SUPPORTING INFORMATION

A Liposomal Platform for Delivery of a Protein Antigen to Langerin-Expressing Cells

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MATERIAL AND METHODS

Lipid conjugation

Ligands and dyes were coupled to PEG-DSPE by NHS conjugation. Alexa Fluor 647 NHS Ester (A647, life technologies) and pHrodo red NHS Ester (pHrodo, life technologies) was conjugated to the primary amine of NH2-PEG-DSPE (PEG MW 2000, Sunbright) via amide coupling. The lipid (1.024 mg) dissolved in 500 µl DMSO was stirred in a pear shaped flask, and 1 mg dye (1.5 equiv.) dissolved in 500 µl DMSO was added dropwise to the lipid. The reaction was stirred overnight in the dark at room temperature. DMSO was freeze dried (Alpha 2-4 LDplus, CHRIST) and the reaction product was dissolved in 2-3 ml buffer containing 0.1 M sodium bicarbonate (Sigma-Aldrich) at pH 8.4. Unconjugated dye was removed by dialysis with a Slide-A-Lyzer cassette (7MWCO, 0.5-3 ml, Thermo Fisher Scientific) first against 500 ml buffer for several hours with two times of buffer exchange as well as an overnight incubation and then three more times dialyzed against water with at least 1 h of dialysis under permanent stirring. Water was removed by freeze drying and the final product was dissolved in DMSO at a concentration of 8 mg/ml. The glycomimetic Langerin ligand contained a terminal primary amine and was coupled to NHS-PE-G-DSPE (PEG MW 2000, NOF Europe) via amide coupling equally as described for A647 conjugation except that 2 mg ligands were dissolved in 900 µl buffer and 0.125 equivalent lipids were dissolved in 100 µl DMF. Lipids were added dropwise in a pear shape flask and DMF was removed in vacuo (Heidolph). The final product was dissolved in DMSO-d6 (Euriso-Top) and the conjugation efficiency was determined by 1H-proton NMR spectroscopy with 265 scans (400 MHz, Variant).

Liposome formulation

Targeted and naked PEGylated liposomes were prepared by hydration film extrusion method as described before (1). Briefly, liposomes contained DSPC: cholesterol: ligand-PEG-DSPE/PEG-DSPE: dye-PEG-DSPE at a mole ratio of 57: 38: 4.75: 0.25. In case of liposomes containing both pHrodo red (Life technologies) and A647, 0.25mol% pHrodo- and 4.5mol% ligand-PEG-DSPE was added. PEGylated lipids were added to a glass tube and DMSO was freeze dried. Next, DSPC and cholesterol were added and Chloroform was removed in vacuo overnight. The dry lipid film was hydrated with DPBS (w/o calcium and magnesium; life technologies) at a concentration of 1.6 mM if not stated otherwise. Lipid containing solution was first vortexed and then sonicated (Ultrabath 1510, Branson) for 3 sec with three repetitions and a short time lag in between. This step was repeated until a homogenous suspension was obtained. Large unilamellar liposomes were produced by pore extrusion (extruder, Avanti Polar Lipids) with 30 strokes first with a polycarbonate membrane of 200 nm and then of 100 nm (Avanti Polar Lipids). Liposome concentration refers to total lipid concentration.

