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Chapter *in* Reviews of Physiology, Biochemistry and Pharmacology · April 2018

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Potential of Cationic Liposomes as Adjuvants/Delivery Systems for Tuberculosis Subunit Vaccines

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Abstract The weakness of the BCG vaccine and its highly variable protective efficacy in controlling tuberculosis (TB) in different age groups as well as in different geographic areas has led to intense efforts towards the development and design of novel vaccines. Currently, there are several strategies to develop novel TB vaccines. Each strategy has its advantages and disadvantages. However, the most important of these strategies is the development of subunit vaccines. In recent years, the use of cationic liposome-based vaccines has been considered due to their capacity to elicit strong humoral and cellular immune responses against TB infections. In this review, we aim to evaluate the potential for cationic liposomes to be used as adjuvants/delivery systems for eliciting immune responses against TB subunit vaccines. The present review shows that cationic liposomes have extensive applications either as adjuvants or delivery systems, to promote immune responses against *Mycobacterium tuberculosis* (Mtb) subunit vaccines. To overcome several limitations of these particles, they were used in combination with other immunostimulatory factors such as TDB, MPL, TDM, and Poly I:C. Cationic liposomes can provide long-term storage of subunit TB vaccines at the injection site, confer strong electrostatic interactions with APCs, potentiate both humoral and cellular (CD4 and CD8) immune responses, and induce a strong memory response by the immune system. Therefore, cationic liposomes can increase the potential of different TB subunit vaccines by serving as adjuvants/delivery systems. These properties suggest the use of cationic liposomes to produce an efficient vaccine against TB infections.

Keywords Adjuvant · Cationic liposome · Delivery system · *M. tuberculosis* · Subunit vaccine

1 TB Infection

Mycobacterium tuberculosis (Mtb) bacterium is a rod-shaped intracellular organism that causes an infectious disease called tuberculosis (TB). Today, TB, as a global public health problem, has infected, although asymptomatically, two billion people worldwide. The bacterium is responsible for nine million new TB cases each year and causes two million deaths. The organism spreads between individuals by coughing or sneezing through the upper respiratory tract, which results in air-borne droplets or dried sputum; both, of which, are contagious. Patients with the active form of TB are the source of Mtb transmission to healthy people. After inhalation of contaminated air-borne droplets of Mtb and migration of the Mtb to the human lung, they are phagocytosed by inactivated alveolar macrophages. Antigen presenting cells (APCs) such as dendritic cells (DCs), alveolar macrophages, and pulmonary epithelial cells are a key part of immunity and engulfment of the

bacterium acts like a bridge between the innate and adaptive immune response. This process ultimately leads to activation of various cell types in the lung by inducing an inflammatory response. The activated innate immune cells, which include neutrophils, blood monocytes, natural killer (NK) cells and the complement system, are recruited to eliminate the pathogen through recognition of pathogen-associated molecular patterns (PAMPs), mycobacterial cell wall components on the bacteria, by receptors, pattern recognition receptors (PRRs), and specifically by the toll-like receptors (TLRs) on APCs. The innate immune response contributes to the defense against Mtb by activation of infected macrophages or by destruction of Mtb-infected macrophages, intracellular bacterial killing, and the recruitment of adaptive immune cells by proinflammatory cytokines. However, the innate immune response is not entirely sufficient and, thus, the adaptive immune response is required at the site of infection. In the alveoli, TB bacilli can actively modulate the activity of inactivated alveolar macrophages using a variety of different mechanisms. One such mechanism includes inhibiting the acidification of the phagosome and its fusion with the lysosome and its resistance to effector molecules, such that the bacterium begins to replicate until there is rupture of the macrophage, with subsequent release into the cytosol. Rapid bacterial growth during the early phase of infection leads to the presence of bacterial antigens and the induction of cell-mediated immunity (CMI) by DC cells in the lymph nodes. Activated T-cells return to the site of infection and serve as the main immune response in controlling bacterial replication, without killing the bacteria, in the solid granuloma, or tubercle. The TB granuloma contains inactive bacteria, necrotic and infected macrophages, and several phenotypes of macrophages (epithelioid cells, multi-nucleated giant cells, and foamy macrophages), DCs, neutrophils, NK cells, B-cells, $CD4^+$, and $CD8^+$ T –cells. A fibrous shell occurs at the latent tuberculosis infection (LTBI) phase and helps to keep bacteria from spreading. Reactivation to active TB disease, in 10% of latently-infected people, especially immunocompromised individuals such as HIV and AIDS-positive patients, occurs due to deficiency in the host's immune system and imbalance between the immune response and the pathogen. Impairment of immune response leads to the formation of a caseous granuloma with a liquified center. As a result, the bacterium begins to multiply uncontrollably and granulomas are destroyed, which leads to the release of virulent bacilli in the lungs, the body, or the environment (active TB) (Fig. 1) (Andersen 2007; Andersen and Kaufmann 2014; Khademi et al. 2016, 2017a, b; Li et al. 2011; Ottenhoff and Kaufmann 2012; Pitt et al. 2013; Wang et al. 2013).

