



Universiteit
Leiden
The Netherlands

Solid-phase synthesis and immunological evaluation of peptide conjugates containing stereochemically defined UPam-based TLR2 ligands and epitopes from SARS-CoV-2 virus

Castello, G.; Isendoorn, M.M.E.; Camps, M.; Meeuwenoord, N.J.; Verhoeks, V.F.H.; Ende T.C. van den; ... ; Filippov D.V.

Citation

Castello, G., Isendoorn, M. M. E., Camps, M., Meeuwenoord, N. J., Verhoeks, V. F. H., Codée, J. D. C., & Ossendorp, F. (2025). Solid-phase synthesis and immunological evaluation of peptide conjugates containing stereochemically defined UPam-based TLR2 ligands and epitopes from SARS-CoV-2 virus. *Bioconjugate Chemistry*, 37(1), 1-10.
doi:10.1021/acs.bioconjchem.5c00345

Version: Publisher's Version

License: [Licensed under Article 25fa Copyright Act/Law \(Amendment Taverne\)](#)

Downloaded from: <https://hdl.handle.net/1887/4289824>

Note: To cite this publication please use the final published version (if applicable).

Solid-Phase Synthesis and Immunological Evaluation of Peptide Conjugates Containing Stereochemically Defined UPam-Based TLR2 Ligands and Epitopes from SARS-CoV-2 Virus

Giulia Castello,⁺ Marjolein M.E. Isendoorn,⁺ Marcel Camps, Nico J. Meeuwenoord, Vincent F. H. Verhoeks, Thomas C. van den Ende, Jeroen D. C. Codée, Ferry Ossendorp,^{*} and Dmitri V. Filippov^{*}



Cite This: *Bioconjugate Chem.* 2026, 37, 1–10



Read Online

ACCESS |



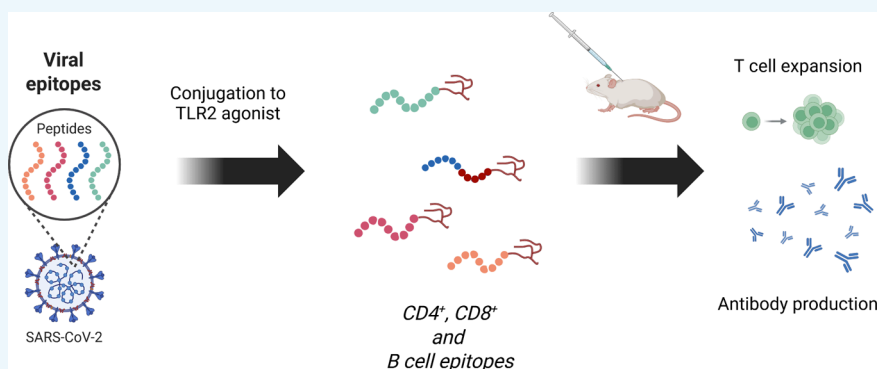
Metrics & More



Article Recommendations



Supporting Information



ABSTRACT: The design of effective vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) relies on eliciting robust cellular and humoral immune responses. In this study, we report the synthesis and immunological evaluation of synthetic long peptide (SLP) vaccines incorporating immunodominant CD4⁺ and CD8⁺ T cell and B cell epitopes from the SARS-CoV-2 proteome, covalently conjugated to stereochemically defined UPam ligands, which serve as synthetic Toll-like receptor 2 (TLR2) agonists. Using solid-phase peptide synthesis, we generated a panel of R- and S-glycerol-based UPam conjugates with varied amino acids adjacent to the palmitoylated cysteine. These conjugates were evaluated for their ability to induce dendritic cell maturation, TLR2 activation, and antigen-specific immune responses. *In vitro* assays demonstrated that R-UPam ligands, particularly those containing Dab and Abu residues, were superior in stimulating IL-12p40 production and TLR2 activation compared with their S-epimer counterparts. *In vivo* immunization studies in mice revealed that covalent conjugation of UPam to CD8⁺ T cell epitopes (VNF and REL) significantly enhanced antigen-specific T cell responses compared to unconjugated mixtures. Furthermore, a UPam-conjugated B cell epitope fused to a universal helper T cell peptide (PADRE) induced strong dendritic cell activation and enhanced PADRE-specific CD4⁺ T and B cell responses, as evidenced by elevated IgG production. These findings underscore the potential of chiral pure UPam-conjugated peptide vaccines to induce balanced, durable immunity against SARS-CoV-2 by combining TLR2-mediated adjuvanticity with precise epitope targeting.

