A matter of delivery: nanocarriers and the engineering of protective immunity in tuberculosis vaccination

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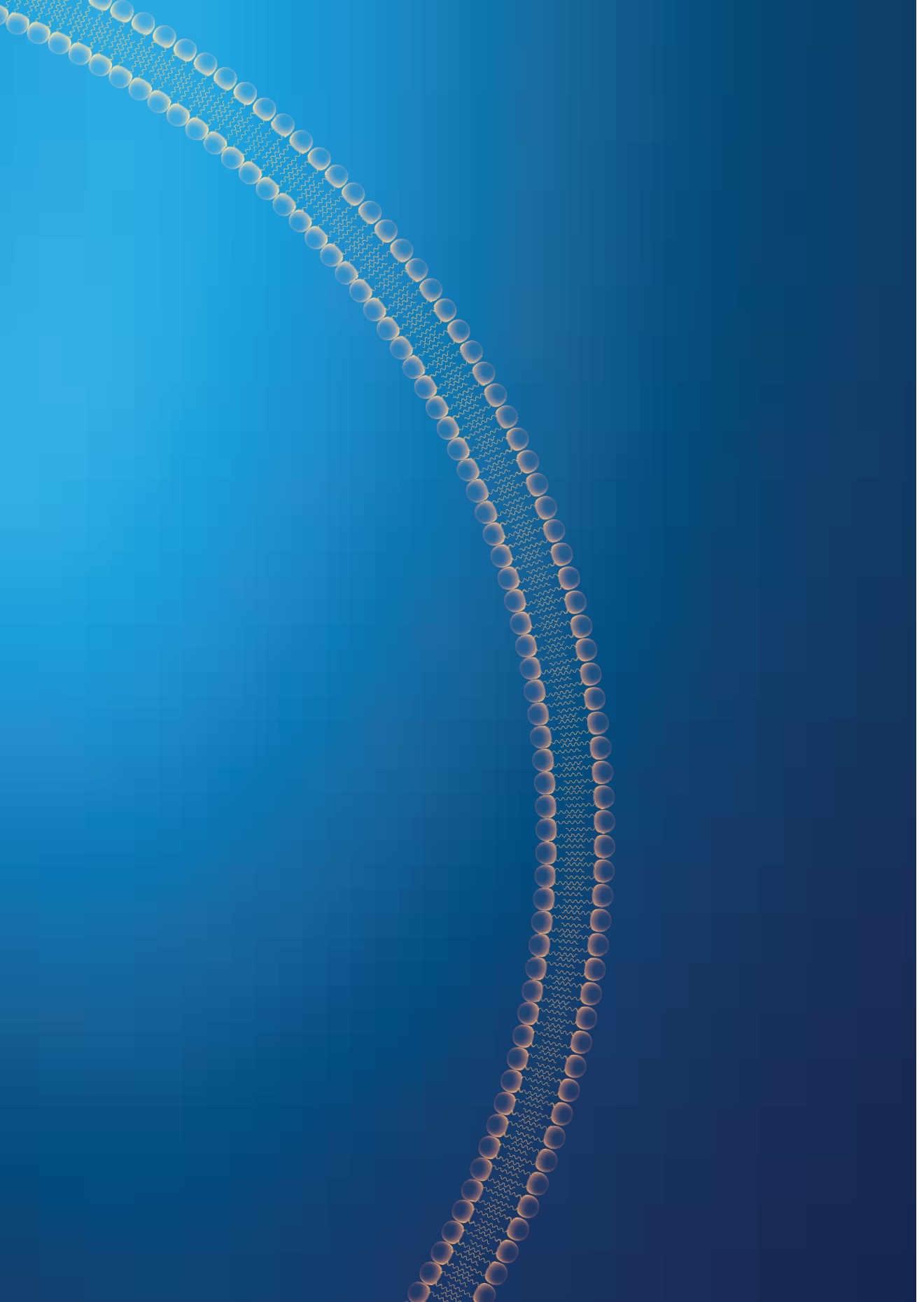

CHAPTER 3

Cationic pH-sensitive liposomes as tuberculosis subunit vaccine delivery systems: effect of liposome composition on cellular innate immune responses

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ABSTRACT

Tuberculosis (TB) is a major global health problem, and the development of effective and safe vaccines is urgently needed. $CD8^+$ T-cells play an important role alongside $CD4^+$ T-cells in the protective immune response against TB. pH-sensitive liposomes are hypothesized to boost $CD8^+$ T-cell responses by promoting class I presentation through a mechanism involving pH-dependent endosomal escape and the cytosolic transfer of antigens. The aim of the study was to explore the potential of pH-sensitive liposomes as a novel delivery system for a multi-stage protein subunit vaccine against TB in primary human cells. The liposomes were formulated with the fusion antigen Ag85b-ESAT6-Rv2034 (AER), which was previously shown to be effective in reducing bacterial load in the lungs HLA-DR3 transgenic mice and guinea pigs. The liposomes were assessed *in vitro* for cellular uptake, cell viability, upregulation of cell surface activation markers, induction of cytokine production using human monocyte-derived dendritic cells (MDDCs), and activation of human antigen-specific T-cells. Liposome DOPC:DOPE:DOBAQ:EPC (3:5:2:4 molar ratio) was effectively taken up, induced several cell surface activation markers, and production of CCL3, CCL4, and TNFα in MDDCs. It also induced upregulation of CD154 and IFNγ in T-cell clones in an antigen-specific manner. Thus, cationic pH-sensitive liposome-based TB vaccines have been demonstrated to be capable of inducing robust protective Mtb-specific immune responses, positioning them as promising candidates for effective TB vaccination.

1. INTRODUCTION

TB is among the leading deadliest infectious diseases worldwide. Until the COVID-19 pandemic at the end of 2019, TB was the number one killing pathogen for the last decades. It is estimated that a quarter of the human population is latently infected with *Mycobacterium tuberculosis* (Mtb), the causative agent of TB. In 2022 1.3 million people died because of it.[1] Unfortunately, the only licensed and available TB vaccine – *Mycobacterium Bovis* Bacillus Calmette-Guérin (BCG) – offers limited protection in adults especially those living in areas of the world where TB is endemic.[2–4] Therefore, there is an urgent need for new TB vaccines.[5]

Liposomes are self-assembled nanovesicles composed of lipids that form a bilayer that surrounds an aqueous core. Liposomes can exhibit many different physicochemical as well as biological properties depending on the lipid composition, size, charge, and surface chemistry.[6-10] One of the subclasses of liposomes is represented by pH-sensitive liposomes. Those liposomes typically respond to a change in the pH in the microenvironment by changing the molecular organization of the bilayer upon acidification. This phenomenon is utilized to deliver their cargo into the cytosol of the cell by avoiding endosomal degradation.[11–15]

Liposomes are often made pH-sensitive by the inclusion of phosphatidylethanolamine (PE) as well as by addition of weakly acidic amphiphiles – i.e. cholesteryl hemisuccinate (CHEMS) or N-(4-carboxybenzyl)-N,N-dimethyl-2,3-bis(oleoyloxy)propan-1-aminium (DOBAQ). PE lipids have a cone-shaped molecular structure that favours the formation of the inverted hexagonal phase instead of stable bilayers, which is why additional amphiphilic lipids are required to stabilize bilayers.[16–22] When exposed to an acidic environment such as inside endosomes, pH-sensitive liposomes undergo destabilization because at lower pH carboxylic groups of the amphiphilic lipids are protonated and therefore are unable to effectively stabilize the bilayer. The unstable lipid phase has a high affinity for other lipids, including those that are present in cellular membranes. Destabilized liposomes fuse with the endosomal membrane such that their cargo is released into the cytosol.[11,15,23–25]

Another mechanism employed by pH-sensitive liposomes is the so-called proton-sponge effect. After liposomes are taken up by the cell through endosome-mediated endocytosis, the endosome fuses with lysosomes, which induces acidification of the

late endosome. Functional groups that can bind free protons (i.e., amines) present on the surface of the liposomes can interfere with this process by capturing the protons and thus preventing the decrease in pH. This prevents liposomal degradation by inhibiting proteolytic and lipase enzymes released by the fusion with lysosomes. Furthermore, osmotic pressure inside the endosome will also increase due to the influx of counter ions (e.g., chloride anions) and subsequently water. This may lead to the rupture of the endosome and the release of its content.[12,26–29]

The unique property of the pH-sensitive liposomes to escape rapid endosomal/lysosomal degradation has potential benefits for vaccination.[30] Other types of liposomes typically remain inside the endosome and undergo degradation.[31,32] Delivered antigens (peptides or proteins) are rapidly degraded and presented mainly via the MHC-II pathway. As a consequence, this would lead to effective $CD4^+$ T-cell responses.[31,32] In contrast, pH-sensitive liposomes due to endosomal escape are capable of protecting antigens from rapid degradation and instead release the antigens into the cytosol. Inside the cytosol, these antigens can be processed by proteasomes instead and presented via the MHC-I pathway, leading to $CD8^+$ T-cell responses.[31,33–35] Such a shift in the balance between the $CD4^+$ and $CD8^+$ T-cell responses can have a potentially significant impact on the type of immune response induced by the vaccine.[36] In the research presented in this paper, we explored the potential of pH-sensitive liposomes for tuberculosis (TB) vaccination.

We investigated the potential of pH-sensitive liposomes as a novel delivery system of a previously designed multi-stage protein subunit vaccine against TB. Mtb is an intracellular pathogen. Such pathogens are specifically eliminated by $CD4^+$ and $CD8^+$ cytotoxic T-cells as well as natural killer cells. It can be hypothesized that if pH-sensitive liposomes further boost $CD8^+$ T-cell responses, it would have an advantage in TB vaccination. The liposomes were formulated with the Mtb fusion antigen AER, which was shown to be effective as a preventive vaccine in HLA-DR3 transgenic mice and guinea pig models.[37] Several stable formulations were developed and we validated their pH-sensitive properties. The liposomes were tested in several *in vitro* assays: cellular uptake by antigen-presenting cells (APCs): human primary MDDCs, and macrophages type 1 and type 2; cellular viability, upregulation of cell surface activation markers, induction of cytokine production, and activation of antigen-specific T-cell clones/lines as assessed by IFNγ secretion and CD154 expression.

2. MATERIALS AND METHODS

2.1 Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP), 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine chloride salt (EPC), N-(4-carboxybenzyl)-N,N-dimethyl-2,3-bis(oleoyloxy)propan-1-aminium (DOBAQ), cholesteryl hemisuccinate (CHEMS), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(Cyanine 5) (18:2 PE-Cy5) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Cholesterol was purchased from Merck KGaA, (Darmstadt, Germany). The chemical structures of unlabeled lipids are shown in Figure S1. Recombinant fusion protein AER was prepared as described previously.[38] AER is a 519-amino acid protein with a molecular mass of 56 kDa, an isoelectric point (pI) of 5.60, and an aliphatic index of 73.64. In short, genes derived from Mtb (lab strain H37Rv) were amplified using polymerase chain reaction (PCR) from genomic DNA and cloned using Gateway technology (Invitrogen, ThermoFisher Scientific, Bleiswijk, Netherlands) in bacteria containing an N-terminal hexa-histidine (His) tag. Successful insertion of the products was confirmed using sequencing. Subsequently, AER was expressed in *Escherichia coli* strain BL21 (DE3) and purified. The quality of the antigen (size and purity) was assessed by gel electrophoresis using Coomassie brilliant blue staining and Western blotting using an anti-His antibody (Invitrogen, ThermoFisher Scientific, Bleiswijk, Netherlands). Endotoxin contamination in the protein was quantified using a ToxinSensor Chromogenic Limulus Amebocyte Lysate (LAL) Endotoxin Assay Kit (GenScript, Piscataway, NJ, USA). The endotoxin contents were below 50 endotoxin units per 1 mg of the protein. Subsequently, the antigen was evaluated to exclude non-specific T-cell activation and cellular toxicity using IFNγ release assay. In 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 liposomes

The liposomes were prepared using the thin-film hydration method as described previously.[39] Appropriate lipids were pre-dissolved in chloroform. The lipids were diluted in chloroform from 25 mg/ml stock solutions. The final total amount of lipids

used per batch was 10 mg (10 mg/ml) in chloroform. The lipid solution was added to a round-bottom flask, and the chloroform was removed using a rotary evaporator (Buchi rotavapor R210, Buchi, Breda, Netherlands). To prepare AER-containing liposomes, the lipid film was rehydrated using 1 ml of 200 µg/ml AER (or 200 µg/ml DQ ovalbumin, Invitrogen, ThermoFisher Scientific, Bleiswijk, Netherlands) in 10 mM phosphate buffer (PB) with 9.8 % sucrose (pH = 7.4). For the preparation of empty liposomes (without AER) and liposomes labeled with 0.1 % 18:2 PE-Cy5 (also without AER), 10 mM PB with 9.8 % sucrose was used for rehydration. After rehydration, the liposomes were downsized by using a tip sonicator (Branson sonifier 250, Branson Ultrasonics, Danbury, UK). The sonication program comprised of eight cycles, 30 s per cycle of sonication at a 10 % amplitude followed by a break of 60 s. The samples were submerged in ice during sonication, which together with short cycles and low amplitude allowed to reduce lipid degradation. After this, the liposomes were spun down (Allegra X-12R, Beckman Coulter, Brea CA, USA) at 1500 RPM for 5 min to remove the metal particles shed by the tip sonicator. To discard the pellet, the liposomal suspensions were transferred to new tubes, and the metal pellets were discarded. To avoid fluorophore degradation by the sonication, fluorescently labeled liposomes were downsized using a 10 ml extruder (LIPEX extruder, Northern Lipids, Evonik, Canada). The liposomal formulations were extruded five times at room temperature. Firstly, through a 400 nm carbonate filter and secondly through a 200 nm filter (Nucleopore Millipore, Amsterdam, Netherlands). The liposomes (5 mg/ml lipids assuming no lipid loss) were stored at 4 °C.

