



## Cross-presentation through langerin and DC-SIGN targeting requires different formulations of glycan-modified antigens



Cynthia M. Fehres<sup>a</sup>, Hakan Kalay<sup>a</sup>, Sven C.M. Bruijns<sup>a</sup>, Sara A.M. Musaafr<sup>a</sup>, Martino Ambrosini<sup>a</sup>, Louis van Bloois<sup>b</sup>, Sandra J. van Vliet<sup>a</sup>, Gert Storm<sup>b,c</sup>, Juan J. Garcia-Vallejo<sup>a</sup>, Yvette van Kooyk<sup>a,\*</sup>

<sup>a</sup> Department of Molecular Cell Biology and Immunology, VUmc, Amsterdam, The Netherlands

<sup>b</sup> Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands

<sup>c</sup> MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands

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### ABSTRACT

Dendritic cells (DCs) and Langerhans cells (LC) are professional antigen presenting cells (APCs) that initiate humoral and cellular immune responses. Targeted delivery of antigen towards DC- or LC-specific receptors enhances vaccine efficacy. In this study, we compared the efficiency of glycan-based antigen targeting to both the human DC-specific C-type lectin receptor (CLR) DC-SIGN and the LC-specific CLR langerin. Since DC-SIGN and langerin are able to recognize the difucosylated oligosaccharide Lewis Y (Le<sup>Y</sup>), we prepared neoglycoconjugates bearing this glycan epitope to allow targeting of both lectins. Le<sup>Y</sup>-modified liposomes, with an approximate diameter of 200 nm, were significantly endocytosed by DC-SIGN<sup>+</sup> DCs and mediated efficient antigen presentation to CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Surprisingly, although langerin bound to Le<sup>Y</sup>-modified liposomes, LCs exposed to Le<sup>Y</sup>-modified liposomes could not endocytose liposomes nor mediate antigen presentation to T cells. However, LCs mediated an enhanced cross-presentation when antigen was delivered through langerin using Le<sup>Y</sup>-modified synthetic long peptides. In contrast, Le<sup>Y</sup>-modified synthetic long peptides were recognized by DC-SIGN, but did not trigger antigen internalization nor antigen cross-presentation. These data demonstrate that langerin and DC-SIGN have different size requirements for antigen uptake. Although using glycans remains an interesting option in the design of anti-cancer vaccines targeting multiple CLRs, aspects such as molecule size and conformation need to be taken in consideration.

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### 1. Introduction

Dendritic cells (DCs), the most efficient antigen presenting cells (APCs) of the immune system, continuously sample their environment for pathogens in order to endocytose, process and, ultimately, present antigens on MHC molecules to T cells. To facilitate antigen recognition, DCs are equipped with a variety of pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and NOD-like receptors (NLRs) [1]. Although the specificity and function of these receptors is rather diverse, the CLR family members detect carbohydrate structures in a Ca<sup>2+</sup>-dependent fashion and mediate antigen uptake to facilitate antigen processing and presentation, whereas TLRs and NLRs are signaling receptors that recognize pathogen-associated molecular patterns and elicit signals that result in proper DC maturation and cytokine production.

Some CLRs are selectively expressed by specific APC subsets and can be used to define APC subpopulations, such as DC-SIGN, expressed on

human dermal DCs (dDCs) and other APCs of the myeloid lineage [2]; and langerin, which is highly expressed on Langerhans cells (LCs) [3] and lower expression levels were recently also detected on intestinal lamina propria DCs [4] and on CD1c<sup>+</sup> myeloid DCs [5]. The glycan binding profiles of DC-SIGN and langerin show some overlap: both receptors are known to bind mannosylated glycans, but have a different specificity towards fucose-containing glycans [6–8]. For example, DC-SIGN shows specificity for all Lewis blood group antigens (Le<sup>a</sup>, Le<sup>b</sup>, Le<sup>x</sup> and Le<sup>y</sup>), whereas langerin only interacts with the difucosylated glycans Le<sup>b</sup> and Le<sup>y</sup> [8].

Additionally, differences in molecular orientation between langerin and DC-SIGN have been described. Langerin forms trimers through a coiled-coil structure in the extracellular neck-region, leading to a rather rigid position in the membrane compared to DC-SIGN, which is organized in tetramers [9]. DC-SIGN forms oligomers via its stem region, which allows for a higher level of flexibility to the carbohydrate recognition domains (CRDs) that facilitate interaction with its ligands [10]. An interesting feature of langerin is its association with Birbeck granules (BGs), which are rod-shaped structures and subdomains of the endosomal recycling compartment uniquely present in LCs [3,11]. The presence of langerin is crucial for BG formation [12]. Antibodies

\* Corresponding author at: Department of Molecular Cell Biology and Immunology, VU University Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands.

E-mail address: [y.vankooyk@vumc.nl](mailto:y.vankooyk@vumc.nl) (Y. van Kooyk).

directed against langerin are internalized in BGs, providing access of antigen to a LC-specific non-classical antigen-processing pathway [3], but how BGs influence the processing and presentation of antigens in MHC class I and II is still not fully understood. Although langerin and DC-SIGN show significant overlap in ligand specificity, they are expressed on distinct DC subsets, have a distinctive structural organization, and target to different intracellular processing machineries and organelles, altogether suggesting that these CLR-mediated pathways mediate different biological responses.

Many CLR-mediated pathways facilitate the internalization of antigens after binding to the receptor, leading to antigen processing and presentation on MHC-II molecules to activate CD4<sup>+</sup> T cells. CLR-mediated uptake of exogenous antigens has also been shown to result in cross-presentation of antigen on MHC-I molecules for the activation of CD8<sup>+</sup> T cells [13–15]. In most of these studies, monoclonal antibodies (moabs) against CLR-mediated pathways were used as targeting agents. For both DC-SIGN and langerin, internalization of the receptors after moab targeting and induction of T cell responses have been described, suggesting that DC-SIGN and langerin targeting routes antigen to MHC class I and II loading compartments [16–18]. However, the K<sub>d</sub> of antibodies is several orders of magnitude lower than that of natural CLR ligands (glycans), which might certainly affect the antigen routing and processing. Therefore, it would be interesting to analyze and compare langerin and DC-SIGN internalization and induction of T cell responses when both CLR-mediated pathways are targeted with antigens conjugated to glycan structures.

The internalization route and T cell stimulating capacity of several CLR-mediated pathways upon targeting with their ligands have already been described. For instance, mannose receptor (MR) targeting using the glycans, 3-sulfo-Lewis<sup>a</sup> and chitotriose conjugated to OVA, resulted in MR-dependent cross-presentation and Th1 polarization *in vivo* [19]. Similar results have been obtained using Le<sup>b</sup>- or Le<sup>x</sup>-modified OVA that target transgenic human DC-SIGN<sup>+</sup> murine DCs [20]. Although most of these experiments have been performed with bone marrow-derived DC or *in vivo* mouse models, little is known about the potency of CLR-mediated pathways in human skin, the primary vaccination site. The complexity of targeting and mobilizing skin APC subsets for the improvement of antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses directed against tumors or viruses are major questions to be addressed [19,21–23].

Although both DC-SIGN and langerin are able to internalize antigen, but little is known on the preferences of these receptors for any form of glycosylated antigen, if there are restrictions related to glycan valency or the size of the vaccine formulation. For DC-SIGN it has been described that glycan multivalency favors the strength of binding, since targeting of DC-SIGN using glycan-modified dendrimers, or glycan-modified liposomes facilitated DC-SIGN-mediated internalization and resulted in the induction of strong CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses [24,25]. On the other hand, less is known about the ligand preferences of langerin. Similar to DC-SIGN, langerin recognizes pathogens such as HIV, *Candida*, *Saccharomyces* and measles virus (MV) in a glycan-dependent manner [26–28]. Although the targeting of antigens to langerin using moabs leads to the development of antigen-specific Th1 and CD8<sup>+</sup> T-cell responses [13], langerin-mediated internalization of MV only induced MV-specific CD4<sup>+</sup> T cell responses, but no antigen cross-presentation occurred [27]. It is currently unclear whether langerin facilitates internalization and induction of T cell responses of glycan-modified antigens of any formulation.

