Simplified Monopalmitoyl Toll-like Receptor 2 Ligand Mini-UPam for Self-Adjuvanting Neoantigen-Based Synthetic Cancer Vaccines

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Synthetic vaccines, based on antigenic peptides that comprise MHC–I and MHC-II T-cell epitopes expressed by tumors, show great promise for the immunotherapy of cancer. For optimal immunogenicity, the synthetic peptides (SPs) should be adjuvanted with suitable immunostimulatory additives. Previously, we have shown that improved immunogenicity *in vivo* is obtained with vaccine modalities in which an SP is covalently connected to an adjuvanting moiety, typically a ligand to Toll-like receptor 2 (TLR2). SPs were covalently attached to UPam, which is a derivative of the classic TLR2 ligand Pam_3CysSK_4. A disadvantage of the triply palmitoylated UPam is its high lipophilicity, which precludes universal adoption of this adjuvant for covalent modification of various antigenic peptides as it renders the synthetic vaccine insoluble in several cases. Here, we report a novel conjugatable TLR2 ligand, mini-UPam, which contains only one palmitoyl chain, rather than three, and therefore has less impact on the solubility and other physicochemical properties of a synthetic peptide. In this study, we used SPs that contain the clinically relevant neoepitopes identified in a melanoma patient who completely recovered after T-cell therapy. Homogeneous mini-UPam-SP conjugates have been prepared in good yields by stepwise solid-phase synthesis that employed a mini-UPam building block pre-prepared in solution and the standard set of Fmoc-amino acids. The immunogenicity of the novel mini-UPam-SP conjugates was demonstrated by using the cancer patient's T-cells.

Introduction

Cancer vaccines aim to induce specific immune responses directed against patients' tumors. Activation of specific T-cells is crucial since they detect and destroy malignant cells by recognition of tumor-expressed antigens.[1–3] A highly specific class of tumor antigens are neoantigens[1,2] which are the result of somatic DNA mutations in tumor cells, translating into amino acid residue changes and antigenic peptides. As these antigens are uniquely expressed on the tumor cells of a patient, these neoepitopes offer an ideal target for personalized cancer immunotherapy and have shown to be more immunogenic than widely expressed tumor antigens.[1,3] The induction of anti-tumor T-cell responses has been reported by vaccination with synthetic peptides (SPs) encoding defined amino acid sequences of various tumor antigens.[1–5] However, SPs are only weakly immunogenic and require an adequate adjuvant to provoke a potent immune response that is able to clear tumors.[1] We have reported that conjugation of an SP with an optimized Toll-like receptor (TLR) 2 ligand, UPam, is an effective strategy to induce functional T-cell responses. Therapeutic vaccination with TLR2 ligands conjugated to SPs, in which the peptide sequence embedded either model or oncoviral tumor antigens, resulted in tumor clearance and increased survival in multiple tumor-bearing mouse models.[6,7] The TLR2 ligand UPam has also been conjugated to SPs containing the oncogenic antigen sequences

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of the human papillomavirus (HPV). These constructs were able to efficiently activate *ex vivo* human T-cells derived from patients with HPV positive cervical cancer.[8]