2.3 Particle size and Zeta-potential measurements

The intensity-weighted average hydrodynamic diameter (Z-average size) and polydispersity index (PDI) of the formulations were quantified by dynamic light scattering, and the Zeta-potential was measured with laser Doppler electrophoresis as described previously.[39] The liposomes 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, Amsterdam, Netherlands). Measurements were carried out in triplicates with a minimum of ten runs per 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 evaluated with Zetasizer Software v7.13 (Malvern Instruments).

2.4 Preparation of dendritic cells and macrophages from peripheral blood mononuclear cells

Buffy coats obtained from healthy donors after written informed consent (Sanquin Blood Bank, Amsterdam, Netherlands) were used to isolate peripheral blood mononuclear cells (PBMCs) as described previously.[39] PBMCs were obtained from buffy coats using the Ficoll-based density gradient centrifugation method. $CD14^+$ cells were isolated using the magnetic cell isolation (MACS) method with an autoMACS Pro Separator (Miltenyi Biotec BV, Leiden, Netherlands). MDDCs, M2, and M1 macrophages were prepared from $CD14^+$ cells by culturing them for six days in the presence of cytokines. To prepare MDDCs, cells were cultured with 10 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; Miltenyi Biotec BV, Leiden, Netherlands) and 10 ng/ml recombinant human interleukin 4 (IL-4; Peprotech, Rocky Hill, NJ, USA). M2 macrophages were generated using 50 ng/ml of macrophage colony-stimulating factor (M-CSF; Miltenyi Biotec BV, Leiden, Netherlands), and M1 macrophages were obtained by using 5 ng/ml GM-CSF (Miltenyi Biotec BV, Leiden, Netherlands) (Verreck et al., 2006). All cell types were incubated at 37 °C / 5 % CO2 in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10 % fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin, and 2mM GlutaMAX (Gibco, Thermo Fisher Scientific, Bleiswijk, Netherlands). MDDCs were harvested by pipetting the medium, and macrophages were harvested with trypsinization (Trypsin-EDTA 0.05 %, phenol red, Gibco, Thermo Fisher Scientific, Bleiswijk, Netherlands).

2.5 LysoSensor acidification assay

To quantify the acidification of acidic cellular compartments LysoSensor green (DND-189 dye) was used (Thermo Fisher Scientific, Bleiswijk, Netherlands). Briefly, MDDCs (30,000 cells/well) in round-bottom 96-well plates (CELLSTAR, Greiner Bio-One GmbH, Frickenhausen, Germany) were treated with empty liposomes (250 µg/ml lipids, in 200 µl medium) and incubated for 2.5 h at 37 ˚C / 5 % CO_2. Subsequently, cells were washed with a medium. 10 µM chloroquine (Sigma-Aldrich, Zwijndrecht, the Netherlands) was used as a control and it was added after washing. Afterwards, cells were incubated overnight at 37 ˚C / 5 % CO_2. The following day the LysoSensor (1 µM in 200 µl medium) dye was added to the cells and incubated for 30 min

at 37 ˚C / 5 % CO_2. Measurement of flow cytometry data was carried out using a BD FACSLyric Flow Cytometer (BD Biosciences, Erembodegem, Belgium). Data were analyzed using FlowJo (version 10.6, FlowJo LLC, BD, USA) software.

2.6 DQ-OVA antigen processing assay

To evaluate antigen processing, MDDCs (30,000 cells/well) in round-bottom 96-well plates (CELLSTAR, Greiner Bio-One GmbH, Germany) were treated with either 5 µg/ml DQ™-Ovalbumin (DQ-OVA, Invitrogen, ThermoFisher Scientific, Bleiswijk, Netherlands) or liposomes containing 5 µg/ml DQ-OVA and 250 µg/ml lipids (assuming no lipid loss during extrusion) in a total volume of 200 µL medium. Cells were incubated at 37 °C with 5 % CO_2 for 1 hour. The DQ fluorophore concentration in each liposomal formulation was quantified by measuring fluorescence intensity (excitation at 490 nm, emission at 520 nm) using an Infinite M1000 microplate reader (Tecan Austria GmbH, Grodig, Austria). To standardize fluorophore concentrations, samples were diluted to match the fluorescence intensity of the DOPC:DOPE (3:5:2:4) formulation. Lipid concentration was kept consistent across all samples by adjusting with buffer and corresponding unloaded liposomal formulations. DQ-OVA, a fluorogenic substrate labeled with BODIPY dyes, exhibits fluorescence quenching that is relieved upon protease-mediated hydrolysis, resulting in the production of brightly fluorescent dye-labeled peptides. Flow cytometry data were collected using a BD FACSLyric Flow Cytometer (BD Biosciences, Erembodegem, Belgium) and analyzed with FlowJo software (version 10.6, FlowJo LLC, Ashland, OR, USA).

2.7 Activation and viability of MDDCs

Cellular viability and adjuvant properties of empty and AER-containing liposomes were evaluated using MDDCs as described previously [39]. The liposome suspensions were added in round-bottom 96-well plates (CELLSTAR, Greiner Bio-One GmbH, Frickenhausen, 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. Afterward, the cells were washed with a complete RPMI medium to remove free liposomes and cultured overnight at 37 °C / 5 % CO_2. The next day, the cells were centrifuged, and the supernatants were harvested and kept at -20 ˚C till further use. For flow cytometry analysis, the cells were first washed with FACS buffer (PBS containing 0.1 % bovine serum albumin; Merck, Amsterdam, Netherlands) and incubated for 5 min with 5 % human serum (Sanquin Blood Bank, Amsterdam, Netherlands) in PBS to block

non-specific Fc-receptor binding. Subsequently, the cells were washed, and the cell surface markers on the MDDCs were stained with monoclonal antibodies for 30 min. We used 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, Amsterdam, Netherlands, all diluted 1:200 in FACS buffer. Subsequently, the cells were washed and stained with SYTOX AADvanced Dead Cell Stain (Invitrogen, Thermo Fisher Scientific, Bleiswijk, Netherlands) diluted 1:2000 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, Erembodegem, Belgium). Data were analyzed using FlowJo (version 10.6, FlowJo LLC, Ashland, OR, USA) software.

2.8 Liposomal uptake study

To evaluate cellular uptake of liposomes, MDDCs, M1, or M2 macrophages were seeded in round-bottom 96-well plates with 30,000 cells/well as described previously.[39] 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., Alabaster, AL, USA) for 1 h. Subsequently, 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.

2.9 T-cell activation

HLA-DR3$^+$, heterozygous MDDCs were exposed with liposomes for 1 h (5 µg/ml AER and 250 µg/ml lipids) in 200 µl RPMI (Gibco, Thermo Fisher Scientific, Bleiswijk, the Netherlands) + 10 % FBS (Hyclone, Cytiva, Medemblik, The Netherlands) as described previously.[39] Cells were washed twice and 2x10^4 liposome-treated HLA-DR3$^+$ MDDCs were cocultured with either 1x10^5 Rv2034-specific[40] T-cells (1B4 clone recognizing peptide 75-105) or Ag85B-specific[41] T-cells (L10B4 clone recognizing peptide 56-65) in a 5 ml Falcon tube in a total volume of 400 µl IMDM 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 additional 16 h. Subsequently,

cells were harvested and stained for flow cytometric analysis with the violet live/dead stain (ViViD, Invitrogen, Thermo Fisher Scientific, Bleiswijk, Netherlands), surface markers CD3-HorizonV500 (UCHT1, BD Horizon, Erembodegem, Belgium), CD4-AlexaFluor 700 (RPA-T4, BD Pharmingen, Belgium), CD8-FITC (HIT8a, BioLegend, Amsterdam, Netherlands) and after fixation and permeabilization with fix/perm reagents (Nordic MUbio, Susteren, the Netherlands) for IFN-Y-PerCP-Cy5.5 (4S.B3, Invitrogen, Thermo Fisher Scientific, Bleiswijk, Netherlands) and CD154-PE (TRAP1, BD Pharmingen, Erembodegem, Belgium).

2.10 IL-12p40 and IL-10 enzyme-linked immunosorbent assay (ELISA)

Production of IL-12p40 and IL-10 by MDDCs exposed to liposomal formulations was tested using supernatants from activation and viability experiments. Biolegend's ELISA MAX Standard Set (London, UK) was used to carry out ELISA assays for human IL-12/IL-23 (p40) and human IL-10. All supernatants were tested in duplicates following the manufacturer's instructions. The assays were performed using Microlon high binding 96-well plates (Greiner Bio-One International, Alphen aan den Rijn, Netherlands), and the absorbance of the samples was measured using a Spectramax i3x spectrometer (Molecular Devices, San Jose, CA, USA).

2.11 Luminex assay

Supernatants 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.

2.12 Statistical analysis

Statistical analyses were performed in GraphPad Prism, version 8.01 (GraphPad Software, Prism, San Diego, CA, 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 of pH-sensitive liposomes

pH-sensitive liposomes were prepared using the thin film hydration method. Initially, the liposomes were prepared empty (without the addition of AER protein). In total 11 different formulations were prepared, which can be divided into two types depending on the amphiphilic lipid used: DOBAQ-containing liposomes and CHEMS-containing liposomes. Moreover, in some of the formulations DOPE was incorporated as an additional pH-sensitive component that displays fusogenic properties at a pH lower than the physiological pH. DOTAP was used as a cationic lipid to introduce a positive charge and DOPC was used as a bilayer-forming zwitterionic lipid. The chemical structures of the used lipids are depicted in Figure S1. Liposomes had to meet arbitrarily set physicochemical selection criteria for further investigations: liposomes should form stable suspensions without visible aggregation and precipitation, Z-average hydrodynamic diameter should be below 200 nm, polydispersity index (PDI) below 0.3, and Zeta-potential between approximately +20 and +30 mV.

The physicochemical characteristics of all prepared liposomes are presented in Table 1. DOPE is a lipid that does not form stable bilayers and needs to be stabilized with other lipids to form stable liposomes. None of the formulations with DOPE but without DOPC met the size criterium: DOPE:DOBAQ:DOTAP 3:2:2 and 3:2:1, and DOPE:CHEMS:DOTAP 3:2:2 and 3:2:1 (Table 1). Additionally, the Zeta-potential of DOPE:DOBAQ:DOTAP 3:2:1 was too low, probably because it contained too little DOTAP. DOPE:CHEMS:DOTAP 3:2:1, and DOPC:DOPE:CHEMS:DOTAP 7:3:2:2 did not meet the Zeta-potential criterium. Their negative Zeta-potential could be caused by the high content of CHEMS which is negatively charged at physiological pH. To circumvent stability issues, we increased the content of DOPC to stabilize liposomes, increased the DOTAP content, and/or decreased the CHEMS content to increase Zeta-potential. All of the selected formulations (that met the selection criteria) had comparable characteristics: hydrodynamic Z-average diameter between 140 and 180 nm, PDI between 0.13 and 0.30, and Zeta-potential between 19 and 26 mV.

Table 1. Physicochemical properties of the selected formulations. The results represent mean ± SD. The number of batches n ≥ 3. Failed batches (marked with *) were prepared once, and SD represents the deviation of 3 measurements.

