MPLA incorporation into DC-targeting glycoliposomes favours anti-tumour T cell responses

Martine A. Boks [a], Martino Ambrosini [a], Sven C. Bruijns [a], Hakan Kalay [a], Louis van Bloois [b], Gert Storm [b,c], Juan J. Garcia-Vallejo [a], Yvette van Kooyk [a,*]

[a] Department of Molecular Cell Biology and Immunology, VU University Medical Center (VUmc), Amsterdam, The Netherlands
[b] Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands
[c] MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, The Netherlands

ABSTRACT

Dendritic cells (DC) are attractive targets for cancer immunotherapy as they initiate strong and long-lived tumour-specific T cell responses. DC can be effectively targeted in vivo with tumour antigens by using nanocarriers such as liposomes. Cross-presentation of tumour antigens is enhanced with strong adjuvants such as TLR ligands. However, often these adjuvants have off-target effects, and would benefit from a DC-specific targeting strategy, similar to the tumour antigen. The goal of this study was to develop a strategy for specifically targeting DC with tumour antigen and adjuvant by using glycoliposomes. We have generated liposomes containing the glycan Lewis(Le)^X which is highly specific for the C-type lectin receptor DC-SIGN expressed by DC. Le^X-modified liposomes were taken up by human monocyte-derived DC in a DC-SIGN-specific manner. As adjuvants we incorporated the TLR ligands Pam$_3$CySK$_4$, Poly I:C, MPLA and R848 into liposomes and compared their adjuvant capacity on DC. Incorporation of the TLR4 ligand MPLA into glycoliposomes induced DC maturation and production of pro-inflammatory cytokines, in a DC-SIGN-specific manner, and DC activation was comparable to administration of soluble MPLA. Incorporation of MPLA into glycoliposomes significantly enhanced antigen cross-presentation of the melanoma tumour antigen gp100$_{280-288}$ peptide to CD8$^+$ T cells compared to non-glycosylated MPLA liposomes. Importantly, antigen cross-presentation of the gp100$_{280-288}$ peptide was significantly higher using MPLA glycoliposomes compared to the co-administration of soluble MPLA with glycoliposomes. Taken together, our data demonstrates that specific targeting of a gp100 tumour antigen and the adjuvant MPLA to DC-SIGN-expressing DC enhances the uptake of peptide-containing liposomes, the activation of DC, and induces tumour antigen-specific CD8$^+$ T cell responses. These data demonstrate that adjuvant-containing glycoliposome-based vaccines targeting DC-SIGN$^+$ DC represent a powerful new approach for CD8$^+$ T cell activation.

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1. Introduction

Dendritic cells (DC) possess the unique ability to induce and modulate antigen-specific immune responses, which makes them likely candidates to exploit for immune modulating therapies against cancer. By effectively targeting DC with tumour antigens, DC can present these antigens to T cells, leading to the development of strong and long-lived tumour-specific T cell responses [1,2]. CD8$^+$ cytotoxic T lymphocytes (CTL) are required for immune-mediated clearance of tumours and DC play a pivotal role in the initiation and regulation of these immune responses [3]. Immature DC reside in peripheral tissues, where they constantly sample their environment in search of pathogens. After recognition of antigens through pattern recognition receptors such as Toll-like receptors (TLR), DC mature and migrate to draining lymph nodes, where naive T cells reside. During maturation, DC enhance the expression of costimulatory molecules that are required for T cell activation [4,5]. Meanwhile, antigens that are internalised by various uptake receptors, are being processed and presented in major histocompatibility complex (MHC) class I and II molecules to CD8$^+$ and CD4$^+$ T cells, respectively [6,7]. Exogenous antigens are presented by MHC class II molecules after processing in the endo-lysosomal route, or can be presented by MHC class I molecules by a process called 'cross-presentation' [8,9].

For immunotherapy, direct and specific targeting of DC in vivo is highly desirable to bring the vaccine only to DC and thereby limiting immune-related adverse side effects. Strategies aimed to develop in vivo DC targeting vaccines require a specific target on DC that favour antigen processing and presentation. Promising targets in this respect are C-type lectin receptors (CLR) such as DC-SIGN, DEC-205, mannose receptor (MR) or CLEC9A [1,2,10]. Pioneering work on DC targeting strategies has been performed on DEC-205. Fusion of tumour antigens

* Corresponding author at: PO box 7057, 1007 MB Amsterdam, The Netherlands.
E-mail address: y.vankooyk@vumc.nl (Y. van Kooyk).

to DEC-205 monoclonal antibodies increased the efficiency of antigen presentation on MHC class I and II [11,12]. In vivo targeting of DC with DEC-205 antibody is very effective at inducing CTL and anti-tumour responses in mice and non-human primates [13,14], and phase I clinical trials are currently underway with the first results showing feasibility and biological activity of the vaccine [15]. A disadvantage of using antibodies for DC targeting could be non-specific uptake via the Fc part and Fc receptor triggering. In addition, even when humanised, these antibodies can elicit adverse immunogenic effects that obstruct the development of a successful anti-tumour immune response. This can be overcome by using carbohydrates, the natural ligands for CLR, which are present on pathogens and self-glycoproteins [16]. In addition, carbohydrates can be produced by organic chemical synthesis, which makes them good candidates for large cost-effective production as compared to antibodies.

CLR are a family of uptake receptors specifically expressed by distinct DC subsets. Their subset-specific expression pattern thus provides the opportunity to target the desired DC subpopulation. Only for those CLR whose ligand specificity has been characterised, this targeting strategy can be pursued. This is the case of DC-SIGN, which binds ligands comprising high-mannose-containing structures and fucose-containing structures, including the Lewis(Le)-type antigens [17,18], present on a wide variety of pathogens as well as host glycoproteins [16]. DC-SIGN is widely expressed on DC at mucosal sites, skin and lymph nodes [19], where DC encounter pathogens, but also any intradermally applied vaccine. Recognition of carbohydrate structures by DC-SIGN results in fast and efficient uptake of antigens and presentation of these antigens by MHC molecules enhancing T cell responses [20,21]. Internalisation via DC-SIGN facilitates routing to the endo-lysosomal pathway, linking antigen uptake to processing and presentation on MHC class II molecules. In addition, the extremely robust CD8$^+$ T cell responses after DC-SIGN targeting demonstrate that exogenous antigens also route to a cross-presentation pathway [22]. We and others have shown that modification of antigens with DC-SIGN-binding glycans leads to improved T cell responses [22–24].

Liposomes are spherical particles consisting of phospholipid bilayers and can encapsulate large quantities of hydrophilic and hydrophobic molecules [25,26]. Consequently, they provide the opportunity to incorporate multiple tumour antigens as well as different DC activating molecules like TLR agonists, and are therefore attractive vaccine candidates. Due to their composition of naturally derived compounds, liposomes are well tolerated by the body and have low toxicity. Modification of liposomes with glycans can be used to facilitate targeting to selected CLR on specific DC subsets. In addition, targeting to CLR ensures the presentation of tumour antigen in MHC molecules, as CLR are efficient uptake receptors that route antigens to the endo-lysosomal compartments. Previously, we have explored this strategy of specifically targeting antigens to DC-SIGN using glycan-modified liposomes [27]. These glycan-modified liposomes are efficiently internalised by DC leading to a massive enhancement in antigen presentation of both in vitro and in vivo CD4$^+$ and CD8$^+$ T cell responses. Importantly, co-administration of the DC maturing agent LPS significantly improved antigen presentation to CD4$^+$ T cells and especially cross-presentation to CD8$^+$ T cells [27]. Indeed it has been demonstrated that efficient cross-presentation of antigen requires signalling via TLR [28,29]. The effective activation of naïve CD8$^+$ T cells requires adequate costimulation and cytokine responses from DC. A large set of TLR ligands are known that act as adjuvants and stimulate cross-presentation [30].