Therefore, we set out to study the preferences of DC-SIGN and langerin for glycan-based vaccine formulations using glycan-modified peptides or liposomes as a model for small sized soluble-based molecular platforms versus large multivalent particulate antigenic carriers.

## 2. Material and methods

### 2.1. Cells

Human immature monocyte-derived dendritic cells (moDCs) were generated from monocytes isolated from buffy coats (Sanquin, The

Netherlands) through a sequential Ficoll/Percoll gradient centrifugation as previously described [29]. Monocytes were cultured for 5–6 days in RPMI 1640 (Invitrogen, USA) supplemented with 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin and 2 mM glutamine (all from BioWhittaker, USA) in the presence of rhIL-4 and rhGM-CSF (both 500 U/ml; BioSource, Belgium). OUW-DC-SIGN and OUW-langerin cells were cultured in RPMI1640 supplemented with 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin and 2 mM glutamine. Primary human LCs were isolated from abdominal resections from healthy donors (Bergman Clinics, Bilthoven, The Netherlands) that were obtained with informed consent within 24 h after plastic surgery. Briefly, skin was cut into slices of approximately 5-mm thickness, containing the epidermis and dermis using a dermatome. The slices were incubated in Iscove's modified Dulbecco's medium IMDM (Invitrogen) supplemented with 10% FCS, Dispase II (1 mg/ml, Roche Diagnostics), and antibiotics (50 U/ml penicillin, 50 µg/ml streptomycin and 10 µg/ml gentamycin; Lonza) overnight at 4 °C and then the dermis and epidermis were mechanically separated using tweezers. The epidermis was washed in PBS and cultured for 2 days in IMDM supplemented with 500 U/ml rhGM-CSF at 37 °C to allow the migration of LCs. After incubation, LCs were further isolated through Ficoll gradient centrifugation in order to reach cell purities above 85%. When indicated, moDCs and LCs were matured in the presence of 20 µg/ml polyinosinic:polycytidylic acid (pI:C; InvivoGen).

### 2.2. Flow cytometry

Phenotypical analysis of isolated LCs was performed by flow cytometry. Cells were washed in PBS supplemented with 1% BSA and 0.02% NaN<sub>3</sub> and incubated for 30 min. at 4 °C in the presence of appropriate dilutions of fluorescent-conjugated moabs to CD1a (BD) and langerin (Beckman Coulter). The cells were subsequently analyzed using a FACSCalibur (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

### 2.3. Liposome preparation

Liposomes were prepared as previously described [30]. The lipophilic fluorescent tracer, 1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine (DiD, Life Technologies) was incorporated to the liposomes during the first step of their preparation. Where indicated, the peptides MART-1<sub>21–36</sub> (YTAEELAGIGILTV) or Gp100<sub>280–288</sub> (YLEPGPVTA) were encapsulated in the liposomes as previously described [25]. Peptides were produced by solid phase peptide synthesis using Fmoc-double-coupling chemistry with a Symphony peptide synthesizer (Protein Technologies Inc., USA).

Coupling of the glycan structures Lewis<sup>b</sup>, Lewis<sup>y</sup> (Dextra labs, UK) or Lewis<sup>x</sup> (Elicityl France) or anti-DC-SIGN (AZN-D1) [31] and anti-langerin (10E2) [26] antibodies to the liposomes was done using thiol-maleimide chemistry. To this end, a thiol group was incorporated to glycans or antibodies through derivatization of the glycans with cysteamine (Sigma-Aldrich) and the antibodies with *N*-succinimidyl *S*-acetylacetate (Thermo Scientific). Briefly, lyophilized glycans were dissolved in dimethyl sulfoxide/acetic acid (8:2) and to this solution 10 equivalents (eq.) of cysteamine were added. After reacting at 65 °C for 20 min, 20 eq. of 2-picoline-borane (Sigma-Aldrich) were added and the mixtures were stirred for 2 h at 65 °C, followed by purification by normal phase HPLC on a Zorbax-NH<sub>2</sub> prep column (Agilent, elution water/acetonitrile, gradient 85% to 15% of acetonitrile in 30 min). After lyophilization of the collected fractions, the resulting dry powder was dissolved in water and treated with 20 eq. of tris(2-carboxyethyl)phosphine (TCEP, Sigma-Aldrich). After 1 h, the thio-glycan solution was purified using disposable sephadex G10 columns equilibrated with 50 mM ammonium formate (Sigma-Aldrich). Glycan derivatization was confirmed by ESI-MS (Thermo Finnigan LCQ-Decca XP Iontrap mass spectrometer in positive mode using nanospray capillary needle, data not shown). Antibodies were dissolved in hepes buffer and 8 eq. of *N*-succinimidyl

S-acetylacetate dissolved in a minimum amount of dimethyl formamide was added. After 45 min at room temperature the protein was washed 3 times over Vivaspin filters (10 kDa cut-off, Sartorius) and then the acetyl group of *N*-succinimidyl S-acetylacetate was removed by reaction with a 1:10 solution of hydroxylamine (Sigma-Aldrich) for 1 h. Subsequently, the yielded thio-glycans or thio-antibodies were coupled to the liposomes through a thiol-ene reaction with maleimide groups of the 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine-N-[4-(*p*-maleimidophenyl) butyramide] lipid as described previously [30]. The presence of the glycans on the liposomes was confirmed by ELISA using antibodies specific for Le<sup>b</sup>, Le<sup>X</sup> and Le<sup>Y</sup> (Supplementary Fig. 1). The calculated amount of glycans per liposome ranges between approximately 500–2000 glycans per liposome. Liposome size, polydispersity index, zeta potential and phospholipid content were determined as previously described [28]; inter-batch variability was kept below 5% as shown in Supplementary Table 1.

#### 2.4. Peptide incorporation determination

The concentration of liposome-encapsulated peptides was determined through an extraction method followed by RP-HPLC analysis. Briefly, 1 volume of liposome suspension (typically 100  $\mu$ l) was diluted with 1 volume of water, then 1 volume of methanol was added to disrupt the liposomes and after a brief stirring, 2 volumes of chloroform were added to remove lipids and cholesterol. After centrifugation at 14,000 rpm for 5 min and the removal of the organic phase, the water/MeOH phase was lyophilized, the peptide pellet was dissolved in 2 volumes of water and analyzed by HPLC. Quantification was done using a calibration curve of free peptides as a reference and the data was corrected after determining the recovery rate (liposome spiked with peptide). The amount of incorporated peptide was 33  $\mu$ g/ml for MART-1 (YTAEELAGIGILTV) and 42  $\mu$ g/ml for GP100 (YLEPGPVTA), corrected for a 6 mM phospholipid suspension.

#### 2.5. Glycan or antibody conjugation to peptides

Le<sup>b</sup>, Le<sup>X</sup> and Le<sup>Y</sup> glycans were conjugated to gp100 and MART-1 peptide sequences on the terminal cysteines with the help of the bifunctional crosslinker 4-*N*-Maleimidophenyl butyric acid hydrazide (MPBH, Thermo Scientific). To both the Gp100 and MART-1 sequences a cysteine is attached at the C-terminus to accommodate the glycan without altering the binding properties of the epitope to MHC-I.

First, the hydrazide moiety of MPBH was covalently linked to the reducing end of the glycan via reductive amination. Shortly, a mixture of MPBH (3 eq.), glycan (1 eq.) and picoline-borane (10 eq.) dissolved in dimethyl sulfoxide/acetic acid (DMSO/AcOH; 7:3) was incubated for 2 h at 65 °C. After cooling down to room temperature (RT), 4 volumes of dichloromethane (Biosolve) were added and the mixture was vortexed thoroughly. Subsequently, 4 volumes of diethyl ether (Biosolve) were added and incubated until glycan-MPBH had completely precipitated. MPBH-glycan was pelleted by centrifugation (2 min at 14,000 g). The supernatant was discarded and the pelleted carbohydrate-MPBH was washed with cold diethyl ether 3 times. The obtained glycan-MPBH pellet was resuspended in 0.1% trifluoroacetic acid (TFA) in MilliQ and lyophilized, followed by purification over a Vydac MS214 prep C18 column 22.1  $\times$  250 mm (Grace Alltech, elution water/acetonitrile, gradient 3% to 50% of acetonitrile in 40 min). The fractions containing the glycan-MPBH were pooled and lyophilized in 1 mg aliquots.

