Molecular Therapy
Methods & Clinical Development
Original Article

Phase 1 Safety and Immunogenicity Trial of Recombinant *Lactococcus lactis* Expressing Human Papillomavirus Type 16 E6 Oncoprotein Vaccine

Sedigheh Taghinezhad-S,[1] Amir Hossein Mohseni,[1] Hossein Keyvani,[2] and Mohammad Reza Razavi[3]

[1]Department of Microbiology, Faculty of Basic Sciences, Science and Research Branch, Islamic Azad University, Tehran 1477893855, Iran; [2]Department of Virology, Faculty of Medicine, Iran University of Medical Sciences, Tehran 1449614535, Iran; [3]Department of Parasitology, Pasteur Institute of Iran, Tehran 1316943551, Iran

The present study purposed to investigate the safety, tolerability, and immunogenicity of the therapeutic NZ8123-HPV16-optiE6 vaccine, following oral vaccination. The safety and tolerability were evaluated. Specific serum immunoglobulin G (IgG) and vaginal IgA antibodies were calculated by ELISA, and E6-specific IFN-γ-secreting T cells were counted by enzyme-linked immune absorbent spot (ELISpot) assay in cervical lymphocytes and PBMC samples. The vaccine was well tolerated, and no serious adverse effects were observed in vaccine recipients. Statistical analysis showed that all vaccine groups had significant increases in antibody levels at day 60 after baseline. The time to peak activation in E6-specific IFN-γ-secreting $CD8^+$ CTL responses was seen at month 1 after last vaccination. According to the results, the humoral immune and cell-mediated responses for the vaccine groups that received 5×10^9 and 1×10^{10} CFU/mL of vaccine were similar and were higher than those of the 1×10^9 CFU/mL group, indicating the dose-dependency of the NZ8123-HPV16-optiE6 vaccine following oral administration. Low antibody levels compared with the placebo groups were recorded at month 6 after the last vaccination. Interestingly, long-term E6-specific CTL responses were observed during follow-up. It was concluded that oral immunization with the NZ8123-HPV-16-optiE6 vaccine is safe, induces persistent immunity, and is reasonably well tolerated.

INTRODUCTION

Human papillomavirus (HPV) is a major risk factor for the development of cervical cancer, the second most common cancer among women.[1] HPV types 16 and 18 cause 70% of all cancers and high-grade pre-cancers. The early E6 and E7 oncoproteins remain uncontrollably expressed, drive cellular immortalization, progress toward cellular transformation, and ultimately result in cancer development.[2] Therefore, the E6 and E7 oncogenes represent ideal targets for gene-specific therapy of cervical cancer.[3,4] The well-established link between HPV and anogenital cancers, high- and low-grade dysplasia, and genital warts has led to the development of prophylactic HPV vaccines.[5] Additionally, these vaccines are not intended to treat pre-existing HPV infections and associated malignancies, which require therapeutic vaccines that primarily target the E6 and E7 HPV oncoproteins. Immunization with E6 or E7 HPV-16 with the resultant generation of antigen-specific CTLs (cytotoxic T lymphocytes) has been a frequent immunotherapeutic approach for HPV-associated neoplasia and has utilized a wide array of potential vaccine delivery systems.[6,7]

Newly, scientists have focused on improvement of new, safe, mucosally administered vaccines for which production is less laborious, less cost-consuming, and more easily usable.[8,9] Live bacteria, including probiotics, can be engineered to the deliver target antigen to stimulate the host immune system. The superiority of these live bacterial vaccine vectors is that they can provoke strong humoral and cellular immunity. Therefore, scientists try to use probiotic bacteria as delivery systems of heterologous antigens, which may help in designing such vaccines.[10,11] Several excellent recent articles detail *Lactococcus lactis* as vaccine vectors and focus on the resulting immune responses generated *in vivo*.[12]

There are several benefits of the use of the *L. lactis* vaccine vector: *L. lactis* is generally regarded as safe, it has intrinsic adjuvant properties, it does not possess endotoxic lipopolysaccharides, it is inexpensive to produce, it can be administered repeatedly because it survives only temporarily in the intestinal tract, and it does not colonize in humans.[13,14] Data from our previous research in mouse tumor models demonstrated that oral immunization with HPV-16 E6 vaccine (NZ8123-HPV16-optiE6) induced clinically active responses leading to regression of established tumor lesions. These responses were associated with the appearance of robust mucosal E6-specific antibody and CTL responses induced by the vaccine.[15]

In the present study, we administered orally escalating doses of an HPV type 16 E6 oncoprotein candidate vaccine to 46 healthy adults

Received 15 April 2019; accepted 14 August 2019;
https://doi.org/10.1016/j.omtm.2019.08.005.

Correspondence: Amir Hossein Mohseni, Department of Microbiology, Faculty of Basic Sciences, Science and Research Branch, Islamic Azad University, Tehran 1477893855, Iran.
E-mail: amho.mohseni@gmail.com
Correspondence: Hossein Keyvani, Department of Virology, Faculty of Medicine, Iran University of Medical Sciences, Tehran 1449614535, Iran.
E-mail: keyvanlab@yahoo.com

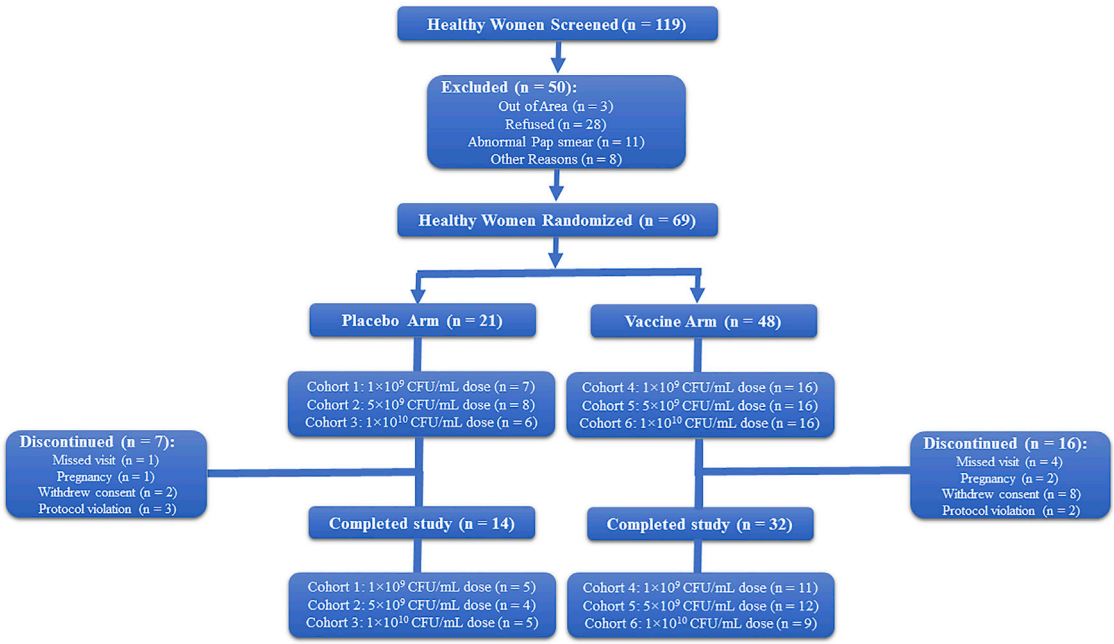

Figure 1. Work-Flow Chart
Flow diagram of patients referred for phase 1 study and reasons for exclusion.

without serologic evidence of previous HPV-16 infection. The purpose of the study was to evaluate the tolerability, safety, and antigenicity of the vaccine. Antigenicity was assessed by measuring antibody levels and by determining cytokine responses in cervical lymphocytes and peripheral blood mononuclear cells (PBMCs) after *in vitro* stimulation.

RESULTS

Characteristics of Study Participants

Of the 119 subjects enrolled, 46 (38.65%) were included in the per protocol population. They had a mean age of 35.5455 years (range 30.1746 to 40.9163 years) and a mean body mass index (BMI) of 22.1308 (range 18.9460 to 25.3156 BMI). A summary of all subjects who participated and discontinued the study is presented in Figure 1. The active vaccine groups were younger (n = 32; mean age = 36.1818 years; range 30.9346 to 41.4290 years; $p < 0.0001$; 95% confidence interval [CI]) than the placebo group (n = 14) with a mean age of 37.0000 (range 31.8131 to 42.1869 years; $p < 0.0001$; 95% CI). All enrolled patients were healthy Iranian females. A few had histories of previous STDs (one patient with chlamydia; two patients with genital herpes). The key demographic characteristics were generally similar between the vaccine and placebo groups (Table 1). One participant had no sex activity, 34 subjects had one sex partner, three had two partners, and one had four partners during the year before the study.