In this study we explored the simultaneous targeting of tumour antigen and adjuvant to DC by using an all-in-one formulation of liposomes that contain the glycan Le^X for specific DC-SIGN targeting, and an adjuvant and a tumour antigen for DC maturation and antigen specificity of the immune response. As adjuvant we compared several TLR ligands and for the induction of tumour antigen specific T cells, we used a melanoma-associated peptide derived from gp100 protein. We

examined DC-SIGN-specific internalisation and activation of DC, as well as the effect of glycan modification and adjuvant incorporation on antigen cross-presentation to CD8$^+$ T cells. This approach combines the specific targeting of the uptake receptor DC-SIGN by well-tolerated glycans with the efficient and flexible encapsulation of tumour antigens and adjuvants by liposomes.

2. Materials and methods

2.1. Liposome preparation

Glycan-modified liposomes containing TLR ligands were prepared from a mixture of phospholipids and cholesterol utilizing the film extrusion method as described previously [31]. Briefly, egg phosphatidylcholine (EPC)-35 (Lipoid): egg phosphatidylglycerol (EPG)-Na (Lipoid): Cholesterol (Sigma-Aldrich, St. Louis, MO, USA) at a molar ratio of 3.8:1:2.5 were mixed, where specified, with MPLA (2 mol%), Pam$_3$CysSK$_4$ (1 mol%) or R848 (4 mol%, all from Invivogen, Toulouse, France). 0.1 mol% of the lipophilic fluorescent tracer DiD (1′-dioctadecyl-3,3,3′,3′-tetramethyl indodicarbocyanine, Life Technologies) was incorporated into the liposomes during the first step of the preparation. Where indicated, the hydrophilic TLR ligand Poly I:C (Invivogen) and the antigenic peptide gp100$_{280–288}$ (YLEPGPVTA) were encapsulated into the liposomes, as previously described, during the hydration step [27]. The peptide was produced by solid phase peptide synthesis using Fmoc-chemistry with a Symphony peptide synthesizer (Protein Technologies Inc., Tucson, AZ, USA). The liposomes were sized by sequential extrusion through two stacked polycarbonate filters (800, 400 and 200 nm) with a high-pressure extrusion device. Non-encapsulated peptide and Poly I:C were removed by sedimentation of the liposomes by means of ultracentrifugation using a Beckman Ultracentrifuge at 200,000 g. Removal of the supernatant and resuspension of the pellet was performed two times. The final resuspension of the liposomes was performed in Hepes buffer pH 7.5.

Le^X-glycolipid (Le^X-hexadecanehydrazide) was prepared from Le^X tetrasaccharide (Elicityl, Crolles, France) and palmitic anhydride (Sigma-Aldrich), the latter undergoing two subsequent chemical transformations, first to tert-butyl N-(hexadecanoylamino) carbamate, then to palmitic hydrazide through common reactivity. Palmitic hydrazide was coupled to Le^X through a reductive amination reaction. Briefly, palmitic hydrazide (2 eq., Sigma-Aldrich) and picoline borane (10 eq., Sigma-Aldrich) were dissolved in DMSO/AcOH/CHCl$_3$ (8:2:1, 200 µl). The mixture was added to Le^X (1 eq.) and the reaction was stirred for 2.5 h at 65 °C. Addition of CHCl$_3$/MeOH/H$_2$O at 8:1:8 v/v ml ratio allowed the extraction of Le^X-glycolipid as white slurry at the interphase. The mixture was centrifuged at 4600 rpm for 20 min, then the aqueous and organic layers were carefully removed and the washing step was repeated once more. The slurry was freeze-dried (methanol/water) to remove residual solvent. Glycan derivatisation was confirmed by ESI-MS (LCQ-Deca XP Iontrap mass spectrometer in positive mode; Thermo Scientific, Fremont, CA, USA) using nanospray capillary needle. Le^X-glycolipid was post-inserted into the liposomes by adding 1 ml of liposome suspension to 0.75 mg of glycolipid, previously dissolved in 15 µl of methanol. After 15 min of vigorous stirring and overnight at 4 °C, the liposome suspensions were centrifuged at 200,000 g and resuspended in Hepes buffer pH 7.5 twice.

Before use, the size, polydispersity index and zeta potential was determined (Table 1) as previously described [31]. The amount of liposomes (total lipid) used in the experiments was calculated based on the determined phospholipid contents (in µmol). The concentration of encapsulated gp100 peptide was quantified by HPLC after extraction with 1 v/v of water, 1 v/v of MeOH and 2 v/v of CHCl$_3$ and was routinely 50 µg/ml. The amount of Le^X was quantified by high pH anion exchange chromatography with pulsed-amperometric detection and was found to be 0.4 mg of glycolipid per 1 ml of liposome suspension.

Table 1
Physical characteristics of liposome formulations.

	Empty	Pam3CysSK4	Poly I:C	MPLA	R848
Mean size (nm)	216 ± 20	229 ± 3	212 ± 7	215 ± 11	214 ± 11
Zeta potential (mV)	−47.3 ± 4.8	−47.0 ± 5.3	−52.7 ± 13.1	−46.6 ± 5.1	−45.5 ± 2.2
Polydispersity index	0.05 ± 0.03	0.09 ± 0.02	0.12 ± 0.08	0.04 ± 0.006	0.05 ± 0.02
	Empty-LeX	Pam3CysSK4-LeX	Poly I:C-LeX	MPLA-LeX	R848-LeX
Mean size (nm)	207 ± 14	221 ± 3	208 ± 17	207 ± 10	209 ± 14
Zeta potential (mV)	−38.8 ± 8.9	−38.5 ± 9.8	−44.5 ± 14.3	−39.8 ± 7.9	−40.5 ± 6.5
Polydispersity index	0.03 ± 0.03	0.05 ± 0.05	0.08 ± 0.04	0.07 ± 0.02	0.06 ± 0.04

Data is represented as mean ± SD.

2.2. DC-SIGN-Fc ELISA

The conjugation and correct orientation of the Lewis^X glycan to the liposomes was confirmed by ELISA using recombinant DC-SIGN-Fc. DC-SIGN-Fc was produced from established transfectants as described previously [32]. The chimeric construct consists of the extracellular domain of DC-SIGN fused to the Fc portion of human IgG1. Liposomes were coated (in concentrations as stated) onto Immuno maxisorp plates (NUNC, Roskilde, Denmark) and incubated 1.5 h at 37 °C. Plates were blocked in Tris–sodium buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$ and 2 mM $MgCl_2$) containing 1% Bovine Serum Albumin (BSA; Fraction V, Fatty acid free, Calbiochem, San Diego, CA, USA). After washing, the liposomes were incubated with DC-SIGN-Fc (2 μg/ml) for 2 h at room temperature, in absence or presence of calcium-chelator EGTA (10 mM) or DC-SIGN neutralising antibody AZN-D1 (10 μg/ml) [19]. Binding was detected using a peroxidase-labelled goat anti-human IgG/Fcγ specific F(ab′)2 (Jackson ImmunoResearch Europe, Suffolk, UK). The reaction was developed with 100 μg/ml 3,3′-5,5′-tetramethylbenzidine (TMB) as a substrate (Sigma-Aldrich) and optical density was measured by a microplate absorbance spectrophotometer (Biorad) at 450 nm.