For the development of new adjuvants with known and improved properties, agonists of pattern recognition receptors (PRRs) such as TLRs, NOD-like receptors (NLRs) and C-type lectin receptors (CLRs) are intensively investigated.[9–12] For some PRRs, structurally well-defined agonistic ligands with a relatively small molecular weight have been discovered, which have been used as synthetic analogues to elicit defined innate immune responses for several purposes.[13] In particular, the ligands for TLR2 have been extensively subjected to structure-activity relationship (SAR) studies, resulting in the often applied Pam_3CSK_4 and Pam_2CSK_4 ligands (Figure 1A) respectively for the TLR2/TLR1 and TLR2/TLR6 heterodimer combinations.[14–18] Interestingly, small molecule ligands prove to be suitable for incorporation in conjugates, in which a peptide epitope is covalently connected to a structurally defined TLR ligand.[19–24] These conjugates outperformed, in terms of immunological properties, mixtures of the non-covalently linked components.[25,7] Thus, the widely used TLR2 ligand Pam_3CysSK_4, which contains a tetralysine linker (K_4), combined with an antigenic peptide in a conjugate (TLR2L-SP) has shown to induce functional T-cell responses.[21] Therapeutic vaccination with a TLR2L-SP, in which the SP encoded a model tumor antigen, resulted in tumor clearance and increased survival of tumor-bearing mice in several mouse models.[22,7] In the context of these studies we found a new TLR2 ligand (UPam, Figure 1A), which upon incorporation in a conjugate with an antigenic SP induced functional T-cell responses.[7] UPam was conjugated to SPs containing antigen sequences of the oncogenic HPV. These constructs were able to efficiently activate human T-cells derived from HPV positive tumor-draining lymph nodes *ex vivo* and are currently used in a phase I clinical vaccination study in $HPV16^+$ cancer patients.[26,8] These favorable properties were an incentive to prepare and evaluate TLR2L-neoantigen conjugates. However, covalent linking of a great variety of antigenic peptides to a lipophilic TLR2 ligand is not always feasible due to solubility problems during synthesis and final preparation as a vaccine. This could hamper the production of personalized cancer vaccines in which a short production time is essential. To tackle this issue, attention was directed to a new design of these conjugates in which the lipophilicity of the TLR2 ligand is minimized while its linker to the antigenic peptide would further contribute to the solubility. Importantly, these modifications should not be detrimental for the TLR2-activating activity of the ligand. SAR studies by David and co-workers resulted in a relatively simple TLR2 ligand (Figure 1B) which contains only one lipophilic tail and is not only water soluble but also human TLR2 (hTLR2) specific.[27–30] Our SAR studies on TLR2 ligands showed that the replacement of the amide by a urea moiety at the N-terminal amine of the Cys residue led to the more potent ligand $UPam_3CysSK_4$.[6] We decided to combine structural features of mono-palmitoyl hTLR2 ligand with the outcome of our studies, resulting in the replacement of the acetyl group at the N-terminal amine of the cysteine in the hTLR2 ligand by a urea moiety, to give a new ureido TLR2 ligand **2** (Figure 1C). To ultimately obtain effective conjugates, we tested three linkers

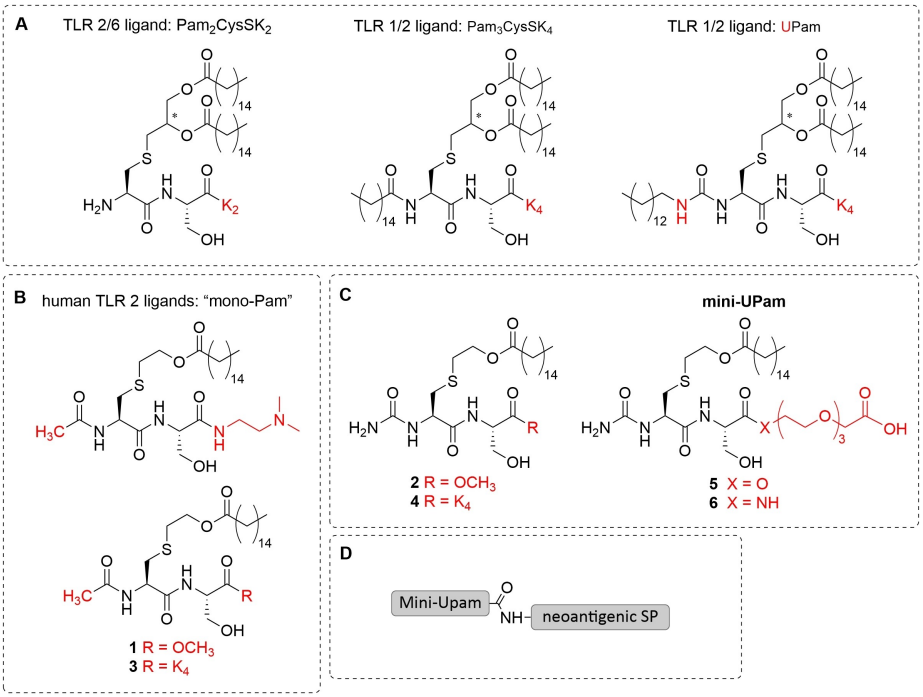

Figure 1. Structures of TLR2 ligands and the projected conjugates. A) Previously reported, highly lipophilic di- and tripalmitoyl TLR2 ligands. B) Previously reported monopalmitoyl derivatives with improved solubility and TLR2-activating potency. C) Ureido-monopalmitoyl TLR2 ligands developed in this work. D) Design of the mini-UPam-synthetic peptide conjugates.