Peptides were glycosylated on their terminal cysteines with the glycan-MPBH through a thiol-ene reaction. Briefly, peptides (3 eq.) were dissolved in 0.05 M phosphate buffer (pH 6.5) and added to the lyophilized glycan-MPBH (1 eq.). After 2 h of incubation at RT, the glycosylated peptides were purified using the Dionex prep 3000 HPLC system and Vydac MS214 prep C18 columns 100  $\times$  250 mm (Grace Alltech, elution water/acetonitrile, gradient 10% to 50% of acetonitrile

in 40 min). The fractions containing the glycosylated peptide were pooled and lyophilized in 50 mg aliquots. The purity of the glycosylated peptides was confirmed by HPLC (Vydac 218MS C18 5  $\mu$ m 4.6  $\times$  250 mm, Grace Alltech) and MS spectrometry (data not shown). Conjugation of the glycans to the glycopeptides was also confirmed by ELISA using antibodies specific for Le<sup>b</sup>, Le<sup>X</sup> and Le<sup>Y</sup>, as previously described (Supplementary Fig. 2) [30].

Peptides were conjugated to antibodies using the bifunctional crosslinker Succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC, Thermo Scientific). Briefly, antibodies were activated with SMCC (8 eq.) in phosphate buffer pH 8.2 for 30 min at RT. After desalting over a G-25 10  $\times$  100 mm single use desalting gel filtration column (Amersham), peptides were dissolved in DMSO and added to the vial containing the antibody. After incubation at RT of 2 h, unconjugated peptide was removed through size-exclusion chromatography using a superdex 75 column (300  $\times$  100 mm, Amersham Biotech) against 50 mM ammonium formate buffer, pH 6.8. The fractions containing the antibody-peptide constructs were pooled and lyophilized.

#### 2.6. DC-SIGN-Fc and langerin-Fc

DC-SIGN-Fc and langerin-Fc were produced from established transfectants as described previously [32]. Both chimeric constructs consist of the extracellular domains of each lectin fused to the Fc portion of human IgG<sub>1</sub>. Langerin-Fc was generated by amplifying the extracellular domains of langerin (aa 63–328) on RNA of LCs by PCR. The products were confirmed by sequence analysis and fused at the C-terminus to human IgG1-Fc in the Sig-pIgG1-Fc vector. Langerin-Fc was produced by stable transfection of CHO cells and langerin-Fc concentrations were determined by ELISA.

#### 2.7. Binding of DC-SIGN-Fc and langerin-Fc to glycan-modified liposomes and peptides using ELISA

The conjugation of the Le<sup>b</sup>, Le<sup>X</sup> and Le<sup>Y</sup> glycans to peptides or liposomes was confirmed by ELISA using anti-Le<sup>b</sup>, -Le<sup>X</sup> or -Le<sup>Y</sup> antibodies (Calbiochem), respectively. Briefly, glycopeptides or glycoliposomes were coated onto NUNC MaxiSorp plates (Roskilde) and incubated o/n at 4 °C. Plates were blocked with 1% BSA (Fraction V, Fatty acid free, PAA laboratories) in TSM (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl<sub>2</sub> and 2 mM MgCl<sub>2</sub>) buffer. After washing 2 times, the glycopeptides were incubated with 2  $\mu$ g/ml DC-SIGN-Fc or langerin-Fc in TSM containing 0.5% BSA for 1.5 h at RT. Binding was detected using a peroxidase-labeled F(ab')<sub>2</sub> goat anti-human IgG/Fc $\gamma$  specific antibody. Binding was visualized with 3,3',5,5'-tetramethylbenzidine (TMB) as a substrate (Sigma Aldrich) and optical density was measured by spectrophotometry at 450 nm. As a positive control polyacrylamide (PAA) conjugates of Le<sup>b</sup>, Le<sup>X</sup> and Le<sup>Y</sup> (Lectinity) were used.

#### 2.8. Liposome uptake by human cells

Uptake of modified liposomes by primary LCs, moDCs or a B cell line (OUW) transformed with DC-SIGN or langerin was determined by FACS after 3 h incubation at 37 °C. Specific uptake via langerin or DC-SIGN was determined by incubating the cells with liposomes in the presence or absence of 20  $\mu$ g/ml blocking antibodies against langerin (10E2) or DC-SIGN (AZN-D1), respectively.

#### 2.9. Antigen presentation assays

The CD8<sup>+</sup> T cell clone specific for MART-1<sub>26–35</sub> (27L) was generated and cultured as described previously [33]. The gp100-specific HLA-DRB1\*0401-restricted T cell line Bridge gp:44 B8 [34] and the GP100-specific CD8<sup>+</sup> T cell clone [35] were cultured as described. HD7, a CD4<sup>+</sup> T cell clone that recognizes a peptide derived from mouse IgG1 Abs in HLA-DR0101/DQw1, was used and cultured as

described [36]. Human MoDCs or LCs ( $2 \times 10^4$ /well) were incubated with indicated concentrations of liposomes or glycopeptides in round bottom 96-well plates in the presence or absence of 20  $\mu\text{g/ml}$  pI:C. After 3 h of incubation at 37 °C, cells were washed and co-cultured with  $10^5$  MART-specific CD8<sup>+</sup> T cells, Gp100-specific CD8<sup>+</sup> T cells or Gp100-specific CD4<sup>+</sup> T cells per well. After 24 h, supernatants were harvested and IFN- $\gamma$  levels were measured by sandwich ELISA (Biosource).

### 2.10. Statistical analysis

Results were analyzed using a one-way ANOVA followed by Bonferroni Multiple Comparison test or a student's T test using GraphPad Prism software (GraphPad Software, San Diego, CA). Results were considered to be significantly different when  $p < 0.05$ .

