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RESEARCH PAPER

Effect of pleurotus sajor-caju polysaccharide encapsulated in poly D, L lactide-co-glycolide nanoparticles for HPV vaccine in murine model

Roopngam Evelyn Piyachat [1*], ***Piyachat Evelyn Roopngam*** [2]

[1]*Department of Clinical Immunology, Faculty of Medical Technology, Western University, Kanchanaburi, Thailand, 71170*

[2]*Head of Clinical Immunology Department, Faculty of Medical Technology, Western University, T.Sralongrua, A.Huay Kra Chao, Kanchanaburi, Thailand, 71170*

ABSTRACT

Objective(s): In the current work, poly D, L lactide-co-glycolide (PLGA) particles were applied for a viral vaccine for the delivery of antigens in cytosolic pathway by increasing the antigen presentation to T-lymphocytes.

HPV-E7 protein with PLGA particles has been reported as a potent adjuvant for HPV vaccine by encapsulating protein into the PLGA particles. Polysaccharide from Pleurotus sajor-caju was also applied as a potent immunomodulator.

Materials and Methods: HPV-E7 protein and Pleurotus sajor-caju polysaccharides (PSC) were encapsulated into PLGA nanoparticles. This combination comprised a strategy to induce helper and cytotoxic T-lymphocytes (CTL) expansion. Mice antibodies and T-lymphocyte expansion were investigated in comparison between encapsulated E7 protein into PLGA nanoparticles (E7PLGA) and E7 protein with PSC encapsulated into PLGA nanoparticles (PSC-E7PLGA).

Results: The results showed that E7PLGA and PSC-E7PLGA could induce antibody response to HPV. The PSC-E7PLGA could increase the level of viral antigen-specific IgG antibodies. The cellular immune responses are also significantly enhanced by expansion of helper T-lymphocytes and CTL. PSC-E7PLGA was shown to be significantly higher in immunomodulating activity than E7PLGA.

Conclusion: Thus, the encapsulation of polysaccharide and HPV-E7 antigen into PLGA nanoparticles is a strategy for development of HPV vaccine.

Keywords: *Antibodies, Cytotoxic T-Cells, HPV, Nanoparticles,Vaccination*

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INTRODUCTION

Cervical cancer is caused by types 16 and 18 high-risk human papillomavirus (HPV) infections. Persistence of infection has been shown in mostly 70% of all cervical cancer patients [1, 2, 3, 4]. The viral genome integrated into the genome of host cell leads to the overexpression of the E6 and E7 proteins, causing the deregulation of retinoblastoma tumor suppressor protein (pRB) and p53 [5]. Several types of HPV vaccines can target HPV proteins, such as type 16-E6 protein (HPV16-E6) and HPV16-E7 proteins, which have been created recently. These include plasmid DNA, viral or bacterial vectors, chimeric virus-like particles, synthetic peptides, and recombinant proteins [6]. Some of these have been found to be safe enough for human testing and clinical studies [1]. Currently, HPV therapeutic vaccines are more powerful than prophylactic vaccines because commercial HPV vaccines can only induce prophylactic antibodies to prevent viral infection of cervical epithelial cells, Further, high efficacy has only been demonstrated in younger women. However, women who have HPV need to use therapeutic vaccines continuously to mitigate cancer symptoms [2]. Therefore, therapeutic

