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

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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_3CySK_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.

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

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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_3CysSK_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_2O 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.