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Dendrimer-conjugated peptide vaccine enhances clearance of *Chlamydia trachomatis* genital infection

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ABSTRACT

Peptide-based vaccines have emerged in recent years as promising candidates in the prevention of infectious diseases. However, there are many challenges to maintaining *in vivo* peptide stability and enhancement of peptide immunogenicity to generate protective immunity which enhances clearance of infections. Here, a dendrimer-based carrier system is proposed for peptide-based vaccine delivery, and shows its anti-microbial feasibility in a mouse model of *Chlamydia trachomatis*. *Chlamydiae* are the most prevalent sexually transmitted bacteria worldwide, and also the causal agent of trachoma, the leading cause of preventable infectious blindness. In spite of the prevalence of this infectious agent and the many previous vaccine-related studies, there is no vaccine commercially available. The carrier system proposed consists of generation 4, hydroxyl-terminated, polyamidoamine (PAMAM) dendrimers (G4OH), to which a peptide mimic of a chlamydial glycolipid antigen—Peptide 4 (Pep4, AFPQFRSATLLL) was conjugated through an ester bond. The ester bond between G4OH and Pep4 is expected to break down mainly in the intracellular environment for antigen presentation. Pep4 conjugated to dendrimer induced *Chlamydia*-specific serum antibodies after subcutaneous immunizations. Further, this new vaccine formulation significantly protected immunized animals from vaginal challenge with infectious *Chlamydia trachomatis*, and it reduced infectious loads and tissue (genital tract) damage. Pep4 conjugated to G4OH or only mixed with peptide provided enhanced protection compared to Pep4 and adjuvant (i.e. alum), suggesting a potential adjuvant effect of the PAMAM dendrimer. Combined, these results demonstrate that hydroxyl-terminated PAMAM dendrimer is a promising polymeric nanocarrier platform for the delivery of peptide

Abbreviations: PAMAM, poly(amidoamine); G4OH, generation 4 hydroxyl-terminated PAMAM dendrimer; Pep4, a peptide with the sequence AFPQFRSATLLL; PID, pelvic inflammatory disease; C. trachomatis, Chlamydia trachomatis; GLXA, glycolipid exoantigen; MHC-II, class II major histocompatibility complex; MW, molecular weight; sulfo-NHS, sulfo-N-hydroxysuccinimide; EDC, ethyl-3-(3-dimethylaminopropyl) carbodiimide; MES, 2-(N-morpholino) ethanesulfonic acid; DIPEA, diisopropylethylamine; Al(OH)$_3$, aluminum hydroxide; 2, 5-DHB, 2, 5-dihydroxy benzoic acid; Fmoc-AHA, fluorenylmethyloxycarbonyl-6-amino-hexanoic acid; Fmoc, fluorenylmethyloxycarbonyl group; AHA, 6-amino-hexanoic acid; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; MWCO, molecular weight cut off; DI water, deionized water; G4OH-yNH$_2$, G4OH dendrimer modified with y amine groups on surface; G4OH-Pep4, dendrimer-Pep4 conjugate; G4OH+Pep4, physical mixture of G4OH dendrimer and Pep4; G4NH2, Generation 4 amine-terminated PAMAM dendrimer; G4COOH, Generation 4 carboxyl-terminated PAMAM dendrimer; DMF, dimethylformamide; DCM, dichloromethane; MeOH, methanol; TLC, thin layer chromatography; ^{1}H NMR, proton nuclear magnetic resonance; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; DLS, dynamic light scattering; HD, hydrodynamic diameter; PBS, phosphate buffer saline; SC, subcutaneous injection; EB, elementary bodies; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; Ab, antibody; H&E, hematoxylin and eosin; DFA, direct fluorescent antibody; IFU, inclusion forming units; OVA, ovalbumin; s.d., standard deviation; mAb, monoclonal antibody; HIV, human immunodeficient virus; HSV, herpes simplex virus; HPV, human papillomavirus virus.

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vaccines and this approach has potential to be expanded to other infectious intracellular bacteria and viruses of public health significance.

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1. Introduction

Subunit vaccines are based on synthetic peptides, isolated proteins, polysaccharides, or antigen encoding DNA sequences that elicit specific immune responses. (Gregory et al., 2013) These vaccines are the preferred strategy in the prevention of infectious diseases currently lacking effective vaccines, such as tuberculosis, human immunodeficiency virus (HIV), group A *Streptococcus*, malaria, hepatitis B, and others. (Heegaard et al., 2010) In addition, vaccines have potential in cancer preventive therapy. (Parmiani et al., 2014) Subunit vaccines can be used as alternatives to attenuated or killed microbes or their toxins in cases where the individual's safety is a concern, such as in the immunocompromised population, the elderly and children. (Gregory et al., 2013) Subunit vaccines can also be employed as an overlap strategy to traditional vaccine formulations that offer low protection levels. (Ivanyi, 2014)