2 BCG and the Advantages and Disadvantages of Current TB Vaccines

It has been proven that to reduce the global burden of TB, as well as the morbidity and mortality rate of TB infection, vaccination is one of the most successful approaches (Andersen and Kaufmann 2014; Girard et al. 2005). Bacillus

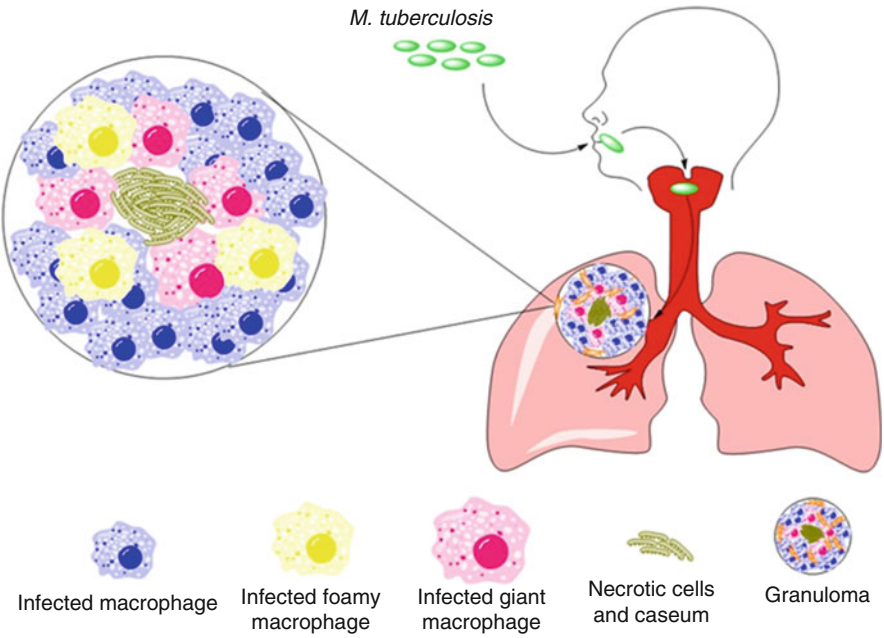

Fig. 1 The TB granuloma. After infection by the aerosol containing *M. tuberculosis* (Mtb), Mtb is first phagocytized by alveolar macrophages of the lungs. Following phagocytosis, Mtb provides a microenvironment within endosomal compartments of these macrophages where it can replicate and disrupt natural macrophage microbicidal mechanisms. Other macrophages and innate immune cells accumulate around these initial sites of Mtb replication, forming the TB granuloma. The center of the granuloma, where the majority of Mtb bacilli are presented, undergoes considerable cell necrosis that builds up necrotic cell debris called caseum, leading to collapse of the granuloma and, thereby, releasing virulent bacilli to infect new host tissues. Around the central necrotic region, granuloma macrophages can fuse to form multi-nucleated giant cells, or alternatively differentiate into foam cells subsequent to the accumulation of lipids

Calmette–Guérin (BCG), a viable attenuated strain of *Mycobacterium bovis*, was developed in 1921 and millions of people around the world have been vaccinated with it. To date, BCG is the only commercially-approved vaccine against TB infection (Girard et al. 2005). However, most studies have shown that the protective efficacy of BCG varies from excellent to no protection at all in different age groups, as well as in different geographic areas (Girard et al. 2005). Studies have proven that the efficacy of BCG vaccination against severe forms of the disease, meningitis, and disseminated TB, in young children is high (46–100%) (Girard et al. 2005). Nevertheless, vaccine efficacy is inconsistent (0–80%) against pulmonary TB in adolescents and adults and with limited effectiveness upon reactivation of the dormant form of a TB infection (Bottai et al. 2015; Girard et al. 2005; Kong et al. 2014). On the other hand, lack of safety in immunocompromised patients, such as HIV-positive individuals, and decreased vaccine efficacy with advancing age (due to inadequate immunological memory) are additional problems for the BCG vaccine (Nor and

Musa 2004). Therefore, it is essential that the research community develop safe, stable, effective, and inexpensive vaccines for BCG and improve its effectiveness by using additional different vaccines to augment the effectiveness of the BCG vaccine (Nor and Musa 2004).

Most successful TB vaccine candidates in clinical trials are divided into two categories: (1) whole mycobacterial vaccines (live and killed vaccines) and (2) non-living vaccines (subunit vaccines and DNA-based vaccines). Live mycobacterial vaccines such as recombinant BCG (rBCG) (VPM1002, phase IIa) and the rMtb deletion mutant (MTBVAC, phase I) are preventative vaccines that are administrated as "pre-exposure" vaccines (before TB infection) in newborns and adolescents. These types of vaccines work through the overexpression of TB antigens or by removal of virulent genes in order to attenuate *M. tuberculosis* (Andersen 2007; Andersen and Kaufmann 2014; Checkley and McShane 2011; Kaufmann 2013). Despite some advantages, the possibility of disseminated disease in HIV-positive patients and interference with the tuberculin skin test (i.e., the Mantoux skin test) are the two main disadvantages of rBCG vaccines (Nor and Musa 2004).

Killed mycobacterial vaccines such as the RUTI (derived from fragmented *M. tuberculosis*, phase I) killed *M. vaccae* (phase III), and *M. indicus pranii* (MIP) (phase III) are therapeutic vaccines and administered against active TB infections and to patients with drug resistant strains of *M. tuberculosis* (Andersen 2007; Andersen and Kaufmann 2014; Checkley and McShane 2011; Kaufmann 2013). However, the protective efficacy of these type of vaccines is no better than BCG (Nor and Musa 2004).

Non-living, DNA-based vaccines are DNA plasmids containing mycobacterial genes, which can induce both humoral and cellular (CD4 and CD8) immune responses against TB infections (Nor and Musa 2004). Some advantages of DNA-based vaccines include increased safety for immunocompromised individuals, ease of vaccine manipulation and administration, and better storage and transport properties, however, there is no DNA-based vaccine in clinical trials at present (Nor and Musa 2004). This may be due to the insertion into the host genome and the risk of autoimmune disease (Nor and Musa 2004).

