



Lipo-Based Vaccines as an Approach to Target Dendritic Cells for Induction of T- and iNKT Cell Responses

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OPEN ACCESS

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Specialty section:

This article was submitted to
Antigen Presenting Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 20 October 2019

Accepted: 27 April 2020

Published: 27 May 2020

Citation:

Stolk DA, de Haas A, Vree J, Duinkerken S, Lübbers J, van de Ven R, Ambrosini M, Kalay H, Bruijns S, van der Vliet HJ, de Gruijl TD and van Kooyk Y (2020) Lipo-Based Vaccines as an Approach to Target Dendritic Cells for Induction of T- and iNKT Cell Responses. *Front. Immunol.* 11:990. doi: 10.3389/fimmu.2020.00990

In this study we developed a liposome-based vaccine containing palmitoylated synthetic long peptides (SLP) and alpha galactosylceramide (α GC) to specifically target dendritic cells (DC) for activation of both innate (invariant natural killer T-cells [iNKT]) and adaptive (CD8⁺ T-cells) players of the immune system. Combination of model tumor specific antigens (gp100/MART-1) formulated as a SLP and α GC in one liposome results in strong activation of CD8⁺ and iNKT, as measured by IFN γ secretion. Moreover, addition of lipo-Lewis Y (Le^Y) to the liposomes for C-type lectin targeting increased not only uptake by monocyte-derived dendritic cells (moDC), dermal dendritic cells and Langerhans cells but also enhanced gp100-specific CD8⁺ T- and iNKT cell activation by human skin-emigrated antigen presenting cells in an *ex vivo* explant model. Loading of moDC with liposomes containing Le^Y also showed priming of MART-1_{26–35L} specific CD8⁺ T-cells. In conclusion, chemically linking a lipid tail to a glycan-based targeting moiety and SLP combined with α GC in one liposome allows for easy generation of vaccine formulations that target multiple skin DC subsets and induce tumor antigen specific CD8⁺ T- and iNKT cells. These liposomes present a new vaccination strategy against tumors.

Keywords: dendritic cell, DC-SIGN, langerin, liposome, targeting, iNKT, cancer vaccine, T-cell priming

INTRODUCTION

During the past decade substantial progress has been made in the field of cancer immunotherapy, especially with the rise of immune checkpoint inhibitor (ICI) therapy (1–3). Unfortunately, as for many other therapies, the amount of patients that respond to ICI is still very limited (1–4), therefore new strategies are warranted. Combining ICI with peptide vaccination strategies could be efficacious since these approaches address synergistic pathways and the combination of both therapies could therefore unleash blockade of effector T-cells and thereby potentially improve anti-tumor outcome.

Dendritic cells (DC) are an attractive target for the initiation of strong immune responses as they possess the capability of capturing antigens and presenting them to specific T-cells for their activation (5, 6). The skin harbors different types of antigen presenting cells, such as

dermal DC (dDC) and Langerhans cells (LC) and is therefore an attractive site for delivery of vaccines (7–10). Delivery of vaccines to DC and LC can be addressed by targeting e.g., C-type lectin receptors such as Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) and Langerin. This can be easily done using their natural glycan ligands, including Lewis Y (Le^Y) (11–14). DC have an additional central role when it comes to activation of invariant natural killer T-cells (iNKT) by presentation of glycolipids such as alpha galactosylceramide (α GC) in CD1d (15). iNKT are of special interest in vaccination strategies since they release a wide variety of pro-inflammatory cytokines upon activation, which aids activation of $CD8^+$ cytotoxic T-cells, NK-cells B-cells and DC (16–18). For this reason they are widely known as typical strong bridgers of innate and adaptive immune responses (19, 20) and numerous pre-clinical studies have emphasized the potential of iNKT activation in anti-tumor responses (21–24). Interestingly, not only moDC but also dDC play an important role in the stimulation of iNKT. We have previously shown that injected α GC is taken up by skin antigen presenting cells (APC) and results in activation of iNKT which enhances antigen cross-presentation by dDC to provide tumor protection (25). Although the potential of iNKT activation in anti-tumor therapies has been widely acknowledged in experimental models, clinical trials with iNKT have thus far revealed that the induction of effective and durable anti-tumor responses is still very limited (26–31). Therefore, more potent strategies are warranted.

In this study we aimed to develop a Le^Y -conjugated liposome-based DC targeting vaccine to initiate strong immune responses by induction of tumor antigen specific $CD8^+$ specific T-cells and iNKT activation. We show simultaneous melanoma antigen-specific $CD8^+$ effector T-cell and iNKT activation by skin emigrated DC upon intradermal delivery of the vaccine, demonstrating its utility for effective cancer vaccination.

RESULTS

Liposomes Are Efficiently Internalized by moDC

Liposomes were generated using a mixture of phospholipids and cholesterol to form so called empty liposomes. In addition, liposomes were generated with palmitoylated synthetic long peptides (SLP) of MHC class I and II epitopes of the melanoma antigen gp100, α GC and a combination of SLP and α GC that were embedded into the bilayer (Figure 1A). Size distribution with dynamic light scattering analysis and zeta potential were measured and showed equal size distribution and charge independent of liposomal content between formulations (Table 1). To assess the capacity of moDC to internalize our nano-carriers, they were incubated with the different liposomes that were fluorescently labeled with the lipophilic dye DiD and flow cytometry was performed. Analysis with imaging flow cytometry showed clearly that after 45 min incubation at 4°C ($t = 0$), most liposomes were located at the membrane of the DC, but after transfer to 37°C, allowing metabolic activity of the DC and rearrangement of the actin filaments,

liposomes were mainly localized intracellularly (Figure 1B). Analysis of DiD signal in moDC as a measurement for uptake of liposomes revealed that moDC most efficiently took up liposomes containing only SLP or a combination of SLP and α GC (Figure 1C) and that the fluorescent content increased over time, suggesting that the amount of liposomes applied did not saturate DC within the given time. In conclusion, liposomes are quickly internalized by moDC.

Liposome Loaded moDC Present Antigen to $CD8^+$ T-cells and α GC to iNKT

After determining the uptake capacity of liposomes by moDC we aimed to investigate the immune related functional properties of the nano-carrier. Hereto, gp100 SLP/ α GC containing liposomes were loaded onto moDC and co-cultured with respectively, gp100 specific $CD8^+$ T-cells or iNKT to test for their potential to activate these T-cell subsets. moDC were shown to present the gp100 epitope from the liposomes, activating gp100 specific $CD8^+$ T-cells as reflected by the high amounts of secreted IFN γ (Figure 2A). Interestingly, addition of α GC increased IFN γ secretion by $CD8^+$ T-cells, even in the absence of iNKT. This might reflect DC activation induced by adjuvant properties of α GC which has been described before (16). When we analyzed the iNKT potentiating capacity of the gp100 SLP/ α GC liposomes we observed that after co-culture with iNKT, moDC properly presented encapsulated α GC to iNKT leading to strong IFN γ secretion (Figure 2B) and induction of CD25 expression (Figure 2C). Remarkably, liposomes containing SLP and α GC gave the highest induction of IFN γ secretion and CD25 expression, which is probably not a direct effect of the SLP itself, but through enhanced uptake of liposomes in the presence of SLP as seen in uptake experiments (Figure 1C). Overall, liposomes that included SLP and α GC have a strong potential to activate both $CD8^+$ T- and iNKT cells upon DC uptake, indicating that our nano-carrier provides dual activation properties of both the innate and adaptive arm of the immune system.