## 3. Results

### 3.1. The CLR DC-SIGN and Langerin show overlapping and different glycan binding patterns

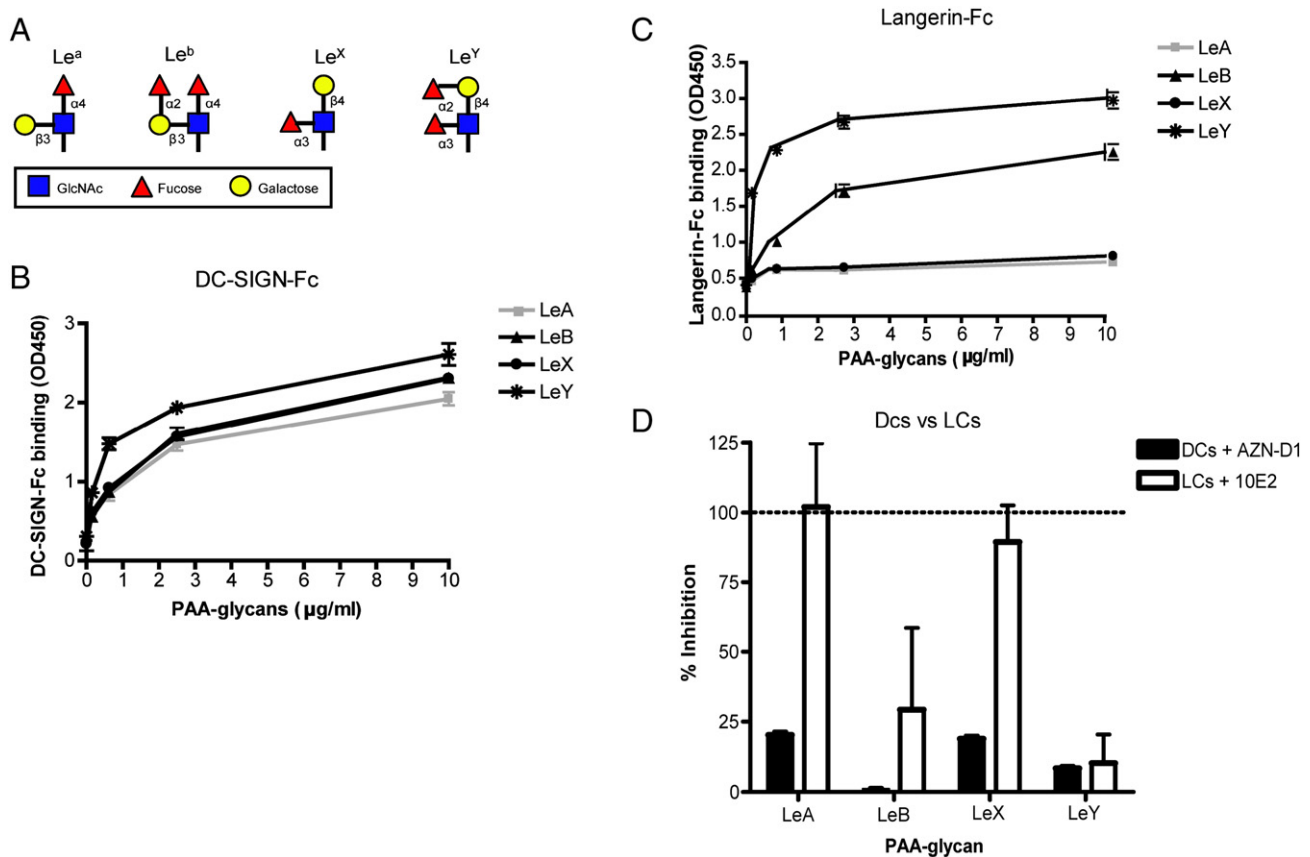
The glycan specificity of DC-SIGN and langerin has been studied before and it had been described that both receptors can bind fucosylated glycan structures. We aim to compare the specificity of these receptors in order to identify potential compounds that can target both receptors. Using DC-SIGN-Fc and langerin-Fc chimeric molecules we determined the glycan specificity of the receptors for the fucose-containing glycans Le<sup>a</sup>, Le<sup>b</sup>, Le<sup>x</sup> and Le<sup>y</sup> (Fig. 1A). These glycans, attached to a PAA backbone, were coated on ELISA plates and binding of DC-SIGN-Fc and langerin-Fc molecules to the plate-bound glycans was analyzed. As

shown in Fig. 1B, DC-SIGN-Fc recognized all Lewis-type glycans tested, whereas langerin-Fc showed a more restricted binding pattern towards Le<sup>b</sup> and Le<sup>y</sup> (Fig. 1C). Furthermore, when DCs were pre-incubated with blocking antibodies to DC-SIGN and LCs with blocking antibodies to langerin, respectively, binding of Le<sup>y</sup>-PAA to DCs and LCs was completely abrogated, demonstrating that no other CLR expressed by human DCs and LCs could bind to Le<sup>y</sup> (Fig. 1D). As expected, blocking DC-SIGN also inhibited the binding of Le<sup>a</sup>, Le<sup>b</sup> and Le<sup>x</sup> to DCs. Blocking langerin partly inhibited the binding of Le<sup>b</sup>-PAA to LCs (Fig. 1D). Since Le<sup>y</sup> is exclusively recognized by DC-SIGN on DCs and langerin on LCs, this glycan can be used to follow the fate of distinct vaccine formulations conjugated to a natural ligand that targets both receptors.

### 3.2. Modification of liposomes with Le<sup>y</sup> results in DC-SIGN-mediated uptake by DCs, but not in langerin-mediated uptake by LCs

We have previously demonstrated that Le<sup>x</sup>-modified liposomes efficiently target DC-SIGN on moDC, thereby inducing specific anti-tumor immune responses [25]. In order to investigate whether Le<sup>b</sup>- or Le<sup>y</sup>-modified liposomes can be used to target both langerin and DC-SIGN expressing cells, we first analyzed the binding of Le<sup>b</sup>, Le<sup>x</sup> or Le<sup>y</sup> modified liposomes to DC-SIGN-Fc and Langerin-Fc. Although DC-SIGN-Fc did bind to Le<sup>b</sup>-, Le<sup>x</sup>- or Le<sup>y</sup>-conjugated liposomes, Langerin-Fc only showed binding to Le<sup>y</sup>-liposomes, but lacked binding to Le<sup>b</sup>- or Le<sup>x</sup>-modified liposomes (Fig. 2A).

To investigate if glycan-modified liposomes were captured by DCs and LCs, we incorporated the lipophilic tracer DiD in the liposomes. As shown in Fig. 2B and C, moDCs were able to capture large amounts of Le<sup>b</sup>-, Le<sup>x</sup>- or Le<sup>y</sup>-modified liposomes. The interaction was



**Fig. 1.** DC-SIGN and langerin bind to Le<sup>y</sup> glycans. A. Schematic representation of Le<sup>a</sup>, Le<sup>b</sup>, Le<sup>x</sup> and Le<sup>y</sup>. B. DC-SIGN binds to all Lewis antigens as determined by an ELISA-based assay using DC-SIGN-Fc. PAA-glycans were coated at indicated concentrations and binding of DC-SIGN-Fc was measured using peroxidase-conjugated anti-human IgG. Mean  $\pm$  SEM is shown,  $n = 2$ , each experiment measured in triplicate. C. Langerin recognized Le<sup>b</sup> and Le<sup>y</sup> as determined by ELISA using langerin-Fc. Mean  $\pm$  SEM is shown,  $n = 2$ , each experiment measured in triplicate. D. Binding of Lewis-PAA glycans to moDCs (black bars) or LCs (white bars) was significantly inhibited when DC-SIGN was blocked using AZN-D1 (10  $\mu\text{g/ml}$ ) and langerin was blocked using 10E2 (10  $\mu\text{g/ml}$ ) respectively. The dotted line represents 100% binding of DC-SIGN and langerin to Le<sup>a</sup>, Le<sup>b</sup>, Le<sup>x</sup> and Le<sup>y</sup>. Mean  $\pm$  SD is shown, measured in triplicate.