Safety and Tolerability

The NZ8123-HPV-16-optiE6 vaccine was well tolerated at all dosage levels. No serious vaccine-related adverse events (AEs) occurred as defined by the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE; version 3.0). The most common systemic clinical AEs were nausea and vomiting, and most of these were mild to moderate in intensity. Few of the immunized subjects at either dose (1×10^9 and 5×10^9 colony-forming units [CFU]/mL) experienced AEs based on the history taken before and after each successive vaccination. However, three subjects (33.33%) in the vaccine group and two (40%) in the placebo group experienced nausea and vomiting after immunization with the 1×10^{10} CFU/mL dose, but this difference was not significantly different ($p = 0.6213$) (Table 2). Consistently, no adverse side effects were recorded in all groups (vaccine or placebo) 6 months after vaccination.

Detection of HPV-16 E6-Specific Antibodies

Induction of HPV-16 E6-specific antibody responses was determined by measuring the antigen-specific serum immunoglobulin G (IgG) and vaginal IgA level by ELISA assay in all patients before vaccination and among patients who had completed the vaccination schedule. No subject had serum antibodies levels before vaccination.

None of the placebo recipients seroconverted; conversely, the paired sample t test analysis proved that all active groups had statistically significant fold increases in IgG antibody level ($p = 0.0563$ for 5×10^9 CFU/mL cohorts and $p = 0.0059$ for 1×10^{10} CFU/mL cohorts), except for the 1×10^9 CFU/mL cohort, at 30 days after vaccination ($p = 0.0123$). IgG responses generally peaked at 60 days after vaccination. They ranged from 0.3021 to 0.5179 in the 1×10^9 CFU/mL dose (mean difference, 0.4100; $p = 0.0005$; 95% CI), 0.2480 to 1.3820 in the 5×10^9 CFU/mL dose (mean difference: 0.8150; $p = 0.0196$; 95% CI),

Table 1. Baseline Demographics of Female Study Participants by Vaccination Group at Enrollment

		Study Arm					
		Vaccine Groups (CFU/mL Dose)			Placebo Groups (CFU/mL Dose)		
		1×10^9 (n = 11)	5×10^9 (n = 12)	1×10^{10} (n = 9)	1×10^9 (n = 5)	5×10^9 (n = 4)	1×10^{10} (n = 5)
Demographic	Status	Number (Percentage)					
Age	17–25 years	2 (18.18)	1 (8.33)	3 (33.33)	2 (40)	1 (25)	1 (20)
	26–35 years	4 (36.36)	5 (41.66)	2 (22.22)	2 (40)	2 (50)	2 (40)
	36–46 years	3 (27.27)	4 (33.33)	3 (33.33)	0 (0)	1 (25)	1 (20)
	47–56 years	2 (18.18)	2 (16.66)	1 (11.11)	1 (20)	0 (0)	1 (20)
Body mass index (BMI)	underweight = <18.5	2 (18.18)	1 (8.33)	2 (22.22)	0 (0)	0 (0)	1 (20)
	normal weight = 18.5–24.9	4 (36.36)	3 (25)	1 (11.11)	0 (0)	1 (25)	0 (0)
	overweight = 25–29.9	2 (18.18)	3 (25)	1 (11.11)	2 (40)	1 (25)	1 (20)
	obesity = BMI of 30 or greater	3 (27.27)	5 (41.66)	5 (55.55)	3 (60)	2 (50)	3 (60)
Marital status	married	6 (54.54)	5 (41.66)	3 (33.33)	1 (20)	1 (25)	3 (60)
	divorce – widow	2 (18.18)	5 (41.66)	3 (33.33)	2 (40)	3 (60)	0 (0)
	single	3 (27.27)	2 (16.66)	3 (33.33)	2 (40)	0 (0)	2 (40)
Age at first sexual intercourse	≤16	1 (9.09)	2 (16.66)	2 (22.22)	1 (20)	0 (0)	0 (0)
	17	5 (45.45)	3 (25)	2 (22.22)	1 (20)	1 (25)	1 (20)
	18	3 (27.27)	5 (41.66)	3 (33.33)	1 (20)	1 (25)	2 (40)
	19	1 (9.09)	2 (16.66)	2 (22.22)	2 (40)	1 (25)	2 (40)
	20≤	1 (9.09)	0 (0)	0 (0)	0 (0)	1 (25)	0 (0)
Smoking status	never smoked	8 (72.72)	10 (83.33)	8 (88.88)	3 (60)	4 (80)	4 (80)
	ex-smoker	1 (9.09)	1 (8.33)	0 (0)	0 (0)	0 (0)	0 (0)
	current smoker	2 (18.18)	2 (16.66)	1 (11.11)	1 (20)	0 (0)	0 (0)

and 0.4882 to 1.2998 in the 1×10^{10} CFU/mL dose (mean difference, 0.8940; p = 0.0036; 95% CI). The month 2 antibody levels for the groups that received 5×10^9 and 1×10^{10} CFU/mL of vaccine were similar (p = 0.4212) and were significantly higher than the month 2 antibody levels for the group that received 1×10^9 CFU/mL of vaccine (p = 0.0044). The humoral response was live with antibodies persisting 1 month after the last vaccination (day 90) (p = 0.0304 for 1×10^9 CFU/mL cohorts, p = 0.0506 for 5×10^9 CFU/mL cohorts, and p = 0.0075 for 1×10^{10} CFU/mL cohorts). Statistically significant differences were seen in all active study vaccine groups compared with the placebo control groups at month 6 after the fourth vaccination (day 240) (p = 0.0326 for 5×10^9 and p = 0.0030 for 1×10^{10} CFU/mL cohorts), apart from the 1×10^9 CFU/mL cohort (p = 0.1260). Surprisingly, a statistically significant decrease was observed in the 1×10^9 CFU/mL cohort compared with the 5×10^9 and 1×10^{10} CFU/mL cohorts in the same volunteers at month 6 after the fourth vaccination (day 240) (p = 0.0024 and p = 0.0006, respectively). Nevertheless, no statistically significant difference was recorded between vaccine groups 5×10^9 and 1×10^{10} CFU/mL cohorts at month 6 after the fourth vaccination (day 240) (p = 0.7716). In comparison with month 1 after the last vaccination (day 90), no statistically significant difference was recorded between vaccine groups 5×10^9 and 1×10^{10} CFU/mL cohorts at month 6 after the fourth vaccination (day 240) (p = 0.0878 and p = 0.0733, respectively), except for the 1×10^9 CFU/mL cohort (p = 0.0019) (Figure 2, left panel).

Vaginal fluids from participants who received complete immunization programs were also evaluated for IgA-specific antibodies. All vaccine recipients became weakly seropositive for IgA; however, relative to IgG responses, the responses to IgA were weak and variable.

Paired sample t test analysis showed that all vaccine groups had statistically significant fold increases in IgA antibody levels at 30 days after vaccination compared with the placebo groups (p = 0.0269 for 1×10^9 CFU/mL cohorts, p = 0.1109 for 5×10^9 CFU/mL cohorts, and p = 0.0505 for 1×10^{10} CFU/mL cohorts). In comparison with the placebo groups, IgA responses generally peaked at 60 days after vaccination were 0.06646 to 0.2895 in the 1×10^9 CFU/mL dose (mean difference, 0.1780, p = 0.0114; 95% CI), 0.08417 to 0.5758 in the 5×10^9 CFU/mL dose (mean difference, 0.3300; p = 0.0235; 95% CI), and 0.2157 to 0.4563 in the 1×10^{10} CFU/mL dose (mean difference, 0.3360; p = 0.0015; 95% CI). The month 2 antibody levels for the groups that received 5×10^9 and 1×10^{10} CFU/mL of vaccine were similar (p = 0.8889) and were significantly higher than the month 2 antibody levels of the group that received 1×10^9 CFU/mL of vaccine (p = 0.0001 for CFU/mL cohorts and

Table 2. Summary of Adverse Effects

Adverse Event	Placebo Arm (n = 14)			Vaccine Arm (n = 32)		
	Cohort 1 1×10^9 CFU/mL	Cohort 2 5×10^9 CFU/mL	Cohort 3 1×10^{10} CFU/mL	Cohort 1 1×10^9 CFU/mL	Cohort 2 5×10^9 CFU/mL	Cohort 3 1×10^{10} CFU/mL
Colitis	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)
Constipation	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)
Diarrhea	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)
Distension/bloating, abdominal	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)
Esophagitis	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)
Gastritis (including bile reflux gastritis)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)
Heartburn/dyspepsia	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)
Hemorrhoids	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)
Nausea	0% (n = 0)	0% (n = 0)	grade 2 20% (n = 1)	0% (n = 0)	0% (n = 0)	grade 2 22.22% (n = 2)
Salivary gland changes/saliva	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)
Taste alteration (dysgeusia)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)	0% (n = 0)
Vomiting	0% (n = 0)	0% (n = 0)	grade 1 20% (n = 1)	0% (n = 0)	0% (n = 0)	grade 1 11.11% (n = 1)

In accordance with the common terminology criteria for adverse events v3.0 (CTCAE v3.0), grades 1 through 5 are displayed with unique clinical descriptions of severity for each adverse event (AE) based on this general guideline: grade 1, mild AE; grade 2, moderate AE; grade 3, severe AE; grade 4, life-threatening or disabling AE; grade 5, death related to AE.

p = 0.0260 for 1×10^{10} CFU/mL cohorts). In comparison with the placebo groups, low IgA antibody levels were observed at month 1 after the last vaccination (day 90) (p = 0.0186 for 1×10^9 CFU/mL cohorts, p = 0.0673 for 5×10^9, CFU/mL cohorts, and p = 0.0044 for 1×10^{10} CFU/mL cohorts) (Figure 2, right panel). In contrast, no statistically significant differences were recorded between any vaccine and placebo groups at month 6 after the fourth vaccination (day 240) (p = 0.1087, p = 0.3910, and p = 0.0751) (Figure 2, right panel).