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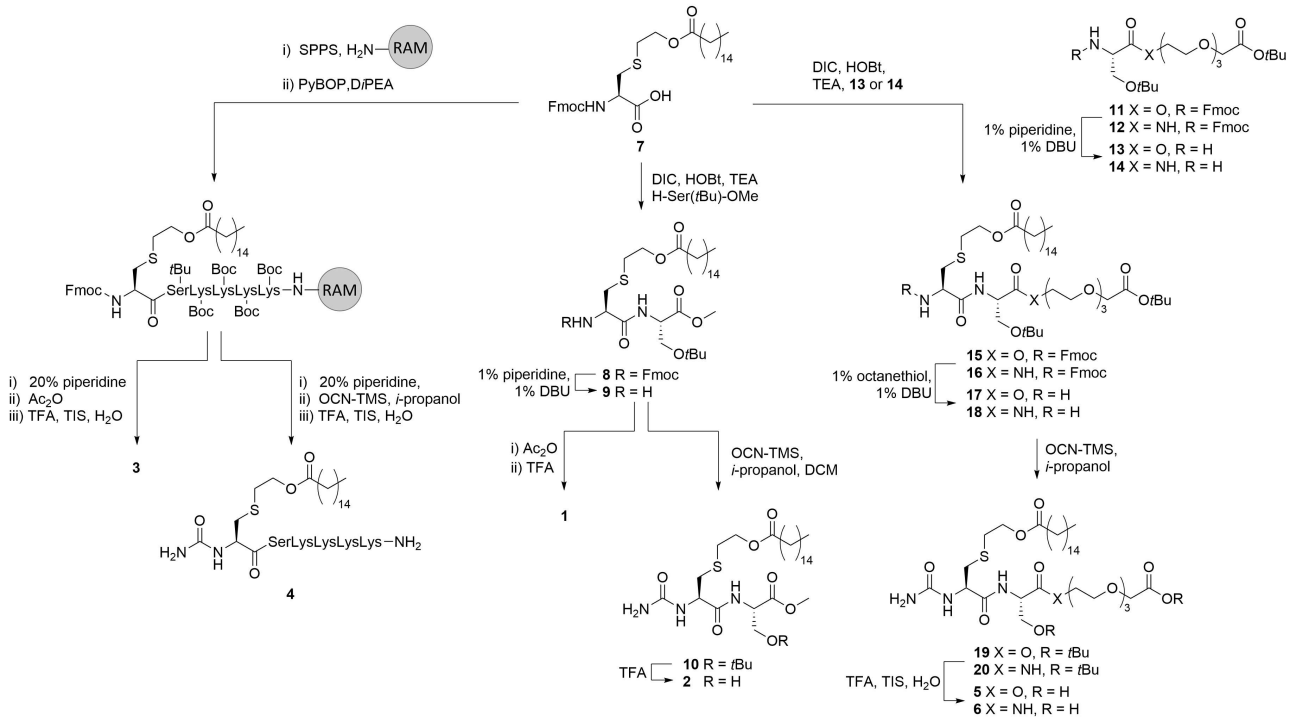
to allow covalent attachment of this new ureido TLR2 ligand to the neoantigen peptides. The conventional tetralysine linker K_4 (resulting in **4**) and a tri(ethylene glycol) linker, connected to new TLR2 ligand **2** via an ester (resulting in **5**) or amide bond (resulting in **6**) were selected.[31] Tri(ethylene glycol) was a selected as a linker to limit the complexity and to minimize the size of the conjugate. It is known that such a linker could be inserted between $Pam_3CysSer$ and an antigenic peptide without detriment to the immunogenicity of the construct.[32] Ligand **1**, previously reported by David and co-workers and its derivative **3** provided with tetralysyl linker K_4 were taken as relevant references. We here present the immunological evaluation of these TLR2 ligands **1**–**6** and the subsequent incorporation of the most favorable ligand (named Mini-UPam) in conjugates with both a $CD8^+$ and a $CD4^+$ T-cell neoepitope originating from a melanoma patient.[33,34] Here we show that these novel conjugates induce improved effective neoepitope-specific human $CD8^+$ T-cell as well as $CD4^+$ T-cells activation compared to the lipophilic UPam lipopeptides.