INTRODUCTION

The development of effective vaccines against infectious diseases and emerging pathogens has been a cornerstone of modern medicine.¹ In this pursuit, a comprehensive understanding of the immune response at the molecular level has become essential. The utilization of peptide epitopes, small antigenic regions of the protein that can be targeted by the immune system, has paved the way for the rational design of vaccines. Since the outbreak of the COVID-19 virus (SARS-CoV-2), much research has been dedicated to finding epitopes to elicit long-lasting, protective immune responses.² Among these, CD4⁺ and CD8⁺ T cell epitopes derived from the SARS-CoV-2 spike protein have been targeted, as they can elicit

potent humoral and cellular immune responses, respectively.^{3,4} The adaptive antiviral immune response to SARS-CoV-2 is characterized by a T cell response and the generation of neutralizing antibodies.^{5,6} The CD8⁺ T cell response plays a pivotal role in the efficient clearance of SARS-CoV-2,⁷ whereas

Received: July 18, 2025

Revised: December 10, 2025

Accepted: December 16, 2025

Published: December 31, 2025



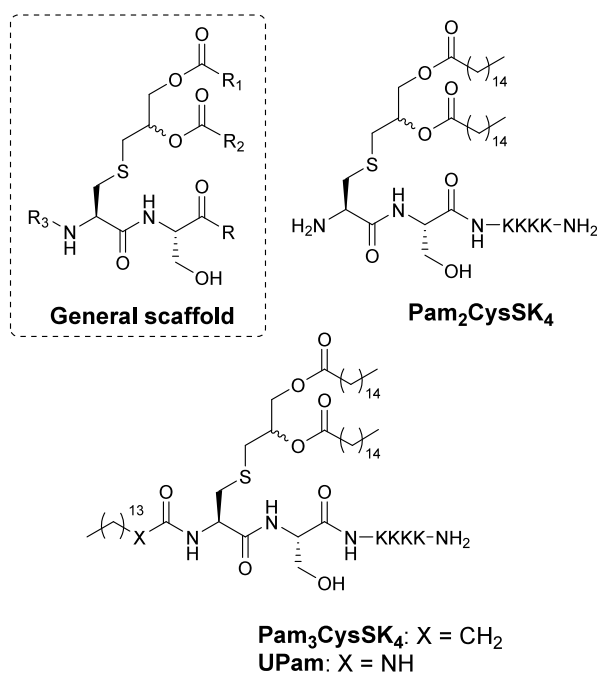


Figure 1. General scaffold representing Pam ligands. Structures of different TLR2 ligands: Pam₂CysSK₄ (TLR2/6), Pam₃CysSK₄ (TLR2/1), and UPam (TLR2).

the CD4⁺ T cell response is involved in potentiating effective viral control by long-lasting virus-specific CD8⁺ and B cell immunity.^{8–10} The integration of conserved dominant B cell epitopes, against which neutralizing antibodies can be targeted, further enhances vaccine efficacy and immunological memory. In recent years, the advent of immunomodulatory molecules, such as Toll-like receptor 2 (TLR2) ligands, specifically lipopeptides, has impacted vaccine design. TLR2, a member of the Toll-like receptor family, plays a crucial role in the recognition of pathogen-associated molecular patterns (PAMPs), triggering innate immune responses. Synthetic lipopeptides, composed of both a lipid and a peptide part, can be used to mimic PAMPs, making them potent activators of the innate immune system. The lipopeptides Pam₃CysSK₄ and Pam₂CysSK₄ have been extensively explored as adjuvants for various vaccine treatments (Figure 1), as they engage with TLR2/TLR1 or TLR2/TLR6 heterodimers, respectively. Such lipopeptides have been covalently attached to antigenic peptides to create self-adjuvanting vaccines.^{11–15} Both ligands consist of a dipalmitoyl functionalized cysteine moiety, of which the C-terminus is coupled to the amino acid serine. Nearly half a century ago, it was demonstrated that the stereochemistry of the glycerol part significantly influences the ligand activity. The naturally occurring form of Pam₃Cys comprises glycerol with the R-configuration. However, for the sake of synthetic convenience, most often, diastereomeric mixtures of Pam₃Cys are employed in vaccine formulations. A previous study has revealed that enantiomerically pure ligands can outperform the diastereoisomeric R,S-mixture in terms of activity.¹⁶ Furthermore, it has been shown that the R-epimer surpasses its S-counterpart in inducing cytokines and stimulating antibody responses when mice were injected with whole protein antigens in combination with the Pam₃Cys adjuvants.^{17–21}