Formulation	Z-average size (nm)	PDI (-)	Zeta-potential (mV)
DOBAQ:DOTAP (1:1)	144 ± 4	0.30 ± 0.02	22.0 ± 0.2
DOPE:DOBAQ:DOTAP (3:2:2)*	>1000 ± 290	0.26 ± 0.05	13.0 ± 0.7
DOPE:DOBAQ:DOTAP (3:2:1)*	>1000 ± 50	0.69 ± 0.20	6.8 ± 0.5
DOPC:DOPE:DOBAQ:DOTAP (7:3:2:2)	143 ± 5	0.13 ± 0.01	19.6 ± 0.4
CHEMS:DOTAP (1:1)	144 ± 3	0.15 ± 0.02	26.5 ± 0.7
DOPE:CHEMS:DOTAP (3:2:2)*	260 ± 19	0.11 ± 0.10	16.1 ± 1.3
DOPE:CHEMS:DOTAP (3:2:1)*	218 ± 6	0.21 ± 0.10	-23.2 ± 0.4
DOPC:DOPE:CHEMS:DOTAP (7:3:2:2)*	159 ± 2	0.12 ± 0.02	-9.6 ± 2.6
DOPC:DOPE:CHEMS:DOTAP (9:3:2:4)	138 ± 2	0.15 ± 0.03	19.2 ± 0.8
DOPC:DOPE:DOBAQ:DOTAP (3:5:2:4)	185 ± 3	0.15 ± 0.02	19.4 ± 0.6
DOPC:DOPE:CHEMS:DOTAP (5:5:2:6)	167 ± 3	0.13 ± 0.04	21.8 ± 1.2
DOPC:cholesterol:DOTAP (3:1:1)	104 ± 6	0.23 ± 0.01	22.5 ± 2.5

3.2. Evaluation of pH-sensitive properties of prepared formulations

The selected liposomes were evaluated in various biological assays to assess their pH-sensitive properties in primary human MDDCs. To assess the pH-sensitive behavior of these formulations, the liposomes were loaded with self-quenched ovalbumin (DQ-OVA) which is used here as a reporter of antigen processing. DQ-OVA in its native form is dimly fluorescent. However, upon degradation self-quenching of the fluorophore is diminished and DQ-OVA becomes brightly fluorescent. The change of this fluorescence can be quantified with flow cytometry. While classical liposomes and their cargo are degraded inside endosomes, pH-sensitive liposomes can protect themselves and therefore protect their cargo from degradation by escaping endosomes. As a control liposome formulation, we used DOPC:cholesterol:DOTAP 3:1:1. We selected this formulation because it is a non-pH-sensitive formulation that contains a comparable amount of DOTAP as the pH-sensitive liposomes. Also, we included cholesterol to account for possible effects of CHEMS used in some of the pH-sensitive liposomes that are associated with its cholesterol-like structure. The results of this assay are depicted in Figure 1a. Free DQ-OVA is freely processed

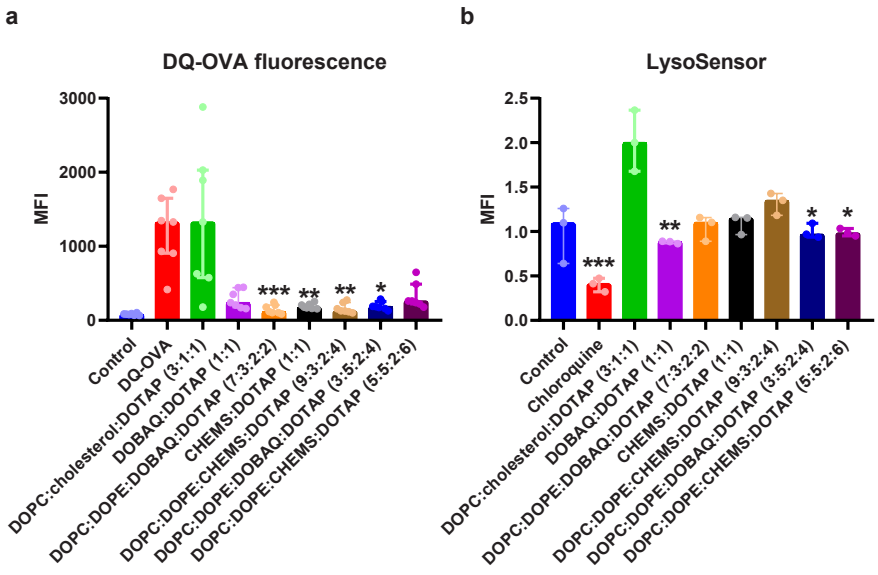

Figure 1. Evaluation of pH-sensitive properties of empty liposomal formulations in human monocyte-derived dendritic cells. (a) Endosomal protein degradation assessed using the DQ™-OVA reporter after 1-hour incubation at 5 µg/ml DQ-OVA (in solution or liposomes) and 250 µg/ml lipids (assuming no lipid loss). Lipid ratios are given as molar ratios. Median fluorescence intensities (MFIs) reflect DQ-OVA intensities, with comparisons made to DOPC:cholesterol:DOTAP (3:1:1). n=7 donors. (b) Cellular acidification measured by the LysoSensor assay. Cells were stimulated for 2.5 hours with liposomes, followed by a 30-minute incubation with LysoSensor the next day. MFIs, normalized to control for donor variability, indicate the reporter intensities. n=3 donors. All results are shown as median ± IQR. Statistical analysis was performed using the Kruskal-Wallis test with uncorrected Dunn's post-hoc test; significance was set at $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

by the MDDCs which is indicated by high fluorescence values. In comparison, the fluorescence of all the selected pH-sensitive liposomes is much lower and in the case of four pH-sensitive formulations, the decrease of fluorescence values was statistically significant in comparison to DOPC:cholesterol:DOTAP 3:1:1. The pH-sensitive properties were also studied with the LysoSensor assay (Figure 1b). LysoSensor assay indicates a decrease in pH inside acidic organelles like lysosomes by an increase in fluorescence intensity of the fluorescent assay reporter. Similarly, the increase in pH is indicated by the decrease in fluorescence. As mentioned, a property of pH-sensitive liposomes is their capability to interfere with the acidification of lysosomes. In this assay, all formulations displayed a pH-buffering effect, which is indicated by the fluorescence that is at the level of the medium control, whereas the control

formulation (DOPC:cholesterol:DOTAP 3:1:1) does not display these properties and this resulted in an increase of the signal. This corresponds to a decrease in pH inside the acidic organelles of MDDCs. To validate the performance of this assay, we used chloroquine which is known to cause an increase in pH within the cells.[42–44] As expected, we observed a significant reduction of the reporter signal in cells treated with chloroquine, which corresponds to increased pH with respect to the medium control. Thus, we demonstrated that the prepared formulations display important properties of pH-sensitive liposomes in the context of vaccination: protection of the antigen from degradation, and disruption of acidification inside endosomes. Non-pH-sensitive liposomes did not display such properties.

3.3 Assessment of uptake, expression of surface activation markers, and viability

The uptake of pH-sensitive formulations was assessed with fluorescently labeled liposomes using MDDCs, M1 (pro-inflammatory), and M2 (anti-inflammatory) macrophages (Figure 2a), which all are antigen-presenting cells (APCs). An increase in median fluorescence intensity is associated with higher uptake of a formulation. The results obtained with MDDCs and both types of macrophages were very comparable. Formulations DOBAQ:DOTAP (1:1), DOPC:DOPE:DOBAQ:DOTAP (7:3:2:2), and DOPC:DOPE:CHEMS:DOTAP (9:3:2:4) were poorly taken up by all tested APCs. Formulation CHEMS:DOTAP (1:1) was moderately taken up, whereas DOPC:DOPE:CHEMS:DOTAP (5:5:2:6) was taken up more by macrophages than MDDCs, although still less effectively than control cationic liposomal formulation (DOPC:chol:DOTAP 3:1:1). The most efficient uptake of pH-sensitive formulation was observed using DOPC:DOPE:DOBAQ:DOTAP (3:5:2:4). It showed the highest uptake in all APCs. All formulations were more taken up by M1 macrophages compared to the other APCs, as indicated by higher median fluorescence values.

Furthermore, the ability of the pH-sensitive liposomal formulations to activate MDDCs was also tested. Most of the tested pH-sensitive formulations were unable to induce measurable activation of MDDCs, as indicated by the lack of increase of activation marker expression in comparison to the medium-only control (Figure 2b, Supplementary Figure S2 and S4). Only DOBAQ:DOTAP (1:1) induced upregulation of activation markers that was significantly different from the negative control condition and as high as or higher than the positive control condition. All cell surface markers except CD80 were upregulated by the DOBAQ:DOTAP (1:1) formulation.

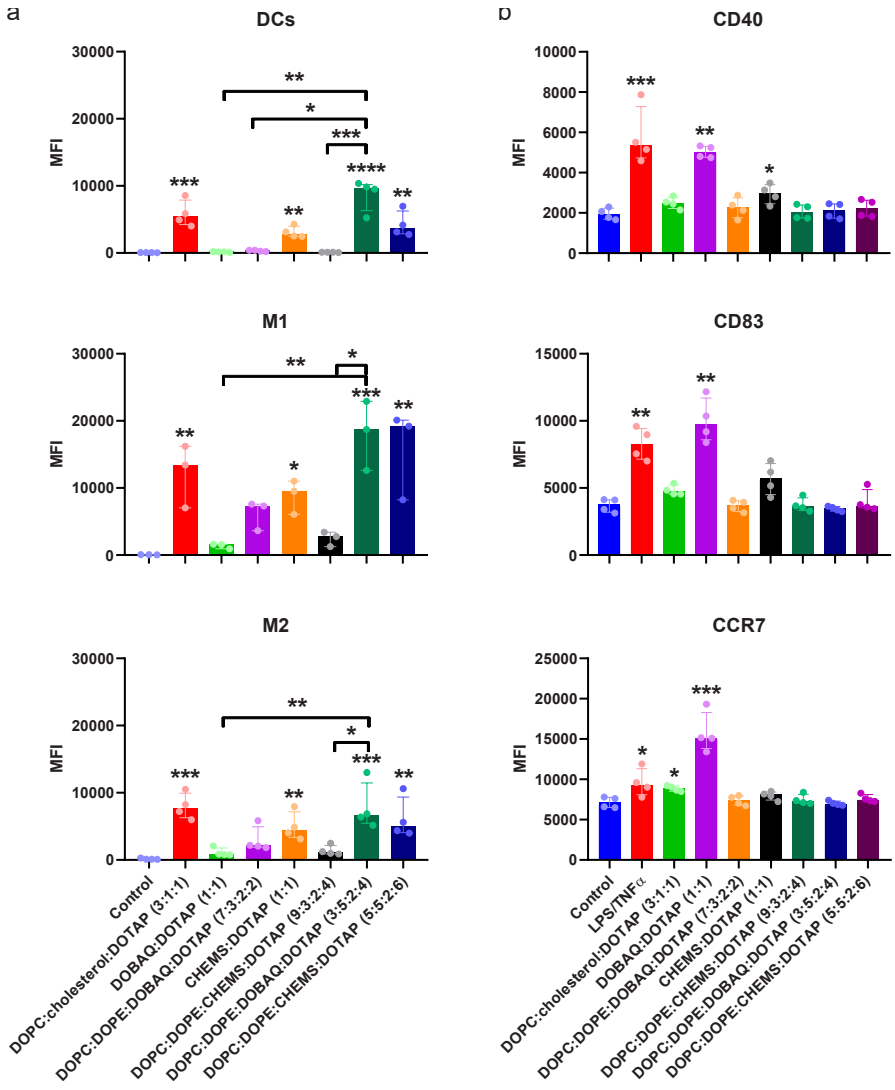

Figure 2. Influence of lipid composition on the uptake of empty liposomes and expression of cell surface activation markers in human monocyte-derived dendritic cells (DCs) and macrophages (M1 and M2). (a) Uptake of Cy5-labeled empty liposomes. Cells were incubated with 1 % v/v liposomes for 1 hour, followed by washing. Median fluorescence intensity (MFI) reflects Cy5 uptake in DCs (n=4), M1 macrophages (n=3), and M2 macrophages (n=4 donors). (b) Expression of activation markers CD40, CD83, and CCR7, shown as MFI. Cells were incubated with 250 µg/ml liposomes (assuming no lipid loss) for 1 hour (n=4 donors). Formulations were compared to a medium-only control unless otherwise specified. Results are presented as median ± interquartile range. Statistical analysis was conducted using the Kruskal-Wallis test with uncorrected Dunn's post-hoc test, with significance at $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

Additionally, the viability of MDDCs after exposure to the pH-sensitive liposomes was quantified (Supplementary Figure S3). In general, pH-sensitive liposomes did not affect the viability of MDDCs. The percentages of viable cells were consistently very similar to those of negative controls, which indicates very low cytotoxicity. Only DOBAQ:DOTAP (1:1) and CHEMS:DOTAP (1:1) formulations caused some decrease in viable cells that was statistically different from the control. However, the observed decrease in MDDCs viability was small. Production of IL-12p40 and IL-10 was quantified with ELISA assays. There was no difference in the cytokine concentrations in supernatants from activation experiments compared to the medium-only controls.

