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Intrinsic immunogenicity of liposomes for tuberculosis vaccines: Effect of cationic lipid and cholesterol

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ABSTRACT

Tuberculosis (TB) is still among the deadliest infectious diseases, hence there is a pressing need for more effective TB vaccines. Cationic liposome subunit vaccines are excellent vaccine candidates offering effective protection with a better safety profile than live vaccines. In this study, we aim to explore intrinsic adjuvant properties of cationic liposomes to maximize immune activation while minimizing aspecific cytotoxicity. To achieve this, we developed a rational strategy to select liposomal formulation compositions and assessed their physicochemical and immunological properties *in vitro* models using human monocyte-derived dendritic cells (MDDCs). A broad selection of commercially available cationic compounds was tested to prepare liposomes containing Ag85B-ESAT6-Rv2034 (AER) fusion protein antigen. 1,2-Dioleoyl-*sn*-glycero-3-ethylphosphocholine (EPC)-based liposomes exhibited the most advantageous activation profile in MDDCs as assessed by cell surface activation markers, cellular uptake, antigen-specific T-cell activation, cytokine production, and cellular viability. The addition of cholesterol to 20 mol% improved the performance of the tested formulations compared to those without it; however, when its concentration was doubled there was no further benefit, resulting in reduced cell viability. This study provides new insights into the role of cationic lipids and cholesterol in liposomal subunit vaccines.

1. Introduction

Tuberculosis (TB) is among the top ten causes of lethality in low-income and lower-middle-income countries with an estimated 3.6 million undiagnosed individuals (World Health Organization, 2020). Approximately a quarter of the entire human population is (latently) infected with *Mycobacterium tuberculosis* (Mtb), and in 2021, 10.6 million people fell ill, and 1.6 million died from TB (World Health Organization, 2022). Moreover, the TB burden is aggravated by the increased occurrence of drug-resistant strains. Thus, TB continues to be a global problem that requires improved (early) diagnosis (Pierneef et al., 2023), treatment, and prevention (World Health Organization, 2018, 2022). In this study, we aim to advance the knowledge of TB prevention by developing novel vaccine modalities.

Abbreviations: AER, Ag85B-ESAT6-Rv2034 antigen; APC, antigen-presenting cell; BCG, *Mycobacterium bovis* Bacillus Calmette–Guérin; CCL, chemokine (C-C motif) ligand; CCR7, C-C chemokine receptor type 7; CD, cluster of differentiation; CXCL, chemokine (C-X-C motif) ligand; DC, dendritic cell; DC-cholesterol, 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol; DDA, dimethyldioctadecylammonium bromide; DODMA, 1,2-dioleyloxy-3-dimethylaminopropane; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; EPC, 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine; FBS, fetal bovine serum; GL-67, N4-cholesteryl-spermine; GM-CSF, granulocyte-macrophage colony-stimulating factor; HLA, human leukocyte antigen; IFN, interferon; Ig, immunoglobulin, IL, interleukin; IQR, interquartile range; MACS, magnetic cell isolation; M-CSF, macrophage colony-stimulating factor; MDDC, monocyte-derived dendritic cell; Mtb, *Mycobacterium tuberculosis*; MVL5, N1-[2-((1S)-1-[(3-aminopropyl)amino]-4-[di(3-amino-propyl) amino]butylcarboxamido)ethyl]-3,4-di[oleyloxy]-benzamide; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cell; PDI, polydispersity index; SA, stearylamine; TB, tuberculosis; Th1, type 1 helper T-cell,TLR, Toll-like receptor; TNF, tumor necrosis factor.

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Vaccines are commonly recognized as the most effective and inexpensive way of solving the burden of infectious diseases (Pollard and Bijker, 2020; Rémy et al., 2015). The complete eradication of smallpox and rinderpest, and the more recent success of SARS-CoV2 vaccines, have proven the efficacy of vaccines in disease prevention (Woodland, 2017). Unfortunately, it is difficult to develop effective and safe vaccines against some infectious diseases, including TB. Currently, the only available vaccine against TB is *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) (Ottenhoff and Kaufmann, 2012). The BCG vaccine confers variable and often inadequate protection, especially against the pulmonary form in adults, which is accountable for Mtb transmission in adolescents and adults (Fine, 1995; Rodrigues et al., 1993; Trunz et al., 2006). Therefore, there is still an urgent need for improved vaccines against TB (Ottenhoff and Kaufmann, 2012). The development of subunit vaccines can contribute to this demand.

Subunit vaccines are based either on synthesized or purified antigens, DNA, or RNA (Christensen et al., 2011). Being non-live, they are one of the safest vaccine types and as a result can potentially be administrated to a very broad population, including immunocompromised individuals (Moyle and Toth, 2013; O'Hagan et al., 2001). Hence, a subunit vaccine is a logical candidate for a TB vaccine, as the countries with the highest TB rates have a substantial incidence of HIV infection (World Health Organization, 2022). However, intrinsically subunit vaccines are often insufficiently immunogenic (Barnier-Quer et al., 2013; O'Hagan et al., 2001; Schmidt et al., 2016) as they lack immune-activating constituents, such as pathogen-associated molecular patterns (PAMPs), which are present in traditional (live-attenuated and inactivated) vaccines. Hence, this vaccine type often cannot induce proficient maturation of antigen-presenting cells (APCs), including dendritic cells (DCs). As a result, they fail to induce adequate protective immunity (Moser and Leo, 2010). Thus, in order to overcome this inherent limitation, the development of subunit vaccine delivery systems is of utmost importance. This study addresses this issue.

Cationic liposomes are excellent subunit vaccine delivery systems that act as particulate adjuvants (Barnier Quer et al., 2012; Christensen et al., 2011; Latif and Bachhawat, 1984; Liu et al., 2016; Yan et al., 2007). Several liposome-based subunit vaccines have been approved for clinical use (Khademi et al., 2018; Luwi et al., 2022; Tretiakova and Vodovozova, 2022). Liposomes can protect their antigenic cargo from degradation, and potentially co-deliver antigens with molecular adjuvants and PAMPs such as Toll-like receptor (TLRs) ligands. In addition, such delivery systems facilitate and enhance antigen uptake by APCs allowing a reduction of the required dose of antigens as well as molecular adjuvants to induce the desired immune responses (Barnier Quer et al., 2012; Christensen et al., 2011; Heuts et al., 2018; Ma et al., 2011; Marasini et al., 2017; Varypataki et al., 2015a). In particular, cationic liposomes can potently enhance antigen-specific immune responses as they have intrinsic immune-stimulatory properties to induce maturation of DCs and can trigger subsequent $CD4^+$–Th1 and $CD8^+$ *T*-cell responses (Barnier Quer et al., 2012; Brgles et al., 2009; Du et al., 2017; Henriksen-Lacey et al., 2010; Nakanishi et al., 1999; Varypataki et al., 2015a). Therefore, cationic liposomes provide a powerful and versatile platform for vaccination.

The full potential of cationic liposomes as vaccine components has not been fully explored yet. It is known that the physicochemical properties of liposomes, like size and surface charge, affect the immunological outcomes, yet the role of the lipids forming the bilayer is still not fully understood (Barnier Quer et al., 2012; Benne et al., 2016; Even-Or et al., 2011; Rosenkrands et al., 2005; Soema et al., 2015). Several studies have compared cationic lipids and investigated their effect on immune responses; however, the available data is not conclusive (Barnier Quer et al., 2012; Chen et al., 2008; Even-Or et al., 2011; Guy et al., 2001; Jiao, 2003; Rosenkrands et al., 2005; Soema et al., 2015). In many of these reports, the evaluated range of cationic lipids was limited. Therefore, it is challenging to draw definitive conclusions in the vaccine field. Moreover, the choice of biological systems, in which these liposomes were tested, varied greatly. Some of those studies used *in vitro* mouse or human models (using primary cells or cell lines) mainly looking at changes in (surface) activation markers of DCs. Others used *in vivo* models and focused on outcomes such as total Ig titers or neutralizing antibody titers. Similarly, the interplay of cationic lipids and liposome components with cholesterol has not been researched thoroughly. It is known that cholesterol can improve uptake of liposomes by APCs and phagocytes; however, the concentration required for this improvement and to what extent the immune response can be improved has not been clearly elucidated (Aramaki et al., 2016; Bakouche and Gerlier, 1986; Barnier-Quer et al., 2013; Batenjany et al., 2001; Ishida et al., 2001; Kaur et al., 2014). Therefore, in this study, we have examined the effect of cholesterol incorporation into various liposomal compositions on the physicochemical properties of liposomes and on various biological outcomes in a systematic way.