Extensive research efforts have been dedicated to increasing the stimulatory efficacy of the Pam₃Cys ligands by systematic

variation of the amino acid sequence flanking the palmitoylated cysteine residue and the introduction of modifications within the lipid moieties. It has been demonstrated that the length of the ester-bound fatty acid chains plays an essential role in human TLR2 activation. Buwitt-Beckmann et al.²² synthesized various lipopeptides, varying both the fatty acid chain length and the lipid chain compositions at the ester and amide positions. It was shown that lipopeptides featuring short-chain O-acetylated fatty acids failed to stimulate responses in human TLR2-transfected HEK293 cells. Longer fatty acid chains (R₁, R₂ > C₈, Figure 1) proved to be essential for TLR2 activation. In contrast, the amide-bound fatty acid was of less importance, as even short-chain lengths (C₅) resulted in adequate activity. Furthermore, it has been demonstrated that a peptide sequence of at least three amino acid residues is essential for the immunogenicity of the Pam₃Cys lipopeptides. The amphiphilic nature of lipopeptides is widely acknowledged as a fundamental physicochemical characteristic.²³ Nevertheless, this can present a significant challenge, as it can lead to aggregation and precipitation in aqueous solutions, resulting in reduced bioactivity. To improve the Pam₃CSK₄ ligand, Willems et al.²⁴ explored the exchange of the N-acyl lipid for an N-tetradecylcarbonyl chain, generating the so-called UPam (Figure 1), which proved to be an improved TLR2/1 ligand. The UPam ligand was further optimized by changing the amino acid flanking the cysteine. UPam was utilized in a phase I clinical trial, where robust HPV16-specific T cell responses were induced.²⁵ The potential formation of a hydrogen bond between the urea and the carbonyl from Phe-312, located inside the hydrophobic pocket of TLR1, can structurally explain the improved TLR2/1 activity.

We here report the synthesis and immunological evaluation of several UPam conjugates as potential SARS-CoV-2 vaccines. Several previously reported UPam ligands were synthesized, as described by Willems et al.²⁴ and van den Ende,²⁶ altering both the stereochemistry of the glycerol and the nature of the amino acid (AA) flanking the cysteine bearing the palmitoylated glycerol (Figure 2A, 1–8). The selection of derivatives was made based on the abovementioned UPam SAR study, from which it became apparent that replacing the amino acid (AA) serine with either 2,4-diaminobutyric acid (Dab), α-amino-butyric acid (Abu), or alanine (Ala) led to improved activity.²⁴ The ligand featuring the S-glycerol and a serine (9) was taken as a negative control. To investigate their suitability as adjuvants in conjugated coronavirus vaccines, four different synthetic long peptides (SLPs) containing epitope sequences were selected (Figure 2A) and used to generate UPam conjugates. The T cell epitopes include the immunodominant CD8⁺ T cell spike_{525–532} epitope (VNF), an ORF1ab_{4736–4743} CD8⁺ T cell epitope (REL), and a CD4⁺ T cell epitope derived from the spike protein_{998–1012} (QQL).^{7–10} The B cell epitope (B1) was derived from a sequence of the receptor-binding domain of the SARS-CoV-2 spike protein, which can interact with the cell receptor ACE-2; this binding is essential for entry into cells.²⁸ The B1 sequence was coupled to universal helper epitope PADRE (PB1). The PADRE epitope has previously been coupled to a linear B cell epitope, showing improved specific antibody responses compared to uncoupled.^{10,29} By harnessing the cellular immunity driven by CD4⁺ or CD8⁺ T cell epitopes and antibody-mediated protection generated against the incorporated B cell epitopes, we aimed to synthesize vaccines that elicit a rapid and potent TLR2-related immune

