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## Special Topic Cluster

## Uptake Kinetics Of Liposomal Formulations of Differing Charge Influences Development of in Vivo Dendritic Cell Immunotherapy



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## ABSTRACT

Dendritic cells (DCs) control adaptive immunity and are therefore attractive for in vivo targeting to either induce immune activation or tolerance, depending on disease. Liposomes, nanoparticles comprised of a lipid bi-layer, provide a nanopatform for loading disease-relevant antigen, adjuvant and DC-targeting molecules simultaneously. However, it is yet not fully understood how liposomal formulations affect uptake by DCs and DC function. Here, we examined monocyte-derived DC (moDC) and skin DC uptake of six different liposomal formulations, together with their DC-modulating effect. Contrary to literature, we show using imaging flow cytometry that anionic or neutral liposomes are taken up more efficiently than cationic liposomes by moDCs, or by skin DCs after intradermal injection. None of the formulations yielded significant modulation of DC function as determined by the upregulation of maturation markers and cytokine production. These results suggest that anionic liposomes would be more suitable as vaccine carriers for a dermal application.

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**Abbreviations:** DC, Dendritic cell; APC, antigen-presenting cell; PS, phosphatidyl-serine; DSPG, 1,2-distearoyl-sn-glycero-3-phosphoglycerol; LC, Langerhans cell; DDC, dermal DC; DC-SIGN, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; DPPS, 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine; DPTAP, 1,2-dipalmitoyl-3-trimethylammonium-propane; CHOL, cholesterol; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DiD, 1,1-Dioctadecyl-3,3,3-tetramethylindodicarbocyanine; PB, phosphate buffer; EPC, Egg L- $\alpha$ -phosphatidylcholine (Egg, Chicken); EPG-Na, ethanolamine phosphoglyceride, Zave, Z-average diameter; Pdl, polydispersity index; DLS, dynamic light scattering; UPLC, ultra-performance liquid chromatography; IMDM,

Iscoe's Modified Dulbecco's Medium; FCS, fetal calf serum; lipo-polysaccharide, (LPS); GM-CSF, granulocyte-macrophage colony-stimulating factor; PE, phycoerythrin; BV421, brilliant violet 421; PerCP, peridinin-chlorophyll-protein; EDTA, ethylenediaminetetraacetic acid; ID, intradermal; PBS, phosphate-buffered saline; FITC, fluorescein-5-isothiocyanate; APC, allophycocyanin; EpCAM, epithelial cell adhesion molecule; MFI, mean fluorescence intensity; BMDC, bone marrow-derived DC.

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## Introduction

Dendritic cells (DCs) are considered important targets for novel immunotherapies due to their crucial role as orchestrators of both tolerogenic and immunogenic adaptive responses.<sup>1,2</sup> *Ex vivo* DC vaccination is an approved treatment against various forms of cancer.<sup>1</sup> Recent studies on several autoimmune conditions have borrowed the approach, replacing tumor antigens and immunogenic adjuvants with auto-antigens and tolerogenic adjuvants.<sup>3</sup> However, *ex vivo* DC vaccination is a costly, cumbersome method that can only be applied in a patient's tailor-made fashion. As an alternative DC-focused strategy, *in vivo* targeting holds promising potential. With this approach, DCs can be reached in their natural niche without external manipulation. Moreover, *in vivo* targeting may also provide the opportunity to target specific DC subsets for immunogenic or tolerogenic manipulation. Liposomes, nanostructures with a lipid bi-layer, are biocompatible and suitable vehicles for loading DC-targeting and modulating compounds.<sup>4</sup>

By shielding the loaded cargo, liposomes may increase half-life, reduce drug-related toxicity, and facilitate the controlled release of therapeutics to desired cellular compartments.<sup>5</sup> Beyond the potential to make immunotherapies more specific, liposomal characteristics, such as surface chemistry (electric charge), lipid composition, rigidity, and size, can be modified with ease during the manufacturing process. These modifications can lead to differential interactions with DCs, providing specific formulations with a bona fide adjuvant effect.<sup>6</sup> Cationic liposomes were shown to preferentially interact with negatively charged membranes of antigen-presenting cells (APCs), which also contributed to their DC activating effects in several *in vitro* studies.<sup>7–9</sup> Anionic formulations were demonstrated to be internalized less by APCs due to electrostatic repulsion.<sup>4</sup> Additionally, certain anionic formulations containing phosphatidyl-serine (PS) or 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG) were attired with tolerogenic effects on bone-marrow-derived DCs (BMDCs) and after *in vivo* injection in mice.<sup>6,10</sup> Thus, depending on liposomal choice, specific formulations are more suitable for DC-activating immunomodulatory treatments, whereas others are preferred for tolerogenic purposes.

A further important aspect to consider for *in vivo* immune modulation is the injection site. The skin provides easy access for vaccination. Moreover, this tissue harbors an intricate network of Langerhans cells (LCs) in the epidermis and several subsets of DCs in the dermis, including CD1a+ and CD14+ dermal DCs (DDCs).<sup>11</sup> These skin DC subsets possess different T cell activating qualities. Human LCs efficiently activate CD8+ T cells and induce strong proliferation of allogeneic naïve T cells in mixed lymphocyte reaction. CD14+ DDCs are less capable of priming for naïve T cell proliferation and were described as efficient activators of humoral responses.<sup>12,13</sup> The specific subsets can be targeted via pattern recognition receptors expressed on their surfaces, such as langerin, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), and others to achieve immune modulation inherent to the qualities of each tissue subset. For example, DDCs were efficiently targeted by liposomes coated with a sugar ligand of langerin and DC-SIGN, Lewis-Y, carrying a tumor-specific antigen, which led to improved cross-presentation to CD8+ tumor-specific T cells.<sup>14</sup>

In this study, we present a thorough systematic analysis of the uptake of six different liposomal formulations of varying lipid composition and surface charge by monocyte-derived DCs (moDCs) and include an *ex vivo* human skin model to validate liposomal choice for a putative skin-based application. We compared results of uptake kinetics between flow cytometry and imaging flow cytometry measurements. Additionally, we investigated whether the liposomal formulations affected moDC maturation, activation, and skewing of human DC skin subsets upon *ex vivo* injection. Contrary to literature

based on flow cytometry, we show that anionic or neutral liposomes are taken up efficiently by moDCs, whereas flow cytometry overestimates uptake of cationic formulations. Moreover, liposomes without additional cargo did not lead to differential activation or cytokine production by moDCs, contrasting findings in many *in vitro* mouse studies. The same results were found when liposomes were applied in an *ex vivo* skin model where cationic liposomes also readily adhered to structural skin cells besides DCs. Hence, we provide valuable fundamental insights using human cells and a human tissue model for the optimal choice of liposomal vesicles in the development trajectory of an *in vivo* DC modulating vaccine.