Today, most TB vaccines in the development pipeline and in clinical trials belong to the subunit protein-based vaccines. Immunogenic antigens or lipid or carbohydrate components of the bacterium are used to create subunit vaccines (Andersen and Kaufmann 2014). Safety and ease of production are the two main advantages of subunit proteins (Checkley and McShane 2011; Nor and Musa 2004). Non-living vaccines, unlike mycobacterial whole-cell vaccines, are not immunostimulatory molecules due to their synthetic nature, and are therefore not able to induce maturation of dendritic cells to stimulate the appropriate immune response and, hence, immunity (Karimi et al. 2016). Non-living vaccines are divided into two categories, (1) viral-based vaccines, and (2) adjuvant-based vaccines (Andersen and Kaufmann 2014). Viral-based, non-living subunit vaccines include Ad5Ag85A (antigen: Ag85A, carrier: Adenovirus 5 vector, phase I), Ad35/MVA85A (antigen: Ag85A, Ag85B and TB10.4, carrier: Adenovirus 35 and modified vaccinia Ankara vector, phase I), Ad35/AERAS-402 (antigen: Ag85A, Ag85B and TB10.4, carrier:

Adenovirus 35 vector + modified vaccinia Ankara vector, phase IIa), and MVA85A (antigen: Ag85A, carrier: modified vaccinia Ankara, phase IIb) and represent pre-exposure vaccines that are administered to newborns and adolescents with the aim of preventing infection with TB (Andersen and Kaufmann 2014).

The development of new TB subunit vaccines remains a challenge due to the lack of proper adjuvants to elicit potent Th1 responses. Adjuvant-based TB vaccines include H4/IC31 (preventive) (antigen: Ag85B and TB10.4, carrier: IC31, phase IIa), M72 (preventive and postexposure) (antigen: Rv1196 and Rv0125, carrier: AS01E (liposomes and MPL), phase IIa), ID93 (preventive, postexposure, and therapeutic) (antigen: Rv2608, Rv3619, Rv3620 and Rv1813, carrier: GLA-SE, phase I), and H1/H56/IC31 (preventive, postexposure, and therapeutic) (antigen: Ag85B, ESAT-6 and Rv2660c, carrier: IC31, phase IIa) and are administered before and during the latent and active phases of TB infection as a "prime" instead of BCG, or as a booster to a BCG "prime" (Andersen 2007; Andersen and Kaufmann 2014). This is because the prime/boost strategy of immunization is more effective for enhancing humoral and cellular immunity that repeated administration of the same vaccine (homologous boosting), which mostly increases the humoral immune response, but not the cellular immune response to target antigens. The prime/boost strategy of immunization entails priming the immune system against a target antigen and then boosting antigen-specific immune responses with a specific immunogen, which is often a recombinant viral vector that expresses the same vaccine antigen.

Another important point is the excellent function of the delivery systems associated with subunit vaccines in terms of enhanced immunity, in vivo stability, conformational integrity, and persistent and prolonged stimulation of the immune system due to the controlled release of the antigen (Kim et al. 2014; Peek et al. 2008).

Thus far in this review, we have focused on recent reports concerning TB vaccines in order to evaluate the potential of using cationic liposomes as delivery systems/adjuvants for TB subunit vaccines.

3 Liposomes

Nanostructures are widely used as drug and vaccine delivery systems (Ahmaditabar et al. 2017; Fasihi-Ramandi et al. 2017). Liposomes are closed, self-assembled vesicular and lipid-based nanostructures that were first discovered by Bangham et al. in 1965 (Alving et al. 2016). These spherical vesicles consist of either a hydrated bilayer, or multilayered lipids, that are composed of phospholipids or amphiphilic non-phospholipids such as cationic lipids (Alving et al. 2016). Biocompatible, and biodegradable, non-toxic liposomes are well tolerated by the human body and have extensive applications in drug delivery, gene delivery, cell delivery, and diagnosis of disease, and they find widespread use in cosmetics and the fields of dermatology and immunology (Garg and Goyal 2014; Vartak and Sucheck 2016). However, their use as adjuvants and, especially, as a vehicle for the successful delivery of vaccines has increased in the last decade (Vartak and Sucheck 2016).

In 1974 and 1976, Allison and Gregoriadis reported that liposomes are able to increase the immune response (Schwendener 2014). They have two simultaneous properties, which include (1) immunostimulant characteristics by their interaction with immune cell receptors such as TLRs, and (2) carrier characteristics due to the depot effect and the subsequent gradual release of vaccine antigens (Schwendener 2014). One of the most important features of liposomes as a vaccine delivery system is their structural versatility and plasticity in terms of size, charge, encapsulation efficiency, and also the location of the entrapped actives that can carry different types of antigens and adjuvants (e.g., hydrophilic compounds, proteins, nucleic acids, and carbohydrates), inside the liquid space, and lipophilic compounds into the lipid bilayer (Schwendener 2014). It has been demonstrated that the physiochemical characteristics of liposomal vaccine adjuvants/delivery systems such as size, charge, lipid composition, and the nature and location of antigens in the particle, all have an important role in cellular uptake, transport to regional lymph nodes, and induction of a Th1 and Th2 immune response (Schwendener 2014; Vartak and Sucheck 2016). Therefore, particles with a size range of 20–200 nm have efficient uptake and transport to lymph nodes. However, for larger particles with a size range of 500–1,000 nm in size, uptake and transport to the lymph nodes is needed so that they can interact with activated dendritic cells that have previously migrated to the lymph nodes to interact with T- and B-cells to initiate the adaptive immune response (Vartak and Sucheck 2016). Previous studies have shown that small and large liposomes were able to induce a Th2 and Th1 immune response, respectively (Badiee et al. 2012). Positively-charged lipids, as well as the composition of lipids themselves, have a significant impact on stability, transfection activity, and induction of strong immune responses. Enhanced interaction, membrane fusion, and improved uptake by dendritic cells occur with positively-charged lipids that comprise cationic liposomes because, compared to neutral and anionic liposomes, cationic liposomes have shown greater uptake by APCs, macrophages, and dendritic cells (Hu et al. 2014; Vartak and Sucheck 2016).