Incorporation of Le^Y as Targeting Ligand for DC-SIGN and Langerin Increases Uptake of Liposomes in moDC and dDC

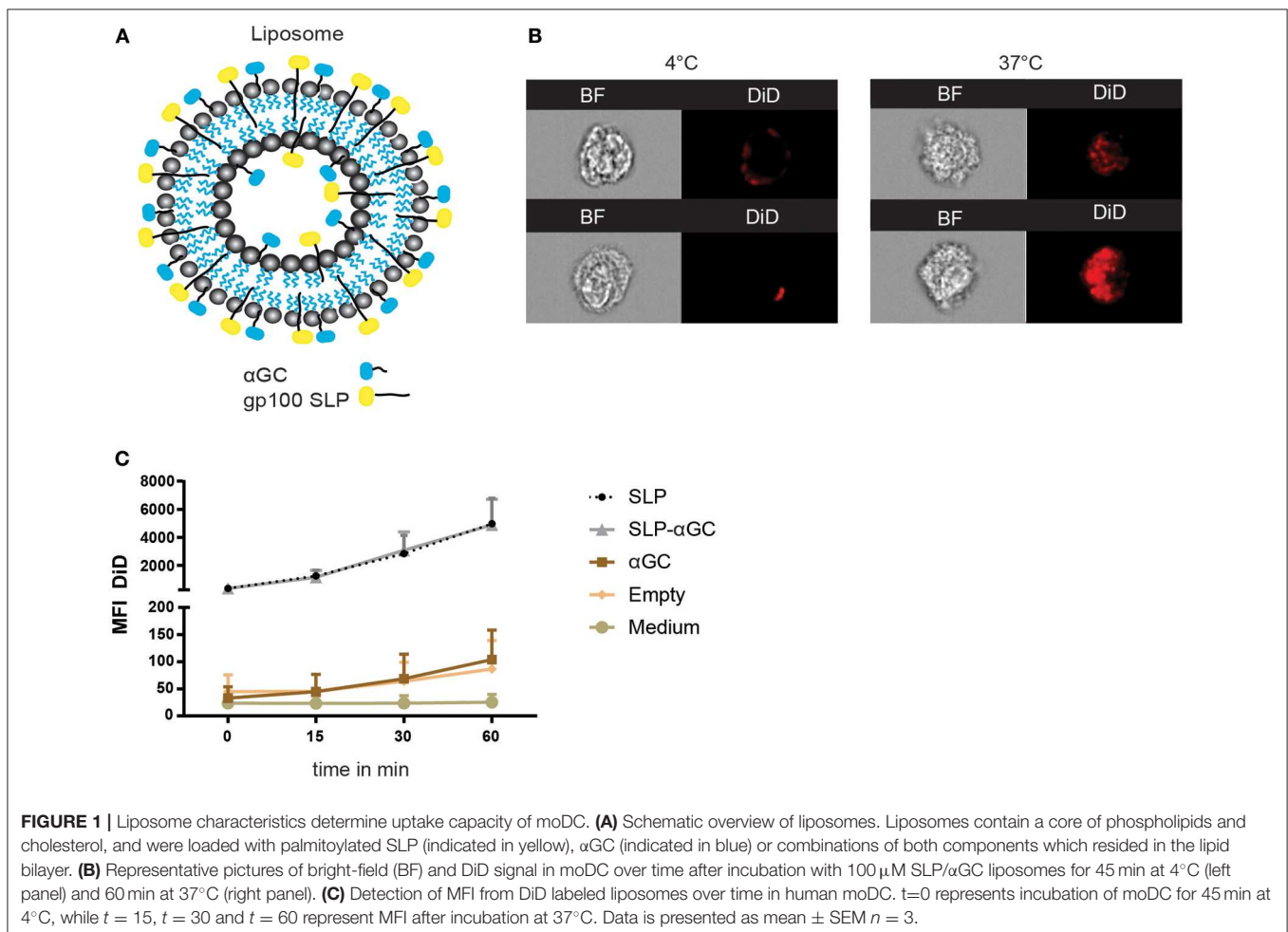
To determine whether the immunogenicity of our nano-carrier could be further improved by enabling cutaneous DC targeting and subsequent cross-presentation, we set out to target the C-type lectin receptors DC-SIGN and Langerin on dDC and LC, respectively, by their carbohydrate ligand Lewis Y (Le^Y). To this end, liposomes were generated and incorporated into the liposome with palmitoylated Le^Y (Figure 3A). Attaching Le^Y to palmitic hydrazide to form lipo- Le^Y , allows for integration into the bilayer of the liposome for multivalent presentation and is thereby easily accessible for Le^Y binding receptors. First, the presence of Le^Y was analyzed by performing binding ELISA using DC-SIGN-Fc and anti-Lewis antibody which confirmed that liposomes had incorporated Le^Y (Figure 3B). Addition of EGTA to chelate calcium (Ca^{2+}) and thereby prevent Ca^{2+} dependent binding of DC-SIGN-Fc, resulted in complete loss of binding. As hypothesized, incorporation of liposomes with Le^Y strongly

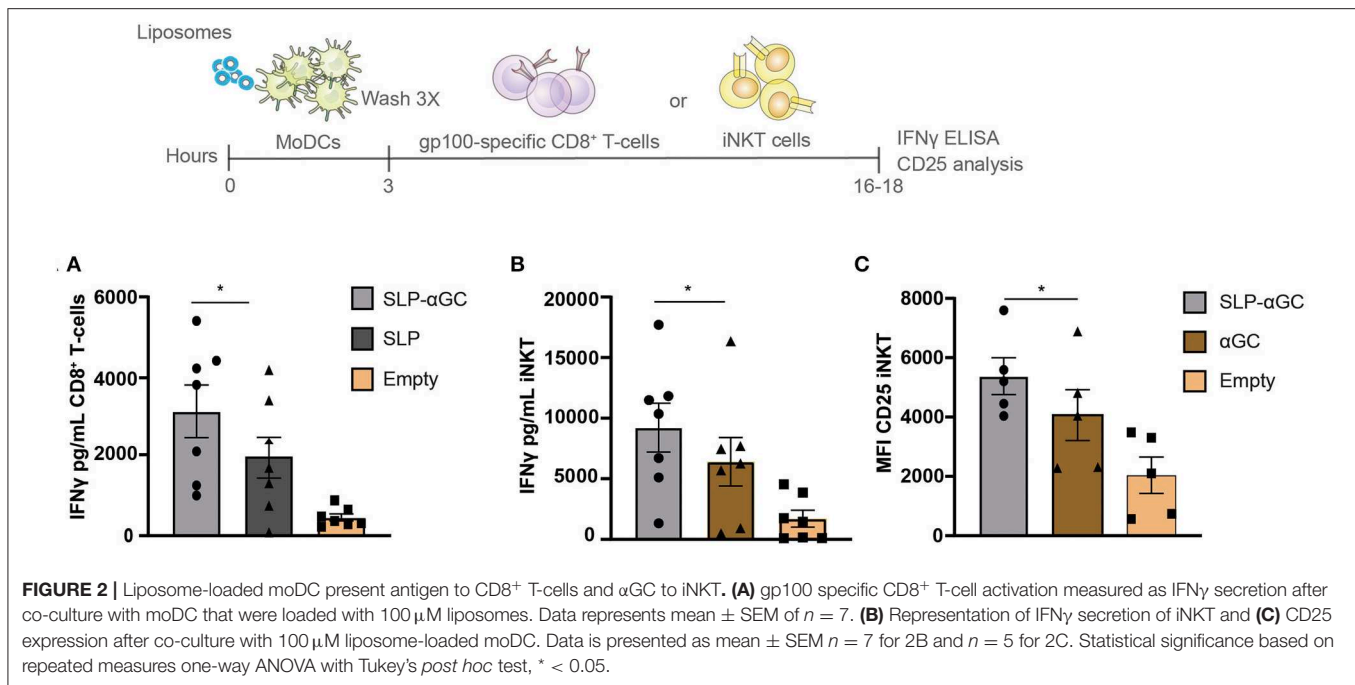
enhanced uptake of liposomes in DC-SIGN⁺ moDC as indicated by the increase in mean fluorescent intensity (MFI) (Figure 3C). Interestingly, the difference in percentage of DiD⁺ cells was most distinct at $t = 0$ at 4°C, where 90% of the moDC were DiD positive after loading with Le^Y modified liposomes compared to 45% after loading with unmodified liposomes (Figure 3D). However, after transfer of moDC to 37°C, it appeared that almost 100% of the moDC also internalized the non-targeted liposomes. Nevertheless, considering the big difference in MFI after 60 min (Figure 3C), we concluded that in presence of the DC targeting ligand Le^Y, the amount of liposomes per DC was highly increased. Next it was investigated whether this increase in particle uptake could be attributed to DC-SIGN targeting. Hereto, moDC were incubated with a DC-SIGN blocking antibody AZN-D1 and decreased uptake of Le^Y decorated liposomes was observed, indicating that uptake was DC-SIGN-mediated (Figure 3E). Next, liposomes were injected intradermal (i.d.) in human skin explants and after 48 hrs. emigrated DC were analyzed for DiD signal (Figure 3F) and gated for DC subset specificity (Figure S1). Also here, Le^Y liposomes were taken up more efficiently than unmodified liposomes (Figure 3F). CD14⁺ dDC from human skin took up most liposomes as reflected by