The goal of this study was to formulate liposomes with different cationic lipids and cholesterol contents, investigate their effect on the physicochemical properties and assess human immune responses *in vitro*. The best-performing formulations were optimized to achieve the most potent immune stimulation while minimizing cellular toxicity. We compared several commercially available cationic lipids formulated in liposomal formulations containing the designed Mtb antigen AER, a hybrid antigen composed of three Mtb proteins with different functions. Previously, we showed that AER can reduce the bacterial load in HLA-DR3 transgenic mice as well as guinea pigs models of acute TB (Commandeur et al., 2014b). The formulations that fulfilled our predefined inclusion criteria were subsequently tested on primary human monocyte-derived DCs (MDDCs), cellular viability, antigen uptake, and cellular activation. The best-performing formulations were selected and optimized to maximize immune activation and minimize cytotoxicity. Since $CD4^+$ Th1 cell responses are an important correlate of immunity and protection against TB, the potential efficacy of the four best-performing vaccine formulations was further determined *in vitro* using the activation of Rv2034 and Ag85B antigen-specific reporter $CD4^+$ *T*-cell clones/lines.

2. Materials and methods

2.1. Materials

1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP), 3ß-[N-(N',N'-*dimethylaminoethane*)-*carbamoyl*]cholesterol hydrochloride (DC-chol), dimethyldioctadecylammonium bromide salt (DDA), 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine chloride salt (EPC), N^4-cholesteryl-spermine hydrochloride (GL-67), 1,2-dioleyloxy-3-dimethylaminopropane (DODMA), N1-[2-((1S)−1-[(3-*aminopropyl*)*amino*]−4-[*di*(3-*amino-propyl*)*amino*]butylcarboxamido)ethyl]−3,4-di[*oleyloxy*]-benzamide (MVL5), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were purchased from Avanti Polar Lipids, Inc. (USA). Cholesterol was obtained from Merck KGaA.(Germany). Recombinant fusion protein AER was produced using the previously described method (Franken et al., 2000). Briefly, MTB genes were amplified using polymerase chain reaction (PCR) from genomic DNA of lab strain H37Rv and cloned using Gateway technology (Invitrogen, USA) in a bacterial expression vector containing an N-terminal hexahistidine (His) tag. Correct insertion of the products was confirmed using sequencing. The recombinant protein was expressed in *Escherichia coli* strain BL21 (DE3) and purified. The quality of the protein in terms of size and purity was evaluated by gel electrophoresis using Coomassie brilliant blue staining and Western blotting using an anti-His-antibody (Invitrogen, USA). The endotoxin level in the protein was measured using a ToxinSensor™ Chromogenic Limulus Amebocyte Lysate (LAL) Endotoxin Assay Kit (GenScript, USA). The endotoxin contents were below 50 EU (endotoxin unit) per mg of a protein. Subsequently, AER was tested to exclude non-specific T-cell stimulation and cellular toxicity in the IFNγ release assay. For this assay

PBMCs of *in vitro* purified protein derivative (PPD) negative, healthy Dutch donors recruited at the Sanquin Blood Bank, Leiden, the Netherlands were used.

2.2. Preparation of liposomal formulations

The liposomal formulations were prepared using the thin-film hydration method. Lipids were dissolved in chloroform and added to round-bottom flasks. Various cationic lipids and zwitterionic lipids were used, and additionally, cholesterol was added to some formulations (Table 1). The lipids of choice were diluted in chloroform from 25 mg/ml stock solutions. The final total amount of lipids used per formulation was 5 mg (10 mg/ml) in chloroform. The lipid solution was transferred to a round-bottom flask, and the chloroform was evaporated using a rotary evaporator (Buchi rotavapor R210, Switzerland). Subsequently, the lipid film was rehydrated with 1 ml of 100 μg/ml AER in 10 mM phosphate buffer (PB) with 9.8 % sucrose (pH = 7.4) to prepare AER-loaded liposomes. For the preparation of empty liposomes (without AER) and fluorescent-labeled liposomes (also without AER), only the buffer was used for rehydration. After the hydration, the liposomes were downsized using a tip sonicator (Branson Sonifier 250, US). The sonication program consisted of eight cycles, each cycle encompassed 30 s of sonication at a 10 % amplitude followed by a break of 60 s. Samples were submerged in ice during the sonication. Short sonication times at a low amplitude alongside submersion in ice allowed to reduce degradation of lipids. Hereafter, the liposomes were centrifuged (Allegra X-12R, US) at 524 g for 5 min to spin down the metal particles shed by the tip sonicator. To remove the metal-particle pellets, the supernatants containing liposomal formulations were transferred to new tubes, and the pellets were discarded. To avoid that the tip sonication would degrade the fluorophore, fluorescent-labeled liposomes (without AER) were downsized using a 10 ml extruder (LIPEX extruder, Northern Lipids, Canada). The liposomal formulations were extruded 5–6 times at room temperature, first through carbonate filters with a pore size of 400 nm and then through a 200 nm filter (Nucleopore Millipore, the Netherlands). Hereafter, the liposomes (5 mg/ml lipids) were stored at 4 °C. To assess the impact of tip sonication on biological results we investigated the effect of pre-sonicated and non-sonicated solutions of AER as controls. We also studied the effects of AER-free non-labeled liposomes that were not sonicated but extruded similarly to fluorescent-labeled liposomes.

Table 1
List of investigated liposomal vaccine formulations.

Cationic lipid	Cholesterol	DOPC/DSPC	Molar lipid ratio
DOTAP	N.A.	DOPC	1:4
DC-chol	N.A.	DOPC	1:4
DDA	N.A.	DOPC	1:4
EPC	N.A.	DOPC	1:4
DOTAP	cholesterol	DOPC	1:1:3
DDA	cholesterol	DOPC	1:1:3
EPC	cholesterol	DOPC	1:1:3
DOTAP	N.A.	DSPC	1:4
DC-chol	N.A.	DSPC	1:4
DDA	N.A.	DSPC	1:4
EPC	N.A.	DSPC	1:4
DOTAP	cholesterol	DSPC	1:1:3
DDA	cholesterol	DSPC	1:1:3
EPC	cholesterol	DSPC	1:1:3
MVL5	N.A.	DSPC	1:4
MVL5	cholesterol	DSPC	1:1:3
GL-67	N.A.	DOPC	1:4
DODMA	N.A.	DOPC	1:4
DODMA	cholesterol	DOPC	1:1:3
GL-67	N.A.	DSPC	1:4
MVL5	cholesterol	DSPC	1:1:3
DODMA	cholesterol	DSPC	1:1:3

2.3. Particle size and Zeta-potential determination

The intensity-weighted average hydrodynamic diameter (Z-average size) and polydispersity index (PDI) of the liposomes were determined by dynamic light scattering, and the Zeta-potential was determined by laser Doppler electrophoresis. For the measurements, the formulations were diluted to 0.25 mg/mL lipid in 10 mM PB (pH = 7.4) and added to 1.5 ml VWR Two-Sided Disposable PS Cuvettes (VWR, the Netherlands). Measurements were conducted in technical triplicates with a minimum of ten runs for each measurement at 20 °C using a nano ZS Zetasizer coupled with a 633 nm laser and 173° optics (Malvern Instruments, Worcestershire, UK). The data were analyzed with Zetasizer Software v7.13 (Malvern Instruments).

2.4. Generation of dendritic cells and macrophages from peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy individuals after written informed consent (Sanquin Blood Bank, The Netherlands). PBMCs were separated from the blood using the Ficoll-based density gradient centrifugation method. Subsequently, $CD14^+$ cells were isolated from the PBMCs using the magnetic cell isolation (MACS) technique with an autoMACS Pro-Separator (Miltenyi Biotec BV, the Netherlands). DCs, anti- (M2), and pro-inflammatory (M1) macrophages were generated from these $CD14^+$ cells by incubating them for six days in the presence of cytokines. To generate MDDCs, cells were incubated with 10 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; Miltenyi Biotec BV, the Netherlands) and 10 ng/ml recombinant human interleukin 4 (IL-4; Peprotech, USA). M2 macrophages were differentiated in the presence of 50 ng/ml macrophage colony-stimulating factor (M-CSF; Miltenyi Biotec BV, the Netherlands), and M1 macrophages in the presence of 5 ng/ml GM-CSF (Miltenyi Biotec BV, the Netherlands) (Verreck et al., 2006). All cell types were cultured at 37 °C/5 % CO_2 in a complete Roswell Park Memorial Institute (RPMI) 1640 medium that was supplemented with 10 % fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin, and 2 mM GlutaMAX (Gibco, Thermo Fisher Scientific, Belgium). MDDCs were harvested by pipetting the medium, and macrophages were harvested with trypsinization (Trypsin-EDTA 0.05 %, phenol red, Gibco, Thermo Fisher Scientific, Belgium).

