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MAJOR ARTICLE

Cellular Immune Responses 6 Years Following 1, 2, or 3 Doses of Quadrivalent HPV Vaccine in Fijian Girls and Subsequent Responses to a Dose of Bivalent HPV Vaccine

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Background. This study examined the cellular immunity of 0, 1, 2, and 3 doses of Gardasil vaccine (4vHPV) in girls after 6 years and their responses to a subsequent dose of Cervarix vaccine (2vHPV).

Methods. A subset of girls (n = 59) who previously received 0, 1, 2, or 3 doses of 4vHPV 6 years earlier were randomly selected from a cohort study of Fijian girls (age 15–19 years). Blood was collected before and 28 days after a dose of 2vHPV. The HPV16- and HPV18-specific cellular immune response was determined by IFNγ-ELISPOT and by measurement of cytokines in peripheral blood mononuclear cell supernatants.

Results. Six years after 4vHPV vaccination, HPV18-specific responses were significantly lower in the 1- (1D) or 2-dose (2D) recipients compared with 3-dose recipients (2D: IFNγ-ELISPOT: $P = .008$; cytokines, IFNγ: $P = .002$; IL-2: $P = .022$; TNFα: $P = .016$; IL-10: $P = .018$; 1D: IL-2: $P = .031$; IL-10: $P = .014$). These differences were no longer significant post-2vHPV. No significant differences in HPV16 responses (except IL-2, $P < .05$) were observed between the 2- or 1-dose recipients and 3-dose recipients.

Conclusions. These data suggest that cellular immunity following reduced-dose schedules was detectable after 6 years, although the responses were variable between HPV types and dosage groups. The clinical significance of this is unknown. Further studies on the impact of reduced dose schedules are needed, particularly in high–disease burden settings.

Keywords. adolescents; cellular immune responses; human papillomavirus vaccine; immune memory; reduced doses.

Cervarix (2vHPV) and Gardasil (4vHPV) HPV prophylactic vaccines have demonstrated remarkably high vaccine efficacy (>96%) against cervical cancer precursors in HPV-naïve individuals, when administered as standard 3-dose schedules [1–5]. The vaccines also induce high levels of type-specific antibody that persist for at least 8 and 9 years for 4vHPV and 2vHPV, respectively [6, 7].

In 2015, The World Health Organization (WHO) revised its recommendation from administering 3 doses of HPV vaccine to 2 doses 6 months apart, in girls <15 years old. This recommendation was based on clinical studies demonstrating non-inferior antibody responses in girls <15 years old who received 2 doses and women aged 16–26 years old who received 3 doses [8, 9]. However, the duration of antibody response following reduced-dose schedules has not been determined, with the longest follow up of antibody responses that were not statistically different between girls who received 1 or 2 doses (0, 6 month) and those who received 3 doses of 4vHPV and 2vHPV being 7 years [10, 11].

The biological basis of vaccination is the generation of immune memory characterized by the induction of specific memory T and B cell populations. The "help" provided by T helper cells are essential for the production of highly-specific neutralizing antibodies and immune memory. Cellular immune responses (ie, induction of memory B and/or T cells) have been reported in individuals vaccinated with 3 doses of either 2vHPV or 4vHPV, 1-month after the last vaccine dose, with only 1 study documenting cellular responses up to 4 years [8, 9, 12–14]. Studies on cellular immune response following reduced-dose HPV schedules, particularly over the long-term, are limited. We recently published data on the neutralizing antibody (NAb) responses to HPV16 and 18 in Fijian girls who were vaccinated with 1, 2, or 3 doses of 4vHPV 6 years previously, and their

Received 3 May 2018; editorial decision 14 June 2018; accepted 18 June 2018.

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DOI: 10.1093/ofid/ofy147

responses to a subsequent dose of 2vHPV [15]. We found that 6 years following their last dose of 4vHPV, 2-dose recipients had similar NAb response as 3-dose recipients. Although lower NAb levels were found in 1-dose recipients when compared with 2- or 3-dose recipients, their levels were at least 5-fold higher than unvaccinated girls after 6 years. We also found that post-2vHPV NAb levels were not statistically different between 1-, 2-, or 3-dose recipients. Here, we report the HPV-specific cellular immune responses in terms of IFNγ producing cells as a surrogate for T cell responses, and cytokine production in a subset of girls from each dosage groups 6 years following last dose of 4vHPV, and 1-month after a dose of 2vHPV.

METHODS

Study Design and Participants

Details of the selection criteria and the study procedures including the informed consent process were previously described in Toh et al. [15]. Briefly, a prospective cohort study was conducted in Fiji between February and March 2015. A total of 200 Fijian girls (now aged 15–19 years old) who were previously vaccinated with 0, 1, 2, or 3 doses of 4vHPV in 2008/9 were recruited. Each 4vHPV dosage groups comprised similar numbers of girls from the two main ethnic groups: indigenous Fijians (iTaukei) and Fijians of Indian Descent (FID). A subset (n = 15/group, pre- and postvaccination) of study samples made up of equal numbers of iTaukei and FID girls from each dosage group (due to our previous study finding ethnic variations in HPV NAb responses [15]) were randomly selected for analysis of cellular immunity to HPV16 and 18.

