be the primary effector of protection conferred by HPV VLP vaccination (Harro et al., 2001; Suzich et al., 1995). These neutralizing antibodies to L1 are predominantly type-specific, with the exception of very closely related types which show weak cross-reactivity (Christensen and Kreider, 1990; Roden et al., 1996a, 1996b). Furthermore, in

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animal studies, vaccination with VLP or virions derived from one papillomavirus type does not protect against experimental infection with heterologous types, indicating that protection is type-specific (Breitburd et al., 1995; Roden et al., 1996a, 1996b). This leads to the idea that effective prophylactic HPV vaccines should be polyvalent and contain the HPV types responsible for most cervical cancers.

In addition to neutralizing antibodies, HPV 16 L1 VLP vaccination has been shown to induce L1-specific T cell responses detectable by proliferation of both CD4 and CD8 T cells and in vitro production of Th1 and Th2 type cytokines (Pinto et al., 2003). The role these responses may play in the prophylactic efficacy of HPV VLP vaccines remains to be elucidated. However, data suggest that CD4 T cells are important for both the induction and maintenance of humoral responses (Yao et al., 2004; Zinkernagel et al., 1996), and might therefore contribute to the efficacy of HPV VLP vaccines. In addition, a protective effect of HPV-16/18 VLP vaccination against infection with other oncogenic HPV types was recently reported (Harper et al., 2006). Natural infection-induced T cell responses to HPV-11, in contrast to antibody responses, have been reported to cross-react with a range of HPV types (Williams et al., 2002). Such cross-reactive T cell responses in the presence of strain-specific antibody responses are a feature of other human viral infections, most notably influenza virus infection (Gelder et al., 1995). Cross-reactive immune responses to heterologous HPV types may be of significance for HPV vaccine strategies and may influence protection against heterologous HPV types.

Finally, little is known about the possible efficacy of HPV VLP vaccines in treating established infections. Animal studies have suggested that L1 VLP vaccination does not induce regression of established papillomas, but provided some evidence that vaccination leads to a reduction in the mean number of lesions observed (Kirnbauer et al., 1996). Furthermore, in one human trial, there is preliminary evidence suggesting that vaccination with HPV-6 VLPs might have a therapeutic effect against genital warts (Zhang et al., 2000). HPV-16 L1-specific T cell epitopes elicited by vaccination have not yet been described and it is not known whether vaccination with HPV-16 L1 VLP induces cellular immune reactivity to other HPV types.

To better understand whether vaccination with HPV-16 L1 VLP induces immune responses against heterologous HPV types, we evaluated proliferative and cytokine (IFN- γ , IL-10, and IL-5) responses to L1 VLP from heterologous HPV types in vitro before and following intramuscular vaccination of healthy young women. We selected as heterologous HPV types of interest HPV-18, HPV-31, and

HPV-53 because of the availability of VLP from these types and also the fact that they represent a broad spectrum of HPV types, including an HPV type within the same species group as HPV-16 (HPV-31), a more distant oncogenic HPV type (HPV-18), and a common non-oncogenic HPV type (HPV-53).

Results

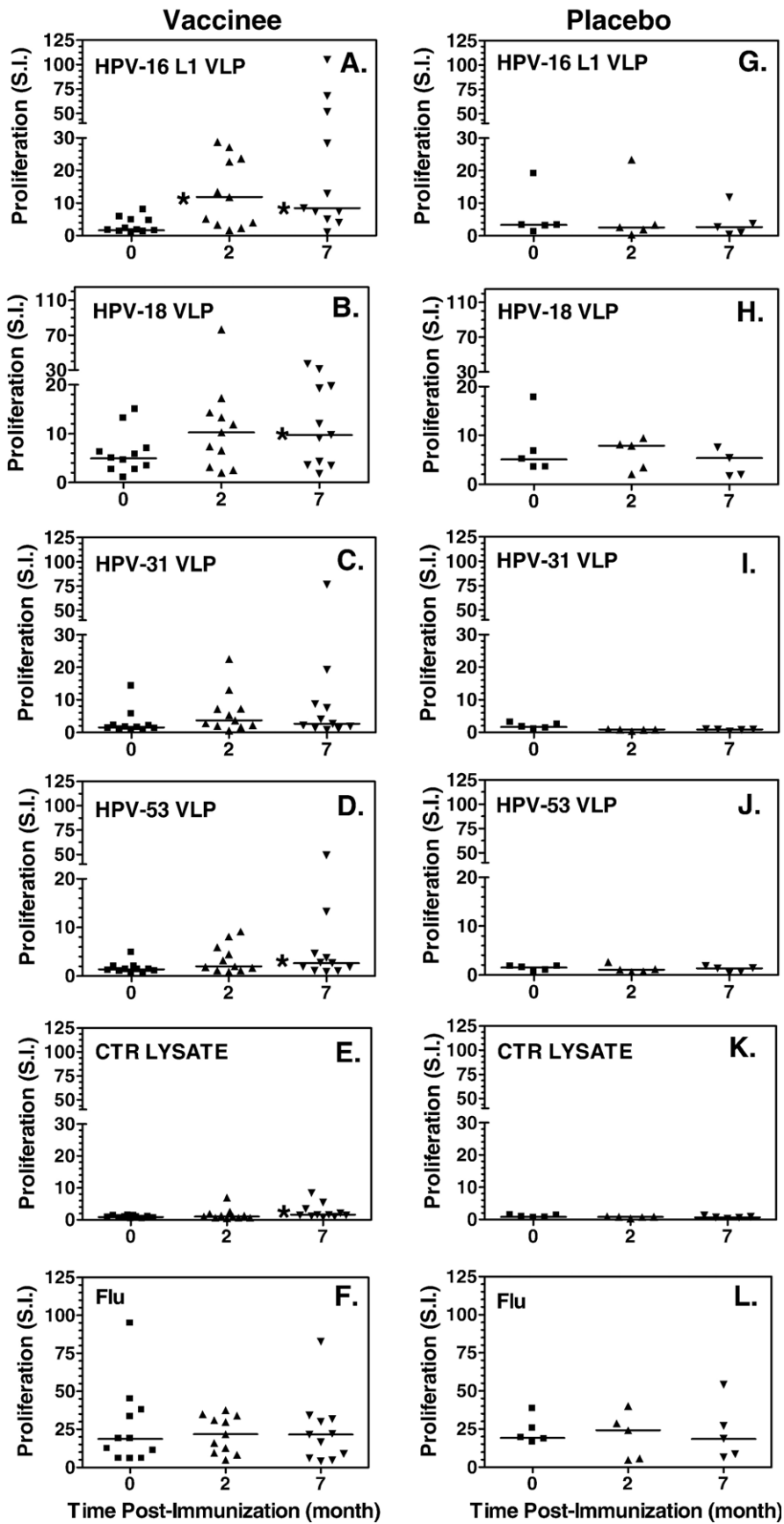
Cellular immune responses to HPV-16 and heterologous-type VLPs following immunization with HPV-16 VLP