Results and Discussion

Synthesis of the TLR2 ligands

For the syntheses of all TLR2 ligands (**1**–**6**, Scheme 1), we used known cysteine derivative **7** as the starting compound.[30] In the solution phase route to ligands **1** and **2**, the carboxylic acid in **7** was condensed with H–Ser (*t*Bu)–OMe, by using the DIC/HOBt combination as coupling reagent and triethylamine (TEA) as a base, to give methyl ester **8** in an yield of 88%. Removal of the Fmoc protecting group with 1% piperidine and 1% DBU in DMF left the methyl ester intact and proceeded effortlessly to give free amine **9**. For the introduction of the urea moiety, treatment of the amine with a solution of trimethylsilyl isocyanate in iso-propanol and CH_2Cl_2 gave the best results in terms of yield and suppression of formation of polymerization products. Finally, the *tert*-butyl ester was cleaved with a mixture of TFA in CH_2Cl_2 and silica gel column chromatography yielded the new ureido ligand **2** in an overall yield of 69%. The corresponding reference compound **1** with N-terminal acetyl was synthesized by using a similar procedure with acetic anhydride to acetylate the amine. Lipopeptide **4**, having the tetralysyl linker (K_4), and corresponding reference compound **3** were assembled by solid-phase peptide synthesis (SPPS). By using Rink amide resin and a standard peptide coupling protocol, four lysines were coupled as commercially available Fmoc-Lys-OH building blocks. Elongation of the immobilized and side-chain-protected K_4 with Ser(*t*Bu) was followed by coupling with cysteine building block **7**, Fmoc removal and the installation of the urea moiety by the procedure described above. After a TFA-assisted cleavage from the resin and HPLC purification, lipopeptide **4** was isolated in an overall yield of 4%. The reference compound **3** was synthesized similarly. PamCysSer derivatives **5** and **6** were synthesized with tri(ethylene glycol) linker in solution as follows. First of all, ester **11** and amide **12** were both synthesized from the corresponding tri (ethylene glycol), by alkylation with *tert*-butyl bromoacetate,



Scheme 1. Synthesis of TLR2 ligands **1**–**6**.

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the replacement of the alcohol with an amine for **13**, and subsequent condensation with Fmoc–Ser(*t*Bu)–OH. The cleavage of the Fmoc group with a DBU/piperidine mixture gave free amines **13** and **14**. Condensation of **13** and **14** with building block **7** went smoothly and provided precursors **15** and **16** in a good yield, after the cleavage of the Fmoc with a mixture of 1-octanethiol and DBU in DMF. Functionalization of the amines with trimethylsilyl isocyanate gave the water soluble urea ligands **19** and **20** in a yield of 90 and 82%, respectively. Finally, removal of the remaining *tert*-butyl groups with TFA in the presence of TIS and purification by silica gel column chromatography yielded ligands **5** and **6** in an overall yield of 35 and 51%, respectively.

Selection of a ligand with the best TLR2-activating potential

The TLR2-activating capacity of the six different ligands (**1**–**6**, Scheme 1) was first evaluated in human embryonic kidney cells (HEK293), which were stably transfected with the human TLR2 gene. Incubation with titrated amounts of these ligands (1 μM–15.6 nM) resulted in the production of reporter cytokine IL-8 by the HEK-TLR2 cells, indicating functional activation of the TLR2 receptor by all the compounds tested (Figure 2A). Compound **2** with the N-terminal urea moiety, proved to be similar or even slightly more active than the known compound **1** with N-terminal acetyl. The introduction of K_4 directly to the C terminus of the mono-Pam ligands as in compounds **3** and **4** proved detrimental for the activity. In contrast, both tri(ethylene glycol) linker-containing ligands **5** and **6** proved highly potent activators of TLR2 in the HEK293 assay. (Figure 2A). The results obtained in the TLR2-transfected HEK293 cells were corroborated in an experiment with immunologically relevant monocyte derived human dendritic cells (moDCs) were incubated with ligands **1**–**6**. Although all ligands were capable of maturing the moDCs, as measured by IL12p40 production, indicative for TLR2-receptor-induced activation, again both ligand **5** and **6** proved to be most active (Figure 2B). The remarkable activity of compounds **5** and **6** could be attributed to the conformational flexibility and smaller size of the triglycol spacer, as compared to the tetralysyl peptide, possibly resulting in a better fit of the ligand to the receptor.