3.4 Optimization of pH-sensitive liposomes

To develop pH-sensitive formulations that are suitable for subunit vaccines, further optimization was necessary. An effective liposomal vaccine delivery system needs to be taken up efficiently and induce APC activation. None of the pH-sensitive formulations we developed met both of these criteria. To further optimize the liposomes we selected DOPC:DOPE:DOBAQ:DOTAP (3:5:2:4) as the starting point, because the formulation was most efficiently taken up in APCs, did not decrease the viability of MDDCs, and its pH-sensitive properties were excellent. The goal of this optimization was to improve adjuvanticity. For this reason, we replaced DOTAP with EPC (we knew from our unpublished work that it induces more activation in APCs), and we decided to incorporate cholesterol (which can improve the immunogenicity of liposomes without a need to change other components).[45–49] We prepared liposomes with 4 different compositions, including the originally selected one. We kept the ratio of DOPE to DOBAQ constant in all formulations, and we incorporated cholesterol at either 20 or 40 mol%. The composition of these liposomes and their physicochemical properties are presented in Table 3. We incorporated AER antigen

Table 3. Physicochemical properties of AER-containing optimized formulations. n≥3 (batches).

Formulation	Z-average size (nm)	PDI (-)	Zeta-potential (mV)
AER/DOPC:DOPE:DOBAQ:DOTAP (3:5:2:4)	160 ± 24	0.18 ± 0.06	20.8 ± 1.7
AER/DOPC:DOPE:DOBAQ:EPC (3:5:2:4)	125 ± 1	0.27 ± 0.01	19.3 ± 1.1
AER/DOPE:DOBAQ:cholesterol:EPC (10:4:11:3)	111 ± 1	0.25 ± 0.01	20.5 ± 0.3
AER/DOPE:DOBAQ:cholesterol:EPC (5:2:3:4)	124 ± 2	0.26 ± 0.01	15.9 ± 0.9

at this stage, so the batches could be directly used for T-cell activation assay. Subsequently, the selected pH-sensitive formulation and 3 new compositions were tested in MDDCs to evaluate if the ability to activate APCs was improved. The results are presented in Figure 3. All of the new formulations induced more activation in comparison to the original formulation but only AER/DOPC:DOPE:DOBAQ:EPC

(3:5:2:4) significantly outperformed the original formulation AER/DOPC:DOPE:DOBAQ:DOTAP (3:5:2:4). A statistically significant increase of expression of surface activation markers was observed for CD40, CD83, and CCR7. In the case of remaining markers, DOPC:DOPE:DOBAQ:EPC (3:5:2:4) induced upregulation of CD86 and HLA-DR that was significantly higher than the control but there was no statistical difference between the optimized and original formulations. CD80 was not upregulated by any of the formulations. Two optimized formulations that contained cholesterol (AER/DOPE:DOBAQ:cholesterol:EPC 10:4:11:3 and AER/DOPE:DOBAQ:cholesterol:EPC 5:2:3:4) induced less activation in comparison to the formulation that did not contain it (AER/DOPC:DOPE:DOBAQ:EPC 3:5:2:4).

The viability of the MDDCs after exposure to the formulations was quantified. All liposomes caused moderate cell death at higher concentrations (5 μg/ml AER, 250 μg/ml lipids) as indicated by viability percentage (Figure 4). On average, the newly developed formulations induced more cell death than the original formulation. Liposomes AER/DOPE:DOBAQ:cholesterol:EPC (10:4:11:3) and AER/DOPE:DOBAQ:cholesterol:EPC (5:2:3:4) tended to be more toxic than AER/DOPC:DOPE:DOBAQ:EPC (3:5:2:4) as the difference in viability observed at already 2 μg/ml AER.

In addition to the expression of activation markers and viability, we quantified cytokines and chemokines produced by MDDCs. Cytokines and chemokines induce immune responses in other cell types and allow the recruitment of certain immune cells. This can modify the induced immune responses and affect T-cell activation. We observed an increase in the production of several cytokines and chemokines (Figure 5). Formulation AER/DOPC:DOPE:DOBAQ:EPC (3:5:2:4) significantly upregulated production of 3 out of 16 cytokines measured:CCL3 (MIP1α), CCL4 (MIP1β), and TNFα. We also observed an increase in CCL2 (MCP1) and IL-1Ra production, but this was not statistically significant. The original formulation containing DOTAP, and two

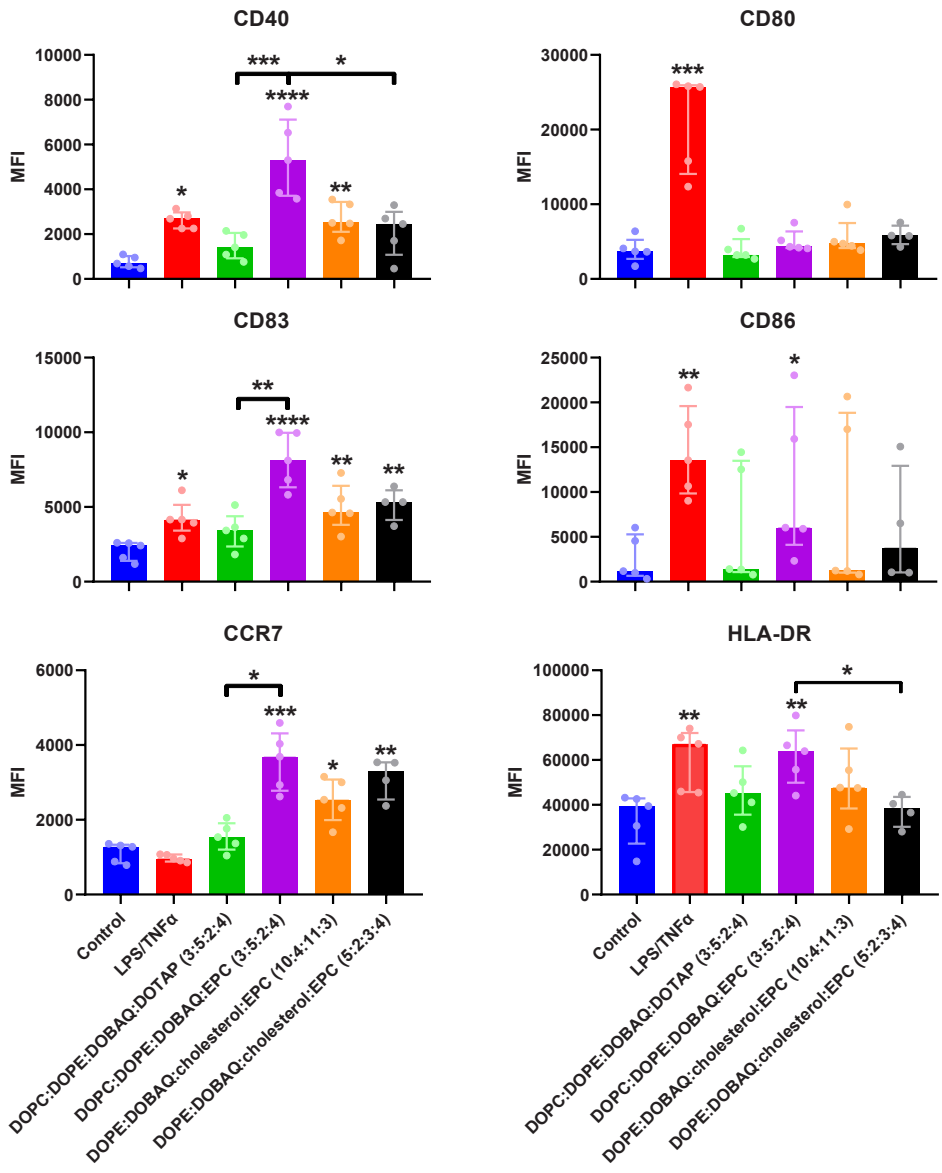

Figure 3. Upregulation of surface activation markers and viability of human monocyte-derived dendritic cells (DCs) following exposure to liposomal formulations loaded with Ag85B-ESAT6-RV2034 antigen for 1 hour. Cells were incubated with formulations containing 5 µg/ml antigen and 250 µg/ml lipids, with lipid ratios specified as molar ratios. Median fluorescence intensities (MFI) indicate expression of activation markers CD40, CD80, CD83, CD86, CCR7, and HLA-DR (n=5 donors). Unless otherwise indicated, formulations were compared to a medium-only control. Results are shown as median ± interquartile range. Statistical significance was determined using the Kruskal-Wallis test with uncorrected Dunn's post-hoc test, with thresholds set at $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

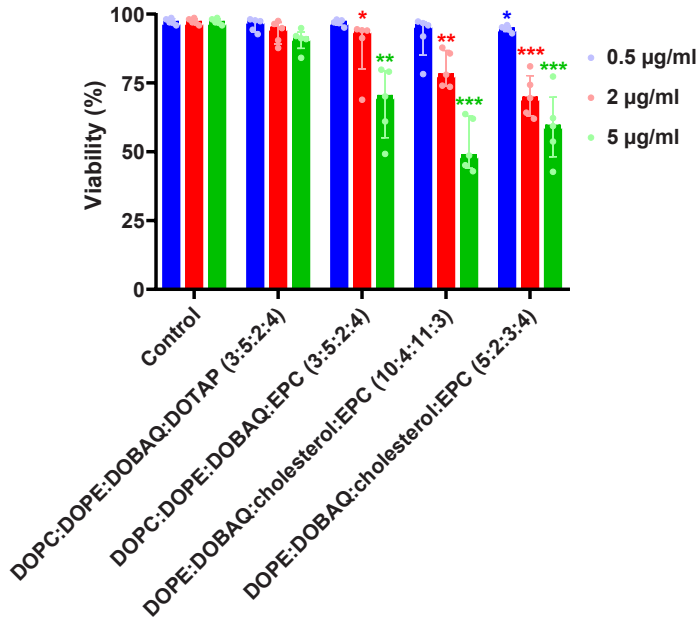

Figure 4. Viability of human monocyte-derived dendritic cells following 1-hour incubation with AER-loaded liposomal formulations. Viability was calculated as the percentage of SYTOX AADvanced-negative cells relative to the total cell population. Antigen concentrations are specified in the figure legend, with lipid concentrations at 50-fold higher than the antigen. Lipid ratios are provided as molar ratios, n=5 donors. Results are shown as median ± interquartile range. Statistical analysis was performed using the Kruskal-Wallis test with uncorrected Dunn's post-hoc test, with significance thresholds at $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). Each formulation was compared to a medium-only control within its respective concentration group.

other formulations containing cholesterol did not induce significant upregulations of any of the cytokines. There was also no significant increase in IL-12p40 and IL-10 production as assessed by ELISA assays.

Lastly, the performance of the optimized pH-sensitive liposomes was tested in a T-cell activation study. We selected for this experiment formulation AER/DOPC:DOPE:DOBAQ:EPC (3:5:2:4) because it induced superior upregulation of activation markers (Figure 3) and caused little cell death. To evaluate the ability to induce activation of antigen-specific $CD4^+$ T-cell clones, we compared liposomes containing the antigen with empty formulations (Figure 6). We used a $CD4^+$ T-cell clone *in vitro* assay to demonstrate the ability of delivery systems to specifically activate T-cells. Furthermore, $CD4^+$ T-cells are crucial to efficiently control the

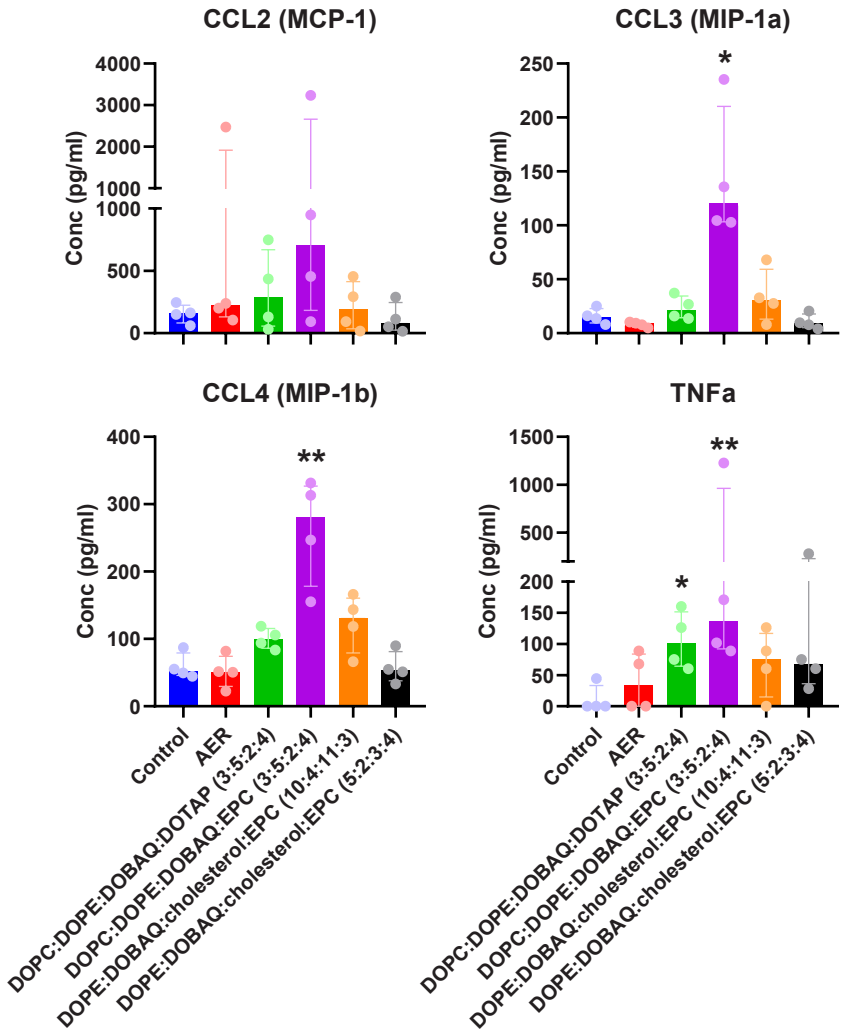

Figure 5. Cytokine production by human monocyte-derived dendritic cells after incubation with liposomal formulations. Cells were exposed to formulations containing 5 µg/ml antigen and 250 µg/ml liposomes for 1 hour (n=4 donors). Formulations were compared to a medium-only control. Results are presented as median ± interquartile range. Statistical significance was assessed using the Kruskal-Wallis test with uncorrected Dunn's post-hoc test, with thresholds at $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