the highest percentage and highest MFI (Figure 3G). However, also in CD1a⁺ and LC we could detect a higher frequency of DiD positive cells and increased MFI after injection of Le^Y modified liposomes compared to unmodified liposomes. This suggests that Langerin, which also has specificity for Le^Y, might be the targeted receptor in LC and therefore responsible for the observed increase in uptake. Overall we can conclude that addition of Le^Y into the bilayer of liposomes enhances uptake in moDC and multiple subsets of skin APCs.

TABLE 1 | Mean size, polydispersity index, and Z potential with SD of five different batches of liposomes.

Liposome content	Size in nm (SD)	Polydispersity (SD)	Z Potential mV (SD)
Empty	201.1 (10.8)	0.03 (0.02)	-56.7(3.9)
SLP	190.3 (10.4)	0.11 (0.04)	-62.8(3.9)
α GC	209.4 (16.5)	0.07 (0.01)	-56.8(3.7)
SLP α GC	196.0 (15.0)	0.17 (0.08)	-65.8(3.1)
SLP α GC Le ^Y	180.2 (17.4)	0.17 (0.04)	-53.9(3.0)





Incorporation of Le^Y in Liposomes Enhances CD8⁺ T-cell and iNKT Activation

Next, it was investigated if enhanced uptake as a result of Le^Y incorporation would result in stronger activation of both iNKT and CD8⁺ cellular responses. Therefore, moDC were loaded with gp100 SLP/αGC liposomes containing Le^Y as targeting moiety and co-cultured with gp100 specific CD8⁺ T-cells or iNKT. Indeed, both CD8 T-cell (**Figure 4A**) and iNKT (**Figure 4B**) activation was increased when moDC were loaded with Le^Y liposomes as reflected by increased secretion of IFN γ . The cross-presentation of antigen in Le^Y modified liposomes by skin DC was investigated next. Liposomes were injected i.d. and 2-day migrated dermal DC were collected and co-cultured with either gp100 specific CD8⁺ T-cells or iNKT (**Figure 4C**). Remarkably, also here incorporation of Le^Y into the bilayer of the liposomes enhanced presentation of gp100 peptide by skin DC to activate gp100 specific CD8⁺ T-cells (**Figure 4D**). Additionally, IFN γ production by iNKT after co-culture of dermal DC migrated from the explant injected with Le^Y liposomes significantly outperformed the conditions where unmodified liposomes were injected (**Figure 4E**). In conclusion, incorporation of Le^Y enhanced uptake of liposomes by moDC and dDC which concomitantly increased activation of iNKT and CD8⁺ T-cells and thereby displayed potential as a tool for boosting anti-tumor responses.

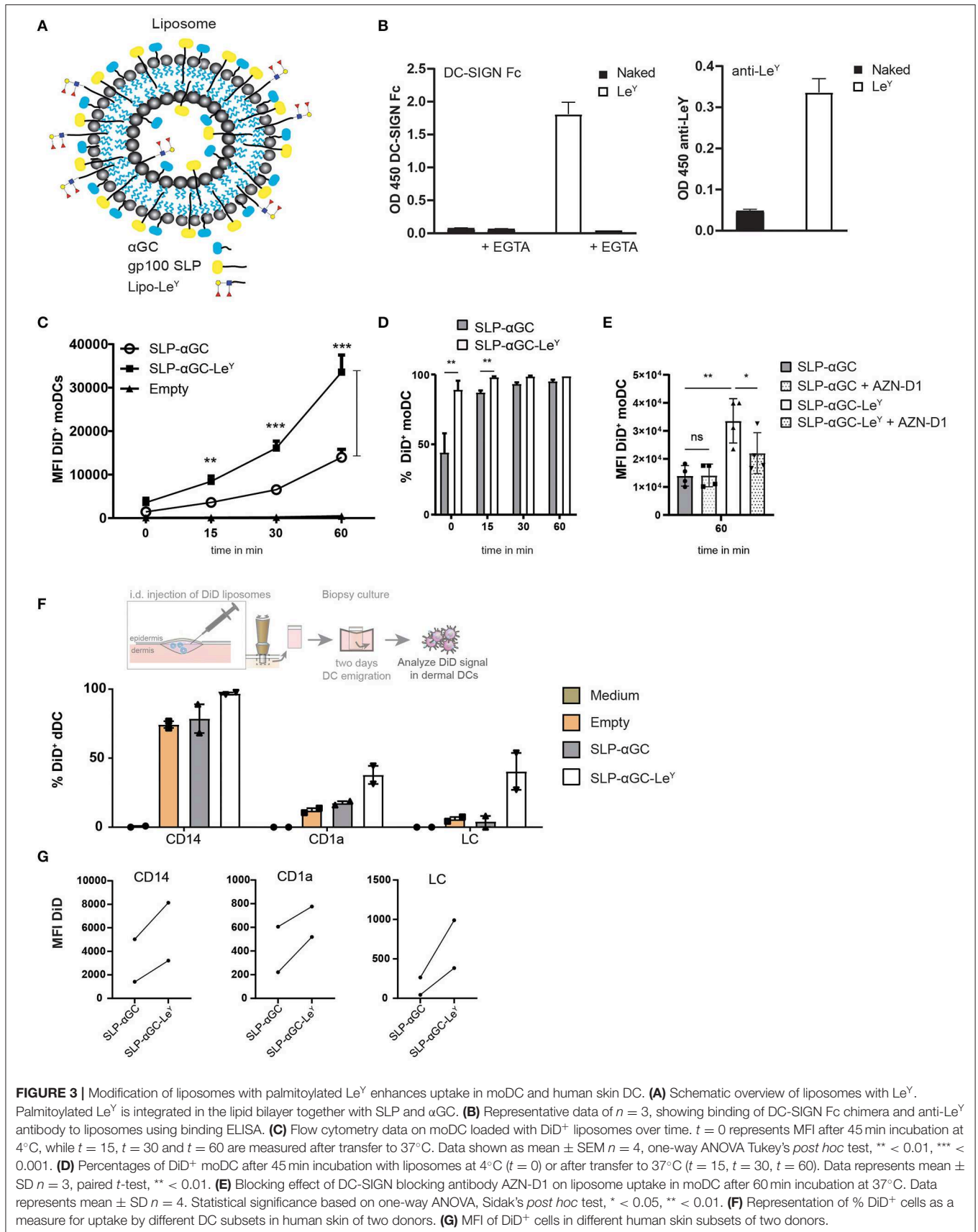
Antigen From Liposomes With Incorporated Le^Y Are Presented by DC to Activate Primary T-cells