Study Procedures

Blood samples were collected on day 0 (6 years following last dose of 4vHPV) and 28 days following a single dose of 2vHPV (Cervarix, GlaxoSmithKline) to determine immunological responses. The blood samples were collected, processed and stored accordingly, as previously described in Toh et al. [15]. All laboratory staff were blinded to the vaccination status of each participant, and each sample was identified according to a unique study number.

Laboratory Procedures

Cryopreserved peripheral blood mononuclear cells (PBMCs) were thawed at 37°C, and diluted in R10 media (RPMI 1640, supplemented with 10% heat inactivated FBS [Hyclone, Utah, USA], and 1% 100x Penicillin/Streptomycin/L-glutamine [Gibco, New York, USA]) to 1 × 10^6 cells/ mL. PBMCs were stimulated with pooled peptides of HPV16 or HPV18 L1 proteins as previously described [14]. The assay controls include negative control: DMSO-PBS (diluent for HPV peptides) and positive control: PHA (5 μg/mL). PBMCs were stimulated for 48 h at 37°C and 5% CO_2 and were then harvested while supernatants were collected and stored at −80°C until used for cytokine analyses.

HPV-Specific IFNγ Producing Response

The number of HPV-specific IFNγ producing cells to HPV16 and HPV18 were measured using an ELISPOT method, according to manufacturer's protocol (BD Bioscience; San Diego, CA). The spots were counted using automated EliSpot Reader and software version 6.0 (AID GmbH, Strassberg, Germany).

Flow Cytometry

A parallel set of PBMC cultures were set up and stimulated as described above to enumerate the memory $CD4^+$ and $CD8^+$ populations based on the phenotype $CD4^+CD45RO^+IFN\gamma^+$ (memory $CD4^+$ T cells) or $CD8^+CD45RO^+IFN\gamma^+$ (memory $CD8^+$ T cells). Five-six hours before the end of the 48-hour incubation, protein inhibitor cocktail (eBioscience Inc, San Diego, CA) was added to each culture condition. The cultured PBMCs were then washed and stained with the following markers: CD4-PE, CD8-FITC, CD45RO-APC, and IFNγ-BUV737. Unstained PBMCs were used as a control and a minimum of 200 000 events per sample were analysed using a BD LSRII flow cytometer. Analyses of flow cytometry data were performed using the FlowJo v10.3 software (FlowJo LLC, Ashland, OR).

Multiplex-Bead Array Assay

A panel of Th1 (IFNγ, TNFα, IL-2) and Th2 (IL-5 and IL-10) cytokines were selected based on previous publications and their roles in vaccine response [16–18]. The concentrations of IFN-γ, TNF-α, IL-2, IL-10, and IL-5 in the supernatants of the PBMC cultures were determined using the Milliplex X_{MAP} kit (EMD Millipore, MA) according to manufacturer's protocol. The plate was then read on a Luminex 200 instrument and analysed using Luminex xPONENT software, version 3.1 (Luminex Corporation, TX).

Statistical Analysis

The primary analysis was the comparison of the number of HPV-specific IFNγ producing cells in girls who 6 years earlier received 1 or 2 doses of 4vHPV, with girls who received 3 doses using the Mann-Whitney *U* test. Secondary analyses were the comparison of the number of HPV-specific IFNγ producing cells 1-month after a dose of 2vHPV between the 1- or 2-dose group and the 3-dose group using the Mann-Whitney *U* test, and the comparison within each dosage groups were also performed using the Wilcoxon matched-pairs signed rank test. The cytokine responses and proportion of HPV-specific T cell populations (as determined by flow cytometry; $CD4^+45RO^+IFN\gamma^+$ or $CD8^+45RO^+IFN\gamma^+$) 6 years after the last 4vHPV dose, and 1-month post-2vHPV between the 1- or 2-dose group and the 3-dose group were also compared using the Mann-Whitney *U* test. Correlations analyses between the cellular (HPV-specific IFNγ producing cells [from ELISPOT] and cytokines) and antibody response (previously determined in Toh et al. [15]) were performed using the Spearman's rank test. All statistical analyses were performed using GraphPad Prism 7.0 software. Based

on previous published data [14], a sample size of 10 and 16 for HPV16 and 18, respectively would provide 60% power to detect a 20% difference in number of HPV16/18-specific IFNγ producing cells with a 2-sided 5% significance level.

Ethics Approval

The study was conducted according to a protocol approved by the Fiji National Research Ethics Review Committee, Fiji National Research Committee (2014.5.FNRERC.5.SU), as well as the Royal Children's Hospital Human Research Ethics Committee, Melbourne, Australia (34239A). The study was registered with clinicaltrials.gov, number NCT02276521.