4 The Role of Cationic Liposomes as TB Adjuvants

Based on their composition and intended use, liposomes can be divided into conventional, pH-sensitive, stealth, immuno, magnetic, heat-sensitive, and cationic liposomes (Garg and Goyal 2014). The structure of cationic liposomes is composed of neutral (e.g., helper lipids, such as cholesterol) and cationic (e.g., cationic head groups, polyamines, tertiary or quaternary ammonium compounds, and linker and hydrophobic tail) lipids (Xiong et al. 2011). The most important cationic lipids are DDA, dimethyldioctadecylammonium, DOTAP, 1, 2-dioleoyl-3-trimethylammonium-propane, DC-Chol, 3B-[*N*-(*N*′, *N*′-dimethylaminoethane)-carbamoyl] cholesterol, DOEPC, 2-dioleyl-sn-glycero-3-ethylphosphocoline, DOTIM, octadecenoyloxy(ethyl-2-heptadecenyl-3-hydroxye-thyl) imidazolinium, CCS, *N*-palmitoyl-D-erythrospingosyl-1-*O*-carbamoyl spermine, diC14-amidine,

3-tetradecylamino-tert-butyl-*N*-tetradecylpropionamidine, DOTMA, and *N*-(1-[2,3-dioleyloxy]propyl)-*N,N,N*-trimethylammonium (Christensen et al. 2009). Although a great number of liposomal adjuvants have been tested in various stages of preclinical or clinical trials, only two liposomal adjuvants (Adjuvant System 01 (AS01) and virosomes) are among the six approved adjuvants for use in human vaccines (Alving et al. 2016; Brito and O'Hagan 2014; Garçon and Van Mechelen 2011; Glück et al. 2004). Currently, the AS01 adjuvant, in combination with the M72 subunit vaccine of TB, which contains antigens Rv1196 and Rv0125, has advanced in phase II clinical trials, and its proposed use is during the latent phase of a TB infection as a booster for a BCG prime (Wang et al. 2013). The high efficacy of Mtb immunodominant antigens is required for adjuvants. Cationic liposomal adjuvants have shown that they are suitable immunostimulatory adjuvants to induce a strong Th1 immune response against a TB infection (Hu et al. 2014) (Figs. 2 and 3).

In this review, we have noted that the most commonly employed cationic liposomal adjuvants utilized to elicit a robust immune response against selected TB antigens included DDA-based adjuvants and DOTAP-based adjuvants.

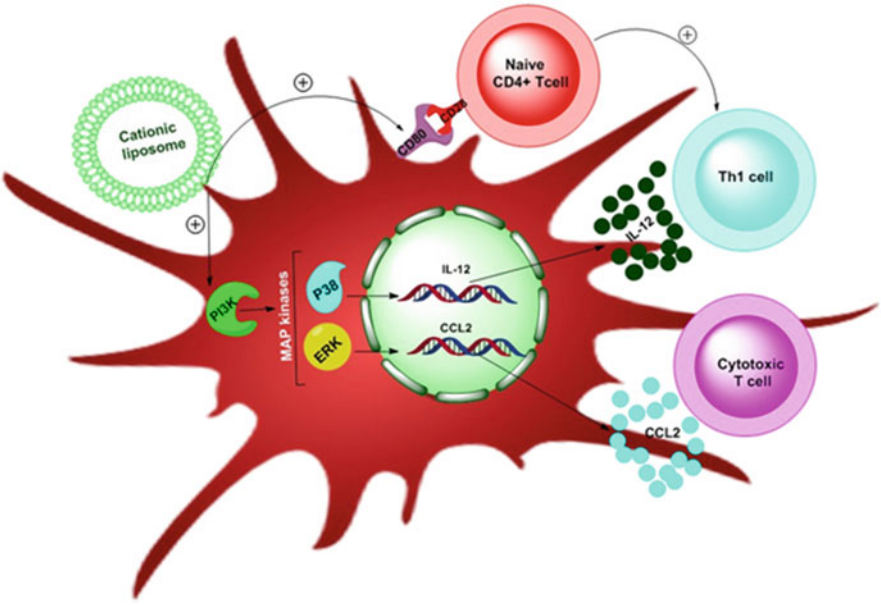

Fig. 2 Cationic liposome-mediated CTL immune response. Cationic liposomes are proposed to cause dendritic cells to mature via activation of CD80/86 that binds to CD26 on the surface of naïve $CD4^+$ T cells. Cationic liposomes also shift naïve T cells to Th1 cells by up-regulating the expression and secretion of Th1-defining cytokines such as IL-12 via activation of PI3 kinase that leads to the enhanced activity of P38, which then up-regulates the expression of IL-12. In addition, cationic liposomes enhance the expression and secretion of the CC chemokine CCL2 that induces migration and maturation of naïve $CD8^+$ cytotoxic T cells. CCL2 is known to be up-regulated via the ERK1/2 pathway, which is induced via cationic liposome-activated PI3K