* *Corresponding Author Email: Piyachat.ro@western.ac.th or pyachat@live.com*

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vaccines targeting HPV proteins have been investigated intensively to improve efficacies, such as by their combination with new adjuvants or delivery vectors [7]. Recently, L1 and L2 capsid proteins have been reported as potential HPV vaccines. However, cytotoxic T-lymphocytes (CTL) responses in L1 and L2 (viral capsid protein) have been found to induce weak responses. On the other hand, E7 oncoprotein has proven to be potent and developed for peptide-based antitumor vaccines [8, 9, 10, 11, 12]. Currently, therapeutic vaccine strategies have focused on targeting E7 specific T-helper type 1 (Th1) and CTL responses [13, 14]. In a previous study by the authors, the immunotherapeutic strategy also focused on E7 protein due to E7 protein amino acid sequences are highly conserved among HPV genotypes and the ability to be continuously expressed by viral integration and cellular transformation [15]. However, the disadvantages of peptides vaccines have been reported such as degradation, ineffective delivery and poor immunogenicity [16]. Therefore, the adjuvants and costimulatory molecules require further investigation to improve the efficacy of the vaccine [17]. Poly D, L lactide-co-glycolide (PLGA) particles have performed as the delivery system of viral vaccine to facilitate antigen presentation in the cytosolic pathway to T-lymphocytes via MHC-I and MHC-II molecules [7]. The E7 peptide vaccine with PLGA particles has also been previously reported to induce stronger CTL response when compared to E7 peptide alone, exhibiting the ability to decrease tumor size in mice [7]. For other immunomodulators, several studies have shown that certain species of edible mushrooms exhibited immunomodulatory abilities [18]. Grey oyster mushroom (*Pleurotus sajor-caju*) is an edible mushroom found in tropical areas. This edible mushroom has reported as the potent immunomodulatory due to the presence of numerous fungal compounds as polysaccharides (mostly β- and some α-glucans) [19]. *Pleurotus sajor-caju* polysaccharides (PSC) has shown to stimulate the immune system. The immunomodulation by PSC is the stimulation of helper T cells and the expansion of CD4+/CD8+ T-cells [18]. In this study, the author hypothesized that encapsulating PSC and HPV-E7 antigen in PLGA nanoparticles (NPs) could increase T-lymphocytes expansion *in vivo*. This novel type of adjuvant possesses the potential to enhance the immune system by PSC-PLGA nanoparticle-based vaccine formulations that deliver HPV-E7 as a potent antigen.

MATERIALS AND METHODS

Mice

Female BALB/c mice (8 weeks of age) were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The mice were maintained under specific pathogen-free conditions and handled following the Animal Care and Handling guidelines by Ethics Committee of the Faculty of Medical Technology, Western University.

Antigens

Recombinant E7 protein of human papillomavirus-16 (Accession: EU430687.1, GI: 167996746) was constructed in *Escherichia coli* (*E.coli*) strain BL21γDE3 (Novagen, Darmstadt, Germany), as previously described [20]. Recombinant protein (His-Tag) was induced to express by isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, MO, USA). *E. coli* was lysed and purified on Ni-NTA agarose column (QIAGEN, Hilden, Germany). The agarose column was washed with 30 mM imidazole to remove lipopolysaccharide contamination and extracted for the recombinant protein by urea (8 M in PBS). Purified proteins were eluted with imidazole (100 mM in 8 M urea) and dialyzed against PBS. The purified protein was lyophilized for ELISA technique.

Polysaccharide extraction

Pleurotus sajor-caju mushrooms were ground and extracted with hot water using percolation and Soxhlet techniques following Kim YH et al. [18]. The extraction was then freeze-dried into lyophilized powder. Subsequently, the powder extract was diluted into carboxymethylcellulose (0.5% viscosity) for the concentration as 1g/kg [18].

Enzyme-linked Immunosorbent Assay (ELISA)

The E7 recombinant protein was evaluated by ELISA. The E7 protein was coated on microtiter plates overnight at 4 °C with 20 µg/well of E7 recombinant protein in PBS. The E7 protein was received from Zheng Yi's lab [20] and bovine serum albumin (BSA) was applied as positive and negative control, respectively, then blocked for 2hr with 200 µl of PBS containing skim milk and washed

three times with PBS. Goat anti-E7 (Biorad, CA, USA) in PBS containing skim milk was incubated for 2 h. After antibody incubation with antigen on microtiter plates, the plates were washed with PBS for three times. The antibodies to the E7 protein on the plates were detected by mouse anti-goat IgG-horseradish peroxidase (e-Bioscience, CA, USA) in PBS. Following washes, the reaction was detected by TMB substrate (Bio-rad, CA, USA). After acid stop solution, the reaction was measured for OD at 450 nm by ELISA reader.