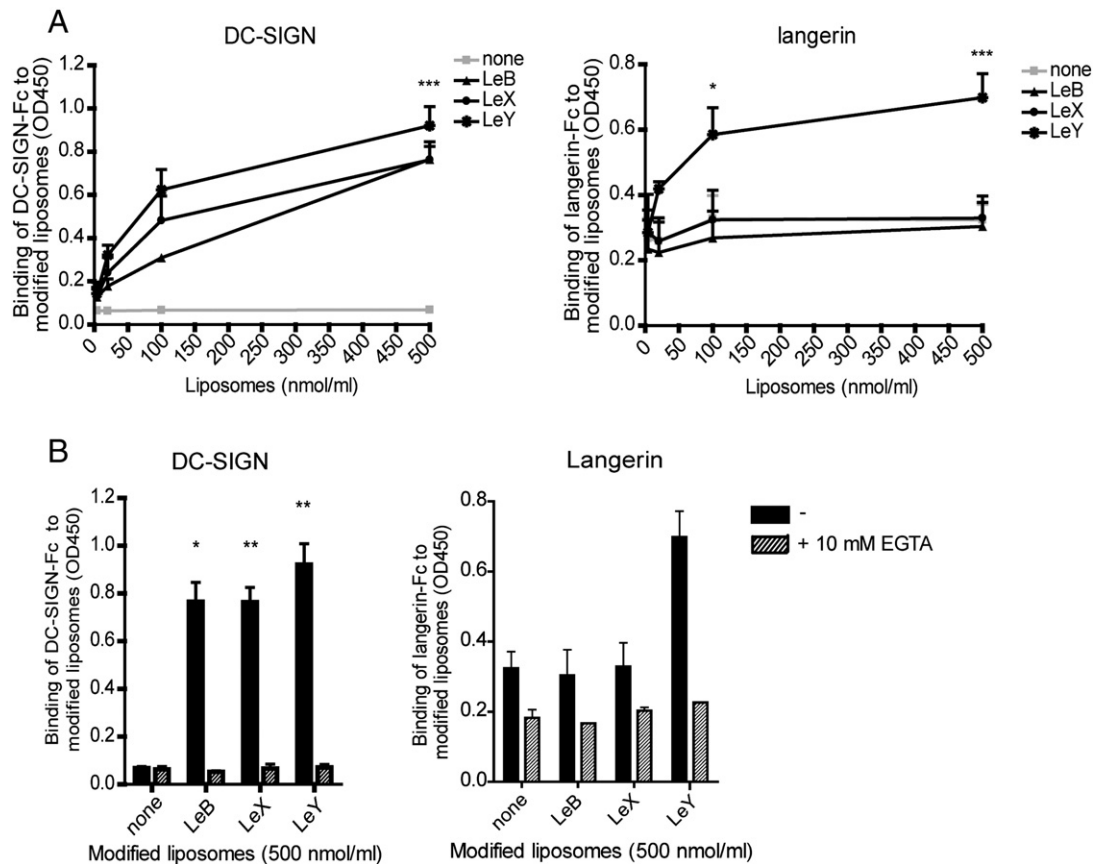
carbohydrate-dependent, since binding of glycan-modified liposomes was completely abolished in the presence of 10 mM EGTA (Fig. 2B). Uptake of unmodified liposomes by DCs was hardly detected (Fig. 2C), which is in line with published data showing that anionic particles are phagocytosed by DCs to a much lower extent than cationic particles [37,38]. However, although langerin was shown to interact with Le<sup>Y</sup>-PAA (Fig. 1C) and Le<sup>Y</sup>-modified liposomes coated on plates (Fig. 2A + B), LCs were unable to take up any type of liposomes (Fig. 2C). To exclude that this phenomenon was caused by a potential inability of langerin to internalize glycosylated liposomes, we also analyzed langerin-mediated internalization in a B-cell line that over expresses langerin (OUW cells). As shown in Fig. 2D, also langerin expressing OUW cells were not able to take up glycan-modified liposomes (right panel), while DC-SIGN expressing OUW cells showed an increased uptake of Le<sup>b</sup>- and Le<sup>Y</sup>-modified liposomes (Fig. 2D, left panel), similar to the internalization capacity of moDCs (Fig. 2B and C).

To investigate whether the lack of langerin-mediated uptake of glycan-modified liposomes was the result of the glycan-langerin interaction, we determined whether LCs or OUW-langerin cells are able to take up liposomes that are modified with anti-langerin antibodies. Incubation of LCs or OUW-langerin with anti-langerin-modified liposomes resulted in significant higher uptake compared to unmodified- or Le<sup>Y</sup>-modified liposomes (Fig. 2E and D (right panel), respectively), providing evidence that LCs can take up liposomes. Together, these

data demonstrate that Le<sup>b</sup>-, Le<sup>X</sup>- or Le<sup>Y</sup>-modified liposomes can be efficiently targeted to and internalized by DC-SIGN, whereas Le<sup>b</sup> and Le<sup>Y</sup>-modified liposomes cannot be used for langerin targeting on LCs, due to a lack of internalization. Instead, only anti-langerin modified liposomes result in langerin targeting and uptake by LCs.

### 3.3. DC-SIGN-mediated uptake of glycan-modified liposomes elicits activation of antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells

Since glycan-modification enhances the internalization of liposomes by moDCs, we examined if this also results in increased antigen presentation to CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Liposomes were generated with encapsulated MART-1<sub>21–36</sub> or GP100<sub>44–59</sub> peptides, which contain a MHC class I binding epitope recognized by the CD8<sup>+</sup> MART-1 T cell clone or MHC class II binding epitope for CD4<sup>+</sup> T cell recognition, respectively. MoDCs were incubated with different concentrations of glycan-modified MART-1 or GP100 liposomes and subsequently co-cultured with MART-1 specific CD8<sup>+</sup> T cells or Gp100-specific CD4<sup>+</sup> T cells, respectively. Exposure of moDCs to Le<sup>X</sup>-modified liposomes resulted in significantly enhanced antigen (cross-)presentation to CD8<sup>+</sup> (Fig. 3A) and CD4<sup>+</sup> (Fig. 3B) T cells as measured by the production of IFN- $\gamma$  by both clones. These data indicate that antigen targeting to DC-SIGN using glycan-modified liposomes efficiently improves liposomal uptake, antigen presentation to CD4<sup>+</sup> T cells and antigen cross-presentation to CD8<sup>+</sup> T cells.



**Fig. 2.** Glycan-modified liposomes are efficiently internalized by DC-SIGN, but not by langerin. A. DC-SIGN-Fc (left panel) binds to liposomes modified with Le<sup>b</sup>, Le<sup>X</sup> and Le<sup>Y</sup> as determined using a DC-SIGN-Fc ELISA where the liposomes were coated at indicated concentrations. Langerin-Fc (right panel) binds to liposomes modified with Le<sup>Y</sup>. B. Binding of DC-SIGN-Fc (left panel) and langerin-Fc (right panel) to modified liposomes is completely abrogated in the presence of 10 mM EGTA. Mean  $\pm$  SEM is shown, n = 2, each experiment measured in duplicate. C. Glycan-modified liposomes (200 nmol/ml) are efficiently internalized by DC-SIGN<sup>+</sup> moDCs but not by langerin<sup>+</sup> LCs, as measured by the fluorescent intensity of DID. Dot plots of 1 representative experiment are shown. D. DC-SIGN-mediated uptake of glycan-modified liposomes was confirmed using a B cell line transfected with DC-SIGN (OUW-DC-SIGN; left panel), whereas OUW-langerin cells were also not able to take up glycan-modified liposomes (right panel). Combined data of at least 2 independent experiments are shown. E. Langerin<sup>+</sup> LCs take up liposomes conjugated to anti-langerin antibodies, as measured by the fluorescent intensity of DID. Means  $\pm$  SEM are shown based on 3 independent experiments, \*\*\*p < 0.001. Statistical significance was determined using an ANOVA combined with a Bonferroni Multiple Comparison test.