2.3. Detection of DiD using flow cytometry

Liposomes were diluted in PBS and analysed by flow cytometry for DiD fluorescence intensity. DiD was quantified by FACS (FACSCalibur, Becton Dickinson, San Jose, USA) and analysed using FlowJo Software (Tree Star, Ashland, OR, USA).

2.4. Human monocyte-derived dendritic cells and macrophages

Human immature DC were generated from monocytes isolated from buffy coats of healthy donors (Sanquin, Amsterdam, The Netherlands). Buffy coats were mixed with PBS containing 0.45% citrate and peripheral blood mononuclear cells (PBMC) were isolated by a ficoll gradient (Lymfoprep; Axis-Shield PoC AS, Oslo, Norway). PBMC were washed and monocytes isolated by a Percoll gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Monocytes were cultured for 5–6 days in RPMI 1640 medium (Invitrogen, Paisley, UK) containing 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM glutamine (all from Lonza, Verviers, Belgium) in the presence of recombinant human IL-4 and GM-CSF (500 U/ml and 800 U/ml, respectively; Immunotools, Friesoythe, Germany). Alternatively, monocytes were cultured for 5–6 days in this medium in the presence of only recombinant human GM-CSF in order to generate macrophages.

2.5. Liposome binding and uptake assay

Internalisation of modified or unmodified (anchor) liposomes (10 nmol/well) was analysed by flow cytometry following incubation with 50,000 DC for 3 h at 4 °C or 37 °C. Specific binding and uptake via DC-SIGN was determined by incubating the DC with liposomes in the presence or absence of 10 mM EGTA or 10 μg/ml neutralising antibody AZN-D1.

2.6. DC maturation and cytokine production

DC (50,000/well) were incubated with liposomes (100 nmol/well) or equivalent concentration of TLR ligand MPLA (Sigma-Aldrich) overnight at 37 °C, in the presence or absence of 10 μg/ml neutralising antibody AZN-D1. Supernatants were harvested for cytokine ELISA and cells were washed and incubated with CD83-PE (Beckman Coulter) or CD86-PE (Becton Dickinson) for analysis of maturation status by flow cytometry. The levels of IL-6 and TNF-α in the cell culture supernatants were quantified using standard sandwich ELISA antibody pairs from Invitrogen following manufacturer's instructions.

2.7. Imaging flow cytometry analysis of internalisation and routing of liposomes by DC

Internalisation and routing of liposomes (20 nmol/well) was analysed by imaging flow cytometry following incubation with 1 million DC or macrophages for 15 and 60 min at 37 °C. After incubation, cells were immediately put on ice and washed in ice-cold PBS. Cells were fixated in ice-cold 4% paraformaldehyde in PBS for 20 min and permeabilised in 0.1% saponin in PBS with 0.05% BSA for 30 min. Subsequently cells were stained for DC-SIGN with CD209-FITC antibody or lysosomes with LAMP-1-FITC antibody (Becton Dickinson) in 0.1% saponin buffer. Cells were acquired on the ImageStream X (Amnis) imaging flow cytometer. A minimum of 15,000 cells was acquired per sample at a flow rate ranging between 50 and 100 cells/s at 60× magnification. At least 2000 cells were acquired from single stained samples to allow for compensation. Analysis was performed using the IDEAS v6.1 software (Amnis) as previously described [33]. Cells were gated based on focus using the Gradient RMS (brightfield) feature, and on size using the features Area (brightfield) vs Aspect Ratio Intensity (brightfield). Internalisation was addressed using the feature Internalisation on a mask calculated by eroding 8 pixels from the circumference of every cell. Co-localisation was calculated using the feature Bright Detail Similarity R3.

2.8. Antigen presentation assay

A CD8$^+$ T cell clone specific for gp100$_{280–288}$ peptide was generated and cultured as described previously [34]. HLA-A2$^+$ DC (30,000/well) were incubated with indicated concentrations of liposomes in a round bottom 96-well plate for 1 h at 37 °C. After extensive washing, cells were co-cultured with gp100-specific CD8$^+$ T-cells (100,000/well). After overnight incubation, supernatants were harvested and IFNγ

levels were measured by sandwich ELISA using specific antibody pairs from Invitrogen.

2.9. Statistical analysis

Results were analysed using a two-way ANOVA, followed by Bonferroni post-test, using GraphPad Prism software (San Diego, USA; version 5.01). Values were considered to be significantly different when $P \leq 0.05$.

3. Results

3.1. Le^X-modified liposomes are specifically bound by human DC-SIGN

As a vehicle for DC-targeting of antigen, we prepared EPC/EPG/cholesterol-based liposomes with a diameter of approximately 200 nm (Table 1) and used DiD as a fluorescent tracer. To confirm that the amount of DiD per liposome was not affected by the incorporation of Le^X or TLR ligand, the DiD content was analysed by flow cytometry (Fig. 1A). The DiD content was similar for all liposomes, irrespective of Le^X or TLR ligand incorporation. The Le^X glycan binds DC-SIGN with high affinity [17] and was coupled to the liposomes via post-insertion of a glycolipid structure that we generated by conjugation of Le^X glycans on their reducing end to a palmitic acid tail as described in the Materials and methods. The presence of Le^X on the liposomes as well as the affinity of Le^X glycan for DC-SIGN was confirmed by ELISA, using a human DC-SIGN-Fc construct on plate-bound liposomes (Fig. 1B). Le^X-coupled liposomes bound dose-dependently to DC-SIGN, whereas

liposomes without glycan did not bind to DC-SIGN. Moreover, binding of Le^X-coupled liposomes was DC-SIGN specific as the calcium-chelator EGTA as well as a DC-SIGN blocking antibody inhibited the binding (Fig. 1C). Thus, all different TLR ligand-modified liposomes with Le^X have high and comparable DC-SIGN-binding capacity.