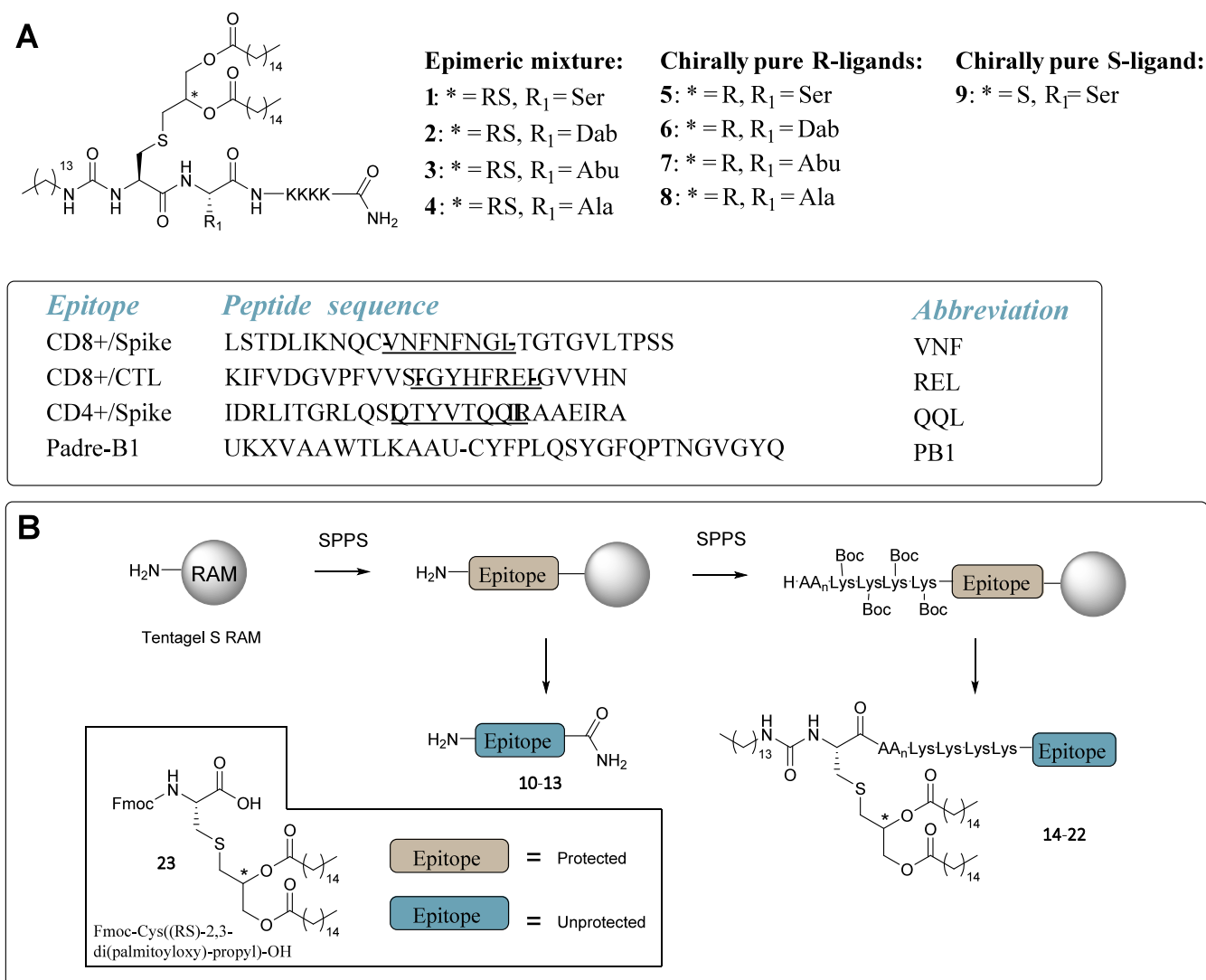


Figure 2. (A) Synthesized UPam derivatives (1–9) and peptide sequences containing the SARS-CoV-2 epitopes (underlined) used in this study. (B) Synthesis of reference peptide epitopes 10–13 and UPam conjugates 14–22 using SPPS.

response and provide robust and long-lasting protection against SARS-CoV-2.

RESULTS AND DISCUSSION

For the assembly of the different UPam conjugates and the reference peptide epitopes, Fmoc-based solid-phase peptide chemistry (SPPS) was used (Figure 2B and Table S1). Starting from RAM Tentagel, the protected epitope-containing peptides were synthesized on a 50 or 100 μ mol scale. From the batches of these intermediate immobilized peptides, 25 μ mol was taken to prepare the reference peptides 10–13. To this end, they were deprotected and cleaved from the resin by using a standard TFA cleavage cocktail and purified by using HPLC with a C₁₈ column. The synthesis of the conjugates was achieved by extension of the immobilized peptides with four protected lysines and the penultimate amino acid, of which the nature was varied. Finally, the appropriate cysteine building block (23) was coupled. After Fmoc removal, treatment with tetradecyl isocyanate, followed by TFA-mediated deprotection and cleavage from the resin, generated conjugates 14–22, which were purified using reversed-phase HPLC with either a

diphenyl or a C₄ column. The yields of the different syntheses are summarized in Table S1.

Immunological Evaluation of the UPam Derivatives and Conjugates

To investigate the potency of the chirally pure R-UPam derivatives, the ligands were tested for their ability to mature murine dendritic cells (DCs) by measuring the IL-12p40 production (Figure 3A). The optically pure R-UPam-Ser (5) performed similar to the diastereomeric mixture (1), and both outperformed the S-UPam-Ser (9) in stimulating DC maturation (Figure 3A). In terms of TLR2 activation, R-UPam-Ser (5) showed activation higher than that of the diastereomeric counterpart (1) (Figure 4A). Upon further analysis of the other modified chirally pure ligands 6–8, it was observed that all induced a similar level of IL-12p40 production as the parent compound 5, with R-UPam-Dab and R-UPam-Abu ligands 6 and 7 outperforming R-UPam-Ser 5. This is in line with previous studies on similar diastereomeric mixtures of UPam ligands.²⁴ Furthermore, all tested ligands were capable of activating the human TLR2 receptor (Figure 4A), with R-UPam-Ala (8) showing the least activation. All chirally pure UPam ligand derivatives 5–8 were