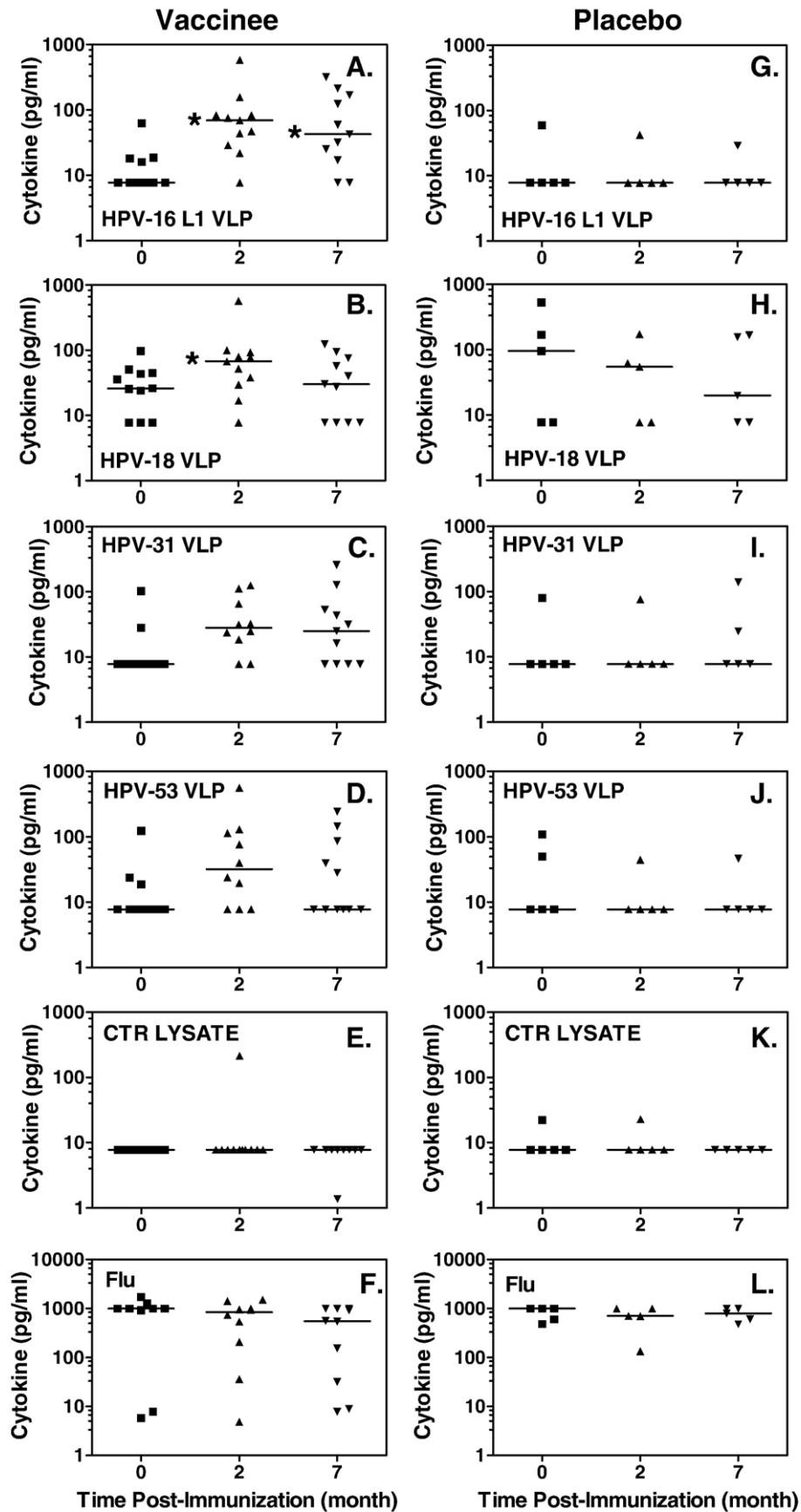
Lymphoproliferative responses to HPV VLPs from various HPV types (HPV-16, -18, -31, and -53) were evaluated before and after vaccination with HPV-16 VLP (Fig. 1). Consistent with our previous publication (Pinto et al., 2003), increases in proliferative responses to HPV-16 VLP were observed following vaccination in most vaccine recipients (Fig. 1A) but not in placebo recipients (Fig. 1G). Median proliferative responses to the heterologous HPV VLPs (18, 31, and 53) were increased after vaccination with HPV-16 L1 VLP (Figs. 1B–F) when compared to responses before vaccination. However, most differences were not statistically significant because increased responses to heterologous VLPs were only seen in some vaccine recipients following vaccination with HPV-16 VLP. No significant increases in lymphoproliferative responses were observed over time for influenza or at month 2 for the control lysate (Figs. 1F and E). At month 7, there was evidence of an increase in lymphoproliferative response for the control lysate, but the median response was below what is typically considered a positive response (SI < 2.0).

When the group of women who received placebo was evaluated, there was no evidence of proliferative response to HPV-16, HPV-18, HPV-31, or HPV-53 VLP postvaccination (Figs. 1G–L).