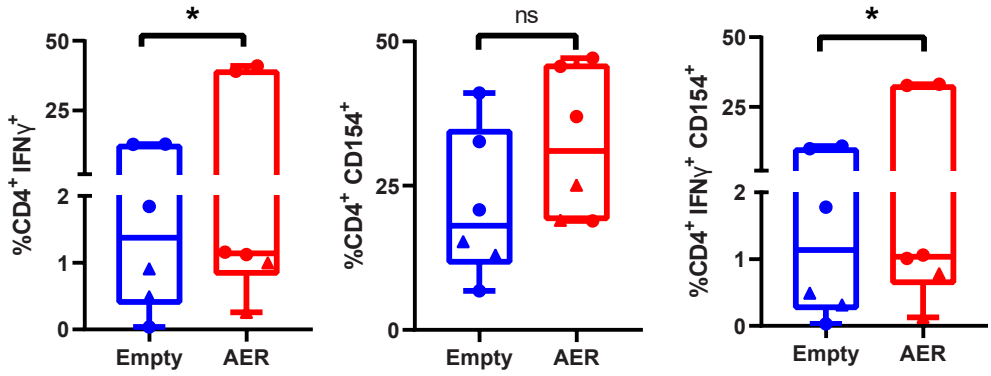

Figure 6. T-cell activation as indicated by the percentage of $CD4^+$ T-cells secreting IFNγ, expressing CD154, or co-expressing IFNγ and CD154 following exposure to empty (without AER antigen) or AER-loaded DOPC:DOPE:DOBAQ liposomes (molar ratio 3:5:2:4) at 5 µg/ml lipids for 1 hour. Circles represent $CD4^+$ T-cell clone L10B4 (specific for Mtb antigen Ag85B peptide 56-65), and triangles represent $CD4^+$ T-cell clone 1B4 (specific for Rv2034 peptide 75-105), with n=6 donors (HLA-$DR3^+$ heterozygous monocyte-derived dendritic cells). Graphs show median ± interquartile range. Statistical comparisons were made using the Wilcoxon matched-pairs signed rank test, with significance thresholds at $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

intracellular pathogen Mtb.[50–52] We observed that antigen-containing liposomes induced significantly higher activation of $CD4^+$ T-cells as indicated by the percentage of cells expressing intracellular IFNγ and $IFN\gamma^+CD154^+$ but not $CD154^+$ alone.

In conclusion, optimized liposome DOPC:DOPE:DOBAQ:EPC (3:5:2:4) overcame the limitations of the previous formulation. It induced significantly higher expression of cell surface activation markers CD40, CD83, and CCR7 compared to the original liposome. The viability of MDDCs after exposure to the liposome decreased when higher concentrations were used. Secretion of cytokines CCL3 (MIP1α), CCL4 (MIP1β), and TNFα was elevated compared to the medium control. HLA-DR3 MDDCs after exposure to DOPC:DOPE:DOBAQ:EPC (3:5:2:4) induced significantly higher activation of antigen-specific $CD4^+$ T-cell clones.

4. DISCUSSION

pH-sensitive liposomes are widely investigated in medical applications such as chemotherapy, immunotherapy, diagnostics, and intracellular drug delivery.[17,53,54] Their unique properties allow for pH-specific release of the delivered cargo, making them an attractive delivery system. In vaccine research, pH-sensitive liposomes are promising for cytosolic delivery and improving $CD8^+$ T-cell responses.

$CD8^+$ T-cells are essential for controlling long-term Mtb infection, both through direct bacterial control and by supporting $CD4^+$ T-cell responses. Inducing Mtb-specific $CD8^+$ T-cells could enhance protective immunity.[5,55,56] Evidence from adoptive transfer,[57] antibody depletion,[58–60] and knockout studies[61–63] demonstrated the necessity of $CD8^+$ T-cells in infection control. Their depletion increased bacterial replication during latency.[64] Additionally, Mtb-specific $CD8^+$ T cell lines can lyse infected macrophages and restrict Mtb growth.[65,66] In animal models, including macaques[67–72] and cattle,[73,74] $CD8^+$ T-cells were vital to Mtb immunity. In humans, $CD8^+$ T-cell function decreased in active TB, both systemically[75] and at infection sites,[76] with a higher antigen burden linked to dysfunction.[77–79] Distinct $CD8^+$ T-cell phenotypes between TB and LTBI subjects,[77,80] especially Mtb-specific $CCR7^-$ $CD45RA^+$ cells, were associated with Mtb control.[81] This evidence underscores $CD8^+$ T-cells as key players in host immunity against Mtb.

$CD8^+$ T-cell responses can be induced by APCs via several pathways including cross-presentation through MHC-class Ia, Ib, and II molecules. Cross-presentation occurs through the cytosolic processing of antigens by proteasomes.[82,83] Protein antigens are typically processed in the endosome compartment, which favors MHC class II antigen presentation that leads to the induction of $CD4^+$ T-cell responses.[31,32] pH-sensitive liposomes can facilitate class I presentation by pH-dependent endosomal escape and delivery of the antigen to the cytosol.

Existing literature on pH-sensitive liposomes shows a significant focus on their application in cancer chemotherapy, immunotherapy, and cancer vaccines, with limited research in the field of vaccines against infectious diseases.[11,17,23,84–86] In the field of TB, three studies are reported in literature where liposomes DOTAP:DOPE (1:1), and/or (egg) PC:DOTAP:DOPE (2:1:1) were used to deliver heat shock 65 plasmid DNA antigen for TB vaccination.[87–89] However, the pH-sensitive properties of these

formulations were not assessed in these studies, and these formulations were not presented as pH-sensitive. Probably these formulations exhibit pH-sensitive properties based on DOPE content.

Delivery of protein-based antigens by pH-sensitive liposomes for vaccination against infectious diseases has not been extensively studied to date. Hence, we wanted to explore this topic for TB vaccination. Several formulations of liposomes that should exhibit such properties due to the incorporation of amphiphilic (CHEMS and DOBAQ) and/or fusogenic (DOPE) lipids were prepared. All pre-selected formulations had similar physicochemical properties (Z-average size, PDI, and Zeta-potential), but varied in their immunological effect. It is unlikely that the observed differences *in vitro* can be attributable to those properties.

Efficient uptake and activation in APCs are crucial for the performance of vaccine delivery systems. We evaluated uptake and activation using MDDCs and macrophage models. DOPC:DOPE:DOBAQ:DOTAP (3:5:2:4) was the most efficiently taken up in MDDCs, M1, and M2 MDMFs, while other formulations were poorly taken up. The majority of the tested formulations failed to upregulate the markers except for DOBAQ:DOTAP (1:1), which induced upregulations in all markers except for CD80. This observation can be explained by the high content of positively charged DOTAP, which is known to cause the activation of DCs.[49,90–93] CHEMS:DOTAP (1:1) liposomal formulation was not efficient probably because CHEMS in contrast to DOBAQ is negatively charged at neutral pH. Therefore, the choice of amphiphilic lipids greatly affected the uptake and activation of MDDCs *in vitro*.

We chose the DOPC:DOPE:DOBAQ:DOTAP (3:5:2:4) formulation based on its efficient uptake and pH-sensitive properties, but it did not effectively activate MDDCs. To improve its immunological effect, we substituted DOTAP with EPC, which we found to be a more potent immune activator in our previous work.[39]

Re-evaluation of MDDCs activation using new formulations showed that DOPC:DOPE:DOBAQ:EPC (3:5:2:4) induced the strongest activation, upregulating all markers except CD80 significantly more than the original formulation. This supports our findings that EPC is a more potent immune activator than DOTAP.[39] Surprisingly, cholesterol-containing formulations were less effective in activating MDDCs, contrary to our previous findings, and other publications[45–49] which showed that cholesterol-containing liposomes show an increased uptake by APCs and subsequently such

liposomes are more effective in activating immune cells. However, in a study by Nakano et al., cholesterol was shown to negatively affect the adjuvanticity of liposomes containing various PE lipids, leading to reduced antigen-specific IgG and preventing IgE production.[94] This suggests that cholesterol in PE-containing liposomes may have a negative effect on APC activation, leading to diminished adaptive immune responses *in vivo*, which might not occur in PE-free liposomes.

Cytokines and chemokine production is crucial for the effective induction of immunity. DOPC:DOPE:DOBAQ:EPC (3:5:2:4) showed the most upregulation in the production of cytokines and chemokines, including CCL3, CCL4, CCL2, IL-1Ra, and TNFα. CCL3 is a chemokine that induces Th1 responses.[95,96] It is essential for the maturation, activation, and migration of DCs to draining lymph nodes. CCL3 depletion results in reduced IFNγ expression by antigen-specific T-cells as well as increased levels of IL-10.[97] Both CCL3 and CCL4 drive Th1 responses[98] by efficient chemoattraction of Th1 cells (but not Th2 cells) in a concentration-dependent manner.[99] They also attract recently activated T-cells[100] and $CD8^+$ T-cells.[101–104] It has been reported that TNFα influences maturation, and recruitment of DCs and activation of T-cells.[105] It has also been demonstrated that TNFα exhibits adjuvant-like properties against viral infections in various models.[106–108] The microenvironment created by the expression of these cytokines can be beneficial for driving protection against Mtb.[109]

MDDCs exposed to AER-containing DOPC:DOPE:DOBAQ:EPC (3:5:2:4) were able to significantly activate two T-cell lines, indicated by increased expression of $IFN\gamma^+$ and $CD154^+$ $IFN\gamma^+$ but not $CD154^+$ alone T-cells whereas the MDDCs exposed to the empty liposome did not induce IFNγ-producing $CD4^+$ $CD154^+$ T-cells. Thus pH-sensitive liposomes and specifically DOPC:DOPE:DOBAQ:EPC (3:5:2:4) formulation are capable of inducing maturation of human MDDCs and enabling these DCs to take up, process, and present two different epitopes to antigen-specific T-cells and induce their activation.

In studies using C57Bl/6 mice, we demonstrated that a DOPC:DOPE:DOBAQ (3:5:2:4)-based TB vaccine, administered subcutaneously with CpG oligonucleotide ODN1826 and monophosphoryl lipid A as molecular adjuvants and AER as the antigen, effectively protected mice against intranasal H37Rv Mtb infection. This was supported by reduced bacterial burdens in the spleen and lungs of infected mice.[110,111] The vaccine elicited robust immune responses, including polyfunctional $CD4^+$

and $CD8^+$ T-cell activation, which are crucial for Mtb control as discussed above. Additionally, after restimulation of splenocytes with AER, we observed activation of B-cell populations, marked by CD69 expression, along with high antigen-specific antibody titers. These findings underscore the vaccine's effectiveness and suggest its potential as a novel TB vaccine. Overall, this study provides valuable insights into the use of pH-sensitive liposomes as carriers for subunit vaccines against TB.

5. CONCLUSIONS

pH-sensitive liposomes are promising for vaccines, as they deliver antigens into the cytosol, bypassing endosomal degradation, and enhance cross-presentation—key for fighting intracellular pathogens like Mtb. This study explores the application of pH-sensitive liposomes for vaccination against TB. We employed a strategy of optimizing liposomal compositions formulated with the Mtb-derived antigen AER using human primary cells for pre-clinical research. We demonstrated the pH-sensitive properties of these formulations and evaluated their immunostimulatory capacities on human cells, including DCs, M1, and M2. The best-performing formulation was DOPC:DOPE:DOBAQ:EPC in a 3:5:2:4 molar ratio. Further research is needed to evaluate the efficacy of this formulation *in vivo* and to incorporate molecular adjuvants to optimize cytokine production induced by these liposomes.