## Materials and Methods

### Liposome Preparation

All lipids were obtained from Avanti Polar Lipids, Alabaster, Alaska, and stored at -20 °C in lyophilized form or rehydrated in chloroform. Liposomes were manufactured following the thin lipid film dehydration-rehydration method, as described elsewhere.<sup>10,15</sup> For the formulations “DSPG”, “DPTAP” and “DPPS”, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) (Tm=54.9 °C),<sup>16</sup> a charged lipid (1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG)(Tm=54.4 °C),<sup>17</sup> 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (DPPS) (Tm=55 °C),<sup>18</sup> or 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP) (Tm=52.8 °C)<sup>19</sup> and cholesterol (CHOL) were dissolved in chloroform and mixed in a round-bottom flask at a molar ratio of 4:1:2 DSPC: charged lipid: CHOL. For the neutrally charged formulation “DPPC”, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (Tm=41.3 °C)<sup>20</sup> and CHOL were mixed in a molar ratio of 1:1. To obtain the cationic formulation “DOTAP” 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Tm=-15 °C), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (Tm=-16 °C)<sup>21</sup> and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (Tm<5 °C)<sup>19</sup> were mixed in a molar ratio of 9:1:2.5, respectively. For fluorescent labeling, the lipophilic tracer 1,1-Dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine (DiD) (Thermo Fisher Scientific, Waltham, Massachusetts) was added to the lipid mix in a molar percentage of 0.1%. The chloroform was evaporated in a rotary evaporator (Rotavapor R-210, Büchi, Switzerland) set at 40 °C for 30 min. 2 ml of 10 mM phosphate buffer (PB), pH 7.4, was used to rehydrate the lipid film, and homogenization was achieved by rotation at 60 °C, using glass beads. The resulting multilamellar vesicles were standardized to a size less than or around 200 nm by high-pressure extrusion at 60 °C (LIPLEX Extruder, Northern Lipids Inc., Canada) by passing the formulation through stacked 400 nm and 200 nm pore size membranes four times. Liposomes were stored at 4 °C and used for further experiments within two months. The formulation “EPG-Na” consisted of Egg L- $\alpha$ -phosphatidylcholine (EPC-35), ethanolamine phosphoglyceride (EPG-Na), and CHOL in a molar ratio of 5:1.5:3.5.

### Quality Control of Liposomes

To confirm the size of the prepared liposomes, Z-average diameter (Zave) and polydispersity index (Pdl) were measured by dynamic light scattering (DLS) using a NanoZS Zetasizer (Malvern Ltd., Malvern, UK). The surface charge of the formulations (Zeta-potential) was determined by laser Doppler electrophoresis, using the same instrument. To confirm stability, measurements were repeated each month after liposome preparation. To measure the lipid concentration of the formulations, reversed-phase ultra-performance liquid chromatography (UPLC) (Waters ACQUITY UPLC, Waters, Massachusetts) was used. For this, 10  $\mu$ l of the sample was injected into a 1.7  $\mu$ m BEH C18 column (2.1  $\times$  50 mm, Waters ACQUITY UPLC). The column temperature and the sample temperature were set at 40 °C

and 4 °C, respectively. The mobile phases were Milli-Q water with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B). For detection, the mobile phases were applied in a linear gradient from 5% to 95% solvent B over 7 min at a flow rate of 0.45 mL/min. Lipids were detected using an Evaporative Light Scattering Detector (Alltech 3300 ELSD, BÜCHI, Switzerland).

#### *In Vitro Generation and Activation of moDCs*

MoDCs were differentiated from peripheral blood monocytes obtained from buffy coats or fresh blood as described elsewhere.<sup>22</sup> To determine liposome uptake, 50–200 × 10<sup>3</sup> immature DCs were incubated with DiD-labelled liposomes (see Table 1 for characteristics) for 1–24 h in Iscove's Modified Dulbecco's Medium (IMDM) (Thermo Fisher Scientific) 5 % fetal calf serum (FCS) (Sigma-Aldrich, St Louis, Missouri) at lipid concentrations of 1, 10 and 30 µg/mL. For assessing liposome-mediated moDC maturation, immature DCs were stimulated for 24 h with 100 ng/mL lipopolysaccharide (LPS) derived from *E. coli* strain O111-B4 (Sigma-Aldrich) and 500 U/mL granulocyte-macrophage colony-stimulating factor (GM-CSF), or GM-CSF only with or without the addition of 1, 10 or 30 µg/mL liposomes. After stimuli were washed away, maturation and morphology were assessed by means of flow cytometry using the following antibody cocktail: anti-CD83-phycoerythrin (PE) (BD Biosciences, Franklin Lake, New Jersey), anti-CD86-brilliant violet 421 (BV421) (BD), anti-HLA-DR-peridinin-chlorophyll-protein-Cy5.5 (PerCP-Cy5.5) (BD), and anti-CD14-PE-Cy7 (Biollegend, San Diego, California).

#### *Evaluation of Mode of Liposome Uptake by moDCs*

To evaluate the mode of uptake, 3 × 10<sup>5</sup> moDCs per condition were preincubated for 1 h at 37 °C with inhibitors of actin polymerization (25 µM Cytochalasin D, Sigma-Aldrich), macropinocytosis (50 µM Imipramine hydrochloride, Sigma-Aldrich), clathrin-mediated endocytosis (10 µM Chlorpromazine hydrochloride, Sigma-Aldrich), the membrane (cholesterol) disruptor nystatin (Sigma-Aldrich) in a concentration of 10 µM, or 2.5 µM ethylenediaminetetraacetic acid (EDTA) to inhibit calcium-dependent processes. After 1 h, liposomes were added at 30 µg/mL lipid concentration and incubated with the DCs for 4–6 h. Subsequently, moDCs were stained with the surface marker CD11c-PE-Cy7, and liposome uptake was measured with flow cytometry.

#### *Analysis of DC-derived Cytokine Production*

Immature DCs (40 × 10<sup>3</sup>) were stimulated with 100 ng/mL LPS (Sigma-Aldrich), (for stimulation of cytokine production of IL-10, IL-6, and TNF-α) or LPS and IFN-γ (for stimulation of IL-12 expression), with or without 1, 10 or 30 µg/mL liposomes. The levels of IL-12p70, IL-6, IL-10, and TNF-α in 24-h culture supernatants were assessed by specific solid-phase sandwich ELISA.<sup>22</sup>

#### *Priming and Extraction of Skin DCs*

Skin was obtained from healthy human subjects undergoing abdominal or breast reduction surgery. Ex vivo crawl-out DCs were obtained from the skin, as described elsewhere.<sup>11</sup> Intradermal (ID) injections were carried out with 50 µl of phosphate-buffered saline (PBS) or 50 µl 628 µg/mL DiD-labeled liposomes. Biopsies were cultured in 1 ml IMDM supplemented with 10% FCS and GM-CSF (500 U/ml). The migrating cells were harvested after three days and stained for skin DC markers with anti-CD11c PE-Cy7 (eBioscience, Thermo Fisher), anti-HLA-DR-PerCP (BD), anti-CD1a- fluorescein-5-isothiocyanate (FITC) (BD), and anti-CD14- allophycocyanin (APC)-Cy7 (BD). For measurement of liposome retention in non-migrating skin cells, single-cell suspensions were prepared of PBS- or liposome-injected biopsies using the GentleMacs human skin dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), following manufacturers' instructions. Skin cells obtained after digestion of non-injected biopsies were incubated with 100 µg/mL liposomes for 2 or 24 h. Single-cell suspensions of liposome-injected, or liposome-incubated skin cells were stained with anti-CD3-APC-Cy7(BD), anti-CD45-BV650 (BD), anti-CD11c-PE-Cy7, anti-HLA-DR-BV421 (BD), anti-CD326 epithelial cell adhesion molecule (EpCAM)-PE (Miltenyi Biotec), and intracellularly with anti-pan-cytokeratin-Alexa-fluor 488 (AF488) (Abcam, Cambridge, UK) for flow cytometric measurement. Previous research found no difference between abdominal or breast-derived skin; hence, data were pooled from all sources.<sup>23</sup>

