

# Effects of Antigen-Expressing Immunostimulatory Liposomes on Chemotaxis and Maturation of Dendritic Cells *In Vitro* and in Human Skin Explants

Anastasia Lanzi · Cynthia M. Fehres · Tanja D. de Gruijl · Yvette van Kooyk · Enrico Mastrobattista

Received: 19 March 2013 / Accepted: 9 August 2013 / Published online: 26 September 2013  
© Springer Science+Business Media New York 2013

## ABSTRACT

**Purpose** Antigen-Expressing Immunostimulatory Liposomes (AnExILs) represent a novel DNA vaccination platform based on the production of protein antigens from DNA templates inside liposomes mediated by an *in vitro* transcription and translation (IVTT) mix. The aim of this study was to analyze the effects of AnExILs on different dendritic cells (DCs) models and to better understand the role of the different components of this formulation on its adjuvanticity.

**Methods** The effect of  $\beta$ -galactosidase-expressing AnExILs on maturation and particle uptake by murine DC cell line, fresh human monocyte-derived DCs or human dermal DCs in skin explants was investigated and compared to the effects of either plain liposomes or IVTT mix alone.

**Results** AnExILs induced efficient DC chemotaxis and promoted up-regulation of maturation markers on murine DCs, due to the presence of IVTT in the formulation. Furthermore, the amount of active  $\beta$ Gal associated with DCs was higher for AnExILs than for free  $\beta$ Gal expressed in IVTT or  $\beta$ Gal encapsulated into non-adjuvanted liposomes. Most interestingly, the same trend was observed with human DCs.

**Conclusions** Both IVTT mix and liposomal vehicles were shown to be key components of the AnExIL formulation responsible for its adjuvanticity. AnExILs combine antigen production, adjuvanticity and delivery in one system, and can efficiently activate both murine and human DCs.

**KEY WORDS** cell-free protein synthesis · chemotaxis · DC activation · DNA-vaccine · liposome

## ABBREVIATIONS

|             |   |
|-------------|---|
| AnExIL      | Antigen-Expressing Immunostimulatory Liposome |
| APC         | Antigen presenting cell                       |
| DC          | Dendritic cell                                |
| FMI         | Forward migration index                       |
| IVTT        | <i>In vitro</i> transcription and translation |
| LAL         | Limulus ameobocyte lysate                     |
| LPS         | Lipopolysaccharide                            |
| MFI         | Mean fluorescence intensity                   |
| Mo-DC       | Monocyte-derived dendritic cell               |
| $\beta$ Gal | $\beta$ -galactosidase                        |

## INTRODUCTION

One of the first requirements that have to be fulfilled for the development of successful vaccines is efficient antigen delivery to antigen presenting cells (APCs), and in particular dendritic cells (DCs). DCs are the most effective APCs and play a critical role in eliciting immune responses to vaccines. They initiate the adaptive immune responses by their unique ability to capture and process antigens through MHC class I and class

**Electronic supplementary material** The online version of this article (doi:10.1007/s11095-013-1179-0) contains supplementary material, which is available to authorized users.

A. Lanzi · E. Mastrobattista (✉)  
Department of Pharmaceutics  
Utrecht Institute for Pharmaceutical Sciences (UIPS)  
Utrecht University, 3584 CG Utrecht, The Netherlands  
e-mail: E.Mastrobattista@uu.nl

C. M. Fehres · Y. van Kooyk  
Department of Molecular Cell Biology and Immunology  
VU University Medical Center, Amsterdam, Netherlands

T. D. de Gruijl  
Department of Medical Oncology, VU University Medical Center  
Amsterdam, Netherlands

II pathways and present the corresponding epitopes to CD4<sup>+</sup> and CD8<sup>+</sup> T cells (1). Importantly, the DCs should themselves be activated by danger signals carried by the vaccine formulation to be able to mature, express co-stimulatory molecules at their surface, migrate to the lymph nodes and eventually deliver all required activation signals to T cells (2). Understanding the interactions of DCs and vaccine candidates is then of highest importance to develop a successful vaccine formulation.

Since soluble purified antigens have been shown to be poorly immunogenic, numerous strategies have been studied to improve *in vivo* antigen delivery, like administration of antigens in combination with adjuvants, or encapsulation/conjugation of antigens in different delivery systems that can introduce antigens into the endosomes and cytoplasm of DCs (3,4). Amongst other carriers, encouraging results have been obtained with virus-like particles (5), polymeric nanoparticles (6) or liposomes (7) delivering either antigen proteins or nucleic acids. These nanocarrier-based vaccines protect the antigen from degradation and allow efficient processing by DCs (8). However, encapsulating protein antigens requires recombinant protein production and purification steps that can be time-consuming and expensive. An alternative is to encapsulate DNA plasmids since they are easier to prepare but their transfection into cells of the vaccine recipient is often limited, leading to low doses of antigens produced and consequently low immune responses generated. Moreover, their poor immunogenicity in humans remains a problem (9,10). Novel vaccine delivery systems are thus still critically needed.

Recently, we have developed a new class of vaccines termed Antigen-Expressing Immunostimulatory Liposomes (AnExILs). This genetic vaccine platform is based on the cell-free production of protein antigens from DNA templates both within and outside submicron-sized liposomes with the use of a bacterial *in vitro* transcription and translation (IVTT) system (Fig. 1a). The advantages of this approach are numerous: i) it mimics transcriptionally active pathogens, yet without the risk of being infectious; ii) the specificity of AnExIL vaccines is exclusively determined by the genetic input, making it flexible and easy to adapt to the intended needs; iii) compared to classical DNA vaccines, AnExIL formulation bypasses the limiting step of plasmid delivery into the nucleus of cells of the vaccinee *in vivo* since antigens are directly produced *in vitro* and can subsequently be taken up by professional APC *in vivo*. AnExIL formulation is thus the unique vaccine candidate combining antigen-production, delivery and adjuvanticity in one system. This formulation has been extensively characterized and optimized for antigen production (11) and its efficacy *in vivo* has been evaluated in previous studies. Vaccination studies in mice demonstrated that AnExIL formulations are able to raise an efficient humoral response against the model antigen  $\beta$ -galactosidase produced *in vitro* inside the AnExIL particles, and that this response was superior to the one induced by a

liposomal formulation in which the protein antigen had been entrapped (12). In another study it was demonstrated that vaccination with AnExILs could also lead to cellular responses against the NP epitope of influenza virus (13).

To be able to explain the superiority of AnExILs compared to conventional liposomal protein vaccines at equal antigen doses, and to further optimize the AnExIL formulation, a deeper understanding of its effects on the immune system is required. Therefore, the aim of the present study was to investigate the effect of these AnExIL particles on DCs *in vitro*. The chemoattractant effect of AnExILs, their uptake by DCs and the DC maturation induction have been characterized for this new vaccine formulation and also separately for its two major components, the IVTT mix and liposomes, in order to analyze the contribution and the importance of these constituents to the adjuvanticity of the AnExIL formulation. A murine DC cell line was first used, before going to human Mo-DCs and finally a more representative human skin model in order to get insight into the potential human application of this vaccine formulation.

