**Improved Adjuvant Based Immune Responses to the HPV-16 VLP Antigen**

Hoorieh Soleimanjahi*, Susan Kaboudanian Ardestani, Zahra Kianmehr, and Fatemeh Fotouhi.

**Abstract**—Almost all cervical cancers are caused by persistent human papillomaviruses (HPV) infection, most often HPV16. Considerable advances have been made in developing HPV prophylactic vaccines based on L1 virus-like particles (VLP). Combination of HPV-16 VLP with LPS isolated from Brucella abortus strain RB51 (R-LPS) as adjuvant or combination of plasmid encoding HPV-16 L1 gene (pcDNA3/L1) with HPV-16 VLP as a DNA priming-VLP boosting regimen have been evaluated in this study. Results showed that although, formulation of VLP with R-LPS adjuvant as well as DNA primed-VLP boosted regimen were led to significantly increasing specific immune responses as compared with VLPs alone, but among them the VLP/R-LPS highly enhanced immune function. Tackling together, the results of our study suggest that re-focusing of research programs on adjuvant development for the next generation of cancer vaccines has potential relevant benefits.

**Keywords**—Adjuvant, Brucella abortus LPS, HPV-16 VLP, Prime-boosted regimen, Prophylactic HPV vaccine.

**I. INTRODUCTION**

Every year, nearly 500,000 new cases and 274,000 deaths occur worldwide from cervical cancer [1]. The identification of a causal association between development of cervical cancer and persistent infection with specific human papillomaviruses (HPV) specially HPV type 16, has triggered considerable interest in the expansion of prophylactic HPV vaccines [2] which represent an important strategy for potentially reducing of incidence and mortality rates of this cancer [3]. A number of vaccine models have been investigated to generate effective immune responses and protection against HPV infections. Nowadays, immunizing agents such as virus-like particles (VLPs), capsomers, recombinant fusion proteins or peptides, live recombinant bacteria, recombinant viruses, and DNA vaccines are being evaluated for vaccination purposes [4]. Besides, although the structure of each model has differed, numerous studies were performed to test different strategies aimed at improving immunostimulatory properties of these vaccines. The higher immune responses to these vaccines can be achieved by different strategies including use of appropriate vaccine administration rout, inclusion of proper adjuvants in vaccine formulation [5, 6] and the prime-boost strategy [7] in which immune responses primed by DNA-encoded antigen were boosted by the administration of similar recombinant protein.

At present, two VLP-based prophylactic vaccines, that is, Gardasil® (Merck, a quadrivalent vaccine against HPV types 6, 11, 16, and 18) and Cervarix™ (GlaxoSmithKline, a bivalent vaccine against HPV types 16 and 18) have been commercialized for human use [8]. VLPs obtained by self-assembly of the HPV L1 major capsid protein subsequent recombinantly expression of L1 protein in eukaryotic expression systems and exhibit the size, shape, and conformational epitopes of native virion capsids [9].

Although HPV-VLPs alone are potent immunogens, several studies have been demonstrated that administration of proper adjuvant can potentially enhance immune response broadening [10]. Selection of a safe, non-toxic and effective adjuvant considered as the prime importance for routine vaccination [11]. Adjuvants exert their effects through different mechanisms. Some adjuvants are natural ligands such as bacterial lipopolysaccharides (LPS). Upon binding of LPS to its ligand, Toll-like receptor 4 (TLR-4), initiate signal transduction and lead to enhancing Th1/Th2 cytokine ratio and increasing antigen uptake, processing and presentation by antigen presenting cells (APCs) [12]. However, endotoxic and pyrogenic activity of LPS have prevented its clinical use and in this condition, employment of natural LPS or detoxified derivatives of current LPS such as MPL (3-O-desacyl-4-monophosphoryl lipid A) with reduced endotoxicity but remained strong adjuvant activity, can resolve this problem [13, 14]. *Brucella abortus* LPS is less toxic and non-pyrogenic in comparison with other bacterial LPS [14].

In the current study, efficacy of two different approaches, combination of HPV-16 VLP with LPS isolated from *Brucella abortus* strain RB51 (R-LPS) as adjuvant and also combination of plasmid encoding HPV-16 L1 gene (pcDNA3/L1) with HPV-16 VLP as a DNA priming-VLP boosting regimen were compared for improving of immune responses to HPV-16 VLP antigen.
II. MATERIALS AND METHODS

A. Vaccine Preparations

The HPV16-L1 full gene was amplified from paraffin embedded tissues, cloned to pTZ57R/T vector (Fermentase, Lithuania) and confirmed by sequencing as described previously [15]. The 1595 bp L1 fragment was subcloned into the pcDNA3 (Invitrogen, Burlington, Canada) under the control of alkaline lysis maxi-preparation [16, 17]. The pcDNA3 (Invitrogen, Burlington, Canada) under the control of the pcDNA3/L1 was purified using a modification of the alkaline lysis maxi-preparation [16, 17]. The HPV16-L1 VLPs were produced by the expression of recombinant baculoviruses encoding full length HPV16-L1 gene in Sf9 insect cells as previously described [18]. The purification of HPV-16 L1 VLPs was performed by subsequent treatment such as detergent lysis using triton X-100, sonication, sucrose cushion, and CsCl equilibrium density centrifugation [19, 20]. The structural integrity of the VLPs was verified by their morphology using electron microscope and their ability to hemagglutinate mouse red blood cells efficiently.