Detection of HPV-16 E6-Specific IFN-γ-Secreting $CD8^+$ T Cell and CTL Responses

The numbers of $E6_{49-58}$- and $E6_{29-38}$-specific interferon (IFN)-γ-producing T cells in PBMCs and cervical lymphocytes were examined separately after vaccination with NZ8123-HPV-16-optiE6.

$E6_{49-58}$-specific IFN-γ-secreting T cells were significantly higher in the active vaccination groups of 1×10^9, 5×10^9, and 1×10^{10} CFU/mL doses than the placebo group at 30 days after vaccination (p = 0.0154, p = 0.0160, and p = 0.0032, respectively) and at 60 days after vaccination (p = 0.0072, p = 0.0068, and p = 0.0004, respectively) in cervical lymphocytes (Figure 3, right panel). Also, the HPV-16 $E6_{29-38}$-specific CTL responses were significantly higher in the active vaccination groups of 1×10^9, 5×10^9, and 1×10^{10} CFU/mL doses than in the placebo group at 30 days after vaccination (p = 0.0025, p = 0.0141, and p = 0.0041, respectively) and at 60 days after vaccination (p = 0.0020, p = 0.0123, and p = 0.0012, respectively) in cervical lymphocytes (Figure 4, right panel).

At 30 days after vaccination, $E6_{49-58}$-specific IFN-γ-secreting T cells and HPV-16 $E6_{29-38}$-specific CTL responses in PBMCs were slightly higher in the same vaccination groups than in the placebo groups (p = 0.0777, p = 0.1027, and p = 0.0086, Figure 3, left panel; and p = 0.0111, p = 0.0426, and p = 0.0304, Figure 4, left panel), respectively. At 60 days after vaccination, barely detectable levels of $E6_{49-58}$-specific IFN-γ-secreting T cells and HPV-16 $E6_{29-38}$-specific CTL responses were detected in PBMCs (p = 0.0402, p = 0.0123 and p = 0.0014, Figure 3, left panel; and p = 0.0062, p = 0.0069, and p = 0.0017, Figure 3, left panel), respectively.

Time to peak responses in $E6_{49-58}$-specific IFN-γ-secreting T cells were seen in cervical lymphocytes at month 1 after the fourth vaccination (day 90) for all active study vaccine groups compared with when the placebo cohorts ranged from 13.5744 to 37.6256 at the 1×10^9 CFU/mL dose (mean difference, 25.6000; p = 0.0041; 95% CI), 21.0231 to 61.4769 at the 5×10^9 CFU/mL dose (mean difference, 41.2500; p = 0.0074; 95% CI), and 24.9441 to 50.6559 at the 1×10^{10} CFU/mL dose (mean difference, 37.8000; p = 0.0012; 95% CI) (Figure 3, right panel). Similarly, HPV-16 $E6_{29-38}$-specific CTL responses generally peaked at day 90 after the last vaccination for all vaccine arms in comparison with the placebo cohorts and ranged from 18.2153 to 45.3847 at the 1×10^9 CFU/mL dose (mean difference, 31.8000; p = 0.0029; 95% CI), 29.3391 to 67.6609 at the 5×10^9 CFU/mL dose (mean difference, 48.5000; p = 0.0040; 95% CI), and 31.5893 to 65.2107 at the 1×10^{10} CFU/mL dose (mean difference, 48.4000; p = 0.0013; 95% CI) (Figure 4, right panel).

Statistically significant increases were seen in all active study vaccine groups compared with the placebo control recipients in the

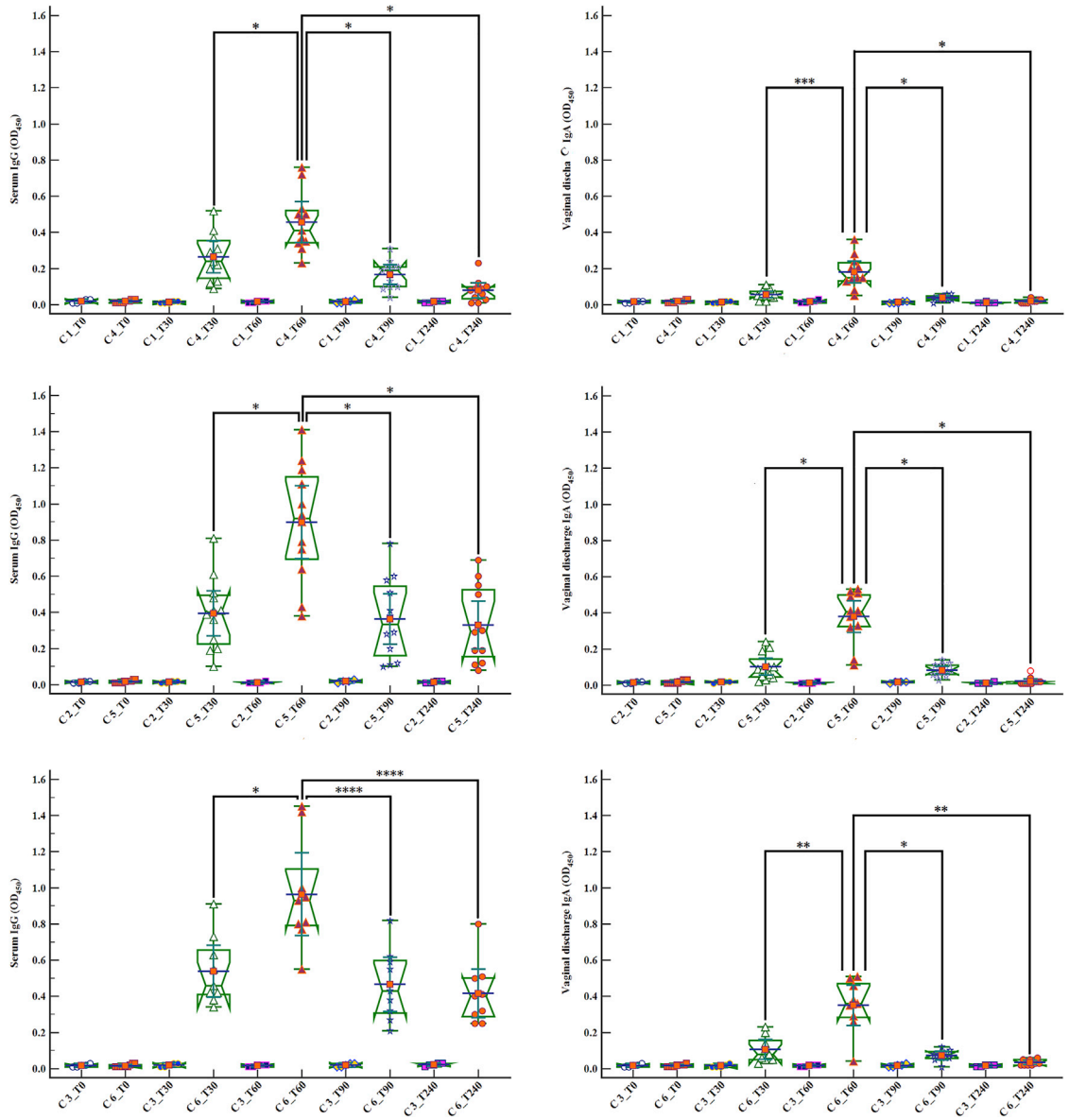

Figure 2. Humoral and Mucosal Immune Responses to HPV-16 E6 Oncoprotein following Immunization

Serum IgG antibody (left panel) and vaginal IgA antibody (right panel) measured by ELISA at a serum dilution of 1:100 and vaginal dilution of 1:10, respectively, at day 0 prior to immunization on days 30, 60, 90, and 240 after vaccination. Placebo groups, cohort 1 (C1), 1×10^9 CFU/mL; cohort 2 (C2), 5×10^9 CFU/mL; cohort 3 (C3), 1×10^{10} CFU/mL. Vaccine groups, cohort 4 (C4), 1×10^9 CFU/mL; cohort 5 (C5), 5×10^9 CFU/mL; cohort 6 (C6), 1×10^{10} CFU/mL. The absorbance of each well was measured at 450 nm. Bars represent the mean ± SE of each group at 95% CI. Statistically significant differences are denoted by asterisk between cohorts 4, 5, 6 T60 and cohorts 4, 5, 6 T30 and between cohorts 4, 5, 6 T60 and cohorts 4, 5, 6 T90 and between cohorts 4, 5, 6 T60 and cohorts 4, 5, 6 T240 (*$p \leq 0.0001$; **$p < 0.0003$; ***$p < 0.0007$; ****$p < 0.005$).