## MATERIALS AND METHODS

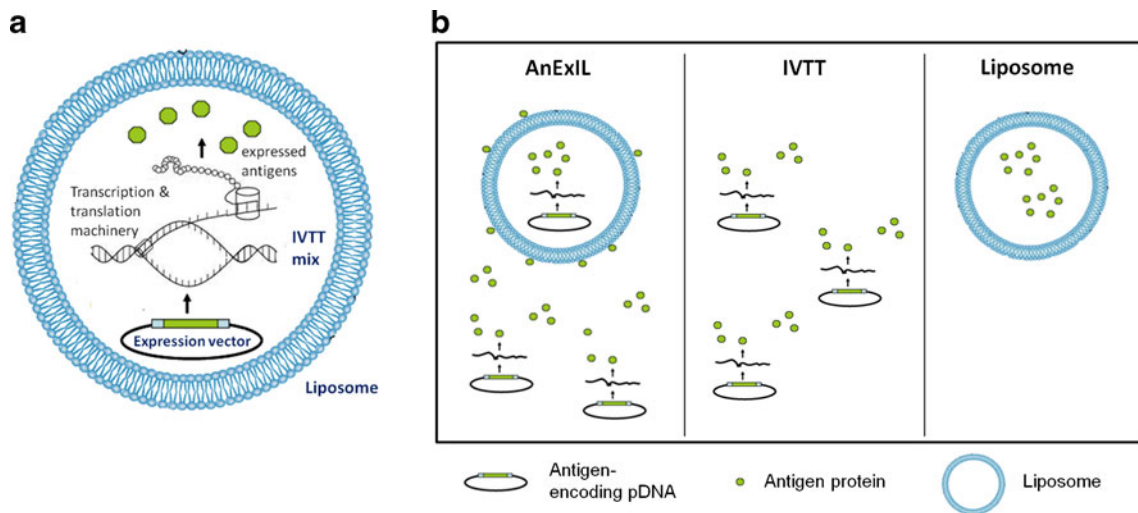
### Materials

Egg-derived L- $\alpha$ -phosphatidylcholine (EPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolaminepolyethylene glycol (PEG) 5000 (DSPE-PEG 5000) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). Luria Broth, 2-mercaptoethanol, adenosine-5'-triphosphate (ATP), phosphoenol-pyruvate (PEP), cytidine-5'-triphosphate (CTP), guanosine-5'-triphosphate (GTP), 3'-5'-cyclic adenosine monophosphate (cAMP), folic acid, cholesterol (CHOL) and  $\beta$ -galactosidase enzyme (400 IU/mg) and each of the 20 encoded amino acids were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Fluorescein di- $\beta$ -D-galactopyranoside (FDG), a fluorogenic substrate for  $\beta$ -galactosidase was obtained from Marker Gene Technologies (Eugene, OR, USA). E-coli tRNA, creatine kinase and creatine phosphate were obtained from Roche (Basel, Switzerland). Uridine 5'-triphosphate (UTP) and T7 polymerase were supplied from Fermentas (Burlington, Ontario, Canada). Dithiothreitol (DTT) and pyruvate kinase (PK) were from Flucka (Seelze, Germany). PEG 8000 was from Promega (Madison, WI, USA).

### Methods

#### Plasmid

The plasmid pIVEX2.2EM-LacZ (14), encoding *E. coli*  $\beta$ -galactosidase under control of the prokaryotic T7 promoter,



**Fig. 1** Schematic representation of the various vaccine formulations tested in this study. **(a)** Antigen-Expressing Immunostimulatory Liposomes (AnExILs) consist of freeze-dried liposomes rehydrated with an *in vitro* transcription and translation mix together with an antigen-encoding pDNA. Transcription and translation of the pDNA leads to *in situ* production of antigens both inside and outside the liposomes. **(b)** Dissection of AnExILs into its two main components, i.e. IVTT mix and liposomes in order to determine which of these components contribute to the adjuvant effect of AnExILs.

was used for *in vitro* production of  $\beta$ -galactosidase ( $\beta$ Gal) as a model antigen for vaccination studies.

### Cell Culture

The D1 cell line, a long term growth factor-dependent immature splenic DC line derived from B6 (H-2<sup>b</sup>) mice, was used for the studies with murine DC (15). The culture medium, called D1 medium, was IMDM (Sigma-Aldrich) containing 10% heat-inactivated fetal calf serum FCS (PAA, GE Healthcare, Little Chalfont, UK), 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine (all from PAA), 50  $\mu$ M 2- $\beta$ -mercaptoethanol, and supplemented with 30% supernatant from the fibroblast cell line R1, containing 10–20 ng/ml recombinant mouse Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF). The R1 cells expressing GM-CSF were cultured in IMDM containing 5% FCS, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 50 mM 2- $\beta$ -mercaptoethanol. 1 mg/mL G418 (Sigma-Aldrich) was added for the R1 cell culture but not for the supernatant production. The supernatant was collected from confluent cultures and filtered (0.2  $\mu$ m, Sartoris Stedim, Goettingen, Germany).

Human monocytes were isolated from the blood of healthy donors (Sanquin, Amsterdam, Netherlands) through a sequential Ficoll/Percoll gradient centrifugation. Immature monocyte-derived DCs (Mo-DCs) were generated by culture of monocytes for 4–7 days in RPMI 1640 (Invitrogen, Gibco, CA) supplemented with 10% FCS, 100 IU/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine in the presence of recombinant human interleukin 4 (IL-4) and rhGM-CSF (500 U/ml and 800 U/ml respectively, Biosource, Camarillo, CA, USA).

### Preparation of Cell-Free Protein Synthesis System

The *E. coli* Rosetta-gami<sup>TM</sup> strain (Novagen, The Netherlands) was used to make S30 extract as described previously (11). Shortly, S30 extract is a cleared bacterial lysate obtained after disruption of bacteria by sonication and centrifugation at 30,000  $\times$  g, followed by a dialysis at 4°C using a Slide-a-Lizer membrane cassette with a MWCO of 10 kD. A coupled *in vitro* transcription/translation reaction mixture (further referred to as IVTT mix), consisted of 30% (v/v) S30 extract, 175  $\mu$ g/mL tRNA, 250  $\mu$ g/mL creatine kinase, 5.8 mM magnesium acetate, 260 U T7 polymerase, and 50% (v/v) low-molecular-weight mix (LM mix) containing 110 mM HEPES, 3.4 mM DTT, 2.4 mM ATP, 1.6 mM CTP, 1.6 mM GTP, 1.6 mM UTP, 0.8 M creatine phosphate (CP), 0.65 mM cAMP, 0.05 mM folinic acid, 0.21 M potassium acetate, 27 mM ammonium acetate, 2 mM each of the 20 amino acids, and 8% (v/v) PEG8000, was used for protein synthesis. All components were mixed on ice prior to use.

### Preparation of Liposomes and AnExIL Formulations

Neutral PEG-liposomes consisting of EPC, CHOL and DSPE-PEG 5000 with a molar ratio of EPC:CHOL:PEG 5000=1.6:0.9:0.025 (corresponding to 1 mol% PEG 5000 relative to total lipid) were prepared as described previously (11) and using the freeze-dried empty liposome method (16). The fluorescent label DiD, which due to its hydrophobicity incorporates into the lipid bilayer of liposomes, was added at a concentration of 0,01% of total lipid during the first step of dissolution of the lipids into dichloromethane:diethylether. The obtained lipid cakes (6  $\mu$ mol of total lipids/batch) were stored in the dark in a desiccator. The liposome formulation,

later referred to as lipo formulation, was obtained by carefully resuspending one batch with 100  $\mu$ l of PBS to allow the formation of the multilamellar liposomes.

