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DC-SIGN mediated antigen-targeting using glycan-modified liposomes: Formulation considerations

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ABSTRACT

Dendritic cells (DCs) are key antigen presenting cells that have the unique ability to present antigens on MHC molecules, which can lead to either priming or suppression of T cell mediated immune responses. C-type lectin receptors expressed by DCs are involved in antigen uptake and presentation through recognition of carbohydrate structures on antigens. Here we have explored the feasibility of modification of liposomes with glycans for targeting purposes to boost immune responses. The potential of targeting glycoliposomal constructs to the C-type lectin DC-SIGN on DCs was studied using either PEGylated or non-PEGylated liposomes. Our data demonstrate that formulation of the glycoliposomes as PEGylated negatively affected their potential to target to DCs.

1. Introduction

Dendritic cells (DCs) are the main professional antigen presenting cells (APCs) of the innate immune system and as such play an important role in steering immune responses towards immunity or tolerance. During their routine surveillance in peripheral tissues, immature DCs take up antigens, which are subsequently processed and presented on major histocompatibility complex (MHC) molecules (Mellman and Steinman, 2001). Upon recognition of antigen–MHC complexes via the T-cell receptor, T cells are either primed or silenced. The outcome of this interaction is dependent on the nature of co-stimulatory signals derived from the DC (Kalinski et al., 1999). This whole process makes DCs the "target of choice" for antigen delivery in vaccination against cancer, AIDS or for treatment of autoimmune diseases (Figdor et al., 2004). Depending on the presence or absence of adjuvant stimuli, affecting costimulatory molecule expression by DC, immune responses can be boosted or inhibited. Thus DC targeting strategies can be used for the treatment of cancer or autoimmune diseases.

DCs present exogenous and endogenous antigens differently. Exogenously administered antigens end up in MHC class II molecules after processing in the endo-lysosomal compartments and can be recognized by $CD4^+$ T cells (Janeway and Medzhitov, 2002). By contrast, endogenous proteins are cleaved by the proteasome into peptide fragments, which are loaded into MHC class I molecules in the Endoplasmic Reticulum, and presented on the cell-surface to $CD8^+$ T cells (Janeway and Medzhitov, 2002). However, DCs have the unique ability to present exogenously administered antigens in MHC class I molecules by a phenomenon termed "cross presentation" leading to $CD8^+$ T cell activation (Heath and Carbone, 2001). However, for soluble antigens the process of cross presentation is highly inefficient and requires very high antigen doses.

Antigen delivery to DCs via particulate carriers (e.g. liposomes) offers several advantages. They mimic pathogens such as bacteria and viruses more closely and thus have a higher chance of properly activating DCs compared to free antigen. Moreover, the particulate carrier protects the antigen against premature degradation, thus reducing the quantity of administered antigen needed to evoke an effective immune response. Indeed, it has been shown that, 10- to 1000-fold lower doses of antigen were required for (cross-) presentation to occur if administered as a particle than as soluble antigen (Arigita et al., 2003; White et al., 2006; Sheng et al., 2008). Moreover, the possibility to incorporate adjuvant along with the antigens in a single formulation, ensures delivery of both the antigen as well as the adjuvant to the same APC, will enhance or

