Interaction of dendritic cells with antigen-containing liposomes: effect of bilayer composition

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(submitted)
Abstract

Vaccine efficacy might be improved by exploiting the potent antigen presenting properties of dendritic cells (DC), since their ability to stimulate specific major histocompatibility complex-restricted immune responses has been well documented during the recent years. In that light, we investigated how the interaction of antigen-containing liposomes with DC was affected by the bilayer composition. Monocyte-derived human DC and murine bone marrow-derived DC were analyzed and compared upon in vitro incubation with liposomes by flow cytometry and confocal microscopy. Anionic liposomes with a bilayer composition of phosphatidylcholine, cholesterol and phosphatidylglycerol or phosphatidylserine interacted with a limited fraction of the total DC population in case of both DC types. Inclusion of mannosylated phosphatidylethanolamine for targeting to the mannose receptor increased the interaction of negatively charged liposomes with both human and murine DC. This increase could be blocked in human DC by addition of the polysaccharide mannan indicating that uptake might be mediated by the mannose receptor. Cationic liposomes containing trimethyl ammonium propane interacted with a very high percentage of both DC types and could be detected in high amounts intracellularly. In conclusion, liposome bilayer composition has an important effect on interaction with DC and might be critical for the vaccination outcome.
Introduction

Dendritic cells (DC) are highly specialized cells of the immune system with antigen presenting properties important for initiating adaptive immune responses [1]. In the developmental state referred to as immature cells, DC patrol and take up antigens in peripheral tissue, and process them into peptides, which are loaded into MHC class I and II molecules. Upon encounter with pathogens and/or inflammatory stimuli, DC maturation is initiated. The cells migrate to secondary lymphoid organs and are turned into efficient presenters of antigens to T-cells resulting in activation of naïve T-cells and thereby in initiation of antigen specific immune responses or immunological tolerance.

Recent advances in the research area of DC biology combined with the urgent need for improved vaccines regarding antigen delivery capacity and adjuvant potency has led to the hypothesis that exploitation of DC properties might be a rational way of improving vaccine efficacy [2,3]. Modulating DC features with appropriately designed vaccines could possibly result in the generation of enhanced specific T-cell responses and thereby more efficient combat of pathogens. One area of DC biology that is of interest when investigating the adjuvant role of DC in vaccine design is antigen uptake and presentation by DC. Enhancement of the interaction between the vaccine formulation and DC could be a potential method for increasing vaccine immunogenicity. In the present study we aim at investigating the interaction of liposomal antigen formulations containing the *Neisseria meningitidis* type B antigen PorA with human monocyte-derived DC (MoDC) and murine bone marrow-derived DC (BMDC) *in vitro*. To our knowledge, no studies so far have reported on the effect of liposomal lipid composition on the interaction with human MoDC and murine BMDC.

Liposomes have potential as efficient presentation form for PorA in the design of vaccines against type B meningococcal infections [4]. Liposomes not only mimic the natural outer membrane environment of PorA but they also allow the manipulation of the formulation, by e.g. the attachment of targeting ligands or the variation of the (membrane) composition.

We analyzed the interaction of different liposomal formulations of meningococcal PorA (see chapter 4 of this thesis) with human Mo-DC and murine BMDC by flow cytometry and confocal laser scanning microscopy (CLSM).
Negatively charged liposomes with phosphatidylglycerol (PG) or phosphatidylserine (PS) and cationic liposomes containing trimethyl ammonium propane (TAP) as charged phospholipids were used. Also, liposomes with mannosylated phosphatidylethanolamine (Man-PE), which is supposed to be recognized by the DC surface molecule mannose receptor (MR), were studied. The possibility to block the interaction of mannosylated liposomes with DC by addition to the culture medium of mannan, a polysaccharide of mannose, was investigated. The data obtained in our studies, and its relevance for the development of efficient vaccine formulations, are discussed.