Liposome loading and purification

Liposomes were loaded with antigens by hydration of the thin film lipid with DPBS containing 5 mg/ml FITC labeled bovine serum albumin (BSA) protein, unless stated otherwise. For Calcein encapsulation, hydration was performed with 300 mM Calcein (Sigma) in 1 M NaOH. Following sonication and pore extrusion, liposomes were purified by ultracentrifugation, unless stated otherwise. To remove free protein or dye by ultracentrifugation, liposomal suspension was transferred into ultracentrifugation tube (Thinwall, Ultra-Clear™, 4 mL, Beckman coulter). The tube was filled up with DPBS to prevent implosion. Liposomes were ultracentrifuged at 60000 rpm for 1 h at 4°C with a SW 60 Ti rotor (Beckman coulter) in an ultracentrifuge (Optima L-80 XP Ultracentrifuge, Beckman Coulter). The supernatant was removed and the pellet was resuspended in DPBS. Ultracentrifugation was repeated twice. Purification by size exclusion was performed with 20 ml sepharose CL gel filtration media (CL-4B, cross-linked, Sigma-Aldrich) that was packed into a chromatography column.
(Econo-column, 1.5x30cm, BioRad). The column was equilibrated with DPBS before liposome solution was loaded to the column. Fractions of 1.5 ml were collected until liposomes and free antigens eluted (10-15 fractions). After fractionation, the column was regenerated with regeneration buffer containing 0.5 M NaCl in 0.1 M NaOH, equilibrated with DPBS until the pH was neutral and the next liposomal solution was separated by size exclusion. To remove free antigens by dialysis, liposomal solution was transferred into a 300 kDa dialysis tube (Spectrum laboratories). Liposomes were dialyzed against PBS (0.137 M NaCl, 2.7 mM KCl, 1.8 mM KH2PO4, 10 mM Na2HPO4) for three days with ten buffer exchanges. After ultracentrifugation, size exclusion or dialysis, liposome concentrations and encapsulation efficiencies were analyzed with a plate reader (SpectraMax M5, Molecular Devices) and calculated on the basis of standard curves. Liposome concentration was measured via Alexa 647 decorated liposomes (ex. 640, em. 670) and antigen concentration via FITC-conjugated BSA (ex. 485, em. 525). Encapsulation efficiencies were calculated per 1 mM liposome.

**Liposome characterization**

Liposomal dispersity and stability were determined to assess liposome quality. Size distributions and zeta potentials were measured by dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern). Liposomes at a concentration of 16 µM diluted in H2O were analyzed in a 2.5 ml disposable UV cuvette (semi-micro cuvettes, NeoLab). Liposomes were then transferred into a disposable, folded capillary zeta cell (DTS1070, Malvern) and the zeta potential was detected. All liposomes were in a size range of 130 to 200 nm and had a zeta potential between approximately -20 to -30 mV.

**Analysis of pH-sensitivity of pHrodo red-labelled liposomes**

The fluorescence intensity of 0.25mol% pHrodo red-labelled liposomes was analyzed in DPBS (w/o calcium and magnesium; life technologies) at different pH values. Therefore, 50 µL of the liposomal suspension were added to 150 µL of DPBS. Relative fluorescence units were measured at wavelengths of λex = 560 nm and λem = 585 nm (SpectraMax M5, Molecular Devices).

**Calcium- and pH-sensitivity of calcein and the effect of self-quenching**

The fluorescence intensity of 30 µM calcein was analyzed in DPBS (w/o calcium and magnesium; life technologies) at different calcium concentrations and pH values respectively. The quenching effect was analyzed by measuring the calcein fluorescence intensity at different calcein concentrations. Relative fluorescence units were measured at wavelengths of λex = 495 nm and λem = 515 nm (SpectraMax M5, Molecular Devices).

**Cell culture**

If not stated otherwise, all media and supplements were purchased from Thermo Fisher Scientific. The human Raji cell line (a gift from Dr. van Sorge, University Medical Center Utrecht, Netherlands) from hematopoietic origin was grown in complete growth medium containing RPMI base medium, 10% FCS, 100 U ml-1 Penicillin-Streptomycin and GlutaMax. Cells were maintained between 0.5 and 3 Mio cells ml-1 by addition or replacement of complete growth medium. Cells were cultured in 10 cm or 15 cm petri dishes (corning) in 10 ml or 20 ml culture medium, respectively. Cells were monitored with a light microscope (IT40 5PH, VWR) and grown under controlled conditions at 37°C and 5% CO2. For cell splitting, cells were centrifuged at 500g for 3 min (Heraeus Megafuge 8R, Thermo Fisher Scientific). The supernatant was aspirated and cells were resuspended in fresh growth medium. Recombinantly expressing cell lines were generated as described before (1). Briefly, human Langerin cDNAs (Sinobiologicals) were cloned into a lentiviral BIC-PGK-Zeo-T2a-mAmetrine:EF1A construct by Gibson assembly (NEB) according to the manufacturer’s protocol. Hek293 cells were transfected
with the lentiviral vector together with third-generation packaging vectors and viral particles were then used for transduction of Raji cell lines.