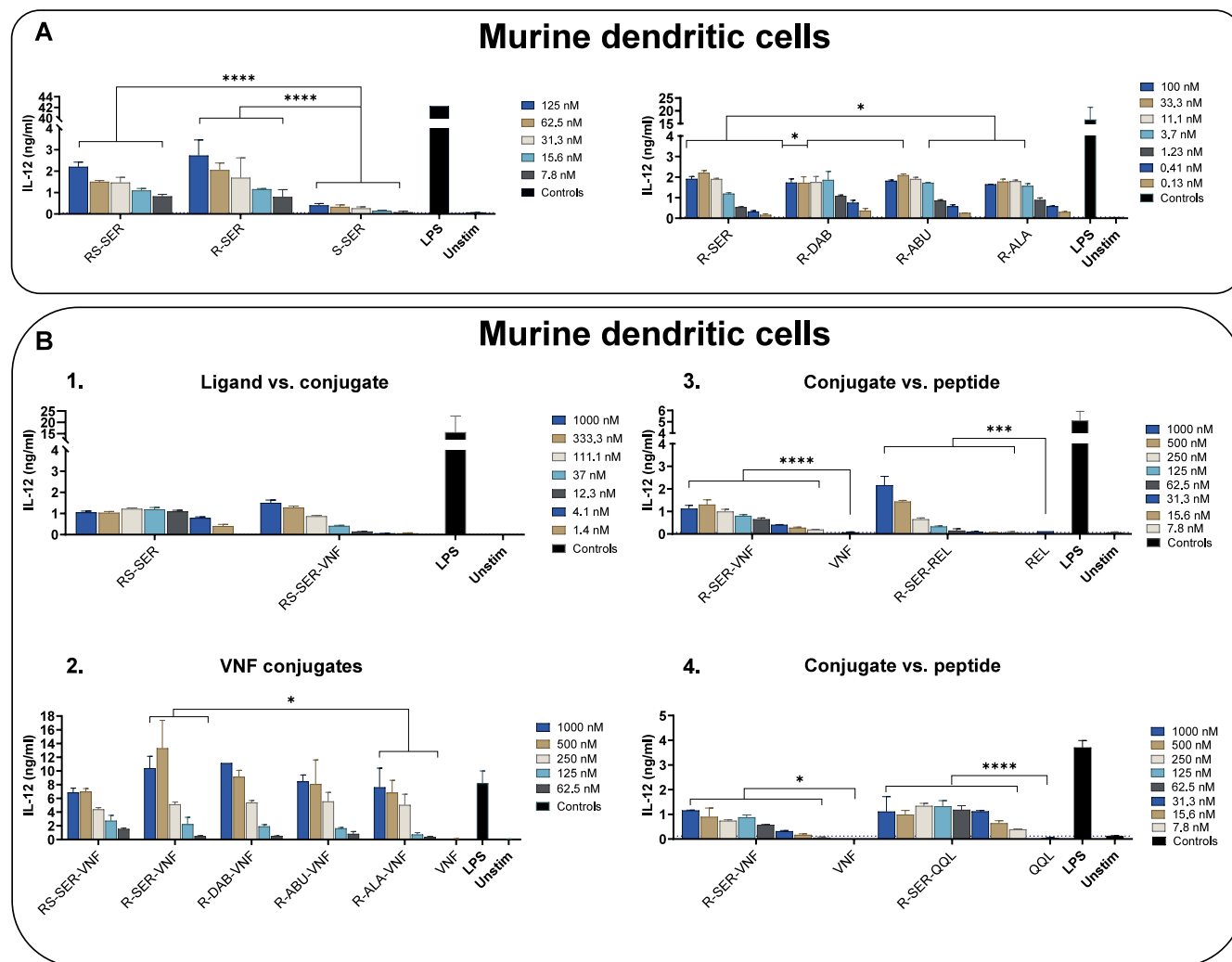


Figure 3. (A) Murine dendritic cell maturation *in vitro* by UPam ligand derivatives at indicated concentrations, as measured by IL-12p40 production: (left) diastereomeric mixture **1** in UPam, R-epimer **5**, and S-epimer **9**; (right) chirally pure UPam derivatives **5–8** and diastereomeric mixture **1**. Nonsignificant differences are not shown. (B) Dendritic cell maturation by UPam conjugates at indicated concentrations, as measured by IL-12p40 production: 1. Diastereomeric mixture conjugated to the VNF epitope (**14**); 2. R-epimer UPam derivatives conjugated to the VNF epitope (**15**, **17–19**); 3. Chirally pure R-epimer conjugated to the VNF epitope (**15**) or REL epitope (**20**); 4. chirally pure R-epimer conjugated to the VNF epitope (**15**) or QQL epitope (**21**).

active only in cells containing human TLR2 and were inactive in wild-type HEK293 cells, as measured by IL-8 production (Figure S1). Conjugating the diastereomeric UPam-Ser to the SARS-CoV-2 spike CD8⁺ T cell VNF epitope (as in conjugate **14**) did not significantly influence its ability to mature DCs (compared to **1** and **14**, Figure 3B-1). Also, the chirally pure R-UPam VNF conjugates, R-UPam-Ser-VNF **15**, R-UPam-Dab-VNF **17**, R-UPam-Abu-VNF **18**, and R-UPam-Ala-VNF **19**, were all capable of inducing DC maturation to similar levels as the R,S-mixture **14** (Figure 3B-2) and could activate the human TLR2 receptor (Figure 4B). The conjugate R-UPam-Ala-VNF **19** could activate the human TLR2 receptor to a lower extent than did the other conjugates (Figure 4B). The conjugates could activate immune cells specifically through TLR2, as seen upon stimulation of TLR2-deficient murine bone marrow-derived dendritic cells (BMDCs) (Figure S2). TLR2-deficient BMDCs do not respond to UPam compounds, while TLR4 signaling is still intact.

