Vaccine xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

A novel liposome adjuvant DPC mediates *Mycobacterium tuberculosis* subunit vaccine well to induce cell-mediated immunity and high protective efficacy in mice

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ARTICLE INFO

Article history:
Received 26 August 2015
Received in revised form
31 December 2015
Accepted 22 January 2016
Available online xxx

Keywords:
Vaccine adjuvant
Cationic liposomes
DDA
Poly I:C
Cholesterol
Mycobacterium tuberculosis

ABSTRACT

Tuberculosis (TB) is a serious disease around the world, and protein based subunit vaccine is supposed to be a kind of promising novel vaccine against it. However, there is no effective adjuvant available in clinic to activate cell-mediated immune responses which is required for TB subunit vaccine. Therefore, it is imperative to develop new adjuvant. Here we reported an adjuvant composed of dimethyl dioctadecylammonium (DDA), Poly I:C and cholesterol (DPC for short). DDA can form a kind of cationic liposome with the ability to deliver and present antigen and can induce Th1 type cell-mediated immune response. Poly I:C, a ligand of TLR3 receptor, could attenuate the pathologic reaction induced by following *Mycobacterium tuberculosis* challenge. Cholesterol, which could enhance rigidity of lipid bilayer, is added to DDA and Poly I:C to improve the stability of the adjuvant. The particle size and Zeta-potential of DPC were analyzed *in vitro*. Furthermore, DPC was mixed with a TB fusion protein ESAT6-Ag85B-MPT64(190-198)-Mtb8.4-Rv2626c (LT70) to construct a subunit vaccine. The subunit vaccine-induced immune responses and protective efficacy against *M. tuberculosis* H37Rv infection in C57BL/6 mice were investigated. The results showed that the DPC adjuvant with particle size of 400 nm and zeta potential of 40 mV was in good stability. LT70 in the adjuvant of DPC generated strong antigen-specific humoral and cell-mediated immunity, and induced long-term higher protective efficacy against *M. tuberculosis* infection ($5.41 \pm 0.38 \log_{10}$ CFU) than traditional vaccine Bacillus Calmette–Guerin (BCG) ($6.01 \pm 0.33 \log_{10}$ CFU) and PBS control ($6.53 \pm 0.26 \log_{10}$ CFU) at 30 weeks post-vaccination. In conclusion, DPC would be a promising vaccine adjuvant with the ability to stimulate Th1 type cell-mediated immunity, and could be used in TB subunit vaccine.

1. Introduction

Tuberculosis (TB) which is mainly caused by infection with *Mycobacterium tuberculosis* (*M. tuberculosis*) or *Mycobacterium bovis* (*M. bovis*) remains one of the most important infectious diseases around the world [1]. The only currently available TB vaccine, *M. bovis* Bacillus Calmette–Guerin (BCG), demonstrates variable levels of efficacy in clinic, and its protective immunity wanes in adult [2]. Therefore, there is an urgent need for developing novel vaccines to replace or supplement BCG. Subunit vaccine is supposed to boost BCG and has demonstrated promising efficacy in various models [3–5].

Adjuvant is needed in the subunit vaccine formulation so as to induce an adequate immune response. Selecting an appropriate adjuvant will not only enhance the level of immune response but also determine the type of immune response [6]. Vaccines target against intracellular pathogens such as *M. tuberculosis* often require

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http://dx.doi.org/10.1016/j.vaccine.2016.01.049
0264-410X/

induction of cellular immune responses, including T helper 1 (Th1) type cells and sometimes cytotoxic T lymphocytes (CTLs) [7]. However, the current used adjuvant in clinic, aluminum based compounds, generally promotes humoral Th2 type immune responses to protein subunit vaccines, and elicits insufficient cell-mediated Th1 type immune responses [6,8]. Therefore, discovering new adjuvant that induced well-defined cell-mediated immune response is crucial for the development of vaccines [9,10]. As for TB subunit vaccines, there are four adjuvants are being evaluated in clinical trials now: (i) AS01E, an adjuvant containing the immunostimulants MPL and Quillaja saponaria fraction 1 (QS21) [11]; (ii) IC31 combining a cationic peptide (KLKL(5)KLK) and a synthetic oligodeoxynucleotide (ODN1a), which is a Toll-like receptor 9 agonist [12,13]; (iii) GLA-SE, a synthetic TLR4 agonist GLA formulated in the squalene-in-water stable emulsion (SE) [14]; (iv) CAF01, composed of dimethyldioctadecylammonium (DDA) liposomal and trehalose 6,6′-dibehenate (TDB), a synthetic analog of the mycobacterial cell wall component trehalose 6,6'-dimycolate (TDM) [15]. These adjuvants for clinical trials are all complex formulations being composed of vehicles in combination with an immunostimulator.