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; CHEMS, cholesteryl hemisuccinate, CXCL, chemokine (C-X-C motif) ligand; DC, dendritic cell; DOBAQ, N-(4-carboxybenzyl)-N,N-dimethyl-2,3-bis(oleoyloxy)propan-1-aminium; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; EPC, 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine; FBS, fetal bovine serum; 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; LAL, limulus amebocyte lysate; MDDC, monocyte-derived dendritic cell; MHC, major histocompatibility complex; Mtb, *Mycobacterium tuberculosis*; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cell; PCR,

polymerase chain reaction; PDI, polydispersity index; PE, phosphatidylethanolamine; PE-Cy5, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(Cyanine 5); PPD, protein purified derivative; TB, tuberculosis; Th1, type 1 helper T-cell; TLR, Toll-like receptor; TNF, tumor necrosis factor.

APPENDICES

Supplementary materials

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CREDIT AUTHOR STATEMENT

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REFERENCES

1. World Health Organization. *Global Tuberculosis Report 2023*. (2023).
2. Fine, P. E. M. Variation in protection by BCG: implications of and for heterologous immunity. *The Lancet* 346, 1339–1345 (1995).
3. Rodrigues, L. C. *et al.* Protective effect of BCG against tuberculous meningitis and miliary tuberculosis: A meta-analysis. *International Journal of Epidemiology* 22, 1154–1158 (1993).
4. Trunz, B. B. *et al.* Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. *Lancet* 367, 1173–1180 (2006).
5. Ottenhoff, T. H. M. & Kaufmann, S. H. E. Vaccines against Tuberculosis: Where Are We and Where Do We Need to Go? *PLoS Pathogens* 8, e1002607 (2012).
6. Inglut, C. T. *et al.* Immunological and Toxicological Considerations for the Design of Liposomes. *Nanomaterials* 10.2: 190 (2020).
7. Akbarzadeh, A. *et al.* Liposome: Classification, preparation, and applications. *Nanoscale Research Letters* 8, 1–9 (2013).
8. Torchilin, V. P. Recent advances with liposomes as pharmaceutical carriers. *Nature Reviews Drug Discovery 2005 4:2* 4, 145–160 (2005).
9. Shah, S. *et al.* Liposomes: Advancements and innovation in the manufacturing process. *Advanced Drug Delivery Reviews* 154–155, 102–122 (2020).
10. Bozzuto, G. & Molinari, A. Liposomes as nanomedical devices. *International Journal of Nanomedicine* 10, 975 (2015).
11. Karanth, H. & Murthy, R. S. R. pH-Sensitive liposomes-principle and application in cancer therapy. *Journal of Pharmacy and Pharmacology* 59, 469–483 (2007).
12. Momekova, D. *et al.* Long-Circulating, pH-Sensitive Liposomes. *Methods in Molecular Biology* 1522, 209–226 (2017).
13. Zhuo, S. *et al.* pH-Sensitive Biomaterials for Drug Delivery. *Molecules* 25, (2020).
14. Mu, Y. *et al.* Advances in pH-responsive drug delivery systems. *OpenNano* 5, 100031 (2021).
15. Balamurali, V. *et al.* pH Sensitive Drug Delivery Systems: A Review. *American Journal of Drug Discovery and Development* 1, 24–48 (2010).

16. Hamai, C. *et al.* Effect of Average Phospholipid Curvature on Supported Bilayer Formation on Glass by Vesicle Fusion. *Biophysical Journal* 90, 1241–1248 (2006).
17. Paliwal, S. R. *et al.* A review of mechanistic insight and application of pH-sensitive liposomes in drug delivery. *Drug delivery* 22:3, 231-242 (2015).
18. Connor, J. *et al.* pH-sensitive liposomes: acid-induced liposome fusion. *Proceedings of the National Academy of Sciences* 81, 1715–1718 (1984).
19. Siegel, D. P. & Epand, R. M. The mechanism of lamellar-to-inverted hexagonal phase transitions in phosphatidylethanolamine: implications for membrane fusion mechanisms. *Biophysical Journal* 73, 3089 (1997).
20. Lai, M. Z. *et al.* Effects of replacement of the hydroxyl group of cholesterol and tocopherol on the thermotropic behavior of phospholipid membranes. *Biochemistry* 24, 1646–1653 (1985).
21. Seddon, J. M. *et al.* Calorimetric studies of the gel-fluid (L beta-L alpha) and lamellar-inverted hexagonal (L alpha-HII) phase transitions in dialkyl- and diacylphosphatidylethanolamines. *Biochemistry* 22, 1280–1289 (1983).
22. Torchilin, V. P. *et al.* pH-Sensitive Liposomes. *Journal of Liposome Research* 3:2, 201-255 (1993).
23. Liu, X. & Huang, G. Formation strategies, mechanism of intracellular delivery and potential clinical applications of pH-sensitive liposomes. *Asian Journal of Pharmaceutical Sciences* 8, 319–328 (2013).
24. Moitra, P. *et al.* New pH-responsive gemini lipid derived co-liposomes for efficacious doxorubicin delivery to drug resistant cancer cells. *Chemical Communications* 53, 8184–8187 (2017).
25. Balazs, D. A. & Godbey, WT. Liposomes for use in gene delivery. *Journal of Drug Delivery* 2011, 1–12 (2011).
26. Yang, S. & May, S. Release of cationic polymer-DNA complexes from the endosome: A theoretical investigation of the proton sponge hypothesis. *Journal of Chemical Physics* 129, 185105 (2008).
27. Agirre, M. *et al.* Low Molecular Weight Chitosan (LMWC)-based Polyplexes for pDNA Delivery: From Bench to Bedside. *Polymers* 6:6, 1727-1755 (2014).
28. Dehshahri, A. *et al.* Gene transfer efficiency of high primary amine content, hydrophobic, alkyl-oligoamine derivatives of polyethylenimine. *Biomaterials* 30, 4187–4194 (2009).

29. Shigeta, K. *et al.* Novel histidine-conjugated galactosylated cationic liposomes for efficient hepatocyte-selective gene transfer in human hepatoma HepG2 cells. *Journal of Controlled Release* 118, 262–270 (2007).
30. Chang, J. S. *et al.* Development of Th1-mediated CD8+ effector T cells by vaccination with epitope peptides encapsulated in pH-sensitive liposomes. *Vaccine* 19, 3608–3614 (2001).
31. Fehres, C. M. *et al.* Understanding the biology of antigen cross-presentation for the design of vaccines against cancer. *Frontiers in Immunology* 5, 149 (2014).
32. Andersen, B. M. & Ohlfest, J. R. Increasing the efficacy of tumor cell vaccines by enhancing cross priming. *Cancer Letters* 325, 155–164 (2012).
33. Bungener, L. *et al.* Virosome-mediated delivery of protein antigens to dendritic cells. *Vaccine* 20, 2287–2295 (2002).
34. Bungener, L. *et al.* Virosome-mediated delivery of protein antigens in vivo: efficient induction of class I MHC-restricted cytotoxic T lymphocyte activity. *Vaccine* 23, 1232–1241 (2005).
35. Melero, I. *et al.* Therapeutic vaccines for cancer: an overview of clinical trials. *Nature Reviews Clinical Oncology* 11:9, 509–524 (2014).
36. Wang, C. *et al.* Self-adjuvanted nanovaccine for cancer immunotherapy: Role of lysosomal rupture-induced ROS in MHC class I antigen presentation. *Biomaterials* 79, 88–100 (2016).
37. Commandeur, S. *et al.* The in vivo expressed Mycobacterium tuberculosis (IVE-TB) antigen Rv2034 induces CD4+ T-cells that protect against pulmonary infection in HLA-DR transgenic mice and guinea pigs. *Vaccine* 32, 3580–3588 (2014).
38. Franken, K. L. M. C. *et al.* Purification of His-Tagged Proteins by Immobilized Chelate Affinity Chromatography: The Benefits from the Use of Organic Solvent. *Protein Expression and Purification* 18, 95–99 (2000).
39. Szachniewicz, M. M. *et al.* Intrinsic immunogenicity of liposomes for tuberculosis vaccines: Effect of cationic lipid and cholesterol. *European Journal of Pharmaceutical Sciences* 195, 106730 (2024).
40. Commandeur, S. *et al.* Clonal Analysis of the T-Cell Response to In Vivo Expressed Mycobacterium tuberculosis Protein Rv2034, Using a CD154 Expression Based T-Cell Cloning Method. *PLoS One* 9, e99203 (2014).

41. Geluk, A. *et al.* A DR17-restricted T cell epitope from a secreted Mycobacterium tuberculosis antigen only binds to DR17 molecules at neutral pH. *European Journal of Immunology* 27, 842–847 (1997).
42. Pascolo, S. Time to use a dose of Chloroquine as an adjuvant to anti-cancer chemotherapies. *European Journal of Pharmacology* 771, 139–144 (2016).
43. Savarino, A. *et al.* Effects of chloroquine on viral infections: an old drug against today's diseases. *Lancet Infectious Diseases* 3, 722–727 (2003).
44. Zou, L. *et al.* Hydroxychloroquine and chloroquine: a potential and controversial treatment for COVID-19. *Archives of Pharmacological Research* 43, 765–772 (2020).
45. Barnier-Quer, C. *et al.* Adjuvant effect of cationic liposomes for subunit influenza vaccine: Influence of antigen loading method, cholesterol and immune modulators. *Pharmaceutics* 5, 392–410 (2013).
46. Benne, N. *et al.* Atomic force microscopy measurements of anionic liposomes reveal the effect of liposomal rigidity on antigen-specific regulatory T cell responses. *Journal of Controlled Release* 318, 246–255 (2020).
47. Aramaki, K. *et al.* Charge boosting effect of cholesterol on cationic liposomes. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 506, 732–738 (2016).
48. Kaur, R. *et al.* Effect of incorporating cholesterol into DDA:TDB liposomal adjuvants on bilayer properties, biodistribution, and immune responses. *Molecular Pharmaceutics* 11, 197–207 (2014).
49. Henriksen-Lacey, M. *et al.* Comparison of the depot effect and immunogenicity of liposomes based on dimethyldioctadecylammonium (DDA), 3β-[N-(N',N'-dimethylaminoethane)carbomyl] cholesterol (DC-Chol), and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP): Prolonged liposome retention mediates stronger Th1 responses. *Molecular Pharmaceutics* 8, 153–161 (2011).
50. Leveton, C. *et al.* T-cell-mediated protection of mice against virulent Mycobacterium tuberculosis. *Infection and Immunity* 57, 390–395 (1989).
51. Flory, C. M. *et al.* Effects of in vivo T lymphocyte subset depletion on mycobacterial infections in mice. *Journal of Leukocyte Biology* 51, 225–229 (1992).
52. Müller, I. *et al.* Impaired resistance to Mycobacterium tuberculosis infection after selective in vivo depletion of L3T4+ and Lyt-2+ T cells. *Infection and Immunity* 55, 2037–2041 (1987).

53. Slepushkin, V. A. *et al.* Sterically stabilized pH-sensitive liposomes. Intracellular delivery of aqueous contents and prolonged circulation in vivo. *Journal of Biological Chemistry* 272, 2382–2388 (1997).
54. Zamani, P. *et al.* MPL nano-liposomal vaccine containing P5 HER2/neu-derived peptide pulsed PADRE as an effective vaccine in a mice TUBO model of breast cancer. *Journal of Controlled Release* 303, 223–236 (2019).
55. Boom, W. H. New TB vaccines: is there a requirement for CD8+ T cells? *Journal of Clinical Investigation* 117, 2092–2094 (2007).
56. Behar, S. M. *et al.* Next generation: tuberculosis vaccines that elicit protective CD8+ T cells. *Expert Review of Vaccines* 6, 441–456 (2007).
57. Orme, I. M. The kinetics of emergence and loss of mediator T lymphocytes acquired in response to infection with Mycobacterium tuberculosis. *The Journal of Immunology* 138, 293–298 (1987).
58. Mogues, T. *et al.* The Relative Importance of T Cell Subsets in Immunity and Immunopathology of Airborne Mycobacterium tuberculosis Infection in Mice. *Journal of Experimental Medicine* 193, 271–280 (2001).
59. North, R. J. & Jung, Y. J. Immunity to Tuberculosis. *Annual Review of Immunology* 22:1, 599–623 (2004).
60. Woodworth, J. S. *et al.* Mycobacterium tuberculosis-Specific CD8+ T Cells Require Perforin to Kill Target Cells and Provide Protection In Vivo. *The Journal of Immunology* 181, 8595–8603 (2008).
61. Behar, S. M. *et al.* Susceptibility of Mice Deficient in CD1D or TAP1 to Infection with Mycobacterium tuberculosis. *Journal of Experimental Medicine* 189, 1973–1980 (1999).
62. Flynn, J. L. *et al.* Major histocompatibility complex class I-restricted T cells are required for resistance to Mycobacterium tuberculosis infection. *Proceedings of the National Academy of Sciences* 89, 12013–12017 (1992).
63. Sousa, A. O. *et al.* Relative contributions of distinct MHC class I-dependent cell populations in protection to tuberculosis infection in mice. *Proceedings of the National Academy of Sciences* 97, 4204–4208 (2000).
64. Van Pinxteren, L. A. H. *et al.* Control of latent Mycobacterium tuberculosis infection is dependent on CD8 T cells. *European Journal of Immunology* 30, 3689–3698 (2000).