#### *Flow Cytometry and Imaging Flow Cytometry*

For flow cytometric analysis, 5–30 × 10<sup>3</sup> cells per condition were measured on a FACS Canto or FACS Fortessa (BD). Compensation was carried out on the machines, using single marker-fluorochrome stained samples, and adjusted per donor if necessary. Analysis of resulting data was done using FlowJo software (Treestar, Ashland, Oregon). For imaging flow cytometry, 3 × 10<sup>5</sup> DCs were used for each liposome concentration and incubation time tested and stained with CD11c-PE-Cy7. 10–30 × 10<sup>3</sup> DCs per condition were acquired on an ImageStreamX Mk II (Amnis ImageStream, X MKII-Luminex, Austin, Texas), and analysis was carried out on IDEAS software. Internalization of liposomes was calculated using the morphology mask applied to brightfield images of the cells, evenly eroded by 6 pixels compared to the cell membrane to represent the inside of cells. IDEAS software calculated internalization scores based on the log-scale ratio between the mean-fluorescence intensity (MFI) of DiD signal falling inside the eroded mask, divided by the MFI of DiD signal in the whole cell. The formula used was the following:

$$\text{Internalization} = \log \left( \frac{a}{1-a} \times \frac{P_i}{P_\beta} \right), \text{ where } a = \frac{M_i}{M_i + M_\beta}$$

M<sub>i</sub> = Mean Intensity of upper quartile pixels in I  
M<sub>β</sub> = Mean Intensity of upper quartile pixels in B  
P<sub>i</sub> = Peak intensity of upper quartile pixels in I  
P<sub>β</sub> = Peak intensity of upper quartile pixels in B

**Table 1**  
Physicochemical properties of liposome formulations included in the study.

| Liposome         | Lipid composition | Mean Z size (nm) | ± SD | PdI  | ±SD  | Mean ζ potential (mV) | ±SD  | (n=) |
|------------------|-------------------|------------------|------|------|------|-----------------------|------|------|
| <b>DPPE</b>      | DPPE:CHOL         | 199              | 0.05 | 0.15 | 0.05 | -1.79                 | 1.89 | 5    |
| <b>DSPG(-)</b>   | DSPG:DSPG:CHOL    | 181              | 8.62 | 0.14 | 0.05 | -39.0                 | 12.7 | 7    |
| <b>DPTAP(+)</b>  | DSPG:DPTAP:CHOL   | 207              | 13.9 | 0.18 | 0.08 | 32.5                  | 2.75 | 6    |
| <b>DOTAP(+)</b>  | DOPC:DOPE:DOTAP   | 168              | 7.47 | 0.17 | 0.04 | 27.1                  | 2.76 | 4    |
| <b>DPPS(-)</b>   | DSPC:DPPS:CHOL    | 176              | 6.35 | 0.07 | 0.02 | -50.9                 | 2.48 | 6    |
| <b>EPG-Na(-)</b> | EPC:EPG-Na:CHOL   | 163              | 5.51 | 0.10 | 0.06 | -53.7                 | 6.04 | 5    |

I is the input mask, and B is the area of the segmentation mask outside the input mask I.

### Statistics

One-way ANOVA was performed for matched measurements with GraphPad Prism software (GraphPad, La Jolla, CA) with Tukey's or Dunnett's corrections for repeated measurements. Values of  $P < 0.05$  were considered significant.

## Results

### Liposome Characteristics

For an extensive evaluation of moDC and skin DC uptake of liposomes, we prepared six different liposomal formulations with the lipid-film dehydration method, incorporating the fluorescent label DiD. As the surface chemistry of liposomes may influence interaction and adjuvant effects on DCs, we included neutrally charged, cationic and anionic lipids in the different formulations to have a balanced range of surface chemical properties. The formulations were neutrally charged DPPC:CHOL (DPPC), cationic DSPC:DPTAP:CHOL (DPTAP), cationic DOPC:DOPE:DOTAP (DOTAP), and three anionic formulations DPPC:DSPG:CHOL (DSPG), DPSC:DPPS:CHOL (DPPS), and EPC:EPG-Na (EPG-Na) (Table 1). All formulations had a size less than 250nm and a polydispersity index (PDI) of less than 0.2, indicating a monodisperse quality. Measured Zeta-potential corresponded to the expected surface charge of the formulations. Formulations were used for 2–3 months after preparation and were stable throughout their use (Supplementary Table 1).

### Anionic or Neutral but not Cationic Liposomes are Efficiently Internalized by DCs

The composition of liposomes and their electric surface charge may influence their uptake by professional APCs, such as DCs. Uptake is frequently measured by flow cytometry which does not distinguish between adherence to the outer membrane and uptake of liposomes. To evaluate liposome uptake, we incubated moDCs with fluorescently labeled liposomes at a lipid concentration of 30  $\mu\text{g/ml}$  for a time span of 1, 2, 4, 6, or 24 h, and measured uptake with flow cytometry (Fig. 1A) or imaging flow cytometry (Fig. 1B). For evaluation of uptake by imaging flow cytometry, we specified the interior of moDCs by eroding the bright field mask created around the cells by six pixels (Fig. 1B). Subsequently, we analyzed uptake using an internalization score, based on the logarithmic ratio of the fluorescent signal inside the eroded mask, divided by the fluorescent signal obtained from the cell's total. Scores with negative numbers signify cell membrane adherence, while positive scores reflect the extent of internalization. When evaluated with flow cytometry after 1 h, the cationic liposomes DPTAP and DOTAP appeared significantly better taken up (50–90% Liposome+ moDCs) compared to the anionic or neutral liposomes (10–50%) (Fig. 1C). The difference was less clear at longer incubation times 4 h (Supplementary Fig. 1) or 6 h (data not shown), although the neutral DPPC liposomes and the EPG-Na formulation were still taken up less than the rest of the formulations. In contrast to the interpretation of uptake by flow cytometry, measurements with imaging flow cytometry revealed that moDCs efficiently internalized the neutral and anionic formulations in an incubation period ranging from 1–4 h (Fig. 1D, Supplementary Fig. 1). Conversely, cationic formulations DPTAP and DOTAP were internalized less, adhering more to the cell membrane. Membrane adherence of cationic formulations could not be prevented at 4 °C and inadvertently was interpreted as uptake by flow cytometry (Fig. 1E). Also, at 4 °C, we found that moDCs were positive for cationic but not neutral or anionic

liposomes. In contrast, internalization scores measured by imaging flow cytometry were negative for both cationic DPTAP and DOTAP uptake, demonstrating that what appeared as uptake with FACS was membrane adherence (Fig. 1F). Notably, cationic liposomes were poorly internalized at 37 °C, as shown by imaging flow cytometry (Fig. 1D).

