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## **Towards the development of synthetic vaccines against tuberculosis**

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# 6

## Summary and prospects

# 6

The research described in this Thesis was aimed at designing and synthesizing nature-inspired compounds as part of TB vaccine discovery. A variety of synthetic analogues of mycobacterial cell wall components, from peptide and glycolipid antigens to glycolipid pathogen-associated molecular patterns (PAMPs) has been accessed. Evaluation of the immune stimulatory activity of the novel compounds in combination with preliminary immunization studies *in vivo*, suggested the potential of selected synthetic conjugates as single molecule vaccines against TB. Further research is needed to verify the efficacy of these vaccine modalities.

**Chapter 1** provides an overview of the interaction of *Mtb* with the human immune system, with emphasis on new TB vaccine strategies in preclinical and in clinical trials. The key players of the human immune system are introduced and a general overview of the tools available for the rational design of vaccines is provided to set the context for the research presented in this Thesis.

**Chapter 2** focuses on the design and synthesis of *Mtb* glycolipid CD1c binding molecules. Three stabilized mannosyl phosphomycoketide (MPM) analogues, comprising a carba-mannose and two C-mannosides, were generated and evaluated for their ability to bind to CD1c molecules and elicit a T cell response. Antigens presented by the CD1c system are of great value for vaccine discovery, given the non-polymorphic nature of the different CD1 (a-e) proteins across humans.<sup>1</sup> This research aimed at generating MPM analogues with improved stability as compared to the natural mycobacterial glycolipid by creating hydrolysis-resistant bonds between the carbohydrate and phospholipid moiety. As it is not possible to determine *a priori* how these modifications impact the binding to the CD1c protein and presentation to T cells, three different stabilized analogues were generated. One of the three MPM analogues, the difluoro-C-mannoside, was shown to be resistant to degradation in APCs and to be cross-reactive with the natural MPM, while the other two were shown to be less effective mimics. Further studies are needed to explore the function and potential of the stabilized difluoro antigen including immunization studies in CD1c-expressing species, such as transgenic mice expressing the human CD1c protein or guinea pigs that naturally express several CD1c genes.<sup>2</sup> Depending on the outcome of these studies, immunization experiments followed by infection with *Mtb* are envisioned to assess the potential of this antigen in prophylactic vaccines.

**Chapter 3** combines the design and synthesis of conjugation-ready *Mtb* glycolipid Mincle ligands with the synthesis and *in vitro/in vivo* immunological evaluation of a fully synthetic single molecule vaccine modality. Two trehalose dimycolate (TDM) and two glucose monomycolate (GMM) analogues are synthesized and their ability to interact with Mincle was assessed. A validated peptide antigen, derived from the *Mtb* Rv1733 latency protein-antigen, was chemically linked to the TDM analogues to generate two synthetic conjugates and the innate stimulatory activity of these compounds was assessed on human DCs, M1 and M2 cells. Finally, one conjugate was employed in a study to immunize HLA-DR3/Ab<sup>0</sup> mice, and the results of these studies indicated the potential of the conjugate to induce humoral and protective immunity against *Mtb*, interestingly and unexpectedly in the absence of detectable T cell responses. In a preliminary immunization study, it was shown that IgG1 and IgG2b antibody responses could be induced accompanied by a reduction of the bacterial load in the spleen of immunized mice. For the synthesis of the conjugates containing Mincle ligands, a maleimido-propionic acid was used to extend the oligopeptide at the N-terminus end and the generated peptide was conjugated to the