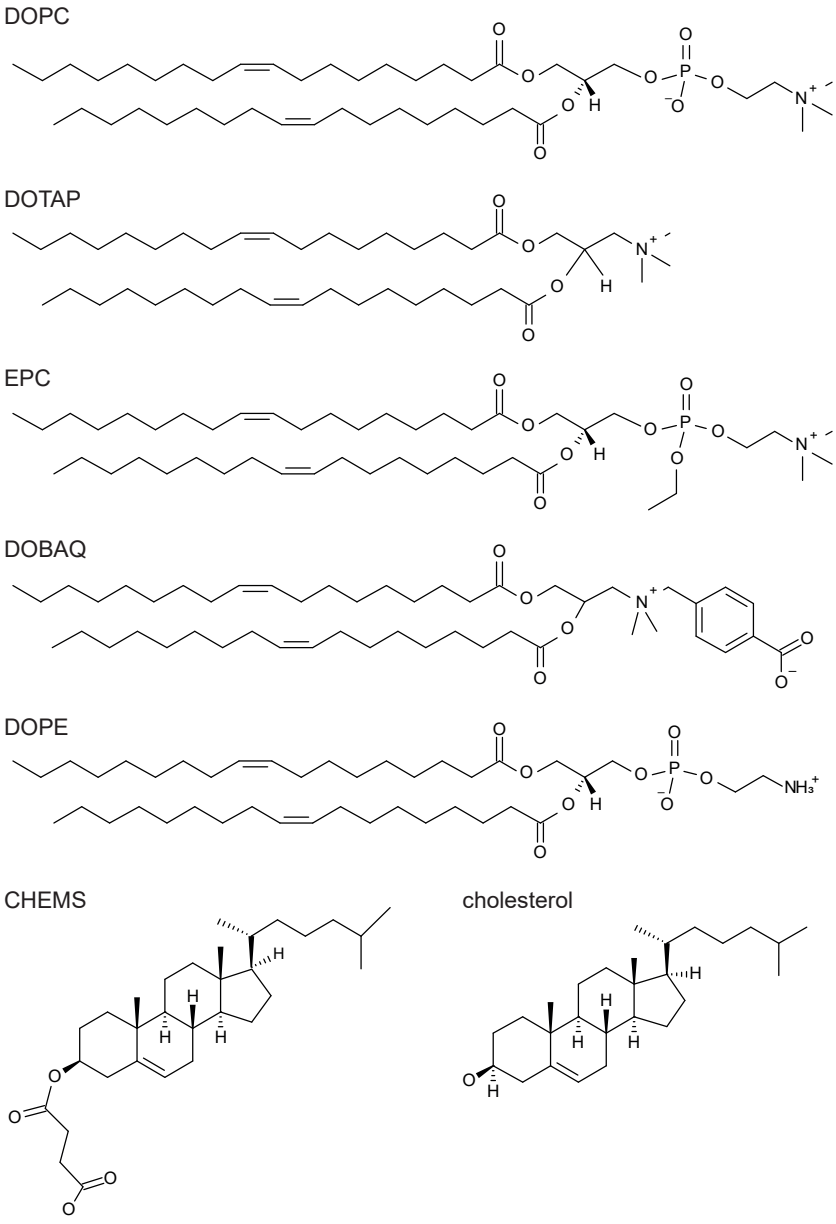
65. Tan, J. S. *et al.* Human alveolar T lymphocyte responses to Mycobacterium tuberculosis antigens: role for CD4+ and CD8+ cytotoxic T cells and relative resistance of alveolar macrophages to lysis. *The Journal of Immunology* 159, 290–297 (1997).
66. Turner, J. & Dockrell, H. M. Stimulation of human peripheral blood mononuclear cells with live Mycobacterium bovis BCG activates cytolytic CD8+ T cells in vitro. *Immunology* 87, 339–342 (1996).
67. Lin, P. L. & Flynn, J. A. L. CD8 T cells and Mycobacterium tuberculosis infection. *Seminars in Immunopathology* 37, 239–249 (2015).
68. Silver, R. F. *et al.* Diversity of human and macaque airway immune cells at baseline and during tuberculosis infection. *American Journal of Respiratory Cell and Molecular Biology* 55, 899–906 (2016).
69. Gideon, H. P. *et al.* Variability in Tuberculosis Granuloma T Cell Responses Exists, but a Balance of Pro- and Anti-inflammatory Cytokines Is Associated with Sterilization. *PLoS Pathogens* 11, e1004603 (2015).
70. Lin, P. L. *et al.* CD4 T Cell Depletion Exacerbates Acute Mycobacterium tuberculosis While Reactivation of Latent Infection Is Dependent on Severity of Tissue Depletion in Cynomolgus Macaques. *AIDS Research and Human Retroviruses* 28, 1693–1702 (2012).
71. Lin, P. L. *et al.* Early events in Mycobacterium tuberculosis infection in cynomolgus macaques. *Infection and Immunity* 74, 3790–3803 (2006).
72. Lin, P. L. *et al.* Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model. *Infection and Immunity* 77, 4631–4642 (2009).
73. Villarreal-Ramos, B. *et al.* Investigation of the role of CD8+ T cells in bovine tuberculosis in vivo. *Infection and Immunity* 71, 4297–4303 (2003).
74. Palmer, M. V. *et al.* Lesion development and immunohistochemical changes in granulomas from cattle experimentally infected with Mycobacterium bovis. *Veterinary Pathology* 44, 863–874 (2007).
75. Smith, S. M. *et al.* Human CD8+ T cells specific for Mycobacterium tuberculosis secreted antigens in tuberculosis patients and healthy BCG-vaccinated controls in the Gambia. *Infection and Immunity* 68, 7144–7148 (2000).
76. Andersson, J. *et al.* Impaired expression of perforin and granulysin in CD8+ T cells at the site of infection in human chronic pulmonary tuberculosis. *Infection and Immunity* 75, 5210–5222 (2007).

77. Day, C. L. *et al.* Functional Capacity of Mycobacterium tuberculosis-Specific T Cell Responses in Humans Is Associated with Mycobacterial Load. *The Journal of Immunology* 187, 2222–2232 (2011).
78. Lancioni, C. *et al.* CD8+ T Cells Provide an Immunologic Signature of Tuberculosis in Young Children. *American Journal of Respiratory and Critical Care Medicine* 185:2, 206-212 (2012).
79. Lewinsohn, D. A. *et al.* Mycobacterium tuberculosis–specific CD8+ T Cells Preferentially Recognize Heavily Infected Cells. *American Journal of Respiratory and Critical Care Medicine* 168:11, 1346–1352 (2012).
80. Rozot, V. *et al.* Mycobacterium tuberculosis-specific CD8+ T cells are functionally and phenotypically different between latent infection and active disease. *European Journal of Immunology* 43, 1568–1577 (2013).
81. Bruns, H. *et al.* Anti-TNF immunotherapy reduces CD8+ T cell–mediated antimicrobial activity against Mycobacterium tuberculosis in humans. *Journal of Clinical Investigation* 119, 1167–1177 (2009).
82. Winau, F. *et al.* Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. *Immunity* 24, 105–117 (2006).
83. Lin, M. & Ottenhoff, T. Host-Pathogen Interactions in Latent Mycobacterium tuberculosis Infection: Identification of New Targets for Tuberculosis Intervention. *Endocrine, Metabolic & Immune Disorders-Drug Targets* 8, 15–29 (2008).
84. Abri Aghdam, M. *et al.* Recent advances on thermosensitive and pH-sensitive liposomes employed in controlled release. *Journal of Controlled Release* 315, 1–22 (2019).
85. Ferreira, D. D. S. *et al.* pH-sensitive liposomes for drug delivery in cancer treatment. *Therapeutic Delivery* 4, 1099–1123 (2013).
86. Gupta, M, *et al.* pH-sensitive liposomes. *Liposomal Delivery Systems: Advances and Challenges* 1, 74–86 (2015).
87. Gaziola De La Torre, L. *et al.* The synergy between structural stability and DNA-binding controls the antibody production in EPC/DOTAP/DOPE liposomes and DOTAP/DOPE lipoplexes. *Colloids and Surfaces B: Biointerfaces* 73, 175–184 (2009).
88. Rosada, R. S. *et al.* Protection against tuberculosis by a single intranasal administration of DNA-hsp65 vaccine complexed with cationic liposomes. *BMC Immunology* 9, 1–13 (2008).

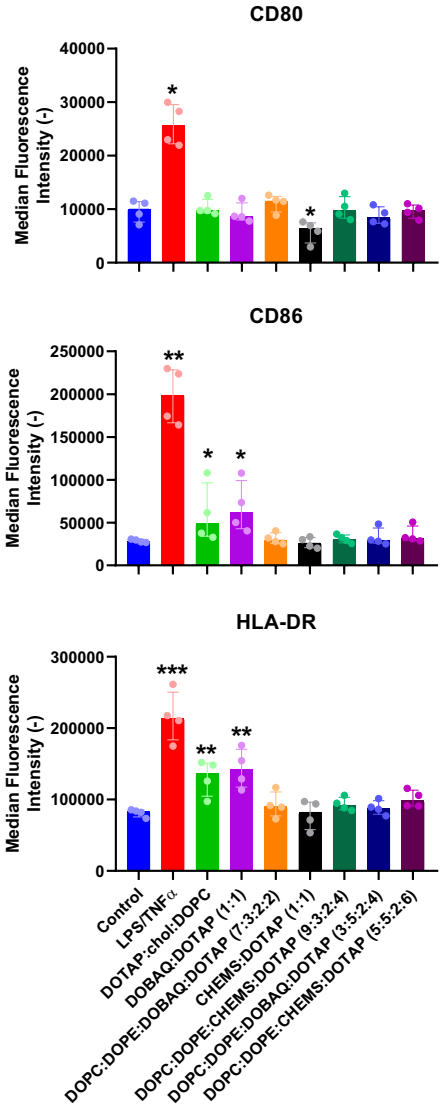
89. De Paula Rigoletto, T. *et al.* Effects of extrusion, lipid concentration and purity on physico-chemical and biological properties of cationic liposomes for gene vaccine applications. *Journal of microencapsulation* 29:8, 759–769 (2012).
90. Vasievich, E. A. *et al.* Trp2 peptide vaccine adjuvanted with (R)-DOTAP inhibits tumor growth in an advanced melanoma model. *Molecular Pharmaceutics* 9, 261–268 (2012).
91. Riehl, M. *et al.* Combining R-DOTAP and a particulate antigen delivery platform to trigger dendritic cell activation: Formulation development and in-vitro interaction studies. *International Journal of Pharmaceutics* 532, 37–46 (2017).
92. Yan, W. *et al.* Mechanism of adjuvant activity of cationic liposome: Phosphorylation of a MAP kinase, ERK and induction of chemokines. *Molecular Immunology* 44, 3672–3681 (2007).
93. Vangasseri, D. P. *et al.* Immunostimulation of dendritic cells by cationic liposomes. *Molecular Membrane Biology* 23, 385–395 (2006).
94. Nakano, Y. *et al.* Cholesterol Inclusion in Liposomes Affects Induction of Antigen-Specific IgG and IgE Antibody Production in Mice by a Surface-Linked Liposomal Antigen. *Bioconjugate Chemistry* 13, 744–749 (2002).
95. Annunziato, F. *et al.* Limited expression of R5-tropic HIV-1 in CCR5-positive type 1–polarized T cells explained by their ability to produce RANTES, MIP-1α, and MIP-1β. *Blood* 95, 1167–1174 (2000).
96. Canque, B. *et al.* Macrophage inflammatory protein-1alpha is induced by human immunodeficiency virus infection of monocyte-derived macrophages. *Blood* 87, 2011–2019 (1996).
97. Trifilo, M. J. & Lane, T. E. The CC chemokine ligand 3 regulates CD11c+CD11b+CD8α− dendritic cell maturation and activation following viral infection of the central nervous system: implications for a role in T cell activation. *Virology* 327, 8–15 (2004).
98. Nath, A. *et al.* Macrophage inflammatory protein (MIP)1α and MIP1β differentially regulate release of inflammatory cytokines and generation of tumoricidal monocytes in malignancy. *Cancer Immunology, Immunotherapy* 55, 1534–1541 (2006).
99. Siveke, J. T. & Hamann, A. Cutting edge: T helper 1 and T helper 2 cells respond differentially to chemokines. *The Journal of Immunology* 160:2, 550-554 (1998).