PBMC of the same volunteers at month 1 after the fourth vaccination (day 90) (HPV-16 $E6_{49-58}$-specific IFN-γ-secreting T cells; 1×10^9 CFU/mL dose group, range 0.5788 to 5.0212; mean difference, 2.8000, p = 0.0249, 95% CI; 5×10^9 CFU/mL dose group, range 2.4530 to 8.5470; mean difference, 5.5000, p = 0.0105, 95% CI; 1×10^{10} CFU/mL dose group, range 2.0880 to 7.9120; mean difference, 5.0000, p = 0.0089, 95% CI; Figure 3, right panel; Figure 3, left panel; and HPV-16 $E6_{29-38}$-specific CTL responses; 1×10^9 CFU/mL dose group, range 3.8344 to 10.1656; mean difference, 7.0000, p = 0.0036, 95% CI; 5×10^9 CFU/mL dose group, range 7.1023 to 14.8977; mean difference, 11.0000, p = 0.0029, 95% CI; 1×10^{10} CFU/mL dose group, range 6.4097 to 12.3903; mean difference, 9.4000, p = 0.0009, 95% CI) (Figure 4, left panel).

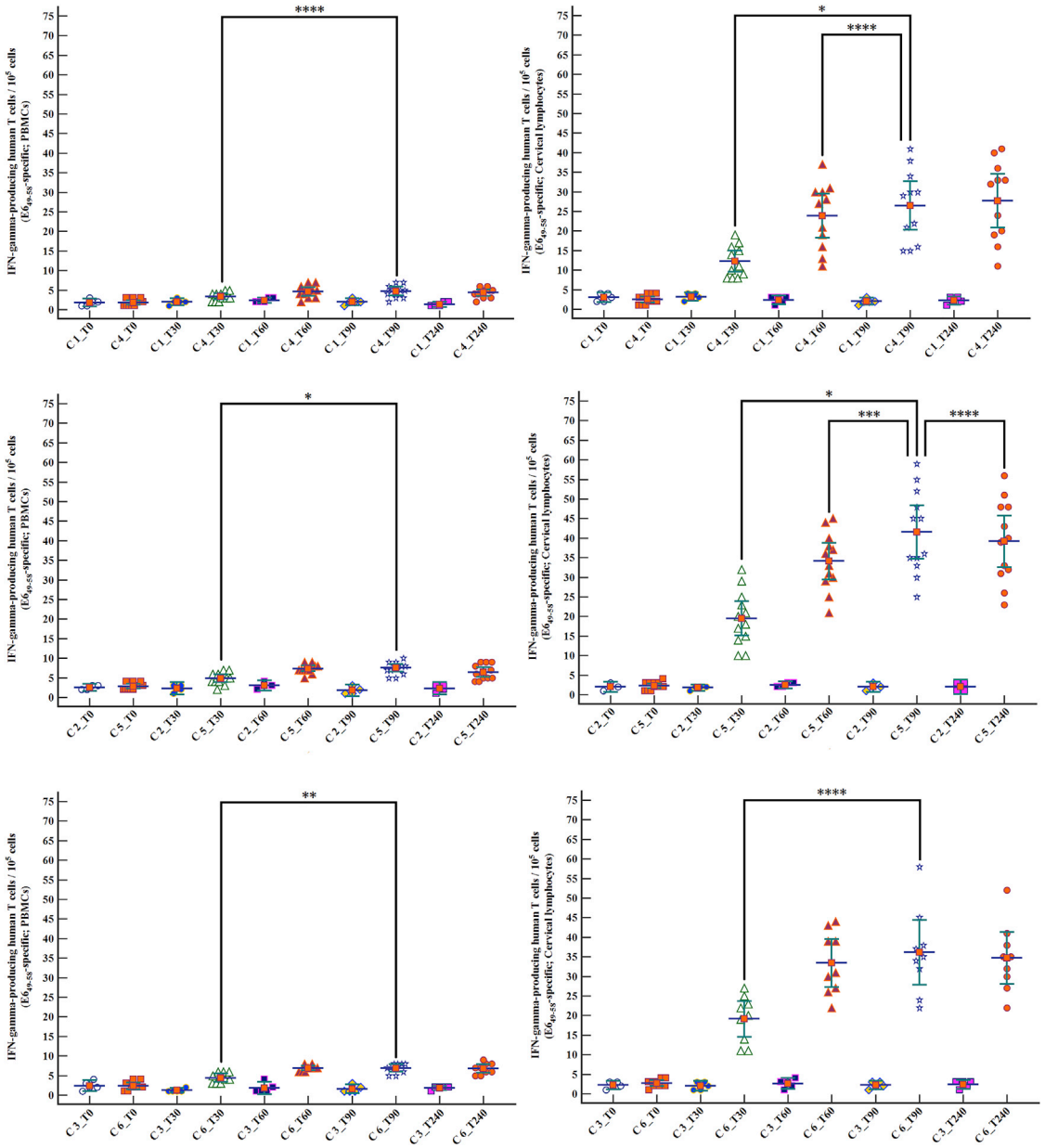

Figure 3. Frequency of $E6_{49–58}$-Specific IFN-γ-Producing T Cells Responding to NZ8123 HPV-16 optiE6 Vaccine

Specific T cell response was measured as by ELISpot in PBMCs (right panel) and cervical lymphocytes (left panel). Results are expressed as spot-forming cells per number of cells at 95% CI for the following time points: day 0 prior to immunization, days 30, 60, 90, and 240 after vaccination. Each sign represents one woman. Placebo groups, cohort 1 (C1), 1×10^9 CFU/mL; cohort 2 (C2), 5×10^9 CFU/mL; cohort 3 (C3), 1×10^{10} CFU/mL. Vaccine groups, cohort 4 (C4), 1×10^9 CFU/mL; cohort 5 (C5), 5×10^9 CFU/mL; cohort 6 (C6), 1×10^{10} CFU/mL. Bars represent the mean ± SE of each group. Statistically significant differences are denoted by asterisk between cohorts 4, 5, 6 T90 and cohorts 4, 5, 6 T30, between cohorts 4, 5, 6 T90 and cohorts 4, 5, 6 T60, and between cohorts 4, 5, 6 T90 and cohorts 4, 5, 6 T240 (*$p \leq 0.0001$; **$p < 0.0003$; ***$p < 0.002$; ****$p < 0.05$).

In comparison with the cervical lymphocytes, however, the number of $E6_{49–58}$- and $E6_{29–38}$-specific IFN-γ-secreting T cells were statistically significantly lower in the PBMCs of the same volunteers in the active vaccination group of 5×10^9 CFU/mL doses at month 1 (day 90) after the fourth vaccination (range 27.5888 to 40.4112, mean difference, 34.0000, $p < 0.0001$, 95% CI; and range 30.3651 to 44.4682, mean difference, 37.4167, $p < 0.0001$, 95% CI, respectively).