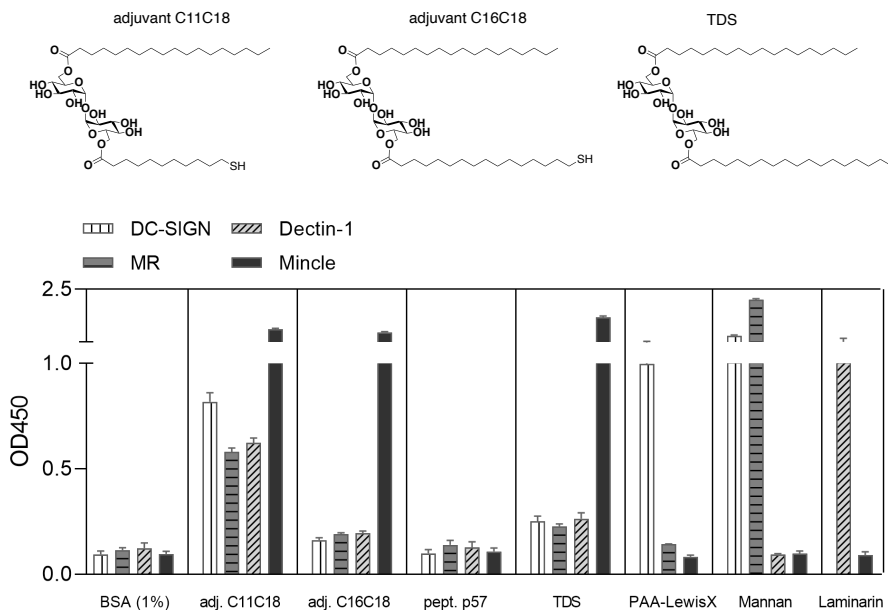
thiol-functionalized adjuvants in solution. This conjugation strategy required the design of conjugation-ready Mincle ligands which had not been described before. Two factors were considered in the design of the ligands: orthogonality of the ligation handle in anticipation of the extension of the single molecule strategy to include multiple PAMPs (in this case to include the TLR2 ligand described in Chapter 4); the position of the ligation handle and the definition of the length (and number) of lipid chains to prevent loss of binding to the receptor. The results from the HEK-murine Mincle revealed that functional binding to murine Mincle was retained for the two synthetic analogues generated. The length of the 6'-OH lipid chain influences the extent of the stimulatory activity of the two Mincle ligands on selected human antigen-presenting cells and further research is needed to determine the relationship between the lipid chain length and the biological activity. Although most studies have been focused on defining the biological function of trehalose glycolipids in relation to Mincle, there is recent evidence that the natural Mincle ligand, TDM, and its well-known analogue TDB are regulating macrophage response in a Mincle-dependent and Mincle-independent way.<sup>3-5</sup> Preliminary experiments performed using soluble human Mincle, DC-SIGN, Dectin-1 and Mannose receptor suggest that the trehalose derivatives used in this research can interact with human Mincle and other C-type lectin receptors (see Figure 1).

However, these results do not formally prove that the two Mincle ligands generated in this Chapter would interact with C-type lectin receptors in a cellular system nor that binding would be functional. To better understand the result of binding to these lectins, further studies could address the binding affinities of TDM analogues to a panel of relevant CLRs, and determine the influence of binding to these receptors on the immunological and functional effects of the different analogues on APCs.

A vaccine formulation containing the TDM analogue TDB has been previously studied and shown to polarize cellular immune responses towards a Th1/Th17 phenotype in a murine model.<sup>6</sup> In that study, TDB was used in combination with the surfactant dimethyldioctadecylammonium (DDA) forming the cationic liposomal CAF01 adjuvant system. However, there is no clear definition, nor consensus, how the formulation affects binding and activation of Mincle. No explanation can yet be provided to account for potential interspecies differences in Mincle. In the case of Mincle-cholesteryl stearate interaction, it has been hypothesized that multivalent presentation of the ligand may be necessary for binding to the human, but not murine receptor.<sup>7</sup>