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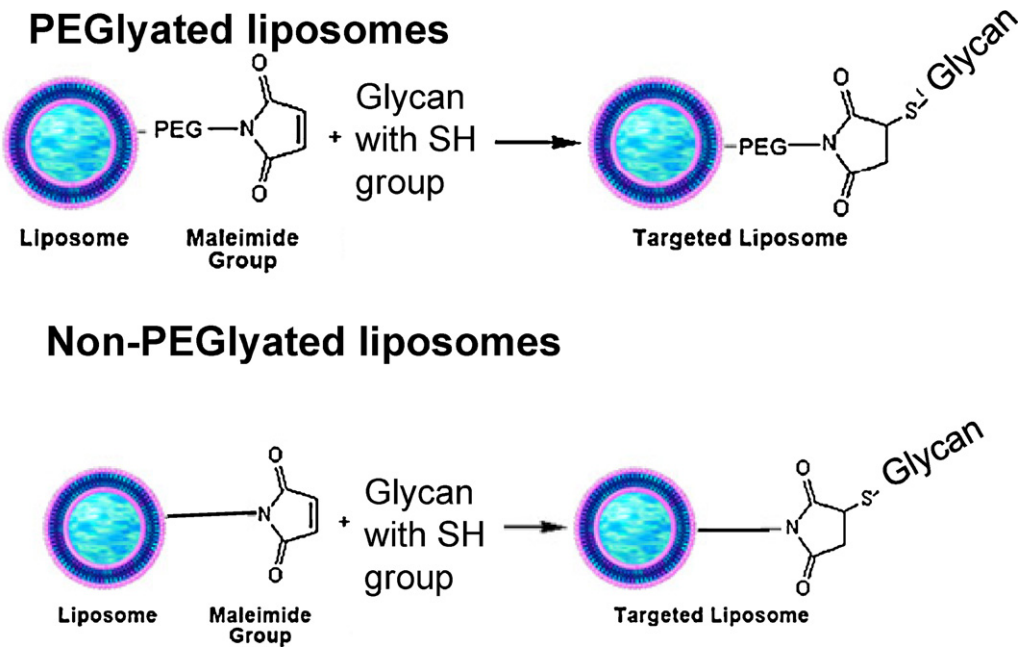

Fig. 1. Reaction schemes for conjugation of glycans on the malemide group of PEGylated and non-PEGylated liposomes.

modulate the immune response more effectively than mixing the components.

In this short research article we have explored the feasibility of incorporating a soluble antigen in a liposomal system for receptor based targeting to DCs. Different receptors (e.g. mannose receptor, Fc receptor, DEC 205) that are expressed by DC have been shown to be involved in the process of antigen binding and uptake. As such, these receptors have been studied for the purpose of targeting of antigens to DCs using antibodies against the receptor as the targeting moieties. However, their expression is not DC-restricted, and therefore formulations may also target other immune cells, resulting in less effective T-cell responses. DCs express C-type lectin receptors (CLR), which are DC-restricted and mediate binding and uptake of carbohydrate structures (e.g. glycans) that are exposed on glycoproteins or glyco-lipids (Figdor et al., 2002). DC-specific intracellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) is a member of the CLR family and expressed on DCs present at mucosal sites, as well as skin, and lymph nodes (Geijtenbeek et al., 2000a). DC-SIGN specifically binds high-mannose glycans and Lewis-type antigens (Appelmelk et al., 2003; van Liempt et al., 2006; Mitchell et al., 2001) which are present on pathogens such as HIV (Geijtenbeek et al., 2000b; Hodges et al., 2007) and Mycobacterium (Geijtenbeek et al., 2003). DC-SIGN recognizes carbohydrate structures through its carbohydrate recognition domain (CRD) in a Ca^{2+}-dependent fashion (Geijtenbeek et al., 2000a).

To mimic physiological recognition of glycoproteins on pathogens, we explored specific targeting of antigens to DC-SIGN using glycan-modified liposomes and compared PEGylated with non-PEGylated glyco-liposomes. We discuss some considerations on the formulation of these glyco-liposomes that affect the specific DC-SIGN targeting potential.

2. Materials

2.1. Lipids

Egg phosphatidyl choline (EPC-35), Ethanolamine Phosphoglyceride (EPG) and cholesterol was obtained from Sigma Chemicals (Sigma, St. Louis, USA), DSPE-PEG maleimide, DSPE-PEG (2000) Maleimide (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (ammonium salt)), MPB-PE (1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide] (sodium salt)) were obtained from Avanti polar lipids (Avanti Polar Lipids, Alabaster, USA).

2.2. Glycans and antibodies

Lacto-N-difucopentose II (Lewis B; Le^B) and lacto-N-fucopentose III (Lewis X; Le^X) were obtained from Dextra labs, UK. Anti-Le^B and anti-Le^X antibodies were obtained from Calbiochem, Damstadt, Germany.

2.3. Chemicals

The soluble antigen ovalbumin (OVA), HEPES, sodium chloride, sodium EDTA and *Salmonella typhosa* lipopolysacharide (LPS) were all obtained from Sigma chemicals (Sigma, St. Louis, MO, USA).