Next, cytokine levels produced by PBMC from vaccine and placebo recipients in response to stimulation with HPV-16 L1 VLP and VLP from the heterologous HPV types were evaluated. Consistent with our previous report (Pinto et al., 2003), increases in cytokine production levels by PBMC in response to HPV-16 VLP were observed following vaccination. We observed a tendency for IFN- γ responses to all three heterologous HPV VLP to increase following vaccination, although the effects did not reach statistical significance for all VLPs because responses were only seen in some individuals (Figs. 2A–D). As seen with proliferative responses, median IFN- γ responses were strongest at month 2 after first vaccination. At month 2, a 2.6-fold, 3.6-fold, and 4.1-fold increase over prevaccination levels was observed for HPV-18,

Fig. 1. Lymphoproliferative responses to HPV-16 L1 VLP and VLPs from heterologous types (HPV-18, -31, and -53) in vaccine (A–F, $n=11$) and placebo (G–L, $n=5$) recipients. PBMCs collected before and after immunization (month 2 and 7) were stimulated in the presence of HPV VLPs (2.5 $\mu\text{g}/\text{ml}$) or a control lysate (0.1 $\mu\text{g}/\text{ml}$) or the recall antigen influenza virus (flu, 1:100). Proliferative responses were evaluated on day 5 after stimulation by a standard ^3H -thymidine incorporation method. Responses are expressed as stimulation indices (S.I.) calculated by dividing the cpm values of cultures in the presence with antigen by cpm values of media background cultures. Median cpm of media background cultures at month 0, 2, and 7 for vaccine recipients were 621, 610, and 529, respectively and for placebo recipients were 628, 801, and 982, respectively. * indicates $p < 0.05$ determined using Wilcoxon signed rank test to compare the median proliferative responses at month 2 or 7 with median proliferative responses at month 0 for each of the antigens within vaccine or placebo recipients.





HPV-31, and HPV-53, respectively (Figs. 2B–D) compared to a median increase of 8.9-fold for HPV-16 (Fig. 2A). No increases in median levels of IFN- γ responses were observed over time to influenza or to the control lysate (Figs. 2F and E). No increases in median levels IFN- γ production were observed in placebo recipients in response to any of the heterologous HPV VLPs evaluated (Figs. 2G–J). Patterns of IL-10 responses to heterologous HPV VLPs were overall similar to those seen with IFN- γ , with the exception of responses to HPV-18 which were not found to be elevated at month 2 following vaccination among individuals who received the HPV-16 VLP vaccine (data not shown). No remarkable increases in IL-5 production were observed at month 2 or 7 in PBMC from vaccine recipients in response to any of the three heterologous HPV VLPs in vaccine recipients (data not shown). In general, placebo recipients did not show responses to the VLPs by the various assays, with the exception of a single individual receiving placebo that exhibited a significant increase in the IL-10 response to HPV-18 VLP (111.3 pg/ml at month 2 compared to 17.2 pg/ml at month 0).

Based on the increase in T cell responses after vaccination, subjects were divided into two groups: vaccine responders and non-responders. Responders were those who had: (1) at least a 2-fold increase in response to VLPs when comparing immune response at baseline (month 0) with responses after vaccination; (2) lymphoproliferative response to VLPs that was positive (SI > 2.0) when compared to media or twice the lowest detectable level of the assay (for cytokines), and (3) ratio of responses at month 2/month 0 for VLP greater than that for the control lysate.

Consistent with our previous publication, 73% of vaccinated women showed evidence of lymphoproliferative response to HPV-16 VLP postvaccination (Table 1) (median increase in response postvaccination among responders = 5.6-fold; median response = SI of 18.0). 64% of vaccinated women produced increased amounts of IFN- γ and IL-10 in response to HPV-16 VLP postvaccination (median increases in response postvaccination among responders = 8.9-fold and 7.4-fold for IFN- γ and IL-10, respectively; median responses = 82.9 pg/ml for IFN- γ and 36.6 pg/ml for IL-10). Also consistent with our previous report, increases in the level of IL-5 production in response to HPV-16 VLP postvaccination were observed in only 36% of vaccinated women (median increases in response postvaccination among responders = 17.5-fold; median response = 56.2 pg/ml). Nearly half (45%) of vaccinated women responded to HPV-16 VLP postvaccination by all three assays, lymphoproliferation, IFN- γ , and IL-10 production. There was little evidence of increasing responses to influenza after vaccination, suggesting that the responses to HPV antigens seen postvaccination were specific (Table 1).

Responses to heterologous HPV VLP types were also observed among vaccinated women. The proportion of vaccinated women who developed T cell responses to heterologous HPV types was lower than that observed for HPV-16 VLP (Table 1). Between 36% (for HPV-18 and HPV-53) and 55% (for HPV-31) of vaccinated women had evidence of a lymphoproliferative response to heterologous HPV types postvaccination. A subset of vaccinated women was also found to produce IFN- γ and IL-10 in response to heterologous HPV types postvaccination (Table 1). The weakest responses were, in general, observed for HPV-18. Few women were defined as responders based on the IL-5 assay, consistent with the modest IL-5 responses seen for HPV-16. Results from the IL-5 assay were therefore not considered in further analyses. 33% of vaccinated women responded to HPV-31 VLP postvaccination by the lymphoproliferation, IFN- γ , and IL-10 assays. The percentages for HPV-18 and HPV-53 were 0% and 22%, respectively.

Immune response to HPV-16 VLP predicts response to heterologous VLPs

Next, we evaluated the proportion of vaccinated women who responded to multiple heterologous HPV types (Table 2). 45% of vaccinated women had evidence of response to more than one heterologous HPV type postvaccination, as measured by the lymphoproliferation assay. 18% responded to all three heterologous types evaluated in our study. The comparable rates (response to >1 type/response to all 3 types) based on IFN- γ and IL-10 measures were 25%/12.5% and 12.5%/0%, respectively. Interestingly, none of the vaccinated women who were non-responders to HPV-16 VLP postvaccination were observed to respond to >1 heterologous HPV type, while a substantial fraction of vaccinated women who responded to HPV-16 VLP postvaccination also responded to multiple heterologous HPV types (Table 2).