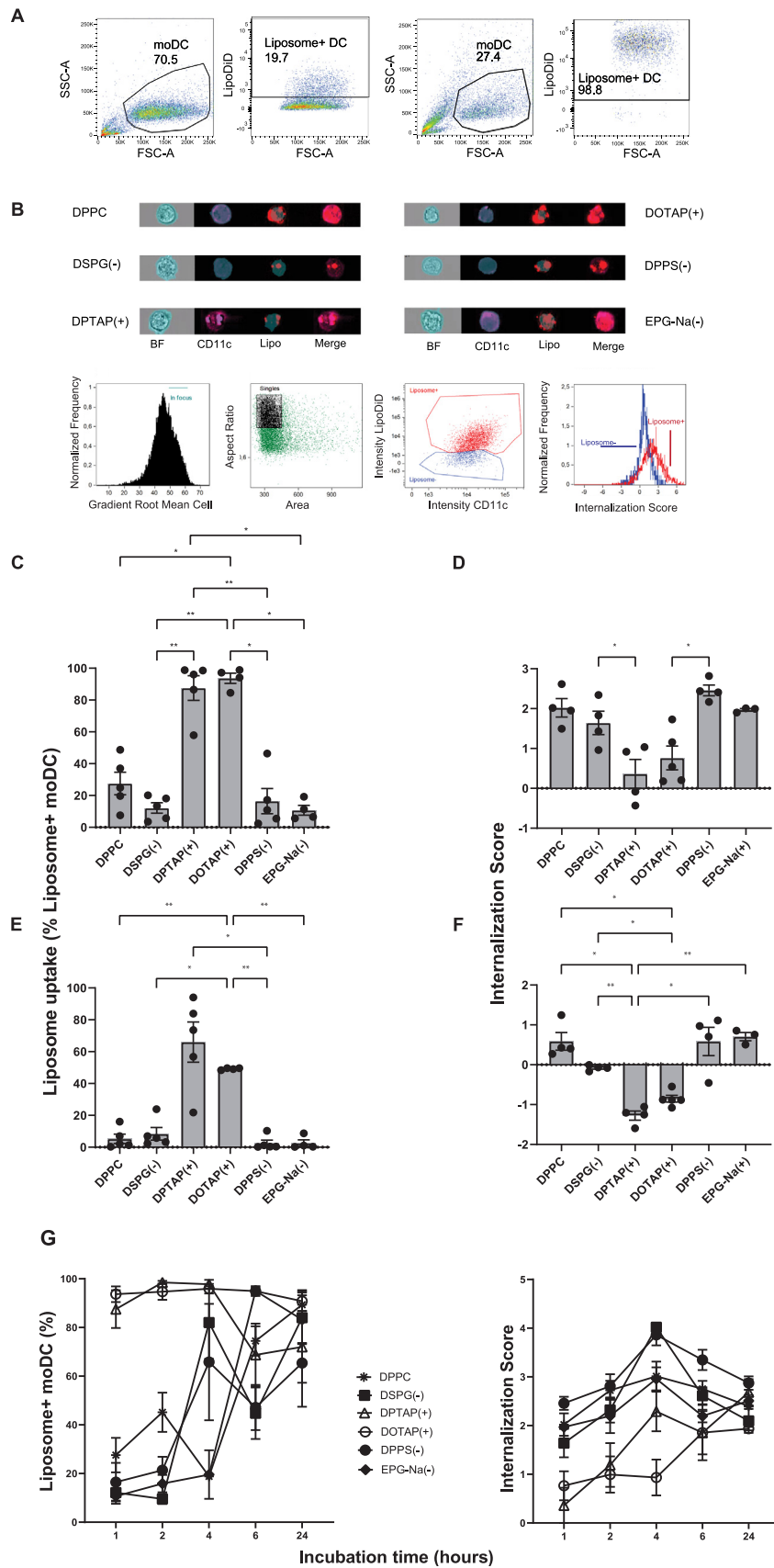
To analyze the kinetics of liposome uptake, we included two further lipid concentrations of 1  $\mu\text{g/ml}$  and 10  $\mu\text{g/ml}$  and observed liposome uptake in time ranging from 1–24 h. Uptake by moDCs at all time points was lipid dose-dependent (Supplementary Fig. 2) and peaked at 4–6 h incubation time for formulations DPPC, DSPG, DPPS on FACS (Fig. 1G, left panel). Cationic DPTAP and DOTAP appeared taken up by close to 100% of moDCs from the earliest incubation time of 1 h up until 24 h when evaluated by FACS. However, following internalization of this formulation in time with imaging flow cytometry indicated internalization lower than the other formulations at incubation times of 1–6 h (Fig. 1G, right panel). Based on internalization scores, all different formulations were internalized the highest at 4–6 h incubation time. Extending incubation time to 24 h reduced the observed differences, and most formulations were taken up close to 100% or with an internalization score that reached a homogenous equilibrium between 2–3 (Fig. 1G, right panel). Thus, measurements with imaging flow cytometry demonstrate that at incubation times between 1–6 h, moDCs efficiently internalize neutral and anionic liposomes, whereas cationic liposomes rather adhere to the cell membrane, reaching their peak of internalization at 24 h only.

### moDCs Internalize Neutrally Charged DPPC and Anionic DSPG Liposomes by Actin Mediated Processes

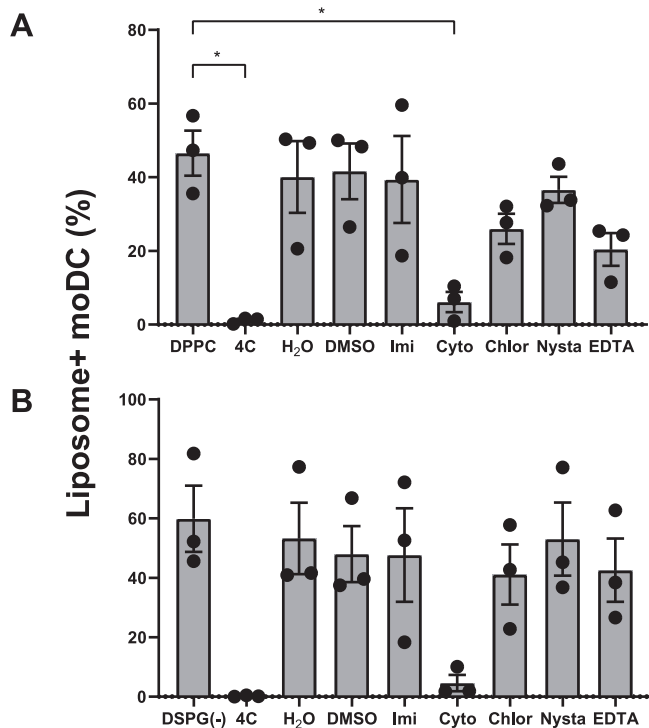
The manner of internalization and intracellular processing of liposomes may influence the fate of immunotherapeutic compounds loaded in them. Given the fact that we found our cationic formulations poorly internalized, we chose neutral DPPC and anionic DSPG to investigate how DCs internalize these formulations using various chemical inhibitors of cellular uptake. Incubation at 4 °C effectively blocked uptake of both DPPC (Fig. 2A) and DSPG (Fig. 2B), suggesting an active uptake process as a mechanism of internalization for these liposomes, as incubation at 4 °C blocks all energy-dependent, vesicular independent forms of cellular uptake.<sup>24</sup> The phagocytosis inhibitor Cytochalasin D also inhibited uptake of DPPC and DSPG liposomes to a level similar to the cold control condition (Fig. 2A, B). With EDTA, we also observed a trend of inhibition for uptake of DPPC liposomes, even though these values were not significant. No effect was found with Imipramine that blocks membrane ruffle formation and thereby macropinocytosis, chlorpromazine that inhibits clathrin-mediated endocytosis, nor nystatin that inhibits caveolin and clathrin-independent processes. Hence, actin-mediated processes, predominantly phagocytosis and macropinocytosis, play the most critical role in the internalization of neutral and anionic liposomes included in this study.