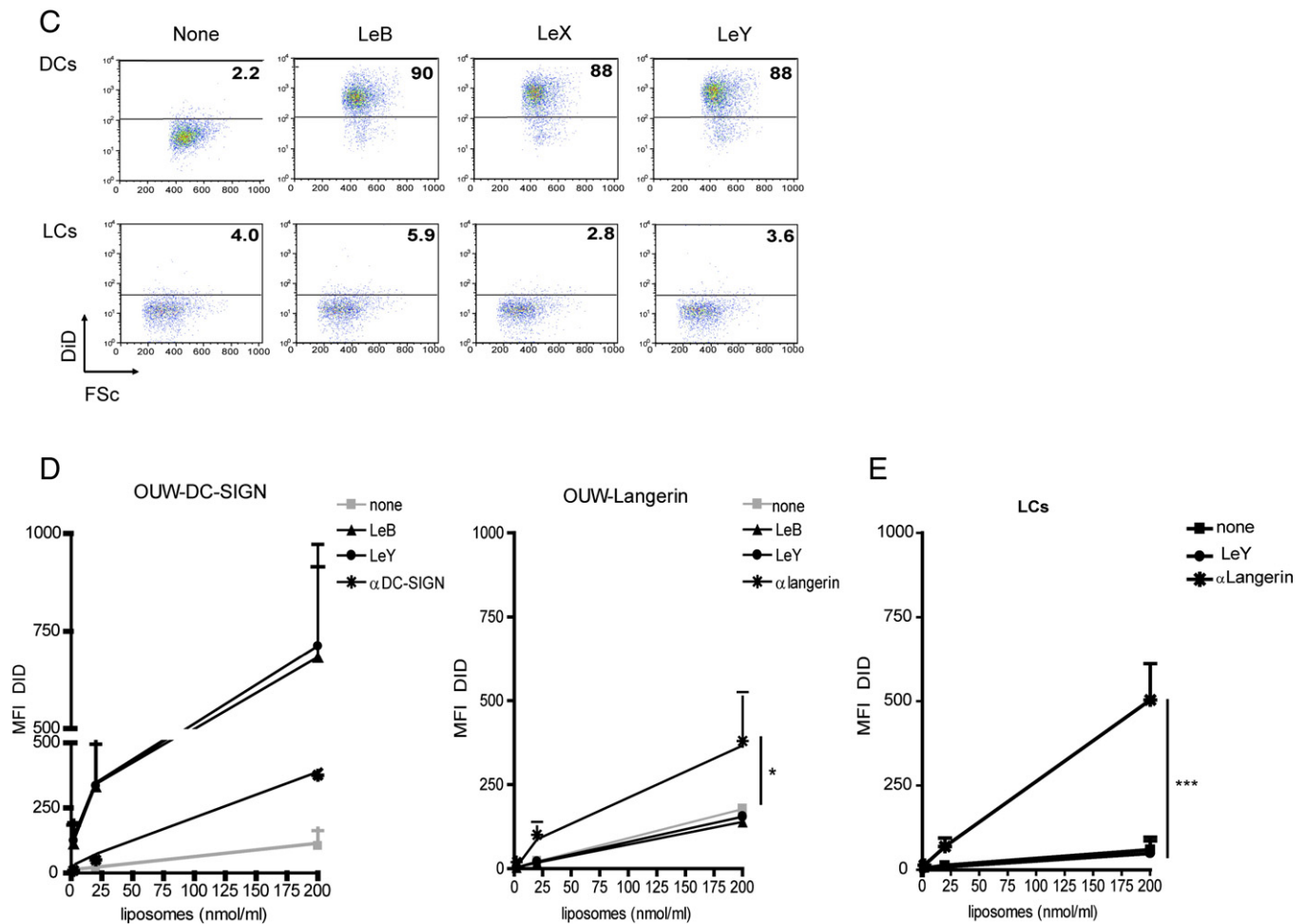


Fig. 2 (continued).

### 3.4. Targeting of Langerin using anti-langerin conjugated liposomes results in increased antigen presentation to CD4<sup>+</sup> T cells, but not to CD8<sup>+</sup> T cells

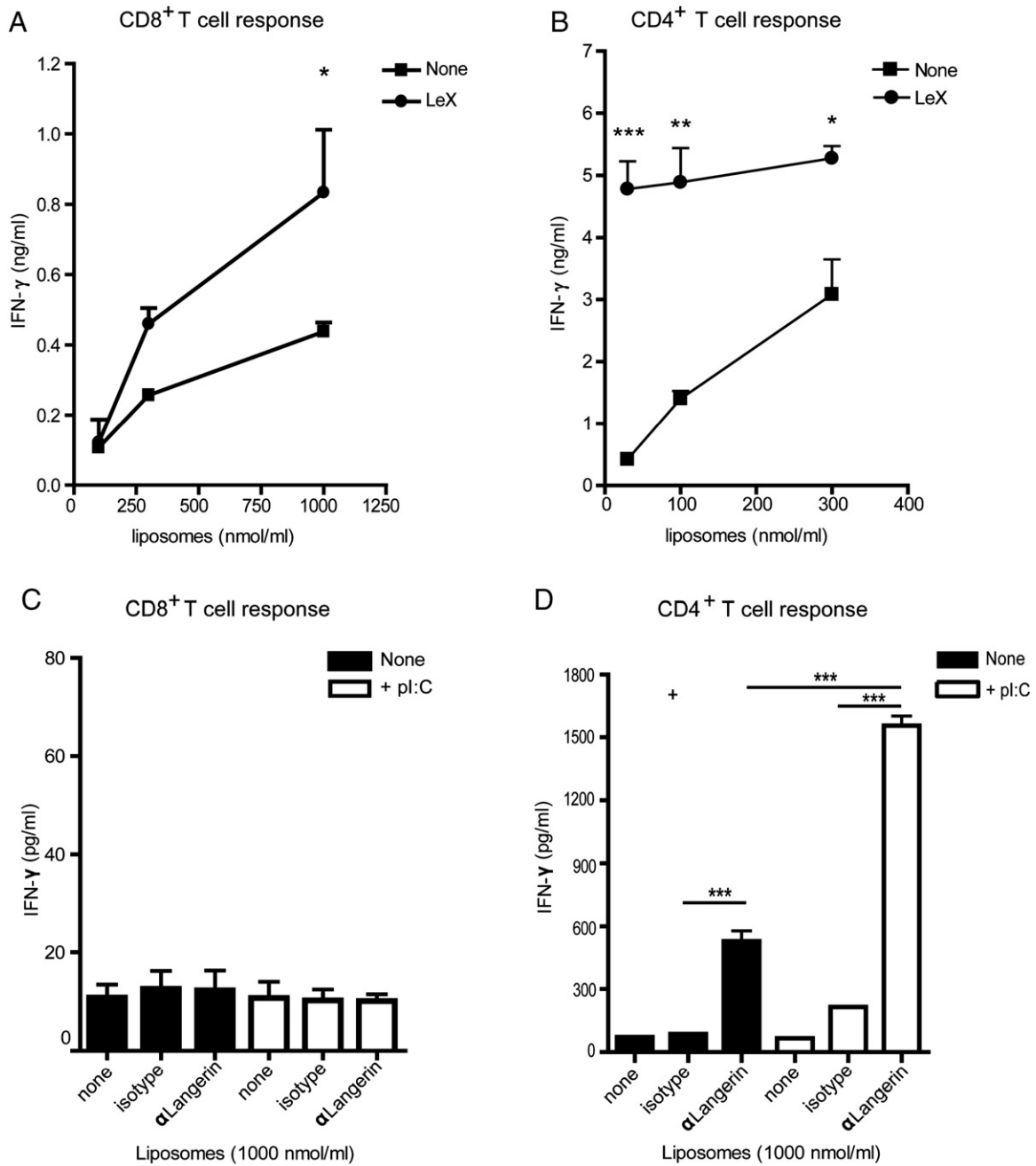
While we have shown antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses after targeting of DC-SIGN using Le<sup>X</sup>-modified liposomes, we were interested to see whether targeting of langerin on LCs would also result in these responses. Because glycan-modified liposomes showed no internalization by langerin, we tested if the anti-langerin modified liposomes that were properly internalized by langerin (Fig. 2E) could induce antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses after langerin-mediated internalization. As shown in Fig. 3C, exposure of LCs to anti-langerin modified liposomes did not result in an increased cross-presentation to the MART-1 specific CD8<sup>+</sup> T cells compared to isotype-conjugated liposomes, despite the significant increase in uptake of the anti-langerin modified liposomes. Furthermore, antigen cross-presentation could not be increased in the presence of the TLR3 ligand pl:C (Fig. 3C). However, langerin-mediated uptake of anti-langerin modified liposomes did result in enhanced antigen presentation to CD4<sup>+</sup> T cells, as determined after co-culture of liposome-pulsed LCs with the HD7 CD4<sup>+</sup> T cell clone [36], which recognizes a peptide derived from the Fc domain of the murine IgG1 antibody. As shown in Fig. 3D, the exposure of LCs to anti-langerin liposomes showed a significant increase in antigen presentation to the CD4<sup>+</sup> T cells compared to LCs exposed to isotype-conjugated liposomes, as measured by the production of IFN-γ. These responses were further increased when LCs were simultaneously stimulated with pl:C (Fig. 3D). Together, these data show that langerin does not facilitate internalization of glycan-modified

liposomes and induction of T cell responses, making liposomes not suitable for LC targeting aiming to induce anti-tumor CD8<sup>+</sup> T cell responses.