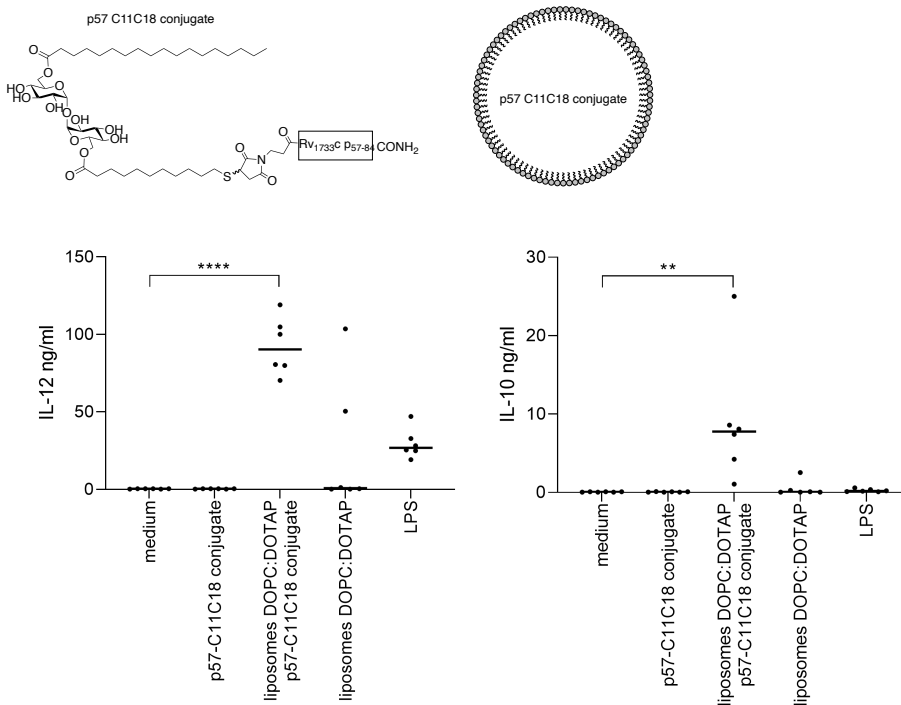
To compare the immune-stimulatory effects of the synthetic conjugate in solution to that of the conjugate incorporated in a cationic liposomal formulation, containing 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and N-1-(2,3-Dioleoyloxy)propyl-N,N,N-trimethylammonium methyl-sulfate (DOTAP) in a 4:1 ratio, the innate stimulatory activity was assessed on human DCs. The extent of cellular activation and expression of T cell co-stimulatory molecules on DCs was significantly increased

upon incorporation of the conjugate in the liposomal formulation (data not shown), and so was the amount of IL-12p40 and IL-10 released by the cells as shown in Figure 2. The evaluation of the vaccine potential of the cationic formulations containing the conjugate in a murine system is ongoing.



**Figure 1 - Binding of compounds to soluble forms of DC-SIGN, Dectin-1, Mannose receptor (MR), Mincle.** ELISA experiment was performed using plate-bound glycolipids (chemical structures shown above) and control compounds. TDS = Trehalose distearate (5 nmol/well), PAA-LewisX (10  $\mu\text{g/ml}$ ), Mannan (10  $\mu\text{g/ml}$ ) and Laminarin (3  $\mu\text{g/ml}$ ) and the reference peptide (5 nmol/well) were chosen as negative controls. The soluble human receptors carrying an Fc-portion were used for assaying binding interaction. Bars represent mean + SEM of duplicates from two independent experiments.

**Chapter 4** describes the synthesis and immunological evaluation of conjugates containing UPam, a known TLR2 ligand, and three antigenic *Mtb*-derived peptides. The ability of the constructs to bind to human TLR2 was demonstrated. The innate immune system stimulatory activity of these conjugates was assessed on DCs, M1 and M2 cells. One conjugate was tested in an antigen-presentation assay with the available T cell clone to verify that the peptide antigen presentation to CD4<sup>+</sup> T cells was not affected by conjugation to the TLR2 ligand. Finally, the *in vivo* vaccine potential of one conjugate was suggested by the ability of the synthetic compound to induce, after subcutaneous immunization of HLA-DR3/Ab<sup>0</sup> mice, a Th17 cellular and a highly diversified humoral immune response, together with protective immunity against *Mtb*.