When the correlation between intensity of response to HPV-16 VLP postvaccination and response to each of the heterologous HPV types was evaluated, the strongest correlations (range 0.73–0.84) were observed between HPV-16 and HPV-31 for all three assays (Table 3). Significant correlations were also observed between HPV-16 and HPV-53 for the three assays. In general, weaker correlations were observed between responses to HPV-16 VLP and HPV-18 VLP, likely due to the high background responses observed against HPV-18 VLP. Correlations among heterologous types themselves (i.e., between HPV-18, HPV-31, and HPV-53 responses) were also observed and ranged from 0.47 (HPV-18: HPV-53) to 0.77 (HPV-31: HPV-53) for lymphoproliferation, 0.3 (HPV-18: HPV-31) to 0.58 (HPV-31: HPV-53) for IFN- γ , and were from –0.08 (HPV-31: HPV-18) to 0.38 (HPV-31: HPV-53) for IL-10. Individuals with

Fig. 2. Cytokine (IFN- γ) responses to HPV-16 L1 VLP and VLPs from heterologous types (HPV-18, -31, and -53) in vaccine (A–F) and placebo (G–L) recipients. PBMC collected before and after immunization (month 2 and 7) were stimulated in the presence of HPV VLPs (2.5 μ g/ml), a control lysate (0.1 μ g/ml) or the recall antigen influenza virus (flu, 1:100) for 3 days. Cytokine levels were determined by ELISA in culture supernatants. Results are expressed as pg/ml of cultures tested in duplicate. * indicates $p < 0.05$ determined using Wilcoxon signed rank test to compare the median proliferative responses at month 2 or 7 with median proliferative responses at month 0 for each of the antigens within vaccine or placebo recipients.

Table 1
Lymphoproliferative and cytokine responses to HPV-16 and heterologous HPV types after vaccination with HPV-16 VLP^a

Antigen	Lymphoproliferation (LPA)				IFN- γ				IL-10				IL-5				LPA/IFN- γ /IL-10 ^b	
	N	% responders ^c	95% CI	Median (SI)	N	% responders	95% CI	Median (pg/ml)	N	% responders	95% CI	Median (pg/ml)	N	% responders	95% CI	Median (pg/ml)	% responders	
HPV-16	11	73% (8)	[39–94%]	18.0	11	64% (7)	[31–89%]	82.9	11	64% (7)	[31–89%]	36.6	11	36% (4)	[11–69%]	56.2	45	
HPV-18	11	36% (4)	[11–69%]	14.6	11	36% (4)	[11–69%]	88.9	10	0% (0)	[0–31%]	36.6	11	0% (0)	[0–28%]	0	0	
HPV-31	11	55% (6)	[23–83%]	7.2	9	56% (5)	[21–86%]	65.6	9	33% (2)	[7–70%]	23.4	10	20% (2)	[2–56%]	23.2	33	
HPV-53	11	36% (4)	[11–69%]	7.0	10	50% (5)	[19–81%]	113.7	9	56% (2)	[21–86%]	26.8	10	20% (2)	[2–56%]	37.1	22	
Influenza	11	18% (2)	[2–52%]	25.2	8	13% (1)	[0–53%]	36.2	8	0% (0)	[0–37%]	36.2	8	0% (0)	[0–37%]	0	0	

SI=Stimulation Index.

CI=confidence interval.

LPA=lymphoproliferation assay.

^a In some instances the total number of women evaluated is <11 due to insufficient material available for assay.

^b % of responders (number of responders).

^c % responders to all of the three assays; IL-5 not considered due to low level of response observed.

Table 2

Distribution of lymphoproliferative and cytokine responses to multiple heterologous HPV types after vaccination with HPV-16 VLP-overall and stratified by response to HPV-16 VLP^a

	N	% responding to >1 heterologous HPV types	% responding to all 3 heterologous HPV types
<i>Lymphoproliferation</i>			
All vaccinees	11	45.0	18.0
HPV-16 non-responders	3	0.0	0.0
HPV-16 responders	8	62.5	25.0
<i>p</i> value		0.18	0.56
<i>IFN-γ</i>			
All vaccinees	8	25.0	12.5
HPV-16 non-responders	3	0.0	0.0
HPV-16 responders	5	40.0	20.0
<i>p</i> value		0.46	1.00
<i>IL-10</i>			
All vaccinees	8	12.5	0.0
HPV-16 non-responders	3	0.0	0.0
HPV-16 responders	5	20.0	0.0
<i>p</i> value		1.00	1.00

The percent of women responding to more than one heterologous HPV type was compared between responders and non-responders to HPV-16 VLP; *p* values were obtained based on the Fisher's exact test.

^a In some instances, the total number of women evaluated is <11 due to insufficient material available for assay.

the highest increases in proliferative responses to HPV-16 had higher average increases to any of the heterologous HPV VLP tested.

No significant correlations were observed between antibody titers and proliferation or IFN- γ responses to any of the HPV types following vaccination. The largest correlation with antibody titers was found for IL-10 response to HPV-16 L1 VLP ($r=0.53$; $p=0.01$). In addition, antibody titers to HPV-16 correlated strongly with HPV-31 neutralizing activity ($r=0.891$, $p=0.0048$), although the HPV-31 neutralizing titers were markedly lower than the HPV-16 titers following vaccination (Table 4) and were only detected in some of the vaccine recipients.

Patterns of response to heterologous VLPs

Patterns of lymphoproliferative responses to heterologous HPV VLP among individuals classified as responders are shown in Fig. 3. Patterns of response to HPV-16 VLP are also presented for comparison. Responses to heterologous HPV VLP were typically lower than those observed against HPV-16 VLP. Response patterns varied between individuals and were most variable at month 7 (one month following the third immunization). While the strongest increases in response were typically observed at month 2 (one month following second immunization), additional increases in response were seen at

Table 3
Correlation between responses to HPV-16 VLP and VLPs from heterologous types at months 2 and 7