3. Methods

3.1. Preparation of liposomes

Glycan-modified PEGylated and non-PEGylated liposomes containing OVA as the model antigen were prepared from a mixture phospholipids and utilizing the film extrusion method. The PEGylated liposomes were made of a mixture of EPC-35:PEG-mal:PEG-DSPE:Chol at a ratio of 1.85: 0.075: 0.075:1 (Koning et al., 1999), whereas non-PEGylated liposomes were prepared from a mixture of EPC-35:EPG:MPB-PE:Chol at a ratio of 1.5: 0.4: 0.075:1. DID was used a fluorescent marker for the lipid bilayer. Briefly, lipids were dissolved in a mixture of chloroform/methanol (1:1, v/v) in a 50 mL round-bottom flask. A lipid film was obtained by evaporation of the solvent under reduced pressure at 35 °C. After flushing with nitrogen, the lipid film was hydrated in HEPES buffer solution pH 7.5 containing OVA (4 mg/mL) yielding a phospholipid concentration of 10 μmol total lipid/mL. Liposomes were sized by sequential extrusion through two stacked polycarbonate filters (800, 400, and 200 nm) with a high-pressure extrusion device. The liposomes were washed

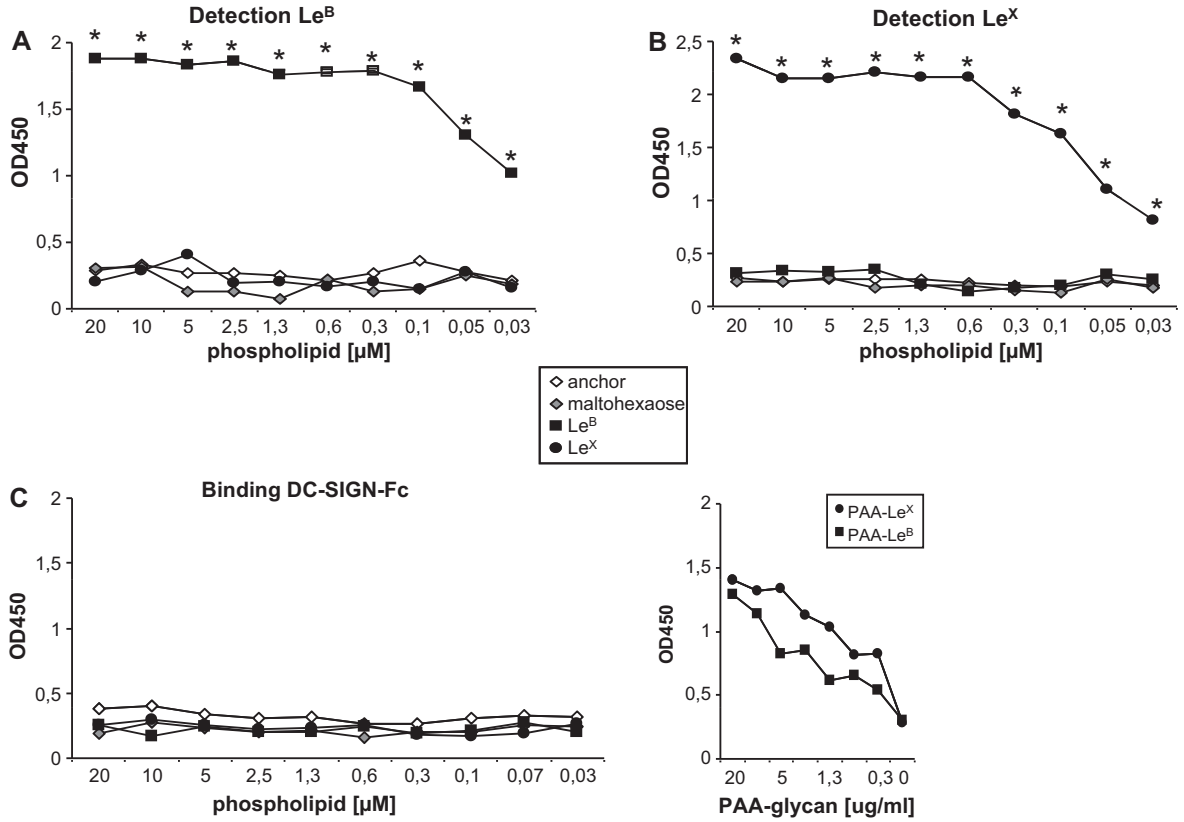

Fig. 2. Conventional PEGylated liposomes can be modified with Le^X and Le^B glycans yet do not bind to DC-SIGN. PEGylated liposomes were prepared and modified with either Le^X or Le^B glycans. (A) Le^B can be detected on Le^B-modified liposomes and (B) Le^X is detected on Le^X-modified liposomes using specific antibodies. (C) Glycan-modified liposomes do not bind DC-SIGN-Fc (left panel); whereas control PAA-glycans do bind DC-SIGN-Fc (right panel). Depicted results are representative of four independent experiments. Significant difference is shown as $^*P < 0.001$ compared to anchor liposomes.

away of non-encapsulated OVA by ultracentrifuging on Beckman Ultracentrifuge at 55,000 rpm and resuspending the pellet three times. The final suspension of liposomes were made in HPEPES buffer pH 7 and labeled as anchor liposomes.

3.2. Coupling of glycans to maleimide activated liposomes

The glycans, Le^X and Le^B, were coupled to both PEGylated and non-PEGylated liposomes through the maleimide group (Fig. 1). Briefly the anchor liposomes were incubated with an excess amount of Le^X and Le^B solution in HEPES buffer 7 and kept overnight. The liposome suspension was washed of the excess of glycans by ultracentrifuging on Beckman Ultracentrifuge at 55,000 rpm and resuspending the pellet three times.