**Cytotoxicity assay**

Cytotoxicity of various inhibitors on human Langerin expressing Hek293 cells was quantified via propidium iodide staining (PI, Sigma). 50000 cells were plated in 100 µl complete growth medium. Mannan (Sigma), Curdlan (Sigma) and Laminarin (Sigma) were added to a final concentration of 50 µg/mL. Serial dilutions of 1:2 of Bafilomycin A1 (Alfa Aesar) and Cytochalasin D (Enzo Life Sciences) were added to a maximum final concentration of 1.6 and 5 mM. After 24 h incubation, cells were washed with DPBS (life technologies), trypsinized and stained with 1 µg/mL PI in DPBS for 15 min at room temperature. PI fluorescence was detected in flow cytometry with a 488 nm laser and a 695/40 nm filter (Attune Nxt, life technologies).

**Cellular liposome internalization assay**

Unless otherwise described, 50000 cells were plated in 100 µl complete growth medium containing 16 µM liposomes. After incubation at 37°C, cells were centrifuged at 500 g for 3 min and the supernatant was discarded. Cells were resuspended in 100 µl ice cold culture medium and analyzed by detecting the co-formulated A647 dye via flow cytometry with a 654 laser and a 670/14 filter (Attune Nxt, life technologies). Encapsulated FITC labeled BSA was measured with a 488 laser and a 574/26 filter (Attune Nxt, life technologies).

**Confocal fluorescence microscopy**

For microscopy, cells were cultured on coverslips (Carl Roth) in 24 culture well plates (Corning). Before cell seeding, coverslips were coated with 500 µl poly-L-Lysine (poly-L-lysine solution, 0.01%, mol wt 70,000-150,000, sterile-filtered, cell culture tested; Sigma Aldrich) by overnight incubation. After washing the coverslips with DPBS, 200000 WT or Langerin+ Hek293 cells were seeded in 500 µl culture medium. The 24 well plate was incubated overnight at 37°C and 5% CO₂. Next day, 16 µM liposomes were added at 37°C. After liposome incubation, cells were fixed with 4% paraformaldehyde (Roti-Histofix, Carl Roth) for 10 min, and the cell membrane was stained with 10 µM DiO (Thermo Fisher Scientific) for 15 min. To stain the nucleus, cells were first permeabilized with 0.1% saponin (Sigma Aldrich) for 10 min and subsequently stained with 1 µg/ml DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride, Sigma Aldrich) for 5 min. For long-term storage, coverslips were fixed on microscope slides (Carl Roth) using a mounting solution (Carl Roth). Slides were analyzed by microscopy (confocal light-sheet microscope DLS-DMi8, Leica).

**Immunofluorescence microscopy to stain endosomal markers**

Human Langerin+ or WT Hek293 cells were plated as described above. Cells were incubated with 16 µM liposomes for 2 h at 37°C. Cells were subsequently fixed with acetone containing 2 mM CaCl₂ for 20 min at 4°C. After washing, primary antibodies were diluted in HBSS buffer containing 1% BSA and 2mM CaCl₂. EEA1, Rab7 and Rab11 were diluted 1:100; Rab5 and Lamp1 were diluted 1:200 (Endosomal marker sample kit, Cell Signaling). Primary antibodies were incubated at 4°C overnight. Cells were washed and an Alexa488 conjugated anti-rabbit IgG secondary antibody (Cell Signaling) was incubated overnight in the dark at 4°C. Finally, the nucleus was stained with 1 µg/ml DAPI for 5 min and the coverslips were mounted on microscopy slides. Slides were analyzed by microscopy (confocal light-sheet microscope DLS-DMi8, Leica).
Life cell microscopy to trace liposomal uptake

20000 human Langerin+ Hek293 cells were cultured in 35 mm glass bottom dishes (ibidi) in 500 μl complete growth medium overnight. The cell culture medium was replaced by freshly prepared medium containing 0.5% (v/v) Hoechst 33342 (Biomol) and 16 uM of targeting liposomes conjugated with pHrodo red and A647. Samples were placed in 5% CO2 conditioned live cell imaging incubator installed on the stage of inverted confocal microscope (DLS-DMi8, Leica). The time-lapse imaging speed was one frame per minute.