2.5. Activation and viability of MDDCs

To assess the potential cellular toxicity and the ability of the empty and AER-containing liposomal formulations to activate MDDCs, the formulations were added to round-bottom 96-well plates (CELLSTAR, Greiner Bio-One GmbH, Germany), seeded with 30,000 MDDCs/well (25 – 250 μg/ml lipids, in 200 μl medium) and incubated for 1 h at 37 °C/5 % CO_2. Hereafter, the cells were washed with a complete RPMI medium to remove the free liposomes and cultured overnight at 37 °C/5 % CO_2. The following day, the cells were spun down, and the supernatants were collected and stored at −20 °C till further use. To stain the cells for flow cytometry, the cells were first washed with FACS buffer (PBS containing 0.1 % bovine serum albumin; Merck, Germany) and incubated for 5 min with 5 % human serum (Sanquin Blood Bank, the Netherlands) in PBS to block non-specific Fc-receptor binding. Next, the cells were washed, and the cell surface markers on the MDDCs were stained for at least 30 min with monoclonal antibodies (CCR7-BB515 (clone 3D12), CD83-PE (clone HB15e), CD40-APC (clone 5C3), CD80-APC-R700 (clone L307.4), HLA-DR-V500 (clone G46–6) from BD Biosciences, Belgium, and CD86-BV421 (clone IT2.2) from BioLegend, the Netherlands) in FACS buffer. Subsequently, the cells were washed and stained with SYTOX AADvanced Dead Cell Stain (Invitrogen, Thermo Fisher Scientific, Belgium) in FACS buffer. Viability was calculated as a percentage of SYTOX AADvanced-negative cell population in relation to

all recorded cells. Acquisition of flow cytometry data was performed using a BD FACSLyric Flow Cytometer (BD Biosciences, Belgium). Data were analyzed using FlowJo (version 10.6, FlowJo LLC, BD, USA) software.

2.6. Liposomal uptake study

MDDCs, M1, or M2 macrophages were seeded in round-bottom 96-well plates at a density of 30,000 cells/well. Afterwards, the cells were exposed to 1 % v/v empty fluorescent-labeled liposomes (containing 0.1 % mol% of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(Cyanine 5) (18:2 PE-Cy5) Avanti Polar Lipids, Inc., USA) for 1 h. Hereafter, the cells were washed with FACS buffer 3 times to remove free liposomes. The acquisition of flow cytometry data was performed using a BD FACSLyric Flow Cytometer. Data were analyzed using FlowJo (version 10.6) software.

2.7. T-cell activation

Similarly, heterozygous HLA-DR3$^+$ MDDCs were exposed for 1 hour with liposomal formulations at 5 μg/ml AER and 250 μg/ml lipids in 200 μl of complete RPMI (Gibco, Thermo Fisher Scientific, Bleiswijk, the Netherlands) at 37 °C/5 % CO_2. Cells were washed twice and 2×10^4 pre-pulsed HLA-DR3$^+$ MDDCs were cocultured with either 1×10^5 T-cells from the Rv2034 specific (Commandeur et al., 2014a)T-cell clone (1B4 recognizing peptide 75–105) or an Ag85B-specific (Geluk et al., 1997) T-cell clone (L10B4 recognizing peptide 56–65) in a 5 ml Falcon tube in a total volume of 400 μl of Iscove's Modified Dulbecco's Medium supplemented with Glutamax, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, Thermo Fisher Scientific, Bleiswijk, the Netherlands) and 10 % pooled human serum (Sigma, Merck, Darmstadt, Germany). After 6 h Brefeldin-A was added (3 μg/ml) (Sigma, Merck, Darmstadt, Germany) and cells were incubated for an additional 16 h at 37 °C/5 % CO_2. Subsequently, cells were harvested and stained for flow cytometric analysis with the violet live/dead stain (ViViD, Invitrogen, Thermo Fisher Scientific, Bleiswijk, the Netherlands), surface markers CD3-HorizonV500 (UCHT1, BD Horizon, Belgium), CD4-AlexaFluor 700 (RPA-T4, BD Pharmingen, Belgium), CD8-FITC (HIT8a, BioLegend, the Netherlands) and after fixation and permeabilization with fix/perm reagents (Nordic MUbio, Susteren, the Netherlands) for IFN-ϒ-PerCP-Cy5.5 (4S.B3, Invitrogen, Thermo Fisher Scientific, the Netherlands) and CD154-PE (TRAP1, BD Pharmingen, Belgium).

2.8. Luminex assay

Supernatants from activation and viability experiments were tested in two Bio-Plex panels (Bio-Rad, Veenendaal, the Netherlands) according to the manufacturer's protocols. In total 16 analytes were measured. The chemokine panel consisted of CXCL9, CXCL11, CCL8, and CCL22. The cytokine panel included CCL11(Eotaxin), GM-CSF, IFN-α2, IL-1β, IL-1rα, IL-6, CXCL10, CCL2(MCP-1), CCL3, CCL4, RANTES and TNF-α. Samples were acquired on a Bio-Plex 200 system and analyzed with Bio-Plex manager software version 6.1.

2.9. Statistical analysis

Statistical analyses were performed in GraphPad Prism, version 8.01 (GraphPad Software, Prism, USA). The results were analyzed with the Kruskal-Wallis test followed by an uncorrected Dunn's post-hoc test when comparing non-parametric data sets of three or more groups to the control group, where $P < 0.05$ was considered as statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). Wilcoxon matched-pairs signed rank test was performed when comparing two non-parametric data groups.

3. Results

3.1. Preparation and characterization of cationic liposomal formulations

A schematic overview of the development of our liposomal vaccine formulations is depicted in Fig. 1. In the first step, we tested the effect of the selected cationic lipids and cholesterol on the physicochemical properties of liposomes. We excluded formulations that were unstable or formed liposomes with Z-average size above 250 nm and PDI above 0.35 from further testing *in vitro*. Liposomal formulations with the various commercially available positively charged lipids (Fig. 2) at physiological pH were prepared. To test the effect of cholesterol (20 mol%), the zwitterionic phospholipid (either DOPC or DSPC) was replaced by cholesterol, while keeping the positively charged molar lipid content constant. The liposomal formulations had an antigen-to-lipid weight ratio of 1:50 and were prepared with the thin-film hydration method followed by tip sonication. The formulations are summarized in Table 1.

Subsequently, all formulations were characterized in terms of their Z-average size, PDIs, and Zeta-potentials. The following selection criteria were included: no visible signs of aggregation or precipitation in the liposomal suspension, Z-average size < 250 nm, PDI < 0.33, and a Zeta-potential between 15 and 40 mV. These inclusion criteria were selected to assure the comparability of tested formulations by minimizing the effect of size differences. The physicochemical properties of the formulations that met these criteria are presented in Table 2. The results of the remaining formulations are presented in Table S1.

The physicochemical properties for most of the selected liposomal formulations were very similar with Z-average sizes between 80 and 100 nm, PDIs between 0.22 and 0.26, and Zeta-potentials between +15 and +24 mV. However, four formulations exceeded these ranges: AER/GL-67:DOPC which had a PDI value of 0.32, and AER/DC-chol:DSPC, AER/DODMA:DOPC, and AER/DODMA:cholesterol:DOPC which had Z-average sizes of 121, 182, and 230 nm, respectively. Although the physicochemical properties differed from the other formulations, we included them for further investigation as the liposome suspensions were stable and did not meet the exclusion criteria. The selected formulations remained stable for at least seven months during storage at 4 °C (remeasured after 4 or 7 months, Table S4). All the formulations were used within six weeks after preparation.

3.2. Effect of composition of liposomal vaccines on the activation of primary human dendritic cells

The selected liposomal formulations were examined on their ability to induce activation of human MDDCs. To assess DC activation, we measured the expression of cell surface DC activation markers. As shown in Fig. 3 and Figure S1 a large variation in expression of activation markers was observed between cells derived from different donors. When compared to the control (medium only), many of the formulations induced a statistically significant upregulation of MDDC surface activation markers *e.g.*, CD40, CD83, and CCR7, evident from both increased median fluorescence intensity values (Fig. 3) and histograms (using concatenation displaying the integrated results of all six donors; Figure S1). Interestingly, the highest expression of surface activation markers was observed in response to formulations containing cholesterol, either as a component: AER/DDA:cholesterol:DOPC, AER/DOTAP:cholesterol:DOPC, and AER/EPC:cholesterol:DOPC, or as a structural part of the cationic constituent (GL-67): AER/GL-67:DOPC. The three latter induced similar or higher upregulation compared to the positive control LPS/TNFα. However, the remaining formulations containing cholesterol or its derivatives, AER/DC-chol:DOPC, AER/DC-chol:DSPC, and AER/DODMA:cholesterol:DOPC did not show the same potency to activate MDDCs, which also applied to the formulations without cholesterol: AER/DOTAP:DOPC, AER/DDA:DOPC, AER/EPC:DOPC, and AER/DODMA:DOPC. When comparing formulations containing the same cationic compound with their cognate formulations containing