For preparing the AnExIL formulation, a batch of freeze-dried liposomes was hydrated with 100  $\mu$ l of IVTT mixture and pIVEX-lacZ at a final concentration of 20 nM. The liposomes were incubated on ice for 10 min to complete the rehydration process, and then at 30°C for 3 h to allow the protein synthesis to complete. This *in vitro* protein production occurs both inside (5–10% of total production) and outside the liposomes. The amount of  $\beta$ Gal produced was determined by measuring enzymatic activity after liposome lysis in the presence of FDG. Substrate conversion rates were compared with a calibration curve of known amounts of  $\beta$ Gal. The amount of LPS was determined by a Limulus amoebocyte lysate (LAL) assay performed at UMCU Utrecht at the Department of Clinical Pharmacy.

The formulation referred to as IVTT corresponds to 100  $\mu$ l of IVTT mixture (3 mg/mL protein) and pIVEX-lacZ at a final concentration of 20 nM.

To prepare a control liposomal protein vaccine, called  $\beta$ Gal lipo formulation, an aqueous solution of  $\beta$ Gal protein (250  $\mu$ g/ml) (Sigma-Aldrich) was prepared in PBS and used to rehydrate a batch of lipid cakes. The liposomes were incubated for 10 min at room temperature. To remove non-encapsulated  $\beta$ Gal, liposomes were washed three times with PBS by centrifugation at 7500  $\times$  g for 10 min at 4°C and subsequent re-suspension in 100  $\mu$ l PBS.

### Chemotaxis Assay

A Chemotaxis<sup>3D</sup>  $\mu$ -Slide (Ibidi, Martinsried, Germany) was used to perform a 3D migration assay with the D1 cells. One hundred and fifty microliters of a collagen gel solution at 1.5 mg/mL (Invitrogen, Gibco) was first mixed with 10  $\mu$ l NaHCO<sub>3</sub> and 20  $\mu$ l MEM 10 $\times$  (both from Sigma-Aldrich), and then with 90  $\mu$ l of a D1 suspension at 12.5  $\times$  10<sup>6</sup> cells/mL. Immediately after mixing, 6  $\mu$ l of this preparation was used to fill the channel of a  $\mu$ -Slide Chemotaxis<sup>3D</sup>. The plate was incubated at 37°C for 1 h until the gel was formed. The two reservoirs on each side of the channel were then filled according to manufacturer's instructions (slow method) to create a concentration gradient, the right chamber containing D1 medium and the left chamber the formulation to be tested (IVTT, AnExIL, plain liposomes) diluted 90 times. Macrophage Inflammatory Protein 1 $\alpha$  (MIP-1 $\alpha$ ) was used as a positive control at a concentration of 2  $\mu$ g/mL. Time-lapse series of moving DCs were recorded every 5 min for 2 h on a Nikon microscope (Eclipse TE2000-U, Nikon Corporation, Japan) equipped with a 10 $\times$  phase-contrast objective, a heating system and a camera. The position of the cells were tracked manually with the ImageJ software and the forward migration

index (FMI) was finally analyzed with the Chemotaxis and Migration Tool (Ibidi).

### Cell Association Assay

D1 cells were seeded in 96-well plates at a density of 2  $\times$  10<sup>5</sup> cells/well in 100  $\mu$ L D1 medium. One hundred microliters of the different formulations (IVTT, AnExIL, lipo, lipo+IVTT), diluted 10 and 50 times in D1 medium were added to the cells in a total volume of 100  $\mu$ l D1 medium in duplicate. For the lipo+IVTT formulation, lipid cakes were first rehydrated in 100  $\mu$ L PBS, then mixed with 100  $\mu$ L IVTT. After addition of the vaccine formulations, cells were incubated for 2 h at 37°C. After one washing with PBS 0.25% BSA (Sigma-Aldrich), FDG was added to the cells for 1 min at 37°C at a final concentration of 1 mM. After two more washing steps, cells were analyzed by flow cytometry (FACS Canto, BD Biosciences, CA, USA). The cell-associated fluorescein fluorescence corresponding to the amount of active  $\beta$ Gal enzyme associated to DCs was normalized to the total amount of  $\beta$ Gal enzyme incubated with the cells.

For the cell association assay with Mo-DCs, the same protocol was used with 5  $\times$  10<sup>4</sup> cells per well.

### Maturation Assay

D1 cells (2  $\times$  10<sup>5</sup> cells/well in 100  $\mu$ L D1 medium) were incubated with 100  $\mu$ L of the different formulations (IVTT, AnExIL, lipo), diluted 100 times. Lipopolysaccharide (LPS, 200 ng/mL) (Sigma-Aldrich) was used as positive control for DC maturation, and D1 medium as negative control. Cells were incubated 24 h at 37°C. After one washing step, cells were stained with APC-labeled anti-mouse CD86 and CD40 antibodies (SouthernBiotech, Birmingham, AL, USA) for 30 min at 4°C. Appropriate Ig isotype controls were included in all experiments and showed no significant non-specific binding. After two more washing steps, cells were analyzed by flow cytometry.

For the maturation assay with Mo-DCs, the same protocol was used with 5.10<sup>4</sup> cells per well, and the cells were stained with anti-human FITC CD86 (Immunotools, Friesoythe, Germany) and PE CD83 antibodies (Beckman Coulter, CA, USA).

### AnExIL Injection in Human Skin Explants

Human skin specimens were obtained after informed consent from patients undergoing corrective breast or abdominal plastic surgery, following hospital guidelines. A detailed description of the skin explant system has been published previously (17). Skin biopsies (6 mm) were intradermally (i.d.) injected with medium (IMDM supplemented with rhGM-CSF at 262.5 U/ml and rhIL-4 at 112.5 U/ml) or with the different

formulations (AnExIL, lipo, IVTT) diluted 4 or 12 times. All the samples were injected into the dermis in a total volume of 10  $\mu$ l. Following injection, the explants (8–12 biopsies/condition) were cultured at 37°C, floating freely on IMDM medium containing 10% FCS, with their epidermal side up, before their removal 2 days later. The explants were discarded, and the medium, containing skin-emigrated DC, was harvested and pooled per test condition. Migrated DCs were counted and 15000 cells/well were seeded for cell association and maturation assessment. FDG was added as described for D1 cells for the antigen uptake evaluation. Cells were stained with anti-human PerCP MHC class II (R&D Systems, Minneapolis, MN, USA), PE CD86 (BD Biosciences) or CD83 antibodies (Beckman Coulter) for the maturation assay. For the CD1a<sup>+</sup> and CD14<sup>+</sup> subpopulation characterization, the total skin-emigrated DCs were also stained with anti-human FITC CD1a and PerCP CD14 antibodies (both from BD Biosciences).

### Statistics

A one-way ANOVA test with a Bonferroni post-test was conducted. Differences were considered significant when  $P < 0.05$ .

## RESULTS

### Cell-Free Protein Synthesis System and AnExILs

A prokaryotic cell-free protein synthesis system was used to transcribe and translate the lacZ gene encoding for *E. coli*  $\beta$ -galactosidase, which was chosen as a model antigen for our studies. The S30 extract used for *in vitro* transcription and translation (IVTT) was derived from *E. coli* BL21 Rosetta-gami<sup>TM</sup> strain, which contains pRARE encoding rare tRNA codons and is devoid of endogenous  $\beta$ Gal enzyme. AnExIL particles were prepared by resuspending freeze-dried liposomes with a mixture of IVTT and plasmid (see [Materials and Methods](#)) (Fig. 1a). Relatively large neutral multilamellar liposomes are obtained (number-weighted mean particle size of 1.2  $\mu$ m), with a heterogeneous size distribution, as described previously [11]. The particles are then incubated at 30°C for 3 h to allow the protein production. It has to be noted that part of the IVTT is not encapsulated inside liposomes and that the antigen production occurs both inside (5–10% of the total production) and outside the particles (Fig. 1b). Approximately 3.5 mg/mL of  $\beta$ Gal is produced in the AnExIL formulation, as compared with around 5 mg/mL in the IVTT formulation. The concentration of LPS was quantified by a LAL assay and correspond to 30.10<sup>6</sup> EU/mL, due to the presence of the bacterial extract.