3.2. Le^X-modified liposomes are specifically bound and taken up via DC-SIGN by human DC

We set out to assess DC-SIGN-mediated binding and uptake of the glycan-modified liposomes with human DC generated from peripheral blood monocytes. Human monocyte-derived DC express high levels of DC-SIGN [19]. Flow cytometry analysis of DC that were incubated with the different types of DiD-labelled liposomes at 4 °C or 37 °C revealed that liposomes modified with the DC-SIGN-binding glycan Le^X were bound and taken up by DC (Fig. 2A). The uptake was strongly enhanced when DC were incubated at 37 °C. Non-modified (anchor) liposomes were taken up significantly lower by DC. The observed internalisation of the Le^X-modified liposomes to DC was DC-SIGN-specific as this was completely abrogated when DC were incubated with liposomes in the presence of EGTA, which interferes with the function of all CLR, or a DC-SIGN blocking antibody, confirming that DC-SIGN is the single CLR involved in this interaction (Fig. 2B, C). In contrast, the uptake of non-modified liposomes was not affected by DC-SIGN blocking antibodies (Fig. 2D, E). These data show that modification of liposomes with the DC-SIGN-binding glycan Le^X results in increased binding and specific uptake by DC through DC-SIGN.
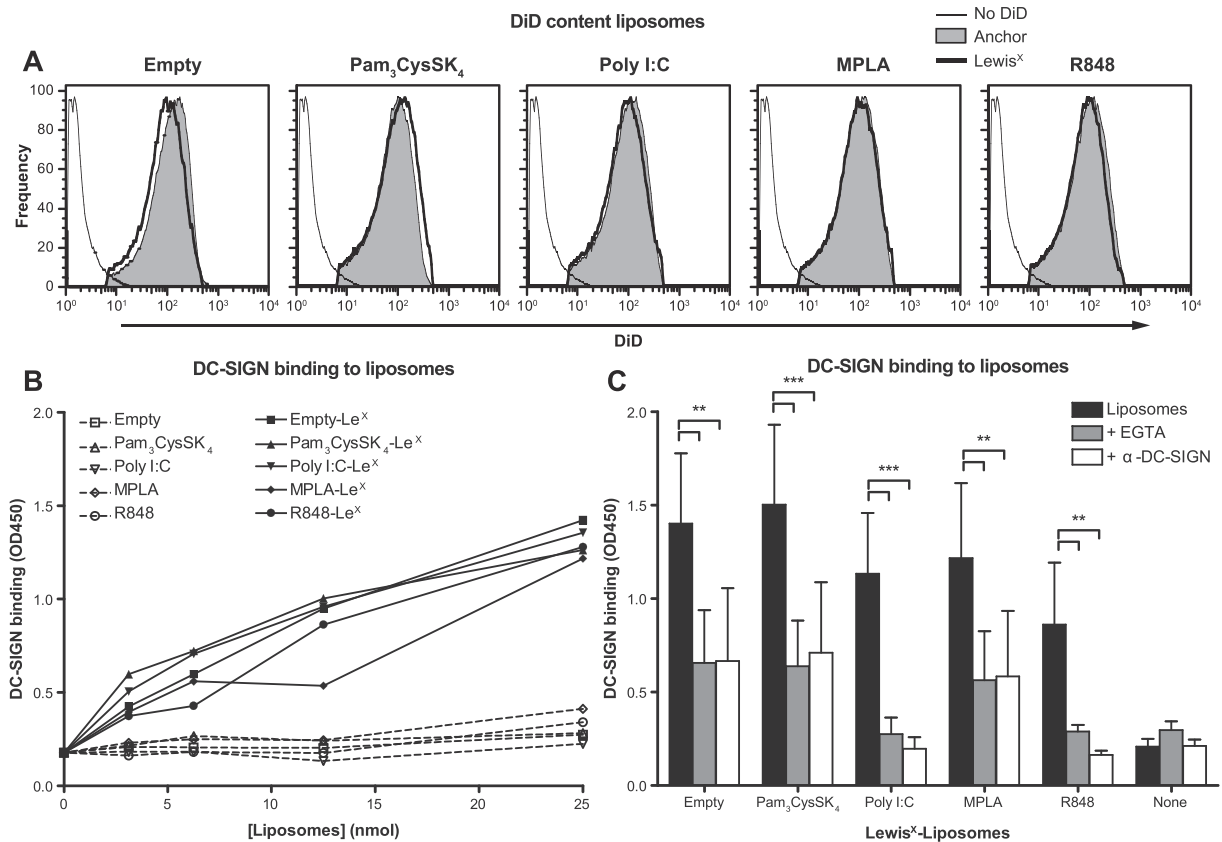
Fig. 1. Physical properties of liposome formulations. A. Liposomes were analysed for DiD fluorochrome content by flow cytometry. Open histograms are empty anchor liposomes (i.e. liposomes with no TLR ligand and no Le^X) without DiD; tinted grey histograms are anchor liposomes; solid thick line histograms are Le^X-coupled liposomes. Data are representative of 2 independent experiments. B. We confirmed the presence of Le^X as well as the affinity of the glycan-modified liposomes for DC-SIGN by ELISA using a DC-SIGN-Fc construct. C. In addition, incubation with DC-SIGN-Fc in the liposome ELISA was performed in the presence of EGTA or blocking antibody against DC-SIGN. Data are shown as mean ± SEM of 3 independent experiments. **$P \leq 0.01$ ***$P \leq 0.001$.
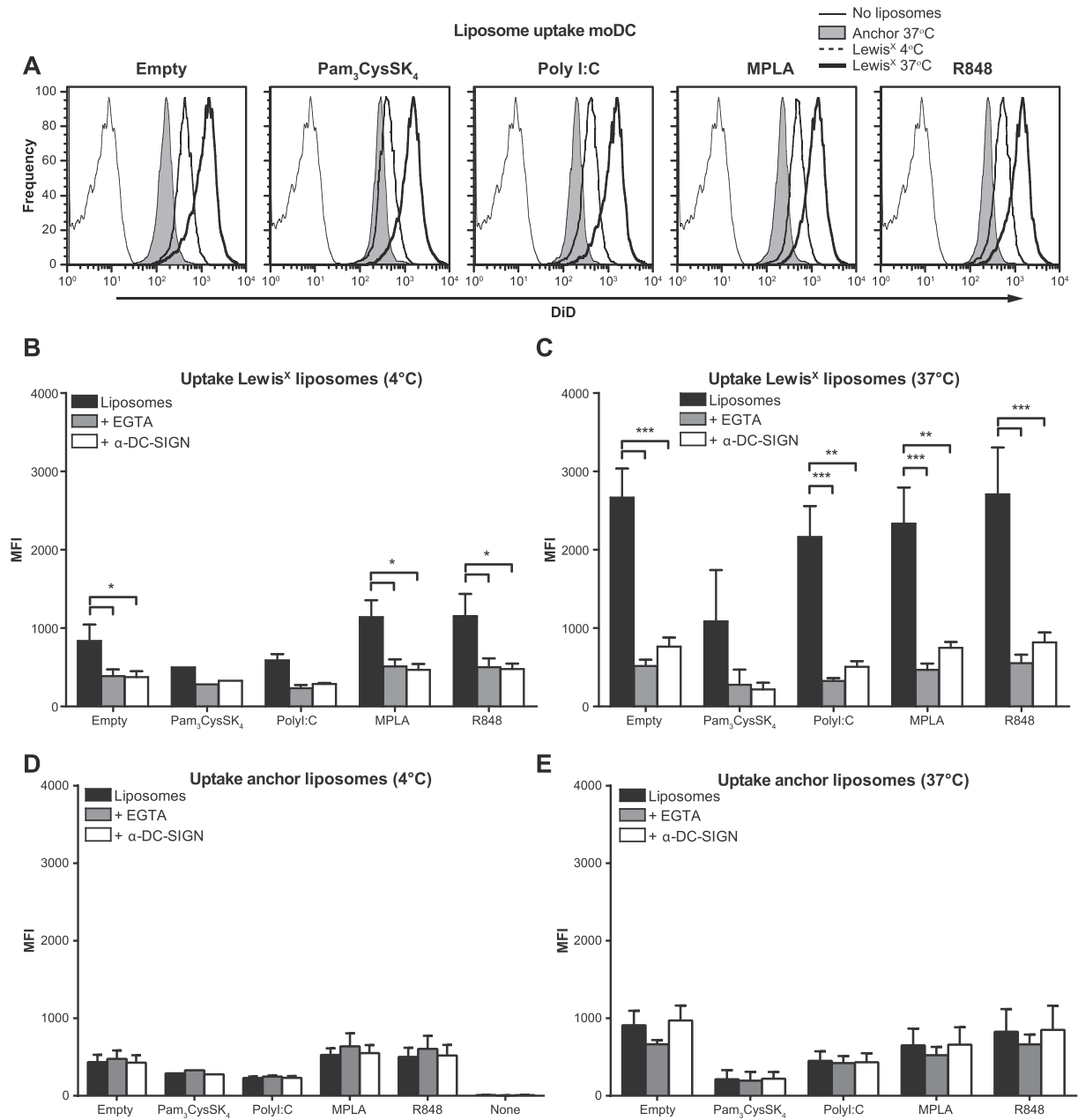
Fig. 2. DC-SIGN-mediated uptake of Lewis^X-coupled liposomes by human DC. Human monocyte-derived DC were exposed to various concentrations of non-modified (anchor) or Le^X-modified liposomes for 3 h at 4 °C or 37 °C and analysed by flow cytometry. A. Open histograms are DC without liposomes; tinted grey histograms are anchor liposomes at 37 °C; dotted line histograms are Le^X-coupled liposomes at 4 °C; solid thick line histograms are Le^X-coupled liposomes at 37 °C. B–E. Incubation with Le^X-modified or non-modified liposomes was performed in the presence of EGTA or blocking antibody against DC-SIGN. Data are shown as mean ± SEM of 3 independent experiments. MFI; mean fluorescence intensity. $*P \leq 0.05$ $**P \leq 0.01$ $***P \leq 0.001$.