**Material and methods**

**Materials**

Dimyristoyl phosphatidylcholine (PC) was purchased at Rhône-Poulenc Rorer (Köln, Germany). Dimyristoyl phosphatidylglycerol (PG) was a gift from Lipoïd GmbH (Ludwigshafen, Germany). Dimyristoyl phosphatidylserine (PS), dimyristooyl trimethylammoniumpropane (TAP) and dimyristoyl phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Texas Red® 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine and DiD, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbo-cyanine, 4-chlorobenzenesulfonate salt, were purchased from Molecular Probes (Leiden, the Netherlands). α-D-mannopyranosyl phenyl isothiocyanate, cholesterol (Chol), n-octyl β-d-glucospyranoside (OG) and Molybdenum Blue spray reagent were obtained from Sigma (Zwijndrecht, the Netherlands). analytical and preparative silica gel 60 F$_{254}$ TLC plates were obtained from Merck, Darmstadt, Germany. Water purified through reversed osmosis was applied throughout the study. All other chemicals were of analytical grade or the best available.

**Synthesis of mannosylated-PE**

\[ N-(3-(4-(\alpha-D-Mannopyranosyl)phenylthiureido)-L-1,2-dimyristoyl-3-phosphatidylethanolamine \ (Man-PE) \] was prepared by ethyldiisopropylamine-promoted coupling of PE and α-D-mannopyranosylphenyl isothiocyanate in
chloroform/methanol (7:1 v/v). The compound was purified by preparative thin layer chromatography. Its nature was confirmed by $^1$H- NMR and LC-MS.

**Preparation of PorA-liposomes**

PorA was purified from outer membrane vesicles obtained from *Neisseria meningitidis* strain F91 (P1.7-2,4, PorB$, RmpM$, low expression of Opa/Opc) as previously described [4]. Prior to incorporation of PorA into liposomes, the protein was precipitated with 80% (v/v) ethanol at -20°C and dissolved in 150 mM OG in Tris-buffered saline (TBS, containing 50 mM Tris-HCl, 150 mM NaCl pH 7.4). Appropriate amounts of each lipid were dissolved in chloroform/methanol (2:1) in a round bottom flask and a lipid film was obtained by solvent evaporation in a rotavapor under reduced pressure. The film was re-suspended in the PorA solution and the resulting mixed micelles were rapidly diluted in TBS, allowing the formation of unilamellar vesicles [5]. Subsequently, the liposomes were pelleted by ultracentrifugation (160,000 g, 1 hour) and resuspended in TBS, obtaining liposomes with an epitope density of approximately 20 µg protein/µmol lipid. PorA liposomes were made with four different lipid bilayer compositions; PC:PG:Chol (8:2:2 molar ratio) (PG-liposomes); PC:PG:Chol:Man-PE (8:2:2:0.6 molar ratio) (Man-liposomes); PC:PS:Chol (8:2:2 molar ratio) (PS-liposomes); PC:TAP:Chol (8:2:2 molar ratio) (TAP-liposomes). All formulations included 0.2 mol% Texas Red-DMPH or DiD as fluorescent marker in the bilayer.

**Characterization of PorA-liposomes**

The average particle size and polydispersity index of PorA-liposomes were determined by dynamic light scattering at 25°C with a Malvern 4700 system using a computer with PCS software (version 1.35, Malvern). For viscosity and refractive index the values of pure water were used. The particle size distribution was reflected in the polydispersity index, which ranges from 0 for a monodisperse to 1.0 for an entirely heterodisperse dispersion. The zeta potential of the liposomes in 3% sucrose was measured with a Malvern Zetasizer 2000 with an aqueous dip-in cell and a computer with PCS software (version 1.35, Malvern). The phospholipid content of the liposomes was determined by the phosphate determination according to Rouser [6]. The protein content was determined according to Peterson [7].
Culture of human DC from peripheral blood