RESULTS

The baseline characteristics of the girls in the whole study cohort were previously described in Toh et al. [15]. A subset of 59 girls were randomly selected from each dosage group (n = 15/group except for 2-dose group, n = 14 [due to limited number of samples]) to examine cellular immune responses. Each dosage group was made up of approximately equal numbers of both ethnic groups (iTaukei and FID). Overall, the subset of girls selected for these analyses had similar baseline characteristics as the whole study cohort, except that the girls in the 3-dose group were older at the time of recruitment, and also when the first dose of 4vHPV was given 6 years previously compared with the girls in the 2- and 1-dose groups (Table 1).

HPV-Specific IFNγ-Producing Cell Response Following 1- or 2-Doses of 4vHPV and Subsequent Responses to a Dose of 2vHPV

Six years after receiving the last dose of 4vHPV (pre-2vHPV), the HPV16-specific IFNγ producing cells in the 1- and 2-dose group were not significantly different from the 3-dose group. However, the HPV18-specific IFNγ producing cells in the 2-dose (P = .004), but not the 1-dose group were significantly lower than the 3-dose group (Figure 1). Following

Table 1. Baseline Characteristics

	Dosage Groups				
Characteristics	3 (n = 15)	2 (n = 14)	1 (n = 15)	0 (n = 15)	*P* Value[a]
Age at recruitment, median (IQR), y	18.0 (17.0–18.0)	16.0 (16.0–17.8)	17.0 (17.0–17.5)	18.0 (17.0–19.0)	.021
Age at first dose of 4vHPV, median (IQR)	12.0 (11.0–12.0)	9.8 (10.0–11.0)	9.0 (11.0–11.0)	NA	.001
Time between:					
Dose 1 and 2, median (IQR), mo	1.0 (1.0–6.0)	6.0 (1.0–11.0)	NA	NA	.008
Dose 1 and 3, median (IQR), mo	6.0 (6.0–8.0)	NA	NA	NA	NA
Time since last dose of 4vHPV, median (IQR), y	5.8 (5.7–5.8)	5.8 (5.4–6.3)	6.3 (6.3–6.3)	NA	<.0001
Height, median (IQR), cm	162.0 (145.0–168.0)	158.5 (153.8–162.0)	162.0 (154.0–169.0)	161.0 (130.0–170.0)	.587
BMI, median (IQR), kg/m^2	25.1 (17.3–29.0)	25.4 (21.2–27.1)	23.4 (19.2–25.7)	24.4 (22.1–28.9)	.763
Ethnicity, No. (%)					
iTaukei	7 (47)	7 (50)	7 (47)	7 (47)	0.997
Fijians of Indian descent	8 (53)	7 (50)	8 (53)	8 (53)	
Participant's education at time of enrollment, No. (%)					0.149
No schooling	1 (7)	1 (7)	1 (7)	3 (20)	
Secondary school	9 (60)	12 (86)	13 (86)	7 (47)	
University	5 (33)	1 (7)	1 (7)	5 (33)	
Partner status at enrollment, No. (%)					
Boyfriend/married	2 (13)	1 (7)	3 (20)	0 (0)	0.644
No. of children	0	0	0	1	1.000
Consumption of alcohol, No. (%)					0.455
Never consumed	10 (67)	8 (57)	10 (67)	8 (53)	
Currently < 1 alcoholic drink per wk	2 (13)	6 (43)	3 (20)	7 (47)	
Currently ≥1 alcoholic drinks per wk	3 (20)	0 (0)	2 (13)	0 (0)	
Consumption of kava, No. (%)					0.191
Never consumed kava	9 (60)	5 (36)	11 (74)	9 (60)	
<1 kava drink per wk	3 (20)	8 (57)	2 (13)	6 (40)	
≥1 kava drinks per wk	3 (20)	1 (7)	2 (13)	0 (0)	
Cigarette smoking, No. (%)					
Smoked ≥100 cigarettes in a lifetime	2 (13)	0 (0)	1 (7)	2 (13)	0.864
Seropositivity to HPV, No. (%)					
HPV 16	15 (100)	14 (100)	13 (95)	1 (7)	
HPV 18	14 (92)	11 (79)	9 (60)	0 (0)	

[a]The characteristics of the different dosage groups were compared using the 1-way analysis of variance test for continuous variables or the χ^2 test for categorical variables.

a dose of 2vHPV, both HPV16 and 18 IFNγ producing cells were not significantly different between the 1- and 2-dose group and the 3-dose group. Significantly higher HPV16-specific IFNγ producing cells were observed compared with HPV18-specific IFNγ producing cells pre- and post-2vHPV for all dosage groups ($P < .01$). Generally, higher HPV-specific IFNγ producing cells were observed in FID girls than in iTaukei girls for all dosage groups, although most comparisons within dosage groups were not significantly different (Supplementary Table 1).

Phenotypic Analysis of HPV-Specific IFNγ T-Cell Responses

Flow cytometric analyses were performed to determine the proportion of IFNγ-expressing memory T cells in girls who received reduced-doses of 4vHPV after 6 years and following a dose 2vHPV. The number of samples for this analysis were lower than for ELISPOT (n ≥7 depending on dosage groups) due to limited number of available cells. Overall, no significant differences in HPV16- and 18-specific $CD4^+CD45RO^+IFN\gamma^+$ cells were observed between all 4vHPV dosage groups pre- and post-2vHPV (Supplementary Figure 1). As expected, low levels of $CD8^+CD45RO^+IFN\gamma^+$+ populations were found across all dosage groups, pre- and post-2vHPV (Supplementary Figure 2).