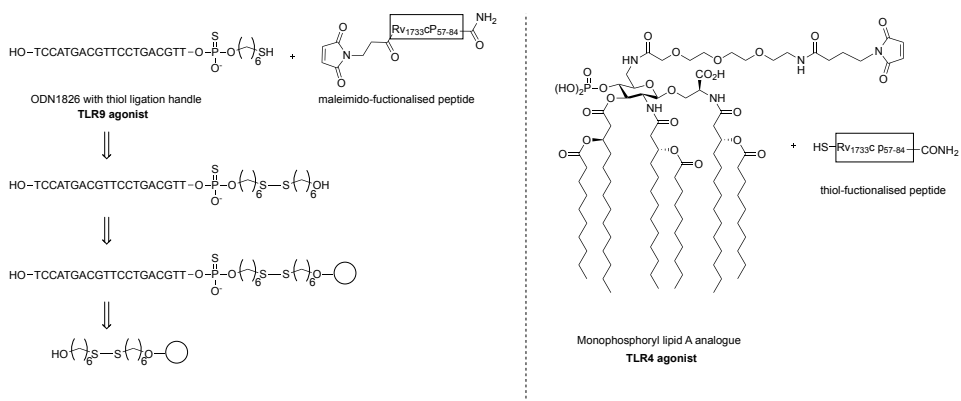


**Figure 2 - Cytokine production profile of human monocyte-derived dendritic cells stimulated with the solubilized p57-C11C18 conjugate or the same conjugate incorporated in a liposomal formulation containing the lipids DOPC:DOTAP in a 4:1 molar ratio.** IL-12p40 and IL-10 amounts present in supernatant were measured by ELISA for  $n = 6$  donors. Each dot corresponds to mean of two replicates from one donor; horizontal line corresponds to the grand median for each treatment group, as calculated using GraphPad Prism.  $p$  values are indicated in the graph above the relative couple of conditions and they are calculated using one-way ANOVA by GraphPad Prism (\*\*\*\* $p < 0.0001$ , \*\* $p < 0.01$ ). LPS (100 ng/ml) is used as a positive control.

A major asset of the use of peptide antigens is that they can be synthesized using automated solid phase synthesis without the need of intermediate purification steps. Final purification of the full-length peptide, usually carried out on an automated HPLC system, delivers a highly pure compound that is free of biological impurities. Chemical methods can be developed to extend the peptide with other molecular components in-line during the automated solid phase synthesis without removal of the peptide from the resin before conjugation. In view of the generation and immunological assessment of a single-molecule vaccine, UPam which is a synthetic lipopeptide analogue of Pam<sub>3</sub>Cys, was selected for conjugation to the three *Mtb*-derived peptides. This TLR2/1 agonist was built via peptide couplings directly on the growing oligopeptide at the N-terminal end. Despite the differences in

physicochemical properties of the three peptides, all synthetic conjugates retained the ability to activate HEK-293 cells via the human TLR2, albeit to a different extent. Overall, the work presented provided evidence for the ability of *Mtb*-derived peptide conjugates containing a TLR2/1 ligand to induce strong *in vitro* activation of human DCs and macrophages, key players in bridging the innate and adaptive immune response. The results further suggested that antigen-presentation by DCs and monocytes to human cloned CD4<sup>+</sup> T cells is neither increased nor decreased by stimulation with the conjugate as compared to stimulation with the reference peptide. Whether the activation of human APCs results in the induction of a protective humoral or cellular adaptive immune response requires further investigation.

