# *In situ* Delivery of Tumor Antigen– and Adjuvant-Loaded Liposomes Boosts Antigen-Specific T-Cell Responses by Human Dermal Dendritic Cells

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Dendritic cells (DCs) have an important role in tumor control via the induction of tumor-specific T-cell responses and are therefore an ideal target for immunotherapy. The human skin is an attractive site for tumor vaccination as it contains various DC subsets. The simultaneous delivery of tumor antigen with an adjuvant is beneficial for cross-presentation and the induction of tumor-specific T-cell responses. We therefore developed liposomes that contain the melanoma-associated antigen glycoprotein $100_{280\text{-}288}$ peptide and Toll-like receptor 4 (TLR4) ligand monophosphoryl lipid A (MPLA) as adjuvant. These liposomes are efficiently taken up by monocyte-derived DCs, and antigen presentation to $CD8^+$ T cells was significantly higher with MPLA-modified liposomes as compared with non-modified liposomes or the co-administration of soluble MPLA. We used a human skin explant model to evaluate the efficiency of intradermal delivery of liposomes. Liposomes were efficiently taken up by $CD1a^+$ and especially $CD14^+$ dermal DCs. Induction of $CD8^+$ T–cell responses by emigrated dermal DCs was significantly higher when MPLA was incorporated into the liposomes as compared with non-modified liposomes or co-administration of soluble MPLA. Thus, the modification of antigen-carrying liposomes with TLR ligand MPLA significantly enhances tumor-specific T-cell responses by dermal DCs and is an attractive vaccination strategy in human skin.

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## INTRODUCTION

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) capable of initiating adaptive immune responses (Banchereau *et al.*, 2000). Immature DCs are sentinel cells located in peripheral tissues, such as mucosa and the skin, and in blood and lymphoid organs. Immature DCs express many pattern recognition receptors, for example Toll-like receptors (TLRs), and internalization receptors, which enables them to recognize and internalize pathogens (Janeway and Medzhitov, 2002; Kawai and Akira, 2010). This is followed by a maturation process and migration to draining lymph nodes where naive T cells reside. Concurrently, internalized antigen is processed and loaded onto major histocompatibility complex (MHC) class I and II molecules for presentation to $CD8^+$ and $CD4^+$ T cells, respectively (Jensen, 2007). Exogenous antigens are internalized and shuttled to the endo-lysosomal pathway for presentation in MHC class II molecules. However, exogenous antigens can also be presented in MHC class I molecules via a process called "cross-presentation" (Carbone and Bevan, 1990; Joffre *et al.*, 2012). Antigen presentation in MHC class I molecules is important for the induction of $CD8^+$ cytotoxic effector T lymphocytes (CTLs), which are essential for clearing the pathogen-infected cells and tumor cells (Appay *et al.*, 2008).

The skin is an immunologically active tissue that harbors various APC subsets, such as Langerhans cells (LCs) in the epidermis, and macrophages and various DC subsets in the dermis. Human LCs efficiently induce $CD4^+$ and $CD8^+$ T–cell responses but in resting state also induce regulatory T cells, thereby maintaining skin homeostasis (Klechevsky *et al.*, 2008; Seneschal *et al.*, 2012). The main populations of dermal DCs (dDCs) that can be found in the human skin are $CD1a^+$ DCs and $CD14^+$ DCs. $CD1a^+$ dDCs are found in the human skin–draining lymph nodes and therefore considered migratory DCs, which have potent T-cell stimulating capacities (Nestle *et al.*, 1993; van de Ven *et al.*, 2011; Segura

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*Abbreviations: APC, antigen-presenting cell; CTL, cytotoxic effector T lymphocyte; DC, dendritic cell; dDC, dermal DC; gp100, glycoprotein 100; LC, Langerhans cell; MHC, major histocompatibility complex; moDC, monocyte-derived DC; MPLA, monophosphoryl lipid A; TLR, Toll-like receptor*

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*et al.*, 2012). $CD14^+$ dDCs have a role in humoral responses but showed a poor ability to stimulate naive $CD4^+$ and $CD8^+$ T–cell proliferation, as well as a poor ability to migrate to the lymph nodes, and are therefore recently proposed as a macrophage-like cell subset (Nestle *et al.*, 1993; Klechevsky *et al.*, 2008; Matthews *et al.*, 2012; McGovern *et al.*, 2014).