Cytokine Responses Following Reduced-Dose 4vHPV Schedules and Subsequent Dose of 2vHPV

The cytokine responses 6 years after receiving the last dose of 4vHPV and post-2vHPV are summarised in Figure 2. The 2-dose group had significantly lower IL-2 in response to both HPV16 ($P = .022$) and 18 ($P = .013$), when compared with the 3-dose group (Figure 2). In addition, significantly lower IFNγ ($P = .002$), TNFα ($P = .016$), and IL-10 ($P = .018$) in response to HPV18 were also observed in the 2-dose group, when compared with the 3-dose group. Following 2vHPV, no significant differences were observed for any cytokines between the 2- and 3-dose groups for both HPV types. Table 2 summarises the results for all cytokines for the comparison of the 0-, 1-, and 2-dose groups with the 3-dose-group. Cytokine levels between the two ethnic groups were similar across the groups, with only a few exceptions (Supplementary Figure 3).

Correlation Analyses of Immune Parameters

We performed correlation analyses to determine the relationship between cellular and antibody measures (previously

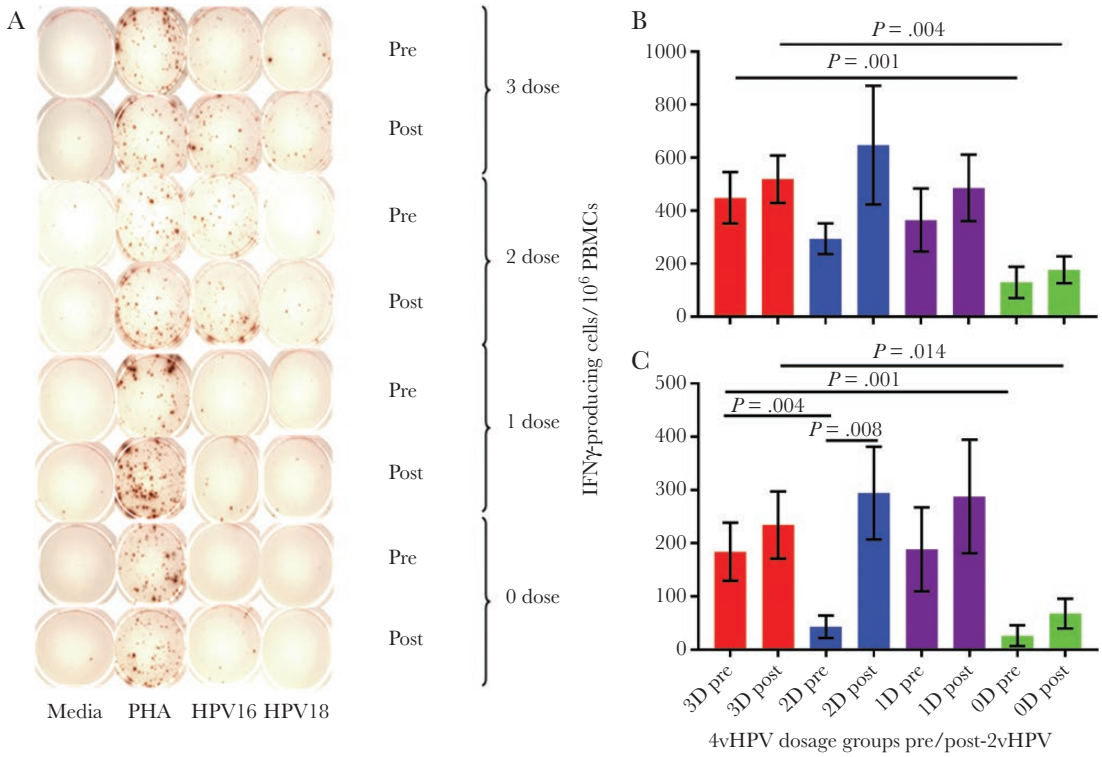

Figure 1. Representative ELISPOT well images for the negative and positive controls and human papillomavirus 16 (HPV16) and HPV18 stimulation for each dosage group (A). Number of HPV-specific IFNγ-producing cells per 10^6 peripheral blood mononuclear cells (PBMCs) to HPV16 (B) and HPV18 (C) 6 years after 0, 1, 2, or 3 doses of 4vHPV and 1 month after a single booster dose of 2vHPV. PBMCs were stimulated with HPV16 or HPV18 pooled peptides for 2 days, and IFNγ-producing cells were measured by ELISPOT. Red bars represent the 3-dose group (n = 15), blue bars represent the 2-dose group (n = 14), purple bars represent the 1-dose group (n = 15), and green bars represent the 0-dose group (n = 15). Bars represent mean ± standard error of mean (SEM).