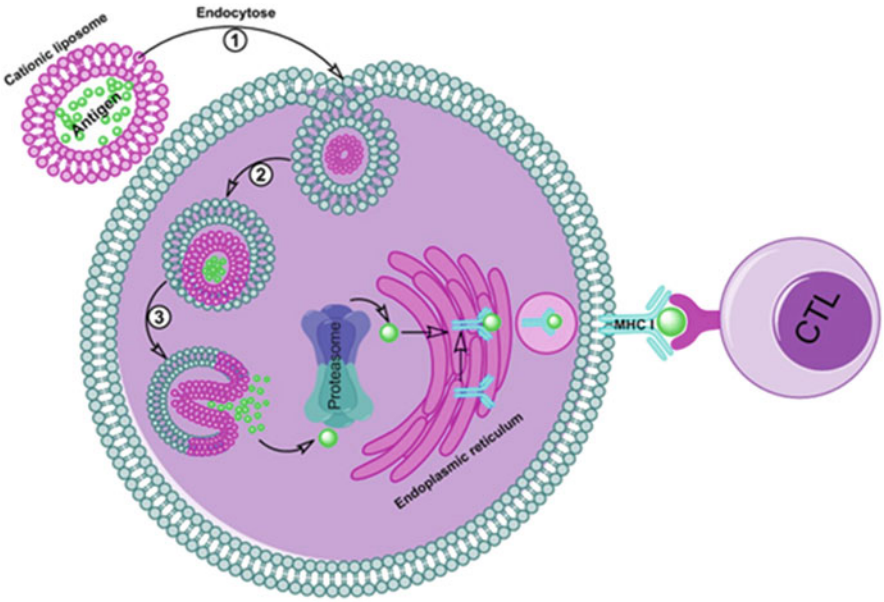

Fig. 3 Cellular delivery mechanism of cationic liposomes. Cationic liposomes can be internalized into the cell through endocytosis via an endosome (step 1). After endocytosis, the cationic lipid destabilizes the endosomal membrane, which leads to a flip-flop of anionic lipids such as phosphatidylserine (step 2) that are predominantly presented in the cytoplasmic face of the endosomal membrane. The anionic lipids diffuse laterally into the liposomal bilayer and form a charged neutralized ion-pair with the cationic lipid (step 3). This process displaces all contained-antigens of the cationic liposome into the cytoplasm. Afterward, released antigens can be processed through proteasome degradation and, then, assembled by MHC I molecules in the endoplasmic reticulum and, finally, presented on the cell surface. Presented antigens can then be recognized by cytotoxic T-cells

5 DDA-Based Adjuvants

In 1966, Gall was the first to discover that DDA, as a quaternary ammonium compound, has potent adjuvant effect (Christensen et al. 2007). In recent years, the use of DDA liposomes as promising TB vaccine adjuvants has been extensively evaluated. The most important features of DDA as a TB adjuvant are: (1) there is strong absorption of antigens, (2) there exists an antigen depot effect at the injection site, and (3) there is more efficient cellular uptake of the antigen (Christensen et al. 2007). As seen in Table 1, the DDA liposomal adjuvant has been used in combination with other non-immunostimulatory molecules, including MPL (monophosphoryl lipid A), TDM (trehalose dimycolate), TDB (trehalose 6, 6′-dibehenate), BCG, and Poly I:C to induce a strong immune response against

Table 1 Characteristics of cationic liposomes as Mtb vaccine adjuvants in some studies

Vaccines			Status	Administration route	Challenge	Animal	Outcome	Reference
Prime	Booster	Adjuvant						
Ag85B-ESAT-6	NA	DDA/MPL	Preclinical study	Intramuscularly	Mtb Erdman	Monkey	Induced both humoral and cellular (CD4 and CD8) immune responses and reduction of bacterial load in lung and protection against TB infection	Langermans et al. (2005)
NA	Recombinant ESAT-6 and Ag85B–ESAT-6	DDA, TDB, MPL	Preclinical study	Subcutaneously	Mtb Erdman	Mice	Synergistically induced strong Th1 cell responses	Holten-Andersen et al. (2004)
BCG	Myc3504 (rBCG)	DDA and MPL	Preclinical study	Subcutaneously Intranasal	Mtb H37Rv	Mice Guinea pig	Induced higher IFN-γ responses as compared to BCG	Badell et al. (2009)
BCG	Ag85A-HspX	DDA and MPL	Preclinical study	Subcutaneously	Mtb H37Rv	Mice	Induced effective protection against progressive TB, especially in the latent phase	Jeon et al. (2011)
BCG	Mtb10.4-HspX	DDA-TDM	Preclinical study	Subcutaneously	Mtb H37Rv	Mice	Induced both humoral and cellular immune responses and able to promote BCG-primed immunity and protective efficacy against TB infection	Niu et al. (2011)
BCG	Mtb10.4-Ag85B ESAT6-Ag85B ESAT6-RpfE ESAT6-Mtb8.4 EAMM MH	DDA-TDM poly(I:C) gelatin	Preclinical study	Subcutaneously	Mtb H37Rv	Mice	Some of the fusion proteins were able to effectively protect against *M. tuberculosis* (EAMM), improve BCG-primed protective efficacy against TB infection, and lower bacterial counts in the lungs and spleens (EAMM+MH)	Xin et al. (2013)

BCG	H1, H4, H28 and H56	CAF01 DDA-MPL	Preclinical study	Subcutaneously	Mtb Erdman	Mice	Induced strong Th1 responses	Hoang et al. (2013)
BCG	CFP-10:HspX: Fcγ2a CFP-10:HspX: His	CAF01	Preclinical study	Subcutaneously	NA	Mice	Adjuvants were safe and induced strong Th1-mediated immune response	Mosavat et al. (2016)
BCG	ESAT-6:Fcγ2a ESAT-6:His	CAF01	Preclinical study	Subcutaneously	NA	Mice	Recombinant protein plus CAF01 adjuvant were induced high level of Th1 immune response more than protein alone	Kebriaei et al. (2016)
BCG	ESAT-6:HspX: mFcγ2a ESAT-6:HspX: His	CAF01	Preclinical study	Subcutaneously	NA	Mice	Induced very strong Th1-mediated immune responses	Soleimanpour et al. (2015)
BCG Ag85B, ESAT-6 and Ag85B-ESAT-6	NA	DDA and MPL	Preclinical study	Subcutaneously	Mtb Erdman and H37Rv	Mice	Induced very strong response and long-term memory immunity to both ESAT-6, Ag85B and the fusion proteins	Olsen et al. (2001)
BCG	Ag85B, ESAT-6, Mtb10.4, Ag85B- ESAT-6, Ag85B-TB10.4	DDA and MPL	Preclinical study	Subcutaneously	Mtb Erdman	Mice	Adjuvants had beneficial effect on the immunogenicity of some antigens	Dietrich et al. (2005)
BCG	MVA/IL-15/ 5Mtb	DDA/MPL	Preclinical study	Subcutaneously	Mtb Erdman	Mice	Induced protective immunity in mouse model of pulmonary TB	Kolibab et al. (2010)
H4	Ad-H4	CAF01	Preclinical study	Subcutaneously	Mtb Erdman	Mice	Induced both CD4 and CD8 T responses as well as increased the protective efficacy against Mtb challenge	Elvang et al. (2009)