The day 90 HPV-16 $E6_{49–58}$- and $E6_{29–38}$-specific IFN-γ-secreting T cell responses for the vaccine groups that received 5×10^9 and

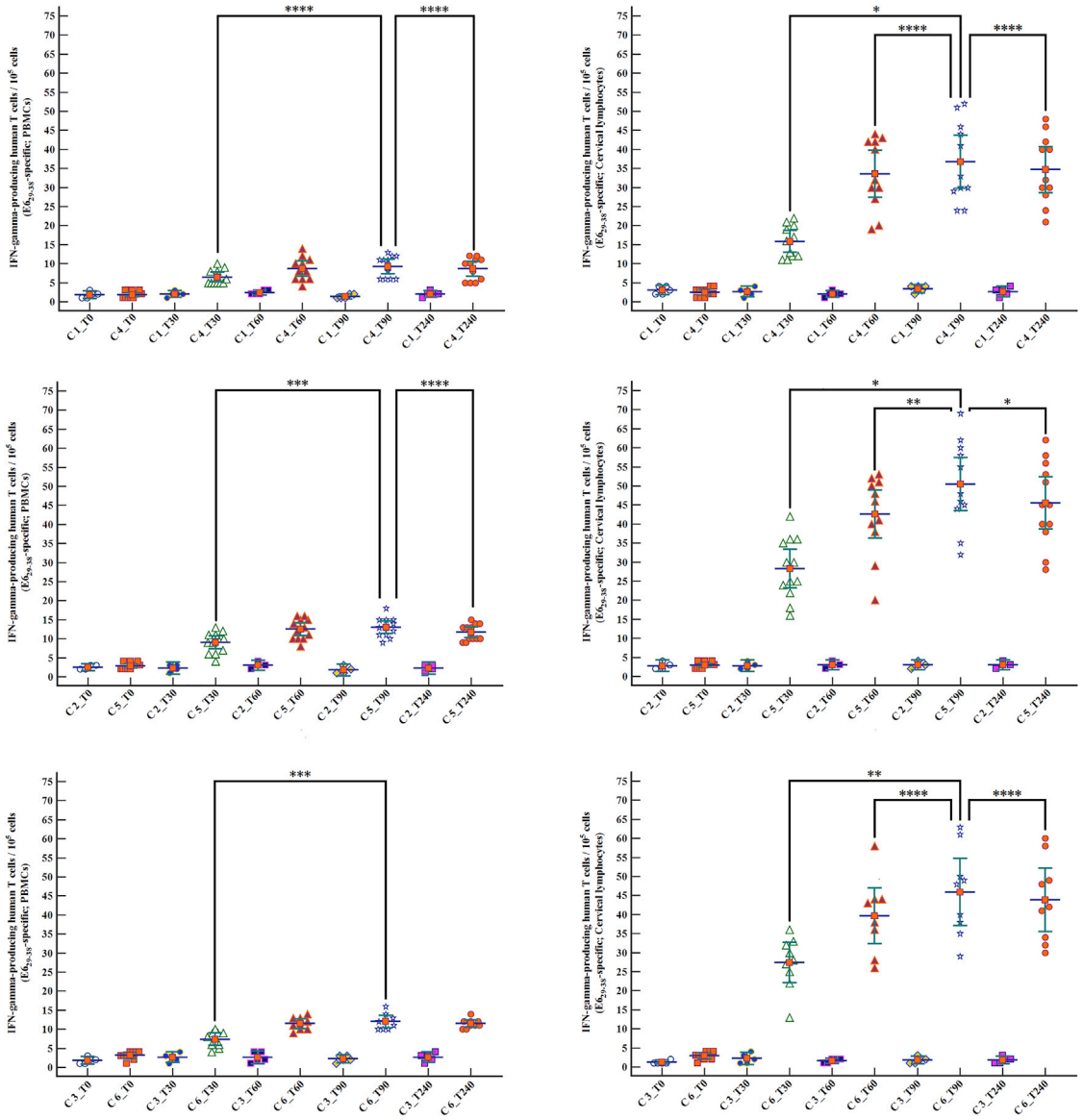

Figure 4. Frequency of $E6_{29–38}$-Specific IFN-γ-Producing T Cells Responding to NZ8123 HPV-16 optiE6 Vaccine

Specific T cell response was measured by ELISpot in PBMCs (right panel) and cervical lymphocytes (left panel). Results are expressed as spot-forming cells per number of cells at 95% CI for the following time points: day 0 prior to immunization, days 30, 60, 90, and 240 after vaccination. Each sign represents one woman. Placebo groups, cohort 1 (C1), 1×10^9 CFU/mL; cohort 2 (C2), 5×10^9 CFU/mL; cohort 3 (C3), 1×10^{10} CFU/mL. Vaccine groups, cohort 4 (C4), 1×10^9 CFU/mL; cohort 5 (C5), 5×10^9 CFU/mL; cohort 6 (C6), 1×10^{10} CFU/mL. Bars represent the mean ± SE of each group. Statistically significant differences are denoted by asterisk between cohorts 4, 5, 6 T90 and cohorts 4, 5, 6 T30, between cohorts 4, 5, 6 T90 and cohorts 4, 5, 6 T60, and between cohorts 4, 5, 6 T90 and cohorts 4, 5, 6 T240 (*$p \leq 0.0001$; **$p < 0.0007$; ***$p < 0.002$; ****$p < 0.05$).

1×10^{10} CFU/mL of the NZ8123-HPV-16-optiE6 vaccine were rather similar in the cervical lymphocytes (range −7.0128 to 10.7906, mean difference, 1.8889, p = 0.6377, 95% CI; and range −9.7231 to 19.5009, mean difference, 4.8889, p = 0.4626, 95% CI, respectively) and in the PBMCs (range −1.3855 to 2.0521, mean difference, 0.3333, p = 0.6666, 95% CI; and range −0.7218 to 3.8329, mean difference, 1.5556, p = 0.1539, 95% CI, respectively) and were statistically significantly higher than the day 90 IFN-γ-secreting T cell responses of the cervical lymphocytes (range 9.8172 to 19.6374, mean difference, 14.7273, p = 0.0001, 95% CI; range 4.3719 to 23.8099, mean difference, 14.0909, p = 0.0090, 95% CI for 5×10^9; and range 1.9665 to 21.3668, mean difference, 11.6667, p = 0.0242, 95% CI; range 0.1687 to 23.1646, mean difference, 11.6667, p = 0.0474 for 1×10^{10} CFU/mL cohorts, respectively) and PBMCs (range 2.0355 to 3.7827, mean difference, 2.9091, p < 0.0001, 95% CI; range 0.5057 to 6.5852, mean difference, 3.5455, p = 0.0265, 95% CI for

5×10^9; range 0.7483 to 4.3628, mean difference, 2.5556, p = 0.0115, 95% CI; and range 1.4117 to 5.2550, mean difference, 3.3333, p = 0.0039, 95% CI for 1×10^{10} CFU/mL cohorts, respectively) in the groups that received 1×10^9 CFU/mL of vaccine.

In comparison with the placebo groups, the somewhat similar HPV-16 $E6_{49-58}$-specific IFN-γ-secreting T cells and HPV-16 $E6_{29-38}$-specific CTL responses were observed at month 6 after the last vaccination (day 240) in the cervical lymphocytes (p = 0.0186 for 1×10^9 CFU/mL cohorts, p = 0.0673 for 5×10^9 CFU/mL cohorts, and p = 0.0044 for 1×10^{10} CFU/mL cohorts; and p = 0.0017 for 1×10^9 CFU/mL cohorts, p = 0.0073 for 5×10^9 CFU/mL cohorts, and p = 0.0011 for 1×10^{10} CFU/mL cohorts) and PBMCs (p = 0.0105 for 1×10^9 CFU/mL cohorts, p = 0.1098 for 5×10^9 CFU/mL cohorts, and p = 0.0021 for 1×10^{10} CFU/mL cohorts; and p = 0.0192 for 1×10^9 CFU/mL cohorts, p = 0.0080 for 5×10^9 CFU/mL cohorts, and p = 0.0004 for 1×10^{10} CFU/mL cohorts, respectively).

DISCUSSION

This trial reported that oral vaccination of recombinant *L. lactis* expressing HPV-16 E6 antigen promoted a clinical response in healthy females and underlined the importance of current strategies for cervical cancer immunotherapy by provoking E6-specific immunity.

The cost of HPV vaccines are an obstacle to worldwide application in developing countries. There is proof that converting the therapy from injection to oral administration of antigens affects the vaccine and offers benefits over other means. These include a decrease in hypersensitivity reactions, decreased costs, ease of use, and potential improvement in uptake rates.[9,16,17]

The gut is a chief immune organ in humans, and an early introduction of lactis acid bacteria to the gut may prime the immune system for a diversity of antigens, leading to development of antibody responses later in life.[15]

In recent years, it has been reported that implementation of antigens produced by *L. lactis* to gut mucosa through the oral route is the greatest significant non-invasive alternative to systemic vaccination.[18] It is presumed that mucosal vaccines through oral routes necessitate the co-administration of adjuvants to induce specific protective responses.[19] *L. lactis* cells seem to be an attractive antigen producer because they have been shown to have intrinsic adjuvant characteristics. Mechanistically, the adjuvant effect of lactic acid bacteria can be explained by the systemic release of specific cytokines after oral ingestion.[9,15]

Few studies have investigated immune effects related to gram-positive bacteria for delivery of recombinant antigens. One trial found that probiotic supplementation consisting of four bacteria strains provided protection from IgE-associated allergies in caesarean-delivered infants.[20] Another trial in infants demonstrated that *L. rhamnosus* GG stimulates oral rotavirus vaccination-induced IgA secretion.[21] Fang et al.[22] reported that *L. lactis* improves the immunologic response to the oral *Salmonella typhii* vaccine in healthy volunteers. The authors are not aware of any publication about stimulation of immune response by recombinant *L. lactis* to HPV-16 in healthy female volunteers. To the best of our knowledge, this is the first trial to investigate the immunomodulating effects of recombinant *L. lactis* expression of the HPV-16 E6 oncogene.