### Liposomes of Differing Charge and Composition do not Affect moDC Maturation or Cytokine Production

Both lipid composition (rigidity) and electric charge have previously been implicated in the immunogenicity of liposomes.<sup>10,25</sup> To assess these adjuvant effects in human cells, we incubated moDCs with 30  $\mu\text{g/ml}$  liposomal formulations with or without LPS stimulation for 24 h and measured surface marker expression of the maturation markers CD83, CD86, and HLA-DR with flow cytometry and production of the cytokines IL-6, IL-12, TNF- $\alpha$ , and IL-10 via ELISA. As incubation with cationic liposomes in a lipid concentration higher than 30  $\mu\text{g/ml}$  proved toxic for moDCs, we did not add liposomes in higher lipid concentrations in our experiments (data not shown). At a lipid concentration of 30  $\mu\text{g/ml}$ , we did not find a significant effect of



**Figure 1.** Anionic and neutral liposomes are internalized better by moDCs compared to cationic liposomes. MoDCs made from buffycoats were incubated for 1 h with DiD-labeled liposomes, washed and measured for liposome uptake with flow cytometry or imaging cytometry. (A) Gating example for liposome uptake measurement by flow cytometry. SSC-A, side-



**Figure 2.** moDCs take up neutral DPPC and anionic DSPG liposomes via active, actin-dependent processes. moDCs were pre-incubated with uptake inhibitors for 1 h after which DiD-labeled liposomes DPPC (A), or DSPG (B) were added for 4 h. Liposome uptake was measured with flow cytometry. Lipid concentration 30  $\mu$ g/ml. N=3. Error bars indicate mean  $\pm$  SEM. \* $p \leq 0.05$ . \*\* $p \leq 0.01$ . Statistical significance was calculated using one-way ANOVA with Dunnett's correction for multiple comparisons. 4C, 4 °C, cold control; H<sub>2</sub>O, water control; DMSO, dimethylsulfoxide control; Imi, Imipramine; Cyto, Cytochalasin D; Chlorpro, Chlorpromazine; Nysta, Nystatin; EDTA, Ethylenediamine tetraacetic acid.

our liposomal formulations on moDC maturation (Fig. 3A, B) or cytokine production (Fig. 3C), neither in LPS-stimulated condition nor without stimulation (data not shown). Similarly, we observed no effect at the lipid concentrations of 1 and 10  $\mu$ g/ml (data not shown). Therefore, our liposomal formulations appear to have no additive adjuvant effects on human moDCs.

#### Cationic Liposomes are Taken Up Less Efficiently by Skin DCs Due To Membrane Adherence to Structural Skin Cells

As the skin is one preferred target site for DC immunotherapy, we examined uptake kinetics of our formulations by total skin crawl-out DCs (CD11c+ HLA-DR+ skin DCs). We injected liposomal formulations in ex vivo human skin obtained from plastic surgery. After 3 days, we harvested crawl-outs and stained them for the skin DC markers CD11c, HLA-DR, CD1a, and CD14. The liposome-DiD label was used within the total CD11c+ HLA-DR+ skin DC population (crawl-out DC) (Fig. 4A) to identify uptake with the three skin DC subpopulations, e. g. CD1a+ DDCs, CD1a++ LCs and CD14+ DDCs. Interestingly, flow cytometry measurements in these skin crawl-out populations aligned perfectly with uptake results obtained from imaging flow cytometry of moDCs. Uptake of liposomes as determined by frequencies of

liposome+ crawl-out DCs were higher after injection of neutral or anionic liposomes compared to injection of the cationic formulations (Fig. 4B). LCs and CD1a+ DDC crawl-outs also internalized less cationic than anionic or neutral formulations, while liposome+ CD14+ DDCs were equally abundant among all liposome conditions (Fig. 4C).

To examine whether the cationic liposomes were retained in the skin biopsies, we digested liposome-injected biopsies and stained them with anti-CD3, CD45, CD11c, HLA-DR (Supplementary Fig. 3A), EpCAM, and anti-pan-cytokeratin (data not shown). Interestingly, both in bulk cells obtained from the biopsies as well as in the stained subpopulations, we found lower frequencies of cationic liposome+ cells compared to the rest of the formulations (Supplementary Fig. 3B, C). However, when we digested non-injected skin biopsies into single-cell suspensions and incubated these cells for 2 h and 24 h with liposomes, the frequencies of cationic liposome+ skin cells were significantly higher, similar to our results with moDCs (60–80% liposome+ cells) (Fig. 4D, Supplementary Fig. 3D). At 4 °C, frequencies of DPTAP or DOTAP liposome+ cells amounted to 40%, indicating that these formulations also readily adhered to skin cells present in the biopsy (Fig. 4D). These data with ex vivo tissue injection show the same pattern as observed in moDCs; neutral and anionic liposomes are efficiently taken up while cationic liposomes mainly adhere to the cell membrane.

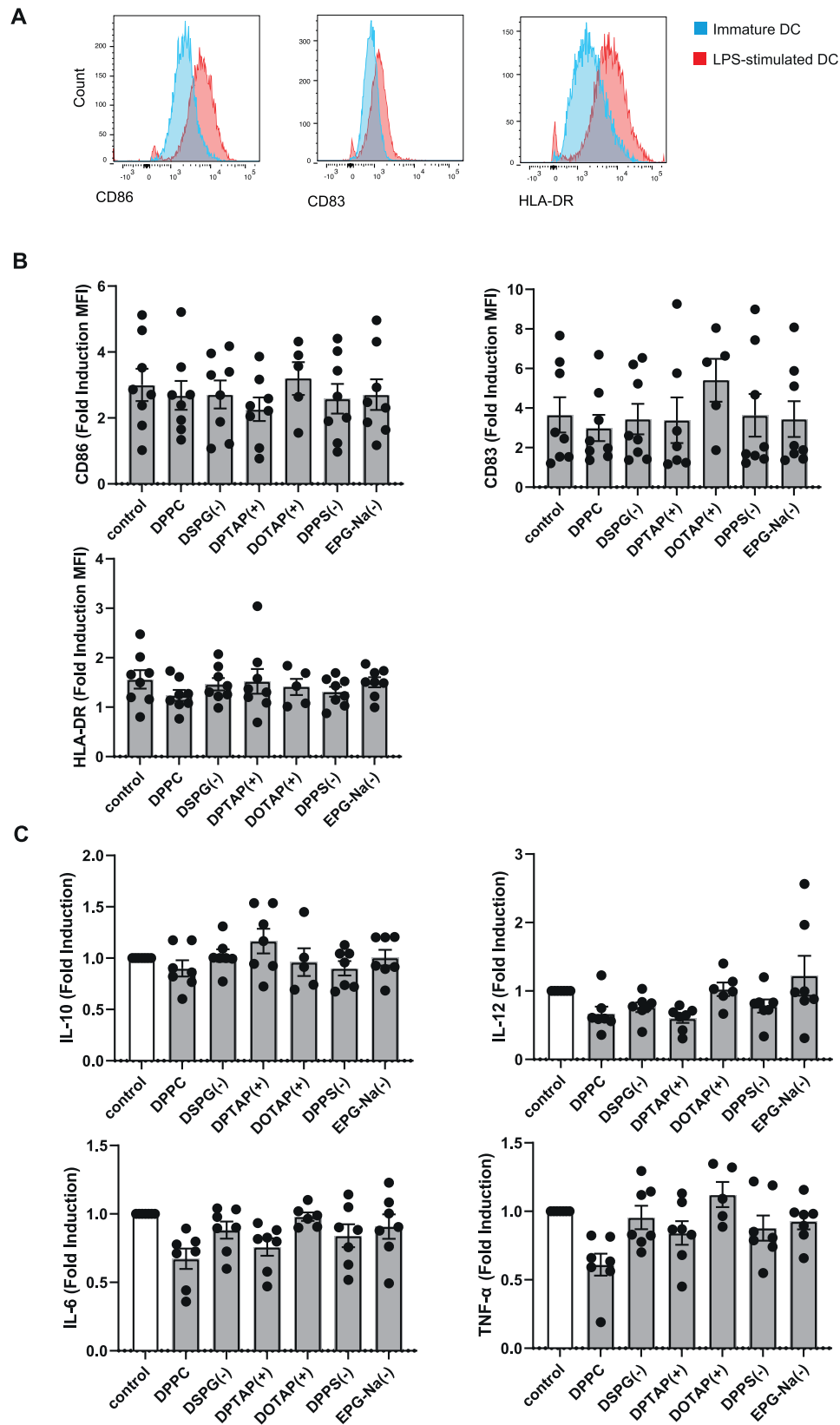
#### Cargo-less Liposomes do not Skew Skin Crawl-out Populations