As there were no major differences in terms of DC maturation and TLR2 activation *in vitro* for different chirally

pure R-UPam derivatives, further studies focused on R-UPam-Ser (**9**). The conjugates, in which the chirally pure R-UPam-Ser was conjugated to the CD8⁺ REL epitope (**11**) and the CD4⁺ QQL epitope (**12**), **20** and **21**, respectively, were also able to stimulate IL-12 production. Additionally, the R-UPam-Ser-VNF conjugate **15**, the R-UPam-Ser-REL conjugate **20**, and the R-UPam-Ser-QQL conjugate **21** outperformed the stand-alone epitopes **10–12** (Figure 3B-3/4). Overall, conjugating chirally pure R-UPam-Ser to either CD4⁺ or CD8⁺ T cell epitopes resulted in conjugates capable of maturing dendritic cells *in vitro*.

Next, the immunodominant CD8⁺ T cell spike epitope (VNF) was used *in vivo* to determine the importance of conjugating T cell epitopes. Mice were injected intradermally at the tail base on day 0 (prime) and received a boost vaccination after 14 days (Figure 5, left). To assess the effect of conjugation, mixtures of the VNF peptide (**10**) and UPam ligands **1** or **2** were evaluated and compared to the RS-UPam-Ser and RS-UPam-Dab VNF conjugates (**14** and **16**, respectively). In addition to the two conjugates, RS-UPam-