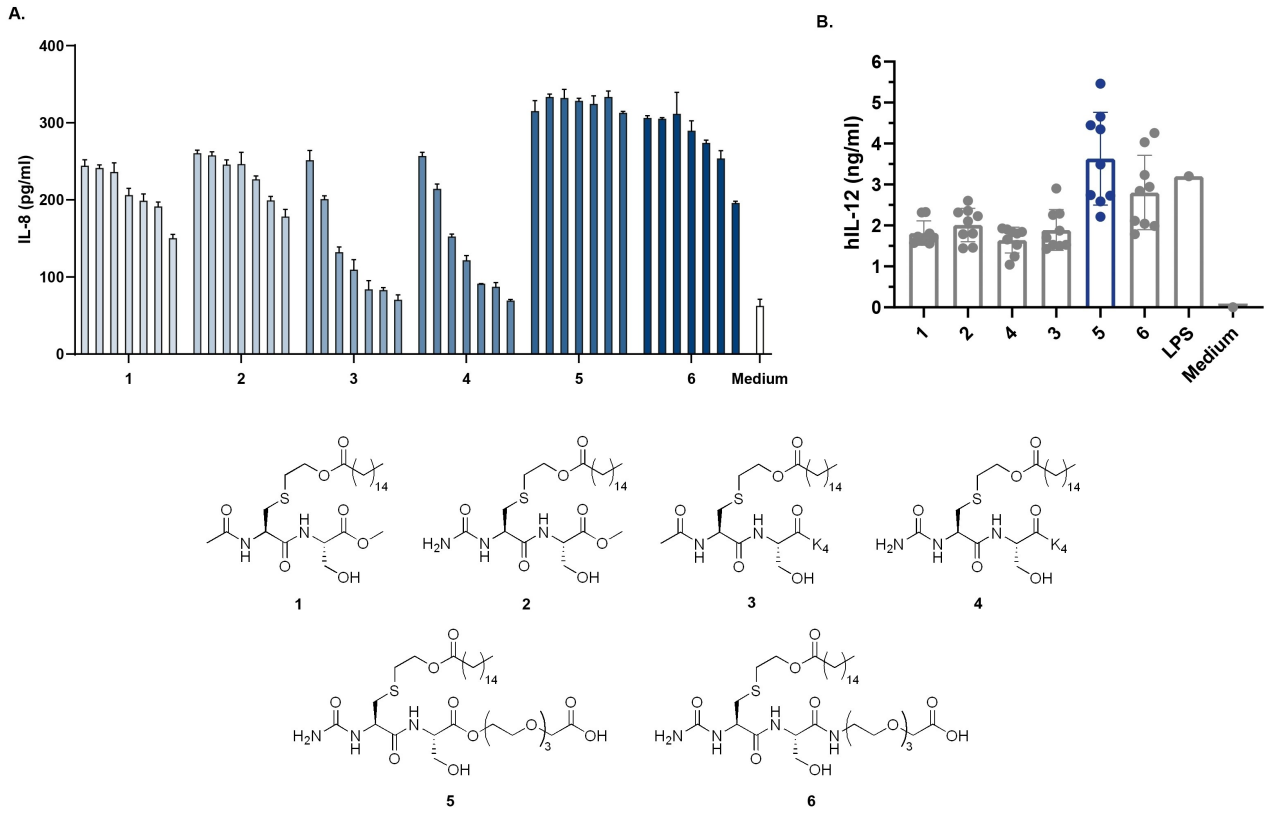

Figure 2. TLR2 activation of the different mini-UPam derivatives in A) HEK-TLR2 cells and B) human moDCs. Cells were incubated with titrated amounts (1 μM–15.6 nM and 1 μM–3.9 nM respectively, twofold titrations) of the indicated compounds (Scheme 1). The lipopolysaccharide (LPS) concentration was 1 μg/mL. After 36 h, supernatants were harvested, and the production of A) IL-8 (data shown as mean ± SD, $n = 3$) or B) IL-12p40 (data shown as mean ± SD, $n = 9$) was determined by specific ELISA.

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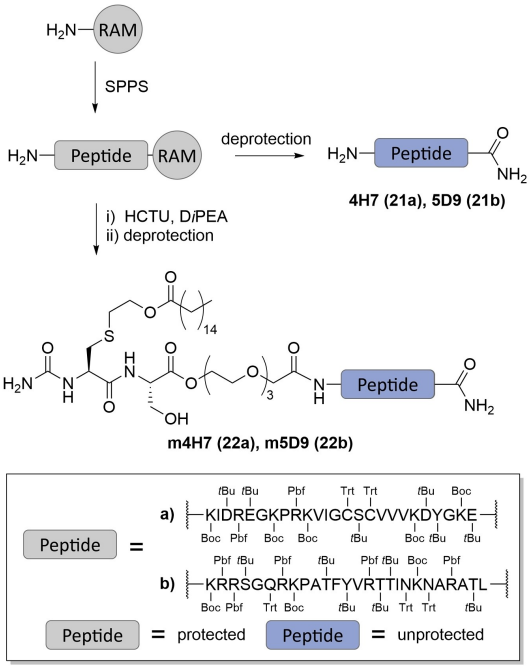
Synthesis of mini-UPam conjugates with neoantigen SPs