3.3. Preparation of micelles

Micelle were prepared by a similar method for preparation of liposomes except that the composition was PEG-mal 2000:PEG-DSPE (0.075:0.075).

3.4. Characterization of liposomes

The liposomes were characterized for size, polydispersity index using Dynamic Light Scattering for Colloidal Size (DLS) measurements. Furthermore, zeta potential, phospholipid contents and amount of encapsulated OVA were determined.

3.4.1. DLS measurements

The average hydrodynamic diameter and the polydispersity index of the liposome dispersions were determined by dynamic light scattering using a Malvern ALV/CGS-3 Goniometer (Malvern Instruments Ltd.; Worcestershire, United Kingdom) at 25 °C using an argon-ion laser (488 nm) operating at 10.4 mW (Uniphase). For data analysis, the viscosity and refractive index of water was used. The system was calibrated with a polystyrene dispersion containing particles of 100 nm. The polydispersity index is a measure for variation in particle size within a liposome population, and varies from 0 (complete monodispersity) to 1 (large variations in particle size), and was calculated according to the method of Zhao et al. (2005).

The size of the PEGylated and non-PEGylated liposomes was between 200 and 220 nm with a polydispersity index below 0.20, indicating a relatively homogenous size distribution.

3.4.2. Zeta potential

Electrophoretic mobility measurements (Zetasizer Nano-Z, Malvern instruments, UK) were performed after dilution of the liposomes in de-mineralized water. The instruments were calibrated using polystyrene latex beads of defined zeta potential. The mean zeta potential of PEGylated liposomes was found to be -25 mV and that of non-PEGylated liposomes was found to be about −55 mV.

3.4.3. Determination of phospholipid content

Phospholipids were quantified spectrophotometrically after destruction with perchloric acid as described by Rouser et al. (1970). The amount of phospholipid content varied from 7 to 8.5 μmol. The lower concentration of the phospholipids than