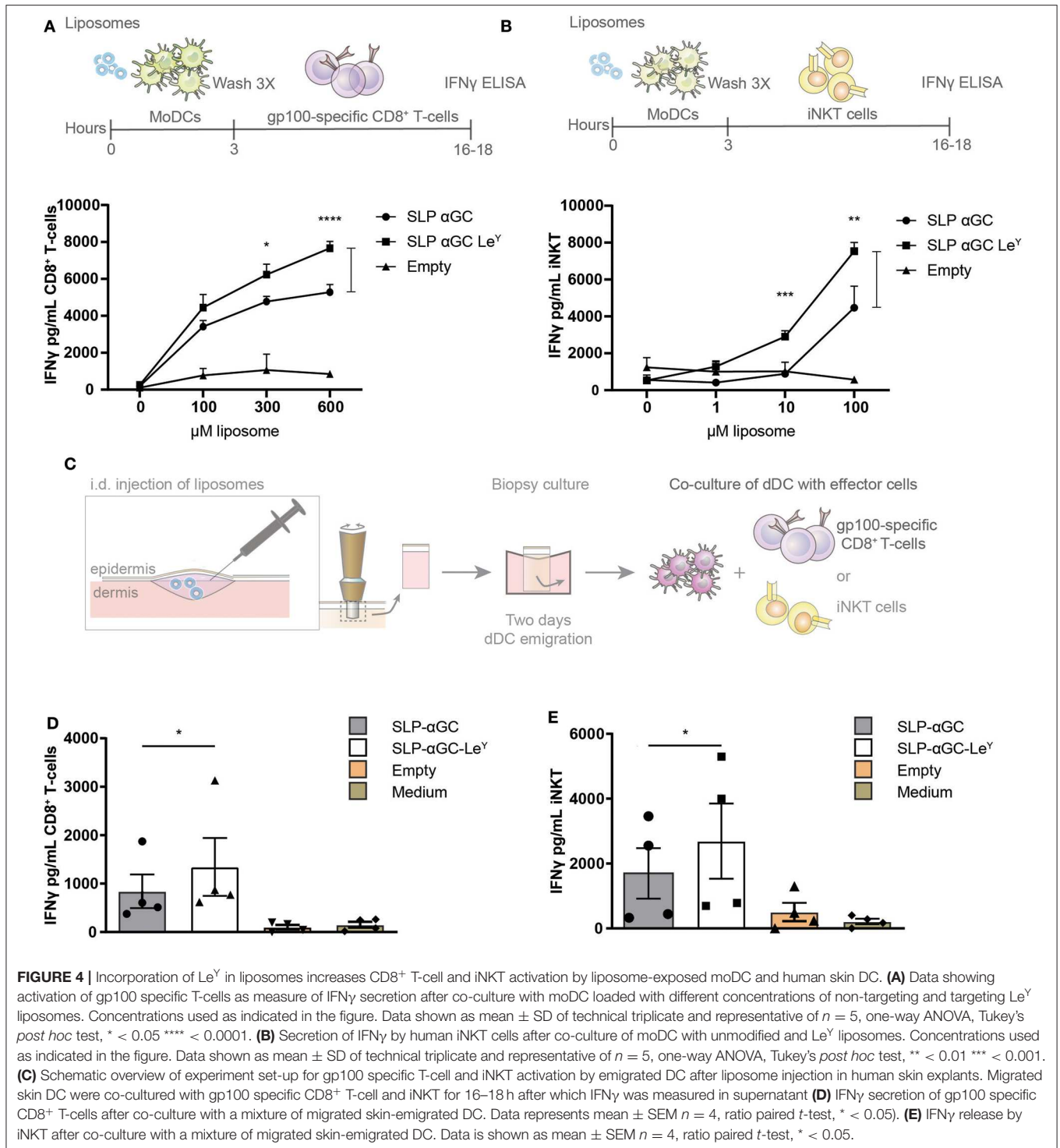
Next, we assessed whether DC loaded with SLP and αGC containing liposomes could cross-prime CD8⁺ T-cells and if proliferation of T-cells would be enhanced after inclusion of Le^Y

into the lipid bilayer of liposomes. Since many healthy individuals harbor a high frequency of circulating naïve MART-1 specific T-cells that are characterized by a typical CD28⁺CD45RA^{hi}/RO⁻ naïve phenotype (32), we have used this as a model system to study the cross-priming capacity of DC after loading with liposomes. Hereto, a 15-mer MART-1 (Melan A) SLP was incorporated in the liposomes as model antigen. CD8 β T-cells were isolated from PBMC and co-cultured with liposome loaded and matured moDC for 10 days after which T-cells were re-stimulated for another 7 days with liposome loaded and matured DC and outgrowth of MART-1_{26–35L} specific CD8⁺ T-cells was measured by HLA-A2 dextramer (D_x) staining (**Figures 5A,B**). Highest rates of primed MART-1_{26–35L} reactive T cells were detected in the condition where moDC were loaded with liposomes containing the Le^Y glycan. In fact this was the only condition that was significantly different compared to the control condition where moDC were loaded with empty liposomes (**Figure 5C**). In cultures without Le^Y, minimal outgrowth was observed. As positive control, DC were loaded with MART-1_{26–35L} short synthetic peptide (**Figure 5D**). To conclude, moDC loaded with SLP-αGC-Le^Y containing liposomes were able to demonstrably cross-prime primary MART-1 specific T-cells, while moDC loaded with all the other liposomes did not. These data thus indicate that targeting with Le^Y could potentially facilitate improved CTL cross-priming.