Assay	HPV type VLP	HPV-18	HPV-31	HPV-53	HPV-16 Ab
Proliferation	HPV-16	0.61 ($p=0.003$)	0.76 ($p<0.0001$)	0.73 ($p=0.0001$)	0.25 ($p=0.26$)
	HPV-18	–	0.53 ($p=0.01$)	0.47 ($p=0.03$)	–0.19 ($p=0.39$)
	HPV-31	–	–	0.77 ($p<0.0001$)	0.07 ($p=0.77$)
	HPV-53	–	–	–	0.31 ($p=0.17$)
IFN- γ	HPV-16	0.31 ($p=0.16$)	0.84 ($p=0.0001$)	0.76 ($p=0.0001$)	0.36 ($p=0.10$)
	HPV-18	–	0.30 ($p=0.18$)	0.57 ($p=0.007$)	0.11 ($p=0.63$)
	HPV-31	–	–	0.58 ($p=0.008$)	0.41 ($p=0.068$)
	HPV-53	–	–	–	0.068 ($p=0.77$)
IL-10	HPV-16	0.17 ($p=0.45$)	0.73 ($p=0.0002$)	0.68 ($p=0.0007$)	0.53 ($p=0.01$)
	HPV-18	–	–0.08 ($p=0.71$)	0.03 ($p=0.91$)	0.08 ($p=0.73$)
	HPV-31	–	–	0.38 ($p=0.10$)	0.35 ($p=0.12$)
	HPV-53	–	–	–	–0.03 ($p=0.90$)

To analyze the relationships between responses to different VLPs, Spearman rank correlations for continuous values were determined. A p value <0.05 was considered significant.

month 7 for HPV-16 VLP in 75% of responders and for heterologous types in 25% (HPV-18) to 67% (HPV-31) of responders. Of the non-responders at month 2, 67% showed increased lymphoproliferative response to HPV-16 at month 7. However, none of the non-responders at month 2 met our criteria for responses to heterologous VLP at month 7. A similar pattern of variability was seen in cytokine responses to the heterologous VLP (data not shown).

Lymphoproliferative response to HPV-16 and heterologous HPV type VLPs includes both CD4 and CD8 T cells

To determine the contribution of CD4 and CD8 T cells in the proliferative responses to VLPs detected after immunization, incorporation of the thymidine analog BrdU was used to measure the relative numbers of CD4 and CD8 T cells progressing through S phase of the cell cycle after in vitro stimulation with the different L1 VLPs. BrdU incorporation into the DNA of CD4 and CD8 T cells was quantified by flow cytometry. Consistent with our previous publication (Pinto et al., 2003), increased levels of proliferating CD4 and CD8 T cells in response to HPV-16 L1 VLP were observed in an independent subset of 6 vaccine recipients tested (Table 5). As previously seen in response to HPV-16 L1 VLPs, responses to the heterologous types (HPV-18, -31, and -53) were also seen in CD4 and CD8 T cells. The level of response to L1 VLPs was

higher among CD4 T cells than among CD8 T cells for HPV-16, -18, and -31 and was similar in both subsets for HPV-53 VLPs.

Discussion

The current results indicate the presence of cross-reactive T cell responses to HPV types in individuals immunized with a monovalent HPV-16 L1 VLP vaccine. PBMC from HPV-16 L1 VLP vaccine recipients showed proliferative (in CD4 and CD8 T cells) and cytokine responses in vitro to L1 VLP from an HPV type that closely resembles HPV-16 (HPV-31), and oncogenic (HPV-18) and non-oncogenic (HPV-53) types that are more distantly related to HPV-16. The proportion of women responding to heterologous HPV VLPs postvaccination was lower than observed for HPV-16 VLP and the patterns of response varied among responders. Response to HPV-16 VLP was predictive of response to heterologous VLP.

As previously reported (Pastrana et al., 2004), sera from HPV-16 L1 vaccine recipients had significant anti-HPV-16 neutralizing titers and no HPV-18 neutralizing activity following immunization. HPV-31 neutralizing activity was weak, detected only in a fraction of the vaccine recipients following vaccination and correlated with HPV-16 neutralizing titers.

Cross-protection between HPV types with vaccines based on the HPV L1 antigen has been considered unlikely, since neutralizing antibodies induced by L1 VLP have been shown to be primarily type-specific in animal models (Breitburd et al., 1995; Roden et al., 1996a, 1996b). Type-specific responses with some cross-reactivity between phylogenetically related types have been shown in studies of natural infection (Combata et al., 2002; Marais et al., 2000; Wideroff et al., 1999).

The data shown here raise the intriguing possibility that by vaccinating with a limited number of HPV types, one might be able to induce immune responses to a broader set of HPV types, and that this response might in turn be involved in protection against heterologous HPV infections. Consistent with this possibility, cross-protection against incident infection with heterologous HPV types (HPV-31 and HPV-45) following vaccination with a bivalent HPV-16/18 VLP vaccine was recently published (Harper et al., 2006). Although the

Table 4
Mean antibody titers at month 0, 2, and 7 in vaccine recipients ($n=11$)

	Mean antibody titers (median)		
	Month 0	Month 2	Month 7
HPV-16	28±94 (0)	6927±16,699 (511.4)	12,663±15,060 (4929)
HPV-18	0 (0)	0 (0)	0 (0)
HPV-31	4±14 (0)	35.6±73.4 (0)	250±438 (50)
BPV	0 (0)	0 (0)	0 (0)

All placebo recipients ($n=5$) had mean titers of 0 at any time post vaccination to any of the HPV types.

Titers were determined using a pseudovirus neutralization assay, as described in Materials and methods.