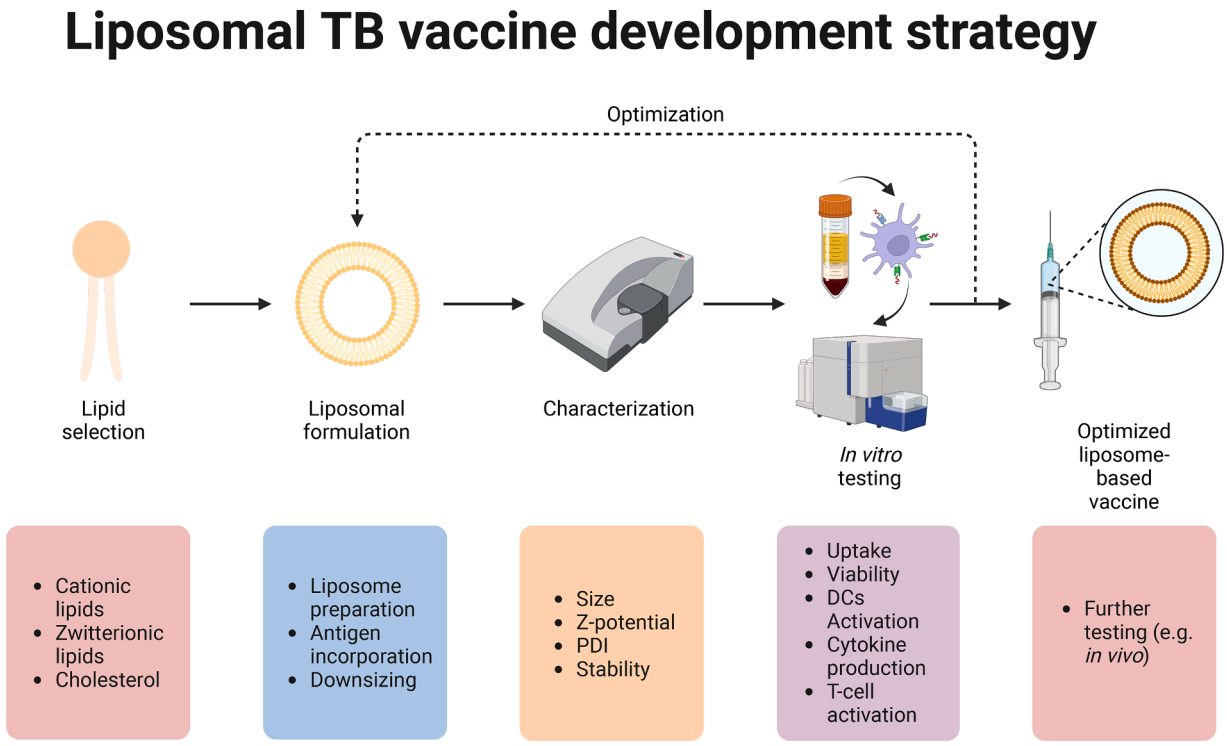

Fig. 1. Schematic overview of the strategy used for the development and optimization of liposomal TB vaccines. Created with BioRender.com.

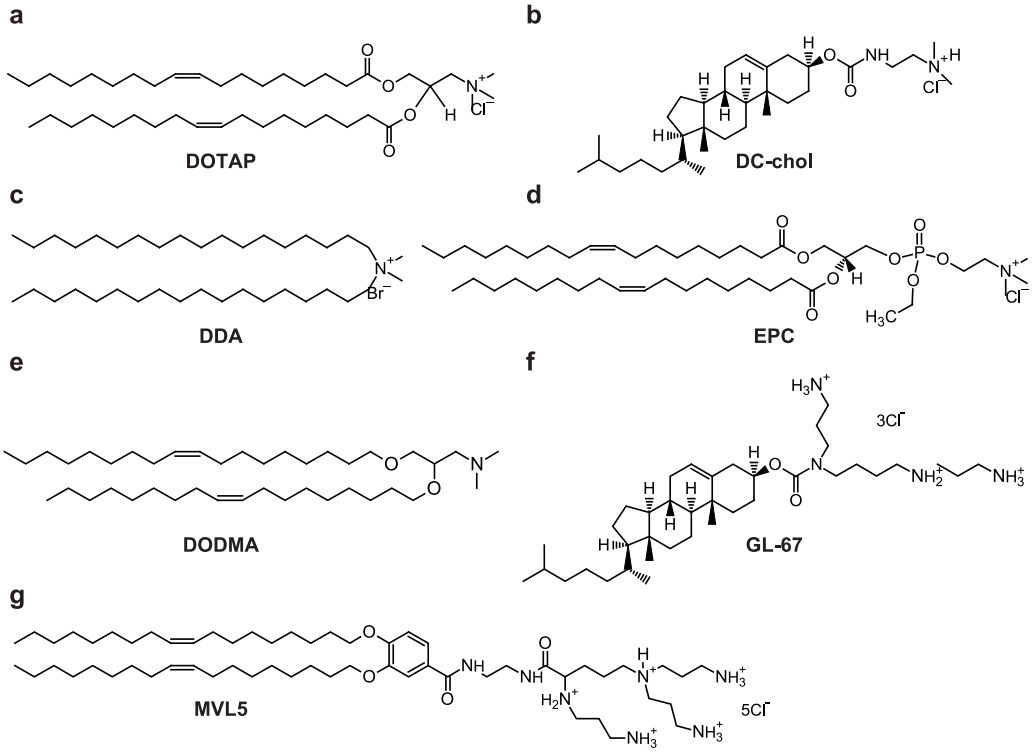

Fig. 2. Molecular structures of cationic compounds. a) DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt), b) DC-chol: 3ß-[N-(N',N'-*dimethylaminoethane*)-*carbamoyl*]cholesterol hydrochloride, c) DDA: dimethyldioctadecylammonium (bromide salt), d) EPC: 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (chloride salt), e) DODMA: 1,2-dioleyloxy-3-dimethylaminopropane, f) GL-67: N^4-cholesteryl-spermine hydrochloride, and g) MVL5: N1-[2-((1S)−1-[(3-*aminopropyl*)*amino*]−4-[*di*(3-*amino-propyl*)*amino*]butylcarboxamido)ethyl]−3,4-di[*oleyloxy*]-benzamide.

cholesterol, the cholesterol-containing formulations tended to increase the expression of the markers; however, only AER/DOTAP:cholesterol:DOPC induced a statistically significant increase of CD83 compared to AER/DOTAP:DOPC ($p < 0.05$) (Table S2). The most potent formulations were AER/DDA:cholesterol:DOPC, AER/EPC:cholesterol:DOPC, and AER/GL-67:DOPC. None of the formulations induced a statistically

Table 2
Physicochemical properties of the selected formulations. The results represent mean ± SD. Number of batches $n \geq 3$.

Formulation	Z-average size (nm)	PDI (-)	Zeta-potential (mV)
AER/DOTAP:DOPC	86 ± 1	0.25 ± 0.01	25.5 ± 0.4
AER/DC-chol:DOPC	102 ± 1	0.27 ± 0.01	24.1 ± 0.3
AER/DDA:DOPC	86 ± 1	0.23 ± 0.01	22.0 ± 0.5
AER/EPC:DOPC	92 ± 1	0.26 ± 0.01	26.4 ± 0.6
AER/DOTAP:cholesterol: DOPC	104 ± 6	0.23 ± 0.01	22.5 ± 2.5
AER/DDA:cholesterol: DOPC	106 ± 4	0.23 ± 0.01	27.0 ± 4.1
AER/EPC:cholesterol: DOPC	98 ± 3	0.26 ± 0.01	26.1 ± 4.3
AER/GL-67:DOPC	95 ± 3	0.32 ± 0.05	24.4 ± 0.4
AER/DC-chol:DSPC	121 ± 3	0.26 ± 0.02	20.2 ± 3.6
AER/DODMA:DOPC	182 ± 5	0.23 ± 0.02	16.1 ± 0.3
AER/DODMA:cholesterol: DOPC	230 ± 11	0.22 ± 0.03	17.3 ± 0.4

significant upregulation of CD80. Unadjuvanted AER did not increase the expression of any of the tested activation markers. The experiment was repeated, and similar results were obtained for batch 2 (Figure S2 and S3). Furthermore, corresponding liposomal formulations without AER were tested and yielded similar results (Figure S4), confirming that the upregulated cell surface expression levels were because of the liposomal constituents and not the loaded antigen. The DC-chol:DSPC formulations were excluded from the following studies because of suboptimal performance in the MDDC activation study. The effect of the sonication method was also investigated. In the repeated experiment (Figure S3) in the AER control group pre-sonicated AER was used. Comparing the results from the original AER batch (batch 1, Figure S3) and the sonicated batch (batch 2) revealed identical outcomes. Empty liposomal formulations (Figure S4), which were downsized with the extrusion method instead of sonication, demonstrated no impact on MDDC activation. From these findings, we concluded that the sonication method did not have any measurable effects on our results.

In summary, these data indicate that DOPC formed more stable liposomes in these formulations compared to DSPC; the most effective cationic lipids were DDA, EPC, and GL-67. Moreover, the addition of cholesterol seemed to increase the DC activation capacity of cationic liposomes.