The immunogenicity of the AnExIL particles was already investigated *in vivo*, by using either the  $\beta$ Gal model antigen or the NP epitope from influenza virus (12,13). This formulation led to efficient results, but because of its complexity, could also raise some questions concerning the necessity of its different constituents, for instance the presence of liposomes or the use of a bacterial extract. The goal of the present study is then to dissect the composition of this specific AnExIL formulation and to characterize the effects of each of its constituents on different models of DCs. Therefore, the AnExILs particles, the IVTT mix and the liposomes will be the only three formulations of interest for the following characterizations (Fig. 1b).

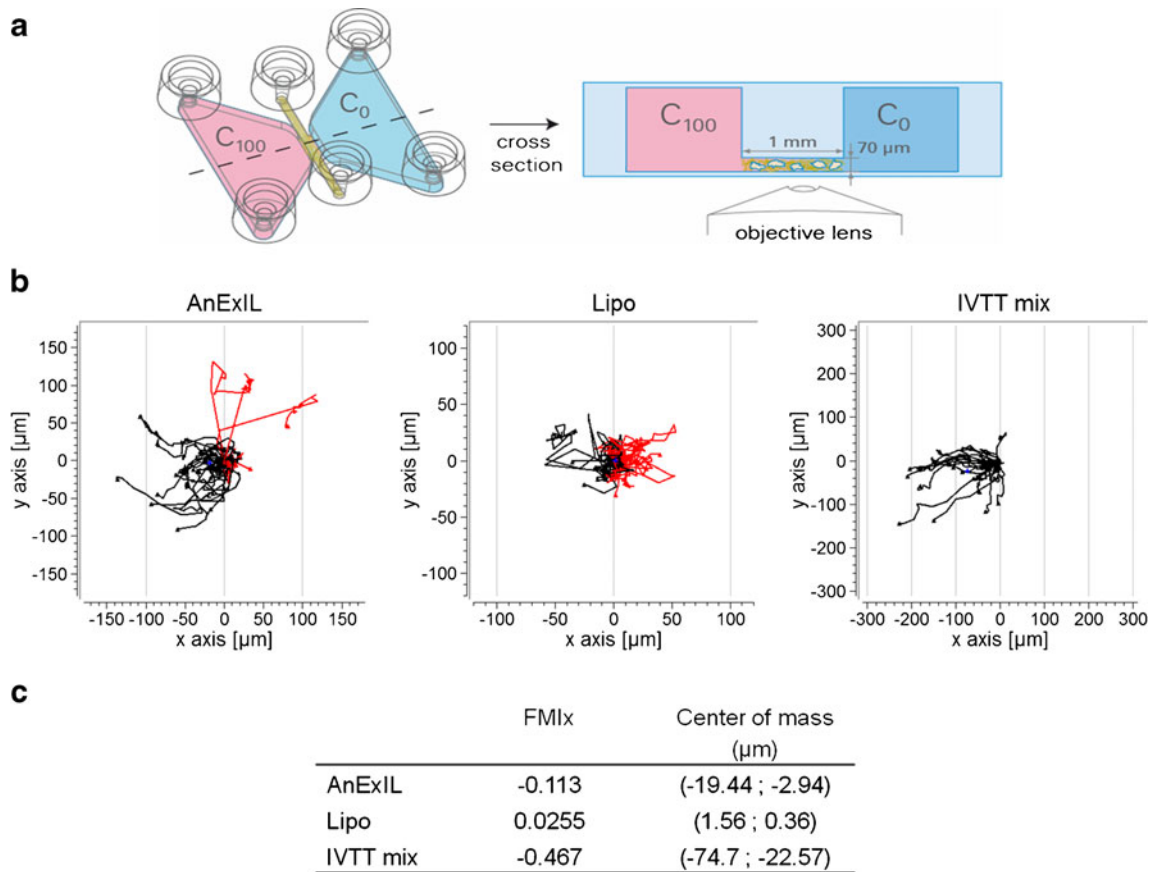
### Chemotaxis Assay

The potential chemoattractant effect of AnExILs on D1 cells (i.e. a murine DC cell line) was evaluated by using the  $\mu$ -Slide Chemotaxis<sup>3D</sup> chamber assay. D1 cells were seeded in the channel separating two reservoirs (Fig. 2a). The left one was filled with AnExILs at a final concentration of 0.67 mM (90 times diluted) and the right one with D1 medium, to establish a concentration gradient. Images were taken for 2 h every 5 min to follow migration of D1 cells in response to chemokines. Most of the D1 cells were moving towards the left compartment (35 out of 44 cells tracked) (Fig. 2b). The Forward Migration Index (FMI) in the x axis, that characterizes the migration efficiency, was  $-0.113$  (Fig. 2c). The center of mass position was displaced to  $(-19.44 \mu\text{m}; -2.94 \mu\text{m})$  2 h after start of migration. The AnExIL formulation is thus able to induce efficient chemotaxis.

To better investigate which component of the AnExIL formulation was responsible for this chemoattractant effect, the chemotaxis assay was repeated with the two main components of the AnExIL formulation separately, i.e. plain liposomes and IVTT mix. With plain liposomes, the FMI<sub>x</sub> was very low (0.0255), and the cells were only moving randomly (Fig. 2b). The center of mass was not displaced (1.56; 0.36) at the end of the 2 h assay. The liposomes alone thus did not have any chemoattractant effect on D1 cells. In contrast, when the IVTT mix was used as source for chemotaxis, the D1 cells were undergoing an efficient migration towards the left chamber, as represented by a FMI<sub>x</sub> of  $-0.466$  and a final center of mass position in  $(-74.7; -22.57)$ . To put these results into perspective, MIP-1 $\alpha$ , a well-known chemokine, was used to fill the left chamber at a concentration of 2  $\mu$ g/mL and the FMI<sub>x</sub> was  $-0.526$ .

### Cell Association Assay

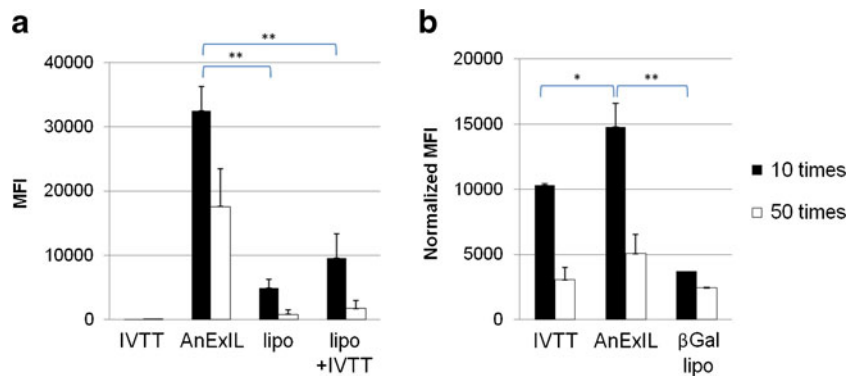
The cell association of the AnExIL particles to the D1 cells was evaluated next, and compared to the cell association of other formulations. The cells were incubated 2 h with IVTT as a