Preparation of E7PLGA and PSC-E7PLGA NPs

The viral protein (HPV-E7) and PSC were encapsulated to PLGA nanoparticles (poly D, L lactide-co-glycolide, 50:50, MW 50,000, Sigma–Aldrich, MO, USA) by using the water-in-oil emulsion technique following Lou Li *et al* [21]. The E7 protein was diluted in PBS at a concentration of 0.5 mg/ml. The PSC solution was diluted in deionized water (20mg/mL). The E7 protein and PSC in solution were used as the internal water phase, and added to the organic phase of PLGA dissolved in DMSO (1 mg/mL). The double emulsion (water-in-oil-in-water) was sonicated for 2 min at 130W and homogenized by pouring the primary emulsion into the external water phase ((Poloxamer 188 (F68) solution), followed by probe sonication for 2min at 150W. Evaporation removed the residual organic solvent for 30min, while the temperature was maintained at 55 OC before collecting the NPs [21].

Characterization of PLGA NPs

E7PLGA and PSC-E7PLGA were measured for the sizes by Malvern Mastersizer 2000 (Zetasizer Nano ZS90, Malvern Instruments Ltd., UK). The particles were newly prepared and diluted appropriately, then subjected to ultrasonication at room temperature before measurement.

E7PLGA and PSC-E7PLGA NPs encapsulated efficiency

E7PLGA and PSC-E7PLGA were digested by 0.02 N NaOH containing 5% (w/v) SDS. The solution was then neutralized with 1 M HCl to pH 7.0 and centrifuged at 3500 rpm for 10 min. E7 protein from PLGA NPs was then subjected to protein quantitation by Bradford assay (Bio-rad, CA, USA) in triplicate by spectrophotometry (UV-Visible, 595 nm). The protein was measured in standard levels of 0.25, 0.50, 0.75, and 1.0 mg of E7 protein to set as a standard curve. The β-glucan level in the extract was measured using a ß - glucan kit (specific for mushroom and yeast, Megazyme, USA). The beta-glucan from the particles was quantified in triplicate and compared with the standard levels of 0.25, 0.50, 0.75, and 1.0 mg of beta-glucan by Spectrophotometry (UV-Visible, 510 nm).

Mice $CD4^+$, $CD8^+$ T-cell analysis

The mice were divided into four groups of three for four subcutaneous immunization (50 µl per injection) with complete Freud's adjuvant on the first day and incomplete Freud's adjuvant on day 14. The first group of mice was administered PBS as a saline control. The second group of mice was administered 1 mg/ml of PLGA NPs. The third and fourth groups of mice were administered E7PLGA (1 mg/ml) and PSC-E7PLGA (1 mg/ml) respectively. After day 28, the heparinized blood was taken from each mouse's heart for 400 µl per sample, and isolated for mononuclear cell separation by Ficoll-Hypaque density gradient centrifugation (Ficoll-PaquePREMIUM 1.084, Sigma–GE, CA, USA). The cells were detected for CTL surface marker ($CD8^+$ T-cell) by using phycoerythrin-cyanine 5 (PE-Cy5)-anti-mouse CD8-antibody (e-Bioscience, CA, USA), and helper T-cell marker ($CD4^+$ T-cell) by PE-anti-mouse CD4-antibody (e-Bioscience, CA, USA). A percentage of mice $CD4^+$ and $CD8^+$ T-cells were reported by FACSortTM flow cytometer (Becton Dickinson, NJ, USA).