DC were generated from human peripheral blood monocytes according to previous reports [8,9]. The study was approved by the ethics committee at the Karolinska Hospital, Stockholm, Sweden, and by the local ethics committee in Copenhagen, Denmark, (KF01-020/00). Briefly, peripheral blood mononuclear cells were isolated from healthy volunteer blood donors, by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). The cells were resuspended to $5 \times 10^6$ cells/ml in complete medium and allowed to adhere for 35 min. at $37^\circ$C in tissue culture flasks. Non-adherent cells were removed by PBS washes. Adherent cells were cultured for 6 days in complete medium (RPMI 1640) supplemented with 2 mM L-glutamine (GibcoBRL, Life Technologies, Stockholm, Sweden), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), 10% heat inactivated fetal calf serum (Sigma-Aldrich, Stockholm, Sweden), in the presence of 60 ng/ml human recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) (Leucomax, Sandoz, Basel, Switzerland) and 50 ng/ml human recombinant interleukin 4 (IL-4) (Schering-Plough, Kenilworth, NJ, USA). For liposome uptake experiments, immature DC were harvested at day 6 of culture and seeded in 24-well tissue culture plates in 0.5 ml complete medium, $2 \times 10^5$ cells/well. For the mannan-inhibition experiment, 3 mg/ml mannan (Saccharomyces cerevisiae, Sigma-Aldrich A/S, Denmark) was added to the medium. Liposomes were added to the wells (20, 80 or 200 nmol lipid per well) and the mixture was incubated 2 or 4 hours at $37^\circ$C. As controls for endocytosis, cells were incubated in medium alone or with non-labeled liposomes (20 or 200 nmol lipid per well) or with Alexa-488-labeled, heat inactivated E. coli (Molecular Probes, Leiden, the Netherlands).

Culture of murine DC

Balb/cOlaHsd mice were obtained from Harlan (Horst, the Netherlands) and maintained under conventional conditions at the Central Animal Laboratory (Utrecht University, the Netherlands). All experiments were done with 8-12 weeks old animals and were approved by the Utrecht University Animal Ethics Committee. BMDC were obtained as described by Inaba et al. [10]. Briefly, bone marrow was flushed from mouse femurs, erythrocytes were lysed and cells were grown at $1 \times 10^6$/ml in filtered
culture medium consisting of RPMI 1640 medium with 10% fetal bovine serum, 50 IU/ml penicillin and 50 µg/ml streptomycin in the presence of 10 ng/ml murine GM-CSF (Immunex, Seattle, WA) and 50 ng/ml murine IL-4 (R&D Systems, Minneapolis, MN). Non-adherent cells were re-plated on day 1 and cells still non-adherent at days 2 and 4 were removed from the cultures, with concomitant refreshment of culture medium. On day 7, non-adherent and loosely adherent cells were harvested. These cells were MHC class II⁺, CD11c⁺ and CD11b⁺. From these, 3x10⁶ cells per well were plated with fresh culture medium together with 80 nmol phospholipid in PorA-liposomes and further incubated for 4 hours at 37°C. As controls, cells were incubated in medium alone or with non-labeled liposomes. After incubation, cells were harvested and analyzed.

Flow cytometry analysis

For FACS analyses of human DC surface markers, cells were incubated with mouse serum (DAKO, Denmark) for blocking of unspecific binding sites and subsequently stained with fluorescein isothiocyanate (FITC) conjugated monoclonal antibody against human leukocyte antigen (HLA) DR (clone L243, Becton Dickinson, San Jose, CA, USA). Mouse IgG2a served as isotype control (clone X39, Becton Dickinson). Fixation was performed with 1% paraformaldehyde. The cells were analyzed by a FACS Calibur flow cytometer (Becton Dickinson) using the CellQuest software (Becton Dickinson). Dead cells were gated out based on their light scattering properties. For the studies with murine DC, the following was used: FITC-labeled monoclonal antibody against CD11b (clone M1/70) and biotinylated mAb against CD11c (clone HL3) were obtained from BD PharMingen (San Diego, CA). Streptavidine-phycoerytrin was purchased from Becton Dickinson (San Jose, CA). M5/114 anti-class II [11] was kindly provided by Dr. Georg Kraal (Free University, Amsterdam, the Netherlands). Murine DC (1x10⁵) were blocked with 5% heat-inactivated mouse serum in PBS for 30 min at room temperature (RT). Blocked cells were then washed with FACS buffer (PBS, 0.1% azide, 1% BSA) and incubated with relevant antibodies for 20 min at room temperature. After this time, cells were washed and, if required, incubated with a specific secondary antibody. Negative controls consisted of unstained cells, cells stained with isotype control antibody, or cells stained with only the secondary antibody. Cells were analyzed by flow cytometry using a FACSCalibur flow cytometer and Cell Quest software (BD
Biosciences). The Win MDI 2.8 program was kindly provided by Joseph Trotter (available on http://facs.scripps.edu/) and was used for further analysis of FACS-data.