The biological activity evaluation of the (linker-containing) TLR2 ligands (**1**–**6**, Figure 2) revealed ligand **5** as the most suitable for the incorporation in conjugates of SPs. For immunological analysis we used SPs that embed neoepitopes 5D9 and 4H7, identified as CD8$^+$ and CD4$^+$ T-cells epitopes, respectively, in a melanoma patient that obtained a complete response after treatment with these neoepitope-specific T-cells.[33] As shown in Scheme 2, both conjugates and the associate reference peptides were assembled with SPPS, by using a standard Fmoc-based protocol. Mini-UPam was installed at the N-terminal end of the immobilized peptide in the final stage of the synthesis by HCTU mediated condensation of building block **5** with the free amino group of the peptide. The conjugates were deprotected and released from the solid support by treatment with a TFA/TIS/H_2O cocktail and finally purified by HPLC to give mini-UPam conjugates **22 a**, **b** to evaluate their immunogenicity. The corresponding peptides **21 a**, **b** were prepared by standard Fmoc-SPPS and used as the essential reference compounds in antigen presentation assays.



Scheme 2. Synthesis of neoantigen-TLR2 ligand conjugates under SPPS conditions.

Immunogenic potential of neoantigen SPs conjugated to mini-UPam

The TLR2-stimulating potency of the SP-mini-UPam conjugates was first evaluated in human embryonic kidney (HEK293) cells that were stably transfected with the human TLR2 gene. Titration of the free ligand as well as the CD8$^+$ (**22 b**) and CD4$^+$ (**22 a**) T-cell epitope conjugates resulted in the production of IL-8 (Figure 3A), whereas no IL-8 production was observed in HEK293 cells that lacked hTLR2 expression (Figure S1), indicating retained biological activity of mini-UPam upon conjugation to a SP. Next, the ability to mature human DCs was tested by titration of both conjugated neoepitopes and incubation of moDCs. Prior to their use, the upregulation of CD11c and loss of CD14 expression was determined to ensure successful differ-

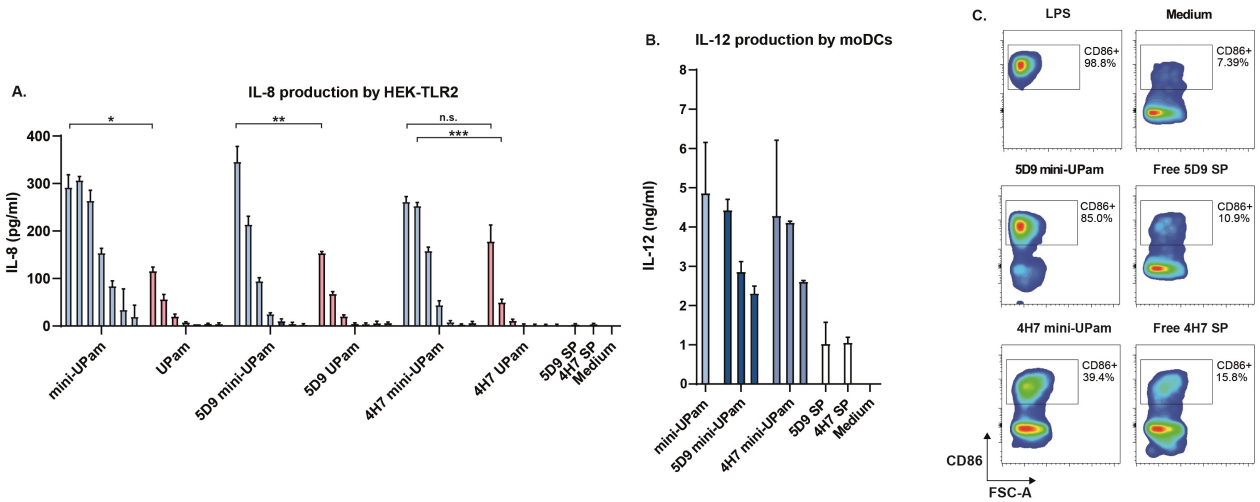


Figure 3. SP–mini-UPam conjugates efficiently target the human TLR2 receptor resulting in moDC activation and maturation. A) IL-8 production determined in the supernatant of HEK-TLR2 cells after 48 h of incubation with free mini-UPam, free 5D9 and 4H7, and the respective conjugates (concentration range: 1 μM; 200 nM; 40 nM; 8 nM; 1.6 nM; 320 pM; 64 pM, fivefold titration) and SPs at 1 μM concentration. B) IL-12p40 production by moDCs. Concentration of mini-UPam: 16 nM; concentration of conjugates: 10 μM; 400 nM; 16 nM and SP 10 μM. C) Upregulation of the maturation marker CD86 by human moDCs after 36 h of incubation with the indicated compounds (concentration of LPS: 1 μg/mL; conjugates and SP: 10 μM; data shown as mean ± SD, $n = 3$). ** $p < 0.01$, *** $p < 0.001$, determined by multiple T-test with Bonferroni-Dunn correction for multiple comparison.