Liposome is a type of adjuvant that can improve antigen uptake as the delivery vehicles [16]. Cationic liposomes were superior to both neutral and anionic liposomes as the proteins will be entrapped within the aqueous compartment of the liposomes and coupled with enhancing their uptake by professional APCs [17]. A few adjuvants based on cationic liposomes have progressed to clinical testing in humans, like CAF01, LPD, JVRS-100 and Vaxfectin [18,19]. The cationic liposome DDA, main component of CAF01, is a synthetic quaternary ammonium compound comprising a hydrophilic positively charged dimethylammonium head-group attached to two hydrophobic 18-carbon alkyl chains [20]. In the aqueous environment, DDA self-assembles into closed vesicular bilayers. The adjuvant effect of DDA is mainly attributed to its ability to associate antigens as the delivery vehicles [21], for it has a net positive charge and is readily attracted to cells that have a high abundance of negatively charged surface molecules. Furthermore, it will bind negatively charged proteins and DNA molecules and is thus capable of bringing these antigens into APC cells [22]. For induction of CD8 T-cell responses against a protein antigen, which has to be presented to T cells on MHC class I molecules, DDA can deliver the endocytosed antigen directly into the cytosol by fusion with endosomal membranes or cross-presentation and then transportation of the generated peptides into the endoplasmic reticulum by the transporter associated with antigen processing [23,24].

With the advanced recognition of the association of innate immune responses with adaptive immunity, researchers have been exploring the potential for enhancing immunogenicity of cationic liposomes through addition of Toll-like Receptor (TLR) agonists [25–27]. TLRs are largely divided into two subgroups depending on their cellular localization and respective ligands of pathogen-associated molecular patterns (PAMP). TLR3 was originally identified as recognizing a synthetic analog of double-stranded RNA (dsRNA), polyinosinic-polycytidylic acid (Poly I:C), which mimics viral infection and induces antiviral immune responses by promoting the production of both type I interferon and inflammatory cytokines [28,29]. Incorporating TLR3 agonists into DDA increased the number of IFN-γ, TNF-α and IL-2 producing antigen-specific CD8+ T cells [30] and facilitated cross-presentation of the antigen on MHC class I molecules [26]. BCG polysaccharide nucleic acid (BCG-PSN), which was extracted by hot phenol method from *M. bovis* bacillus Calmette–Guerin and contains polysaccharides and nucleic acid (such as CpG motif), was found to improve the protective efficacy of DDA liposome [31]. CpG motifs (CpG ODN) can directly activate B cells and plasmacytoid dendritic cells (pDC) *via* Toll-like receptor 9 pathway [32]. TDM often referred to as cord factor, which is an immunostimulatory component of the mycobacterial cell wall [33]. CAF01, consisting of DDA and TDB, analog of TDM was reported to induce Th1-type cellular immune responses [15].

In our study, we combined Poly I:C to DDA liposome to construct the novel adjuvant. Mice were primed with BCG and boosted with subunit vaccine Ag85B-MPT64(190-198)-Mtb8.4 (AMM) in the adjuvant of DDA plus Poly I:C (DP). We found that antigen AMM delivered in a combination of DDA plus Poly I:C presented smaller lesions compared to adjuvant DDA alone following challenge with *M. tuberculosis* H37Rv. However, cationic liposomes are easy to flocculate [34], and result in highly unstable and heterogeneous formulations, which limit the application of DP in clinic. At last, cholesterol was incorporated into the cationic adjuvant DP to construct the new adjuvant consisting of DDA, Poly I:C and cholesterol (DPC for short). Cholesterol is an abundant component of mammalian cell membranes and has been extensively studied as an artificial membrane stabilizer in a wide range of phospholipid liposome systems [35]. The novel adjuvant DPC with particle size of 400 nm showed good stability. We mixed the adjuvant DP and DPC respectively with a novel fusion protein ESAT6-Ag85B-MPT64(190-198)-Mtb8.4-Rv2626c (LT70 for short), and the immune responses and protective efficacy against *M. tuberculosis* H37Rv infection of the subunit vaccines were investigated. DPC and DP induced almost similar immune responses and protective efficacy, and DPC provided less pathologic lesions than DP following *M. tuberculosis* challenge.

2. Materials and methods

2.1. Materials

DDA, cholesterol, β-cyclodextrin, lecithin, gelatin and PLGA were obtained from Sigma-Aldrich (Poole, UK). Poly I:C was obtained from Kaiping Genuine (γ-irradiated, Guangdong, China). The antigens AMM and LT70 were prepared in our lab [36].

2.2. Animals

C57BL/6 female mice (6–8 weeks old) were obtained from Slaccas, Inc. (Beijing, China). Mice were housed in special pathogen-free conditions in Gansu University of Traditional Chinese Medicine. For *M. tuberculosis* H37Rv challenge experiments, animals were kept in ABSL-3 Lab at Wuhan University. Animal experiments were performed in compliance with the ethical and experiment regulations for animal care at Gansu University of Traditional Chinese Medicine and Wuhan University.

2.3. Particle size and zeta potential characterization of liposomes

The particle size distribution of the liposomes was measured in Tris buffer (1/10 dilution; 1 mm, pH 7.4) using a Malvern Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) *via* dynamic light scattering. The liposome surface charge was determined by assessing the zeta potential on samples diluted 300-fold in Tris buffer (1 mm, pH 7.4). Both measurements were done at 25 °C.

2.4. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) measurements

Sample was prepared as follows: a drop of the liposome solution was placed onto a SEM grid (copper grid, 3.02 mm, 200 mesh); Then the sample was transferred to a vacuum cup of liquid nitrogen and frozen at −80 °C for 2 h; subsequently, the sample was put into a lyophilizer for 24 h for freeze-drying. Then the size of liposome

Please cite this article in press as: Liu X, et al. A novel liposome adjuvant DPC mediates *Mycobacterium tuberculosis* subunit vaccine well to induce cell-mediated immunity and high protective efficacy in mice. Vaccine (2016), http://dx.doi.org/10.1016/j.vaccine.2016.01.049