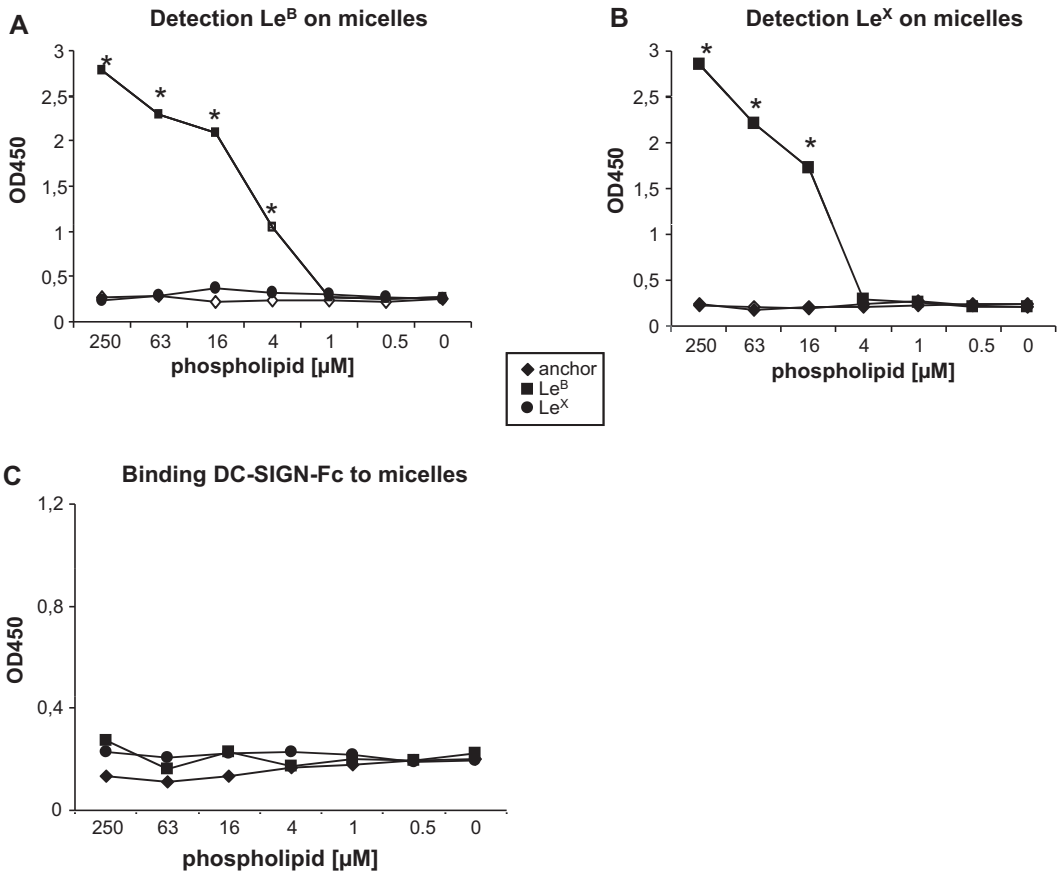

Fig. 3. Glycan-modified PEG containing micelles do not bind to DC-SIGN. PEGylated micelles were prepared and modified with either Le^X or Le^B glycans. (A) Le^B can be detected on Le^B-modified micelles using an anti-Le^B-specific antibodies and (B) Le^X is detected on Le^X-modified micelles an anti-Le^X-specific antibodies. (C) Glycan-modified micelles do not bind DC-SIGN-Fc. Depicted results are representative of two independent experiments. Significant difference is shown as $^*P < 0.001$ compared to non-modified micelles.

the actual may be because of the losses during extrusion and washings.

3.4.4. Determination of OVA content

OVA encapsulated inside the liposomes was quantified using an indirect protein determination method as described by Lowry et al. (1951). The amount of OVA encapsulated in the liposomes varied from 200 to 300 μg/mL.

3.5. Detection of glycans using enzyme linked immunosorbant assay (ELISA)

The linkage of the Le^X and Le^B glycans to the liposomes was confirmed in an ELISA by staining the liposomes with anti-Le^X and -Le^B antibodies, respectively, and correct orientation was assessed using DC-SIGN-Fc molecules. Briefly, glyco-liposomes were coated onto NUNC maxisorb plates (Roskilde, Denmark) and incubated o/n at 4 °C. Plates were blocked with 1% BSA in PBS for aspecific binding. After extensive washing, the glyco-liposomes were incubated either with anti-Le^X and -Le^B antibodies or DC-SIGN-Fc for 1.5 h at RT. Binding was detected using peroxidase-labeled F(ab′)2 fragment goat anti-mouse IgG, Fcγ fragment specific antibody; peroxidase-labeled goat anti-mouse IgM, Fcγ fragment specific antibody or F(ab′)2 fragment goat anti-human IgG, Fcγ fragment specific antibody, respectively. The reaction was developed and optical density was measured at 450 nm. As a positive control, Le^X and Le^B attached to polyacrylamide (PAA) (Lectinity, Moscow, Russia) was used.