3.3. Le^X- and MPLA-modified liposomes induce DC maturation and cytokine production comparable to administration of soluble TLR ligand

For specific activation of DC we incorporated TLR ligands into the liposomes. We used different ligands to investigate the activation via different TLR; Pam$_3$CysSK$_4$, a lipopeptide as a ligand for TLR2; Poly I:C, double-stranded RNA that binds to TLR3, MPLA (monophosphoryl lipid A), a derivative of *Salmonella Minnesota* LPS that activates TLR4, and R848, an imidazoquinoline compound for TLR7/8 activation. DC were incubated with the different liposomes overnight and their phenotype analysed by flow cytometry. Especially the targeting of MPLA-modified liposomes to DC showed very efficient DC activation with increased expression of the maturation marker CD83 and the

costimulatory molecule CD86 (Fig. 3A, B). This activation was dependent on the targeting of MPLA to DC, as non-targeted MPLA liposomes and the blocking of Le^X-targeted MPLA liposomes with a DC-SIGN blocking antibody did not induce DC maturation. In addition, production of TNFα and IL-6 was increased with Le^X-targeted MPLA liposomes in a DC-SIGN-dependent manner (Fig. 3C, D).

Next, we compared this stimulatory ability of MPLA-containing liposomes to that of soluble MPLA. MPLA that is targeted to DC in Le^X-modified liposomes showed equal induction of DC maturation (Fig. 4A, B) and cytokine production (Fig. 4C, D) as compared to soluble MPLA. Liposomes without MPLA or the non-targeted liposomes with MPLA induced no, or low, activation of DC. Together, these results demonstrate that targeting of adjuvant MPLA to DC-SIGN using
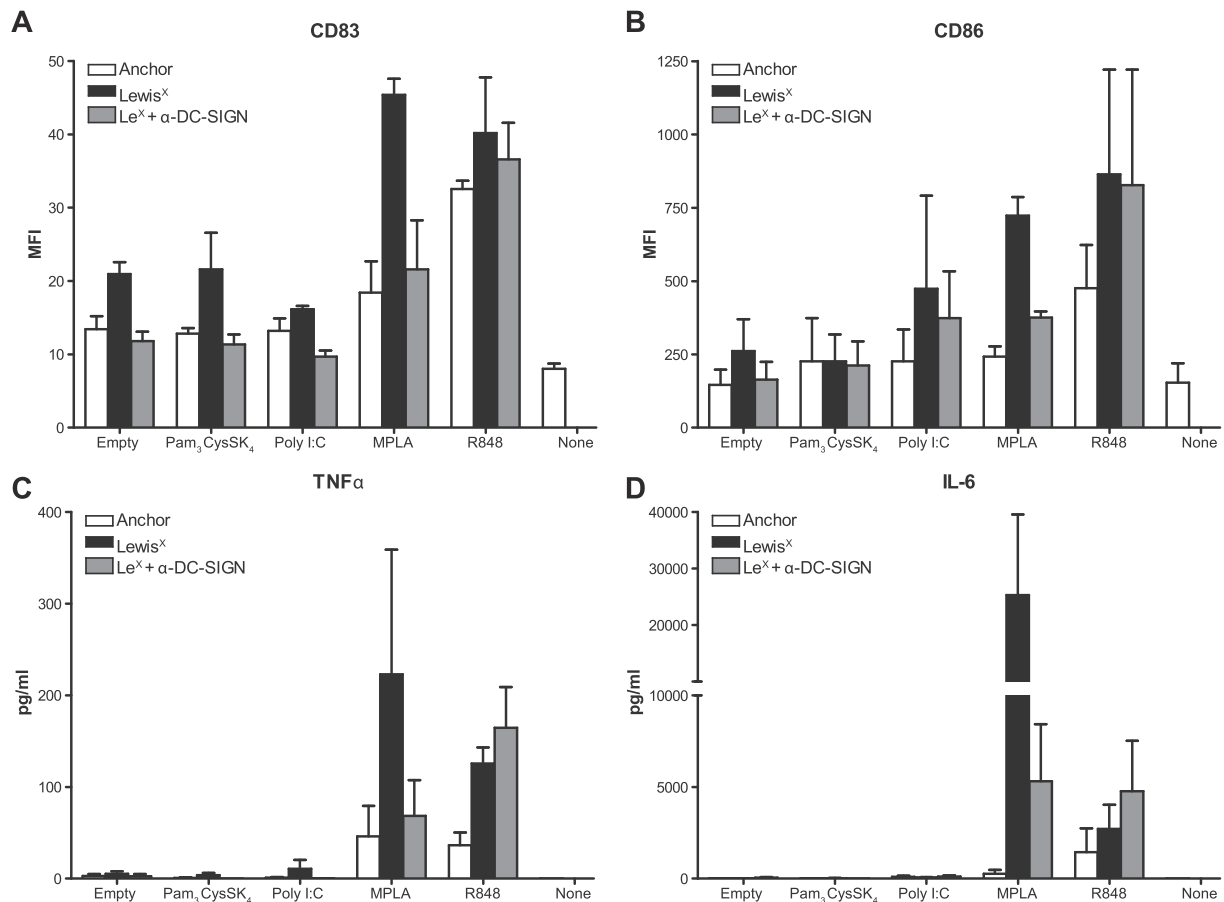
Fig. 3. DC-SIGN-mediated targeting of Lewis^X- plus MPLA-modified liposomes induces DC maturation and cytokine production. After overnight incubation with Le^X-modified or non-modified (anchor) liposomes, with or without TLR ligand incorporation DC were analysed by flow cytometry for CD83 and CD86 expression (A, B) or cytokines TNFα and IL-6 production was measured in culture supernatants by ELISA (C, D). Incubation with liposomes was performed in the presence of DC-SIGN blocking antibody. Data are shown as mean ± SEM of duplicate cultures. Results are 2 representative experiments of 4 independent experiments. MFI; mean fluorescence intensity.