With various DC subsets, the human skin is an attractive and an easily accessible site for antigen-based vaccination. Delivery of antigen to dDCs *in situ* enhances antigen cross-presentation and CTL responses (Schneider *et al.*, 2012; Fehres *et al.*, 2013; Oosterhoff *et al.*, 2013). The simultaneous delivery of antigen together with a potent adjuvant, such as TLR ligands, will enhance the cross-presentation capacity of DCs and the generation of T-cell responses (Joffre *et al.*, 2012). Liposomes are spherical particles that consist of phospholipid bilayers that are ideal as delivery vehicles, as large amounts of hydrophilic and hydrophobic molecules can be encapsulated, and may serve as antigen reservoir prolonging MHC class I antigen presentation (Torchilin, 2005; Joshi *et al.*, 2012). Using liposomes offers the opportunity to include various tumor antigens, as well as different types of DC-activating molecules—for example, TLR agonists. Liposomes have low toxicity and are well tolerated by the body because of their assembly with naturally derived compounds.

Our aim is to develop a therapy using liposomes for the simultaneous delivery of tumor antigen and adjuvant to human skin–resident DCs. We used a human skin explant model to evaluate DC function in the natural complex tissue environment and to explore the potential of intradermally delivered vaccine. We generated liposomes with a melanoma-associated peptide derived from glycoprotein 100 (gp100) protein together with the TLR4 ligand monophosphoryl lipid A (MPLA), which easily incorporates into the lipid bilayer of liposomes. MPLA is a detoxified, but still immunostimulatory, derivative of lipopolysaccharide that has been shown effective in different studies as vaccines to malaria, HIV, and several types of cancer (Steinhagen *et al.*, 2011). Incorporation of MPLA into liposomes resulted in efficient DC maturation and enhanced tumor antigen cross-presentation to gp100-specific $CD8^+$ T cells. Importantly, cross-presentation was only enhanced when MPLA was incorporated in the liposomes, as soluble MPLA did not affect cross-presentation. Our data demonstrate the effective delivery of tumor antigen and TLR ligand *via* liposomes to dDCs and the potency as anti-cancer vaccine for stimulating the skin APCs to instruct tumor-specific $CD8^+$ T–cell responses.

## RESULTS

### MPLA-modified liposomes are taken up by human monocyte–derived DCs and induce maturation and cytokine production

As a carrier for tumor antigen and adjuvant, we prepared liposomes on a phosphatidylcholine, phosphatidylglycerol, and cholesterol basis as described in Materials and Methods section. The size, the polydispersity index, zeta potential, phospholipid concentration, and the amount of encapsulated fluorochrome DiD were determined (Table 1) as previously described (Joshi *et al.*, 2011). The amount of liposomes (total lipid) used in the experiments was calculated based on the determined phospholipid contents. The fluorochrome DiD was incorporated for research purposes. We incorporated an MHC class I-restricted $gp100_{280\text{-}288}$ melanoma-associated peptide for presentation by HLA-$A2^+$ DC to gp100-specific T cells. Furthermore, we incorporated the TLR4 agonist MPLA, a derivative of *Salmonella Minnesota* lipopolysaccharide, which as a lipid easily incorporates into the lipid bilayer of the liposomes. The purpose of TLR ligand incorporation into liposomes is to enhance the adjuvanticity of the liposomes and to increase vaccine efficacy in inducing T-cell responses.

DCs are phagocytic cells that efficiently take up (particulate) antigens from their surroundings. We first assessed whether human DCs take up these MPLA-loaded liposomes. Incubation of monocyte-derived DCs (moDCs) with non-modified or MPLA-containing liposomes resulted both in highly efficient dose-dependent uptake of the liposomes (Figures 1a and b). As expected, incorporation of MPLA did not significantly interfere with the uptake of liposomes by DCs. Next, we analyzed the effect of MPLA-modified liposomes on moDC maturation and cytokine production. MPLA incorporation into liposomes showed a tendency to induce the expression of maturation marker CD83 and costimulatory molecule CD86 (Figure 1c). Also, production of pro-inflammatory cytokines TNF-α and IL-6 seemed to be induced by MPLA-modified liposomes (Figure 1d). Although not significantly different because of donor variations in the expression and production levels, we observed upregulation of CD83, CD86, TNF-α, and IL-6 in every donor when treated with MPLA-modified liposomes. Importantly, DC activation was evident both after treatment with liposomal MPLA and soluble MPLA, albeit at slightly lower levels for liposomal MPLA. These data show that modification of liposomal carriers with adjuvant MPLA results in effective DC maturation and cytokine production.

**Table 1. Physical characteristics of liposome formulations**

| | Mean size (nm) | Polydispersity index | Zeta potential (mV) | Concentration (mM) | DiD (MFI)[1] |
|---|---|---|---|---|---|
| Empty | 214 ± 16 | 0.06 ± 0.02 | −52.0 ± 6.3 | 6.5 ± 0.5 | 58.6 ± 10.8 |
| MPLA | 210 ± 14 | 0.05 ± 0.03 | −52.7 ± 7.4 | 7.5 ± 1.1 | 53.0 ± 1.0 |

Abbreviations: MFI, mean fluorescence intensity; MPLA, monophosphoryl lipid A.
Data of seven liposome batches represented as mean ± SD.
[1]MFI measured by flow cytometry.