We previously reported that the marked induction of humoral and cellular immune responses after oral administration of *L. lactis* NZ9000 expressing HPV-16 E6 in C57BL/6 mice could neutralize HPV-16 infection and have developed this clinical trial in response.[15] In this randomized placebo-controlled trial, it has been shown that oral administration of NZ8123-HPV-16-optiE6 vaccine for 8 weeks with three doses was reasonably safe and tolerable in healthy females. This vaccine is thought to provide protection through the production of serum and vaginal anti-HPV-16 IgG and IgA antibodies, respectively, plus CTL response in vaginal secretion and PBMCs, as measured by ELISA and enzyme-linked immune absorbent spot (ELISpot), respectively. In other words, NZ8123 HPV-16 optiE6 vaccination elicited high levels of antibodies, which likely required T cell help and was capable of inducing T helper 1 (Th1) (IFN-γ). These findings are consistent with those of the few studies on the probiotic effect on vaccine response in humans and similarly support enhanced protective effects of NZ8123-HPV-16-optiE6 vaccine in healthy females.[23,24]

Increasing evidence suggests the role of $CD8^+$ cells in providing protective immunity in controlling the pathogenesis of HPV and against HPV-16-related diseases. Although humoral responses to HPV-16 are very important, E6-specific $CD8^+$ CTLs are essential for viral control and clearance. Human leukocyte antigen (HLA) class I-restricted HPV-16 E6 peptides are most likely to produce a CTL response that has been defined elsewhere. Therefore, it is supposed that selected restricted epitopes resulting from the HPV-16 E6 oncoprotein may be used to stimulate memory $CD8^+$ T cells. Evans et al.[25] proved that the HPV-derived CTL HPV-16 $E6_{29-38}$ epitope is appropriate for the immunotherapy of cervical cancer. In the current experiment, there was stimulation of CTL response to the peptides HPV-16 $E6_{49-58}$ and $E6_{29-38}$ after vaccination, demonstrating that the use of CTL peptide-epitopes results in strong $CD8^+$ T cell responses.

This study was designed to determine an optimal vaccine dose. There was an evident dose-response relationship in all groups, although the NZ8123-HPV-16-optiE6 vaccine induced high-level anti-HPV-16 in the serum IgG, vaginal IgA, and cytokine of all study participants who received the 5×10^9 and 1×10^{10} CFU/mL doses than of most study participants in the 1×10^9 CFU/mL groups. Nevertheless, no significant differences were observed among the recipients of the 5×10^9 and 1×10^{10} CFU/mL dose groups.

It is encouraging to note that, with the higher 5×10^9 CFU/mL dose of NZ8123-HPV-16-optiE6 vaccine, the final IgG, IgA, and IFN-γ

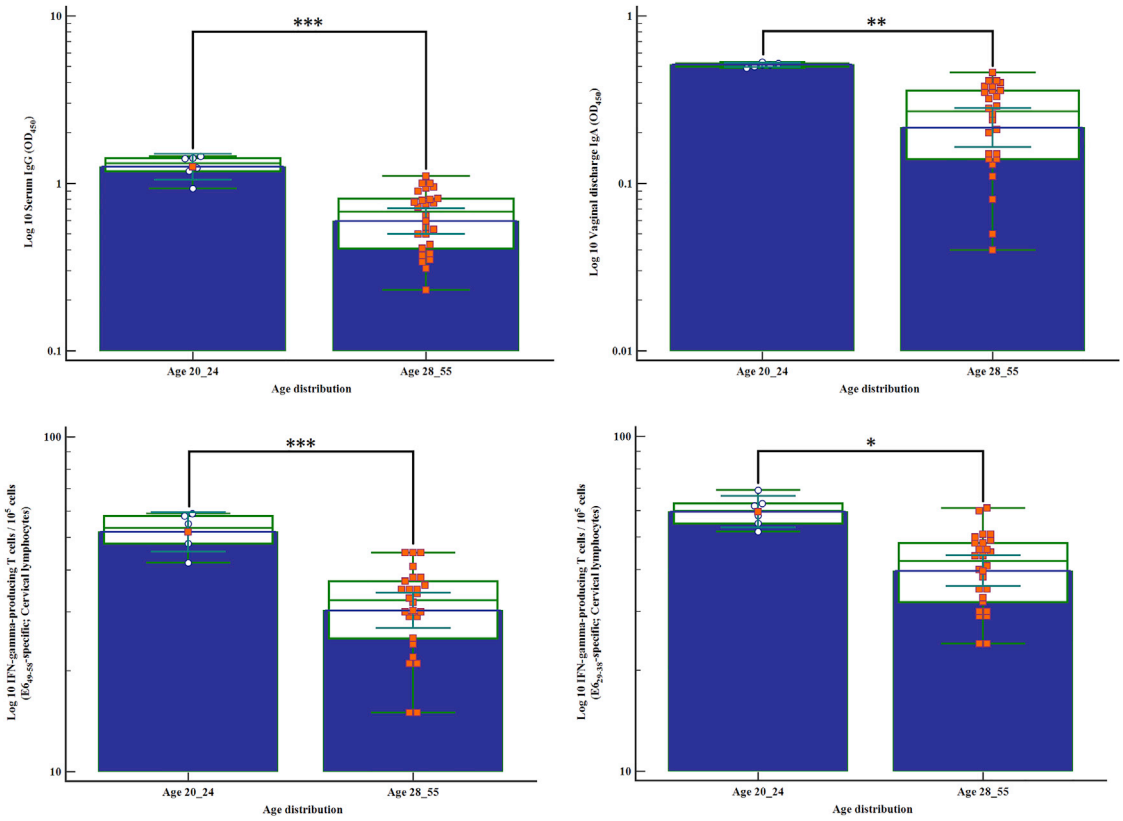

Figure 5. Age Distribution

Comparison of logarithm 10 serum IgG (left, above panel), vaginal IgA (right, above panel), $E6_{49–58}$-specific IFN-γ-producing T cells (left, bottom panel), and $E6_{29–38}$-specific IFN-γ-producing T cells (right, bottom panel) responding to NZ8123 HPV-16 optiE6 vaccine between subjects of two age groups (20 to 24 years and 28 to 55 years). Statistically significant differences are denoted by asterisk between participants aged 20 to 24 years and participants aged 28 to 55 years (*$p \leq 0.002$; **$p < 0.008$; ***$p < 0.05$).

levels were higher than those detected systemically and mucosally in the subjects who were seronegative before vaccination. The similar levels seen at the 1×10^{10} CFU/mL dose suggest that even higher doses of vaccine would probably not induce substantially higher antibodies and cytokine levels. Because the consumption of the 1×10^{10} CFU/mL dose was associated with increased nausea and vomiting by recipients of the vaccine, the optimal immunogenicity and reactogenicity profile in the current study was obtained with the 5×10^{9} CFU/mL dose of NZ8123-HPV-16-optiE6 vaccine.

These outcomes indicate that participants aged 17 to 26 years who received the three-dose regimen of active NZ8123-HPV-16-optiE6 vaccine produced more robust antibody and cytokine responses than subjects aged 27 to 56 years. These data indicate that the vaccination becomes less cost-effective with an increase in age above 26 years of the target vaccination group as is recommended by other public health organizations (Figure 5).[26,27]

In addition to establishment of the safety and immunogenicity of NZ8123-HPV-16-optiE6, this study provides information about the magnitude of mucosal-cell-mediated immune response in the cervix, which was large enough to allow comparison of different antigen strategies. This information will be useful for designing subsequent investigations. The results showed a strong mucosal-cell-mediated immune response in the cervix to HPV-16 E6 by oral vaccination of the NZ8123-HPV-16-optiE6 vaccine at intestinal mucosal inductive sites (Peyer's patches). However, the vaccine had a poor ability to stimulate systemic cell-mediated immune response to the HPV-16 E6 oncogene. These observations suggest that induced mucosal effector T cells by NZ8123-HPV-16-optiE6 vaccine in the gut enter the peripheral circulation and then migrate and settle in the cervical mucosa. Regardless of the NZ8123-HPV-16-optiE6 dose, spot-forming units of stimulated lymphocytes isolated from PBMCs were negligible in the ELISpot assay. This could be due to the dilution and low concentration of lymphocytes in the circulation. This information will be of critical importance to future studies investigating the use of NZ8123-HPV-16-optiE6 as an immune agent for mucosal vaccines.

The current study has several limitations. The first is the potential differences in the sensitivity of serological assays that test for HPV antibodies and cytokines. Another is that the sample size was small for

this proof-of-concept study, and we had insufficient power to detect small and moderate effects on vaccine responsiveness. Moreover, pending results from follow-up at months 12, 18, 24, and 48 will also be important. The safety and immunogenicity profile obtained in this study encourages further clinical investigation of HPV therapeutic vaccines.