DISCUSSION

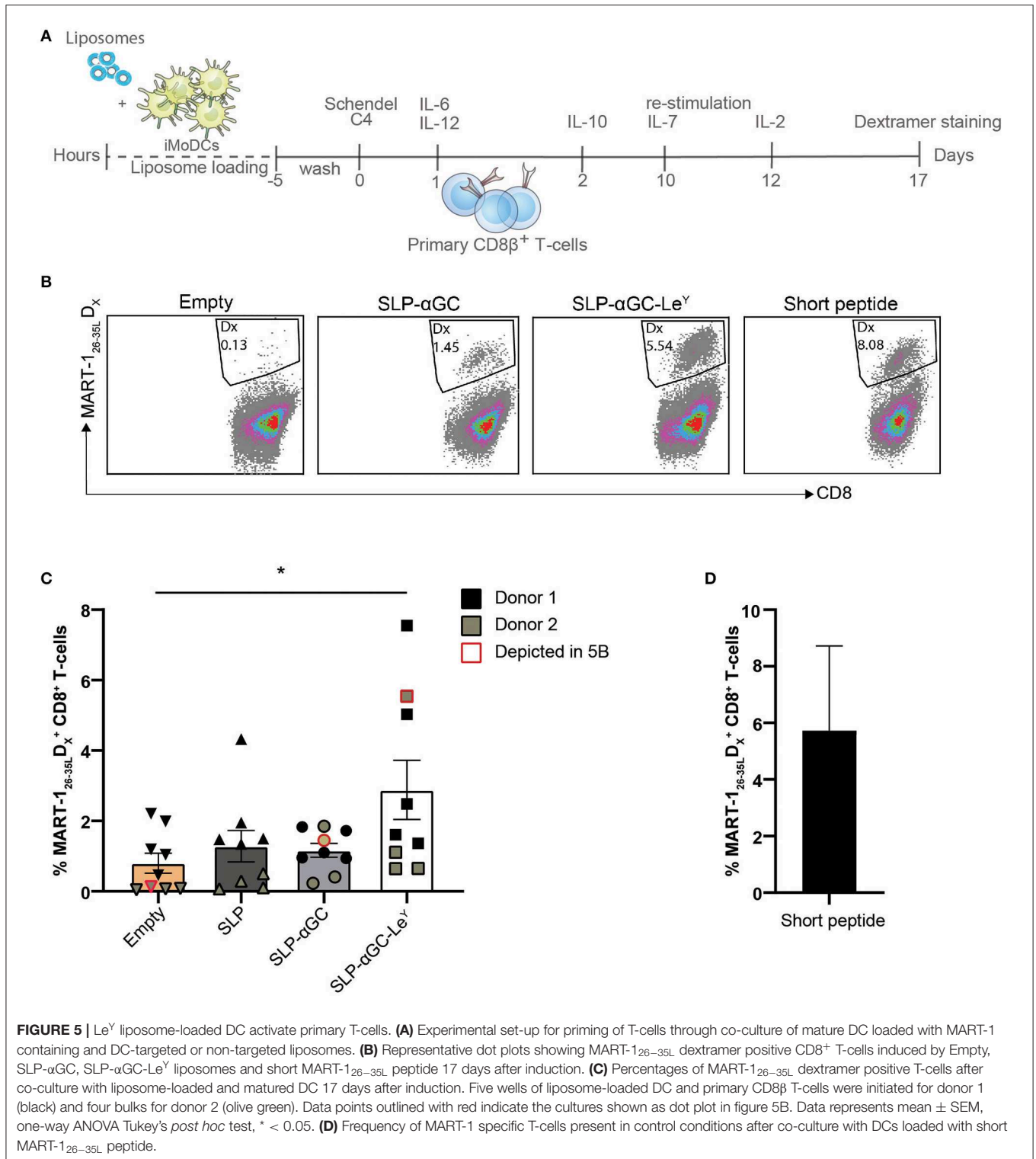
Immune checkpoint inhibition unleashes already existing specific T-cell responses against tumor epitopes in T cell-infiltrated tumors by lifting molecular brakes imposed by the tumor-conditioned tissue microenvironment (33–35). However, more





recent pre-clinical studies have shown the importance of the generation and recruitment of *de novo* responses that together with ICI could provide a greater anti-tumor efficacy, especially in so-called “immunological desert” tumors that lack lymphocyte infiltrates (36, 37). Novel vaccination strategies represent a promising approach to convert these immunologically “silent”

tumors into effector T cell-infiltrated “hot” tumors. In this study we have generated a liposome-based vaccine with incorporated palmitoylated SLP containing MHC-I and MHC-II epitopes of the melanoma associated antigen gp100, the glycolipid α GC and the palmitoylated natural ligand for C-type lectin receptors DC-SIGN and Langerin, i.e., lipo-Le^Y. We showed that these 200 nm



sized, negatively charged liposomes efficiently delivered SLP and α GC to both moDC, dDC, and LC. We have shown that delivery to moDC and skin DC can be enhanced by inclusion of the targeting moiety Le^Y into the liposomal bilayer. As a result of

enhanced uptake we could detect improved T-cell responses with potential anti-tumor activity, not only by activation of a gp100 specific CD8⁺ T-cell clone, but also by activation of iNKT and priming of MART-1_{26-35L} reactive T-cells. Therefore, Le^Y

modified liposomes provide a promising vaccination platform for the induction of multifactorial anti-tumor immunity.

To the best of our knowledge this is the first report using palmitoylated tumor antigens and palmitoylated Le^Y in combination with α GC as components in liposomes. We have shown that palmitoylated antigens incorporated in liposomes are efficiently cross-presented to CD8⁺ T-cells and that inclusion of palmitoylated Le^Y increases activation of CD8⁺ T-cells as a result of enhanced antigen loading in DC. Evidence is emerging that vaccination with neopeptides might elicit stronger anti-tumor responses (38–41) than vaccination with common shared tumor antigens and combination of multiple antigens in one vaccine formulation has been reported to efficiently elicit a multi-targeted T cell response (41, 42). Addition of a single palmitic acid is a relatively inexpensive and non-laborious process that can be applied to both antigens and DC-targeting ligands. Therefore, our strategy of incorporating palmitoylated antigens opens a window for development of a personalized vaccine in which identified tumor specific neo-epitopes could be included in the form of SLP, palmitoylated and potentially combined in liposomes as vaccine delivery vehicle to DC by targeting any receptor that might be deemed appropriate.

Interestingly, enhanced uptake of Le^Y modified liposomes was not only observed in dDC subsets but also in LC. It is well-known that DC-SIGN and Langerin, which are present on dermal DC and LC respectively, share glycan binding specificity for Le^Y (43) but the fact that uptake in LC is observed with palmitoylated Le^Y modified liposomes emphasizes that multivalency of the ligand and spatial orientation influences internalization capacity: as reported previously, Langerin mediated uptake of glycan modified liposomes with a thiol-maleimide linker in LC could not be observed (44), whereas glycan-dendrimers bearing Le^Y, could perfectly target both DC-SIGN and Langerin (45). Apparently, spatial orientation of Le^Y in the lipid bilayer of liposomes influences Langerin binding and Langerin-mediated internalization of the vehicle. These results therefore indicate that not only size of the particle, but, more importantly, also the presentation and/or conjugation of glycans on vaccination vehicles such as liposomes or dendrimers should be considered for dual targeting of DC-SIGN and Langerin and the design of glyco-vaccines in general.

For both gp100 specific CD8⁺ T-cells and iNKT, responses were increased after co-culture with SLP- α GC-Le^Y loaded DC. However, it remains to be determined whether this increase in IFN γ secretion is due to the fact that frequency of activated cells is higher or that the magnitude of the activation is increased, i.e., secretion of IFN γ per cell. We have earlier reported that stimulation of DC with a different vaccine modality, modified with Le^Y, induced a higher percentage of degranulating gp100 specific CD8⁺ T-cells, based on CD107a/b cell surface expression (45). Therefore, we speculate that the activation in number of T-cells is higher after co-culture with SLP- α GC-Le^Y loaded DC rather than an elevated production of cytokine per cell.