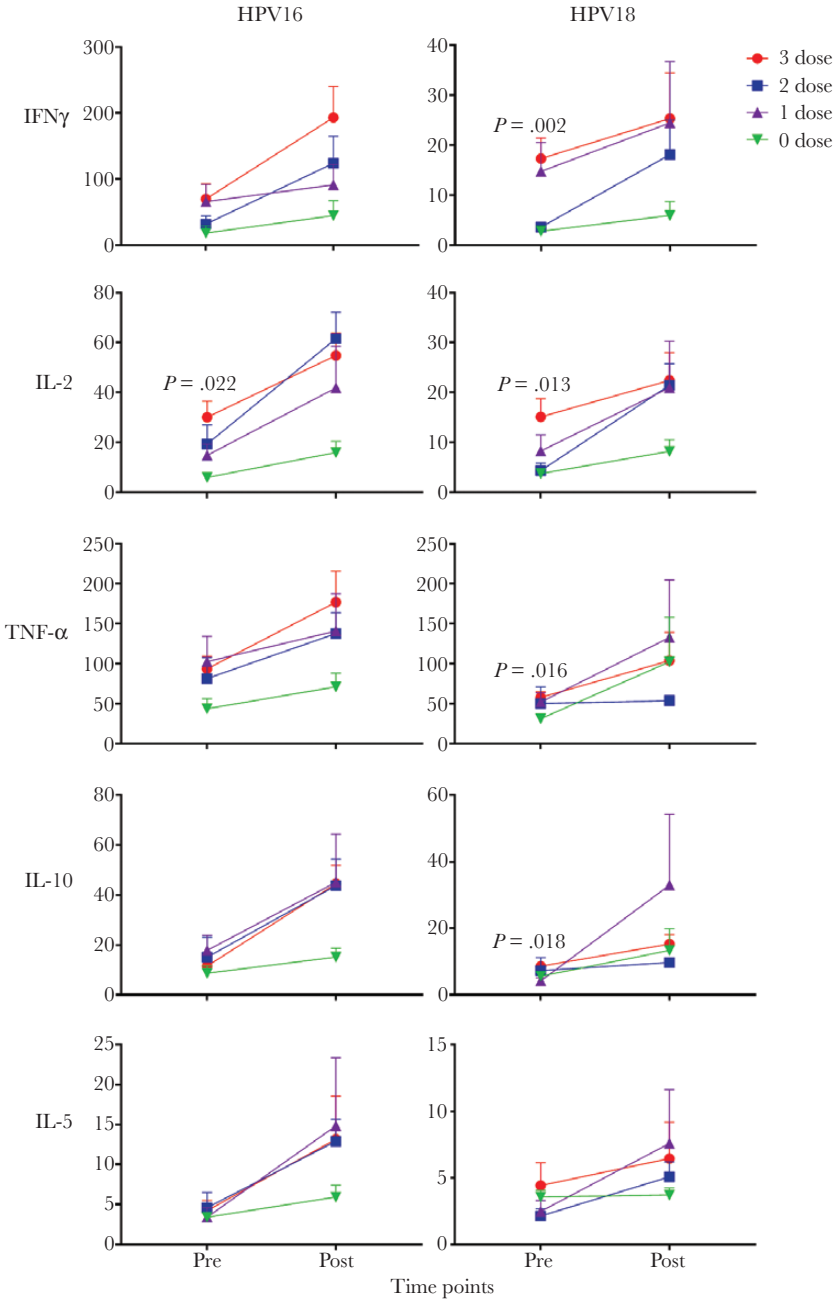

Figure 2. Peripheral blood mononuclear cells (PBMCs) cytokine responses to human papillomavirus 16 (HPV16) and HPV18 pre- (6 years after last vaccine dose) and post-2vHPV. The red symbol and line represent the 3-dose group (n = 15), the blue symbol and line represent the 2-dose group (n = 14), the purple symbol and line represent the 1-dose group (n = 15), and the green symbol and line represent the 0-dose group (n = 15). The error bars represent mean ± SEM. *P* values represent significant difference between the 3- and 2-dose groups.

published in Toh et al. [15]) of HPV immunity. The number of HPV-specific 16/18 IFNγ producing cells measured by ELISPOT had a moderate correlation with the NAb when the dosage groups were pooled (Figure 3). When stratified by dosage groups, this is only observed for the 2-dose group (Supplementary Figure 4). Although almost all HPV16-specific cytokines measured showed significant correlations with HPV16-specific NAb titres for all 4vHPV dosage groups (except for the 1-dose group), only some HPV18-specific cytokines in the 2-dose and 0-dose group had moderate statistically significant correlation with the HPV18-specific NAb titres (Supplementary Table 2). A moderate to strong correlation between the HPV-specific 16/18 cytokines measured and the number of HPV-specific 16/18 IFNγ producing cells were observed in most cases between the 4vHPV dosage groups pre- and post-2vHPV (Supplementary Table 3).