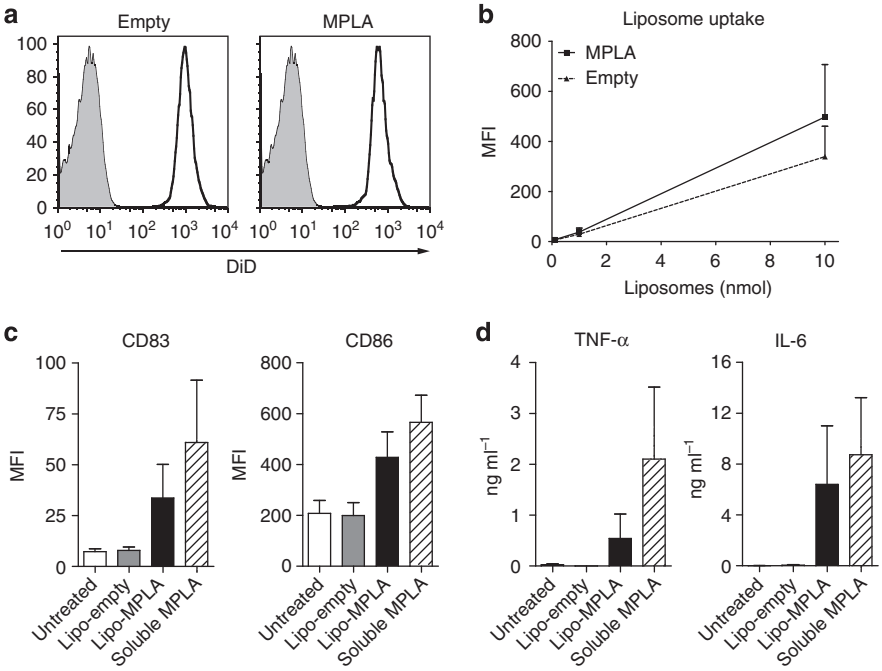


**Figure 1. Monophosphoryl lipid A (MPLA)-modified liposomes are taken up by human monocyte–derived dendritic cells (DCs) and induce DC maturation and cytokine production.** Human monocyte-derived DCs (moDCs) were exposed to various concentrations of non-modified or MPLA-modified liposomes for 3 hours at 37 °C and analyzed by flow cytometry. (**a**) Histograms of liposome uptake. Tinted gray: DC only; solid lines: DC with liposomes. (**b**) Data are shown as mean ± SEM of six independent experiments. (**c** and **d**) moDCs were stimulated with liposomes (100 nmol) or soluble MPLA (concentration as used for liposome preparation) overnight at 37 °C. DCs were analyzed by flow cytometry for maturation, (**c**) or cytokine production was measured in culture supernatants by ELISA (**d**). Data are shown as mean ± SEM of duplicate cultures of 4 independent experiments. MFI, mean fluorescence intensity.

### Administration of MPLA-modified liposomes, but not soluble MPLA, enhances antigen presentation to CD8$^+$ T cells

The uptake of liposomes and subsequent activation of DCs may facilitate antigen cross-presentation to CD8$^+$ T cells. In contrast to soluble short peptide that will directly load onto MHC molecules, liposomal short peptides will be taken up by DCs and enter the cross-presentation route. TLR ligand–modified liposomes containing an MHC class I-restricted gp100$_{280\text{-}288}$ peptide were incubated with HLA-A2$^+$ moDC. Subsequently, DCs were co-cultured with a gp100-specific CD8$^+$ T–cell clone. Loading of DCs with liposomes that contain MPLA markedly enhanced presentation of the gp100 peptide to CD8$^+$ T cells as revealed by significantly higher IFN-γ production, even at lower liposome concentrations (Figure 2). We observed no IFN-γ production without administration of the gp100 peptide (data not shown), indicating that the production of IFN-γ is tumor antigen specific. Administration of soluble MPLA together with gp100-containing liposomes did not enhance T-cell IFNγ production. These results demonstrate that simultaneous administration of tumor antigen and adjuvant, incorporated into the same particle, to DC increases cross-presentation to CD8$^+$ T cells.