Confocal laser scanning microscopy

For staining of CD1a the human cells were harvested, washed with ice-cold PBS and fixed for 15 min. in 1% paraformaldehyde on ice. Staining was performed with a primary monoclonal mouse anti human CD1a antibody (clone BL6, Immunotech, Beckman Coulter AB, Sweden) followed by a FITC-conjugated donkey anti-mouse IgG (AffiniPure, Jackson Immunoresearch Laboratories, Inc., West Grace, PA, USA) in PBS with 4% BSA. The cell preparations were washed with PBS and mounted for CLSM. Visualization of fluorescent signals and transmitted light was performed with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, GmBH, Germany) equipped with an argon laser (458 and 488 nm) and a HeNe laser (543 nm) using the LSM 510 software. Murine cells (1x10^5), incubated with PorA-liposomes under similar conditions as for flow cytometry studies, were washed with 2 ml PBS and subsequently fixed in 4% formaldehyde in PBS for 20 min at room temperature. After fixation, cells were washed twice with PBS and mounted on glass slides with FluorSave reagent (Calbiochem, San Diego, CA). Fluorescent and transmitted light microscope images of cells were taken simultaneously using a Leica TCS-SP microscope equipped with an 488 nm Argon, 568 nm Krypton and 633 nm HeNe laser and analyzed using Leica TCS-SP Power Scan software (Leica Microsystems, Rijswijk, the Netherlands).

Results

Characterization of liposomes

The composition and characteristics of the liposome formulations are summarized in Table 1. The PorA-containing liposomes had a mean particle size of around 200 nm. Incorporation of mannosylated phospholipid resulted in formation of liposomes with less negative surface charge and larger size as compared to PG-liposomes. TAP-liposomes exhibited, as expected, a high positive surface charge.
Table 1. Characteristics of PorA liposomes.

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>Composition</th>
<th>PorA:lipid ratio (µg/µmol)</th>
<th>Sizea (nm)</th>
<th>PDb</th>
<th>Zeta potentiala,c (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>PC:PG:Chol (8:2:2)</td>
<td>15.0</td>
<td>180 ± 0.4</td>
<td>0.2</td>
<td>-54.2 ± 1.6</td>
</tr>
<tr>
<td>Man</td>
<td>PC:PG:Chol:Man-PE (8:2:2:0.6)</td>
<td>26.0</td>
<td>279 ± 1.2</td>
<td>0.3</td>
<td>-37.3 ± 2.5</td>
</tr>
<tr>
<td>PS</td>
<td>PC:PS:Chol (8:2:2)</td>
<td>21.3</td>
<td>203 ± 2.3</td>
<td>0.3</td>
<td>-50.0 ± 0.1</td>
</tr>
<tr>
<td>TAP</td>
<td>PC:TAP:Chol (8:2:2)</td>
<td>29.5</td>
<td>204 ± 1.9</td>
<td>0.3</td>
<td>44.2 ± 1.4</td>
</tr>
</tbody>
</table>

aValues represent mean ± S.D., n = 3.
bPolydispersity: indication of the size distribution of the liposomes; ranges from 0.0 for a monodisperse to 1.0 for an entirely heterodisperse suspension.
cZeta potential was measured in a 3% solution of sucrose.