Subunit vaccines may offer new opportunities in the development of a preventive vaccine for *Chlamydia* infections, a goal that has remained elusive in spite of the significant vaccine development efforts over the past decades. (Yu et al., 2016) *Chlamydia trachomatis* (*C. trachomatis*) is an important vaccine target. It is the most prevalent sexually transmitted bacterium worldwide, with the latest statistics showing more than 1.4 million cases annually in the United States alone. (Centers for Disease Control and Prevention, 2015) However, there is currently no commercially available vaccine for human chlamydial infection. In an acute infection, *C. trachomatis* can infect men and women causing urethritis and cervicitis. However, up to 70–75% of infections in women are asymptomatic and, without treatment, might lead to chronic infection and damage to the genital tract. (Hafner et al., 2008) *Chlamydia* genital infection is directly related to chronic pelvic inflammatory disease (PID), infertility, and adverse pregnancy outcomes, in addition to being a risk factor for other sexually transmitted diseases. (Shaw et al., 2011) Treatment for *C. trachomatis* infection is currently based on antibiotics, but some studies have shown that *C. trachomatis* may enter a persistent infectious state before or during treatment, which is also linked to long-term sequelae affecting the reproductive tract as well as joints, in reactive arthritis. (Carter et al., 2010) As for most bacteria, primary chlamydial infection does not prevent reinfection. Protective immunization is, therefore, an essential and widely accepted strategy to control infection by this bacterium. (Yu et al., 2016)

A peptide mimic of the glycolipid exoantigen (GLXA) – a genus-wide chlamydial antigen, has been regarded as a promising antigen candidate. (Stuart and Macdonald, 1989) We previously derived a series of peptides (including Pep4) from a phage display peptide library by selection of peptides bound by an anti-GLXA antibody (mAb1). (Hamzeh-Mivehroud et al., 2013; Petrenko, 2008; Whittum-Hudson and Hudson, 2011) These peptide sequences retain the genus-wide characteristic of GLXA (Stuart and Macdonald, 1989; Vora and Stuart, 2003) and may provide protection against not only the sexually transmitted chlamydial serovars, but against trachoma (eye infection) (Abu El-Asrar et al., 1998) and some human diseases associated with other *Chlamydia* species such as cardiovascular disease, (Wong and Ward, 1999) chlamydial pneumonia and asthma, (Hahn et al., 1991) some subsets of Alzheimer's disease, (Balin et al., 1998) multiple sclerosis, (Stratton and Sriram, 2003) and reactive arthritis. (Gerard et al., 1998) In addition, the genus-wide nature of Pep4 confers potential for use in veterinary vaccines for cattle, swine and goats, all of which have native chlamydial species that cause spontaneous abortions, infertility and reactive arthritis. Koala bears which are being devastated by chlamydial infections would also benefit from a genus-wide vaccine. (Waugh et al., 2016)

Micro- and nanoparticle vaccine carriers have been proposed to overcome the inherent challenges associated with free peptide-based subunit vaccines. (Dobrovolskaia, 2017; Gregory et al., 2013; Keegan et al., 2003; Moon et al., 2012; Torres-Sangiao et al., 2016) For instance, micro- and nanoparticles as vaccine delivery systems can enhance antigen delivery to the target tissue and cell populations of interest, (Gregory et al., 2013), or act as immunostimulatory adjuvants to activate or augment specific immune responses. (Sahdev et al., 2014) The incorporation of antigenic peptides into the particles are mainly divided into two strategies: encapsulation in carriers and chemical conjugation. (Singh et al., 2007; Zhao et al., 2014) The peptide/protein encapulation in micro- and nanocarriers is a facile and efficient method to prepare nanoscale vaccine formulatoins. Dixit et al. (2014) encapsulated a recombinant peptide of *Chlamydia trachomatis* major outer membrane protein (MOMP) into a biodegradable poly(lactic acid)–poly (ethylene glycol) (PLA-PEG) nanoparticle. Immunization of mice with encapsulated peptide elicited higher specific T-cell cytokines and potentiated crucial adaptive immunity against *Chlamydia*. (Dixit et al., 2014; Jiang et al., 2017) designed a vault (ribonucleoprotein) nanoparticle vaccine that loaded recombinant *Chlamydia* proteins such as the major outer membrane protein (MOMP) or polymorphic membrane protein G-1 (PmpG-1). The vaccines delivered via intranasal delivery induced strong anti-chlamydial immunity at distant genital mucosal sites and significantly attenuated bacterial burden following challenge infection, while avoiding destructive inflammation. (Champion et al., 2009; Jiang et al., 2017) One limitation of these MOMP-loaded nanovaccine formulations is the lack of genus-wide specificity. Whittum-Hudson et al. (2001) developed a monoclonal anti-idiotypic antibody (a molecular mimic of GLXA)-encapsualted polylactide microsphere vaccine which demonstrated significant protection against topical vaginal challenge with *C. trachomatis* after either mucosal (oral or intranasal) or systemic (subcutaneous) immunization. (Whittum-Hudson et al., 2001) However, encapsulated formulations may face the challenge of less spatially/temporally controlled release of peptides/proteins. Chemical conjugation of antigens to nanoparticles is an alterantive strategy to overcome this issue as it enables stronger interaction between nanoparticles and antigenic peptides (i.e. covalent bonding), potentially leading to a stronger protection of peptide from degradation. Additionally, the linkers between the therapeutic molecule and nanoparticles can be designed to allow for the release of the cargo in a controlled fashion, and within specific environments such as the cell cytosol or acidic endolysosomes, thus affording further spatial and temporal control of the release of the therapeutic cargo. (Kurtoglu et al., 2010; Zhong and da Rocha, 2016) However, nanoparticle-conjugated peptide vaccines have not been reported for *Chlamydia*. Of various nanoparticles, PAMAM dendrimers — a type of synthetic hyperbranched polymer — are of particular interest as they are highly monodisperse, and their size (ca. 3–10 nm) and molecular weight can be controlled easily. (Esfand and Tomalia, 2001) More importantly, they possess a high density of functionalizable peripheral groups (Svenson and

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