Image processing

The confocal microscopy images were processed by ImageJ (NIH) and custom Python code. The peak fluorescence intensity values of liposome were identified by applying local maximum finding algorithm (SciPy) on the averaged and Gaussian filtered raw images. To distinguish between single and multiple liposome, the sum of the mean integrated fluorescence intensity value of A647 and its standard error of the mean during the initial stage of uptake is used as the threshold value. Therefore, if one liposome has lower integrated value than the threshold, then it is estimated as a single liposome. The integrated liposome fluorescence intensity was calculated by integrating the fluorescence intensity value over estimated individual liposome circular region.

Kinetics of pHrodo containing liposomes

Black 384 well glass bottom plates (Greiner) were coated with Poly L-Lysine (Sigma) overnight. 25000 Hek hLangerin cells were cultured in 100 μl complete growth medium with 25 mM HEPES (Carl Roth) overnight. Old medium was replaced by fresh containing 6.25 nM Bafilomycin or 100 nM Cytochalasin D. Targeted liposomes co-formulated with pHrodo red conjugated lipids were added to a final concentration of 16 μM. Cells were incubated at 37°C and images were taken hourly with an RFP filter at 10-fold magnification (Cytation 5, BioTek). Image analysis was performed with Matlab R 2016A.

Fluorescein labeling of bovine serum albumin

20 mg BSA (VWR Chemicals) was diluted in 9 ml DPBS (life technologies) and transferred into a 50 ml glass flask with septum and stir bar. Fluorescein isothiocyanate (Thermo Fisher Scientific) was dissolved in DMSO (1 mg/ml) and 1 ml were added slowly to BSA. The flask was covered in aluminium foil and stirred overnight. The reaction was quenched by adding a final concentration of 50 mM ethanolamine (Sigma-Aldrich) and stirred for 1 hour at room temperature. Unconjugated fluorescein was removed by a Slide-A-Lyzer cassette (7MWCO, 0.5-3 ml, Thermo Fisher Scientific) first against 500 ml buffer containing 0.1 M sodium bicarbonate (Sigma-Aldrich) at pH 8.4 for several hours with two times of buffer exchange as well as three more times dialyzed against water. The concentration of FITC conjugated BSA was measured with absorbance at 280 nm and the conjugation efficiency was determined at 495 nm with a nanodrop (Implen Nanophotometer).

Kinetics of liposomal cargo release

50000 Raji hLangerin cells were incubated with 16 μM Calcein encapsulated liposomes in full growth medium for 1 h at 4°C. After extensive washing, cells were transferred to 37°C for the indicated time-points. Calcein fluorescence was detected via flow cytometry with a 488 nm laser and a 695/40 nm filter. Co-formulated A647 was detected with a 638 nm laser and 670/14 filter (Attune Nxt, life technologies).
Epidermal suspension cells

Epidermal suspension cells were prepared as described before (1). Briefly, skin samples from healthy donors (after informed consent and in regard to the local ethics committee (AN 5003 360/5.22 of 15/04/2016)) were removed from subcutaneous fat with a scalpel. Skin parts were incubated in RPMI1640 medium (Lonza) supplemented with 1.5 U/ml dispaseII (Roche) and 0.1% trypsin (Sigma-Aldrich) overnight at 4°C. Peeled off epidermis was filtered through a 100 µm cell strainer (Thermo Fisher Scientific) to obtain single cell suspensions. Cell suspensions were incubated with 16 µM liposomes in RPMI1640 medium containing 10% FCS for 2 h at 37 °C. Before analyzing cells by flow cytometry (Attune Nxt, life technologies), cells were stained with an eFluor® 780 viability dye (eBioscience) and fluorochrome-conjugated antibodies (CD1a, clone: HI149; CD14, clone: HCD14; HLA-DR, clone: L243; CD45, clone: HI30 - Biolegend; Langerin, clone: MB22-9F5 - Miltenyi Biotec; isotype-matched control antibodies) for 30 min at 4°C.
SUPPLEMENTARY FIGURES

Figure S1. Control liposomes containing either A647 or pHrodo red. Human Langerin expressing Hek293 cells were incubated with 16 µM targeted liposomes co-formulated with A647 or pHrodo red for 4 h at 37°C. The cell nucleus was stained with Hoechst 33342 and cells were analyzed by microscopy.