FACS analysis of liposome association with DC

FACS analysis allows assessment of cell-associated fluorescence and was used to examine interaction of Texas Red- or DiD-labeled liposomes with DC. Immature DC were generated from human peripheral blood mononuclear cells and from murine bone marrow cells. We have previously reported the characteristic surface marker expression of normal human DC upon culturing under the described conditions (see Material and Methods). The human DC lack lineage-specific markers expressed on other leukocyte populations (CD3, CD14, CD19 and CD56) and express CD1a, CD40, MHC class I and II [12]. The level of CD83 expression is low on immature DC. The murine cultures were MHC class II⁺, CD11c⁺ and CD11b⁺ and had a purity of approximately 90% (results not shown) comparable to DC described by Inaba et al. [10]. The human DC were incubated with the fluorescent liposome formulations at 37°C for 2 or 4 hours, harvested and stained with a FITC-labeled monoclonal mouse-anti-human HLA-DR antibody. By FACS-analysis, the percentage of double-positive cells (HLA-DR⁺, liposome-Texas Red⁺) of the gated cells was determined. For comparison, we also studied the interaction of liposomes with murine BMDC. Incubation was done with DiD-labeled liposomes for 4 hours and the percentage of DiD-positive cells was determined after gating of the DC similarly to the human DC.
In case of human DC, a striking difference in the percentage of double positive cells was observed after 2 hours incubation between the negatively charged PG- or PS-liposomes and the positively charged TAP-liposomes. While almost the entire DC population incubated with TAP-liposomes had high levels of the fluorescent marker associated, only less than 2% of the cell population had slightly increased levels of fluorescent marker associated when incubated with PS/PG-liposomes (Fig. 1). Increasing the incubation time to 4 hours did not change the number of double positive cells significantly. The same pattern was seen with murine bone marrow-derived DC (Fig. 2) although the frequency of fluorescence positive cells
varied. These results indicate that the charge of the liposome formulation plays an important role for the level of association of the liposomes with DC.

![Diagram](image)

**Figure 2.** FACS analysis of murine DC after 4h incubation of $3 \times 10^6$ cells with DiD labeled or unlabeled liposomes (80 nmol phospholipid) followed by staining for MHCII (or second antibody alone as control in A, lower right) with a FITC-labeled antibody. Experiments were repeated at least twice. Panel A: dot plots showing double immunofluorescent cells of gated DC populations; upper left: unlabeled TAP-liposomes; upper right: labeled TAP-liposomes; lower left: labeled PG-liposomes; lower right: secondary antibody control. Panel B: histograms showing interaction of liposomes with gated, HLA-DR-positive DC (---- unlabeled liposomes, — DiD-labeled liposomes), upper panel: PG-liposomes; lower panel: TAP-liposomes. Panel C: percentage of DiD positive cells of gated cells incubated with non-labeled liposomes was determined (corresponds to M1 in B)

**Effect of liposome mannosylation on their binding with and uptake by DC**

In order to study whether mannose receptor (MR)-targeting could enhance liposome binding to and uptake by DC, a mannosylated lipid was incorporated into the liposomes. A concentration-dependent increase in the percentage of double positive cells was observed in human and murine DC cultures (Fig. 3). Moreover, a time dependent increase in the percentage of double positive cells was seen after 4 hours (results not shown). To test whether the MR could mediate the enhanced interaction, uptake of Man-liposomes was measured in the presence or absence of 3
mg/ml of mannan in the medium, a bacterial polysaccharide that binds with high affinity to MR. At this mannan-concentration, maximum inhibition is achieved [13]. As shown in Fig. 3, mannan reduced the percentage of double-positive human DC to background levels when adding 200 nmol Man-liposomes to $2 \times 10^5$ DC. Addition of mannan to the medium of human DC cultures pulsed with TAP-liposomes had no effect on the percentage of double positive cells, while uptake of fluorescently labeled *E. coli* (i.e. whole bacteria with sugar residues in the surface) is completely inhibited by inclusion of mannan in the medium (data not shown). These results obtained with human DC indicate that uptake is mediated by MR. However, the same effect was not observed in the murine BMDC cultures (Fig. 3).

**Figure 3:** FACS analysis of human and murine DC after 2 hours incubation of $2 \times 10^5$ cells with Texas-red labeled Man-liposomes (20 or 200 nmol phospholipid) or unlabeled Man-liposomes (200 nmol phospholipid) or Man-liposomes (200 nmol phospholipid) where 3 mg/ml mannan was included in the medium, followed by staining for HLA-DR or MHCII with a FITC-labeled antibody. Experiments were done in triplicate. **Panel A:** Histogram showing interaction of liposomes with gated, HLA-DR-positive DC (.... unlabeled Man-liposomes, --- Texas-red labeled Man-liposomes). **Panel B:** Percentage of HLA-DR/Texas red positive cells of gated cells incubated with non-labeled liposomes (corresponds to M1 in A). **Panel C:** FACS analysis of murine DC after 4 hours incubation of $3 \times 10^6$ cells with DiD-labeled Man-liposomes (concentrations as indicated) with or without 3 mg/ml mannan in the culture medium.
**Intracellular localization of liposomes by CLSM**