### 3.5. Glycan modified peptides give strong langerin-mediated internalization and presentation to CD4<sup>+</sup> and CD8<sup>+</sup> T cells

Based on our findings that langerin could bind and internalize glycans, but not glycoliposomes, we hypothesized that langerin might prefer the binding and internalization of small molecules over large glycan-modified liposomes. Therefore, we generated synthetic long MART-1<sub>21–36</sub> peptides and conjugated a single glycan to the C-terminus of the peptide to allow targeting to DC-SIGN and/or langerin. To confirm that DC-SIGN and langerin could bind glycopeptides, an ELISA using DC-SIGN-Fc and langerin-Fc was performed. As expected, both DC-SIGN-Fc and langerin-Fc recognized Le<sup>Y</sup>- and Le<sup>b</sup>-modified peptides, whereas Le<sup>X</sup>-modified peptides were only recognized by DC-SIGN-Fc (Fig. 4A). Correct conjugation of Le<sup>b</sup>, Le<sup>X</sup> or Le<sup>Y</sup> to the peptides was confirmed using specific anti-Le<sup>b</sup>, -Le<sup>X</sup> and -Le<sup>Y</sup> antibodies in the ELISA (Suppl. Fig. 2).

To investigate whether langerin targeting using a small monovalent molecule could result in antigen internalization and antigen-specific T cell responses, we incubated human LCs with glycan-modified MART-1 peptides for 3 h, washed, and then co-cultured with a MART-1 specific CD8<sup>+</sup> T cell clone. As presented in Fig. 4B, conjugation of MART-1 peptides with Le<sup>b</sup> or Le<sup>Y</sup> resulted in enhanced cross-presentation to the CD8<sup>+</sup> T cells by LCs. These responses were langerin-mediated,

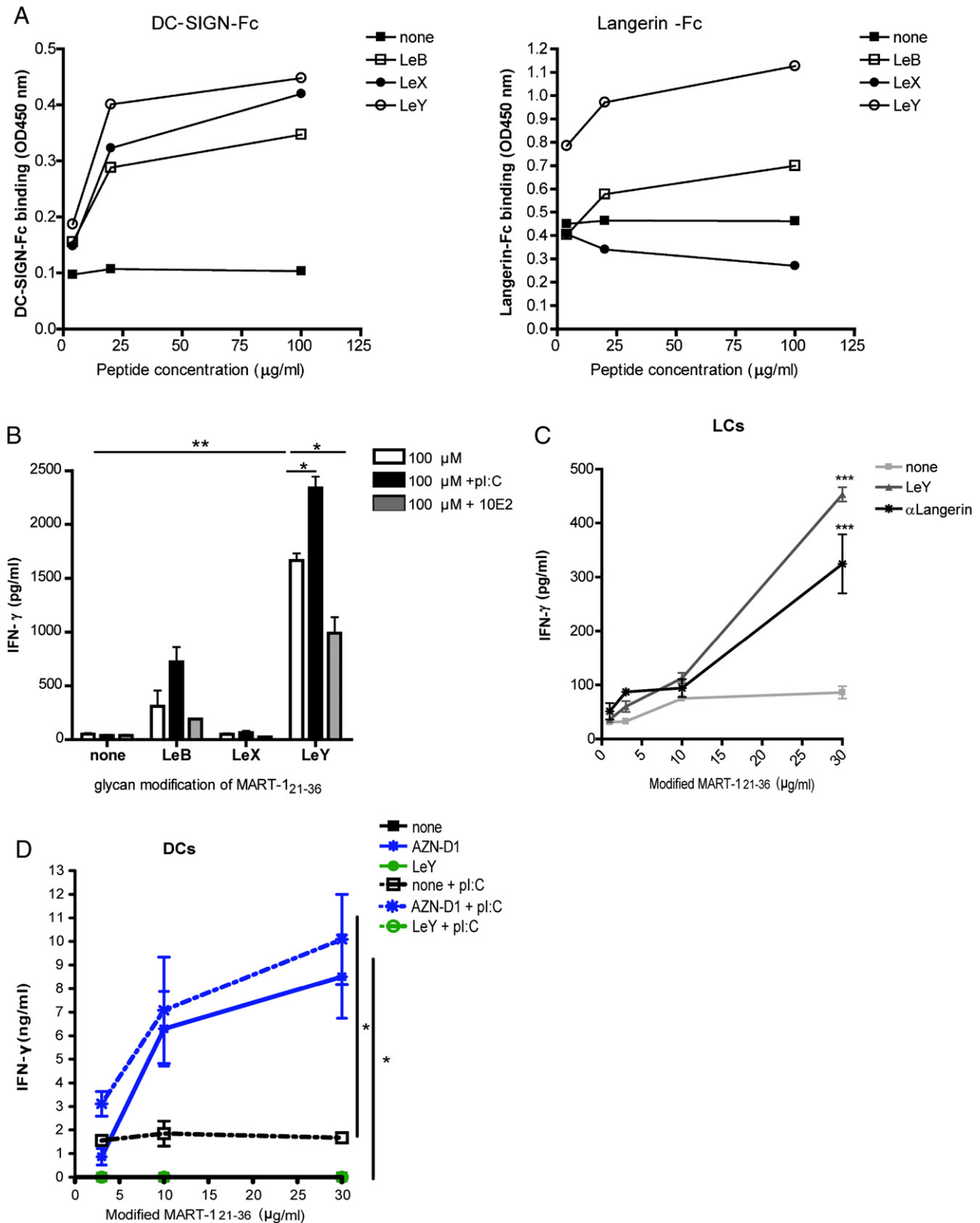


**Fig. 3.** DC-SIGN-mediated uptake of Le<sup>X</sup>-modified liposomes results in significantly enhanced CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses, whereas langerin-mediated uptake of anti-langerin modified liposomes only induces CD4<sup>+</sup> T cell activation. DC-SIGN<sup>+</sup> moDCs were pulsed with Le<sup>X</sup>- or unmodified liposomes containing MART-1 or Gp100 peptides and subsequently co-cultured with MART-1 specific CD8<sup>+</sup> T cells (A) or Gp100 specific CD4<sup>+</sup> T cells (B). Langerin<sup>+</sup> LCs were pulsed with anti-langerin, isotype or unmodified liposomes in the presence or absence of pl:C and subsequently co-cultured with MART-1 specific CD8<sup>+</sup> T cells (C) or mlgG1-specific CD4<sup>+</sup> T cells (D). Activation of T cells was determined using an IFN-γ ELISA on supernatants taken 24 h after co-culture. Data are shown as means ± SEM of triplicate cultures. Results shown are representative of 2 independent experiments. \*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001. Statistical significance was determined using a two-way ANOVA combined with a Bonferroni Multiple Comparison test.

since simultaneous administration of blocking antibodies reduced cross-presentation significantly. Additionally, simultaneous administration of pl:C enhanced cross-presentation (Fig. 4B). pl:C stimulation in the absence of langerin triggering using Le<sup>b</sup>- or Le<sup>y</sup>-modified MART-1 did not directly promote the LC-T cell co-culture, since T cell responses after LC stimulation with unmodified or Le<sup>X</sup>-modified MART-1 were not increased in the presence of pl:C compared to antigen stimulation without TLR ligand (Fig. 4B), providing evidence of an existing synergy between langerin and TLR3 triggering.

The results from Fig. 2E demonstrated a clear difference in langerin-mediated internalization when liposomes were conjugated to glycans or moabs. However, when langerin was targeted with glycan-

modified peptides we could observe langerin-mediated internalization and T cell responses. Therefore, we were interested to see if moab-modified peptides show the same langerin targeting potential as the glycan-modified peptides. Indeed, targeting LCs with MART-1 peptides conjugated to either Le<sup>y</sup>- or an anti-langerin Ab had similar effects on antigen cross-presentation to CD8<sup>+</sup> T cells (Fig. 4C). Together, these data show that targeting of langerin, expressed by human LCs, using synthetic long anti-tumor peptides conjugated to Le<sup>y</sup> or anti-langerin antibodies enhances LC cross-presentation. No significant differences were observed between langerin targeting using a glycan or an ab. This is the first study wherein moab and glycan targeting of langerin are directly compared.



