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Lipid-enveloped zinc phosphate hybrid nanoparticles for codelivery of H-2K^b and H-2D^b-restricted antigenic peptides and monophosphoryl lipid A to induce antitumor immunity against melanoma

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ABSTRACT

Nanoimmunotherapy, the application of nanotechnology for sustained and targeted delivery of antigens to dendritic cells (DCs), has attracted much attention in stimulating antigen-specific immune response for antitumor therapy. In order to *in situ* deliver antigens to DCs for efficient antigen presentation and subsequent induction of strong cytotoxic T lymphocytes (CTL) response, here we developed a multi-peptide ($TRP2_{180–188}$ and $HGP100_{25–33}$) and toll-like receptor 4 agonist (monophosphoryl lipid A) codelivery system based on lipid-coated zinc phosphate hybrid nanoparticles (LZnP NPs). This delivery system equips with the chelating property of zinc to realize the high encapsulation efficiency with antigenic peptides and the influence on immune system with adjuvant-like feature. The combination of H-2K^b and H-2D^b-restricted peptides could provide multiple epitopes as the target of specific MHC alleles, making tumor more difficult to escape from the surveillance of immune system. The formulated LZnP nano-vaccine with the size of 30 nm and outer leaflet lipid exhibited antitumor immunity as the secretion of cytokines *in vitro* and increased $CD8^+$ T cell response from IFN-γ ELISPOT analysis *ex vivo*. The antitumor effects were further evidenced from the prophylactic, therapeutic and metastatic melanoma tumor models compared with free antigens and single peptide-loaded nano-vaccines. These results validate the benefit of LZnP-based vaccine for antitumor immunity and indicate that co-delivery of tumor antigens along with adjuvant may be an optimized strategy for tumor immunotherapy.

1. Introduction

Cancer immunotherapy is prospected to recognize and selectively kill tumor, prevent the occurrence and development of tumor, inhibit tumor metastasis and avoid tumor recrudesce by stimulating the human's own immune system [1,2]. Antigen *ex vivo* pulsed DC-based cancer vaccine remains insufficient and costly but has paved the way for developing next generation of more efficient, productive and low cost cancer vaccine formulations. The ideal vaccine formulation should comprise three main components, antigen, adjuvant and delivery system. Antigens applied until now mainly focused on synthetic peptides, recombinant proteins, tumor cells or tumor lysates, *etc.* [3]. Libraries of synthetic peptides with high purity, no toxicity or infectious factors can be presented directly by DCs without processing inside the cells and have been widely used for vaccination [4,5]. Of note, most of the tumor antigens are non-mutated self-antigens with poor immunogenicity. Tumor cells could develop multiple mechanisms to suppress immune response, such as down expression of tumor antigens, dysfunction of T-cells, and MHC allelic losses [6–8]. Different altered MHC phenotypes that originate from multiple molecular mechanisms have been identified in tumor cells. A single epitope specific to a particular MHC type might be inadequate to overcome immune suppression and induce effective immune response. Compared with single epitope peptide, the combination of peptides would provide multiple epitopes as the target of specific MHC alleles, making tumor more difficult to escape from the surveillance of immune system [9,10]. The immunological adjuvants could boost the immune response by activating innate immunity, optimizing antigen presentation, recruiting DCs, and creating a cytokine environment [11]. Toll-like receptor (TLR) agonists were commonly used as they could act as danger signal to alert immune system and promote the persistent and magnified immune response. When it comes to delivery system, nanoparticles have shown great potential in codelivering antigens and adjuvants as they can not only efficiently load and transport antigenic peptides, prevent the payload from inactivation, but also equip with immune regulation and specific

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targeting ability [12,13]. With controllable size, regular morphology, modifiable surface and sufficient antigen and adjuvant co-encapsulation, inorganic nanoparticles have been investigated for their utilization in vaccines [14–17].

Zinc and phosphate both are essential elements for life, cofactors for functional enzymes and transcription factors [18]. It has been reported that zinc can tightly combine with protein by chelating and also influence the immune system by promoting the activity of antigen-presenting cells (APCs) [19–21]. H-2K^b-restricted epitope peptide TRP2$_{180–188}$ and H-2D^b-restricted epitope peptide HGP100$_{25–33}$ have been demonstrated to be highly expressed in melanoma cells, and extensively applied in tumor vaccine to stimulate specific immune response [22–24]. TLR4 agonist, monophosphoryl lipid A (MPLA) has been already used as adjuvant in approved vaccine formulations. In this work, to take use of the chelating properties and influence on the immune system of zinc, we developed a novel kind of lipid-coated zinc phosphate hybrid nanoparticles for co-encapsulating the antigenic peptides (TRP2 and HGP100) and MPLA. This system combined the unique coordinative binding property of Zn^{2+} with peptides for the high encapsulating capacity of multi-peptides, and the lipid-doped structure for the improved particles stability, biocompatibility and entrapment capability of lipid-like adjuvant of MPLA. The co-adjuvant potency of this LZnP NP was demonstrated as the synergistic antitumor immunity with MPLA. The remarkable effect of LZnP-based vaccine for antitumor immunity was further validated through the prophylactic, therapeutic and metastatic melanoma models. To the best of our knowledge, this is the first report to develop a peptide-based vaccine system delivered by LZnP NPs with high encapsulating efficiency and adjuvant-like property to enhance DC maturation, increase cytokines secretion and promote $CD8^+$ T cell response (Scheme 1). This nanovaccine based on LZnP NPs and multi-peptides possesses great application prospect in cancer immunotherapy.

