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Engineering detoxified pneumococcal pneumolysin derivative ΔA146PLY for self-biomineralization of calcium phosphate: Assessment of their protective efficacy in murine infection models

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ABSTRACT

Vaccine design ushered in the era of nanotechnology, as the vaccine is being developed toward particulate formulation. We have previously shown that the attenuated pneumolysin mutant (ΔA146PLY) was a safe and effective pneumococcal vaccine candidate. Here, to further optimize the formulation, we fused calcium phosphate (CaP) binding domains with ΔA146PLY so that the biocompatible CaP can mineralize with the protein automatically, allowing simple production of nanoparticle antigen during preparation. We fabricated four different nanoparticles, and then we compared the characteristics of different CaP-ΔA146PLY nanoparticles and demonstrated the influence of CaP binding domains on the size, shape and surface calcium content of the nanoparticles. It was found that these self-biomineralized CaP-ΔA146PLY nanoparticles varied in their capacity to induce BMDCs and splenocytes production of cytokines. We further demonstrated that, compared to free proteins, nanoparticle antigens induced more efficient humoral and cellular immune responses which was strong enough to protect mice from both pneumonia and sepsis infection. Also, the integration of CaP to protein has no significant impairment on body weight of animals, and subcutaneous injection of ΔA146PLY-peptides@CaP nanoparticles did not lead to permanent formation of nodules in the skin relative to Alum adjuvant formulated antigens. Together, our data sufficiently suggest that soluble ΔA146PLY vaccine candidate could be processed into nanoparticles by self-biomineralization of CaP, the immunogenicity of which could be efficiently improved by the CaP binding domains and biomineralization.

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1. Introduction

The development of protein vaccine has been the research focus of next generation vaccine, primarily due to its favorable safety and manufacturing traits. Both academia and pharmaceutical companies are interested in the development of protein-based vaccines, including bivalent or multivalent protein formulations [1], suggesting that the protein subunit vaccine is an effective and attractive alternative strategy to prevent infections. However, the drawbacks of the protein antigen in its immunogenicity and bioavailability [2], possibly due to their small size and soluble characteristic, highlight the need of sustaining efforts in the development of protein-based vaccines.

In general, adding an adjuvant to protein preparations is an efficient strategy to improve the immunogenicity of the target protein antigens. However, only a few numbers of adjuvants have been approved in human vaccine, including aluminum adjuvant, MF19 and AS04 [2]. Of the adjuvants available, the aluminum adjuvant is the most widely being used one [2], but this adjuvant has shown limits in terms of induction of cell-mediated Th1 and cytotoxic CD8 T-cell immune responses, and could cause autoimmune or neurological diseases [3]. Enabled by advances in genetics and biomaterials, vaccine/adjuvant design is more flexible and

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could be tailored to enhance specific immune responses [4,5]. Particulate/nanoparticle subunit vaccine represents an exciting vaccine formulation being developed, which could be easily tailored to enhance the immunogenicity of the target antigens and hence induce specifically the humoral immunity, cellular immunity, or both [6–8]. There are several ways for antigens to be processed into particles, including adsorption or attachment to biodegradable materials, such as polyesters [9,10], inorganic substances [8,11], polyanhydride and polysaccharides [12], and self-assembly [13].

Although several synthetic or natural nanoparticles have been used as carriers for vaccination [14–16], using calcium phosphate (CaP) nanoparticles to deliver target antigens holds some advantages. CaP is naturally present in the organism [17,18], and has been approved in human vaccination in some Europe countries [19]. CaP is biodegradable material which could be caught via endocytosis by macrophage or dendritic cells and degraded in lysosome in the host. Using CaP as a carrier, dendritic cells and antigen-specific $CD4^+$ and $CD8^+$ T-cell responses could be efficiently induced [5,8]. The discovery of self-biomineralization related peptides for CaP has greatly simplified the preparation process of nanoparticles and makes the preparation reproducible [8,20]. Thus, CaP may be an attractive adjuvant candidate to be incorporated in vaccine formulations.

Pneumolysin derivatives are attractive vaccine candidates for targeting pneumococcal infection [21,22]. ΔA146PLY is a derivative of pneumolysin which is minimally toxic but immunogenic [23,24]. We have previously shown that the Ply mutant protein (ΔA146PLY) was an effective vaccine candidate to protect against pneumococcal infections [24]. Other groups have also shown that this protein can produce a protective immune response in mice [23]. Meanwhile, it is known that pneumolysin is a toll-like receptor 4 (TLR4) ligand, and deletion mutant in 146 aa of pneumolysin did not impair its ability as a TLR4 agonist [25]. It has been shown that nanoparticles have synergistic effect with agonist to elicit more profound immune responses [26]. Inspired by these observations, we proposed to fabricate nanoparticles of CaP-ΔA146PLY in order to circumvent the problems for soluble antigens regarding their weak-to-moderate immunogenicity alone. Thus, in the present study, several main CaP biomineralization peptides containing PA44 [27], NW, N6, W6 [20] were fused to the C-terminal of protein ΔA146PLY to fabricate biomineralized protein nanoparticles. Next, the size, shape, and surface calcium content of CaP-ΔA146PLY nanoparticles were compared and their immuno-protective effects were investigated to screen for the most efficient forms *in vitro* and *in vivo*.