The vaccine potential of one of the UPam-peptide conjugates described in this Thesis was preliminarily assessed using a murine model system, suggesting that a cellular Th17 immune response is initiated and that antigen-specific IgG antibodies are present in the sera as a result of immunization. This response corresponded to the induction of protective immunity against a live *Mtb* challenge in the same transgenic murine model. While differences in the murine and human immune system limits the generalizability of the results, this approach provides new insight in the efficacy of a single molecule vaccination strategy. The versatility and modularity that is intrinsic to peptide-conjugate vaccines represents a powerful tool to screen for different adjuvant-antigen couples. To expand this strategy to known synthetic PAMPs recognized by either TLR9 or TLR4, the synthetic strategies shown in Figure 3 were devised and partly implemented for the generation of two other synthetic conjugates.



**Figure 3 – Synthetic strategies devised for the generation of novel TLR9 ligand-peptide and TLR4 ligand-peptide conjugates.**



In summary, the 3'-disulfide modified CpG oligonucleotide was synthesized using an automated solid phase synthesizer, then purified using ion-exchange chromatography and treated with dithiothreitol to reduce the disulfide. A conjugation to the maleimide-functionalized peptide would lead to the generation of desired target ODN1826-peptide conjugate. The CpG oligonucleotide ODN1826, whose sequence is shown in Figure 3, has previously been shown to function as molecular adjuvant in combination with SLPs and induce protective responses against *Mtb*.<sup>8</sup> To target TLR4, a conjugate containing a peptide linked to a monophosphoryl lipid A (MPL) analogue, CRX-527, can be readily generated via a maleimide-thiol conjugation, as previously demonstrated for a different peptide.<sup>9</sup> MPL has been shown to possess strong adjuvanticity and diminished toxicity as compared to the naturally occurring TLR4 agonist, lipid A.<sup>10,11</sup>

**Chapter 5** provides insight in the effects of Mincle/TLR2 co-stimulation on murine and human dendritic cells. For this purpose, two synthetic adjuvants were generated, namely trehalose distearate, a Mincle agonist,<sup>12</sup> and Upam-SK<sub>4</sub>, a TLR2 agonist.<sup>13</sup> To improve the composition of current or novel vaccines, it is of relevance to understand possible synergistic/antagonistic effects of different PAMPs. With the overarching goal of eventually generating single molecule conjugate vaccines containing a peptide and two or more different adjuvants, explorative studies were performed to identify the innate stimulatory/inhibitory activity of the combination of different synthetic PAMPs. Although studies using murine macrophages suggest potential synergistic activation by TLR2 and Mincle stimulation,<sup>14</sup> co-stimulation of murine or human dendritic cells using well-defined agonists for these two receptors has not yet been explored. In murine dendritic cells, signaling via both receptors increases the expression of activation markers with respect to single TLR2 or Mincle activation, although to a limited extent. The studies performed on human monocyte-derived dendritic cells showed that an increase in production of IL-6 and TNF- $\alpha$  could be detected, but only at a specific concentration range. However, the absence of cytokine production when only Mincle was activated, points to possible synergistic activity of TDS with UPam. To better define the role played by the two ligands in the activation of human DCs and understand the interaction between the two receptors, mechanistic studies are required. Experiments using blocking antibodies for TLR2 and Mincle would be a logical first step to obtain further molecular proof of ligand receptor interaction.

Overall, the research described in this Thesis encompasses design and synthesis of several synthetic vaccine components, antigens and adjuvants, of relevance in the context of mycobacterial infection. Organic synthesis is combined to immunological assays to identify relevant biologically active analogues of naturally occurring compounds to be used as ingredients in soluble or liposomal subunit vaccines. This research resulted in the identification of novel interesting CD1c ligands, which are stable to most common hydrolytic conditions, novel analogues of the Mincle-binding

trehalose dimycolate and a library of Mincle-ligand or TLR2-ligand peptide conjugates with *in vitro* and/or *in vivo* activity.