Various adjuvants of immunotherapy may alter migration of skin DCs or skin DC subpopulations.<sup>26</sup> To investigate whether any of the formulations included in this study had adjuvant effects on skin DCs via skewing cell migration, we stained harvested crawl-out cells with anti-CD1a for distinguishment of CD1a+ DDCs and CD1a++ LCs and with anti-CD14 to identify CD14+ DDCs. We examined the relative abundance of the subpopulations among skin DC crawl-outs, which did not differ in counts between the different conditions of injection (Fig. 5A). Injection of cargo-less liposomes did not lead to enhanced migration of any of the skin DC subsets out of the biopsies (Fig. 5B). This was similarly true for percentages of the subpopulations (Fig. 5B, upper panel), as well as absolute counts of each skin DC subset (Fig. 5B, lower panel). Similarly, we observed no differences in distribution of liposome+ CD1a+ DDCs, LCs or CD14+ DDCs, as among liposome+ crawl-outs, we consistently observed 60–80% CD1a+ DDCs, 10–20% LCs and 20–40% CD14+ DDCs (Supplementary Fig. 4A, B).

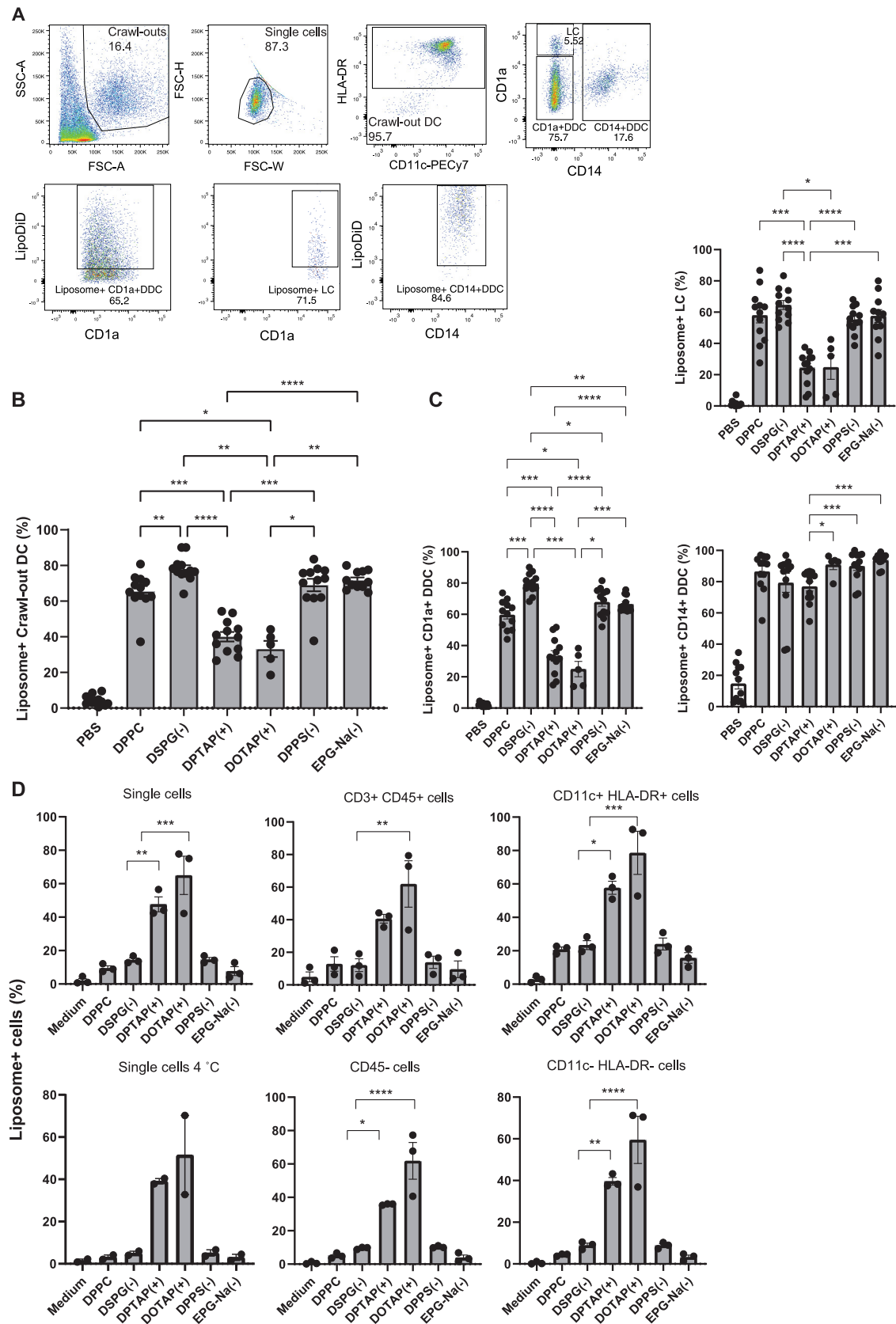
#### Discussion

It is crucial to investigate how liposomes without cargo interact with DCs, before adding pharmacological cargo to nanoparticles that modulate DC function and subsequent adaptive immune responses. In this study, we present a systematic analysis comparing uptake and adjuvant effects of six differing liposomal formulations on human moDCs and skin DCs. With the aid of imaging flow cytometry, we clearly show that neutrally or negatively charged liposomes are more efficiently taken up compared to cationic liposomes DPTAP and DOTAP. This is important as the cargo (adjuvant or antigens) of liposomes that merely adhere to the outer membrane will not affect DC function and subsequent adaptive immune responses. Additionally, we showed that uptake of neutral DPPC and anionic DSPG formulations by human DCs is an active and actin-dependent process.