As a result of enhanced uptake, increased gp100 specific CD8⁺ T-cell activation was observed after i.d. injection of Le^Y modified liposomes in human skin. This indicates that targeting DC-SIGN and/or Langerin results in effective cross presentation of antigen

by skin DC to T-cells. Interestingly, all DC subsets (CD14⁺, CD1a⁺, LC) in skin were taking up Le^Y liposomes to a larger extent than unmodified liposomes. Since DC-SIGN is mostly expressed on CD14⁺ cells (13, 45) and they have been described to be either monocyte derived macrophages (46) or macrophage-like cells trans-differentiated from cutaneous CD1a⁺ DC (47–49), it is no surprise that the highest MFI and percentage of DiD⁺ cells could be observed in this subset as a result of their phagocytic capacity and their relatively high expression of DC-SIGN. Although CD14⁺ cells in skin have been defined to be inferior antigen presenters compared to LC and CD1a⁺ cells, they are not incapable of inducing proliferation of allogeneic CD4⁺ and CD8⁺ T-cells (50) and they also have been shown to induce expansion of MART-1⁺ specific T-cells (9) and cross present antigen to CD8⁺ specific gp100 T-cells (13). Additionally their role in activation of memory T-cells has been acknowledged (46). Also in CD1a⁺ dDC, which have a lower expression of DC-SIGN compared to CD14⁺ cells (13, 45), the percentage of DiD⁺ cells increased upon injection of Le^Y modified liposomes. Interestingly, Menares et al. proposed a model in which the counterpart of human CD1a dDC in mice, CD11b⁺ DC, mature and migrate to draining lymph nodes (LN) upon tissue resident memory T-cell (T_{rm}) activation (51). This might especially be of interest if besides a tumor specific SLP and α GC, another skin memory epitope is included in the vaccine as additional natural adjuvant, whereby CD14⁺ cells potentially could locally activate T_{rm} to in their turn activate CD1a⁺ dDC to carry tumor antigens to the LN for subsequent activation of cytotoxic T-cells. Overall, we think that developing a vaccine that targets multiple skin APC subsets, which all have different functions and advantageous features in the induction of strong anti-tumor immunity, might be a strategic and effective approach for therapeutic vaccination against cancer.

Although little uptake of α GC liposomes could be observed at 60 min, iNKT activation assays with moDC and dDC showed clear iNKT responses. This suggests that little α GC is needed for proper iNKT activation, which is supported by multiple papers reporting that IFN γ secretion could already be measured with doses as low as 100 ng/mL (52, 53). The enhanced effect in activation can be attributed to increased loading of α GC in CD1d as a result of increased uptake in moDC and dDC. The question remains which subset in the skin is responsible for the enhanced iNKT activation after injection of Le^Y liposome in human skin. Since CD1d expression on LC is inhibited by TGF β secretion of keratinocytes (54) and because no expression could be observed in epidermal cell suspensions (55), it is unlikely that the iNKT activation observed after co-culture with DC from liposome injected human skin can be attributed to LC. More likely, iNKT activation after injection in skin can be attributed to one of the dermal HLA-DR⁺ APC subsets (55).

The fact that enhanced activation of iNKT was observed upon co-culture with moDC and dDC, which were loaded with α GC and SLP containing and Le^Y-modified liposomes, is promising for future *in vivo* studies that yet need to be conducted and will show whether the combination of CD8⁺ T-cells and iNKT activation will lead to enhanced anti-tumor efficacy. Of note, it has already been shown that co-delivery of tumor antigen and

α GC can promote strong anti-tumor responses in a prophylactic and therapeutic setting (56). The combination of α GC and SLP in one vaccine formulation evoked strong antigen specific CTL and could either delay or even prevent tumor progression (57, 58). Additionally, multiple studies showed for the efficacy of PD1/PDL-1 blocking antibodies in terms of overcoming attenuation of iNKT activation (59, 60). Therefore, combining liposomes containing SLP and α GC with ICI might also elicit a stronger and more durable iNKT response. Whether iNKT activation induced via delivery of α GC encapsulated in SLP and Le^Y containing liposomes to DC also leads to increased anti-tumor responses *in vivo* still needs to be determined.

Different clinical trials with peptide vaccines have shown the efficacy of adjuvants like Montanide and Freud's adjuvant (61–63). Also addition of Addavax and poly I:C or other TLR ligands like CpG to peptide vaccines has shown good efficacy in pre-clinical models (64–67). Furthermore, the role of α GC as adjuvanting component has been extensively described in multiple studies (68–72). Likewise, we demonstrated that inclusion of α GC in liposomes enhanced CD8⁺ T-cell responses compared to liposomes containing SLP alone and that responses of CD8⁺ T-cells but also of iNKT can be further enhanced by addition of Le^Y. The question remains how these immune responses compare to SLP vaccination with other existing adjuvants. Remarkably Dölen et al. investigated the effect of OVA- α GC nanoparticles (NPs) in comparison to OVA NPs with conventional TLR ligands R848 and poly I:C and found that OVA- α GC outperformed TLR containing NPs (58). Having a direct comparison of α GC NP to other existing adjuvants would be of great value to better understand and implement α GC in SLP containing vaccines. Moreover, it would be very interesting to resolve whether immune responses induced by SLP- α GC-Le^Y liposomes could be further enhanced by inclusion of additional adjuvanting components. Interestingly, we have shown in mouse models that 2 h after s.c. injection of Addavax mouse DC-SIGN⁺ cells could be found in the skin (73). Therefore, combining SLP- α GC-Le^Y liposomes with Addavax might be a great strategy to recruit DC-SIGN⁺ DCs to even further strengthen antigen specific immune responses.

In summary, combining palmitoylated antigen and palmitoylated Le^Y together with a α GC in one liposome constitutes a promising peptide vaccination platform for *in situ* delivery to skin APCs which allows for increased uptake and antigen presentation to CD8⁺ T-cells together with iNKT activation and therefore may contribute to improve ICI anti-cancer immunotherapy.

MATERIALS AND METHODS

Antibodies

CD1a (Clone HI149, BD Biosciences, New Jersey, U.S.), CD14 (Clone M5E2 Sony, San Jose, U.S.), CD141 (Clone 1A4 RUO, BD Biosciences), HLA-DR (Clone G46-6, BD Biosciences), EPCAM (Clone EBA-1, BD Biosciences), anti-V α 24 (Clone C15, Beckman Coulter, Brea, U.S.) anti-TCR V β 11 (Clone C21 Beckman Coulter), CD8 (clone RPA-T8, BD Biosciences), CD8 β (Clone

2ST8.5H7, Beckman Coulter) Fixable Viability Dye (FVD) eFluor 780 (Invitrogen, Waltham, U.S.).

Peptide Synthesis

The antigenic peptides gp100 (VTHTYLEPGPVTANRQLYPEWTEAQLDC), MART-1 (YTAEELAGIGILTV) containing the immune dominant epitope MART-1_{26–35L} and MART-1_{26–35L} (ELAGIGILTV) were synthesized by solid phase peptide synthesis using Fmoc chemistry on a Symphony peptide synthesizer (Protein Technologies Inc., Tucson, U.S.). In the last step of the synthesis, prior to the cleavage of the peptide from the resin, a palmitic tail was added at the N-terminus of the gp100 and MART-1 peptide through a reaction with palmitic anhydride in dichloromethane (DCM). The resulting lipopeptides were cleaved and purified on a preparative Ultimate 3000 HPLC system (Thermo Fisher) over a VYDAC 214MS1022 C4 25 × 250 mm column (Grace Davidson). Mass and purity were confirmed by UPLC-MS on a Ultimate 3000 UHPLC system (Thermo Fisher, Waltham, U.S.) hyphenated with a LCQ-Deca XP Iontrap ESI mass spectrometer (Thermo Finnigan, Waltham, U.S.) using a Prosphere HP C4 5 μ m 150 × 4.6 mm column and ionizing the sample in positive mode.