3.6. Liposome binding to DC-SIGN on murine bone-marrow derived DC

Bone marrow-derived DC (BMDC) were generated from DC-SIGN transgenic and non-transgenic littermates using a method previously described by Lutz et al. (1999) DC-SIGN and control BMDC (5×10^4/well) were incubated with 50 nM liposomes in RPMI1360 Gibco, CA, USA supplemented with 10% FCS (BioWhittaker, Walkersville, MD) in the presence or absence of 10 nM EGTA for 30 min at 4 °C. Cells were washed and stained with APC-labeled anti-CD11c for 15 min at RT, and analyzed by flow cytometry (Calibur, BD Biosciences).

3.7. Statistical analysis

Results of experiments were analyzed either using a Two-way ANOVA, followed by Bonferroni post-test or unpaired Student *t*-test. Values were considered to be significantly different when $P < 0.05$.

4. Results

The efficiency of coupling of the maleimide group of the phospholipids to the thiol group of the glycans was checked using ELISA. Using a specific antibody against Le^B, we were able to detect the presence of the glycan on the surface of the modified liposomes (Fig. 2A), indicating efficient coupling of the glycan. Moreover, the glycan was equally well detectable irrespective of high or low amounts of liposomes used. Liposomes modified with Le^X or a

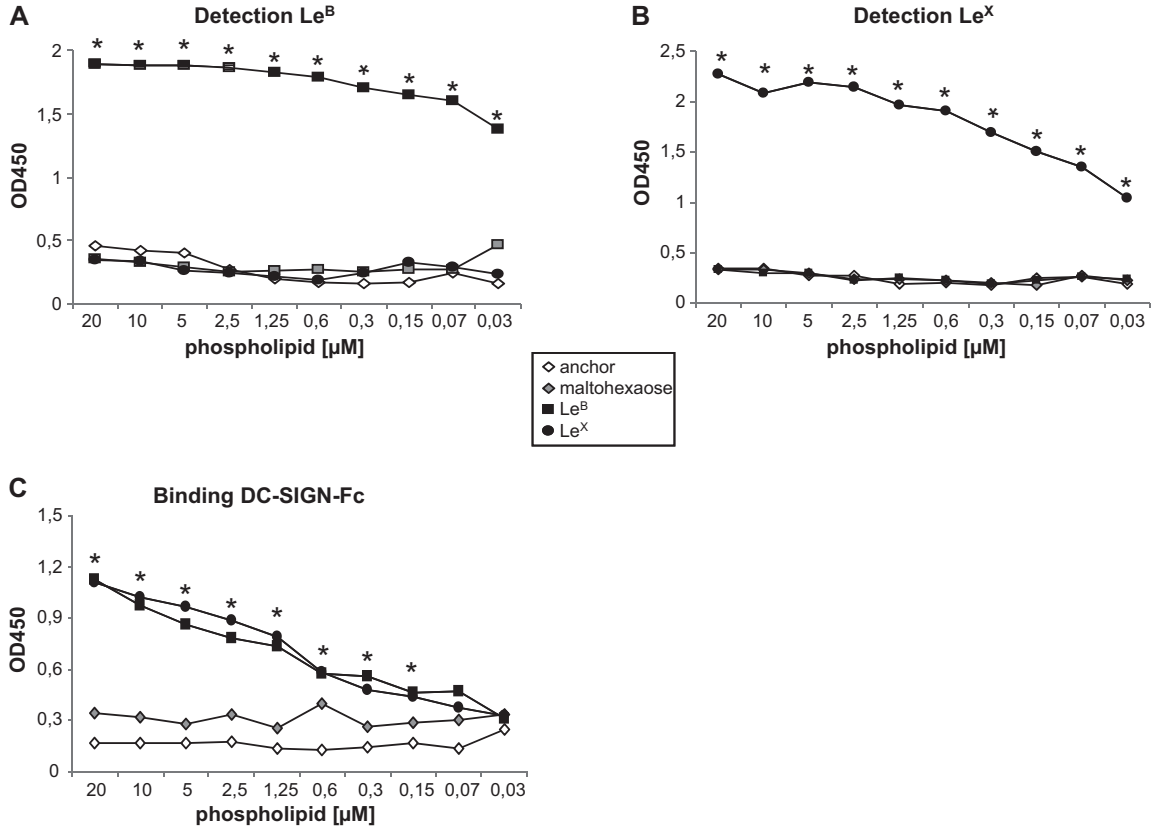

Fig. 4. Removal of PEG from glycan-modified liposomes ensures binding to DC-SIGN. Non-PEGylated Le^X or Le^B modified liposomes were analyzed (A) using an anti-Le^B antibody or (B) using anti-Le^X antibody and (C) binding to DC-SIGN using DC-SIGN-Fc chimeric molecules. Depicted results are representative of four independent experiments. Significant difference is shown as $^*P < 0.001$ compared to anchor liposomes.