**Fig. 2** Chemotaxis assay. **(a)** Schematic representation of the  $\mu$ -Slide Chemotaxis <sup>3D</sup>. **(b)** Plots of individual DC migration paths. The left reservoir was filled with AnExILs, liposomes or IVTT mix and the position of the D1 cells was tracked for 2 h. **(c)** FMIx values and final center of mass position after the 2 h migration for each formulation tested. One out of two experiments was shown.

negative control or with the different DiD-labeled liposome or AnExIL formulations diluted 10 or 50 times. The cell-associated DiD fluorescence was efficiently detected for the AnExIL formulation in a dose-dependent manner (Fig. 3a). Notably, the Mean Fluorescence Intensity (MFI) was approximately 6 times higher for the AnExIL group than for plain liposomes resuspended in PBS after 2 h of incubation with the

D1 cells. This hierarchy was also reflected by the higher percentage of DiD<sup>+</sup> D1 cells after incubation with AnExIL (39%) *vs* liposomes (17%) (Supplementary Material Fig. 1). To further explore the role of the IVTT mix in enhancing the uptake of AnExILs by D1 cells, preformed plain liposomes were mixed with IVTT, such that the IVTT is only present on the outside of the liposomes as opposed to the AnExIL



**Fig. 3** Cell association assay. D1 cells were incubated with IVTT, AnExIL, lipo,  $\beta$ Gal lipo or lipo+IVTT formulations for 2 h at 37°C and then analyzed by flow cytometry for the liposomal **(a)** or antigen **(b)** cell association. Expression levels in MFI of DiD or fluorescein are respectively represented. For the antigen uptake, the MFI values are normalized to the total amount of  $\beta$ Gal enzyme incubated with the cells.  $N = 2$ , \* $P < 0.05$  and \*\* $P < 0.01$  *versus* AnExIL formulation.

formulation that also contains IVTT entrapped, and incubated with the D1 cells. The MFI of DiD was comparable to the one detected for plain liposomes (Fig. 3a).

Since it was shown that AnExILs are efficiently taken up by DCs, the next step was to investigate whether this would translate into enhanced uptake of antigen produced inside the AnExILs as compared to soluble antigen produced with bulk IVTT mix. The amount of  $\beta$ Gal associated to the D1 cells was evaluated by adding an FDG substrate to the cells. The MFI of fluorescein was higher for the AnExIL formulation than for the IVTT one, at equal amount of  $\beta$ Gal incubated with the cells (Fig. 3b). The AnExIL formulation was then compared to a control liposomal protein vaccine (i.e.  $\beta$ Gal lipo) formulation, corresponding to  $\beta$ Gal protein encapsulated in liposomes. The amount of antigen associated with DCs was much lower with this formulation than with AnExILs (Fig. 3b).

Altogether, the AnExIL formulation proved superior in delivering antigens to D1 cells in culture as compared to conventional liposomal formulations or soluble antigen produced with IVTT mix.

### DC Maturation Assay

To investigate the effect of AnExIL formulation on DC maturation state, the D1 cells were incubated 24 h with the AnExIL, IVTT, lipo formulations or with LPS, and the level of CD86 and CD40 markers at their surface was evaluated by flow cytometry. As expected, LPS-pulsed D1 showed clear maturation, with up-regulation of all markers in comparison to the immature state (medium condition) (Fig. 4). After incubation with the AnExIL formulation, a significant increase of all markers expression levels was also observed. This up-regulation was either similar to the one induced by LPS, or even higher for the CD86 marker. Interestingly, the maturation induced by AnExIL formulation was comparable to that induced by the IVTT formulation. By contrast, incubation of

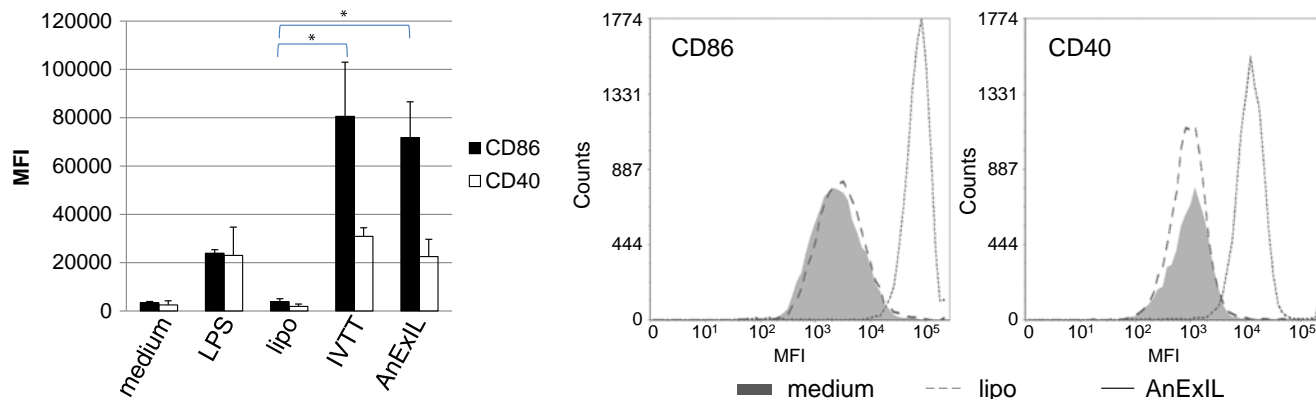
D1 with the lipo formulation did not lead to any maturation (Fig. 4).

### Effect of AnExILs on Human Mo-DCs

After this first investigation of the effect of AnExILs on a murine DC line, human Mo-DCs were used to determine if the preceding results were reproducible with human cells. Cell association and maturation assays were performed in a similar way with the Mo-DCs. Again, the AnExIL particles were better taken up by Mo-DCs than the other formulations (lipo and lipo+IVTT), in keeping with the results with D1 cells (Fig. 5a). This was also reflected by the higher percentage of DiD<sup>+</sup> cells incubated with AnExIL formulation (Supplementary Material Fig. 2). Besides, the cell association of lipo and lipo+IVTT formulations to Mo-DCs was comparable. Concerning the antigen uptake, the amount of  $\beta$ Gal associated with Mo-DCs was higher with AnExILs than with IVTT mix (Fig. 5b). AnExILs were also able to induce a significant maturation of the Mo-DCs, as visualized by an increase in the CD86 and CD83 markers expression at the surface of the Mo-DCs (Fig. 5c). Both AnExILs and IVTT mix could induce higher levels of CD86 and CD83 in comparison to lipo formulation, which did not lead to any maturation of the cells.