Preparation of Lewis^Y Glycolipid

Lewis Y (Le^Y)-hexadecanohydrazide (palmitic hydrazide) *ea.* palmitoylated Le^Y (from here on, shortly, lipo-Le^Y) was prepared from Le^Y pentasaccharide (Elicityl, Crolles, France) and hexadecanohydrazide through a reductive amination reaction. Addition of CHCl₃/MeOH/H₂O at 8:1:8 v/v ratio, followed by vigorous stirring and centrifugation, allowed the extraction of lipo-Le^Y as a white slurry at the interphase. The slurry was freeze-dried to remove residual solvent. The correct mass of the glycolipid was confirmed by ESI-MS (Thermo Finnigan LCQ-Deca XP Iontrap mass spectrometer in positive mode) using nanospray capillary needle.

Liposome Preparation

Liposomes containing palmitoyl-gp100 or palmitoyl-MART-1 lipopeptides and lipo-Le^Y targeting glycan were prepared from a mixture of phospholipids and cholesterol utilizing the film extrusion method. Briefly, EPC-35 (Lipoid):EPG-Na (Lipoid):Cholesterol (Sigma-Aldrich, St. Louis, U.S.) at a ratio of 3.8:1:2.5 in mol were dissolved in a mixture of chloroform/methanol. The lipophilic fluorescent tracer DiD (1'-dioctadecyl-3,3,3'-tetramethyl indodicarbocyanine; Life Technologies, 0.1% in mol) was added to the mixture (where indicated) as well as palmitoyl-gp100 (400 μ g) or palmitoyl-MART-1 (400 μ g), lipo-Le^Y (1.5 mg) and the NKT cell activator α -galactosylceramide, KRN7000 (Funakoshi, Tokyo, Japan) (30 μ g). A lipid film was obtained by evaporation of the solvent under reduced pressure at 50°C that was hydrated in HEPES buffer solution pH 7.5 yielding a liposome preparation of phospholipids at a concentration of ~8 μ .mol total lipid/mL. After extrusion through two stacked polycarbonate filters of 200 nm, the non-encapsulated antigen and lipo-Le^Y were washed away by two consecutive ultracentrifugation steps on

Beckman Ultracentrifuge at 55,000 rpm. The final suspension of liposomes was obtained in Hepes buffer pH 7.5. After 15 min of vigorous stirring and overnight incubation at 4°C, the liposome suspensions were centrifuged at 55,000 rpm and resuspended in Hepes buffer pH 7.5 twice. Size, polydispersity index, zeta potential and amount of encapsulated fluorochrome DiD was determined prior to use, as previously described (74). After destruction with perchloric acid, phospholipid content (concentration in molar) was quantified spectrophotometrically as described before (75). The amount of liposomes used in the experiments was calculated based on the phospholipid contents in mol as previously described (76). Comparison of lipid concentration before (8.7 mM) and after (5.6 mM) extrusion revealed an overall yield of 64%. The content of α GC and lipopeptide was calculated from the phospholipid concentration. The concentration of α GC and lipo-SLP was 19.2 μ g/mL and 256 μ g/mL, respectively.

Generation of moDC

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy volunteers (Sanquin, Amsterdam, The Netherlands) by centrifugation on a Ficoll gradient. First, blood was gently mixed with PBS containing 1% citrate and carefully layered on top of Ficoll. After 30 min centrifugation at $700 \times g$, the interphase containing monocytes and lymphocytes was collected. Next, monocytes and lymphocytes were washed with PBS/Citrate at $400 \times g$ for 10 min and the pellet was resuspended in complete RPMI. To isolate monocytes, PBMCs were carefully added on top of a Percoll layer (GE Healthcare, Chicago, U.S.) at a concentration of 10×10^6 cells/mL and centrifuged at $400 \times g$ for 40 min. Again the interphase was collected and tubes were filled with PBS/Citrate prior to a 10 min centrifugation at $400 \times g$. After washing three times with PBS/Citrate, pellets were resuspended in complete RPMI. MoDC were generated by culturing monocytes for 5–7 days at a concentration of 1.25×10^6 /mL in complete RPMI (Lonza, Basel, Switzerland) containing 500 μ /mL IL-4 (ImmunoTools, Friesoythe, Germany) and 800 μ /mL Granulocyte Macrophage Colony stimulating Factor (GM-CSF) (ImmunoTools) using T75 flasks (Greiner, Kremsmünster, Austria).

Isolation and Culture of iNKT

iNKT cell lines were generated from PBMCs of healthy donors by magnetic activated cell sorting (MACS). iNKT were enriched by using a murine anti-human TCR V α 24 mAb in combination with magnetic beads labeled with a polyclonal goat-anti-mouse Ab (Miltenyi, Bergisch Gladbach, Germany). Initial iNKT expansion was achieved by co-culturing iNKT for 7 days with α GC 100 ng/mL (Funakoshi) loaded and LPS (Sigma Aldrich, 100 ng/mL) pulsed moDC in the presence of cytokines rhIL-7 10 μ /mL and rhIL-15 10 ng/mL. Purification of iNKT was achieved by repeating magnetic sorting with the murine anti-human TCR V β 11 mAb. For evaluation of iNKT purity, cells were incubated for 30 min at 4°C with FITC-labeled anti-human TCR V α 24 mAb and PE-labeled anti-human TCR V β 11 mAb, washed twice and analyzed by flow cytometry on a FACS BD LSRFortessa based on double positive TCR V α 24/ V β 11 selection. Purified

(V α 24⁺ V β 11⁺ cells) iNKT were weekly re-stimulated with irradiated feeder-mix, consisting of allogeneic PBMCs from two healthy donors and JY cells, an EBV transformed lymphoblastoid cell line, in Yssel's medium supplemented with 50U/mL rhIL-2 (Proleukin, Clinigen, Burton upon Trent, U.K.) and 50 ng/mL PHA (Vector Laboratories). iNKT purity used for all experiments was >90%.

Intradermal Injection of Liposomes and Culture of Skin Biopsies

Liposomes were diluted in serum-free IMDM (Lonza) supplemented with antibiotics (50 U/mL penicillin, 50 μ g/mL streptomycin and 10 μ g/mL gentamycin) and injected intradermal in human abdominal skin explants from healthy donors (Bergman Clinics, Bilthoven, The Netherlands) in a volume of 20 μ l as previously described (13). Human skin samples were obtained after informed consent from patients that underwent corrective breast or abdominal plastic surgery, according to hospital guidelines and in accordance with the "Code for Proper Use of Human Tissues" as formulated by the Dutch Federation of Medical Scientific Organizations. Biopsies of 8 mm were taken with a biopsy punch (Microtec, Zutphen, The Netherlands) and cultured in a 48-well plate in 1 mL/well complete IMDM (10% FCS, 2 mM L-glutamine, 100 U/mL penicillin (Lonza), 100 U/mL streptomycin (Lonza) and 10 μ g/mL gentamycin). After 48 h of culture, biopsies were removed from wells and crawl-out DC were collected, pooled and used for experiments.

Liposome Binding to and Uptake via DC-SIGN in moDC

Liposomes labeled with the lipophilic dye DiD (Invitrogen), were added at a concentration of 100 μ M to moDC, which were either pre-incubated with 20 μ g/mL of a neutralizing α DC-SIGN antibody (AZN-D1 in house production) for 30 min at 37°C or left untreated before loading. Loading of moDC with labeled liposomes continued for 45 min at 4°C after which a moDC sample was collected and washed two times with PBS prior to fixation on ice with 4% PFA (Aurion) for 20 min ($t = 0$). Immediately after collecting the $t = 0$ sample, the remaining moDC were incubated at 37°C for 15 ($t = 15$), 30 ($t = 30$), or 60 ($t = 60$) minutes. Samples were washed three times with PBS containing 1% BSA (PBA) and fixed in 4% PFA before analysis on LSR Fortessa (BD Biosciences).