Figure S2. Fluorescence intensity of (A) A647 and (B) pHrodo was monitored over 4h in live cell imaging. The error bars represent the standard error of the mean of triplicates from one experiment.
Figure S3. Liposome routing in human Langerin expressing Hek293 cells. Human Langerin expressing Hek293 cells were incubated with 16 µM targeted liposomes for 2 h at 37°C. After incubation, cells were immune-fluorescently stained with endosomal markers, including Rab5, Rab7, Rab11, EEA1 and Lamp-1. Primary antibodies were then labeled with an Alexa488 conjugated secondary antibody. The cell nucleus was stained with DAPI and cells were analyzed by microscopy.

Figure S4. Cytotoxic effect of inhibitors over 24 h. Hek hLangerin cells were cultured with (A) Bafilomycin or (B) Cytochalasin D for 24 h. Cytotoxicity was evaluated in flow cytometry via propidium iodide (PI) staining. Green: concentrations selected for kinetic assay.
Figure S5. Quenching effect of calcein. (A) Liposomal integrity at different pHs was analyzed by measuring size and dispersity (Poly-dispersity-Index) by DLS. (B) Calcein encapsulated liposomes were dissolved by addition of 50% DMSO. The effect of calcein release was analyzed by measuring the fluorescence in a plate reader. (A-C) Fluorescence of calcein was measured at different calcein concentrations (C), at different pH values (B) and different calcium concentrations (E) in a plate reader. (F) Self-quenching effect of calcein encapsulated liposomes. Raji hLangerin cells were incubated with targeted liposomes co-formulated with A647 and encapsulated with calcein for 1 h at 4°C. Fluorescence of A647 and calcein were measured via flow cytometry following incubation at 37°C for indicated time points. The error bars represent the standard deviation of triplicates from one experiment.
Figure S6. FITC-BSA encapsulation into liposomes. (A) Schematic presentation of FITC-BSA encapsulated liposome. (B) Size exclusion, ultracentrifugation and dialysis with a cutoff of 300 kDa were tested to remove free antigen from encapsulated antigen. FITC-BSA fluorescence was measured with a plate reader. (C) Quality of liposomes was measured by DLS after different purification methods. Size and zeta potential (ZP) were analyzed. The error bars represent the standard deviation from the indicated number of independent experiments.

|                  | Size [nm] | Peak STD [nm] | PdI  | ZP [mV] |
|------------------|-----------|---------------|------|---------|
| Size exclusion   | 145.9 ± 2.0 | 40 ± 6        | 0.056 ± 0.021 | -29.0 ± 1.4 |
| Ultracentrifugation | 138.3 ± 3.8 | 58 ± 6        | 0.124 ± 0.025 | -28.9 ± 5.3 |
| Dialysis         | -         | -             | -    | -       |
Figure S7. Optimization of protein encapsulation with a test protein FITC-BSA. (A) The initial FITC-BSA concentration that was used to rehydrate the thin lipid film and (B) the initial liposome concentration of the rehydrated lipid film were varied to detect optimized encapsulation efficiencies. (C) Quality report, including size and zeta potential (ZP), were analyzed by DLS. The encapsulation efficiency was calculated after ultracentrifugation with a plate reader. Encapsulated FITC-BSA antigen (AG) was calculated per 1 mM liposome. The error bars represent the standard deviation of one representative experiment with triplicates.
REFERENCES

1. Wamhoff E-C, Schulze J, Bellmann L, Bachem G, Fuchsberger FF, et al. 2018. bioRxiv