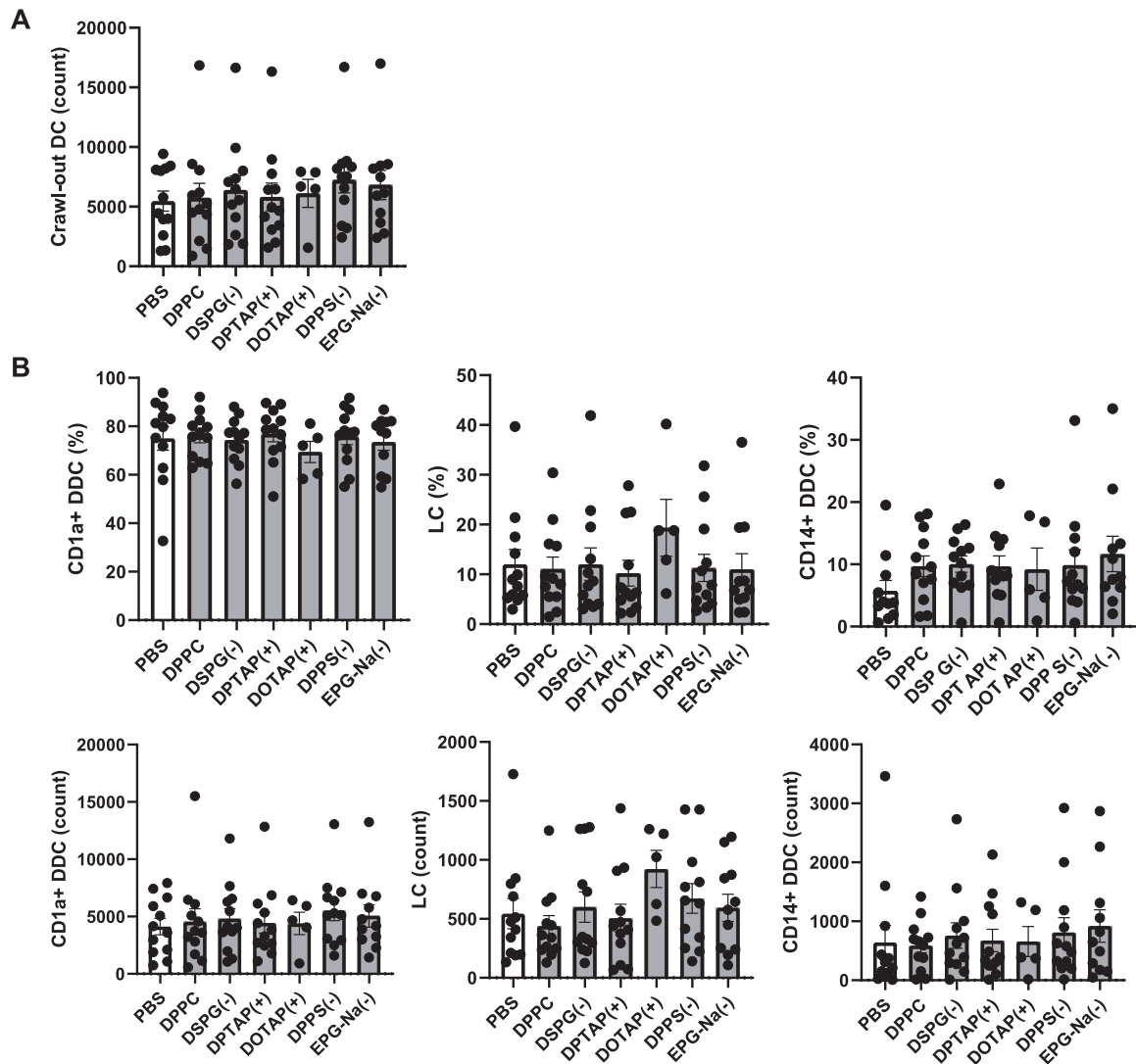
scatter; FSC-A, forward scatter; LipoDiD, Liposomal fluorescent label DiD. (B) Example images of liposome+ CD11c+ moDCs as visible with imaging cytometry, and gating example for internalization scores. MoDC mask was based on brightfield image and eroded by six pixels for expression of liposome uptake. BF, BrightField. (C) Uptake of six different liposomal formulations measured with flow cytometry as percentage DiD+ (Liposome+) DCs, or (D) with the ImageStream internalization score after 1-hour incubation at 37 °C (C, D), or 1-hour incubation at 4 °C (E, F). G) moDC uptake of liposomes in time, as revealed by flow cytometry (left panel), or imaging cytometry (right panel) measurement. Lipid concentration 30  $\mu$ g/ml. N=3–5. Error bars indicate mean  $\pm$  SEM. \* $p \leq 0.05$ . \*\* $p \leq 0.01$ . \*\*\* $p \leq 0.001$ . Statistical significance was calculated using mixed-effects analysis with Tukey's correction for multiple comparisons.



**Figure 3.** Expression of moDC surface markers of maturation and moDC cytokine production does not change upon overnight incubation with cargo-less liposomes. (A) Representative examples of moDC maturation after overnight stimulation with LPS compared to unstimulated control (GM-CSF only) (in blue immature DCs, in red LPS-stimulated DCs). (B) Induction of CD86, CD83 or HLA-DR MFI in LPS-stimulated DCs compared to the respective MFIs of the immature GM-CSF DC-condition, with or without liposomes. Lipid concentration 30  $\mu$ g/ml. N=5-8. Mean and SD of CD86 MFI, CD83 MFI and HLA-DR MFI for unstimulated moDCs were  $2072 \pm 912$ ,  $6015 \pm 1948$ ,  $737 \pm 149$  respectively, while for LPS-stimulated moDCs the values were  $5759 \pm 2239$ ,  $8616 \pm 2297$  and  $2575 \pm 1310$ . (C) Induction of cytokine production of IL-10, IL-12, IL-6 or TNF- $\alpha$  in LPS- or for IL-12 LPS + IFN- $\gamma$ -stimulated moDCs, normalized to the control condition without liposomes. Lipid concentration 30  $\mu$ g/ml. N=5-7. Mean  $\pm$  SD of LPS-stimulated IL-10 production, IL-6 or TNF- $\alpha$  production were  $553 \text{ pg/ml} \pm 390 \text{ pg/ml}$ ,  $9.7 \text{ ng/ml} \pm 7.3 \text{ ng/ml}$ ,  $1.74 \pm 1.6 \text{ ng/ml}$ , while for LPS + IFN- $\gamma$  stimulated IL-12 production it was  $3.8 \text{ ng/ml} \pm 2.9 \text{ ng/ml}$ . Error bars indicate mean  $\pm$  SEM. Statistical significance was calculated using mixed-effects analysis with Dunnett's correction for multiple comparisons.



**Figure 4.** Neutral or anionic liposomes associate better with crawl-out DCs migrating from skin biopsies compared to cationic liposomes, while adhering less to cultured skin-cells obtained from skin biopsies. PBS or DiD-labeled liposomes were injected in ex vivo human skin, after which a biopsy was taken from the injection site and cultured for 3 days in IMDM supplemented with GM-CSF. Harvested skin crawl-outs were stained for CD11c and HLA-DR to identify crawl-out DCs and expression of CD1a and CD14 was used to identify CD1a+ DDCs, CD1a++ LCs and CD14+ DDCs among crawl-out DCs. Liposome uptake was measured with flow cytometry. (A) Representative example for gating crawl-out DCs and the skin DC subpopulations present in them. In each subpopulation, liposome+ DCs were gated based on % liposome+ cells. (B) Percentage liposome+ crawl-out DCs per liposome-



**Figure 5.** Injection of cargo-less liposomes does not differentially affect migration of skin crawl-out populations. (A) Counts of CD11c+ HLA-DR+ crawl-out DCs. (B) Percentages (upper panels) and counts of CD1a+ DDCs, CD1a+ LCs and CD14+ DDCs present in crawl-out DCs, per liposome-injection condition. Lipid concentration injected 628  $\mu\text{g}/\text{ml}$ . N= 5-12. Error bars indicate mean  $\pm$  SEM. Statistical significance was calculated using mixed-effects analysis with Dunnett's correction for multiple comparisons.

However, we could not confirm adjuvant effects of any of our formulations on human moDCs or skin DCs.