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entiation of the monocytes into moDCs (Figure S2). After 36 hours of incubation, the production of IL-12p40, a Th1-inducing cytokine (Figure 3B), and the upregulation of the maturation markers CD83 and CD86 were determined (Figures 3C and S3). Both the 5D9-mini-UPam and the 4H7-mini-UPam conjugates were able to efficiently mature moDCs and the conjugation did not compromise the bioactivity of the UPam.

Finally, the uptake, processing, and subsequent antigen presentation and T cell stimulation of the conjugated $CD8^+$ and $CD4^+$ T-cell epitopes (**22a**, **b**) by antigen-presenting cells (APCs) were tested by making use of human moDCs and neoepitope-specific human T-cells obtained from the melanoma patient who was successfully treated with these neoepitope-specific T-cells. The tumor-reactive T-cell cultures used for this successful treatment were established and stored in the LUMC (Leiden) and used for this study.[33–35] The APCs used in these experiments were derived from HLA-matched donors or, if available, autologous cells were used. After differentiation, the APCs were loaded overnight with titrated amounts of the $CD8^+$ T-cell epitope-containing SP (**22b**) or $CD4^+$ T-cell epitope-containing SP (**22a**) conjugates. The patient's T-cells, containing the neoepitope-specific $CD8^+$ and $CD4^+$ T-cells, were added, followed by overnight incubation of the APCs. T-cell activation was determined by the expression of the activation marker CD137 and the production of the cytokine IFNγ. The 5D9-mini-UPam conjugate (**22b**) was able to significantly activate the 5D9-specific $CD8^+$ T-cells, whereas the equimolar mix of the peptide and free mini-UPam only marginally resulted in T-cell activation (Figures 4A, B and S4). This indicates adequate processing and MHC class I presentation of the conjugated epitope and the relevance of the conjugation of a TLR2 ligand to an antigenic peptide to improve its immunogenicity.[7,8] The activated $CD8^+$ T-cells were capable of producing IFNγ upon stimulation with the 5D9-mini-UPam conjugate, indicating their functionality/activation of their effector function (Figure 4C). The APCs loaded with the 4H7-mini-UPam conjugate were also able to activate neoepitope-specific $CD4^+$ T-cells (Figures 4B and S4), showing that the conjugated $CD4^+$ T-cell epitope was properly processed and correctly loaded into the MHC class II complex followed by presentation on the cell surface. However, as we have observed previously for $CD4^+$ T-cell epitopes conjugated to a TLR2 ligand,[8] the 4H7-mini-UPam (**22a**) conjugates do not outperform free SP or mixtures in T-cell activation in *in vitro* settings.

Conclusion

We have reported mini-UPam as an optimized TLR2 ligand that is able to efficiently bind and activate the human TLR2 receptor in HEK-hTLR2 cells and human DCs. Conjugation of the mini-UPam to two different amino acid sequences each containing a human cancer neoepitope did not hamper the ability of the ligand to trigger TLR2 signaling. Our aim was to design a TLR2 ligand with less lipophilic characteristics compared to UPam that allows reliable covalent attachment to SPs, resulting in vaccine conjugates with better solubility than the UPam conjugates but with retained immunogenicity. Mini-UPam being much less lipophilic than UPam has a lesser impact on the physicochemical properties of the peptide vaccines and allows the synthesis and the chromatographic purification of the mini-UPam conjugates much in the same way as the conventional peptide-based synthetic