(continued)

Table 1 (continued)

Vaccines			Status	Administration route	Challenge	Animal	Outcome	Reference
Prime	Booster	Adjuvant						
BCG	Ag85B AMM	DDA-BCG PSN	Preclinical study	Subcutaneously	Mtb H37Rv	Mice	AMM in combination with adjuvant induced both strong humoral and cellular immune responses, enhanced BCG-primed immunity and protection against TB infection	Luo et al. (2009)
BCG	Ag85B AMH AMM AMH+ AMM	DDA-BCG PSN	Preclinical study	Subcutaneously	Mtb H37Rv	Mice	Induced high levels of humoral and cellular immune responses and increased clearance of Mtb in the lungs of TB-challenged mice	Li et al. (2011)
BCG	CFP-10:Fcγ2a CFP-10:His	CAF01	Preclinical study	Subcutaneously	NA	Mice	Induced strong Th1-mediated immune response	Baghani et al. (2017)
BCG	ESAT-6:CFP-10:Fcγ2a ESAT-6:CFP-10:His	CAF01	Preclinical study	Subcutaneously	NA	Mice	Induced strong Th1-mediated immune response	Farsiani et al. (2016)
CFP-25, CFP-20.5, CFP-32, Ag85A and Ag85B	NA	DDA/MPL	Preclinical study	Subcutaneously	Mtb H37Rv	Mice	Induced both strong humoral and cellular immune responses	Sable et al. (2005)
Ag85B-ESAT-6	NA	CAF01	Preclinical study	Subcutaneously	Mtb H37Rv	Mice	Induced both strong humoral and cellular immune responses and elicited significant protective immunity against TB challenge	Agger et al. (2008)

BCG	LT70	DDA Poly I:C	Preclinical study	Subcutaneously	Mtb H37Rv	Female C57BL/6 mice	Fusion protein in combination with adjuvants strongly induced both humoral and cellular immune responses	Liu et al. (2016)

Myc3504 (rBCG) Ag85B-ESAT-6, *EAMM* ESAT6-Ag85B-MPT64$_{190\text{-}198}$-Mtb8.4, *MH* Mtb10.4-HspX, *CAF01* DDA-TDB, *H1* Ag85B-ESAT-6, *H4* Ag85B-TB10.4, *H28* Ag85B-TB10.4-Rv2660c, *H56* Ag85B-ESAT-6/Rv2660c, *MVA/IL-15/5Mtb* a recombinant modified vaccinia Ankara overexpressing Ag85A, Ag85B, ESAT6, HSP60, Mtb39 and IL-15, *Ad-H4* recombinant replication-deficient adenoviral 5 based vaccine, *AMM* Ag85B-Mpt64$_{190–198}$-Mtb8.4, *AMH* Ag85B-Mpt64$_{190–198}$-HspX, *LT70* ESAT6-Ag85B-MPT64$_{-(190–198)-}$Mtb8.4-Rv2626c, *NA* not available

TB subunit vaccines. One reason for this is that the induced immune response against the subunit vaccine containing weak antigens, in combination with DDA adjuvant, is insufficient (Christensen et al. 2007). However, it has been shown that DDA in combination with the MPLA adjuvant led to induction of both a humoral and cellular (CD4 and CD8) immune response, reduction of the bacterial load in the lung, and protection against TB infection in various animal models when compared with DDA alone (Table 1). Induction of a strong T-cell response to TB subunit vaccines by DDA/MPL adjuvants is due to stimulation of APC cells through TLR-4 and induction of an antibody isotype switching to IgG2a, differentiation of Th cells to Th1, Th2, and Th17, and induction of a CD8 T-cell response (Christensen et al. 2007). Moreover, the adjuvant activity of DDA increased when used with TDM and induced both a humoral and cellular immune response, which was promoted by BCG-primed immunity and, therefore, protecting various animal models against a TB infection (Table 1).

A combination of DDA and TDB known as "CAF01" showed a long-lasting depot effect at the site of injection, which induced both a strong humoral and cellular immune response, especially a Th1 response, as well as elicited significant protective immunity against a TB challenge (Table 1). CAF01 (DDA-TDB) is only one cationic liposome in combination with Hybrid 1, which contains Ag85A and ESAT-6 and is currently in phase II clinical trials (Wang et al. 2013). Use of this type of vaccine is as a prime or booster for immunotherapy against a TB infection (Wang et al. 2013). Furthermore, DDA, in combination with other adjuvants (Poly I:C), also triggered immunity against TB infections (Table 1). The present review suggests that simultaneous administration of cationic liposomes (DDA) with Poly I:C adjuvant (as a type I IFN-inducing TLR ligand) leads to a potent CD8 T-cell response to TB subunit vaccines.

In the present review, we could not identify a study that used other cationic liposomal adjuvants to promote immune responses against TB subunit vaccines.

6 The Role of Cationic Liposomes as a TB Delivery System

Using cationic liposomes as a carrier system to enhance the immune response against TB protein subunit vaccines is well documented due to three reasons: (1) inherent immunogenicity, (2) an antigen depot effect, and (3) optimal antigen delivery characteristic (Henriksen-Lacey et al. 2010a). Among cationic, anionic, and neutral liposomes used in the development of vaccine delivery systems, cationic liposomes interact more completely with the antigen. In addition, there is better retention of the vaccine at the site of injection, and thus, prolonged presentation of the antigen is achieved to induce Th1 and Th17 immune responses (Henriksen-Lacey et al. 2010b). Potent cell-mediated immune (CMI) responses, especially a Th1-type response, is a key factor with which to induce protection against a TB infection. Research has shown that replacement of cationic liposomes

with anionic and neutral liposomes resulted in the production of a Th1 bias response (Hussain et al. 2014).