Mice antibody response

The antibodies against E7 was evaluated from mice serum by indirect ELISA. The E7 protein in PBS was coated on microtiter plates overnight at 4 °C with 25 µg/well of antigen. Afterwards, they were blocked with 200 µl of PBS containing skim milk for 2 h at 37 °C and washed three times with PBS. Then, the mouse serum was diluted to 1: 20 with skim milk in PBS and incubated for 2 h with E7 antigen on the plates. After incubation, the plates were washed with PBS for three times. Mice antibodies to E7 protein bound to the E7 antigen on the plates were detected by goat anti-mouse IgG-horseradish peroxidase (e-Bioscience, CA, USA) in PBS after incubating for 2 h at 37 °C. Following washes, the reaction was detected by TMB substrate (Bio-rad, CA, USA). After acid stop solution, the plates were measured for OD at 450 nm by ELISA reader.

Statistical analysis

The statistical analysis was determined by a two-tailed Student's t-test for significant differences between the means. The statistically significant is P-values <0.05.

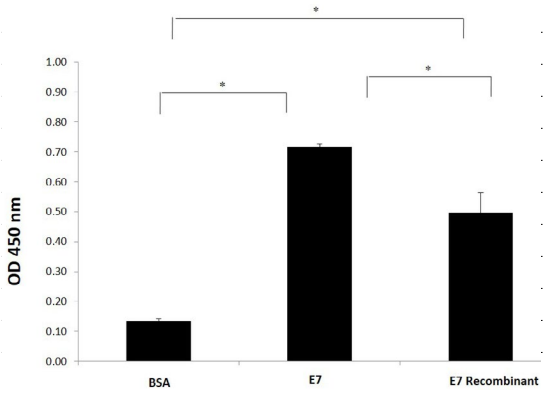

Fig 1. The antigenicity of E7 recombinant protein was determined by indirect ELISA. The E7 protein and BSA were applied as positive and negative control, respectively. The bars represent the arithmetic mean value (n = 3) ± SD.*, P < 0.05 between BSA, E7 and E7 recombinant protein

RESULTS

E7 recombinant protein has antigenicity

The lyophilized E7 protein from eluates was subjected to testing by ELISA. Antibody response to the positive, negative control and E7 recombinant protein was shown in OD450 nm as 0.716 ± 0.06, 0.174 ± 0.01 and 0.496 ± 0.04, respectively (Fig 1). Antibody response to the positive control and E7 recombinant protein showed significantly higher levels of OD (n=3, p<0.05) than the negative control (Fig 1).

This data indicates that the purified E7 protein obtained from Ni-NTA agarose column has antigenicity.

3.2 Sizes of PLGA E7PLGA and PSC-E7PLGA NPs The size of E7PLGA and PSC-E7PLGA NPs was evaluated by Malvern Mastersizer 2000, and the NPs are in the size of 632.47 and 781.23 nm, respectively in colloidal systems (Fig 2). Polydispersion (PDI) of PLGA NPs is 0.492 and 0.532, respectively.

Encapsulation efficacy of E7PLGA and PSC-E7PLGA

The E7 protein (1 mg) was encapsulated in PLGA NPs and quantitated by Bradford Assay before and after encapsulation. The beta-glucan (1 mg) was also encapsulated in PLGA NPs and quantitated by beta-glucan test kit before and after encapsulation. The quantitation of encapsulated E7 protein is shown as 0.8 mg, and beta-glucan into PLGA NPs is shown 0.75 mg (Fig 3).

The encapsulation efficacy of E7PLGA and PSC-E7PLGA are 80% for E7 protein and 75 % for beta-glucan.

PSC-E7PLGA could induce higher humoral immune response

Antibody response to PBS, PLGA, E7PLGA and PSC-E7PLGA was evaluated in mice serum since mice carry the E7 antibody. The antigenicity of E7PLGA and PSC-E7PLGA was evaluated by mice immune response for the same reason. Mice antibody response to PBS, PLGA, E7PLGA and PSC-E7PLGA were shown in OD450 nm as 0.132 ± 0.01, 0.134 ± 0.01, 0.566 ± 0.07 and 0.918 ± 0.04, respectively (Fig 4).