3.3. Effect of lipid composition on the uptake, viability, and cytokine production by MDDCs

Empty fluorescently labeled liposomes were used to evaluate the uptake of vaccine formulations by human MDDCs (Fig. 4a). The uptake depended on the composition of the formulations. The formulations that induced the highest uptake contained either cholesterol, DOTAP: cholesterol:DOPC and EPC:cholesterol:DOPC, or contained the cationic cholesterol-based derivative GL-67:DOPC. This correlated with the profile of the activation markers (Fig. 3). DC-chol:DOPC and DDA: cholesterol:DOPC were not taken up effectively, and neither were DODMA:cholesterol:DOPC liposomes. Therefore, the MDDCs do not take up all formulations equally, demonstrating clear selectivity. Compared to their cholesterol-free counterparts, EPC:cholesterol:DOPC and DOTAP:cholesterol:DOPC liposomes were taken up significantly better than the corresponding liposomes without cholesterol ($p < 0.05$). The full statistical comparison of the formulations is summarized in Table S3.

Liposome uptake was also studied for human macrophages. These were (GM-CSF differentiated) human (pro-inflammatory) M1 macrophages and (M-CSF differentiated) human (anti-inflammatory) M2 macrophages (Figure S5), both of which are APCs and can locally play a role in processing and presenting antigens. Importantly, macrophages are the predominant habitat of Mtb and thus must be recognized by T-cells for bacterial control (Ottenhoff and Kaufmann, 2012). While sharing the same uptake pattern, both M1 and M2 macrophages had a higher liposome uptake than MDDCs, and GL-67:DOPC liposomes were taken up to the highest degree by the M1 and M2 macrophages.

Subsequently, the effect of the antigen-loaded liposomes on the viability of human MDDCs was tested. Each liposomal formulation was tested in three lipid concentrations: 25, 100, and 250 μg/mL with an AER-to-lipid weight ratio of 1:50. The viability of the cells depended substantially on the formulation added (Fig. 4b) as at the lowest concentration, none of the formulations reduced cellular viability. At the highest concentrations AER/DOTAP:cholesterol:DOPC, AER/DDA: cholesterol:DOPC, AER/EPC:cholesterol:DOPC caused intermediate cell death (between 25 % and 35 %) while also inducing the highest upregulation of the activation markers and the uptake in MDDCs and M1 and M2 macrophages. The formulations that increased the upregulation of the surface markers to a low degree also had a low impact on cellular viability (>85 % viability). Only the AER/GL-67:DOPC liposomes caused an unacceptable reduction of viability as less than 20 % of cells remained viable at the highest concentration. In general, cellular viability decreased as the concentration of AER and lipid concentration increased.

3.4. Antigen-specific T-cell responses

The three most promising liposomal formulations: AER/DOTAP: cholesterol:DOPC, AER/DDA:cholesterol:DOPC, AER/EPC:cholesterol: DOPC were selected to examine T-cell activation. GL-67-containing liposomes were highly toxic and did not improve the upregulation of surface markers in MDDCs substantially better than the other formulations, therefore we decided that these liposomes were not appropriate for further testing. Two HLA-DR3 restricted AER-specific T-cell clones were exposed to HLA-DR3$^+$ MDDCs from different donors, that had been incubated with the formulations. When MDDCs take up the liposomes, they will process the antigen and present it to T-cell clones that recognize the relevant peptide epitope presented via HLA-DR3 (Commandeur et al., 2014a). If the MDDCs receive costimulatory signals, they will mature and interact with the T cells, which will upregulate antigen-specific surface markers (CD154) and start producing cytokines (IFNγ) as a result, which can be detected by flow cytometry using intracellular staining (Fig. 4c). Two of the AER-loaded formulations: DOTAP:cholesterol:DOPC and EPC:cholesterol:DOPC induced statistically significant increases in T-cell clone activation by an increase of the percentage of IFNγ$^+$ CD154$^+$ double-positive cells compared to the empty liposomes. No statistical difference in the activation of T-cells was observed between the two AER-containing formulations. However, it has to be noted that variability in expression between different HLA-DR3$^+$ MDDCs donors was considerable.

3.5. Optimization of the best-performing formulations

The best-performing compositions in regard to cellular toxicity, uptake, and stimulatory capacities contained cholesterol and either DDA or EPC. DOTAP liposomes with cholesterol performed equally well, however, in a parallel unpublished work we saw that DOTAP liposomes were intrinsically less immunogenic, hence we did not include them. In the subsequent optimization step, we doubled the molar content of the cationic compound and/or cholesterol compared to formulations discussed above resulting in cationic lipid:cholesterol:DOPC molar ratios of 2:1:2, 1:2:2, and 2:2:1. The Z-average sizes, PDIs, and Zeta-potentials of the formulations are summarized in Table 3. We observed that the new

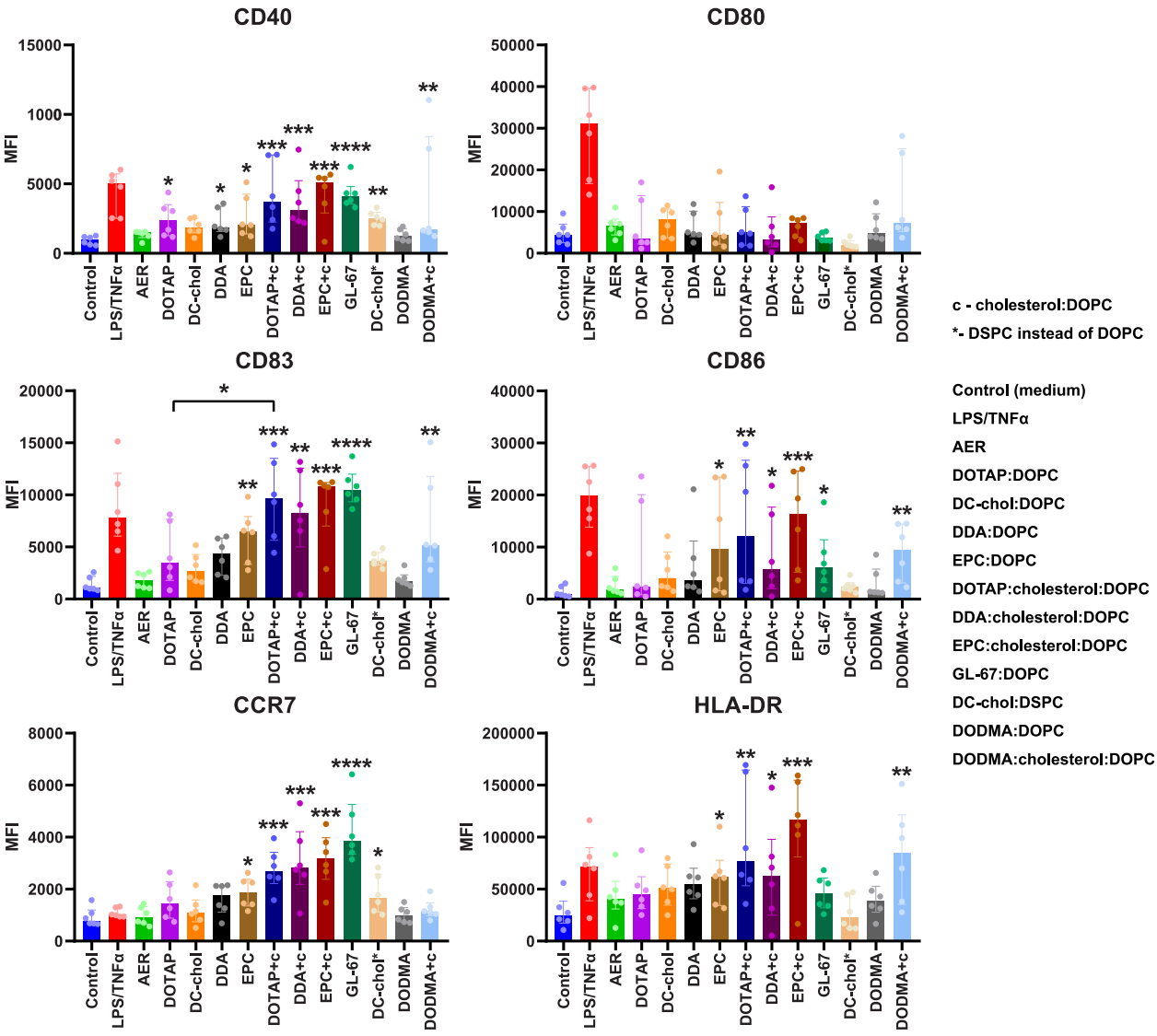

Fig. 3. Cell surface activation marker expression levels in MDDCs after stimulation with medium (control), a combination of LPS and TNFα (100 and 5 ng/ml, respectively) as the positive control, unadjuvanted AER (5 μg/ml), and liposomal formulations (5 μg/ml AER, 250 μg/ml liposomes). Median fluorescence intensities related to the expression of indicated activation markers: CD40, CD80, CD83, CD86, CCR7, and HLA-DR. The formulations are compared to the control in the significance testing. The results represent median ± IQR. n = 6 (cell donors).

variants of the AER/DDA:cholesterol:DOPC formulation did not meet our above-specified criteria in regard to the physicochemical properties and therefore were excluded from further analysis.