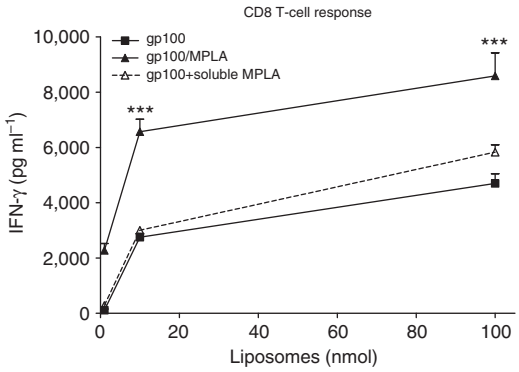


**Figure 2. Liposome-incorporated monophosphoryl lipid A (MPLA) enhances antigen presentation by dendritic cells (DCs) to CD8$^+$ T cells.** HLA-A2$^+$ monocyte-derived DCs (moDCs) were exposed to various concentrations of non-modified or MPLA-modified liposomes loaded with gp100$_{280\text{-}288}$ peptide for 1 hour. Non-modified liposomes were used in the presence or absence of soluble MPLA. After extensive washing, an HLA-A2-restricted gp100-specific CD8$^+$ T–cell clone was added, and after 24 hours supernatants were taken and analyzed for IFN-γ production by ELISA. Data are shown as mean ± SEM of triplicate cultures. Results are representative of three independent experiments. $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ significant difference to non-modified gp100 liposomes. NS, not significant.

### MPLA-modified liposomes are taken up by human dermal DCs after intradermal injection

Next, we investigated whether MPLA-modified liposomes are internalized by dDCs *in situ* in a human skin explant model. Two days after intradermal injection of liposomes, dDCs emigrated from skin biopsies were collected, and liposome internalization was studied. Figure 3a shows our gating strategy for CD1a$^+$ and CD14$^+$ dDC. Total (HLA-DR$^+$) dDCs were

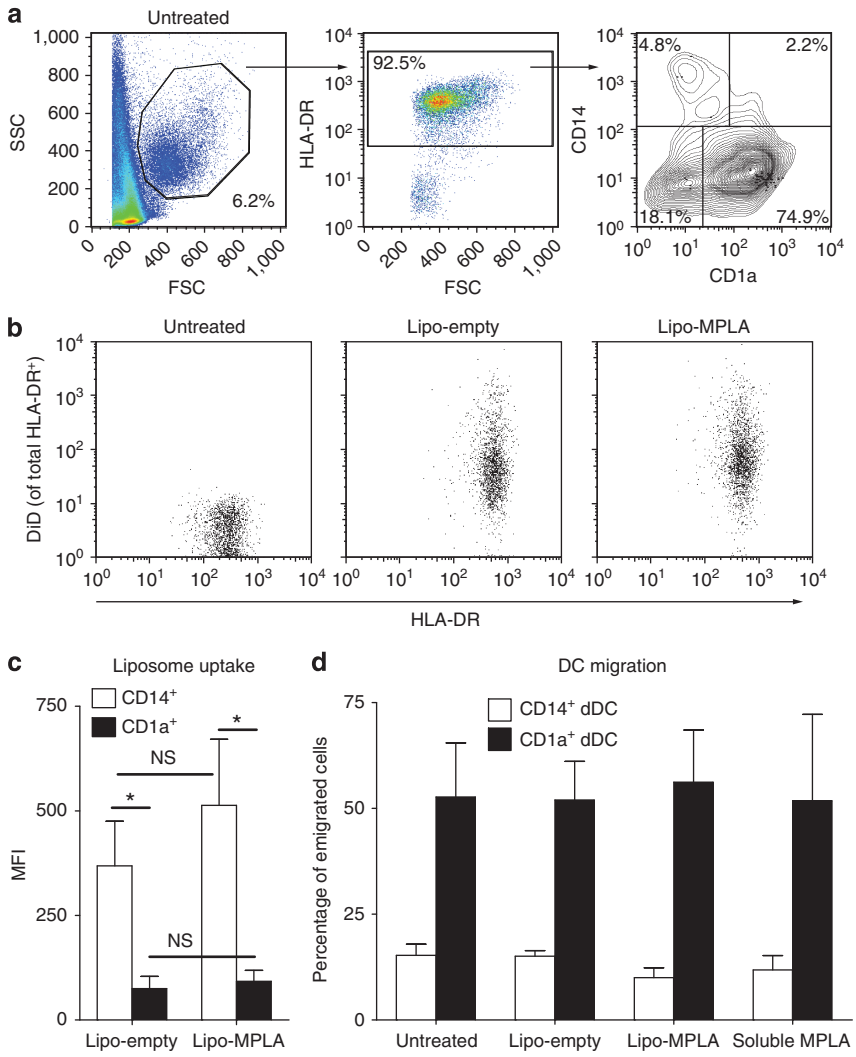


**Figure 3. *In situ* liposome uptake and emigration of dermal dendritic cell (DC) subsets.** Liposomes (50 nmol; **b**–**d**), medium (**a**, **b** and **d**) or soluble monophosphoryl lipid A (MPLA) (**d**), were injected intradermally. Biopsies were taken and after 2 days of culture dermal DCs (dDCs) were collected and stained for HLA-DR and dDC subset markers CD1a and CD14 and analyzed for liposome uptake. (**a**) Gating strategy after cell migration out of skin biopsies. (**b**) Dotplots of liposome internalization by total HLA-DR$^+$ dDC. Representative of three experiments is shown. Data are shown as mean ± SEM of three (**c**) or two (**d**) independent experiments. MFI, mean fluorescence intensity.