The transfection mechanism by cationic liposomes is not fully understood. However, Felgner et al. demonstrated the ability of cationic liposomes to deliver immunogenic peptides into cells, and that this process occurs through simple fusion with the plasma membrane. However, others have reported that it occurs through endocytosis (Vangasseri et al. 2006; Xiong et al. 2011). Positively-charged cationic liposomes undergo electrostatic interactions with negatively-charged proteins, hydrophilic compounds, nucleic acids, carbohydrates, and mammalian cell membranes (Xiong et al. 2011).

Cationic liposomes, after absorption by macrophages and dendritic cells, cause disruption of the endosomal membrane, which leads to release of antigens to the cytosol and subsequent induction of a potent cellular immune response (Vartak and Sucheck 2016).

7 DDA-Based TB Delivery System

The synthetic amphiphilic lipid DDA has frequently been used to deliver different antigens of Mtb as subunit vaccines (Tables 1 and 2). The results of different studies presented in this review show that adsorption of antigen into DDA leads to enhanced uptake and presentation of the vaccine antigen to APCs, as compared with antigen alone (Table 2). Furthermore, simultaneous delivery of antigens and immunomodulators by a carrier leads to limited use of immunostimulatory components and reduces any toxic effects (Table 2). Various reports, along with the current review, have shown that separately administered delivery vehicles and single immunomodulators do not induce powerful immune responses and protection against a TB infection and, thus, are not an efficient TB vaccine (Li and Szoka 2007). As shown in Table 2, subunit vaccine delivery of TB by DDA liposomes, either alone, or in combination with immunomodulators, leads to induction of a Th1 response, maintains prolonged immunological memory, and a significant level of protection against TB infection. As it pertains to a DDA:TDB (CAF01) delivery vehicle, it should be noted that its effectiveness can be attributed to the synergistic effect of the components on the immune response, in which the cationic lipid component targets the TB antigen to APCs and the immunostimulatory component induces a proinflammatory response and the Th1 immune response (Li and Szoka 2007). It is also noteworthy that a DDA-based TB delivery system, with the same antigen, not only induces a strong IgG2a and IFN-γ response relative to other cationic liposomes (DOTAP and DC-Chol) and both neutral and anionic lipid liposomes, but also elicits long-term memory due to DDA's depot effect and the slow or protracted release of the antigen (Table 2).

Table 2 Characteristics of cationic liposomes as Mtb vaccine delivery systems in some studies

Vaccines		Carrier	Status	Administration route	Challenge	Animal	Descriptions	Reference
Prime	Booster							
Ag85B-ESAT-6	NA	DDA:MPL DDA:TDB	Preclinical study	Subcutaneously	Mtb Erdman and H37Rv	Guinea pig	Induce significant level of protection against TB infection, as compared with antigen alone, close to BCG level	Olsen et al. (2004)
Ag85B-ESAT-6	NA	DDA/BCG lipid (or MPL) DOTAP/BCG lipid DC-Chol/BCG lipid	Preclinical study	Subcutaneously	Mtb Erdman	Mice	Induce a powerful IgG2a and Th1 responses and maintain prolonged immunological memory superior to BCG	Rosenkrands et al. (2005)
Ag85B-ESAT-6	NA	DDA:TDB, DOTAP:TDB, DC-Chol:TDB	Preclinical study	Intramuscularly	NA	Mice	Cationic liposomes showed long-term retention, slow release of antigen and potent Th1 immune response	Henriksen-Lacey et al. (2010a)
Ag85B-ESAT-6	NA	DDA:TDB	Preclinical study	Intramuscularly	NA	Mice	Prolonged antigen retention and antigen presentation as well as induce strong immune responses (Th1 and Th17) more than anionic and neutral liposomes. Also, induce low level of Th2 responses	Henriksen-Lacey et al. (2010b)
Hybrid56	NA	DDA:TDB	Preclinical study	Intramuscularly	NA	Mice	Produced higher IgG1, IgG2a, IL-2, and IFN-γ responses compared with antigen. Also, decrease in Th2 (IL-5 and IL-10) responses	Hussain et al. (2014)

Ag85B-ESAT-6	NA	DSPC:Chol: DDA and DSPC:Chol: DDA:TDB	Preclinical study	Subcutaneously	Mtb Erdman	Mice	Addition of the cationic lipid to neutral liposomes leads to decrease in size, increase in entrapment efficiency and high levels of IFN-γ responses	McNeil et al. (2011)
Ag85B-ESAT-6	NA	PLGA+DDA and PLGA +DDA+TDB	Preclinical study	Subcutaneously	NA	Mice	High antigen entrapment efficiency, prolonged release profile and induce both strong humoral and cellular immune responses	Kirby et al. (2008)
Ag85B MPT-64, MPT-83	NA	PLGA+DDA	Preclinical study	Intramuscularly	Mtb H37Rv	Mice	Induce potent Th1 immune response due to the effect of DDA stimuli or sustained release DNA vaccine and a significant protection in mice after challenge with Mtb H37Rv	Cai et al. (2005)
DNA-hsp65	NA	EPC/DOPE/ DOTAP	Preclinical study	Subcutaneously Intramuscularly Intranasal	Mtb H37Rv	Mice	Adjuvants/delivery systems/ DNA vaccine induced immune responses however do not prevent from TB infection	Rosada et al. (2008)
DNA-hsp65	NA	L-α-PC/ DOTAP/DOPE	Preclinical study	Intranasal	Mtb H37Rv	Mice	Promising vaccine for Mtb treatment	Rosada et al. (2012)
DNA-hsp65	NA	EPC/DOTAP/ DOPE and DOTAP/DOPE	Preclinical study	Intramuscularly	NA	Mice	Induced IgG2a antibody production	de la Torre et al. (2009)