3.6. Effect of the increased cationic lipid and cholesterol contents on human DC activation, viability, T-cell activation, and cytokine production

The MDDCs were exposed to the best-performing formulation: AER/EPC:cholesterol:DOPC (molar lipid ratio 1:1:3 used in the first series of studies) and its three variations are reported in Table 3. The upregulation of the activation markers was evaluated (Fig. 5 and Figure S6, statistical data is shown in Table S5). The initially developed AER/EPC:cholesterol:DOPC 1:1:3 liposomes induced all the evaluated activation markers, except for CD80 (Table S4). The two variations that contained a double amount of EPC induced a more robust activation indicated by increased Median Fluorescence Intensities values, *e.g.*, CD40 and CCR7 (Fig. 5a), and histograms shifted towards high-intensity values (Figure S6). At the highest tested concentration (5 μg/ml AER with 250 μg/ml total lipids), both the 2:1:2 and 2:2:1 formulations induced a statistically significant upregulation of CD80 when compared to the control. This was not achieved by any of the liposome formulations in the prior MDDC activation experiments. Interestingly, no difference was observed between 2:1:2 and 2:2:1 variants (that contain the double amount of EPC), and between 1:2:2 and 1:1:3 variants. We observed a decrease in cellular viability for these double-amount formulations, especially for 2:2:1.

The formulation AER/EPC:cholesterol:DOPC 2:1:2 selected as the best upregulator of surface activation markers was tested again for T-cell recognition. Indeed, this formulation showed a significant increase in the percentage of $IFN\gamma^+$ $CD154^+$ double-positive cells when compared to the empty liposomes.

Finally, we measured the levels of cytokines and chemokines with multiplex assays in the supernatants from MDDC cultures exposed to the original formulation of AER/EPC:cholesterol:DOPC and the three variations. We assessed the levels of several cytokines and chemokines (Fig. 6) and observed that formulations 2:1:2 and 2:2:1 induced significantly increased levels of CCL3, CCL4, CXCL10, and CCL11, compared to the AER alone and the 1:1:3 and 1:2:2 variants. For CCL2 and CCL22 we observed a (statistically non-significant) trend towards upregulation of the cytokine levels. For IL-12p40, IL-10, IL-1β, and TNFα we did not

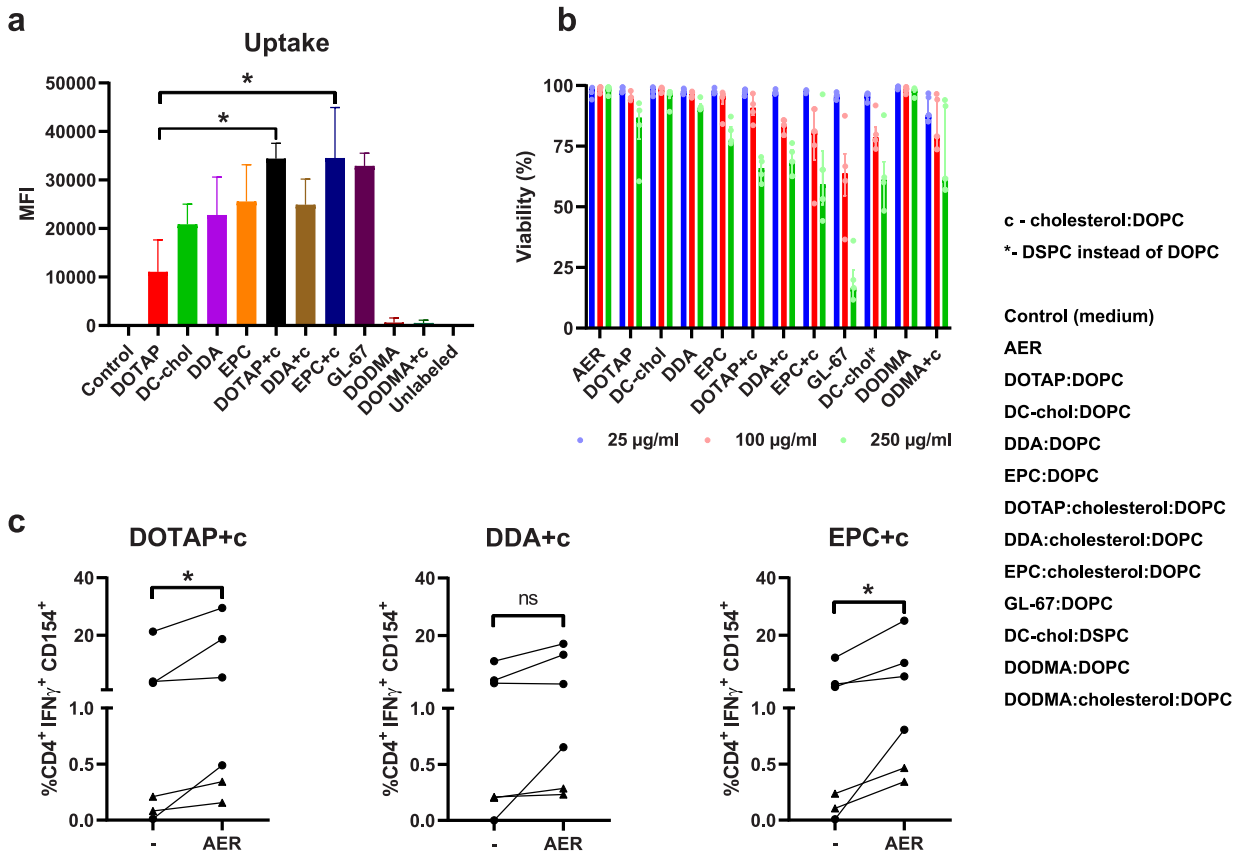

Fig. 4. Effect of lipid composition on the uptake of liposomes, the viability of MDDCs, and the T-cell activation. a) Uptake of Cy5-labeled (empty) liposomes in MDDCs, n = 12, b) viability of MDDCs after exposure to AER-containing liposomal formulations, n = 6 (cell donors), c) T-cell activation as a percentage of $CD4^+$ *T*-cells that produce IFNγ and express CD154. Comparison between empty (-) and AER-loaded (AER) liposomes (5 µg/ml AER, 250 µg/ml liposomes), exposure 1 h. Circles represent the L10B4 clone (Ag85B p56–65) and triangles the 1B4 clone (Rv2034 p75–105). The results in panels a and b represent median ± IQR.

Table 3
Physicochemical properties of optimized formulations. $n \geq 3$ (batches).

Formulation	Molar lipid ratio	Z-average size (nm)	PDI (-)	Zeta-potential (mV)
AER/DDA: cholesterol:DOPC *	2:1:2	141 ± 2	0.44 ± 0.01	33.5 ± 1.0
AER/DDA: cholesterol:DOPC *	1:2:2	136 ± 2	0.33 ± 0.01	30.1 ± 1.4
AER/DDA: cholesterol:DOPC *	2:2:1	156 ± 1	0.33 ± 0.01	31.7 ± 1.0
AER/EPC: cholesterol:DOPC	2:1:2	95 ± 6	0.28 ± 0.02	30.9 ± 0.8
AER/EPC: cholesterol:DOPC	1:2:2	109 ± 2	0.28 ± 0.01	31.6 ± 0.4
AER/EPC: cholesterol:DOPC	2:2:1	89 ± 1	0.26 ± 0.01	36.4 ± 3.6

* Visibly aggregated formulation. The sample for the measurement of the Z-average size, PDI and Zeta-potential was taken from the upper part of the solution that was free of visible aggregates.

observe changes in the concentration of detectable cytokines compared to the medium control.

4. Discussion

Cationic liposomes are not only potent delivery systems for subunit vaccines but also exhibit intrinsic adjuvant properties. In this study, we explored these properties with an extensive list of commercially available cationic lipids and different cholesterol concentrations to evaluate their role in the physicochemical properties of liposomes and immunological outcomes as summarized in Fig. 1. In this way, we aim to fill the gap in publicly available data. We used a rationalized selection of assays that allowed us to perform a head-to-head comparison of multiple liposomes to identify the optimal formulation based on human *in vitro* immune responses.

4.1. The selection of lipids and stability of liposomes

The selected cationic lipids differ substantially in terms of their chemical structures, which can affect the stability of liposomes and, consequently, the interaction between the liposomes and APCs. We observed that the mean size of liposomes prepared with DODMA is larger compared to the other formulations, and this is likely because of its head group structure, which is smaller compared to quaternary ammonium cations. Liposomes prepared with MVL-5 and GL-67 were large and unstable in the presence of the AER. Their massive cationic head groups might interact with the antigen and cause aggregation, resulting in unstable suspensions.