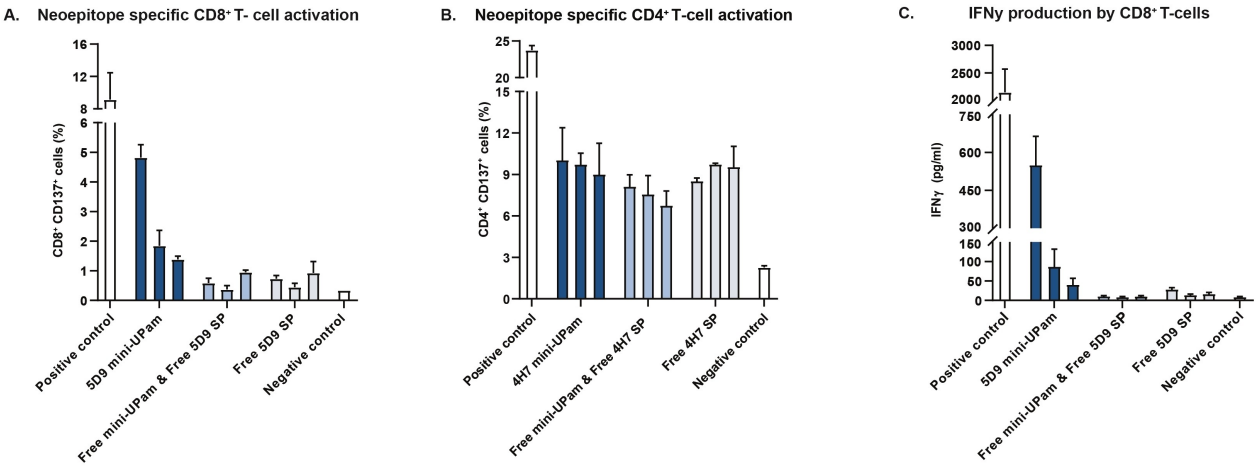

Figure 4. Activation of neoepitope-specific human $CD8^+$ and $CD4^+$ T-cells by the SP-mini-UPam conjugates. Percentages of total A) $CD8^+$ and B) $CD4^+$ T-cells expressing the activation marker CD137 after overnight co-culture with monocytes loaded with 5D9/4H7–mini-UPam conjugates, the free mini-UPam mixed with the SP or the SP alone (concentration range: 2, 1, 0.5 μM). Positive controls were A) a SP containing the minimal $CD8^+$ T-cell epitope (1 μg/mL) or B) a SP containing the $CD4^+$ T-cell epitope with the natural flanking amino acids (10 μg/mL). Negative controls were unloaded monocytes. Data from a representative experiment out of three independent experiments are shown. C) IFNγ production determined in the supernatant of the activated $CD8^+$ T-cells. The positive control is a SP containing the minimal $CD8^+$ T-cell epitope (1 μg/mL). A representative experiment is shown as mean ± SD ($n = 4$) out of three independent experiments.

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vaccines. Both conjugates reported here were able to functionally stimulate human DCs and activate neoepitope-specific human T-cells. For the construct with a CTL epitope the conjugation of the peptide and mini-UPam strongly enhanced activation of neoepitope-specific $CD8^+$ T-cells. The equimolar mix of the free mini-UPam with the corresponding SP did not result in activation of the specific $CD8^+$ T-cells, which is consistent with our earlier observations with murine conjugates versus a mix of the free components.[7,25] The CD4 mini-UPam-SP conjugate with a T-helper epitope, however, was not superior in antigen presentation and $CD4^+$ T-cell activation as compared to the mix of free SP and TLR2 ligand or free SP alone. We have observed this before in our studies with mouse and human $CD4^+$ T-cell epitope-containing TLR-L-SPs and we explain this by the different uptake and routing of MHC class II presented peptides as compared to MHC class I processing routes.[8,7] Apparently, *in vitro* the continuous presence of SP in the DC culture allows endosomal uptake and processing of antigenic peptides in the MHC II processing route. Based on our results this process cannot be improved *in vitro* by conjugation of the antigenic peptide to a TLR-targeting adjuvant. This is in contrast to the MHC class I processing route which requires uptake and endosomal escape to a cytosolic route which may be improved by TLR-ligand targeting and signaling.[36] Importantly, we still prefer TLR2 ligand adjuvanting for MHC class II presented peptide in the synthetic vaccine, as we have shown that *in vivo* not only $CD8^+$ but also $CD4^+$ T-cell activation and tumor control obtained by conjugates is superior to that obtained by physical mixtures of free TLR2 ligands and SPs.[6,7]

To conclude, taking inspiration from the known monopalmitoyl cysteine derivative and having introduced a urea and tri(ethylene glycol) moiety into its structure, we developed a new simplified and chirally pure TLR2 ligand that is significantly less lipophilic than Pam_3CSK_4 and UPam. Next, two neoantigen-containing elongated SPs were conjugated to our most potent TLR2L to provide molecular constructs that can efficiently activate human cancer-specific T-cells. We propose the mini-UPam as a broadly applicable immunogenic modifier for antigenic synthetic peptides particularly for future applications in the rapid synthesis of multiple lipopeptides crucial for personalized immunotherapy of cancer.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: cancer vaccines · dendritic cells · lipopeptides · neoepitopes · solid-phase synthesis · TLR2 ligand

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