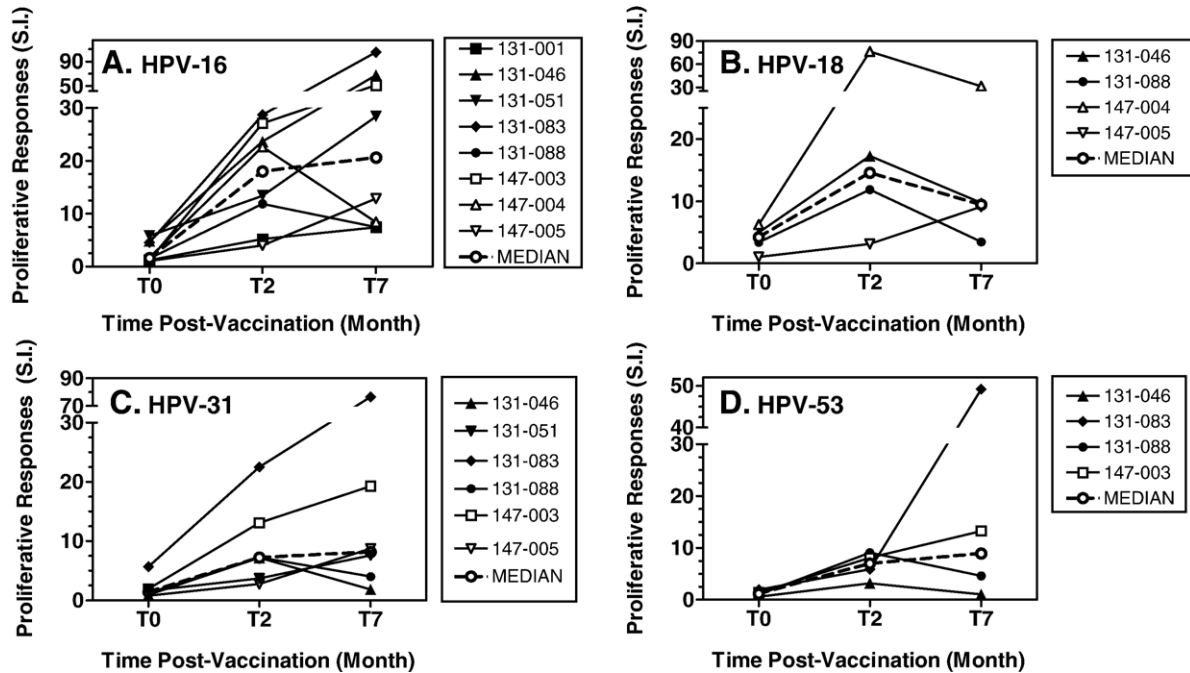


Fig. 3. Lymphoproliferative responses to HPV-16, -18, -31, and -53 VLPs before and over time after vaccination with HPV-16 L1 VLPs among responders. All vaccine recipients were tested but only responders to respective VLPs are shown. Results are expressed as Stimulation Indices (SI). Responders were classified as defined in Materials and methods. PBMC were cultured for 5 days in the presence of each of the antigens and proliferation was determined using a standard ^3H -thymidine incorporation method, as indicated in Materials and methods.

mechanism of cross-protection has not been yet determined, it is possible that a strong antibody and cellular immune response may contribute to this *in vivo* effect.

While sterilizing immunity mediated by neutralizing antibody responses remains the main goal for prophylactic HPV vaccination, cell-mediated immune responses should not be overlooked. CD4 T cell responses have been shown to play a role in the induction and maintenance of humoral responses (Yao et al., 2004; Zinkernagel et al., 1996) and CD4 and CD8 T cells or cytokines induced by HPV VLP vaccination (Emeny et al., 2002; Pinto et al., 2003) might well be capable of targeting HPV-infected cells, thereby potentially participating in the clinical benefit from HPV VLP vaccination. Future evaluation, in ongoing large-scale HPV VLP vaccine trials, of the efficacy of HPV VLP vaccination in the eradication of prevalent HPV infection is needed to directly address this possibility. Several questions remain to be answered, including: (1) whether cross-reactive T help induced by the vaccine would help in the rapid induction of an antibody response to infection by an heterologous HPV type; (2) whether T cell responses against the L1 protein of HPV can effectively target HPV infections at the basal layer of the epithelium, where levels of L1 expression are typically below detectable levels (De Bruijn et al., 1998; Firzlaff et al., 1988); (3) whether the levels of response demonstrated herein are sufficient to induce protection, and if so to what HPV types.

Responses to VLPs from heterologous types following immunization were in general weaker and more variable than those seen for HPV-16 VLP. Nevertheless, responses to HPV-31 correlated with responses to HPV-16 VLP (Table 3). HPV-31 is the most closely related heterologous HPV type to HPV-16

investigated, having approximately 83% homology (Chan et al., 1995; Roden et al., 1996a, 1996b). A significant correlation was also seen between HPV-16 L1 and HPV-53 VLP cellular immune responses, consistent with the fact that L1 shares substantial sequence homology between different HPV types and therefore, it is possible that T cell epitopes may be common across serologically distinct types (Chan et al., 1995; Roden et al., 1996a, 1996b). Weaker correlations were seen between HPV-18 and HPV-16. This reduced correlation might be explained by the high baseline responses to HPV-18 VLP, which made difficult the evaluation of changes over time for this VLP type. These high background responses may be related to the presence of contaminants from the system of production that were not present in the lysate controls used. Alternatively, responses may be related to a general immunogenicity of the HPV-18 VLP structure (Rudolf et al., 2001) or to previous T cell priming after exposure to the virus in some instances. The inclusion of additional controls for the system of VLP production, such as VLPs from unrelated viruses produced in a similar system may be of interest in future studies.

The inter-individual differences in the ability to respond to VLPs from different HPV types could be due to HLA differences. We did not see any clear patterns between responses and HLA class I or class II haplotypes (data not shown) but the number of individuals studied here was too small to address this possibility adequately. Alternatively, differences in T cell reactivity may be explained by differences in exposure to microbial antigens. The T cell repertoire available at the time of viral infection may affect the response to a particular virus (Welsh et al., 2004). Recent studies have revealed that each

Table 5
Percentage of proliferating (BrdU+) cells within CD4 and CD8, CD3 lymphocytes from vaccine recipients in response to HPV VLPs before and after immunization

Time ^a	Condition	%CD3+CD4+BrdU+ (mean±SD, %, n=6)	%CD3+CD8+BrdU+ (mean±SD, %, n=6)	
Pre	Media	0.017±0.028	0.025±0.022	
	HPV-16 VLP	0.022±0.023	0.005±0.008	
	HPV-18 VLP	0.369±0.210	0.474±0.446	
	HPV-31 VLP	0.016±0.017	0.001±0.003	
	HPV-53 VLP	0.144±0.188	0.215±0.402	
	Control lysate	0.011±0.012	0.018±0.021	
	Post	Media	0.023±0.0024	0.017±0.010
		HPV-16 VLP	1.293±1.285	0.474±0.447
		HPV-18 VLP	1.605±0.92	0.801±0.361
HPV-31 VLP		0.157±0.224	0.110±0.153	
HPV-53 VLP		0.793±0.699	0.767±0.442	
Control lysate		0.032±0.035	0.028±0.021	

Percentages were determined as described in Materials and methods.

^a Time of PBMC collection, before (pre) or after (post) immunization (months 2 or 7).

individual experiences a series of bacterial or viral infections, which shape the quality and quantity of the memory T cell pool. This preexisting T cell (memory) pool (Selin et al., 1994; Stockinger et al., 2004) may be activated and expanded by subsequent viral infections.