The choice of lipids affects the stability, size, and PDI of the liposomes. We observed that liposomes that consisted of unsaturated lipids, *e.g.*, AER/DOTAP:DOPC and AER/EPC:DOPC, were smaller than AER/DOTAP:DSPC and AER/EPC:DSPC liposomes that consisted of a mix of unsaturated and saturated lipids. Liposomes containing DDA, which is a saturated lipid, or cholesterol-based compounds like DC-chol and GL-67 should be stable. As expected, DC-chol formed stable liposomes when formulated with DOPC and DSPC. Surprisingly, AER/GL-67:DSPC and AER/DDA:DSPC formulations did not, this could be ascribed to the addition of the antigen.

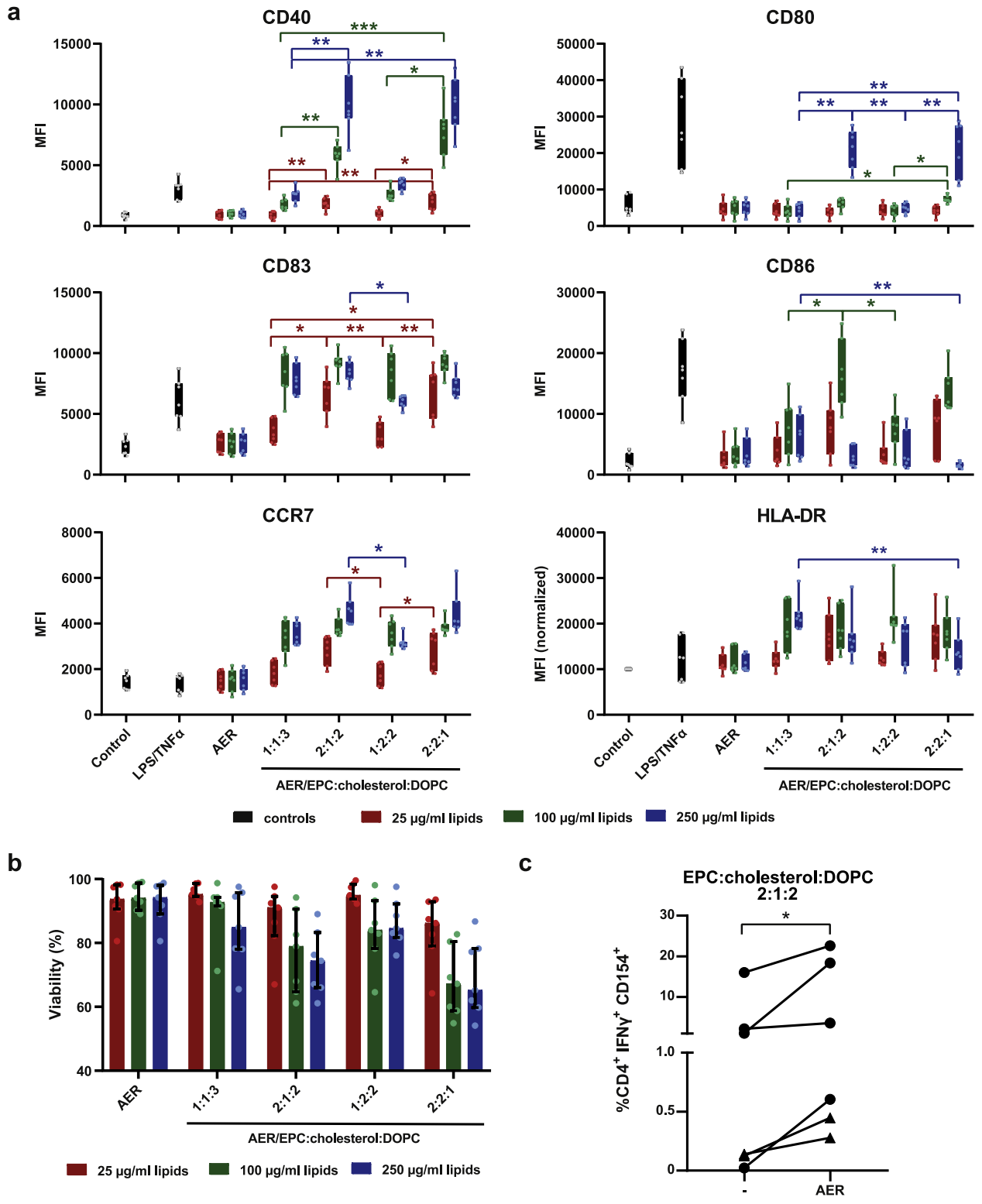

Fig. 5. Upregulation of cell surface activation markers and the viability of MDDCs exposed to liposomal formulations containing AER/EPC:cholesterol:DOPC in different molar ratios. a) Median fluorescence intensities related to the expression of indicated activation markers: CD40, CD80, CD83, CD86, CCR7, and HLA-DR, n = 7 (cell donors). b) Viability of MDDCs after 1-h exposure to the liposomal formulations, n = 7 (cell donors). c) T-cell activation as a percentage of $CD4^+$ *T*-cells that produce IFNγ and express CD154. Circles represent the L10B4 clone (Ag85B p56–65) and triangles the 1B4 clone (Rv2034 p), n = 6 (cell donors). The results represent median ± IQR.

4.2. The choice of lipid and consequent immunological response

The formulations that fulfilled our predefined selection criteria for further immunological evaluation were tested in several biological assays. We observed that liposomes containing cholesterol were taken up more efficiently by human MDDCs and also induced a higher expression of activation markers, but also increased cellular toxicity, which did not lead to massive cell death, compared to liposomes without

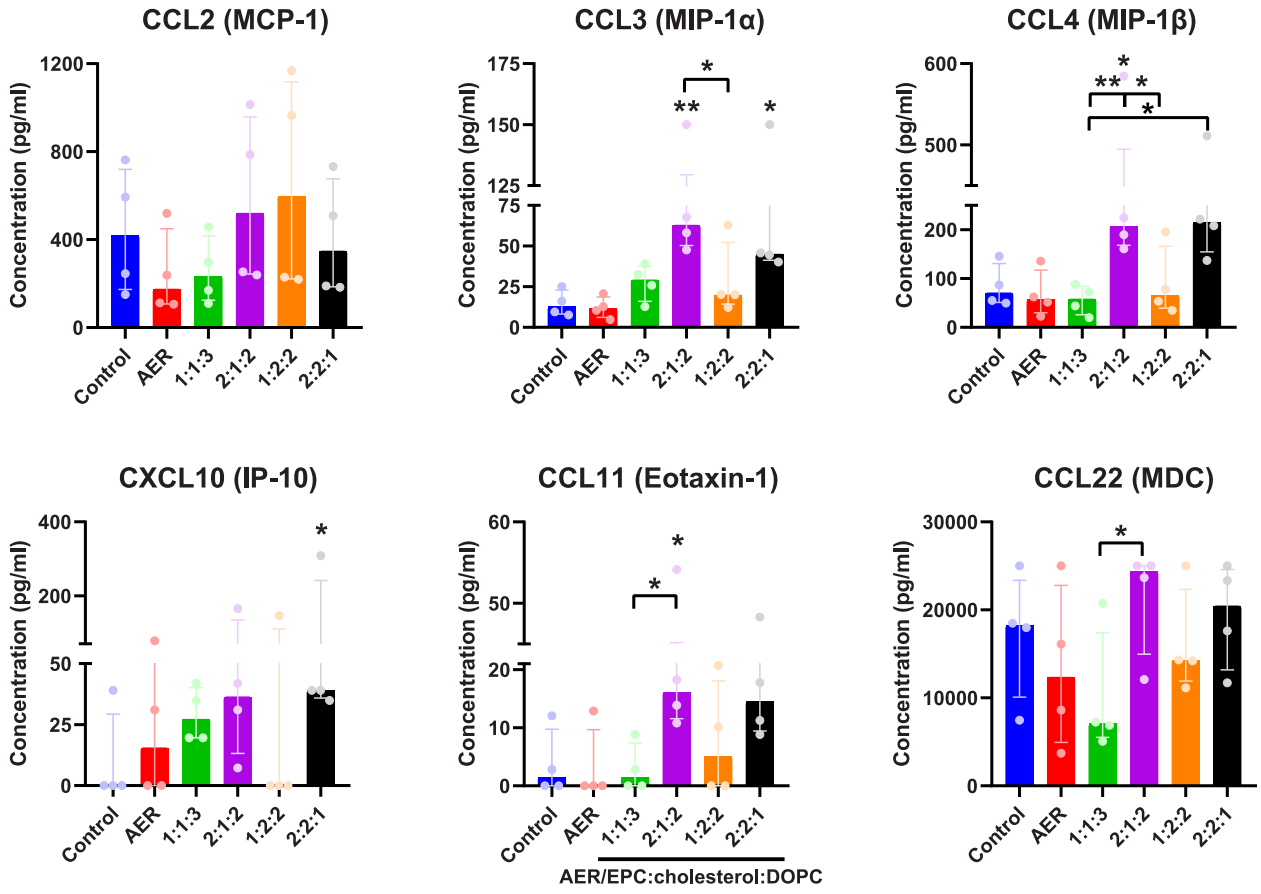

Fig. 6. Production of cytokines by MDDCs exposed to liposomal formulations (5 μg/ml AER, 250 μg/ml liposomes, exposure 1 h), n = 4 (cell donors). The results represent median ± IQR.

cholesterol. This is likely because of the liquid-ordered organization of the bilayer of liposomes, which is more rigid than a liquid-disordered phase in liposomes without cholesterol (Benne et al., 2020; Krause and Regen, 2014; Martinez-Seara et al., n.d.; Takechi-Haraya et al., 2016). This finding is in line with reports that more rigid liposomes with higher cholesterol content are more efficiently taken up by DCs (Barnier-Quer et al., 2013) and macrophages (Christensen et al., 2012; Kaur et al., 2014; Takano et al., 2003).