To obtain information about the cellular localization of DC-associated liposomes, CLSM was used. In order to study the localization of liposomes in DC, either intracellularly or on the DC membrane surface, human cells were incubated with fluorescent liposomes, then fixed and stained for surface CD1a expression. CD1a is considered as a human DC-specific marker under the used culture conditions. While incubation of DC with Texas red-labeled PG-, PS- and Man-liposomes gave no detectable signal in CD1a-positive cells by CLSM, incubation with TAP-liposomes heavily stained the intracellular region of the CD1a-positive cells (Fig. 4). The nuclei were free of fluorescence. This shows that at least the fluorescent label is present intracellularly. The localization was examined in murine DC as well (Fig. 5). After 4h incubation of negatively charged (PG and PS) liposomes with murine DC, low levels of cell-associated fluorescence were observed. Incubation with mannosylated liposomes resulted in increased cell-associated fluorescence in contrast to what was observed in the human MoDC. In murine BMDC, the fluorescence was punctated and located intracellularly (Fig. 5). When positively charged liposomes were used, high levels of fluorescence associated with the cells were observed, both in the intracellular compartment and on the cell surface (Fig. 5).

**Figure 4** Two-color immuno-fluorescence of the association of TAP-liposomes with DC. Human DC were incubated 2 hours with Texas-red labeled liposomes (Panels A-C) (red) or unlabeled liposomes as control (Panel D), fixed and stained with a primary antibody to CD1a (Panels A-C) or control IgG (Panel D) and a secondary FITC-labeled anti-CD1a antibody (green). Visualization was done by CLSM.
Interaction of dendritic cells with antigen-containing liposomes

Discussion

Liposomes have the ability to enhance immune responses against incorporated peptide and protein antigens making liposomes interesting adjuvants for vaccine delivery [14,15,16]. Liposomal vaccine formulations are usually given as a limited number of small doses, either by intramuscular or subcutaneous injection. A part of their adjuvant effect is believed to be caused by the formation of an antigen depot at the injection site and/or by the facilitated delivery of antigen to antigen presenting cells [17].

A prerequisite for antigen presentation is the introduction of exogenous antigen into the cytosol or the endosomes of antigen presenting cells. Exogenous material including liposomal antigens is usually taken up by endocytosis and presented by the MHC class II presentation pathway. In addition, liposomes (especially pH-sensitive and fusogenic) have the ability to deliver antigens to the cell cytoplasm, and liposome-associated antigens are thus presented by the MHC class I [18]. The main research focus has until now been on macrophage-liposome interaction. The powerful antigen presenting function of DC, which is considered as the most important antigen presenting cell, led us to investigate how two types of DC cultures (murine bone-marrow derived and human monocyte-derived) interact with liposomes with different liposome-bilayer composition.
Our results establish that bilayer composition has a pronounced impact on the level of interaction and uptake of liposomes by human monocyte-derived and murine bone marrow-derived DC. While anionic PG- and PS-liposomes hardly interacted with human DC at all, we observed an intensive interaction of cationic TAP-liposomes with both human and murine DC, resulting in dense intracellular localization of fluorescent label. This is in accordance with the published observation that cationic microparticles are phagocytosed by DC to a much greater extent than anionic particles [19]. The mechanism behind this is suggested to be an electrostatic interaction between the negatively charged cell surface and the net positively charged liposomal surface.

This charge-effect is observed despite the presence of serum proteins in the medium that may adsorb onto microparticulate surfaces and modify their surface characteristics [20,21]. Specific serum proteins such as IgG, complement factors and fibronectin facilitate recognition and phagocytosis by antigen presenting cells of microparticles by adsorption to the surface [22]. Thiele et al. [21] hypothesize that the positive surface charge provides an initial strong trigger mediating recognition and uptake by DC, which is even superior to the presence of opsonins adsorbed to the particle surface.

The differences we observe between human MoDC and murine BMDC in degree of liposome-DC interaction might be caused by differential interaction between the two cell types with serum proteins adsorbed onto the liposomal surface. Bias of results could be due to the presence of species-different serum components or serum-interacting components on the cell surfaces. Other factors that should be considered are the differences on the labels used, source of cells, culture conditions and differentiation patterns.