Table 2. Comparison of HPV16/18-Specific Cytokine Responses in the 0-, 1-, or 2-Dose Group with the 3-Dose Group 6 Years After 4vHPV and 1 Month After a Booster Dose of 2vHPV

	Pre-2vHPV							Post-2vHPV						
	[a]3 Dose (n = 15)	2 Dose (n = 14)		1 Dose (n = 15)		0 Dose (n = 15)		[a]3 Dose (n = 15)	2 Dose (n = 14)		1 Dose (n = 15)		0 Dose (n = 15)	
Dosage Groups/ Cytokines	Mean (95% CI), pg/mL	Mean (95% CI), pg/mL	*P* Value	Mean (95% CI), pg/mL	*P* Value	Mean (95% CI), pg/mL	*P* Value	Mean (95% CI), pg/mL	Mean (95% CI), pg/mL	*P* Value	Mean (95% CI), pg/mL	*P* Value	Mean (95% CI), pg/mL	*P* Value
HPV16														
IFNγ	69.9 (20.9 to 119.0)	31.9 (5.7 to 58.2)	.093	65.8 (10.2 to 121.5)	0.430	18.3 (–5.5 to 42.1)	**.001**	193.2 (92.1 to 294.2)	123.8 (34.9 to 212.8)	.146	91.1 (24.2 to 157.9)	**.029**	44.6 (–4.4 to 93.5)	**<.001**
IL-2	30.1 (16.4 to 43.8)	19.4 (3.0 to 35.8)	**.022**	14.8 (5.1 to 24.5)	**0.040**	6.0 (2.6 to 9.3)	**<.001**	54.8 (35.4 to 74.3)	61.6 (38.8 to 84.4)	.591	41.9 (6.0 to 77.7)	.057	16.0 (6.4 to 25.6)	**<.001**
TNFα	93.5 (60.0 to 127.1)	81.4 (24.7 to 138.1)	.217	102.4 (34.8 to 170.1)	0.595	43.7 (16.9 to 70.5)	**.003**	176.7 (93.6 to 259.8)	137.7 (81.5 to 193.9)	.354	140.8 (41.3 to 240.4)	.098	70.9 (34.3 to 107.5)	**.004**
IL-10	11.6 (7.9 to 15.2)	15.1 (–2.1 to 32.3)	.120	17.8 (5.0 to 30.7)	0.751	8.7 (2.3 to 15.1)	**.046**	44.5 (28.9 to 60.2)	43.8 (20.9 to 66.6)	.591	45.1 (3.8 to 86.4)	**.032**	15.2 (7.7 to 22.7)	**<.001**
IL-5	4.2 (1.4 to 7.0)	4.6 (0.5 to 8.7)	.659	3.4 (0.9 to 5.9)	0.298	3.4 (2.4 to 4.4)	.736	13.2 (1.6 to 24.7)	12.9 (7.0 to 18.8)	.41	14.8 (–3.5 to 33.1)	.055	5.9 (2.7 to 9.1)	.164
HPV18														
IFNγ	17.3 (8.6 to 26.1)	3.6 (1.7 to 5.6)	**.002**	14.8 (2.3 to 27.2)	0.132	2.8 (0.7 to 4.9)	**<.001**	25.3 (5.8 to 44.9)	18.1 (3.1 to 33.1)	.331	24.4 (–1.8 to 50.7)	**.043**	6.0 (0.0 to 11.9)	**<.001**
IL-2	15.1 (7.3 to 22.9)	4.4 (1.2 to 7.6)	**.013**	8.3 (1.3 to 15.2)	**0.031**	3.8 (1.5 to 6.0)	**.026**	22.4 (10.5 to 34.3)	21.5 (12.2 to 30.7)	.949	21.0 (0.9 to 41.0)	.068	8.2 (3.3 to 13.2)	**.015**
TNFα	57.9 (42.9 to 72.9)	50.3 (5.5 to 95.04)	**.016**	52.1 (27.8 to 76.3)	0.461	31.2 (21.1 to 41.4)	**.005**	103.9 (28.1 to 179.8)	53.6 (39.3 to 67.9)	.377	132.8 (–21.7 to 287.3)	.098	102.5 (–16.4 to 221.4)	.267
IL-10	8.6 (5.6 to 11.7)	7.3 (–1.0 to 15.7)	**.018**	4.2 (2.3 to 6.1)	**0.014**	5.6 (3.3 to 8.0)	.099	15.3 (9.2 to 21.3)	9.7 (6.9 to 12.6)	.158	33.0 (–12.6 to 78.5)	.060	13.4 (–0.5 to 27.2)	**.044**
IL-5	4.4 (0.8 to 8.1)	2.1 (1.0 to 3.3)	.470	2.5 (0.7 to 4.2)	0.530	3.6 (2.5 to 4.6)	.243	6.5 (0.6 to 12.3)	5.1 (2.7 to 7.5)	.723	7.6 (–1.1 to 16.2)	.289	3.8 (2.6 to 4.9)	.943

Abbreviations: 2vHPV, Cervarix, GSK; CI, confidence interval; pre-2vHPV, 6 years after 4vHPV; post-2vHPV, 1 month after a booster dose of 2vHPV.

[a]Comparison group.