Consistent with previous findings (Pinto et al., 2003), the highest increments in responses were seen after the second immunization (i.e., at month 2). In a sizeable subset of individuals with evidence of increased response after the second immunization, however, further increases were observed one month following the third immunization. These results suggest that repeated immunizations might be effective at boosting cellular immune responses in a subset of recipients. Determinants of whether or not a vaccine recipient is likely to benefit from multiple booster immunizations are currently not known and will be of interest to investigate should ongoing trials indicate that HPV VLP-based vaccines can effectively target established HPV infections.

This is the first demonstration of generation of *in vitro* cross-reactive cellular immune responses to heterologous HPV types in individuals vaccinated with an HPV-16 L1 VLP vaccine. Cross-reactive lymphoproliferative responses to HPV-6 and HPV-16 have previously been reported in individuals vaccinated with HPV-11 VLP (Evans et al., 2001). This accumulating data suggest the presence of conserved T cell epitopes in L1 across serologically distinct HPV types. Larger studies are needed to identify specific L1 epitopes common across HPV types, and to evaluate the potential role of L1-directed T cell

responses in the induction and maintenance of neutralizing humoral responses and in the resolution of established HPV infections.

Materials and methods

Study design

Details of the study design have previously been reported (Pinto et al., 2003). In brief, a double-blind, randomized, placebo-controlled phase II trial was conducted at The John Hopkins University Center for Immunization Research (Baltimore, MD) to examine the safety and immunogenicity of three intramuscular injections (at 0, 1, and 6 months) of 50 µg of the L1 HPV-16 VLP vaccine, without adjuvant, in a group of healthy female volunteers 18–25 years of age who reported no more than four sexual partners in their lifetime. Subjects were evaluated clinically and blood specimens were collected prior to the initial vaccination (month 0) and 1 month following each subsequent vaccination (months 2 and 7). The vaccine was well tolerated and consistently induced high levels of antibodies, as reported previously (Harro et al., 2001).

The protocol for this study was approved by The John Hopkins University Institutional Review Board. The blood specimens earmarked for cell-mediated assays were shipped fresh to the monitoring laboratory where peripheral blood mononuclear cells (PBMC) were separated by density centrifugation over a Ficoll–Hypaque gradient and cryopreserved. Cryopreserved PBMC were available for the present study from 16 participants (11 vaccine and 5 placebo recipients).

HPV VLPs

Recombinant human papillomavirus (HPV) type 16 L1 virus-like particles (VLPs) expressed in the baculovirus system were used to investigate the cellular immune response to VLP vaccination. HPV-16 L1 VLP used for vaccination was expressed in baculovirus-infected Sf9 insect cells (Novavax, Rockville, MD), in accordance to GMP guidelines, as previously reported (Harro et al., 2001). The HPV VLPs used for *in vitro* assessment of CMI were produced by Dr. Viscidi (JHU) in insect cells (High Five, Invitrogen, Carlsbad, CA) from recombinant baculovirus expressing the L1 gene of HPV-18 and 53 and L1 and L2 of HPV-31, as previously described (Viscidi et al., 2003).

Lymphoproliferation assays

Lymphoproliferation assays were performed on cryopreserved PBMC collected before (month 0) and after (months 2 and 7) vaccination from a total of 11 vaccine and 5 placebo recipients (Harro et al., 2001). PBMC were plated in triplicate at 2×10^5 cells per well in 96 well round bottom plates (Costar, Cambridge, MA) AIM-V serum-free media (Gibco, Invitrogen). Cells were cultured in the presence or absence of VLPs from HPV-16, -18, -31, and -53 (2.5 µg/ml) diluted AIM-V serum-free media. Stocks of VLP preparations were provided from the

vaccine manufacturer to the laboratory at 0.8 mg/ml and 1.0 mg/ml. The purity of the HPV-16 VLPs was >96% as determined by SDS-PAGE. PHA (1:100, Sigma, St. Louis, MO) and influenza virus (1:100, ATCC, Manassas, VA) were used as controls for the assay. Cultures containing mitogens or antigens were pulsed with 1 μ Ci of 3 H-thymidine (Perkin Elmer, Wellesley, MA) for 18 h after 48 or 96 h of culture, respectively. Cultures were harvested and counted in an automated scintillation counter (Microbeta, Perkin Elmer). Results were expressed as cpm or stimulation indices (S.I.) determined as: cpm of cultures in the presence of antigen or mitogens/cpm of cultures in the presence of media alone.

Because the VLPs were purified from a baculovirus expression system, a Sf-9/baculovirus insect cell lysate (control lysate I) or a lysate from High Five cells (control lysate II, 0.1 μ g/ml) was used as control antigen for the system of production of the L1 VLPs in experiments performed to determine specificity of the VLP responses.

Cytokine induction assays

PBMC (at a final concentration of 1.5×10^6 /ml) were incubated in the absence or presence of PHA-M (1:100), Influenza virus (1:100), VLPs from HPV-16, -18, -31, and -53 (2.5 μ g/ml) for 3 days at 37 °C and 6% CO₂ in RPMI-1640 (Gibco, Invitrogen Life Technologies, Carlsbad, CA) supplemented with penicillin/streptomycin (100 μ g/ml/100 U/ml, Gibco, Invitrogen), Glutamine (2 mM), HEPES buffer (10 mM), and 10% FCS (Gibco, Invitrogen). Cell free supernatants were harvested and frozen at -20 °C. As described above for the lymphoproliferation assays, a Sf-9/baculovirus or High Five cell insect cell lysate (0.1 μ g/ml) was used as a control antigen in experiments performed to evaluate specificity of the VLP responses.

Cytokine determinations

Supernatants from the cytokine induction assay were thawed and tested in duplicate wells for IFN- γ , IL-10 and IL-5 by ELISA (Endogen, Woburn, MA), following manufacturers' instructions. The lower levels of detection for IFN- γ , IL-10 and IL-5 were 15.6, 7.8 and 6.4 pg/ml, respectively. Levels lower than the lowest detection levels were considered arbitrarily as half of the lowest detection level (7.8, 3.9 and 3.2 pg/ml, respectively).