## Materials and methods

### *ELISA using C-type lectin receptors*

Recombinant Human DC-SIGN/CD209 Fc Chimera Protein and recombinant Human CLEC4E Fc Chimera Protein were purchased from RD systems and used at concentrations of 1.5 µg/ml and 0.83 µg/ml respectively. Fc-hDectin-1a was purchased from InvivoGen and used at a concentration of 3 µg/ml. Cys-MR-Fc fusion protein (hydrophobic signal sequence and the first 139 residues of human MR, a 2-residue linker, and the Fc region of human IgG1) was produced in 293T cells, according to published procedure<sup>15</sup> and supernatant was directly used in the ELISA experiments without further purification. Control compound PAA-Lewis X was purchased from Lectinity (MW approx. 20 KDa, Carbohydrate content around 20% mol). Laminarin (from *Laminaria digitata*) and Mannan (from *Saccharomyces cerevisiae*) were purchased from Sigma-Aldrich. All synthetic compounds were dissolved in iso-propanol and were transferred to 96 well plates (Nunc MaxiSorp, Biolegend) at three different concentrations (5 - 1 - 0.2 nmol/well) for a total volume of 50 µL/well. The solvent was evaporated at 50°C. Control compounds Laminarin (3 µg/ml), PAA-Lewis X (1 µg/ml) and Mannan (1 µg/ml) were diluted in PBS and used to coat remaining wells for a total volume of 50 µL/well. After coating for 2 hours at room temperature, all the wells were washed twice with TMS (20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8.0; 150 mM NaCl; 1 mM CaCl<sub>2</sub>; 2 mM MgCl<sub>2</sub>) (150µL) and blocked with 100 µL TMS with 1% of BSA (Fraction V, Merk Millipore) for 30 min at room temperature. The plates were then incubated for 1 hour at room temperature with 50 µL soluble receptor in TMS with 1% of BSA. The wells were washed two times with TMS (150µL) and incubated at room temperature with 50 µL of Goat-anti human HRP (0.8 µg/mL, JacksonImmuno) in TMS with 1% of BSA for 30 min. After two washes with TMS (150µL), 50 µL of substrate solution (3,3',5,5'-Tetramethylbenzidine, TMB, in citric/acetate buffer, pH=4, and H<sub>2</sub>O<sub>2</sub>) were added and after 2 min at room temperature the reaction was stopped with 50 µL of H<sub>2</sub>SO<sub>4</sub> (0.8M). Sample absorbance at 450 nm was measured using a Spectramax i3x (Molecular Devices, CA, USA) spectrometer. All samples were tested in duplicate.

### *ODN1826 and p57-ODN1826 synthesis*

Thiol-functionalized ODN1826 was synthesized on an Expedite automated solid phase synthesizer (PerSeptive Biosystems), using 3' Thiol Modifier C6 SS Glyc CPG (Biosearch technologies) as solid support. Reaction scale 10 µmol based on solid support loading. Oligonucleotide elongation was performed using an established protocol.<sup>16</sup> In summary, elongation was performed using 5'-DMT-protected 3'-phosphoramidite derivatives in the presence of dicyanoimidazole as coupling agent. After each coupling, remaining free 5'-hydroxyls were capped using a solution of t-butylphenoxyacetic anhydride/1-methylimidazole in THF/pyridine, followed by sulfurization of the phosphite to phosphorothioate using the Beaucage reagent. The DMT protecting groups on the 5'-position were then removed using trichloroacetic acid, after which the elongation process was