Our study is one of the very few comparing the interactions of a range of liposomal formulations with human moDCs and skin DCs. A large number of studies have examined liposome uptake by bone-marrow-derived DCs (BMDCs) in mice, with more minor studies focusing on human cells.<sup>10,27–30</sup> Moreover, a majority of studies measure liposome internalization with flow cytometry, unable to distinguish cellular uptake from membrane adherence. Hence, the question remained of which type of liposomes may be taken up most by DCs, thereby showing the best potential for offloading therapeutic cargo. Imaging flow cytometry has the additive value of integrating microscopy with flow cytometry. Indeed, comparative measurements with this technique enabled us to visualize that negatively or neutrally charged formulations (DPPC, DSPG, DPPS, and EPG-Na) were efficiently internalized in an incubation range of 2–24 h. Cationic formulations DPTAP and DOTAP, on the other hand, tended to adhere to

the moDC membrane. Even though we did not standardize all six included formulations for rigidity, three of the formulations were of standard rigidity, only differing in the charged lipid incorporated in them (DSPG, DPTAP, and DPPS), emphasizing that differential uptake of these liposomes can be attributed to electric charge.<sup>10,25</sup> However, we can not guarantee this is true for the rest of the formulations, where we only standardized for size, leaving potential differences in rigidity as an alternate explanation for differential interactions with DCs. A higher percentage of skin crawl-out DCs was also positive for the anionic and neutral formulations compared to cationic DPTAP or DOTAP. These cationic formulations readily adhered to single cells from skin biopsies, either DCs or structural cells. Hence, in contrast to the prevailing message in literature, we highlight that cationic formulations may not provide a therapeutic advantage over anionic or neutrally charged liposomes, as these formulations rather adhere to cell membranes and are therefore internalized less swiftly by DCs. However, DCs take up anionic and neutral formulations efficiently,

type. Lipid concentration for injections was 628  $\mu\text{g}/\text{ml}$ . N= 5-12. (C) Percentage liposome+ CD1a+ DDC (left panel), liposome+ LC (upper panel), and liposome+ CD14+ DDC. N= 5-12. (D) Liposome+ cells in bulk single cells of digested biopsies or in biopsy cell subpopulations as indicated. Empty biopsies were enzymatically digested and cultured for 2 h with liposomes. After incubation cells were washed and stained with anti-CD3, CD45, CD11c and HLA-DR. Lipid concentration 100  $\mu\text{g}/\text{ml}$ . N= 2-3. Error bars indicate mean  $\pm$  SEM. \* $p \leq 0.05$ . \*\* $p \leq 0.01$ . \*\*\* $p \leq 0.001$ . \*\*\*\* $p \leq 0.0001$ . Statistical significance was calculated using mixed-effects analysis with Tukey's correction for multiple comparisons.

which yields an essential argument for their use in DC-modulating in vivo therapies. Surprisingly, when making single-cell suspensions of biopsies that were injected with liposomes, we could not detect more cationic liposome+ cells not in the DCs nor in the non-immune cells. We speculate that these liposomes also adhere to the extracellular matrix present in the biopsies, which is lost after digestion. Indeed, when incubating skin-derived single-cell suspensions with different types of liposomes and analyzing the uptake by flow cytometry, we found high binding of cationic liposomes both in DCs as well as structural cells.

An additional disadvantage of utilizing cationic liposomes lies in their toxicity, as these particles form pores in cell membranes and cause membrane disruption.<sup>30,31</sup> In anticancer in vivo vaccine platforms targeting cancer cells, this may be considered an advantage. With regards to DC modulation, however, toxicity adds an unwanted variable to the therapeutic platform. These findings imply that therapeutic cargo would have to be loaded very efficiently into cationic formulations to avoid high lipid concentrations. However, peptide and protein antigens can often only be loaded with encapsulation efficiencies of 1–10% in liposomal formulations, making cationic liposomes the less optimal choice for antigen-specific immune modulation.

Considering mode of internalization, we observed significant inhibition of internalization of both DPPC and DSPG liposomes with Cytochalasin D to levels of the cold control condition. This points to an essential role of an active process of uptake involving phagocytosis and macropinocytosis. We also observed a slight involvement of calcium-dependent processes in DC-uptake of these formulations, suggesting a possible role for C-type lectins or other calcium-dependent receptors, which could be exploited in the future for targeting DCs in vivo. Depending on particle size and cell types used, neutral or anionic liposomes have been described to be internalized with various mechanisms, including actin-dependent but also actin-independent processes.<sup>32</sup> This may be of influence on how therapeutic cargo is processed in DCs.<sup>33</sup> Therefore, establishing the mode of uptake of the specific formulations we used in human DCs contributes to a well-established selection of the formulation we will select for loading immune-modulating compounds.

Favorable uptake kinetics together with a bona fide adjuvant effect could synergize with immune modulation affected by adjuvants and antigens. Cationic liposomes are generally regarded as DC-activating while some anionic formulations, such as PS or DSPG, also featured in this study, were attired with tolerogenic effects on BMDCs as well as human DCs.<sup>6,10,25,34</sup> These results suggest that cationic formulations may be used for DC-activating therapies, while anionic formulations could be selected for tolerogenic DC-modulation. Thus, we also investigated potential adjuvant effects of the empty formulations included in this study and, to our surprise, could not confirm adjuvant effects on moDCs, as measured by maturation markers CD83, CD86, and HLA-DR, and cytokine production of IL-10, IL-12, TNF- $\alpha$  or IL-6. We could also not detect a modulating effect of our formulations on migration of skin DCs after injection, repeatedly confirming their status as carrier vesicles without additional immune-modulating properties. This discrepancy could partly be explained by species differences in DCs used.<sup>35</sup> Furthermore, liposomes referred to as 'empty' often carry a model antigen, such as OVA or targeting moieties attached to the lipid membrane,<sup>7,9,28</sup> complicating deductions on adjuvant qualities of the lipid vesicles. Finally, liposomes can be prepared in various sizes, with a large plethora of lipid compositions, which can lead to differing adjuvant effects. For example, liposomes containing bile salts smaller than 200nm induced T helper type 2 immunity in mice, while vesicles with a larger, polydisperse size range (Z-average diameter 980nm) induced T helper type 1 immunity.<sup>36</sup> Similarly, cationic liposomes composed of dimethyldioctadecylammonium and trehalose dibehenate larger than 2 $\mu$ m activated

IL-10 production upon in vivo injection, while vesicles around 500nm promoted IFN- $\gamma$  production by mouse splenocytes.<sup>37</sup> Hence, we can not exclude different results with different liposomes. Lack of adjuvanticity in our experimental setting may be advantageous, as the loaded therapeutic cargo will determine DC-activating or tolerizing qualities instead of the formulation chosen for the therapy.

Thus, we thoroughly evaluated liposome uptake results and adjuvant effects in a human setting with a close examination of DC-liposome kinetics. Even though we did not confirm the adjuvant effects of the formulations, we showed that both moDCs and skin DCs favorably interacted with our anionic or neutral liposomes. Based on our results, we suggest that neutral or anionic liposomes may be more suitable for DC-modulation and DC-targeted skin vaccination.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Payment to institute by Health Holland and Samenwerkende Gezondheidsorganisaties (SGF) Grantnr: LSHM18065-SGF. Payment to institute by Health Holland – TKI-LSH PPP Allowance – Grant nr. LSHM19073, European Commission, NWO-TKI, AB Enzymes, Angany Inc.

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## Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.xphs.2022.01.022.

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