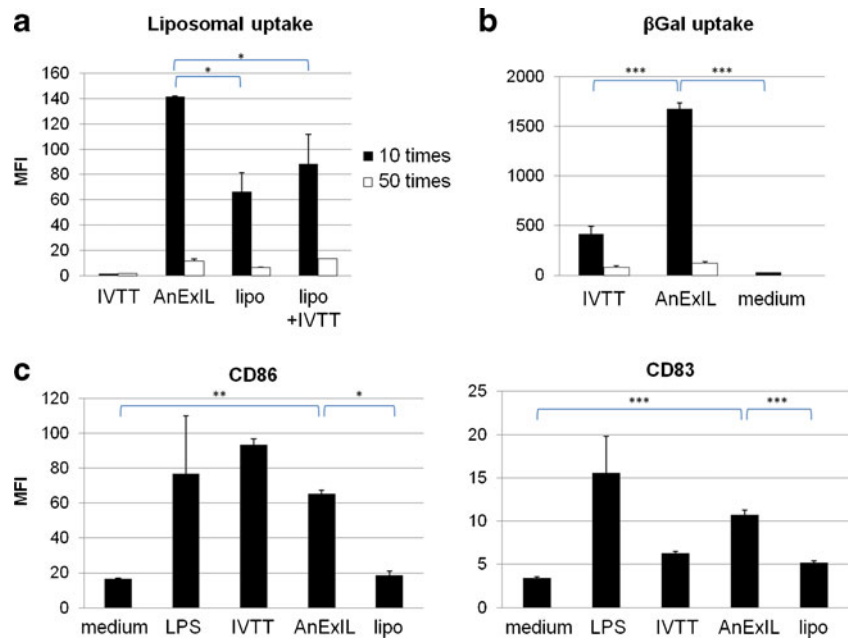
### Human Skin-Explant Assay

Since the AnExIL formulation is able to activate both murine and human DCs *in vitro*, we next evaluated its effect on primary human DCs using a near-physiological human skin explant model. Human skin was injected with the different formulations, the biopsies were incubated in medium for 2 days, and the skin-emigrated DCs harvested after this period. The same number of cells was collected for all the groups, meaning that the incubation with vaccine formulation does not influence the migration of DCs out of the skin. The cell association and maturation state were then measured. Also in



**Fig. 4** Maturation assay. D1 cells were incubated with medium (negative control), LPS (positive control), lipo, IVTT or AnExIL formulations for 24 h at 37°C, stained with anti-CD86 and CD40 antibodies and analyzed in flow cytometry.  $N = 2$ , \* $P < 0.05$  versus lipo formulation.

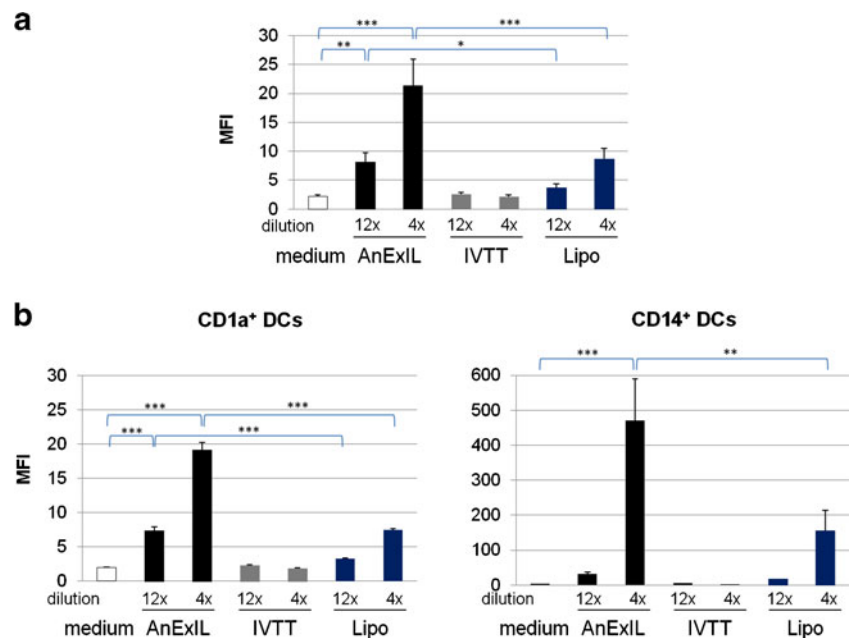
**Fig. 5** Investigation of the AnExIL effect on human Mo-DCs. **(a)** Liposomal or antigen **(b)** cell association was evaluated by incubating Mo-DCs with IVTT, AnExIL, lipo or lipo+IVTT formulations for 2 h at 37°C. **(c)** Maturation state of Mo-DCs after 24 h incubation with medium (negative control), LPS (positive control), lipo, IVTT or AnExIL formulations. The cells were stained with anti-CD83 and CD86 antibodies and analyzed in flow cytometry. *N* = 2, \**P* < 0.05, \*\**P* < 0.01 and \*\*\**P* < 0.001 versus AnExIL formulation.



this model, we could demonstrate that AnExIL particles were efficiently taken up by the human dermal DCs, and this uptake was more efficient than the one of plain liposomes, as indicated by the higher MFI and higher percentage of DiD<sup>+</sup> cells for the AnExIL group (Fig. 6a and Supplementary Material Fig. 3a). The skin-emigrated DCs were then stained to differentiate the two main subsets of human dermal DCs, the CD1a<sup>+</sup> and CD14<sup>+</sup> DCs. CD1a<sup>+</sup> CD14<sup>-</sup> DCs represented around 70% of the population of skin-emigrated DCs, and CD14<sup>+</sup> CD1a<sup>-</sup> DCs around 3% (Supplementary Material Fig. 4). Interestingly, both subsets were able to take up the AnExIL particles, and the trend AnExIL>lipo was conserved

(Fig. 6b). The uptake by CD14<sup>+</sup> DCs was higher, with for instance 90% of DiD<sup>+</sup> CD14<sup>+</sup> DCs after incubation with AnExILs diluted 4 times, compared to 70% of DiD<sup>+</sup> CD1a<sup>+</sup> DCs (Supplementary Material Fig. 3b, 4). Also, a higher MFI was detected for the CD14<sup>+</sup> population than for the CD1a<sup>+</sup> (Fig. 6b). Concerning the maturation of the DCs, the level of CD83 and CD86 was similar between cells injected with medium, AnExILs, liposomes or IVTT mix (Supplementary Material Fig. 5). In conclusion, even under near physiological conditions of human skin biopsies, AnExILs can be efficiently taken up by skin resident DCs *in situ*. As such, AnExILs show promise as a vaccine delivery system for intradermal administration.

**Fig. 6** Cell association assay in a human skin model assay. Biopsies were injected with medium, AnExIL, IVTT or lipo formulations and were incubated for 2 days. Skin-emigrated DCs were collected in the supernatant and the MFI of DiD was evaluated for the global DC population **(a)** or separately for the two main subsets of human dermal DCs, CD1a<sup>+</sup> and CD14<sup>+</sup> DCs **(b)** to evaluate the liposomal uptake efficiency. *N* = 2, \**P* < 0.05, \*\**P* < 0.01 and \*\*\**P* < 0.001 versus AnExIL formulation.





## DISCUSSION

In this study, the effects of Antigen-Expressing Immunostimulatory Liposomes on different DC models were characterized. The two main objectives of this investigation were: i) to analyze the contribution of the two major constituents of the AnExIL formulation (IVTT and liposomes) to its adjuvanticity, ii) to evaluate the effect of AnExILs on human DC in culture and in human skin explants as a first step towards human use. To understand the influence of the liposome component on the adjuvanticity and antigen uptake efficiency of the AnExIL formulation, we compared AnExIL with IVTT, in order to have the same concentration of IVTT in both formulations. Therefore, we did not include in this study any of the washed or purified AnExIL formulations that were previously tested in our *in vivo* experiments (12,13). We eventually plan to use AnExILs as personalized cancer vaccine.