R-LPS from B. abortus RB51 was purified using some modification of previously described procedures [21].

B. Experimental Design

Female C57BL/6 mice (6-8 weeks old) were purchased from Pasteur Institute of Iran (Tehran, Iran), housed under filter top conditions with water and food, and were used for experimental purposes with approval of the animal ethics committee of the ministry of health and medical education (Tehran, Iran). Groups of five C57BL/6 mice were injected subcutaneously with corresponding vaccines in a total volume of 100 μl at 2-week intervals as indicated in Table I. Two weeks after the final immunization, blood was collected from the animals by puncture of the superficial temporal vein and serum samples were taken for evaluating antibody responses and kept at −70°C until use. Then, all mice were sacrificed and the spleens were removed for cytokine assays.

C. Cytokine ELISA Assay

Single spleen cell suspensions were obtained by gentle homogenization. Red blood cells were lysed, and the resulting splenocytes were resuspended at a density of 2×10⁶ cells/ml in RPMI1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin. Cells were seeded in flat-bottom 24-well plates (Nunc, Denmark) and were stimulated with the appropriate antigen at 37°C with 5% CO₂. Culture media were collected for assays after 72 h. Cytokine levels were measured by using commercial ELISA cytokine assay kits (R&D system, Minneapolis, MN) according to the manufacturer’s instruction. Values were presented as pg cytokine/ml (mean ± SD, n = 5).

D. Detection of Anti-VLP Antibodies by ELISA

VLPs-specific IgG antibodies in individual serum samples were determined in the HPV16-VLPs coated 96-well ELISA plates (Nunc, Denmark) by overnight incubation at 4°C. The VLP coated plates were incubated sequentially with serially diluted mice sera and HRP-conjugated goat anti-mouse IgG. The enzyme reaction was evaluated by substrate solution (TMB) and measurement of absorbance at 450 nm.

E. Statistical Analysis

The statistical significance of differences between experimental groups was determined using one-way ANOVA followed by Turkey’s post-test. *p<0.01 were considered statistically significant. All statistical analyses were performed using the GraphPad Prism 6.01 software (La Jolla, CA, USA).

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III. RESULTS

A. Cytokine Assay

Spleen cells of immunized mice were cultured; re-stimulated in vitro and different cytokine profiles such as IL-4, IL-10, IFN-γ, TNF-α and IL-17 secreted by T-cells were assessed by ELISA kits.

Results showed that while immunization of mice with R-LPS adjuvant alone did not induce any increase in IFN-γ production, formulation VLP antigen with R-LPS as adjuvant resulted in significantly improving IFN-γ production in comparison with VLPs alone (p<0.01). In addition, administration of one dose pcDNA3/L1 and two doses VLP in prime-boosted group led to raising IFN-γ levels than administration of three doses VLP (Fig. 1a). As shown in Fig. 1b, all of vaccine preparations except R-LPS, had almost similar effect on IL-10 production. Between our examined vaccines, VLP in combination with R-LPS could induce the highest level of IL-4 in comparison with other vaccines (Fig. 1c). As illustrated in Fig. 1d, formulation of VLP with R-LPS adjuvant and also DNA primed-VLP boosted regimen similarity were led to significantly increasing TNF-α level as compared with other vaccine preparations (p<0.01). The level of IL-17 secreted by splenocytes from mice given VLP in combination with R-LPS adjuvant was significantly higher than those immunized with other vaccines (p<0.01) whereas there were no significant difference in ability for producing of
IL-17 between VLP alone and or DNA primed-VLP boosted regimen (Fig. 1e).

These data confirm that R-LPS as adjuvant capable of promoting Th1, Th2 and Th17-type immune responses to HPV-16 L1VLPs in mice.

C57BL/6 mice were subcutaneously immunized three times biweekly as described in Table 1, and two weeks post-immunization, spleens were harvested. Splenocytes were cultured and stimulated ex vivo in the presence of the mitogen for 72 h. Cytokines released into culture media were determined by ELISA kit. Data are mean ± SD, n = 5. The levels of statistical significance for differences between test groups were determined using ANOVA followed by Turkey’s post-test.

* indicates statistical significance (**p < 0.01, *p < 0.001).

B. Influence of the Different Vaccine Formulations on the VLP-Specific Antibody Responses

Fig. 1. Measurement of cytokine levels secreted by splenocytes of immunized mice.