When focusing on the cationic lipids, formulations containing DOTAP, DDA, EPC, and GL-67 induced the highest upregulation of surface activation markers. This might be a consequence of the higher uptake by MDDCs. We observed that liposomes containing DDA, EPC, and GL-67 tended to be more toxic as they reduced the viability of MDDCs more than those with DOTAP and DC-chol. Induction of cell death and activation of MDDCs can be mechanistically linked: apoptotic vesicles from dying cells can interact with TLRs on viable DCs, which can lead to cross-priming and induction of $CD8^+$ *T*-cells *in vivo* (Caruso and Poon, 2018; Winau et al., 2006). The liposomal formulation with the cation GL-67 reduced the viability pronouncedly, even at lower concentrations. This is most probably caused by the induction of necrosis by the primary amines in GL-67 (Arisaka et al., 2010; Mayhew et al., 1987). It has been reported that liposomes containing cholesterol and eDPPC (ethyl dipalmitoylphosphatidylcholine), are taken up by APCs to a higher degree than liposomal formulations with both cholesterol and DDA or DC-chol (Barnier-Quer et al., 2013). This may suggest that cationic compounds having ethyl phosphocholine head groups, such as eDPPC and EPC, increased liposomal uptake. Vangasseri and colleagues reported that EPC-containing liposomes were superior in stimulating bone marrow-derived DCs (namely in upregulation of the surface expression of CD80) compared to liposomes containing other compounds *e.g.*, DOTAP (Vangasseri et al., 2006). Based on these results, we selected three formulations for further evaluation: DOTAP:cholesterol:DOPC, DDA:cholesterol:DOPC and EPC:cholesterol:DOPC.

To gain more insights into the immunomodulatory capacity of these formulations, we used human T -cell activation assays, a step not commonly reported in adjuvant/delivery literature. The specific interaction of the liposome-treated DCs and T-cells is essential to protective immunity against TB. We observed that all AER-loaded liposomes induced a higher activation of two different antigen-specific T-cell clones compared to the empty liposomes. This indicates that the AER-containing liposomal formulations were not only efficiently taken up by MDDCs, but were processed and their epitopes presented to activate the T-cell clones. DOTAP:cholesterol:DOPC and EPC:cholesterol:DOPC induced a statistically significant increase of antigen-specific T-cell activation compared to the empty counterparts, demonstrating clear antigen specificity. This was, however, not observed for DDA:cholesterol:DOPC, suggesting that this formulation was not as effective in delivering the antigen and activating MDDCs as the DOTAP- and EPC-based liposomes.

To further improve the quality of the immune response, we selected the cationic lipids DDA and EPC formulations and doubled the cationic lipid and/or cholesterol content. Because of the unfavorable physicochemical properties of the DDA formulation when increasing the cationic or cholesterol levels, we focused on the EPC formulations. Liposomes that contained a double amount of EPC, but not double cholesterol induced an increased upregulation of surface activation markers CD40 and CD80. This is in line with the literature showing that increasing the content of the cationic compound leads to stronger DC maturation (Ma et al., 2011) and increased IgG titers *in vivo* (Barnier Quer et al., 2012). Similar to the initial set of formulations, the liposome

variants that upregulated DCs activation markers induced also more cell death. Doubling the cholesterol content did not affect surface marker expression, however, there was reduced viability when a higher cholesterol content variant was used. We speculate that once a more rigid liquid-ordered organization occurs at 2:1:2 liposomal composition a further increase of cholesterol provides no additional beneficial effect. Therefore, we decided to only test the 2:1:2 liposome in the T-cell activation assay. We observed a statistically significant increase of CD154 and IFNγ double positive T-cells when MDDCs were pre-treated with AER-containing liposome compared to the empty one. This indicates that increased EPC content did not negatively affect the ability of the liposome to activate T-cells and it is likely to induce effective antigen presentation to T-cells *in vivo*.

Lastly, the capacity of the AER-containing liposomes to induce cytokine production in MDDCs was assessed and we observed increased cytokine production for a few cytokines, especially CCL3 (MIP-1α), CCL4 (MIP-1β), CXCL10 (IP-10), and CCL11 (Eotaxin-1). CCL3 and CCL4 have been shown to actively chemoattract $CD8^+$ *T*-cells (Honey, 2006), modulate the interactions between T-cells and APCs in the draining lymph nodes after immunization, and enhance memory T-cell responses (Askew et al., 2016; Castellino et al., 2006; Hugues et al., 2007). CXCL10 is reported as a specific chemoattractant for effector T-cells (Khan et al., 2000) and is thought to be directly involved in the generation of antigen-specific $CD8^+$ *T*-cell responses after vaccination (Majumder et al., 2012). Moreover, it is a marker of trained immunity, mediating the inhibition of mycobacterial growth in human macrophages (Joosten et al., 2018). Therefore, this may indicate that liposomes containing 40 mol% EPC favor a microenvironment that is beneficial for TB vaccination, as both $CD4^+$ and $CD8^+$ *T*-cell responses are important to prevent TB (Ottenhoff and Kaufmann, 2012). CCL11 is an eosinophil-specific chemoattractant (Lacy, 2017). We observed a small increase in the CCL2 (MCP-1) production, which promotes the trafficking of effector cells including monocytes, memory T-cells, and natural killer cells from the circulation across the endothelium (Deshmane et al., 2009), and CCL22 (MDC). Expression of CCL22 induces cellular contacts of DCs with regulatory T-cells through the CCR4 receptor (Rapp et al., 2019) and inhibits the T-cell activation capacities of DCs by decreasing the expression of HLA molecules and CD80 (Kühnemuth et al., 2017). Expression of CCL22 may therefore reduce T-cell activation *in vivo*. We did not detect any production of IL-12, IFNα, which concurs with previous reports that cationic liposomes without molecular adjuvants do not induce IL-12 production in DCs (Varypataki et al., 2015b). The lack of these cytokines combined with the low production of pro-inflammatory cytokines (CCL3, CCL4, CXCL10) indicates that cationic liposomal formulations require additional adjuvants *e.g.*, TLR agonists, to achieve robust immune responses *in vivo*.

5. Conclusions

TB is still among the leading causes of death and it has been the deadliest infectious disease worldwide for decades. Therefore, additional measures that can control and combat this disease are highly needed. This study presents a strategy to compare, optimize, and select cationic liposomal compositions formulated with the multivalent Mtb antigen AER, based on a rational pipeline of *in vitro* testing and down-selecting using human cells, as a prelude further pre-clinical investigations, thus reducing animal experimentation. The best-performing formulation was comprised of an AER-containing formulation containing the lipids EPC:cholesterol:DOPC in a molar ratio of 2:1:2, as assessed by an increase in cell surface activation markers, cellular uptake, antigen-specific T-cell activation, cytokine production, and cellular viability. Moreover, the addition of cholesterol improved the performance of the formulations. The liposomal TB vaccine development strategy described in this paper can be used to elucidate which molecular adjuvants should be incorporated in the liposomal formulations before evaluating the effect of the composition in animal models and can be extended to other pathogens besides Mtb.

Appendices

Supplementary materials.

CRediT authorship contribution statement

M.M. Szachniewicz: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **M.A. Neustrup:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **K.E. van Meijgaarden:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing, Supervision. **W. Jiskoot:** Conceptualization, Supervision. **J.A. Bouwstra:** Conceptualization, Writing – review & editing, Project administration, Funding acquisition, Supervision. **M.C. Haks:** Conceptualization, Supervision. **A. Geluk:** Conceptualization, Writing – review & editing, Project administration, Funding acquisition, Supervision. **T. H.M. Ottenhoff:** Conceptualization, Writing – review & editing, Project administration, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2024.106730.

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