Le^X-modified liposomes enhances the activation of DC, comparable to soluble MPLA.

3.4. Effective internalisation by DC and intracellular routing to lysosomes of Le^X- and MPLA-modified liposomes

DC-SIGN is specifically expressed by DC [19] and Le^X glycans are highly specific for this receptor [17,18]. To confirm the specificity of Le^X-modified liposomes for DC, we have investigated the ability of macrophages and lymphocytes to internalise Le^X liposomes. Cells exposed to liposomes were analysed for the presence and intracellular localisation of liposomes using imaging flow cytometry. As expected, only DC expressed high levels of DC-SIGN (Fig. 5A), which correlated to the binding and uptake of Le^X liposomes (Fig. 5B). Lymphocytes had negligible levels of liposome uptake and, although macrophages showed some internalisation, there was no significant difference attributable to Le^X (Fig. 5B). These data clearly show that binding and uptake of Le^X liposomes only occurs in DC-SIGN-expressing cells, demonstrating the specificity of Le^X liposomes to DC-SIGN expressing DC.

To verify the binding and uptake of Le^X- and MPLA-modified liposomes, we performed imaging flow cytometry analysis on the internalisation of the liposomes by DC. DC were incubated with liposomes for indicated time periods and subsequently fixated for analysis of internalisation. Both Le^X- and MPLA/Le^X-modified liposomes showed highly efficient internalisation by DC compared to non-targeted liposomes (Fig. 5C). This internalisation took place at 37 °C only, as shown by the negative internalisation score of Le^X-modified liposomes incubated at 4 °C, and was very rapid for glycan-modified liposomes. To

determine the intracellular routing of DC-SIGN-targeting liposomes, we performed a pulse-chase assay with the liposomes in combination with staining for the lysosomal marker LAMP-1. Upon internalisation of Le^X- and MPLA/Le^X-modified liposomes, we observed high colocalisation with lysosomes (Fig. 5D). Non-targeted liposomes did not show any colocalisation with lysosomes, as they do not internalise. Thus, this indicates that DC-SIGN targeting with glycan-modified liposomes routes antigenic cargo to lysosomal compartments, necessary for proper antigen processing prior to presentation.

3.5. Targeting antigen to DC with MPLA-modified liposomes, but not with soluble MPLA, enhances antigen presentation to CD8$^+$ T cells

Since glycan-modification of liposomes enhances the internalisation and routing to lysosomal compartments, and MPLA-modification of liposomes enhances DC activation, we examined whether this resulted in increased antigen presentation to CD8$^+$ T cells. For these experiments, liposomes were loaded with the melanoma-associated antigen peptide gp100$_{280–288}$, allowing the detection of antigen presentation using a gp100-specific CD8$^+$ T cell clone [34]. HLA-A2$^+$ DC were incubated with different concentrations of liposomes and subsequently cocultured with the T cells. Loading of DC with MPLA-modified liposomes markedly enhanced presentation of the gp100$_{280–288}$ peptide to CD8$^+$ T cells as revealed by significantly higher IFNγ production compared to liposomes without MPLA (Fig. 6). We observed no IFNγ production without administration of the gp100 peptide (data not shown) indicating that the production of IFNγ is tumour antigen specific. Specific targeting of gp100$_{280–288}$ peptide and MPLA using Le^X-modified liposomes
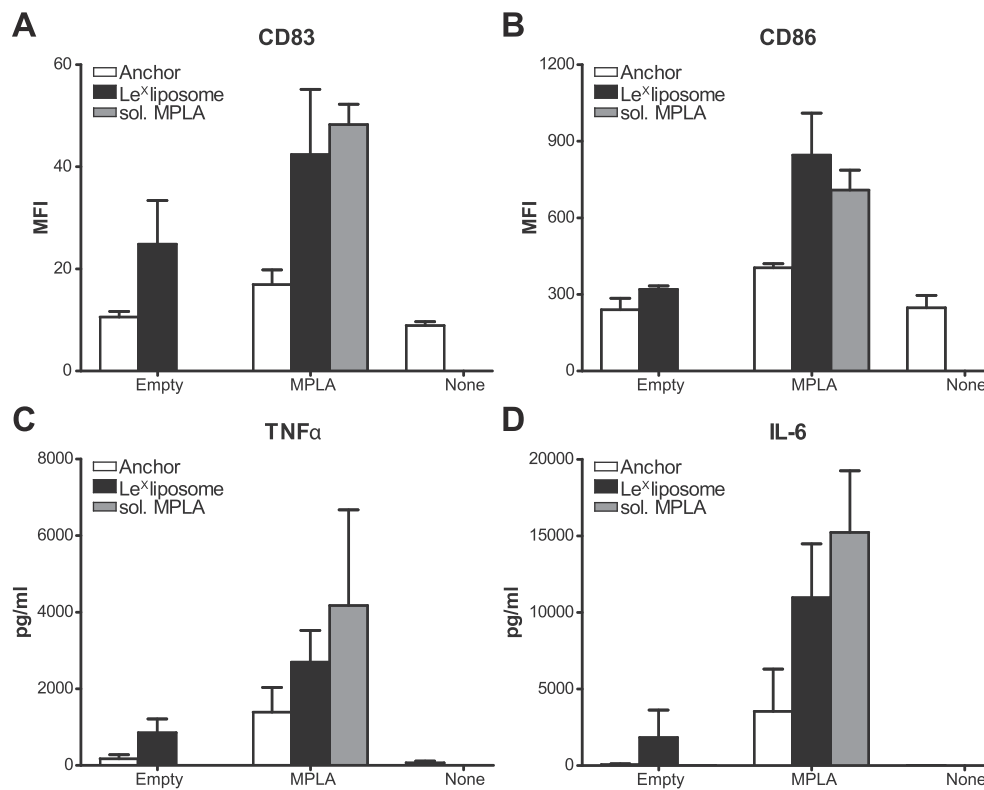
Fig. 4. DC-SIGN-mediated targeting of Lewis[X]- plus MPLA-modified liposomes induces DC maturation and cytokine production similar to soluble MPLA. DC were stimulated with Le[X]-modified (black bars) or non-modified (white bars) liposomes, with or without MPLA incorporation. Incubation with liposomes was compared to stimulation with soluble MPLA (concentration as used for liposome generation) (grey bars). After overnight incubation DC were analysed by flow cytometry for CD83 and CD86 expression (A, B) or cytokines TNFα and IL-6 production was measured in culture supernatants by ELISA (C, D). Data are shown as mean ± SEM of 3 independent experiments (with duplicate cultures). MFI; mean fluorescence intensity.

resulted in significantly increased IFNγ production compared to MPLA liposomes without Le[X] glycan. Furthermore, we compared the induction of antigen presentation of MPLA inside liposomes to the co-administration of MPLA with liposomes. Although DC activation was not different between liposomal MPLA and soluble MPLA (Fig. 4), specific targeting of MPLA and antigen together in one liposome, either targeted or non-targeted, significantly increased antigen presentation to CD8^{+} T cells when compared to co-administration of liposomes and soluble MPLA (Fig. 6). Together, these data show that simultaneous targeting of antigen and adjuvant to DC-SIGN using glycan-modified liposomes efficiently improves cross-presentation to CD8^{+} T cells.