The MR is a surface 175 kD C-type lectin with 8 carbohydrate recognition domains and has broad substrate specificity for sugars [23]. The MR is expressed on several different cell types, among them monocyte-derived human DC [13] and human DC derived from cord blood CD34+ hemopoietic progenitors [24], where it mediates efficient antigen presentation as well. The MR mediates phagocytosis of mannose-coated particles such as bacteria (i.e. *E. coli*) and yeast and endocytosis of mannosylated proteins that are taken up into vesicular structures where the ligand is released, and the MR is transported back to the cell surface [25]. The MR is thereby constitutively recycled between endosomes and the plasma membrane resulting in a
sustained capacity for antigen capture, concentrating large amounts of antigen intracellularly. Uptake of glycosylated antigen via the MR can enhance 100-10,000 fold the uptake of soluble antigen in vitro [25,26]. After release from the MR, antigens are transported to MHC class II compartments and delivered to the MHC class II presentation pathway. We attempted to take advantage of this uptake mechanism by actively targeting the PorA liposome formulation to the DC mannose receptor by attaching a mannose residue to the phospholipid head-group. We found a time- and dose-dependent increase in uptake of Man-liposomes compared to conventional PG-liposomes and in agreement with the results of Copland et al. [27]. Yet the interaction between DC and Man-liposomes was much weaker that in case of the cationic TAP-liposomes.

Whether the enhancement of the interaction by mannosylated liposomes is sufficient to affect the immunological outcome remains to be tested. In vivo, Man-liposomes are not expected to show strong interactions with other, irrelevant cells, whereas TAP-liposomes may bind to any cell in a non-specific way. Thus, the net effect of mannosylation and the use of negatively charged liposomes might still be advantageous over the use of positively charged liposomes. Our results demonstrate that the interaction of a liposomal vaccine formulation with DC can be tuned in vitro by variation of the surface charge of the liposome and by the addition of targeting moieties. Other parameters such as liposome size and antigen/liposome ratio might be important for DC interaction as well. Another important question is how liposome-mediated antigen delivery to DC influences the ability of DC to stimulate T-cells. Overloading of DC with antigen could induce tolerant T-cells [1]. Also, the liposomal antigen formulation may interfere in different ways (positively or negatively) with processes such as intracellular processing pathways, DC-maturation and antigen presentation. Human co-culture systems of antigen-loaded DC and peripheral blood mononuclear cells are currently investigated for a functional T-cell stimulation read-out in response to antigen.

Addition of mannan to the medium reduced the interaction with human monocyte derived DC to background level suggesting that uptake was specifically mediated by the MR. However, no blocking by mannan was observed in case of murine BMDC. The question arises whether the MR is involved in uptake of mannosylated entities by the latter DC type. Reis e Sousa et al. could not detect MR in lysates of purified murine Langerhans cells by Western blotting, although a
mannose specific uptake by the cells was identified [28]. The mannose-associated binding activity observed by us and by others in murine DC might thus be due to another (yet unidentified) receptor. Linehan et al. found no expression of MR on DC in vivo in thymus, lymph node, spleen and Peyer's patch of normal mice by in situ hybridization and immunocytochemistry [29]. This brings up the question to which extent cultured DC reflect the properties of DC in situ. Differences in MR-expression patterns in cultures of DC and by DC in situ are important to notice when investigating targeting potentials. A prerequisite for benefiting from a MR-targeting strategy to reach DC more efficiently is that the receptor is expressed by DC at the vaccine administration site and that this expression is sustained for a time period sufficient for antigen uptake.

Though liposomes can be efficient in in vitro delivery of antigens to DC, it should be realized that additional co-stimulatory signals are necessary to successfully induce an immune response in vivo. Liposomes by themselves act as adjuvants. Furthermore, other immunostimulatory adjuvants can be incorporated into the lipid structures to further increase the adjuvant activity of liposomes. The concept of active targeting immunostimulatory drug delivery systems to antigen presenting cells is of evident interest for vaccinology, where an urgent need for the development of more effective vaccines exists [30].

In conclusion, liposome bilayer composition has an important effect on interaction with DC and might be critical for the vaccination outcome.

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References