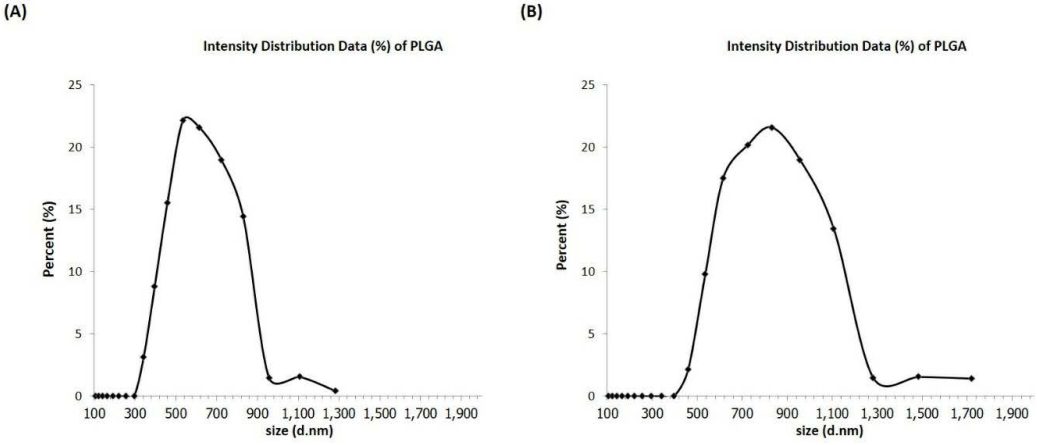

Fig 2. Size distribution of E7PLGA (A) and PSC-E7PLGA NPs (B). The NPs are in colloidal systems with a size of 632.47 and 781.23 nm, respectively

It was observed that the mice exhibited no immune response to PBS and PLGA, whereas the mice into which E7PLGA and PSC-E7PLGA were administered produced significantly higher levels of antibodies (n=3, $p<0.001$) than the mice administered PBS and PLGA. The data indicates that the E7PLGA and PSC-E7PLGA administered mice could generate the humoral immunity by producing specific antibody to E7 protein. The highest level of antibodies was observed in PSC-E7PLGA NPs treated mice (Fig 4).

Expansion of helper and cytotoxic T-cells was triggered by PSC-E7PLGA

The expressions of T-lymphocytes induced by PBS, PLGA, E7PLGA and PSC-E7PLGA NPs were evaluated in vivo for mice helper (CD4) and cytotoxic (CD8) T-cells (Fig 5). The significant expansion of mice $CD4^+$ and $CD8^+$ T-cells was shown in the mice immunized with E7PLGA and PSC-E7PLGA by flow cytometry analysis data (Fig 5). The percentages of $CD4^+$ T-cells from PBS, PLGA, E7PLGA and PSC-E7PLGA immunized mice were 1.47 ± 0.38 %, 2.32 ± 0.12 %, 6.87 ± 0.71 % and 8.26 ± 0.65 %, respectively. The percentages of CD8+ T-cells from PBS, PLGA, E7PLGA and PSC-E7PLGA immunized mice were 1.93 ± 0.46 %, 2.32 ± 0.12 %, 4.87 ± 0.90 %, and 6.48 ± 0.71 % respectively. There was a significant increase in mice CD4+ and CD8+ T-cells population from PSC-E7PLGA immunized mice when compared to the E7PLGA immunized group. This data suggests that PSC-E7PLGA could significantly enhance the expansion of mice helper and cytotoxic T-cells at a higher level than E7PLGA (n=3, $p<0.05$) (Fig 5).

DISCUSSION

PLGA particles have been an intensive focus of research for several decades in nanotechnology due to their desirable properties such as biodegradable synthetic polymers. They have been approved for human and veterinary use by the American Food and Drug Administration (FDA) [22]. Furthermore, PLGA particles have been reported to magnify the potency of vaccine [23,24]. In recent vaccinology, PLGA particles are the effective delivery systems for vaccination. Studies by Newman et al. showed that PLGA NPs could improve immune responses of poor immunogens. The particulate property of PLGA demonstrated rapid internalization due to its charge and pathogen-mimicking size. In general, the particulate form of antigens is higher for uptake by antigen presenting cells (APCs) than soluble form [25].