Results of VLP-specific IgG titers identification in mice serum revealed that employment of R-LPS as adjuvant in combination with VLPs developed significantly the highest antibodies titers in comparison with other groups (p<0.01). Also, administration of combined pcDNA3/L1 and VLP as prime-boosted regimen resulted in significantly elevating of VLP-specific IgG titers as compared with VLP alone (Fig. 2).
Immunizations were carried out three times at 2-week intervals by subcutaneously injecting 100 μl corresponding vaccines. At two weeks post-immunization, mice were bled and individual sera assayed by end point dilution ELISA. Data are means ± SD, n =5. *indicates statistical significance (*p < 0.01). Groups shown as abbreviated per Table 1

Fig. 2. Induction of specific IgG antibodies titers.

IV. DISCUSSION

Since upon HPV infections, L1 proteins are detectably expressed only in the more superficial layers of the epithelium, L1-specific CD8 T cells generally are not considered to be of particular relevance in prevention or therapy of HPV-related diseases [22]. However, antiviral cytokines released by antigen-specific T cells, such as IFN-γ and TNF-α, may contribute to inhibition and control of infection [23]. In this context, previous studies have shown that immunization with HPV-16 L1VLPs elicits a substantial T and B cells responses [24, 25] and also that TLRs play a central role in the recognition of HPV-L1VLPs by immune system. In the presence of “alert” signals such as TLR ligands or inflammatory cytokines, dendritic cells (DCs) are stimulated to mature and differentiate into potent activators of antigen-specific T cells [26]. Among several adjuvants, detoxified LPS from Gram-negative bacteria or its Lipid A component have been used as a potent Th1 adjuvant in human studies. LPS act as “alert” signals and a potent natural adjuvant when administered in combination with protein antigens [27]. LPS have been shown to exert a variety of effects on the innate immune system through binding to TLR4 and lead to releasing a number of cytokines such as TNF-α, IL-6, IL-12, IL-10 and IFN-γ, and generating antibody responses towards non-associated Ag [28]. Less toxic and non-pyrogenic nature of B. abortus LPS in comparison to other bacterial LPS can consider its potential use as adjuvant in vaccine design without passing detoxifying procedure. Jamalan et al. investigated effectiveness of B. abortus S19 LPS as an adjuvant for extracted tuberculin PPD (purified protein derivative) from Mycobacterium tuberculosis and demonstrated that effect of this LPS in skewing of the immune response to Th1 or Th2 pathway is antigen-dependent [28].

In an attempt to improve HPV16 VLP immunogenicity via a subcutaneous route, the effectiveness of administration of VLPs in combination with R-LPS isolated from B. abortus RB51 as adjuvant and also DNA priming-VLP boosting regimen were compared in this study. VLP-specific IgG titers in mice immunized with three doses VLP, VLP plus R-LPS and or primed with pcDNA3/L1 and boosted with VLP were measured by endpoint dilution ELISA as described in section 2. Formulation of VLP with R-LPS adjuvant led to more increasing specific IgG titers in comparison with DNA priming-protein boosting regimen.

The adaptive immune system contains a balanced repertoire of Th1 and Th2 CD4+ cells. Relative amounts of Th1 cytokines (e.g., IL-12, IFN-γ, IL-2 and TNF-α) and Th2 cytokines (e.g., IL-4, IL-5 and IL-10) in the beginning of an immune response may essentially decide whether the response will turn act to be of the Th1 or Th2-type. Th1 cells promote inflammatory responses through the secretion of cytokines such as IFN-γ, while Th2 cells provide help to antibody-producing B cells and secrete certain cytokines, such as IL-10 that is critical for the maintenance of immune homeostasis.

Analysis of different cytokine profiles revealed that formulation of VLP with R-LPS adjuvant and also DNA primed-VLP boosted regimen were led to significantly increasing IFN-γ level as compared with VLPs alone, and among them the VLP/ R-LPS extremely enhanced immune responses. To better quantify the Th1/Th2 cytokine profiles, IFN-γ/IL-10 ratios were determined (Fig. 3). VLP plus R-LPS vaccine associated with higher IFN-γ response (IFN-γ /IL-10 ratio of 1.5) rather than other candidate vaccines (Fig. 3). Furthermore, combination of VLP and R-LPS adjuvant exhibited increased level of IL-4 compared with VLP alone or combined pcDNA3/L1 and VLPs. In addition, both strategies i.e. use of R-LPS adjuvant and DNA priming-VLP boosting
induced significantly higher level of TNF-α secretion in comparison with VLP alone.

Interestingly, VLP along with R-LPS adjuvant could promote the highest IL-17 level by splenocytes when compared with other examined vaccines. Since IL-17 as an inflammatory cytokine released by Th17 cells; provide protection from microbial infection [29], elevated IL-17 level in mice given VLP plus R-LPS vaccine may confer effective protection from HPV infection at the first site of virus entry.

Taken together, our data consistent with other studies suggest that administration of proper adjuvants efficiently improve VLP immunogenicity than prime-boost strategy and also B. abortus RB51 LPS can be serve as an effective and promising adjuvant for an HPV prophylactic vaccine based on HPV L1 VLPs.

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REFERENCES