100. Sallusto, F. *et al.* Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. *Immunol Today* 19, 568–574 (1998).
101. Honey, K. CCL3 and CCL4 actively recruit CD8+ T cells. *Nature Reviews Immunology* 6, 427 (2006).
102. Castellino, F. *et al.* Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. *Nature* 440, 890–895 (2006).
103. Wang, A. *et al.* CCL3 and CCL4 secretion by T regulatory cells attracts CD4+ and CD8+ T cells (P1077). *The Journal of Immunology* 190, 121.10-121.10 (2013).
104. Sektioglu, I. M. *et al.* Basophils promote tumor rejection via chemotaxis and infiltration of CD8+ T cells. *Cancer Research*`77, 291–302 (2017).
105. Calzascia, T. *et al.* TNF-α is critical for antitumor but not antiviral T cell immunity in mice. *Journal of Clinical Investigation* 117, 3833–3845 (2007).
106. Brunner, C. *et al.* Enhanced Dendritic Cell Maturation by TNF-α or Cytidine-Phosphate-Guanosine DNA Drives T Cell Activation In Vitro and Therapeutic Anti-Tumor Immune Responses In Vivo. *The Journal of Immunology* 165, 6278–6286 (2000).
107. Chen, Z. *et al.* Enhanced HER-2/neu-specific antitumor immunity by cotransduction of mouse dendritic cells with two genes encoding HER-2/neu and alpha tumor necrosis factor. *Cancer Gene Therapy* 9:9, 778–786 (2002).
108. Nimal, S. *et al.* Enhancement of immune responses to an HIV gp120 DNA vaccine by fusion to TNF alpha cDNA. *Vaccine* 24, 3298–3308 (2006).
109. Salgame, P. Host innate and Th1 responses and the bacterial factors that control Mycobacterium tuberculosis infection. *Current Opinion in Immunology* 17, 374–380 (2005).
110. Szachniewicz, M. M. *et al.* Evaluation of PLGA, lipid-PLGA hybrid nanoparticles, and cationic pH-sensitive liposomes as tuberculosis vaccine delivery systems in a Mycobacterium tuberculosis challenge mouse model – A comparison. *International Journal of Pharmaceutics* 666, 124842 (2024).
111. Szachniewicz, M. M. *et al.* Cationic pH-sensitive liposome-based subunit tuberculosis vaccine induces protection in mice challenged with Mycobacterium tuberculosis. *European Journal of Pharmaceutics and Biopharmaceutics* 203, 114437 (2024).

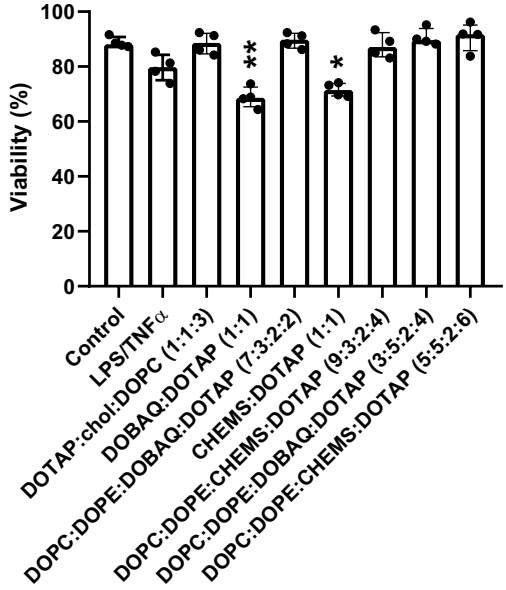
SUPPLEMENTARY MATERIALS



Supplementary Figure S1. Chemical structures of lipids used in production of pH-sensitive liposomes.

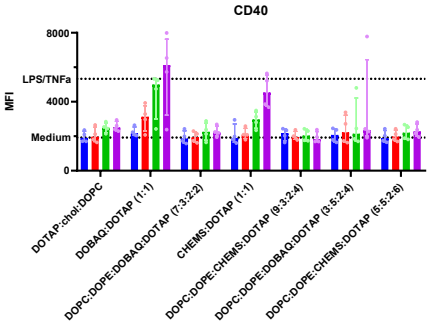


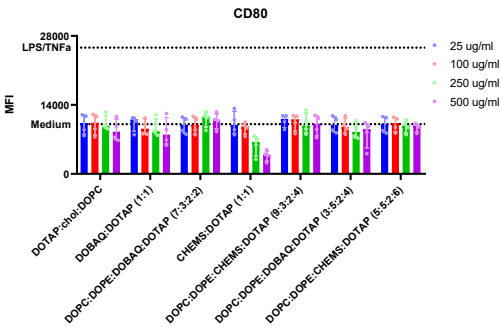
Supplementary Figure S2. Upregulation of surface activation markers and viability of human monocyte-derived dendritic cells (DCs) following exposure to liposomal empty formulations for 1 hour. Cells were incubated with formulations 250 µg/ml lipids, with lipid ratios specified as molar ratios. Median fluorescence intensities (MFI) indicate expression of activation markers CD80, CD86, and HLA-DR (n=4 donors). Formulations were compared to a medium-only control. Results are shown as median ± interquartile range. Statistical significance was determined using the Kruskal-Wallis test with uncorrected Dunn's post-hoc test, with thresholds set at $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

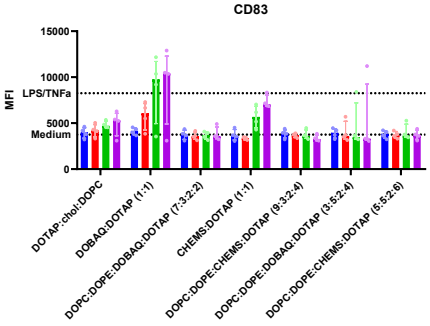


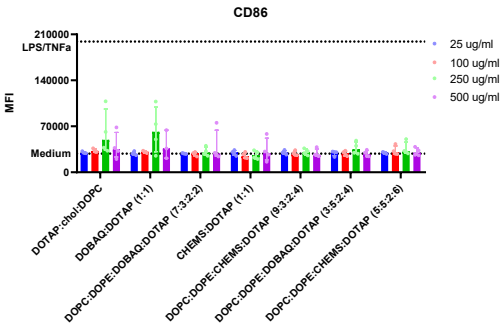
Supplementary Figure S3. Viability of human monocyte-derived dendritic cells following 1-hour incubation with empty liposomal formulations. Viability was calculated as the percentage of SYTOX AADvanced-negative cells relative to the total cell population. Cells were incubated with formulations containing 250 µg/ml lipids, with lipid ratios specified as molar ratios. Results are shown as median ± interquartile range, n = 4 (cell donors). Statistical analysis was performed using the Kruskal-Wallis test with uncorrected Dunn's post-hoc test, with significance thresholds at P < 0.05 (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

→ **Supplementary Figure S4.** Upregulation of surface activation markers and viability of human monocyte-derived dendritic cells (DCs) following exposure to empty liposomal formulations. Cells were incubated with formulations containing 250 µg/ml lipids, with lipid ratios specified as molar ratios for 1 hour. Median fluorescence intensities (MFI) indicate expression of activation markers CD40, CD80, CD83, CD86, CCR7 and HLA-DR (n=4 donors). Viability was calculated as the percentage of SYTOX AADvanced-negative cells relative to the total cell population. Results are shown as median ± interquartile range.


CD40
MFI
8000
LPS/TNFa
4000
Medium
0
DOTAP:chol:DOPC
DOBAQ:DOTAP (1:1)
DOPC:DOPE:DOBAQ:DOTAP (7:3:2:2)
CHEMS:DOTAP (1:1)
DOPC:DOPE:CHEMS:DOTAP (9:3:2:4)
DOPC:DOPE:DOBAQ:DOTAP (3:5:2:4)
DOPC:DOPE:CHEMS:DOTAP (5:5:2:6)


CD80
MFI
28000
LPS/TNFa
14000
Medium
0
25 ug/ml
100 ug/ml
250 ug/ml
500 ug/ml
DOTAP:chol:DOPC
DOBAQ:DOTAP (1:1)
DOPC:DOPE:DOBAQ:DOTAP (7:3:2:2)
CHEMS:DOTAP (1:1)
DOPC:DOPE:CHEMS:DOTAP (9:3:2:4)
DOPC:DOPE:DOBAQ:DOTAP (3:5:2:4)
DOPC:DOPE:CHEMS:DOTAP (5:5:2:6)


CD83
MFI
15000
10000
LPS/TNFa
5000
Medium
0
DOTAP:chol:DOPC
DOBAQ:DOTAP (1:1)
DOPC:DOPE:DOBAQ:DOTAP (7:3:2:2)
CHEMS:DOTAP (1:1)
DOPC:DOPE:CHEMS:DOTAP (9:3:2:4)
DOPC:DOPE:DOBAQ:DOTAP (3:5:2:4)
DOPC:DOPE:CHEMS:DOTAP (5:5:2:6)


CD86
MFI
210000
LPS/TNFa
140000
70000
Medium
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25 ug/ml
100 ug/ml
250 ug/ml
500 ug/ml
DOTAP:chol:DOPC
DOBAQ:DOTAP (1:1)
DOPC:DOPE:DOBAQ:DOTAP (7:3:2:2)
CHEMS:DOTAP (1:1)
DOPC:DOPE:CHEMS:DOTAP (9:3:2:4)
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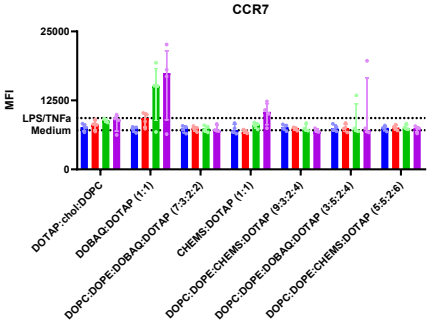
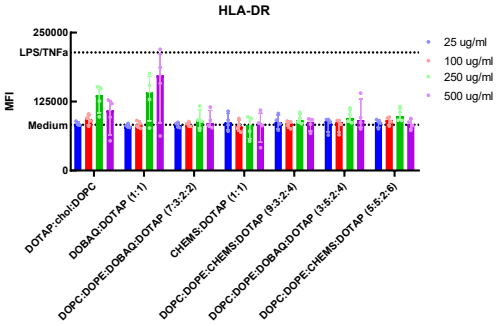
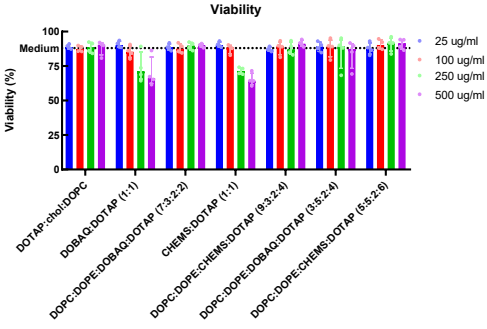
DOPC:DOPE:CHEMS:DOTAP (5:5:2:6)


CCR7
MFI
25000
12500
LPS/TNFa
Medium
0
DOTAP:chol:DOPC
DOBAQ:DOTAP (1:1)
DOPC:DOPE:DOBAQ:DOTAP (7:3:2:2)
CHEMS:DOTAP (1:1)
DOPC:DOPE:CHEMS:DOTAP (9:3:2:4)
DOPC:DOPE:DOBAQ:DOTAP (3:5:2:4)
DOPC:DOPE:CHEMS:DOTAP (5:5:2:6)


HLA-DR
MFI
250000
LPS/TNFa
125000
Medium
0
25 ug/ml
100 ug/ml
250 ug/ml
500 ug/ml
DOTAP:chol:DOPC
DOBAQ:DOTAP (1:1)
DOPC:DOPE:DOBAQ:DOTAP (7:3:2:2)
CHEMS:DOTAP (1:1)
DOPC:DOPE:CHEMS:DOTAP (9:3:2:4)
DOPC:DOPE:DOBAQ:DOTAP (3:5:2:4)
DOPC:DOPE:CHEMS:DOTAP (5:5:2:6)


Viability
Viability (%)
100
Medium
75
50
25
0
25 ug/ml
100 ug/ml
250 ug/ml
500 ug/ml
DOTAP:chol:DOPC
DOBAQ:DOTAP (1:1)
DOPC:DOPE:DOBAQ:DOTAP (7:3:2:2)
CHEMS:DOTAP (1:1)
DOPC:DOPE:CHEMS:DOTAP (9:3:2:4)
DOPC:DOPE:DOBAQ:DOTAP (3:5:2:4)
DOPC:DOPE:CHEMS:DOTAP (5:5:2:6)