Hybrid56 Ag85B-ESAT6-Rv2660c, *NA* not available

8 DOTAP-Based TB Delivery System

DOTAP is a liposomal carrier/adjuvant with a strong positive charge. This quaternary ammonium compound has been used to induce protective immune responses against microbial infections in several studies (Christensen et al. 2007). These studies have demonstrated the therapeutic potential of DOTAP-based liposomes, as compared with antigen alone, in inducing both a humoral and cellular-mediated immune response, especially a Th1 response (Christensen et al. 2007). Compared with the DDA adjuvant, a few studies have used DOTAP-based delivery systems to enhance the immune response against TB subunit vaccines (Tables 1 and 2).

9 DC-Chol-Based TB Delivery System

DC-Chol cationic liposome has been used as a carrier for the targeted delivery of subunit vaccines (DNA and protein antigens) (51). However, very few studies have evaluated the immunostimulatory capacity of the cationic lipid DC-Chol as a TB delivery system. Nevertheless, a DC-Chol-based TB subunit vaccine demonstrated long-term retention (antigen depot) at the site of injection, slow release of the antigen, and a potent Th1 immune response similar to the DDA-based TB delivery system (51).

10 Challenges of Cationic Liposomes

Despite the many potential applications for the use of cationic liposomes as extremely useful adjuvants/delivery systems against TB infection, their use is associated with the following limitations. First, they possess relatively weak adjuvant activity. Some studies have shown that many types of cationic liposomes, when used alone, exhibit relatively weak adjuvant activity. Therefore, to solve this problem, they are used in combination with other components (Alving et al. 2016). Another limitation of cationic liposomes (e.g., DDA as an adjuvant) is its instability in vivo (Christensen et al. 2007). Studies have shown that incorporation of immunostimulators (TDB (CAF01) or MPL or TMD) into DDA, in addition to the immunogenicity, also increases the stability of cationic liposomal adjuvants (Christensen et al. 2007). A third challenge to the use of cationic liposomes is their recognition and rapid elimination from the circulation by the reticuloendothelial system (RES) or the lung endothelial capillaries or proteins that function as opsonins and thereby make the adjuvant more susceptible to phagocytosis (Hashida et al. 2002; Li and Szoka 2007). Studies have shown that intravenous delivery of the cationic liposomes leads to interactions with serum proteins (e.g., serum albumin), an increase in particle size, and subsequent rapid elimination (Li and Szoka 2007).

To overcome the rapid elimination in vivo, the surface of cationic liposomes can be covered with either a polymeric compound, be manufactured to have a size <100 nm, or by neutralizing the positive charge on the surface of the cationic liposome (Li and Szoka 2007). Lastly, cationic liposomes exhibit cytotoxicity when used at high concentrations. Previous research indicates that cytotoxicity is one of the greatest challenges to utilizing cationic liposomal vaccines, which affects in vivo stability and the capacity to elicit an improved immune response (Fan et al. 2015). To solve this problem, surface modification of particles can be achieved by using neutral phospholipids and also anionic or inert polymers such as polyethylene glycol (PEG). The surface modification strategy appears to improve the efficacy of cationic liposomal vaccines in terms of providing a long circulation time, enhanced stability and immunogenicity, and toxicity (Alving et al. 2016; Fan et al. 2015; Joshi et al. 2013; Li and Szoka 2007). However, it should be noted that such changes can decrease the Th1, and increase the Th2, immune responses (Alving et al. 2016).

11 Conclusion

In the present review, we have provided evidence that DDA, when used either as an adjuvant or as a delivery system, is the most commonly employed cationic liposome to elicit an immune response against Mtb subunit vaccines. DDA is either used alone or in combination with other immunostimulatory factors. The immunostimulatory capacity of cationic liposome-forming lipids, despite their inherent immunogenicity, is rather low. Therefore, this review has provided literature justification for the combination of different cationic lipids and immunomodulators (TDB, MPL, TDM, Poly I:C, etc.), which leads to (1) long-term storage (i.e., a depot effect) of the vaccine at the injection site, which is facilitated by inclusion of the cationic lipid component, (2) strong electrostatic interactions with APCs by the cationic lipid component, (3) potentiation of both the humoral and cellular (CD4 and CD8) immune responses by the immunomodulator component, and (4) a strong memory immune response. In this review, different cationic liposomes with different immunogenicity properties were discussed. These different immunogenicity properties of various cationic liposomes can be attributed to the cationic lipid type (e.g., different size, charge, depot effect, uptake mechanisms, and intracellular stimulation pathways), the injection site, and the inclusion of immunostimulatory molecules. In general, the immunostimulatory capacity of cationic liposomes was found to be greater than neutral and anionic liposomes and other vaccine delivery systems such as polymeric particles, which makes them excellent candidates for use as adjuvants/delivery systems for TB subunit vaccines. The properties of cationic liposomes that make them an excellent choice for adjuvants/delivery systems for TB subunit vaccines are the fact that there is better interaction with antigens and superb entrapment efficiency (negatively charged-DNA and proteins), improved retention at the site of injection, prolonged antigen presentation to APCs, and enhanced interaction and uptake by APCs.

By controlling liposome's physicochemical properties by the addition of cationic lipids or immunomodulatory components, both, of which, are critical factors in inducing an appropriate immune response, medical researchers and pharmaceutical scientists can increase the therapeutic potential of cationic liposomes as adjuvants/delivery systems for tuberculosis subunit vaccines.

Acknowledgements None.

Funding No funding was received for preparing this review.

Compliance with Ethical Standards This is a review article not involving any cellular, animal or human test.

Conflict of Interest None.

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