These trials are expected to offer more information and obvious insight into how this vaccine can contribute to the treatment of cervical cancer. As discussed, it is well established that future randomized, placebo-controlled trial studies must be done to evaluate the clinical efficacy of oral vaccination with the NZ8123-HPV-16-optiE6 vaccine in treating HPV-16-associated cervical cancer.

MATERIALS AND METHODS

Study Subjects

Between June and August 2018, 119 healthy female volunteers aged 17 to 56 years were enrolled after referral to Keyvan Virology Specialty Laboratory (KVSL). All volunteers were tested for the absence of HPV contamination. Accordingly, residual ThinPrep cervical cytologic samples were centrifuged at 12,000 rpm for 1 min, and the concentrated cell pellet was used for DNA extraction. Total DNA was extracted from ThinPrep specimens using a high pure viral nucleic acid kit (Roche Diagnostics, Mannheim, Germany). Genomic DNA of the samples was used in PCR using MY09 and MY11 degenerate consensus primers. DNA quality and lack of PCR inhibitors in samples were verified using beta-globin PCR assay.[28]

The criteria for eligibility was determined by medical history; physical examination, including genital and pelvic examination; electrocardiogram and routine laboratory tests, including complete blood cell count, platelet count, alanine aminotransferase, serum creatinine, normal urinalysis findings, and aspartate aminotransferase, alkaline phosphatase, bilirubin, calcium, phosphorus, total protein, albumin, serum electrolytes, glucose, blood urea nitrogen, and creatinine values within normal range, hepatitis B surface antigen, and HIV and HCV antibody tests.

All aspects of the protocol were explained to the subjects who met the eligibility criteria, and informed consent was obtained before vaccination from all participants. Exclusion criteria included abnormal serum immunoglobulin G, A, or M levels, allergy to any vaccine component, positive urine pregnancy test or abnormal Pap smear, history or clinical manifestation of genitourinary disease, having received any other vaccination in the previous 30 days, a history of cancer or chronic hepatitis, current use of immunosuppressive medication or a history of immunodeficiency, having received any blood product or component in the previous 6 months, and anogenital warts within the previous year. Some participants left the area and failed to complete the vaccination regimen. The Institutional Review Board for Iran University of Medical Sciences approved the study protocol. This trial is registered with the Iranian Registry of Clinical Trials (IRCT) (https://www.irct.ir/trial/39227), registration number: IRCT20190504043464N1.

Composition of Vaccine and Placebo

Recombinant *Lactococcus lactis* strain NZ9000 expressing the codon-optimized full-length E6 oncogene of HPV-16 (NZ8123-HPV-16-optiE6) was developed using the nisin-controlled expression (NICE) system. The details have been reported elsewhere.[15] The NZ8123-HPV-16-optiE6 was grown from a master cell bank according to good manufacturing practice conditions in a fermenter under carefully controlled conditions within a clean room offered by H.K.

The NZ8123-HPV-16-optiE6 was purified by washing several times with PBS, then resuspended at concentrations of 1×10^9, 5×10^9, and 1×10^{10} CFU/mL based on optical density 600 (OD_{600}) readings and the colony count from serial dilutions in GM17 agar containing chloramphenicol antibiotics (10 μg/mL) in duplicate in PBS at the appropriate concentrations. It was then placed in vials and stored at 2°C–8°C. The placebo used in this study contained PBS carrying *L. lactis* harboring empty vectors (NZ8123) that were similar to those in the vaccine at total concentrations of 1×10^9, 5×10^9, and 1×10^{10} CFU/mL. The vaccine and placebo were not visually distinguishable. One significant point in the Good Manufacturing Practice (GMP) guidelines is quality control (QC) during manufacturing to guarantee that the non-sterile pharmaceutical products are free of microbial contamination. QC was set as total aerobic microbial count (TAMC), total combined yeasts and molds count (TYMC), and objectionable microorganisms. Microbial examination of the non-sterile product was performed according to the methods given in the text on microbiological examination of non-sterile products: microbial enumeration test < 61 > and test for specified microorganism < 62 >. The acceptance criteria for microbiological quality of product were 100 CFU/mL for TAMC and 10 CFU/mL for TYMC, and the absence of bile-tolerant gram-negative bacteria, *Escherichia coli*, *Salmonella*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans*, in accordance with USP-41 NF-36 (chapter < 1111 >).[29]

Study Design

This was phase 1 of a 2-month randomized, double-blind, placebo-controlled, dose-ranging immunogenicity and tolerability study of three dose formulations. The subjects were randomized using a computer-generated randomization schedule at a 2:1 ratio at the study center to receive (1 mL) four rounds of oral vaccination of either NZ8123-HPV-16-optiE6 vaccine or a placebo at weeks 1, 2, 4, and 8. Each dose was administered orally once each morning after overnight fasting for 5 days per treatment week.

To determine whether or not the dose of NZ8123-HPV-16-optiE6 vaccine would influence reactogenicity or immune response, the trial was conducted in a dose-escalation manner starting with 1×10^9 CFU/mL of NZ8123-HPV-16-optiE6 vaccine. When this dose was determined to be safe, we then evaluated 5×10^9 and 1×10^{10} CFU/mL of NZ8123-HPV-16-optiE6.

Adverse Event Monitoring

Study subjects were seen and evaluated at 1 h before and 2, 5, and 7 days after each vaccination. Also, AEs that occurred or worsened up to 240 days after the first scheduled administration of the medication were assessed blindly for severity and relationship to study drug.

Evaluations consisted of a medical history, physical examination, and performance of routine laboratory tests as outlined above. In addition to the monitoring of the laboratory parameters, the safety and tolerability of NZ8123-HPV-16-optiE6 was evaluated through the collection and review of AEs after each vaccination. Any AEs were recorded on a separate diary card after each vaccination. These were graded according to the National Cancer Institute CTCAE version 3.0.

Immunogenicity Assessments to HPV-16 E6

Whole blood and vaginal fluids were collected as described previously on day 0 before the initial vaccination, at days 30 and 60 after the first vaccinations, and at months 1 and 6 after the last vaccination. The specific serum IgG and vaginal IgA antibodies were calculated by ELISA as described elsewhere[15] using goat anti-human IgG H&L (horseradish peroxidase [HRP]) antibody (ab6858; Abcam, Canada; 1:1,000 dilution) and goat anti-human IgA alpha chain (HRP) (ab97215; Abcam, Canada; 1:1,000 dilution).

Approximately 10^5 PBMCs and 10^5 cervical lymphocytes were isolated from each subject as described previously[23] on day 0 before the initial vaccination, at days 30 and 60 after first vaccinations, and at months 1 and 6 after the last vaccination. Accordingly, Th1 type IFN-γ and antigen-specific CTLs (HLA-A*0201-restricted CTL)[25] against HPV-16 E6 were measured after stimulation with 10 μg/mL major histocompatibility complex (MHC) class I and HPV-16-derived CTL epitopes (synthesized HPV-16 $E6_{49-58}$ and HPV-16 $E6_{29-38}$ peptides, respectively) using a human IFN-γ ELISpot kit according to manufacturer instructions (R&D Systems, USA). and spots were counted under a dissection microscope with digital assistance. All assays were performed in triplicate.

Statistical Analysis

The primary endpoint was to assess any adverse side effects in order to determine the safe dosage of the vaccine, and the secondary endpoint was to evaluate the existence of a vaccine-specific mucosal and systemic HPV-16 E7 response in relation to the dosage.

All participants who received the study vaccine were included in the analysis of safety. A sample size of 46 healthy female volunteers in all treatment cohorts were selected (vaccine cohorts, n = 32; placebo cohorts, n = 14) who finished the study. The data was analyzed in MedCalc software (version 17.6; MedCalc, Belgium).[30] Specific antibodies of a participant who received NZ8123-HPV-16-optiE6 vaccine was deemed positive if the OD_{450} was greater than the mean OD_{450} plus three standard deviations from the mean for a panel of participants who received the placebo and vaccine was at least 0.100. Specific T cell response to HPV-16 E6 was considered positive when specific T cell frequencies were greater than 3 in 10^5 cervical lymphocytes or PBMCs.

The results are presented as the mean ± SE. Group comparison was made using a paired sample t test, and $p < 0.05$ was considered statistically significant. The assessment of a dose response was implemented using visual plots and a step-down no-statistical-significance-of-trend procedure to recognize the lowest vaccine dose level with proof of immunogenicity. Therefore, the antibodies and cytokine levels were computed with exact 95% CIs for the treatment group.