5-Bromodeoxyuridine (BrdU) labeling and flow-cytometric analysis

This assay was performed as previously described (Pinto et al., 2003). Briefly, BrdU incorporation into CD4 and CD8 T cells from a subset of six vaccine recipients was determined before the initial immunization (i.e., at month 0) and after the second or third immunization (i.e., at months 2 or 7, respectively). PBMC cultured for 5 days at 37 °C in the presence of L1 VLPs (2.5 μ g/ml), control baculovirus lysate, and control media were incubated with 10 μ M BrdU (Sigma)

for the final 4.5 h of culture. Cell surface staining was performed with either anti-human CD3 PE (Becton Dickinson, San Jose, CA), anti-human CD4 PC5 (Beckman Coulter, Fullerton, CA), or anti-human CD8 ECD (Beckman Coulter) antibodies. The stained cells were treated with OptiLyse C lysing solution (Beckman Coulter) for 10 min at room temperature, followed by incubation, for 15 min at 37 °C, with 1% paraformaldehyde and 1% Tween-20 in PBS, to fix and permeabilize the cells. Cellular DNA in the permeabilized cells was partially digested, for 30 min at 37 °C, with DNase-I (Boehringer-Mannheim, Roche Applied Science, Indianapolis, IN) in DNase buffer (PBS with 4.2 mM MgCl₂, pH 5) and then was stained, for 30 min, with anti-BrdU FITC (Becton Dickinson) antibody in PBS containing 1% bovine serum albumin and 0.5% Tween-20. Cells were washed twice before flow-cytometric analysis. A target of approximately 100,000 CD3+ T cells was collected. Samples were stained and analyzed in parallel with unlabeled cells (without BrdU) from the same individual, and this value was subtracted from the value obtained for BrdU-labeled cells. Data are presented as the percentage of cells in the specific lymphocyte pool that are BrdU positive. The high sensitivity (0.01% BrdU+ cells) of this assay derives from analysis of large numbers of events (50,000–100,000), strong anti-BrdU-antibody staining of labeled cells, and low background binding of anti-BrdU antibody to unlabeled cells (Lempicki et al., 2000).

Neutralization assay

HPV-16, HPV-18, and HPV-31 neutralizing antibody titers were determined using a pseudovirus-based neutralization assay performed as previously described (Pastrana et al., 2004). Briefly, diluted pseudovirus (HPV-16, HPV-18, HPV-31, and BPV) carrying a secreted alkaline phosphatase (SEAP) reporter gene was combined with diluted serum for 1 h at 4 °C. This mixture of pseudovirus-antibody mixture was incubated with preplated 293 TT cells for 72 h, at 37 °C. At the end of the incubation, supernatant was harvested and clarified. The SEAP content in the clarified supernatant was determined using the Great EscAPE SEAP Chemiluminescence Kit (Becton Dickinson) as manufacturer's directions. Twenty minutes after the substrate was added, samples were read in a chemiluminescence reader (Molecular Devices, Sunnyvale, CA). Serum neutralization titers were defined as the reciprocal of the highest dilution that caused at least a 50% reduction in SEAP activity. A serum was considered to be positive for neutralization in the HPV-16, HPV-18, or HPV-31 assay if it was neutralizing at a dilution at least 4-fold higher than the titer observed in the BPV control neutralization assay. Neutralizing activity against HPV-53 was not determined because SEAP HPV-53 VLPs were not available. Undetectable antibody levels were considered 0. As shown in Table 4, sera from all HPV-16 recipients had detectable anti-HPV-16 neutralizing antibodies after vaccination. Median antibody titers in vaccine recipients ($n=11$) before vaccination were 0 (mean 28 ± 94 , ranges 0–313). Only one of the 11 vaccine recipients had detectable neutralizing antibodies at entry for

HPV-16 and -31 with an antibody titer of 313 and 46, respectively. Following vaccination, median antibody levels were 511 and 4929 at months 2 and 7 (means of $6927 \pm 16,699$; ranges 168 to 56,632 and of $12,663 \pm 15,060$; ranges of 997 to 55,930), respectively. Sera from vaccine recipients had no detectable anti-HPV-18 neutralizing activity before and after vaccination. Median anti-HPV-31 neutralizing titers was 0 at month 2 after vaccination (mean of 36 ± 73 , ranges 0 to 247) and 50 at month 7 (mean of 250 ± 438 , ranges 0 to 1248) after vaccination. HPV-31 neutralizing activity was detectable in 5 and 7 of the vaccine recipients at month 2 and 7, respectively. This activity was detectable in the serum samples with the highest median HPV-16 neutralizing titers (median HPV-16 antibody titers in sera with HPV-31 neutralizing activity = 9386, ranges 1223–56,632 versus median HPV-16 antibody titers in sera with undetectable HPV-31 neutralizing activity = 997, ranges 168–2271).

Median antibody titers in placebo recipients ($n=5$) at enrolment, months 2 and 7 were 0 (undetectable levels for all placebo recipients) for any of the types tested.

Statistical analysis

We defined responders as individuals who fulfilled the following criteria: (1) a minimum of 2-fold increase in response (measured as stimulation index-SI-for lymphoproliferation assay and pg/ml for cytokines) seen at month 2 relative to month 0, (2) a minimum response at month 2 of 2-fold relative to media control (SI > 2.0 for lymphoproliferation assay) or twice the lowest detectable level of the assay (for cytokines), and (3) ratio of responses at month 2/month 0 for VLP greater than that for the control lysate. This type of definition accounts for the variability on the VLP responses of these assays and has been used in other vaccine studies (Kang et al., 2004).

When evaluating responses to influenza, the first two criteria were applied. Based on the above definition, percent responders were estimated for each HPV type and for influenza. 95% confidence intervals (95% CI) were computed using exact methods. The percent of women responding to more than one heterologous HPV type was also estimated, and compared between responders and non-responders to HPV-16 VLP; p values were obtained based on the Fisher's exact test. For these estimates, only the lymphoproliferation, IFN- γ , and IL-10 assays were considered, given the low levels of IL-5 responses observed. Wilcoxon signed rank test was used to determine statistical differences between median to antigens before and after vaccination. To determine the relationships between responses to the different VLPs following vaccination, Spearman rank correlations were computed, along with their corresponding p values.

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