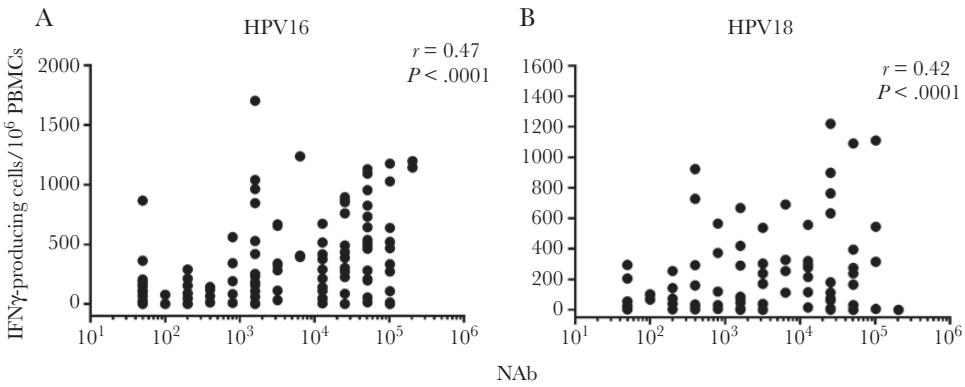

Figure 3. Correlation between IFNγ-producing cells (from ELISPOT) and neutralizing antibody titers (NAb) specific to human papillomavirus 16 (HPV16) (A) and HPV18 (B), regardless of dosage group (n = 59).

DISCUSSION

This study examined the cellular immune responses in girls who received fewer than 3 doses of 4vHPV 6 years previously and their subsequent responses to a single dose of 2vHPV. To our knowledge, this is the first study that has examined cellular immunity in girls who received only 1 dose of 4vHPV. Reduced-dose HPV schedules, particularly 1-dose schedule is of significant interest globally. A single dose of HPV vaccine were found to induce antibodies that last for 7 years (albeit lower levels than 2 or 3 doses) and are protective against vaccine type HPV infections [10, 11]. Whilst the NAbs are thought to be the mechanism of action for HPV vaccine and correlate with protection, the role of cellular immunity in long-term protection is poorly understood.

Our study found that although there was no significant difference in the number of HPV16-specific IFNγ-producing cells between the 1- or 2-dose group and 3-dose group after 6 years, significantly lower number of IFNγ-producing cells to HPV18 were observed in the 2-dose group when compared with the 3-dose group. Interestingly, this was not the case for the 1-dose group. Additionally, both 1- and 2-dose groups also had significantly lower cytokine (ie, IL-2 and IL-10) levels to HPV18 when compared with the 3-dose group. The lower HPV18 responses observed in the 2-dose group could be due to a chance finding associated with the small sample size, hence variability in responses. This explanation is supported by the fact that the number of IFNγ-producing cells in the 3- and 1-dose groups in this study were similar to previous studies of $CD4^+$ T cell responses 4 years following 4vHPV vaccination [12], and that no statistically significant differences in antibody levels were observed between the 2 and 3 dose groups in this study [15]. A longitudinal study of this cohort including both antibody and cellular immune responses may help to address this finding. Another possible explanation could be due to increased priming of cellular immune responses from prior or current exposure to HPV18 among girls in the 1-dose group, although this is unlikely based on low HPV16/18 prevalent infections among pregnant Fijian women who previously received 2 or 1 dose of 4vHPV 6 years earlier (F. Russell, MBBS, PhD, personal communication, 2017). However, it is still possible that prior exposure and subsequent clearance of infection may have also occurred. These results are consistent with the generally lower immune responses induced to HPV18 than to HPV16 observed in this study and other previous studies [19–22]. It remains to be determined whether these lower cellular immune responses (IFNγ-producing cells and cytokine responses) to HPV18 among girls who received <3 doses of 4vHPV are associated with an increased risk of HPV infection, although there are currently no data that suggest this.

Following a dose of 2vHPV, cellular immunity was not significantly different between girls who received 1-, 2-, or 3-doses of 4vHPV 6 years previously. This suggests that while the priming response following reduced-dose 4vHPV may have differed, this did not affect the ability to boost cellular immune responses following 2vHPV. Larger studies are needed to confirm this finding. We also found a moderate correlation between the NAb and IFNγ-producing cells for both HPV16 and 18, suggesting a possible relationship between these two parameters, and highlight the role of cellular immune response (IFNγ-producing cells) in antibody production. This correlation was less pronounced when the responses were stratified by dosage groups, which is likely due to the smaller sample size.