control glycan (maltohexaose) or liposomes without any surface modification (designated as "anchor liposomes") were not detected by the anti-Le^B antibody (Fig. 2A). Similarly, using a Le^X-specific antibody we established efficient coupling of LeX to the liposomes as inferred from a high OD signal. Again, the other glycoliposomes were not binding with this antibody. Thus, the coupling between the maleimide group of phospholipids and thiol group of glycans is occuring efficiently.

We next analyzed whether the glycans were properly oriented allowing recognition by DC-SIGN. Thereto, binding of a chimeric DC-SIGN molecule to the glyco-liposomes was determined, Surprisingly, neither Le^X- nor Le^B-modified liposomes were bound by the chimeric DC-SIGN receptor (Fig. 2C, left panel). The absence of DC-SIGN binding was not due to any functional defect in the chimeric molecule as glycans present on PAA co-polymers were efficiently bound (Fig. 2C, right panel). These data suggest that some hinderence is causing the inability of the glycans to interact functionally with DC-SIGN.

It is possible that the PEGylated phospholipids influence either the orientation or the flexibility of the glycan, herewith affecting DC-SIGN binding in a negative manner. To determine whether this is the case, we prepared micelles containing the PEGylated phospholipids of the liposome composition. Le^X and Le^B glycans were coupled to the activated maleimide linker on PEG in the micelles. Using specific antibodies, both Le^B and Le^X glycans could be detected on the surface of Le^B- and Le^X-modified micelles, respectively (Fig. 3A and B). Control micelles were not detected. When the micelles were incubated with the chimeric DC-SIGN-Fc molecule, no binding of DC-SIGN to the glycan-modified micelles was detectable (Fig. 3C), similar to our observations using the glycan-liposomes (Fig. 2C).

Anticipating that PEG sterically hindered the interaction between glycan decorated liposomes and DC-SIGN, we prepared nonPEGylated liposomes. Glycans were coupled to these liposomes using the maleimide anchor MPB-PE. Both Le^X and Le^B glycans were detected by ELISA on the surface of these non-PEGylated liposomes, similar to the PEGylated liposomes (Fig. 4A and B vs Fig. 2A and B). However, in contrast to our findings with the PEGylated glycoliposomes the non-PEGylated glycoliposomes could be recognized by DC-SIGN-Fc (Fig. 4C). As expected control liposomes are not bound by the DC-SIGN-Fc molecule.

The chimeric Fc molecule used to assess the binding of the glyco-liposomes to DC-SIGN contains two DC-SIGN molecules per Fc molecule. However, on the surface of a DC very high amounts of DC-SIGN molecules are present. It can thus be hypothesized that the higher availability of DC-SIGN molecules might facilitate binding of PEGylated glycoliposomes. To examine this, we determined binding of PEGylated and nonPEGylated liposomes to DC-SIGN expressed on murine BMDC. We observed that only non-PEGylated Le^X- and Le^B-modified liposomes bound to DC-SIGN BMDC (Fig. 5). PEGylated glycoliposomes did not bind to DC-SIGN expressed on BMDC. The nonPEGylated glycoliposomes bound to DC-SIGN specifically, as binding could be abrogated by addition of EGTA and no binding was detected when non-transgenic control BMDC, that lack any DC-SIGN expression, were used. Control liposomes did not bind to either DC-SIGN or control BMDC.