shown to efficiently take up liposomes (Figure 3b). Inclusion of MPLA in the lipid bilayer of the liposomes did not significantly alter the uptake. Moreover, analysis of liposome internalization by the CD14$^+$ and CD1a$^+$ dDC subsets showed that CD14$^+$ DCs had taken up a higher amount of liposomes on a per cell basis (Figure 3c). Further analysis of the percentage of emigrated DC subsets revealed that it was, however, primarily the CD1a$^+$ DC subset that migrated out of the skin (Figure 3d), with no difference between TLR-stimulated or non-stimulated conditions. Thus, dDCs are capable of taking up TLR ligand–modified liposomes and migrate out of the skin.

### Injection of MPLA-modified liposomes, but not soluble MPLA, induces pro-inflammatory cytokine production

We investigated the potential of intradermally injected TLR ligand–modified liposomes to activate dDCs. Both CD14$^+$ and CD1a$^+$ dDC subsets express TLR4, the receptor for MPLA (Figure 4a), as shown previously (van der Aar *et al.*, 2007; Harman *et al.*, 2013). Two days after injection, biopsy culture supernatants were collected and investigated for cytokine production. Injection of MPLA-modified liposomes in the skin showed a tendency to enhance the production of pro-inflammatory cytokines IL-6, IL-8, and IL-1β (Figure 4b). Importantly, this enhancement of cytokine production was not seen when soluble MPLA was injected into the skin, in keeping with our previous observations (Oosterhoff *et al.*, 2013; Fehres *et al.*, 2014). These results suggest that the administration of liposomal MPLA, but not soluble MPLA, enhances the production of pro-inflammatory cytokines in the skin microenvironment.

### Intradermal delivery of MPLA-modified liposomes enhances antigen presentation by dermal DCs

As MPLA-modified liposomes are efficiently taken up by dDCs and induce the production of pro-inflammatory

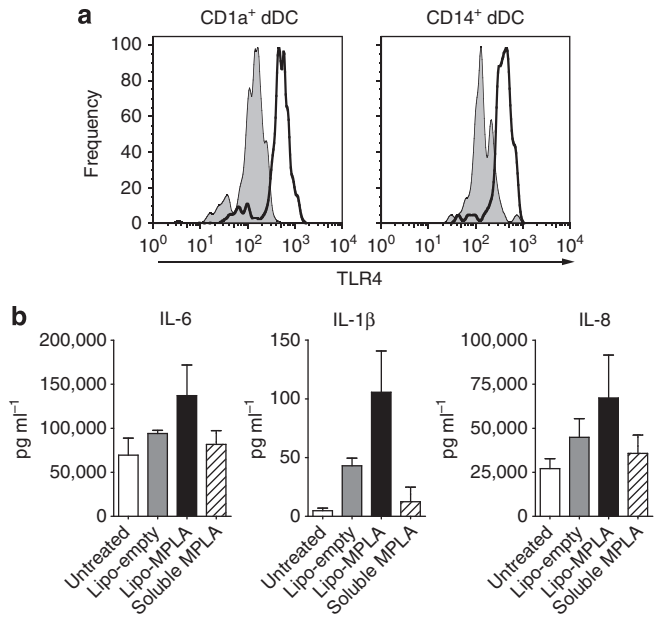


**Figure 4. Increased production of pro-inflammatory cytokines after intradermal injection with monophosphoryl lipid A (MPLA)-modified liposomes.** (**a**) Toll-like receptor 4 (TLR4) expression by emigrated $CD1a^+$ and $CD14^+$ dDCs. Intracellular staining of dermal dendritic cells (dDCs) after 2-day spontaneous migration out of skin biopsies. Tinted gray: isotype controls; solid lines: TLR4 staining. (**b**) Liposomes (50 nmol) or soluble MPLA was injected intradermally. After 2 days of skin biopsy culture, supernatants were collected, and cytokine production was measured by ELISA. Data are shown as mean ± SEM of three or two (IL-1β) independent experiments.