2. Materials and methods

2.1. Ethics statement

Specific pathogen-free, female and male C57BL/6 (6–8 weeks old) were purchased from Beijing HFK Biotechnology, and raised at Chongqing Medical University, Chongqing, China. All experimental protocols were approved by the Ethics Committee of Chongqing Medical University. The research was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

2.2. Bacterial strains and growth conditions

E. coli strains were grown in Luria–Bertani medium at 37 °C. *S. pneumoniae* strain D39 (NCTC7466, serotype 2) was obtained from the National Collection of Type Culture (London, UK). *S. pneumoniae* 19F (CMCC 31693) was purchased from the National Center for Medical Culture Collections (Beijing, China), and was cultured on Columbia plates containing 5% sheep blood or in Casitone-Yeast extract (C + Y) medium both in an atmosphere of 5% CO_2 at 37 °C.

2.3. Preparation of recombinant proteins

The primers used in this study were synthesized by Takara Bio, Dalian and listed in Table S1. Primer pairs F-Ply-N and R-PA44Ply-C, F-Ply-N and R-NWPly-C, F-Ply-N and R-N6Ply-C, and F-Ply-N and R-W6Ply-C, respectively, were used to generate the coding sequences of ΔA146PLY -PA44, -NW, -N6, and -W6 by PCR. The resulting PCR products were digested with *BamH* I and *Xho* I and cloned into the prokaryotic expression vector pET28α (Novagen). Correct clones were identified by sequencing and subjected to the following use.

The plasmids pET28α-ΔA146PLY-PA44, pET28α-ΔA146PLY-NW, pET28α-ΔA146PLY- N6 and pET28α-ΔA146PLY-W6 were transformed into the prokaryotic expression host of *E. coli* BL21 (DE3) (Stratagene), respectively. All fusion proteins were overproduced in *E. coli* BL21 (DE3) and expression was induced at an OD_{600} of 2.0 by addition of 0.5 mM isopropyl-D-thiogalactoside (IPTG) at 25 °C. Eight hours after induction, cells were harvested by centrifugation, washed once with the appropriate disruption buffer, and disrupted by ultrasonication. Proteins were purified by nickel chelate affinity chromatography (GE Healthcare) according to the established protocol. Polymyxin B-agarose (Sigma) was used to remove lipopolysaccharide (LPS) contamination after purification. The purity of all the fusion proteins was determined by 10% SDS-PAGE. Aliquots were stored −80 °C until use.

2.4. Preparation of biomineralized nanoparticles

CaP-ΔA146PLY nanoparticles were prepared essentially as described elsewhere [27]. In brief, 1 mL solution containing 8.8 μmol $CaCl_2$, 5.28 μmol $(Na)_2HPO_4/NaH_2PO_4$ (pH 7.5) and either 360 μg of ΔA146PLY-PA44, ΔA146PLY-NW, ΔA146PLY-N6, ΔA146PLY-W6 or ΔA146PLY, was gently mixed with micro magnetic stirring bars. The mixtures were held at 4 °C for 2 h and then placed at room temperature for 24 h for stabilization.

2.5. Physical properties of biomineralized protein nanoparticles

Equal amount of proteins ΔA146PLY-PA44, ΔA146PLY-NW, ΔA146PLY-N6, ΔA146PLY-W6 and ΔA146PLY were spotted on polyvinylidene fluoride films and incubated with excessive $CaCl_2$ and $(Na)_2HPO_4/NaH_2PO_4$ for 2 h. These polyvinylidene fluoride films were washed twice by deionized water, followed by von Kossa staining according to the manufacturer's instructions (Showbio), and visualized under light microscope.

For transmission electron microscopy (TEM), original protein samples were diluted with 0.9% NaCl (pH 7.5), and then processed by the Electron Microscopy Research Service of Chongqing Medical University. For visualization, cells were sectioned and imaged with a Hitachi H-7500 transmission electron microscope. For X-ray photoelectron spectroscopy (XPS), small drips of samples were onto cover glasses and allowed to air dry. X-ray photoelectron spectroscope (ESCALAD-250) was used to analyze the surface content of calcium, phosphorus and oxygen. The levels of Ca^{2+}, PO_4^{3-} and Ca/P ratio in the whole particles were detected after dissolution by acids (PH 5.0) using reagent kits (Biosino bio-technology and science inc) following the manufacture's instruction.

2.6. Generation of murine bone marrow-derived dendritic cells (BMDCs)

BMDCs were prepared as described previously [26]. Briefly, the

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