Liposome Uptake by dDC

DiD labeled liposomes containing palmitoylated gp100 peptide, α GC and Le^Y were diluted in serum-free IMDM (Lonza), supplemented with antibiotics (50 U/mL penicillin, 50 μ g/mL streptomycin and 10 μ g/mL gentamycin), and injected intradermal in human abdominal skin explants from healthy donors (Bergman Clinics, Bilthoven, The Netherlands) in a volume of 20 μ l. Next, 8 mm biopsies were taken with a biopsy punch (Microtec) and biopsies were cultured in a 48-well plate (Greiner) in 1 mL complete IMDM (10% FCS, 2 mM L-glutamine, 50 U/mL penicillin, 50 μ g/mL streptomycin and 10 μ g/mL gentamycin). After 48 hrs. of culture, biopsies were

removed from wells and crawled out dDC were collected and stained with a multi-color antibody panel containing anti-CD1a, anti-CD14, anti-HLA-DR, anti-EPCAM, FVD.

Antigen Presentation of moDC to CD8⁺ gp100 Specific T-cells

Day 5–7 moDC or skin migrated DC (20.000/well) were incubated with different concentrations of liposomes containing gp100 SLP, α GC and Le^Y in triplicate for 3 h at 37°C in a 96-well U bottom plate in a total volume of 100 μ l. After incubation with the liposomes, DC were washed three times with serum-free RPMI and centrifuged for 3 min at 400 \times g. gp100-specific CD8⁺ T-cells [generated and cultured as described previously (77)] were added to the liposome-loaded moDC or migrated dDC/LC at a concentration of 100.000 cells/well (ratio 1:5) in enriched IMDM, i.e., Yssel's medium (20 μ g/mL human transferrin, 5 μ g/mL insulin, 2 μ g/mL linoleic acid, 2 μ g/mL palmitic acid, 0.25% BSA, 1.8 μ g/mL 20-amino ethanol). The DC and T-cell co-culture was left overnight at 37°C/5%CO₂ and subsequently 75 μ l supernatant was collected for IFN γ ELISA. Samples were stored at –20°C.

iNKT Activation Assay

Day 5–7 moDC or skin migrated DC (20.000/well) were incubated with different concentrations of liposomes containing gp100 SLP, α GC and Le^Y in triplicate for 3 h at 37°C in a 96-well U bottom plate in a total volume of 100 μ l. After incubation with the liposomes, moDC were washed three times with serum-free RPMI and centrifuged for 3 min at 300 \times g. iNKT (isolated and cultured as described above) were added to the liposome-loaded moDC or migrated skin DC at a concentration of 100.000 cells/well (ratio 1:5) in enriched IMDM (Lonza), i.e., Yssel's medium. The DC and T-cell co-culture was left overnight and subsequently 75 μ l supernatant was collected for IFN γ ELISA. Samples were stored at –20°C. CD25 levels were measured by flow cytometry and cells were gated on live V β 11⁺ V α 24⁺ cells.

Cytokine ELISA

1 μ g/mL anti-human IFN γ (Invitrogen) was coated overnight in 50 mM Na₂CO₃, pH 9.7. Plates were washed with PBS containing 0.05% Tween and blocked for 30 min at 37°C with 1% BSA in PBS. Supernatants were added together with the corresponding IFN γ detection antibody for 2 h at RT while shaking. Presence of the cytokines was detected with Streptavidin-PO (Biosource Finnigan, Waltham, U.S.). Binding was determined by adding substrate buffer (110 mM citric acid (Merck), 110 mM sodium acetate (Fisher scientific, Waltham, U.S.), pH 4 and 100 μ g/mL fresh TMB (Sigma) and 0.006% H₂O₂. Absorbance was measured at 450 nm on an ELISA reader (Bio-Rad Benchmark, Hercules, U.S.).

Priming of MART Specific CTLs

PBMCs isolated from a buffy coat (Sanquin) were pre-stained with an unlabeled CD8 β ⁺ mAb and incubated with magnetic

polyclonal goat-anti-mouse beads (Miltenyi). CD8 β ⁺ CTLs, were positively selected by MACS sorting. Monocytes were isolated from the CD8 β - fraction through the use of CD14 magnetic sorting beads (Miltenyi) and in complete RPMI (Lonza) containing 500 U/mL IL-4 (ImmunoTools) and 800 U/mL GM-CSF (ImmunoTools) for 5 days. At day 5, immature moDC were pre-cultured in the presence of 25 μ M of liposomes and loaded for 5–6 h. Maturation of moDC, was induced by using the “Schendel-C4” maturation cocktail, consisting of the following cytokines: 500IU GM-CSF 5 ng/mL IL4 (Strathmann, Hamburg, Germany), 480IU/mL TNF α (Miltenyi), 10 ng/mL IL-1 β (Miltenyi), 250 ng/mL PGE2 (Sigma), 1000IU/mL IFN γ (Calbiochem, Darmstadt, Germany) and 1 μ g/mL R848 (Invivogen). As a control mature moDC were loaded with 1 μ g/mL MART-1_{26–35L} short 9mer synthetic peptide (EAAGIGILTV) in the presence of β 2-microglobulin (Fitzgerald Industries, Acton, US) for 4 h. Ten day cultures were started with 1 \times 10⁵ liposome- or peptide-loaded mature moDC, 1 \times 10⁶ CD8 β ⁺ CTLs and 1 \times 10⁶ CD8 β - autologous irradiated (50Gy) feeder cells, in Yssel's medium supplemented with IL-12 10 ng/mL (Novartis, Basel, Switzerland) and IL-6 10 ng/mL (R&D systems, Minnesota, U.S.) in a 48 wells plate. Twenty-four hours after start of culture IL-10 10 ng/mL (Ebioscience, Waltham, U.S.) was added. At day 10 cultures were re-stimulated with 1x10⁵ liposome- or peptide loaded mature moDC and supplemented with 10 UI/mL IL-7. Forty-eight hours after re-stimulation 10IU/mL IL-2 (Proleukin) was added to the cultures. The % of MART-1 specific CD8⁺ T cells, was analyzed by flow cytometry using a MART-1_{26–35L} PE-labeled HLA-A2 Dextramer (Immudex, Copenhagen, Denmark), APC-labeled CD8 mAb and FVD.

Statistics

Statistical analysis was accomplished by the use of Graphpad 8.0.2 Prism software. Data was analyzed using one-way or two-way ANOVA with *post hoc* analysis as indicated in the figure legend or with use of (ratio paired) *t*-test.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

DS, AH, and JV performed research. DS, TG, HV, and YK designed the experiments. Data collection was performed by DS, AH, JV, SD, JL, and RV. SB optimized assays. Products were synthesized by AH, MA, and HK. DS and JV analyzed data. Manuscript was prepared by DS and corrected by YK, TG, and HV. All authors have read the article and gave their approval for publication.

FUNDING

This research was supported by KWF VU2014-7200 (DS, JV, YK, TG, and HV), LSH-TKI project DC4Balance LSHM18056-SGF (AH) and by European Research Council (ERC advanced 339977- Glycotreat SD, JL, YK).

ACKNOWLEDGMENTS

We thank members of the O₂ Flow Cytometry and GlycO₂peptide Facilities of Amsterdam UMC,

location VUmc, for excellent technical support and synthesis of constructs. We also thank Bergman clinics, Bilthoven, The Netherlands for supply of healthy donor skin.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.00990/full#supplementary-material>

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- Conflict of Interest:** HV is employed by and acts as chief scientific officer of Lava Therapeutics.
- The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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