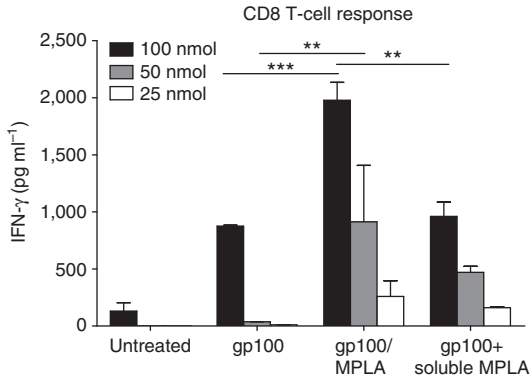


**Figure 5. Liposome-incorporated monophosphoryl lipid A (MPLA) enhances antigen presentation by dermal dendritic cells (DCs) to $CD8^+$ T cells after intradermal injection in human skin.** Various concentrations of non-modified or MPLA-modified liposomes loaded with $gp100_{280\text{-}288}$ peptide were injected intradermally in $HLA\text{-}A2^+$ skin. Non-modified liposomes were also co-injected with soluble MPLA. Emigrated dendritic cells (DCs) were collected after 2 days of skin biopsy culture. Emigrated dDCs were co-cultured with an HLA-A2-restricted gp100-specific $CD8^+$ T–cell clone. After 24 hours, supernatants were taken and analyzed for IFN-γ production by ELISA. Data are shown as mean ± SEM of duplicate cultures. Results are representative of three independent experiments.

cytokines, we examined the effect of liposomal MPLA on antigen cross-presentation of a liposome-incorporated $gp100_{280\text{-}288}$ peptide by dDCs. Liposomes were injected intradermally into the skin of an $HLA\text{-}A2^+$ donor, and after 2 days emigrated dDCs were collected and co-cultured with a gp100-specific HLA-A2-restricted $CD8^+$ T–cell clone. Incorporation of MPLA into liposomes enhanced antigen presentation by dDCs, as measured by increased IFN-γ production by the T cells (Figure 5). Of note, co-administration of soluble MPLA with gp100-containing liposomes did not enhance antigen presentation, indicating that antigen and adjuvant need to be present in the same formulation to allow most efficient antigen presentation of skin APCs. These results demonstrate that simultaneous delivery of tumor antigen and the adjuvant MPLA to dDC augments antigen cross-presentation to $CD8^+$ T cells.

## DISCUSSION

Here we evaluated a two-component vaccine strategy in a human skin explant model for induction of cross-presentation by dDCs. The incorporation of the clinically applicable TLR4 ligand MPLA into liposomes resulted in enhanced DC maturation and cytokine production. Moreover, the uptake of MPLA-containing liposomes induced efficient presentation of the $gp100_{280\text{-}288}$ peptide to $CD8^+$ T cells by dDCs. Importantly, presentation was only enhanced upon simultaneous delivery of tumor antigen and adjuvant, whereas co-administration of soluble MPLA with liposome-encapsulated tumor antigen did not enhance cross-presentation.

The skin is an attractive immunological target for vaccination purposes, because of its relatively high content of APC. In our study, intradermal vaccination of antigen and adjuvant is mimicked *ex vivo* by using skin explants from healthy donors that underwent corrective plastic surgery. In this model, DC function can be studied in their natural complex tissue environment and is a highly representative model of the *in vivo* situation (Fehres *et al.*, 2013; Oosterhoff *et al.*, 2013). We here show that intradermal vaccination with tumor antigen– and adjuvant-containing liposomes results in the uptake of liposomes by dDCs. Especially, the $CD14^+$ dDC subset showed high phagocytic capacity, but it was mainly the $CD1a^+$ dDC subset that migrated out of the skin. $CD14^+$ dDCs may therefore be important at inducing T-cell responses locally in the skin. Indeed, it was shown that inflammatory DCs may shape adaptive immunity *in situ* by activating tissue-resident effector memory $CD8^+$ T cells, thereby showing that a T-cell response mechanism in extra-lymphoid tissues can act to control localized infection (Wakim *et al.*, 2008). For $CD14^+$ dermal cells, it was shown that they efficiently activate memory T–cell responses (Klechevsky *et al.*, 2008; McGovern *et al.*, 2014). Thus, the uptake of therapeutic liposomes by both the resident $CD14^+$ dermal cells and the migratory $CD1a^+$ subset might be beneficial for inducing primary T-cell responses in skin-draining lymph nodes as well as activating memory T cells locally in the skin.