For HPV vaccine, E7 protein PSC-E7PLGA on PLGA NPs has been applied as the adjuvant to magnify immune responses in mice [7]. Due to its efficiency and safety profile, PLGA adjuvant has the potential to be an effective immunomodulator in existing and experimental vaccines [26]. In this study, E7 protein and PSC-E7PLGA were applied into PLGA NPs. Here, E7 antigen protein has shown its antigenicity by ELISA, which is one of the best methods to determine antigenicity and the quantity of protein on PLGA NPs. Moreover, the size of NPs in the current study could be suitable in both potencies of immunomodulator as the size of 600-800 nm in the colloidal system. Mice antibody response evaluated the antigenicity of E7PLGA and PSC-E7PLGA for studying the humoral and cellular immunity.

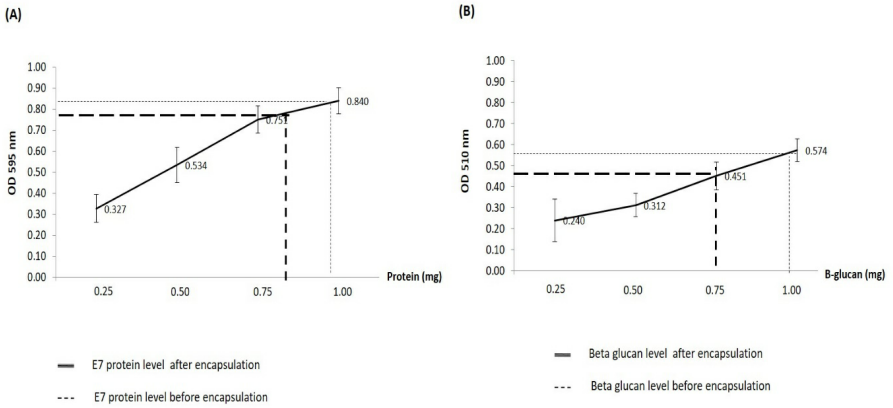

Fig 3. Encapsulation efficacy of E7PLGA (A) and PSC-E7PLGA (B). The E7 protein and beta-glucan of 1.0 mg were encapsulated in PLGA NPs and quantitated by Bradford Assay and beta-glucan test kit before and after encapsulation. The quantitation of encapsulated protein and beta-glucan into PLGA NPs is shown as 0.8 mg and 0.75 mg, respectively

For viral vaccines, the primary focus is on human lymphocytes to represent the whole adaptive immune system and guard against the virus as well as eradicate cancer.

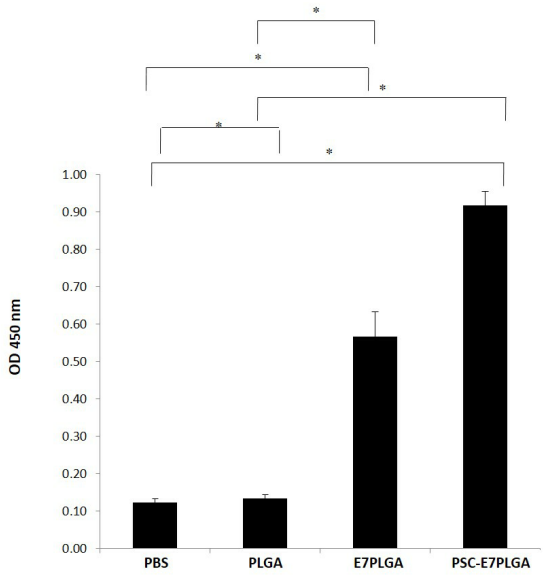

Fig 4. Groups of mice (n=3) were subcutaneously vaccinated twice on days 0 and 14 with PBS, PLGA, E7PLGA and PSC-E7PLGA. Mice antibody levels were determined by indirect ELISA. Bars represent the arithmetic mean value (n=3) ± SD.*, P<0.005 between PBS, PLGA, E7PLGA and PSC-E7PLGA