A 3D migration assay demonstrated a chemoattractant effect of AnExILs on murine DCs. This chemotaxis-induced recruitment of DCs to the vaccine formulation can be considered as an alternative approach in comparison to ligand-targeted vaccine formulations. The latter relies on the use of APC-specific targeting ligands attached to the vaccine delivery system to stimulate vaccine uptake by these cells. However, this will only be effective if the targeted vaccine formulation passively encounter the DC, which is hampered by the limited diffusion capacity of some vaccine formulations at the site of injection. Our system is therefore anticipated to be more effective in delivering the antigens than non-adjuvanted, actively targeted vaccine formulations. DC migration induced by AnExILs was further investigated by repeating the experiment with the two main components of AnExIL formulation separately: the IVTT mix and the plain liposomes. Our results demonstrate that the AnExIL ability to attract DCs is most probably mediated by the presence of the bacterial components from the IVTT mix in the formulation.

Chemotaxis should lead to efficient uptake of the vaccine formulations. A cell association assay could indeed demonstrate that AnExIL particles are taken up efficiently by D1 cells, and a dose-dependent effect was observed. Notably, AnExILs were taken up more efficiently than plain liposomes by murine DCs. Again, this could be attributed to the presence of IVTT mix in AnExIL formulation. But interestingly, adding IVTT to plain liposomes resuspended in PBS does not allow any increase of the liposomal uptake. This result shows that the IVTT mix present outside the liposomes is not enough to induce efficient liposomal uptake, but that the IVTT mix should be encapsulated in the liposomes in order to stimulate cellular uptake. Slow release of bacterial chemokines from the AnExIL formulations may have contributed to the establishment of a chemokine gradient required for chemotaxis, which in turn resulted in better uptake of AnExILs compared to plain liposomes.

Since the AnExIL particles are better taken up by DCs than plain liposomes, they represent a more efficient delivery system for antigen. The antigen uptake was then evaluated, and the AnExIL formulation indeed led to higher cell-associated fluorescein fluorescence than the control  $\beta$ Gal lipo formulation, at equivalent quantity of  $\beta$ Gal incubated. Also,  $\beta$ Gal uptake was higher for AnExILs than for IVTT mix alone, demonstrating that free antigens are less efficiently taken up by DCs than when formulated in AnExILs. Even if only part of the antigen production is encapsulated inside the particles, this result highlights the fact that liposomes are necessary components of the AnExIL formulation. It could be hypothesized that part of the antigen is adsorbed at the surface of the liposomes and also benefits of a better uptake. Liposomes are then crucial not only for establishing a chemokine gradient but also for favoring antigen uptake by DCs. Notably, other teams have demonstrated that the cross-presentation of nanoparticle-encapsulated antigens is much more efficient than for soluble antigens (18,19). Even if not studied here, this is another important criterion to justify the presence of liposome in the AnExIL formulation, since it would increase its efficiency in case of cancer vaccine application. Altogether, both the delivery system and the IVTT adjuvanticity are required for an efficient uptake of AnExIL particles by DCs.

AnExILs could also induce maturation of the murine DCs, and this induction was similar to the one obtained with IVTT. On the contrary, no increase in CD86 or CD40 levels could be observed with plain liposomes. The IVTT mix is thus responsible for the maturation of DCs in presence of AnExILs. In summary, the chemoattractant effect of AnExILs, their efficient uptake by DCs and the DC maturation are all intimately linked to the presence of IVTT mix in the formulation. Indeed, this IVTT mix is prepared from a *E.coli* bacterial extract and therefore contains pathogen-associated molecular patterns (PAMPs) like lipopolysaccharide (LPS) that will provide danger signals and activate the DCs through different toll-like receptors (TLR) pathways (20). The DC chemotaxis induced by IVTT could also be explained by the presence of formylated peptides in the mixture, such as N-formylmethionyl-leucyl-phenylalanine (fMLP), which are known to be the most potent bacterial chemoattractants for immature DCs (21). IVTT mix is then not only necessary for the antigen synthesis *in vitro* but also acts as a powerful adjuvant. However, for further vaccine development, the presence of a bacterial extract with high level of endotoxins could lead to problems of reactogenicity. To prevent endotoxin-related toxicities, different strategies can be applied, like removing LPS from the formulation (22), or using other sources of IVTT mixes, like extracts prepared from probiotic bacteria such as *Lactobacillus* sp., rabbit reticulocytes or wheat germ (23,24). Alternatively, the fully characterized PURExpress system which is based on a mixture of recombinant factors

needed for protein expression and devoid of endotoxins or other potentially reactogenic compounds could be used (25). Special attention should be paid to the protein production yield and the type of immune responses generated. Nevertheless, it is important to note that a number of commercialized vaccines against bacteria-induced diseases like tuberculosis or cholera are based on live-attenuated or killed bacteria (26) and that many other formulations based on bacterial extracts or attenuated recombinant bacteria have been used safely in clinical trials (27–29). Similarly, a recent clinical trial with mixed bacterial vaccine demonstrated that high endotoxin levels are well tolerated after s.c. injection as long as the dose was slowly increased (30). As a result, safe AnExIL administration in patients could also be conceivable and we consider using this formulation as a cancer vaccine where LPS could act as an important component to overcome tumor immune suppression.

Altogether, AnExILs induce better DC chemotaxis, DC maturation and liposomal and antigen uptake than conventional liposomal vaccine formulations. Since DC activation is a very important first step towards activation of adaptive immune responses, these findings may explain the higher levels of antibody titers that were reached with AnExIL formulations compared to liposomal formulations after i.m. vaccination in mice (at equal antigen doses) in our previous study (12).

To get a first insight whether AnExIL formulation could also work in humans, the immunostimulatory effect of AnExILs was then evaluated on human Mo-DCs. The results obtained are similar to the ones observed with murine DCs, which is a promising outcome giving hope for potential efficiency of AnExIL in humans. Finally, to get even closer to the *in vivo* situation in humans, an assay was performed with a human skin explant model. By intra-dermally injecting AnExILs in human skin flaps and subsequently culturing the skin biopsies, the study of human cutaneous DCs and their functions in their natural complex tissue environment was possible. Similar uptake results were again obtained with this model. Concerning the maturation state, no significant increase of CD86 or CD83 expression could be detected. However, it is important to note that the injection of medium supplemented with GM-CSF and IL-4 already leads to DC maturation (17). The skin-emigrated DCs were then stained to differentiate the two main subsets of human dermal DCs, namely the CD1a<sup>+</sup> and CD14<sup>+</sup> DCs. Both populations could take up the particles, with CD14<sup>+</sup> DCs being more efficient. This can be due to the fact that the CD14<sup>+</sup> DCs are in a more immature state than the CD1a<sup>+</sup> DCs, and have then a greater ability for antigen capture (31,32). Interestingly, these two subpopulations have distinct roles: CD14<sup>+</sup> DCs have been described to be able to prime humoral immunity, by priming CD4<sup>+</sup> T cells into cells that induce naïve B cells to switch isotype and become plasma cells, whereas CD1a<sup>+</sup> DC is more linked to the cellular immunity and can activate CD8<sup>+</sup> T cells

(33). We can then expect both sides of immunity to be primed by injecting AnExILs to human patients. This first result in a skin model is then very promising.

## CONCLUSION

AnExILs can induce DC chemotaxis, DC maturation and efficient uptake of both carrier and encapsulated antigen. Both IVTT mix and liposomes were key components of the AnExIL formulation responsible for these effects, with IVTT mix playing an important adjuvant role and the liposomes contributing to the uptake efficiency. Despite of its complexity, there is now deep evidence that the AnExIL formulation should be kept intact. These results were established both in murine and human DCs, which is promising for potential applicability of this formulation in humans for specific indications like cancer vaccine.

## ACKNOWLEDGMENTS AND DISCLOSURES

We thank Dr Maryam Amidi for our fruitful discussions. We thank Vincent Atteveld for having performed the LAL assay. This work was financially supported by the Faculty of Science of Utrecht University.