Generally, the maturation of DCs enhances the efficacy to cross-present antigen. A large number of TLR ligands act as adjuvants and thereby augment cross-presentation (Nair-Gupta *et al.*, 2014). However, administration of soluble TLR ligands in the dermis does not induce maturation of dDCs as

seen with *in vitro*-generated moDC, except for modest effects of MPLA, Poly I:C, and several combinations of TLR ligands (Schneider *et al.*, 2012; Oosterhoff *et al.*, 2013; Fehres *et al.*, 2014). Using liposomal carriers gives the opportunity to co-deliver adjuvants along with antigens to enhance maturation and cross-presentation of the DCs. A study by Schlosser *et al.* (2008), demonstrated that *in vivo* cross-priming and CTL responses were enhanced when an antigen, i.e., OVA, was co-encapsulated together with an adjuvant, CpG or Poly I:C, in biodegradable nanoparticles, as compared with inoculation of antigen-containing nanoparticles with soluble adjuvant or separately encapsulated adjuvant. Therefore, we used liposomes to simultaneously deliver tumor antigen and adjuvant to DCs *in situ*. Our data show that *in vitro* activation of moDC was similar for liposomal and soluble MPLA ligands. However, cross-presentation was enhanced with co-delivery of MPLA and antigen in liposomes. Remarkably, cross-presentation was enhanced even at low concentrations of gp100/MPLA liposomes. 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 result in superior antigen presentation to T cells. This in contrast to gp100 liposomes administered with soluble MPLA, where MPLA predominantly triggers cell-surface TLR4, possibly resulting in a different effect on the antigen presentation route. Furthermore, it is not assured that the liposomes and MPLA given soluble reach the same cell for simultaneous TLR4 triggering and antigen presentation. Also cross-presentation by dDCs after intradermal injection of liposomes containing both tumor antigen and MPLA was enhanced compared with injection of soluble MPLA, demonstrating the necessity of co-delivery of antigen and adjuvant in one particulate formulation.

The potency of liposomal delivery of tumor antigen and TLR ligand in the skin was shown in an *in vivo* study by Jerome *et al.* (2006), where they used a melanoma peptide tyrosinase–related protein 2 in combination with CpG as adjuvant. After intradermal prime-boost vaccination, reactive CTL populations were generated even against poorly immunogenic tumors, resulting in a potent anti-tumour immune response *in vivo*. The authors observed a reduction in tumor volume and prolonged survival times upon prophylactic vaccination with low-dose TPR2-encapsulated liposomes with CpG compared with untreated animals or free peptide-treated mice. Although the results with CpG delivery are very promising for determining vaccination strategies, it might not be the best human adjuvant for skin vaccination as TLR9 is not expressed on human myeloid skin APCs as it is a ligand for TLR9, which is only expressed by plasmacytoid DCs.

Concluding, the uptake of liposomal antigens and the simultaneous DC activation by liposomal MPLA resulted in enhanced tumor antigen cross-presentation to $CD8^+$ T cells by moDC and dDCs. Our data demonstrate the effective delivery of liposomes *in situ* to dDCs and the potency as anti-tumor vaccine for inducing tumor-specific T-cell responses. Future research is needed to investigate whether liposomal vaccination could also induce *de novo* tumor-specific T-cell responses from primary naive T cells. We propose that liposomes can be used to deliver tumor antigen and adjuvants directly to dDCs *in vivo* and can be exploited as a vaccine platform to induce potent anti-tumor immune responses.

## MATERIALS AND METHODS

### Liposome preparation and MPLA and peptide incorporation

Liposomes containing TLR ligands were prepared from a mixture of phospholipids and cholesterol, utilizing the film extrusion method as described previously (Joshi *et al.*, 2011). Briefly, EPC-35 (Lipoid, Ludwigshafen, Germany):EPG-Na (Lipoid):cholesterol (Sigma-Aldrich, St Louis, MO) at a molar ratio of 3.8:1:2.5 in mol were dissolved in a mixture of chloroform/methanol. Overall, 0.1% in mol of the lipophilic fluorescent tracer DiD (1′-dioctadecyl-3,3,3′,3′-tetramethyl indodicarbocyanine; Life Technologies, Bleiswijk, The Netherlands) was added to the mixture and, where specified, MPLA (2% mol; Invivogen, Toulouse, France) was added. 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 containing the antigenic peptide $gp100_{280\text{-}288}$ (YLEPGPVTA), yielding a liposome preparation of 14.6 μmol total lipid $ml^{-1}$. The peptide was produced by solid-phase peptide synthesis using Fmoc-chemistry with a Symphony peptide synthesizer (Protein Technologies, Tucson, AZ). After extrusion through two stacked polycarbonate filters of 200 nm, the non-encapsulated peptides were washed away by two consecutive ultracentrifugations on Beckman Ultracentrifuge at 55,000 r.p.m. The final suspension of liposomes was made in Hepes buffer pH 7.5. 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 $ml^{-1}$.