continued. Cleavage from the resin was performed using a solution of 25% NH<sub>4</sub>OH in water to give the desired 3'-disulfide modified ODN (ODN-SS-hexyl-OH). This compound was purified with ion exchange chromatography on an AKTA Explorer system using a Q-Sepharose column (source-Q 10 x 100 mm) and the following running buffer solutions: buffer A = 25 mM NaOAc with 25 mM NaClO<sub>4</sub>; buffer B = 25mM NaOAc with 500 mM NaClO<sub>4</sub>. Gradient used for purification: 25% to 75% B in A, over 50 minutes (flow 4 ml/min). Fractions containing the pure product were combined and lyophilized three times using new aliquots of Endotoxin-free water (Sigma) to yield disulfide ODN compound as a white solid (9.1 mg, 1.35 $\mu$ mol, 13.5%). Quantification was performed by UV absorbance at 260 nm. LC-MS analysis: (C18 column with 10 mM NH<sub>4</sub>OAc, linear gradient 00 $\rightarrow$ 50% B, 11 min): Rt = 5.980 min. CpG-SS-hexyl-OH (3.4 mg, 0.5 $\mu$ mol, 1 eq) was deprotected overnight in 2 ml dithiothreitol (DTT) buffer (350 mg DTT, 260 mg of NaOAc 3 H<sub>2</sub>O in 10ml endotoxin-free water). The resulting reduced CpG-SH was purified using a PD-10 column which was pre-equilibrated using a 50 mM phosphate solution (25 mM Na<sub>2</sub>HPO<sub>4</sub>, 25 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA) which was already degassed and flushed with Argon. The filtrate (3.25 ml) was directly transferred to a tube containing the maleimide-functionalized peptide (10 mg, 3 $\mu$ mol, 6 eq). The resulting solution was degassed, sonicated and stirred under Argon for two days at RT. The CpG-peptide conjugate was isolated after gel-filtration on a superdex 75 column (16 x 60 mm) with isocratic gradient of 0.15 M NH<sub>4</sub>OAc and further purified by RP-HPLC using a C18-column (10 x 250 mm) with 10 mM NH<sub>4</sub>OAc and a gradient of 5 to 30% acetonitrile in water over 15 minutes. Fractions containing the pure product were combined and lyophilized three times using new aliquots of Endotoxin-free water to yield the synthetic conjugate as a white powder (2.6 mg, 272 nmol, 54%). Quantification was performed by UV absorbance at 260 nm. LC-MS analysis: (C18 column with 10 mM NH<sub>4</sub>OAc, linear gradient 50 $\rightarrow$ 90% B, 15 min): Rt = 7.222 min, ESI-MS [M+H]<sup>10+</sup>=972.5 found, 971.7 calculated, ESI-MS [M+H]<sup>8+</sup>= 1215.7 found, 1214.4 calculated. MALDI-TOF [M+NH<sub>4</sub>]<sup>+</sup>: 9724.051 found, 9725.164 calculated.

### **Generation of DOPC:DOTAP liposomes**

DOPC/DOTAP (4:1 molar ratio) liposomes were prepared using the thin-film hydration method. First, a thin lipid film was formed by adding stock solutions of DOTAP and DOPC in chloroform (25 mg/mL) to a round-bottomed flask, followed by evaporation of the organic solvent of the lipids using a rotary evaporator. Hereafter, p57-C11C18 conjugate in DMSO (nothing was added for the empty liposomes) was added to the round-bottomed flasks, and the formulation was homogenized by adding 5 glass beads and swirling them around to remove the lipid film from the glass wall. The formulations were transferred to 15 ml tubes, and the round-bottomed flask was rinsed 4 times, each time with 1 mL of Milli-Q water, which was added to the tubes to make sure that the main part of the formulation was transferred. The liposomes were snap-frozen in liquid nitrogen followed by lyophilization overnight. The resulting lipid cakes were rehydrated in 10 mM phosphate buffer (PB) (pH 7.4) to reach a total lipid concentration of 10 mg/mL and 77.2 nmol of p57-C11C18 conjugate (305  $\mu$ g/mL). Rehydration was performed in three steps, where 25%, 25%, and 50% of the total volume (2 mL) were added with 30 min intervals at 60°C. After the last addition, the formulations equilibrated for 1 hour at 60°C, the obtained liposomes were extruded (Lipex 1.5 mL extruder, TransferraNanosciences, Burnaby, Canada) simultaneously through stacked polycarbonate filters with a pore size of 400 and 200 nm, six times at 60°C. Free molecules were removed by dialysis (24 hours, RT) using a Float-A-Lyzer G2 with a 100 kDa molecular weight cut-off

(MWC0) (Repligen, Waltham, MA, USA) and 10 mM PB in the dialysate. The obtained liposomal formulations were stored at 4°C until further use.