5. Discussion

In this study we examined the feasibility of antigen targeting to DC-SIGN on DC using liposomes modified with glycans. We assessed that glycans could efficiently be conjugated to the sur-

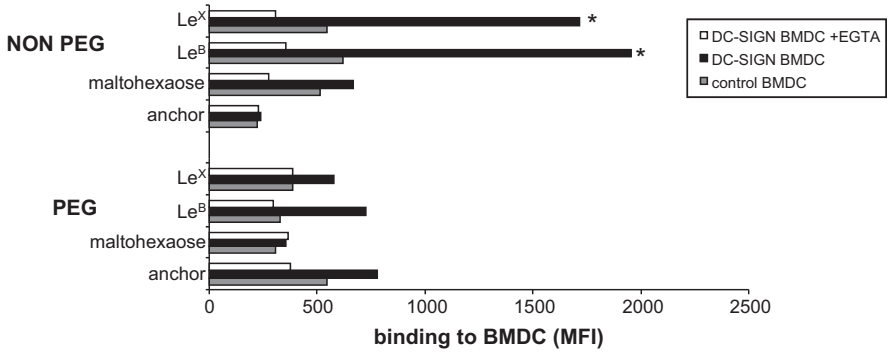

Fig. 5. Only glycan-modified nonPEGylated liposomes bind to DC-SIGN on DC. Glycan-modified PEGylated and nonPEGylated liposomes were DiD labeled and binding to DC-SIGN on BMDC was assessed. Binding was compared with control liposomes. To this end, 5×10^4 DC-SIGN$^+$ BMDC and control (WT) BMDC were incubated with 50 nM of the indicated liposomes for 30 min at 4 C. High Mean Fluorescence Intensity (MFI) reflects binding of the liposomes to BMDC. Binding of the liposomes in the presence of EDTA, blocks glycan binding by DC-SIGN, and inhibits the liposome binding to DC-SIGN$^+$ BMDC. One representative experiment out of three is shown. *P*-value <0.001 was considered significantly different from DC-SIGN$^+$ BMDC binding control liposomes.

face of conventionally used PEGylated liposomes. However, these PEGylated liposomes could not be bound by DC-SIGN indicating inability of the glycans to bind DC-SIGN. Recent evidence indicates that the nature of the surface and the space between the glycan and the surface can affect the interaction with glycan-binding proteins (Taylor and Drickamer, 2009). In our case, the lack of binding may be caused by PEG chains due to altered mobility and proper orientation of the glycans herewith hampering interaction with DC-SIGN. Indeed, glycoliposomes prepared using a nonPEG-containing maleimide anchor (i.e. MPB-PE) could successfully bind to DC-SIGN.

Only a few reports describe similar findings (Kawamura et al., 2006). Here, an IgG antibody was conjugated to the surface of liposomes via the mobile PEG arms. The IgG-coating of the PEGylated liposomes failed to promote uptake of the liposomes by DCs. The authors suggested that the PEG molecules caused steric hindrance, resulting in diminished interaction between IgG on the liposomes and FCγR receptors on DCs. Although in these studies the ligand was an antibody, which consists of a much larger entity than the glycans, in both cases PEG may modify the biological ligand interaction with immune receptors.

Besides PEG itself, also the spacer length and density of the PEG might interfere in the interaction of a modified liposome with a DC-expressed receptor. It has been shown previously that cellular uptake of folate-modified liposomes was increased when a longer PEG spacer and a higher density of PEG-DSPE were used (Yamada et al., 2008). In all our formulations, the occupancy of the maleimide linkers by the glycans was 100%. Thus, the density of glycans on the surface of both PEGylated and non-PEGylated liposomes was equal and therefore is not considered to be a confounding factor. The length of the PEG spacer used in our study was 2000. It is possible that PEGs with different lengths (e.g. PEG 3400 or PEG 5000) do not interference with targeting. This is currently under investigation.

The aim of the project was to assess the feasibility to design liposomal formulations modified with glycans that specifically target the C-type lectin receptor DC-SIGN on DC for enhanced antigen uptake and presentation. We conclude that PEG interferes with glycan-DC-SIGN interactions and should therefore be avoided in a CLR-targeting liposomal vaccine.

6. Conclusion

Our results demonstrate that non-PEGylated glycan-modified liposomes can specifically interact with DC-SIGN expressed by DC. By contrast, glycan-modification of conventionally used PEGylated liposomes fail to bind to DC-SIGN. Our data emphasize that the formulation of the liposomes used for specific targeting purposes should be carefully considered.

Acknowledgments

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