### MoDC culture

Human immature DCs were generated from monocytes isolated from buffy coats of healthy donors (Sanquin, Amsterdam, The Netherlands). Informed consent was obtained from all blood donors for the use of their blood. Monocytes were isolated by subsequent ficoll gradient (Lymfoprep; Axis-Shield PoC AS, Oslo, Norway) and percoll gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Monocytes were cultured for 5–6 days in RPMI1640 medium (Invitrogen, Paisley, UK) containing 10% fetal calf serum, 50 U $ml^{-1}$ penicillin, 50 μg $ml^{-1}$ streptomycin, and 2 mM glutamine (all from Lonza, Verviers, Belgium) in the presence of r.h.IL-4 and r.h.GM-CSF (500 U $ml^{-1}$ and 800 U $ml^{-1}$, respectively; Immunotools, Friesoythe, Germany; Romani *et al.*, 1994; Sallusto and Lanzavecchia, 1994; Gluckman *et al.*, 1997).

### Liposome uptake by moDC

Uptake of modified liposomes (in concentrations as stated) was analyzed by flow cytometry following incubation with 50,000 moDCs for 3 hours at 37 °C. Uptake was quantified by FACS (FACSCalibur, Becton Dickinson, San Jose, CA) and analyzed using FlowJo Software (Tree Star, Ashland, OR).

### moDC maturation and cytokine production by MPLA-modified liposomes

A total of 50,000 moDCs were incubated with liposomes (100 nmol) or equivalent concentrations as used for liposome generation of MPLA (Sigma-Aldrich) overnight at 37 °C. Supernatants were collected for cytokine ELISA, and cells were washed and incubated with monoclonal antibodies (mAb) specific for CD83 (Beckman Coulter, Woerden, The Netherlands) or CD86 (Becton Dickinson,

San Jose, CA) 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.

### Antigen presentation of gp100 peptide to CD8$^+$ T cells

An HLA–A2-restricted CD8$^+$ T–cell clone specific for gp100$_{280\text{-}288}$ was generated and cultured as described previously (Schaft *et al.*, 2003). HLA-A2$^+$ DCs were incubated with indicated concentrations of liposomes in a round bottom 96-well plate for 1 hour at 37 °C. After extensive washing, DCs were co-cultured with 100,000 gp100-specific CD8$^+$ T cells in a 1:3.33 ratio. After overnight incubation, supernatants were collected, and IFN-γ levels were measured by sandwich ELISA using specific antibody pairs from Invitrogen.

### Intradermal injection of liposomes, culture of skin biopsies, and analysis of emigrated skin cells

Human skin explants were derived from abdominal resections from healthy donors (Bergman Clinics, Bilthoven, The Netherlands) within 24 hours after surgery. Material was obtained with informed consent (information leaflet for use of 'left-over' material), which was approved by the Medical Ethical Committee of VUmc. Liposomes or equivalent concentrations of MPLA (Sigma-Aldrich) were diluted in serum-free Iscove's modified Dulbecco's medium (Invitrogen) containing 50 U ml$^{-1}$ penicillin, 50 μg ml$^{-1}$ streptomycin, 2 mM glutamine (Lonza) and 10 μg ml$^{-1}$ gentamycin (Invitrogen), and r.h.IL-4 and r.h.GM-CSF (500 U ml$^{-1}$ and 800 U ml$^{-1}$, respectively; Immunotools, Friesoythe, Germany) and injected intradermally in a volume of 20 μl per biopsy (50 nmol or as indicated). Biopsies were taken immediately after injection using a 6 mm biopsy punch (Microtek Medical Malta, Zutphen, The Netherlands) and cultured with epidermal side up floating in a 48-well plate containing 1 ml of IMDM medium supplemented with 10% fetal calf serum, penicillin, streptomycin, glutamine, and gentamycin at 37 °C. In each experiment, 8 biopsies were taken per experimental condition. After 48 hours of culture, the biopsies were discarded, and emigrated dDCs were collected and used for experiments. Supernatants of biopsy cultures were collected and analyzed for cytokine production.

Internaliation of liposomes by emigrated skin cells was analyzed using flow cytometry. Fluochrome-conjugated mAbs used were specific for CD1a, CD14 (Becton Dickinson), and HLA-DR (R&D Systems, Minneapolis, MN), and biotin-conjugated goat Ab used specific for TLR4 or goat IgG Ab as isotype control (R&D Systems) with donkey anti-goat Alexa 647 (Molecular Probes, Bleiswijk, The Netherlands). For antigen presentation, emigrated HLA-A2$^+$ skin cells were collected and co-cultured with 100,000 gp100-specific CD8$^+$ T cells in a 5:1 ratio. After overnight incubation, supernatants were collected, and IFN-γ levels were measured by ELISA.

### Statistical analysis

Results were analyzed with one-way or two-way analysis of variance, followed by the Bonferroni post-test, using GraphPad Prism software (San Diego, CA; version 5.01). Values were considered to be significantly different when $P \leqslant 0.05$.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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