**Fig. 4.** Le<sub>B</sub>- and Le<sub>Y</sub>-modification of peptides enhances antigen (cross-)presentation of LCs in the presence of pl:C, but not of moDCs. A. DC-SIGN binds to Le<sub>B</sub>-, Le<sub>X</sub>- and Le<sub>Y</sub>-modified MART-1 peptides (left panel); langerin binds moderately to Le<sub>B</sub>- and highly to Le<sub>Y</sub>-modified MART-1 peptides (right panel), as was determined using a DC-SIGN-Fc or langerin-Fc ELISA where the peptides were coated at indicated concentrations. B. Le<sub>B</sub>- or Le<sub>Y</sub>-modification of MART-1 enhances langerin-mediated uptake and cross-presentation by LCs to MART-1 specific CD8<sup>+</sup> T cells. Simultaneous administration of pl:C further increased antigen cross-presentation, whereas addition of anti-langerin blocking antibodies (10E2) abrogated the CD8<sup>+</sup> T cell response. Data are shown as means ± SEM of 3 independent experiments, each measured in triplicate. \**p* < 0.05. \*\**p* < 0.01. C. Conjugation of anti-langerin antibodies to MART-1 resulted in an equally increased cross-presentation by LCs compared to Le<sub>Y</sub>-modification. Result shown is representative of 1 independent experiment, measured in triplicate. \*\*\**p* < 0.001. D. Le<sub>Y</sub>-modification of MART-1 SLP did not enhance DC-SIGN-mediated uptake and cross-presentation by moDCs, not even in the presence of pl:C. However, conjugation of anti-DC-SIGN antibodies to MART-1 did enhance antigen cross-presentation compared to unmodified peptides. Data are shown as means ± SEM of triplicate cultures of 2 independent experiments. \**p* < 0.05 and \*\*\**p* < 0.01. Statistical significance was determined using a two-way ANOVA combined with a Bonferroni Multiple Comparison test.



Because glycan-modified liposomes effectively targeted DC-SIGN and stimulated antigen cross-presentation, we questioned if similar findings could be observed using glycan-modified and antibody-modified MART-1 peptides. Despite the high binding of DC-SIGN-Fc to glycan-modified peptides (Fig. 4A), we could not detect enhanced antigen cross-presentation by moDCs targeted with Le<sup>Y</sup>-modified MART-1 compared to unmodified MART-1 peptides. Surprisingly, targeting of DC-SIGN using anti-DC-SIGN antibodies conjugated to MART-1 peptides resulted in significantly increased cross-presentation and subsequent CD8<sup>+</sup> T cell activation (Fig. 4D). Similar to langerin stimulation on LCs, simultaneous administration of pI:C as a maturation stimulus enhanced DC-SIGN-mediated antigen cross-presentation by the DCs. No positive effects of pI:C on cross-presentation were observed with the Le<sup>Y</sup>-modified peptides, suggesting that TLR3 triggering does not improve DC-SIGN-mediated cross-presentation.

#### 4. Discussion

In the present study, we compared different targeting strategies for langerin and DC-SIGN using glycans or moabs conjugated to SLPs or antigen-encapsulated liposomes. We have shown that human moDCs are well equipped to internalize relatively large and multivalent glycan-modified liposomes in a DC-SIGN-dependent manner, which stimulated both antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses. Strikingly, DC-SIGN targeting using glycan-modified peptides did not result in antigen internalization and subsequent T cell responses. Conversely, LCs were efficiently targeted using glycan-modified peptides in a langerin-dependent process resulting in enhanced antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses, but no langerin-mediated uptake of the glycan-modified liposomes could be observed.

The differences we observed between human LCs and MoDCs in their capacity to bind, internalize and (cross-)present antigens of various sizes might be caused by cell subset intrinsic capacities [39]. Studies by Bond et al. showed that human LCs displayed a significantly higher uptake of fluorescently labeled OVA (45 kDa) and dextran (70 kDa) compared to dermal DCs [40]. In addition, others have shown that intrinsic differences between LCs and DCs exist with regard to the route of antigen internalization [41]. Although human monocyte-derived LCs and DCs were equally able to take up human papillomavirus-like particles (HPV-VLPs), MoDCs took up the antigen via phagocytosis and the formation of clathrin-coated pits, whereas uptake of VLP by LCs was clathrin-independent and relied on caveolae [41]. Moreover, processing of HPV-VLPs antigens by LCs was less efficient than by DCs, but both APCs cross-presented antigens *in vitro*. Besides, human LCs highly express langerin and BGs, langerin<sup>+</sup> organelles that are part of the endosomal recycling pathway [11]. Although not much is known about BGs and antigen processing, they might have an influence on antigen internalization and (cross-) presentation by LCs and thereby contribute to the differences observed between DCs and LCs.

In addition, we have described differences between human LCs and MoDCs and langerin and DC-SIGN, respectively, with respect to the uptake of 200 nm sized glycan-modified liposomes. Although langerin could recognize Le<sup>Y</sup>-modified liposomes, no increased internalization was observed in both primary human LCs as in langerin-transfected OUW B cells. Our data suggests that the interaction between langerin and Le<sup>Y</sup> coupled to liposomes is not sufficient to allow liposome internalization. Interestingly, conjugation of anti-langerin antibodies to liposomes facilitated uptake by human LCs. In general, the interaction between antibodies and their ligand occurs with a higher affinity than receptor–glycan interactions. In contrast to langerin, DC-SIGN was very well capable to internalize the glycan-modified liposomes, suggesting that the binding between DC-SIGN and Lewis glycans occurs with a higher avidity. The multivalency of the compound and the activation status of the receptor after binding can influence the capacities of a cell to internalize bound antigen. For example, DC-SIGN has been shown to exhibit the highest affinity enhancement when the second and third

mannose residues were added to mannose-BSA of all CLR tested, included langerin, which provides evidence that DC-SIGN binding is more affected by multivalency compared to langerin [42]. Moreover, DC-SIGN is organized in 200 nm sized nanodomains on the surface membrane of DCs [43]. DC-SIGN cluster formation is required for the binding and internalization of viral particles and interestingly, DC-SIGN molecules acquire a higher avidity for multimeric ligands when organized in multimolecular assemblies [43]. This receptor distribution is advantageous to DC-SIGN, because it favors multiple interactions with pathogens that differ greatly in size [44]. For langerin, the formation of nanodomains on the plasma membrane is not described.

Langerin and DC-SIGN not only differ with regard to the formation of microdomains on the plasma membrane, but also in multimerization. Langerin forms trimers on the cell surface, whereas DC-SIGN is expressed as a tetramer. Thus, the organization of DC-SIGN at two levels of clustering (tetramerization and formation of nanodomains) favors the synergistic enhancement of the strength of single glycan–receptor bonds. Multivalent ligands benefit from this phenomenon, as the strength of the glycan–DC-SIGN interactions increases from low avidity to high avidity in the submicromolar and nanomolar range [44]. These differences in both levels of clustering might contribute to the differences we have observed between langerin<sup>+</sup> LCs and DC-SIGN<sup>+</sup> moDCs with regard to glycoliposome internalization.

Another striking difference between LCs and DCs is the location within human tissues. LCs are located in the outermost layers of the skin in close contact with the bacterial skin flora, whereas the DC reside in the dermis underneath the epidermis. Therefore, it is hypothesized that LCs should not participate in the recognition of bacteria to prevent inflammation, whereas dDCs should quickly respond to bacteria [45]. This is further supported by data showing that LCs do not express TLR2, 4 and 5, the TLRs responsible for the recognition of extracellular bacteria, in contrast to dDCs that express these TLRs and respond to extracellular bacteria [46,47]. In addition, previous publications showed that LCs inefficiently internalized, degraded and presented bacterial antigens compared to the dDCs, thereby inducing tolerance to the bacterial skin flora [45,48].

Altogether, we have shown that in contrast to glycan-modified liposomes, human langerin internalizes antibody- and glycan-modified peptides efficiently into LCs. On the other hand, DC-SIGN bound and internalized glycan-modified liposomes to a high extent, but failed to endocytose glycan-modified peptides. Thus, targeting APCs using vaccine formulations with specific properties, like size and multivalency, allows for DC subset specific targeting, thereby greatly influencing the efficacy of DC-targeting vaccines.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jconrel.2015.01.040>.

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