4. Discussion

Since DC and tumour-specific CTL are of vital importance for effective anti-tumour immune responses, the objective of this study was to develop a therapeutic strategy to target DC with tumour antigen combined with a DC activating adjuvant in order to induce efficient tumour-specific T cell responses and to reduce off-target effects. For this, we generated glycoliposomes to specifically target DC-SIGN. DC-SIGN is a DC-specific CLR and is known to bind high-mannose and fucose structures [17,18], making DC-SIGN an ideal target for DC-specific glycan-based targeting. DC-SIGN is expressed by myeloid DC located at distinct sites, including skin, mucosa and lymph nodes [19] and therefore an attractive target and easily accessible for vaccines.

In vitro and in vivo targeting of antigen coupled to DC-SIGN antibodies induced effective antigen-specific T cell responses and inhibited tumour growth [35,36]. However, since antibodies can elicit immunogenicity and require costly production, the use of natural glycans is preferred. Moreover, it was shown that targeting DC-SIGN with glycan structures led to enhanced antigen uptake, whereas targeting antibodies showed poor ability to accumulate within DC [37]. Modification of

soluble antigen with DC-SIGN-binding glycans not only improved targeting to DC-SIGN as shown by enhanced antigen uptake by DC, but also increased (cross-) presentation to antigen-specific CD8^{+} and CD4^{+} T-cells [22–24]. In contrast to soluble antigens, antigens delivered in a particulate form, such as pathogens, are far more efficiently cross-presented [38,39]. Previously we showed that glycan modification of both soluble antigens or liposomes resulted in increased internalisation via DC-SIGN and increased presentation of the ingested antigen in MHC molecules [22,27]. However, the activation of CD4^{+} and CD8^{+} T cells induced by glycan-modified liposomes seems to be much more efficient than by glycan-modified soluble antigens, especially with simultaneous TLR triggering. This difference could be explained by the binding preferences of DC-SIGN for large particles as DC-SIGN is renowned for its capacity to bind pathogens [40]. Also, it has been shown that OVA coupled to 50 nm particles was routed to acidic compartments where it was more rapidly degraded and inefficiently cross-presented than when bound to larger particles of 500 nm [41]. It could be hypothesized that due to the difference in size, glycan-modified soluble antigens or liposomes are routed to different intracellular compartments, or processed at different rates. As such, a particle may lead to more efficient antigen (cross-) presentation than soluble antigens.

Liposomes are among the most extensively investigated vaccine delivery systems owing to their biocompatible, biodegradable and non-toxic nature [25,26]. Many liposome-based therapies carrying chemotherapeutic drugs are currently in phase I or II clinical trials for several solid tumours [42]. However, so far only a few studies exist that show targeting of liposomes specifically to DC-expressed CLR. In some of these studies, liposomes were modified with antibodies against DEC-205 or DC-SIGN which improved uptake by human monocyte-derived and CD1c^{+} blood DC in vitro [43,44]. When modified with mannosyl-lipid derivatives or oligo-mannose glycans, liposomes were efficiently endocytosed in a MR-dependent manner by human
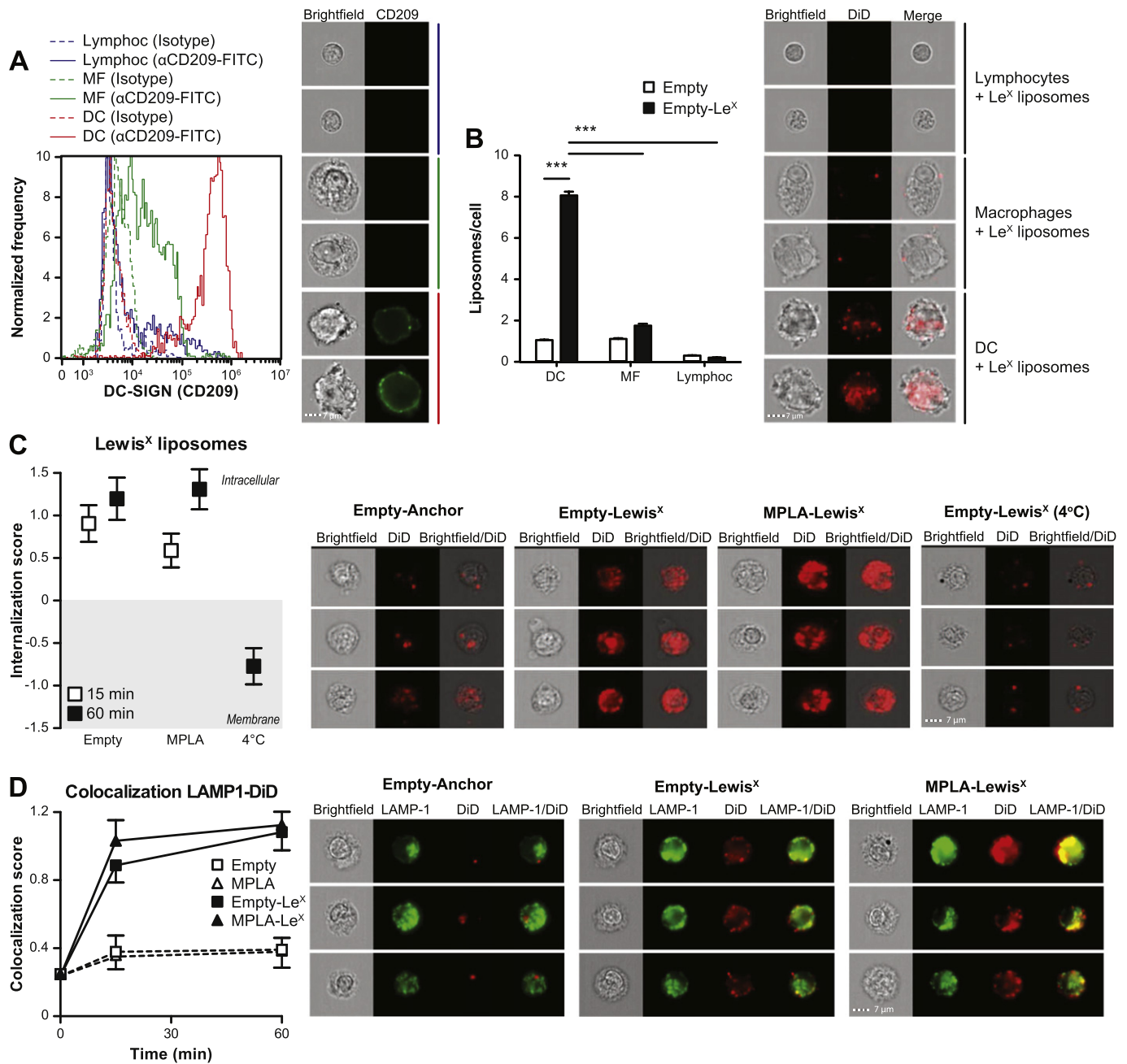
Fig. 5. Lewis[X]- plus MPLA-modified liposomes are efficiently internalised by DC-SIGN expressing DC only and route to lysosomes. DC, macrophages and lymphocytes were analysed with imaging flow cytometry for DC-SIGN expression after staining for CD209 or isotype control (A) or were incubated with Le[X]-modified or non-modified (anchor) liposomes for 15 min at 37 °C and were analysed for liposome internalisation (B). DC were incubated with liposomes for 15 or 60 min at 37 °C, or at 4 °C as control for no uptake (C, D). After incubation DC were fixated and analysed with imaging flow cytometry for liposome internalisation (C) and colocalisation of liposomes with lysosomes after staining for LAMP-1 (D). Right panels show representative cells after 15 min incubation. Data are shown as mean ± SD of a representative experiment of 1 (A, B), 3 (C) or 2 (D) independent experiments. Lymphoc; lymphocytes, MF; macrophages.