Studies evaluating cellular immune responses following vaccination with HPV prophylactic vaccines (ie, 2vHPV, 4vHPV and 9vHPV) are limited, particularly in the context of reduced-dose HPV schedules. Although NAb are recognised as the primary marker of protection against HPV infection, cellular immune responses are also likely to have an important role and is suggested to better predict long-term immunity following vaccination [23]. Einstein et al. compared the immunogenicity induced by 3 doses of either 2vHPV or 4vHPV in women aged 18–45 years

and found higher T cell responses (characterised by the expression of 2 of these 4 markers on $CD4^+$ cells: CD40L, IFNγ, TNFα, IL-2) induced by 2vHPV than 4vHPV to both vaccine (HPV16 and 18) and nonvaccine HPV types (HPV31 and 45), 1-month and at 2 and 4 years following the last vaccine dose [12, 24, 25]. The induction of T cell responses to nonvaccine types in unvaccinated individuals has also been demonstrated, suggested to be due to shared peptide epitopes between different HPV types [26]. Whether this is also true following vaccination is unknown. In terms of reduced-dose 4vHPV schedules, Puthanakit et al. found similar HPV-specific $CD4^+$ T-cell responses in girls aged 9–14 years who received 2 doses of 2vHPV and women aged 15–25 years who received 3 doses, 1- and 6-month after the last dose [27]. However, Smolen et al. found both HPV16 and 18-specific T cells by IFNγ- ELISPOT assay were lower in girls who received 2 doses compared to 3 doses, 1-month after the last dose of 4vHPV [14]. Our results extend these findings to 6 years. However, it is important to note that the differences in the study populations, methodologies and experimental procedures used in these studies makes comparison of results difficult.

In contrast to our results by IFNγ-ELISPOT assay, we did not observe any significant differences in the proportion of $CD4^+$ memory T cells that express IFNγ by flow cytometry. This could be due to differences in the specificity and sensitivity of the methods. The IFNγ ELISPOT assay measures total IFNγ secretion by different immune cell populations and are more sensitive in detecting low level responses [28, 29], whereas the flow cytometry assay measures intracellular IFNγ production by specific immune cells.

The significant increase in Th1 (IFNγ, IL-2 and TNFα) or Th2 (IL-10 and IL-5) cytokines measured in this study is consistent with previous studies using either the first generation HPV16 VLP vaccine (which did not contain any adjuvant) or following 3 doses of 2vHPV [16, 17, 30]. The Th1 cytokines are important as they promote antiviral activity and are involved in memory responses, whereas the Th2 cytokines promote robust antibody responses [18]. Detailed studies that investigate the role of different immune cell populations following HPV vaccination may elucidate important mechanisms associated with protection against HPV infection. It is also important to note that the different adjuvants in 4vHPV/9vHPV and 2vHPV may contribute to the differences in immunogenicity observed and may explain the higher immune responses observed for 2vHPV than 4vHPV [31, 32]. It is therefore possible that a difference in magnitude of cellular immune response may be found if the same vaccine were used for priming and boosting.

There are some limitations in this study. First, the sample size for each group is relatively small, particularly for comparison of responses between ethnicities, and therefore these results should be interpreted with caution. However, the novelty of this study represents an important contribution to our knowledge of how HPV vaccines induce robust NAb responses. Secondly, the lack of a defined HPV epitope for T-cell stimulation assays may have underestimated the actual response. Nevertheless, we were still able to detect significant differences in cellular immune responses using this approach. Thirdly, we did not have any sexual activity data throughout the follow-up period (6 years) to correlate with these immune parameters due to ethical considerations of self-swabbing in an otherwise healthy female cohort. Further studies incorporating both long-term HPV prevalence and immunological data will be critical to help our understanding of the mechanisms of HPV vaccine-induced protection.

In summary, this is the first study of its kind to document the elevated cellular immune responses in girls who were previously vaccinated with at least 1 dose of 4vHPV when compared with unvaccinated girls, 6 years following the last dose of 4vHPV, and also following a dose of 2vHPV. Our results suggest that reduced-dose 4vHPV schedules may be effective at inducing long-lasting immune memory, even after only 1 dose, although larger clinical studies are needed to validate these findings. Furthermore, it will be critical to determine whether the lower cellular immunity observed for HPV18 following reduced doses of 4vHPV is associated with any increase in risk of HPV infection over the long-term, especially in high-risk populations. These data highlight the continued need for rigorous HPV vaccination programmes in settings where there remains a high burden of disease.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Acknowledgments

We thank the study's participants and families for their involvement in this study, as well as the Fiji Ministry of Health and Medical Services, all the staff in Mataika House, and all the National Vaccine Evaluation Project staff. We would also like to thank Matthew Burton for his help with flow cytometry and analysis. We would like to thank the staff of Fiji Health Sector Support Program (FHSSP), in particular, Kylie Jenkins and Karen Kenny.

Financial support. This work was supported by the Department of Foreign Affairs and Trade of the Australian Government and FHSSP. FHSSP is implemented by ABT Associates on behalf of the Australian Government. This work was also supported in part by the Victorian Government's Operational Infrastructure Support Program. Z.Q.T. is a recipient of the Melbourne Research Scholarship from The University of Melbourne. F.M.R. is a recipient of the National Health and Medical Research Council (NHMRC) Early Career Fellowship. P.V.L. is a recipient of the NHMRC Career Development Fellowship.

Potential conflicts of interest. S.M.G. reports grants from Merck, GSK, CSL, and the Commonwealth Department of Health; reports nonfinancial support from Merck, outside the submitted work; and delivered lectures and received speaking fees from Merck for work performed during personal time. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Prior presentation. The study results were presented at the 31st International Papillomavirus Conference (Cape Town, South Africa, 2017) and EUROGIN (Amsterdam, Netherlands, 2017).

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