## REFERENCES

1. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1998;392(6673):245–52.
2. Gallucci S, Matzinger P. Danger signals: SOS to the immune system. *Curr Opin Immunol*. 2001;13(1):114–9.
3. Peek LJ, Middaugh CR, Berkland C. Nanotechnology in vaccine delivery. *Adv Drug Deliv Rev*. 2008;60(8):915–28.
4. Guy B. The perfect mix: recent progress in adjuvant research. *Nat Rev Microbiol*. 2007;5(7):505–17.
5. Buonaguro L, Tagliamonte M, Tornesello ML, Buonaguro FM. Developments in virus-like particle-based vaccines for infectious diseases and cancer. *Expert Rev Vaccines*. 2011;10(11):1569–83.
6. Caputo A, Sparnacci K, Ensoli B, Tondelli L. Functional polymeric nano/microparticles for surface adsorption and delivery of protein and DNA vaccines. *Curr Drug Deliv*. 2008;5(4):230–42.
7. Henriksen-Lacey M, Korsholm KS, Andersen P, Perrie Y, Christensen D. Liposomal vaccine delivery systems. *Expert Opin Drug Deliv*. 2011;8(4):505–19.
8. Nandedkar TD. Nanovaccines: recent developments in vaccination. *J Biosci*. 2009;34(6):995–1003.
9. Saade F, Petrovsky N. Technologies for enhanced efficacy of DNA vaccines. *Expert Rev Vaccines*. 2012;11(2):189–209.
10. Stevenson FK, Mander A, Chudley L, Ottensmeier CH. DNA fusion vaccines enter the clinic. *Cancer Immunol Immunother*. 2011;60(8):1147–51.
11. Amidi M, de Raad M, de Graauw H, van Ditmarsch D, Hennink WE, Crommelin DJ, *et al*. Optimization and quantification of protein synthesis inside liposomes. *J Liposome Res*. 2010;20(1):73–83.
12. Amidi M, de Raad M, Crommelin DJ, Hennink WE, Mastrobattista E. Antigen-expressing immunostimulatory liposomes as a genetically

- programmable synthetic vaccine. *Syst Synth Biol*. 2011;5(1–2):21–31.
13. Amidi M, van Helden MJ, Tabataei NR, de Goede AL, Schouten M, de Bot V, *et al*. Induction of humoral and cellular immune responses by antigen-expressing immunostimulatory liposomes. *J Control Release*. 2012;164(3):323–30.
  14. Mastrobattista E, Taly V, Chanudet E, Treacy P, Kelly BT, Griffiths AD. High-throughput screening of enzyme libraries: *in vitro* evolution of a beta-galactosidase by fluorescence-activated sorting of double emulsions. *Chem Biol*. 2005;12(12):1291–300.
  15. Winzler C, Rovere P, Rescigno M, Granucci F, Penna G, Adorini L, *et al*. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J Exp Med*. 1997;185(2):317–28.
  16. Kirby C, Gregoriadis G. Dehydration-rehydration vesicles: a simple method for high yield drug entrapment in liposomes. *Nat Biotechnol*. 1984;2:979–84.
  17. de Gruijl TD, Luyckx-de Bakker SA, Tillman BW, van den Eertwegh AJ, Buter J, Loughheed SM, *et al*. Prolonged maturation and enhanced transduction of dendritic cells migrated from human skin explants after *in situ* delivery of CD40-targeted adenoviral vectors. *J Immunol*. 2002;169(9):5322–31.
  18. Waeckerle-Men Y, Groettrup M. PLGA microspheres for improved antigen delivery to dendritic cells as cellular vaccines. *Adv Drug Deliv Rev*. 2005;57(3):475–82.
  19. Belizaire R, Unanue ER. Targeting proteins to distinct subcellular compartments reveals unique requirements for MHC class I and II presentation. *Proc Natl Acad Sci U S A*. 2009;106(41):17463–8.
  20. Diebold SS. Activation of dendritic cells by toll-like receptors and C-type lectins. *Handb Exp Pharmacol*. 2009;188:3–30.
  21. Yang D, Chen Q, Stoll S, Chen X, Howard OM, Oppenheim JJ. Differential regulation of responsiveness to fMLP and C5a upon dendritic cell maturation: correlation with receptor expression. *J Immunol*. 2000;165(5):2694–702.
  22. Magalhaes PO, Lopes AM, Mazzola PG, Rangel-Yagui C, Penna TC, Pessoa Jr A. Methods of endotoxin removal from biological preparations: a review. *J Pharm Pharm Sci*. 2007;10(3):388–404.
  23. Takai K, Sawasaki T, Endo Y. The wheat-germ cell-free expression system. *Curr Pharm Biotechnol*. 2010;11(3):272–8.
  24. He M. Cell-free protein synthesis: applications in proteomics and biotechnology. *N Biotechnol*. 2008;25(2–3):126–32.
  25. Shimizu Y, Inoue A, Tomari Y, Suzuki T, Yokogawa T, Nishikawa K, *et al*. Cell-free translation reconstituted with purified components. *Nat Biotechnol*. 2001;19(8):751–5.
  26. Plotkin SA. Vaccines: past, present and future. *Nat Med*. 2005;11(4 Suppl):S5–S11.
  27. Berstad AK, Oftung F, Korsvold GE, Haugen IL, Froholm LO, Holst J, *et al*. Induction of antigen-specific T cell responses in human volunteers after intranasal immunization with a whole-cell pertussis vaccine. *Vaccine*. 2000;18(22):2323–30.
  28. Cripps AW, Peek K, Dunkley M, Vento K, Marjason JK, McIntyre ME, *et al*. Safety and immunogenicity of an oral inactivated whole-cell *Pseudomonas aeruginosa* vaccine administered to healthy human subjects. *Infect Immun*. 2006;74(2):968–74.
  29. Le DT, Brockstedt DG, Nir-Paz R, Hampl J, Mathur S, Nemunaitis J, *et al*. A live-attenuated *Listeria* vaccine (ANZ-100) and a live-attenuated *Listeria* vaccine expressing mesothelin (CRS-207) for advanced cancers: phase I studies of safety and immune induction. *Clin Cancer Res*. 2012;18(3):858–68.
  30. Karbach J, Neumann A, Brand K, Wahle C, Siegel E, Macurer M, *et al*. Phase I clinical trial of mixed bacterial vaccine (Coley's Toxins) in patients with NY-ESO-1 expressing cancers: immunological effects and clinical activity. *Clin Cancer Res*. 2012;18(19):5449–59.
  31. Angel CE, Lala A, Chen CJ, Edgar SG, Ostrovsky LL, Dunbar PR. CD14<sup>+</sup> antigen-presenting cells in human dermis are less mature than their CD14<sup>+</sup> counterparts. *Int Immunol*. 2007;19(11):1271–9.
  32. de Gruijl TD, Sombroek CC, Loughheed SM, Oosterhoff D, Buter J, van den Eertwegh AJ, *et al*. A postmigrational switch among skin-derived dendritic cells to a macrophage-like phenotype is predetermined by the intracutaneous cytokine balance. *J Immunol*. 2006;176(12):7232–42.
  33. Klechevsky E, Morita R, Liu M, Cao Y, Coquery S, Thompson-Snipes L, *et al*. Functional specializations of human epidermal Langerhans cells and CD14<sup>+</sup> dermal dendritic cells. *Immunity*. 2008;29(3):497–510.