monocyte-derived DC, in contrast to non-targeted liposomes [45–47]. A major drawback in targeting the MR could be that apart from DC, the MR is also expressed by monocytes, macrophages, subsets of endothelial cells, retinal pigment epithelium, kidney mesangial cells, and tracheal smooth muscle cells [48]. Indeed, oligo-mannose-modified liposomes were found to be predominantly ingested by macrophages upon injection into mice [49]. In contrast, DC-SIGN is expressed exclusively on DC [19] and Le[X]-modified liposomes target only to DC-SIGN+ bone marrow (BM)-derived DC and not DC-SIGN− BM-DC nor macrophages or monocytes [31]. Although targeting of CLR with liposomes shows good results with regard to antigen uptake, data on improved antigen-specific T cell activation is limited. One study with mannosylated

liposomes and encapsulated tetanus toxoid showed effective T cell proliferation compared to non-targeted liposomes [46]. An in vivo study with mannosylated liposomes containing OVA antigen demonstrated cytotoxic activity of spleen cells against OVA-expressing tumour cells and efficient anti-tumour immunity [47].

The goal of in vivo DC targeting is two-fold; firstly to accumulate antigen to DC in a cell-specific manner and secondly to promote DC maturation and cross-presentation. Although the cross-presentation route is still poorly characterised, it has been demonstrated that this pathway is sensitive to TLR stimulation [28,29]. We here show that CD8+ T cell activation was greatly enhanced when DC-SIGN targeting was performed in the context of TLR4 activation with adjuvant MPLA.

CD8 T cell response
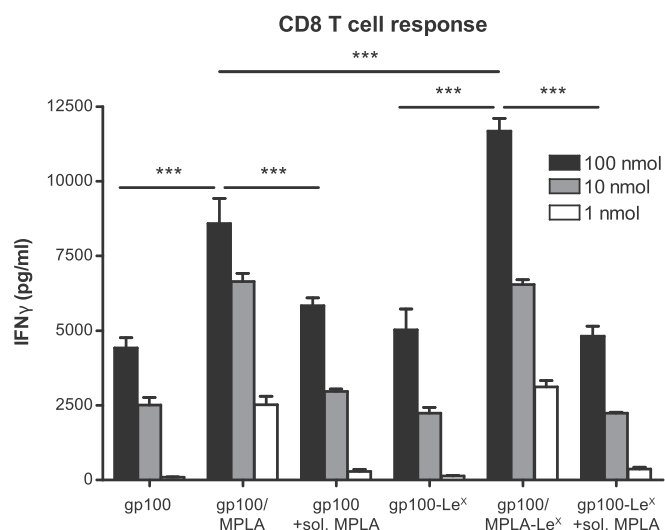
Fig. 6. Targeting DC with Lewis^X- and MPLA modified liposomes enhances antigen presentation to CD8$^+$ T cells. HLA-A2$^+$ DC were exposed to various concentrations of non-modified or Le^X- and/or MPLA-modified liposomes, all loaded with gp100$_{280-288}$ peptide, for 1 h, in the presence or absence of soluble MPLA (concentrations as used for liposome generation). After extensive washing, a gp100-specific HLA-A2-restricted CD8$^+$ T cell clone was added and after 24 h supernatants of the co-culture were taken and analysed for IFNγ production by ELISA. Data are shown as mean ± SEM of triplicate cultures. Results are representative of 3 independent experiments. ***$P \leq 0.001$.

Remarkably, cross-presentation was significantly enhanced with liposomal MPLA, but not with soluble MPLA, whereas DC activation was similar for liposomal MPLA and soluble MPLA. It is possible that the delivery of antigen and the triggering of TLR4, both on the cell surface as well as intracellular, on the same cell results in superior antigen presentation to T cells. This in contrast to liposomes administered with soluble MPLA, where MPLA predominantly triggers cell surface TLR4, possibly resulting in a different effect on the antigen presentation route.

Simultaneous delivery of antigen and adjuvant in one particle to the same APC seems to be crucial for inducing potent T cells responses and to reduce off-target effects of the adjuvant [50]. Non-targeted liposomes containing tumour antigen and TLR ligand CpG, MPLA or Poly I:C showed effective T cell responses and potent anti-tumour immune responses when compared to liposomes without adjuvant or to liposomes plus soluble adjuvant [51–53]. These liposomes promoted DC activation, CTL responses, reduction of tumour growth, and a higher frequency of CD8$^+$ T cells infiltrating the tumour. However, the approach of combining adjuvant and a DC targeting moiety in one liposome has not been investigated in detail. Our results demonstrated that CD8$^+$ T cell activation was greatly enhanced when MPLA was targeted to DC in Le^X-modified liposomes, whereas the co-administration of soluble MPLA did not enhance cross-presentation. A recent study by Chen et al. demonstrated the adjuvant effect of IDO siRNA incorporated in mannosylated liposomes [54]. Vaccination with these liposomes and the subsequent gene silencing of IDO, thereby inhibiting the enzyme that normally leads to T cell inhibition and regulatory T cell induction, displayed a delayed onset time of melanomas, increased survival time of the mice, reduced tumour size and increased T cell reactivity against melanoma antigens. Thomann et al. used mannosylated liposomes with TLR2 ligands Pam$_2$CysSK$_4$ or Pam$_3$CysSK$_4$ which were more efficient in the eradication of tumours than liposomes together with soluble TLR agonist or than non-targeted liposomes [55]. Together with our data, these studies show the potential of targeting antigen and adjuvant specifically to APC-expressed uptake receptors. Addressing in vivo targeting of DC-SIGN is challenging, as the homologue of DC-SIGN is not expressed by any murine DC subset. However, targeting glycoliposomes or glycosylated antigen to DC-SIGN$^+$ DC in DC-SIGN transgenic mice was very effective and induced strong antigen-specific

T cell responses [22,27]. This targeting was done with Le^b-containing liposomes, since in mice the lectin MGL1 also recognizes Le^X.

In summary, the incorporation of TLR ligands into glycan-modified liposomes resulted in enhanced DC maturation. The increased uptake by DC and the increased activation of DC by MPLA-incorporated glycoliposomes resulted in enhanced tumour antigen cross-presentation to CD8$^+$ T cells. Our data demonstrate the effective targeting of TLR ligand-glycoliposomes to DC-SIGN$^+$ DC and the potency as anti-tumour vaccine for inducing tumour-specific T cell responses. We propose that glycoliposomes can be used to deliver tumour antigen and adjuvants directly to DC in vivo and be exploited as a vaccine platform to induce potent anti-tumour immune responses.

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