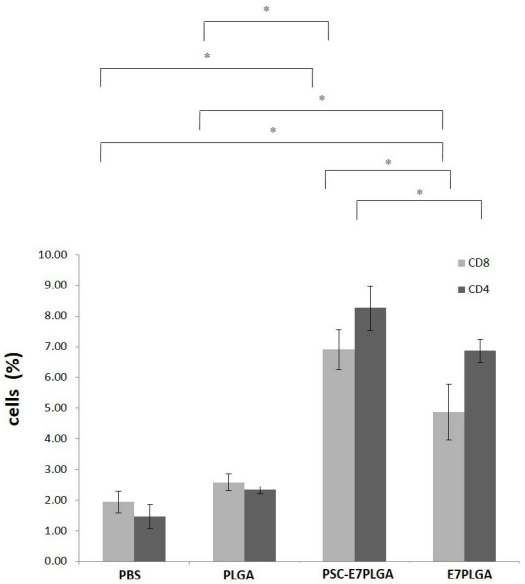

Fig 5. PBS, PLGA, E7PLGA and PSC-E7PLGA immunized mice were monitored for CD4+ and CD8+ T cells by flow cytometry analysis. Percentage of CD4+ and CD8+ T-cells is shown as bars. The bars correspond to the arithmetic mean value (n=3) ± SD. *, P<0.05 between PBS, PLGA, E7PLGA and PSC-E7PLGA

The immunized mice with E7PLGA have shown the specific antibody response to E7 protein. Therefore, E7PLGA is potent for enhancing the protective immunity and recommended for application in vaccines to prevent HPV16.

The results of mice immunization are related to the expansion of helper T-lymphocytes by PSC-E7PLGA. A higher expression of helper T-cells was induced by PSC encapsulation onto PLGA NPs when compared to E7 encapsulation of PLGA NPs. Mature T-helper cells express the surface marker CD4 referred to as CD4+ T-cells [27, 28]. Helper T-cells are a type of T-cell that plays as important role particularly in the adaptive immune system. They increase the activity of immune cells by released cytokines to suppress or regulate immune responses and promote B-cell antibody immune cell populations [29].

The expansion of cytotoxic T-cells is also significantly higher in PSC-E7PLGA immunized mice. The cytotoxic T- cell is a T-lymphocyte that kills cancer cells as well as cells that are infected mainly with viruses [33,34]. Cytotoxic T-cells also play a critical role in cancer cell elimination. CD8+ T-cells recognize an antigen on MHC class-I molecules, which is the peptide from protein degradation inside the cell. These are given at the cell surface to cytotoxic T-cells, enabling the cytotoxic T-cells to scan for cellular alterations [30, 31]. To date, effective vaccines are also focused on cytotoxic T-cells for immunotherapy [32, 33], which are aimed at modulating the immune response against viruses. The protein antigens must enter antigen-presenting cells, where peptides are processed and presented by MHC class-II molecules to CD4+ T cells [34]. Biodegradable particles are applied as the delivery of exogenous antigen to antigen presenting cells. The cross-presentation is required for exogenous antigen by increasing antigen presentation to CD8+ T-cells by MHC-I [35,36]. This cytosolic delivery of intracellular antigen for cross-presentation is the advantage of PLGA NPs. This PLGA NPs combination with immunomodulating polysaccharide from mushrooms could potentially perform as effective immunostimulatory and delivery systems for vaccines [26, 33, 37].

CONCLUSION

E7PLGA and PSC-E7PLGA exhibited the ability to induce an adaptive immune response to HPV. PSC-E7PLGA is significantly higher in immunomodulator properties for HPV vaccine by

enhancing the humoral immunity consisting of viral antigen-specific IgG virus-neutralizing antibodies and increasing the cellular immune response characterized by expansion and activation of viral antigen-specific CTL.

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