### **Particle size and zeta potential measurements**

The formulations were diluted twenty-fold in 10 mM PB. The intensity-weighted average hydrodynamic diameter (Z-average) and polydispersity index (PDI) of the particles were determined by dynamic light scattering (DLS) and the zeta potential was determined by laser Doppler electrophoresis. Measurements were conducted in triplicates at 25°C using a nano ZS zetalyzer coupled with a 633 nm laser and 173° optics (Malvern Instruments, Worcestershire, UK) and analyzed using Zetasizer Software v7.12 (Malvern Instruments).

<b>Liposomes (DOPC:DOTAP 4:1)</b>	<b>Size (nm)</b>	<b>PDI</b>	<b>ZP (mV)</b>
<i>Empty</i>	121,2333	0,0959	33,69
<i>p57-C11C18 conjugate</i>	136,3667	0,1216	28,39

### **Generation and stimulation of immature human moDCs using liposomes**

Buffy coats of healthy human Blood Bank donors were purchased from Sanquin Amsterdam. CD14<sup>+</sup> monocytes were isolated from whole blood using Ficoll-Paque density gradient followed by purification on autoMACS<sup>®</sup> Pro Separator instrument using CD14 MicroBeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). The monocytes were differentiated to monocyte-derived dendritic cells (moDCs) at a concentration of 10<sup>6</sup> cells/mL in RPMI 1640 (Gibco, PAA, Linz, Austria) medium containing 10% FCS (Hyclone, GE Healthcare Life Sciences, Eindhoven, the Netherlands), 2 mM GlutaMAX<sup>™</sup> (Gibco, PAA, Linz, Austria), 100 U/ml penicillin, 100 µg/ml streptomycin and 10 ng/mL GM-CSF (Life Technologies-Invitrogen, Bleiswijk, the Netherlands) and 10 ng/mL IL-4 (Peprotech, Rocky Hill, NJ). On day 3, all cultures were replenished with fresh culture media with the appropriate concentrations of GM-CSF and IL-4. Cells were incubated for 5 days at 37°C and in a 5% CO<sub>2</sub> atmosphere incubator. Immature dendritic cells were then harvested, counted and transferred to 96 well plates (round bottom, Corning Costar TC-Treated Microplates, Corning, NY) at 50.000 cells/well. The next day cells were stimulated for three hours, before the supernatant was discarded and replaced by medium before overnight incubation, using synthetic compounds (20 µM concentration) or liposomes (100 µg/ml concentration of the lipids DOPC:DOTAP in a 4:1 molar ratio; 0.89 µg/ml = 0.22 nmol/ml synthetic conjugate concentration in the final formulation). The synthetic compounds were dissolved in DMSO at a concentration of 5 nmol/µL, further diluted and premixed in RPMI 1640 medium containing 10% FCS, 2 mM GlutaMAX<sup>™</sup>, 1% Pen-Strep; the liposomes were diluted in the same medium at described concentration. LPS (100 ng/ml) was used as positive control for stimulated cells. Supernatants were harvested 20 hours after the initial addition of stimuli for subsequent analysis of cytokines.

***Human IL-12(p40) and IL-10 ELISA***

Human IL-12/IL-23 (p40) and human IL-10 ELISA kits were purchased from Biolegend (ELISA MAX™ Standard Set; London, UK). All supernatants were tested in triplicates according to manufacturer's instructions. Sample absorbance was measured using a Spectramax i3x (Molecular Devices, CA, USA) spectrometer.

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