AUTHOR CONTRIBUTIONS

S.T.-S. and A.H.M. conceptualized and designed the study; S.T.-S. and M.R.R. drafted the manuscript; S.T.-S. and A.H.M. acquired, analyzed, and interpreted data; H.K. performed critical revision of the manuscript for important intellectual content; M.R.R. provided administrative, technical, and material support; H.K. supervised the study.

ACKNOWLEDGMENTS

We are indebted to the study participants who volunteered their time and support for this clinical trial. The authors are grateful to Narges Ghobadi for her insight into and guidance of the use of live *L. lactis* vaccines, for her technical advice on ELISpot assays, and for her active support during the study. We also acknowledge the expert assistance of the KVSL staff when coordinating the clinical studies and their thoughtful discussions on these studies. Informed consent has been obtained from the participants involved in this clinical trial study. This randomized, double-blind, placebo-controlled clinical trial was registered in the Iranian website for registration of clinical trials as https://www.irct.ir/trial/39227: IRCT (20190504043464N1). This research was carried out in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Iran University of Medical Sciences (IUMS; Tehran, Iran).

REFERENCES

1. Burd, E.M. (2003). Human papillomavirus and cervical cancer. Clin. Microbiol. Rev. *16*, 1–17.
2. Yim, E.-K., and Park, J.-S. (2005). The role of HPV E6 and E7 oncoproteins in HPV-associated cervical carcinogenesis. Cancer Res. Treat. *37*, 319–324.
3. Taghinezhad-S, S., Razavilar, V., Keyvani, H., Razavi, M.R., and Nejadsattari, T. (2017). Codon optimization of Iranian human papillomavirus Type 16 E6 oncogene for Lactococcus lactis subsp. cremoris MG1363. Future Virol. *12*, 499–511.
4. Mohseni, A.H., Razavilar, V., Keyvani, H., Razavi, M.R., and Khavari Nejad, R.A. (2017). Codon usage optimization and construction of plasmid encoding Iranian human papillomavirus type 16 E7 oncogene for Lactococcus lactis Subsp. Cremoris MG1363. Asian Pac. J. Cancer Prev. *18*, 783–788.
5. Lowy, D.R. (2016). HPV vaccination to prevent cervical cancer and other HPV-associated disease: from basic science to effective interventions. J. Clin. Invest. *126*, 5–11.
6. Lin, J., Xu, J., Albers, A.E., and Kaufmann, A.M. (2012). New Developments in Therapeutic HPV Vaccines. Curr. Obstet. Gynecol. Rep. *1*, 106–115.
7. Lin, K., Doolan, K., Hung, C.F., and Wu, T.C. (2010). Perspectives for preventive and therapeutic HPV vaccines. J. Formos. Med. Assoc *109*, 4–24.
8. Uddin, M.N., Kouzi, S.A., and Hussain, M.D. (2015). Strategies for Developing Oral Vaccines for Human Papillomavirus (HPV) Induced Cancer using Nanoparticle mediated Delivery System. J. Pharm. Pharm. Sci. *18*, 220–234.

9. Mohseni, A.H., Razavilar, V., Keyvani, H., Razavi, M.R., and Khavari-Nejad, R.A. (2019). Oral immunization with recombinant Lactococcus lactis NZ9000 expressing human papillomavirus type 16 E7 antigen and evaluation of its immune effects in female C57BL/6 mice. J. Med. Virol. *91*, 296–307.

10. Taghinezhad-S, S., Razavilar, V., Keyvani, H., Razavi, M.R., and Nejadsattari, T. (2018). Extracellular overproduction of recombinant Iranian HPV-16 E6 oncoprotein in Lactococcus lactis using the NICE system. Future Virol. *13*, 697–710.

11. Mohseni, A.H., Taghinezhad-S, S., Keyvani, H., and Razavilar, V. (2019). Extracellular overproduction of E7 oncoprotein of Iranian human papillomavirus type 16 by genetically engineered Lactococcus lactis. BMC Biotechnol. *19*, 8.

12. Bahey-El-Din, M., and Gahan, C.G. (2011). Lactococcus lactis-based vaccines: current status and future perspectives. Hum. Vaccin. *7*, 106–109.

13. Mohseni, A.H., Razavilar, V., Keyvani, H., Razavi, M.R., and Khavari-Nejad, R.A. (2017). Efficient production and optimization of E7 oncoprotein from Iranian human papillomavirus type 16 in Lactococcus lactis using nisin-controlled gene expression (NICE) system. Microb. Pathog. *110*, 554–560.

14. Medina, M., Vintiñi, E., Villena, J., Raya, R., and Alvarez, S. (2010). Lactococcus lactis as an adjuvant and delivery vehicle of antigens against pneumococcal respiratory infections. Bioeng. Bugs *1*, 313–325.

15. Taghinezhad-S, S., Mohseni, A.H., Keyvani, H., and Razavilar, V. (2019). Protection against human papillomavirus type 16-induced tumors in C57BL/6 mice by mucosal vaccination with Lactococcus lactis NZ9000 expressing E6 oncoprotein. Microb. Pathog. *126*, 149–156.

16. Clendinen, C., Zhang, Y., Warburton, R.N., and Light, D.W. (2016). Manufacturing costs of HPV vaccines for developing countries. Vaccine *34*, 5984–5989.

17. Cyriac, J.M., and James, E. (2014). Switch over from intravenous to oral therapy: A concise overview. J. Pharmacol. Pharmacother. *5*, 83–87.

18. Bermúdez-Humarán, L.G. (2009). Lactococcus lactis as a live vector for mucosal delivery of therapeutic proteins. Hum. Vaccin. *5*, 264–267.

19. Lavelle, E.C., and O'Hagan, D.T. (2006). Delivery systems and adjuvants for oral vaccines. Expert Opin. Drug Deliv. *3*, 747–762.

20. Korpela, K., Salonen, A., Vepsäläinen, O., Suomalainen, M., Kolmeder, C., Varjosalo, M., Miettinen, S., Kukkonen, K., Savilahti, E., Kuitunen, M., and de Vos, W.M. (2018). Probiotic supplementation restores normal microbiota composition and function in antibiotic-treated and in caesarean-born infants. Microbiome *6*, 182.

21. Praharaj, I., John, S.M., Bandyopadhyay, R., and Kang, G. (2015). Probiotics, antibiotics and the immune responses to vaccines. Philos. Trans. R. Soc. Lond. B Biol. Sci. *370*, 20140144–20140156.

22. Fang, H., Elina, T., Heikki, A., and Seppo, S. (2000). Modulation of humoral immune response through probiotic intake. FEMS Immunol. Med. Microbiol. *29*, 47–52.

23. Kawana, K., Adachi, K., Kojima, S., Taguchi, A., Tomio, K., Yamashita, A., Nishida, H., Nagasaka, K., Arimoto, T., Yokoyama, T., et al. (2014). Oral vaccination against HPV E7 for treatment of cervical intraepithelial neoplasia grade 3 (CIN3) elicits E7-specific mucosal immunity in the cervix of CIN3 patients. Vaccine *32*, 6233–6239.

24. Maciag, P.C., Radulovic, S., and Rothman, J. (2009). The first clinical use of a live-attenuated Listeria monocytogenes vaccine: a Phase I safety study of Lm-LLO-E7 in patients with advanced carcinoma of the cervix. Vaccine *27*, 3975–3983.

25. Evans, M., Borysiewicz, L.K., Evans, A.S., Rowe, M., Jones, M., Gileadi, U., Cerundolo, V., and Man, S. (2001). Antigen processing defects in cervical carcinomas limit the presentation of a CTL epitope from human papillomavirus 16 E6. J. Immunol. *167*, 5420–5428.

26. Bonanni, P., Bechini, A., Donato, R., Capei, R., Sacco, C., Levi, M., and Boccalini, S. (2015). Human papilloma virus vaccination: impact and recommendations across the world. Ther. Adv. Vaccines *3*, 3–12.

27. Liu, Y.-J., Zhang, Q., Hu, S.-Y., and Zhao, F.-H. (2016). Effect of vaccination age on cost-effectiveness of human papillomavirus vaccination against cervical cancer in China. BMC Cancer *16*, 164.

28. Keyvani, H., Taghinezhad Saroukalaei, S., and Mohseni, A.H. (2016). Assessment of the human cytomegalovirus UL97 gene for identification of resistance to ganciclovir in iranian immunosuppressed patients. Jundishapur J. Microbiol. *9*, e31733.

29. Monographs, N.P.G. (2018). United States Pharmacopeia and National Formulary USP 41–NF 36. United States Pharmacopeial Convention, Inc.

30. Mohseni, A.H., Taghinezhad-S, S., Keyvani, H., and Ghobadi, N. (2018). Comparison of Acyclovir and Multistrain Lactobacillus brevis in Women with Recurrent Genital Herpes Infections: a Double-Blind, Randomized, Controlled Study. Probiotics Antimicrob. Proteins *10*, 740–747.