2. Materials and method

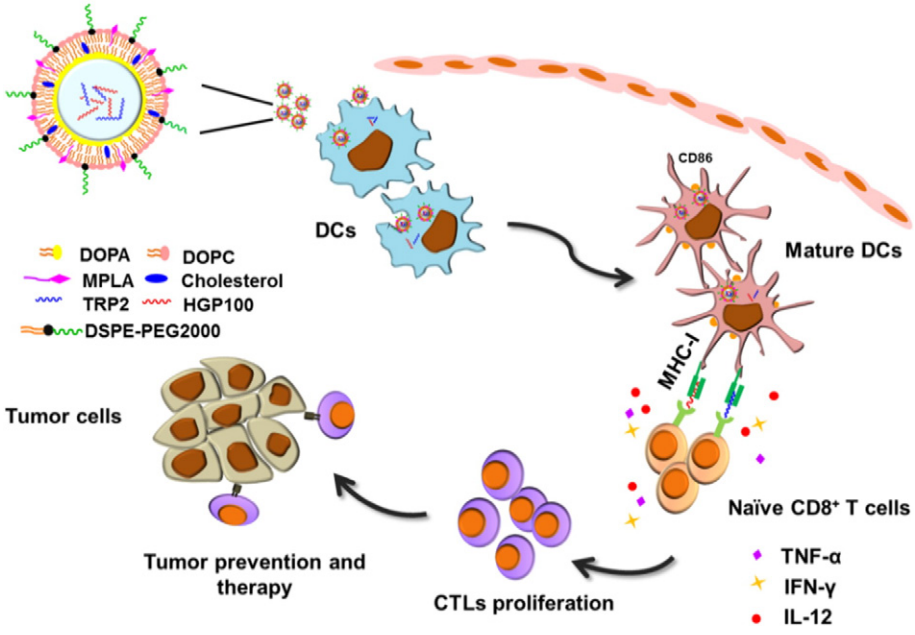
2.1. Materials

Zinc nitrate ($Zn(NO_3)_2 \cdot 6H_2O$, AR), disodium hydrogen phosphate (Na_2HPO_4, AR) and cyclohexane were purchased from Aladdin®. Igepal CO-520 and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich (St. Louis, MO). Dioleoyl phosphatidic acid (DOPA), 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolami-ne-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000), monophosphoryl lipid A (MPLA) and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Beyotime Institute of Biotechnology. 1,1-Dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine (DiD) was obtained from KeyGen Biotechnology (Nanjing, China). HGP100 (KVPRNQDWL), P15E (KSPWFTTL), AFP (FMNKFIYEI), FITC-HGP100, TAMRA-TRP2 and modified peptides p-TRP2 (pSpSSSVYDFFVWL) were synthesized by Bioyears (Wuhan Bioyeargene Biosciences Co. Ltd., China). Purified Anti-Mouse CD4 (GK1.5) antibody was purchased from Tianjin Sungene Biotech Co., Ltd. Anti-mouse monoclonal antibodies against B220, CD11b, CD11c, CD86 and CD3e were purchased from BD Pharmingen™.

B16-F10 murine melanoma cancer cells and DC2.4 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/mL of penicillin and 100 μg/mL of streptomycin in a humidified atmosphere incubator with 5% CO_2 at 37 °C. Bone marrow-derived dendritic cells (BMDCs) were extracted and cultured as previously reported [25]. Briefly, the BMDCs were collected from marrow cavities of femurs and tibias of mice and cultured in RPMI 1640 medium supplemented with 10% FBS, 100 IU/mL of penicillin, 100 μg/mL of streptomycin, 10 ng/mL GM-CSF and 5 ng/mL IL-4 at 37 °C in 5% CO_2 humidified atmosphere. Six to eight week-old female C57BL/6 mice were purchased from the Experimental Animal Center of Wuhan University, China. All animal experiments were performed under specific pathogen-free (SPF) condition in the Animal Center of HuaZhong University of Science and Technology, China.

2.2. Preparation and characterization of zinc phosphate nanoparticles (ZnP NPs)

One hundred microliter of $Zn(NO_3)_2$ (500 mmol) and 100 μL of Na_2HPO_4 (100 mmol) were dispersed in 4 mL cyclohexane/CO520 and stirred at room temperature to form reverse water-in-oil microemulsion, respectively. Thirty minutes later, the P phase was dropped slowly (1 mL/10 min) into the Zn phase and left to stir for 2 h. An equal volume of absolute ethanol was added to disrupt the emulsion and the mixture was centrifuged at 13,000 g for 15 min to remove



Scheme 1. Schematic of antitumor immunity induced by LZnP NPs based peptide vaccines.