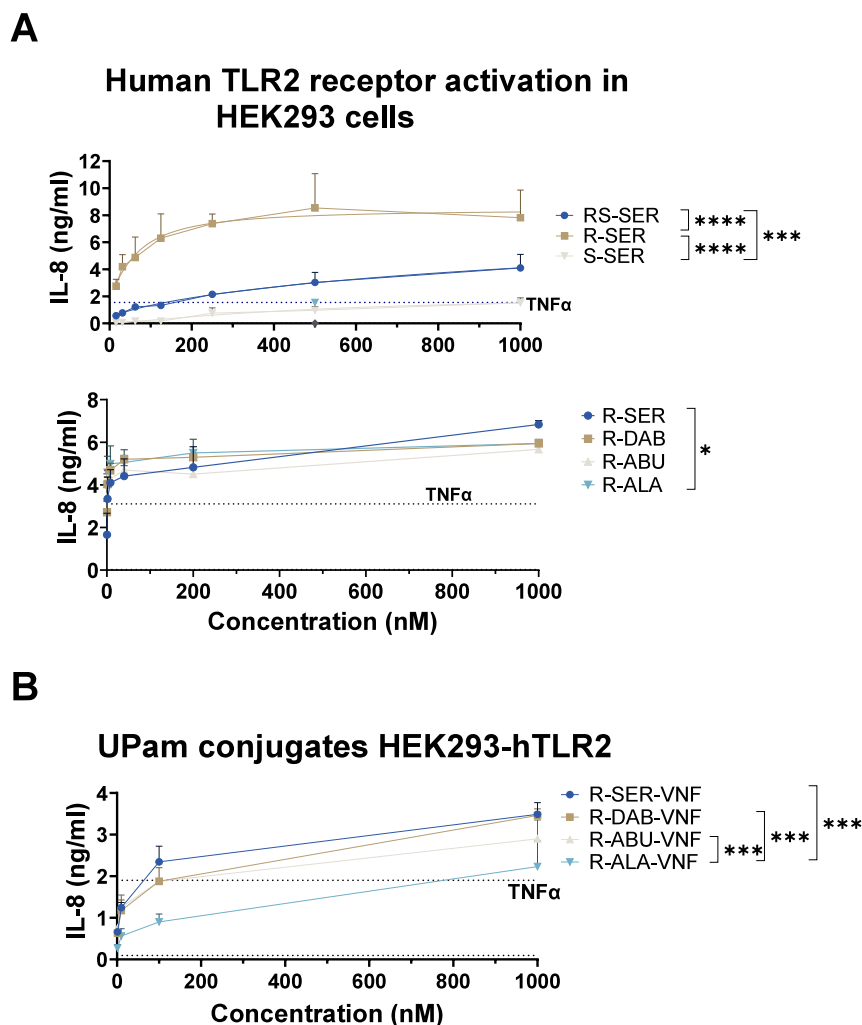


Figure 4. (A) Human TLR2 receptor activation in HEK293 cells *in vitro* by UPam ligand derivatives at indicated concentrations, as measured by IL-8 production: (top) diastereomeric mixture **1** in UPam, R-epimer **5**, and S-epimer **9**; (bottom) chirally pure UPam derivatives **5–8** and diastereomeric mixture **1**. Nonsignificant differences are not shown. (B) Human TLR2 receptor activation by UPam conjugates at indicated concentrations, as measured by IL-8 production: R-epimer UPam derivatives conjugated to the VNF epitope (**15**, **17–19**).

OVA24, RS-UPam-Ser conjugated to a synthetic ovalbumin-derived SLP containing the MHC-I epitope SIINFEKL (OVA24: DEVSGLEQLSIINFEKLAAAAAK), was used as a control compound as no corona-specific T cell responses can be raised by this conjugate.³⁰ The levels of VNFNFNGL- or SIINFEKL-specific CD8⁺ T cells (control experiment) in the blood of the vaccinated mice were determined at days 10 and 20 after initial injection using either an MHC class I-VNFNFNGL or MHC class I-SIINFEKL tetramer. As shown in Figure 5, there is no difference in spike-specific CD8⁺ T cell responses *in vivo* between the two different UPam ligands mixed with a VNF epitope (**1** + **10** vs **2** + **10**). 10 days after initial injection, these spike-specific T cell responses were similar to what was observed when injecting mice with the UPam conjugate containing the unrelated ovalbumin-derived epitope (RS-UPam-OVA24). However, 20 days after the initial injection, the responses increased compared to the negative control RS-UPam-OVA24. The conjugates outperformed the mixtures with regard to specific T cell responses for both UPam derivatives (**1** + **10** vs **14** and **2** + **10** vs **16**), in line with what has been seen in previous studies,^{30,31} with RS-UPam-Dab **16** slightly outperforming the original RS-UPam-Ser **14**.

To further demonstrate the importance of UPam conjugation for CD8⁺ T cell responses *in vivo*, the chirally pure R-UPam-Ser-VNF (**15**), R-UPam-Ser-REL (**20**), and R-UPam-Ser-QQL (**21**) were injected intradermally in the tail base in mice. Mice received a prime vaccination at day 0 and a boost vaccination after 14 days (Figure 6, left).³⁰ The levels of antigen-specific CD8⁺ T cells in the blood of the vaccinated mice were determined at days 10 and 20 using either an MHC class I-VNFNFNGL or MHC class I-TGYHFREL tetramer (Figure 6, right). The R-UPam-Ser-QQL CD4 epitope conjugate (**21**) was used as a negative control, as it was not expected to give any MHC class I-specific T cell response seen by the two aforementioned tetramers. 20 days after the initial vaccination, we see that the TLR2 ligands conjugated to either the VNF or the REL CD8⁺ epitopes were both capable of inducing two independent SARS-CoV-2-specific CD8 T cell responses. The control conjugate QQL (**21**) did not induce detectable CD8 T cell responses. Therefore, conjugating T cell epitopes to UPam can augment specific T cell responses *in vivo*.

It has previously been shown that the production of neutralizing antibodies is of importance in SARS-CoV-2 vaccination.⁷ Therefore, the antibody production in response

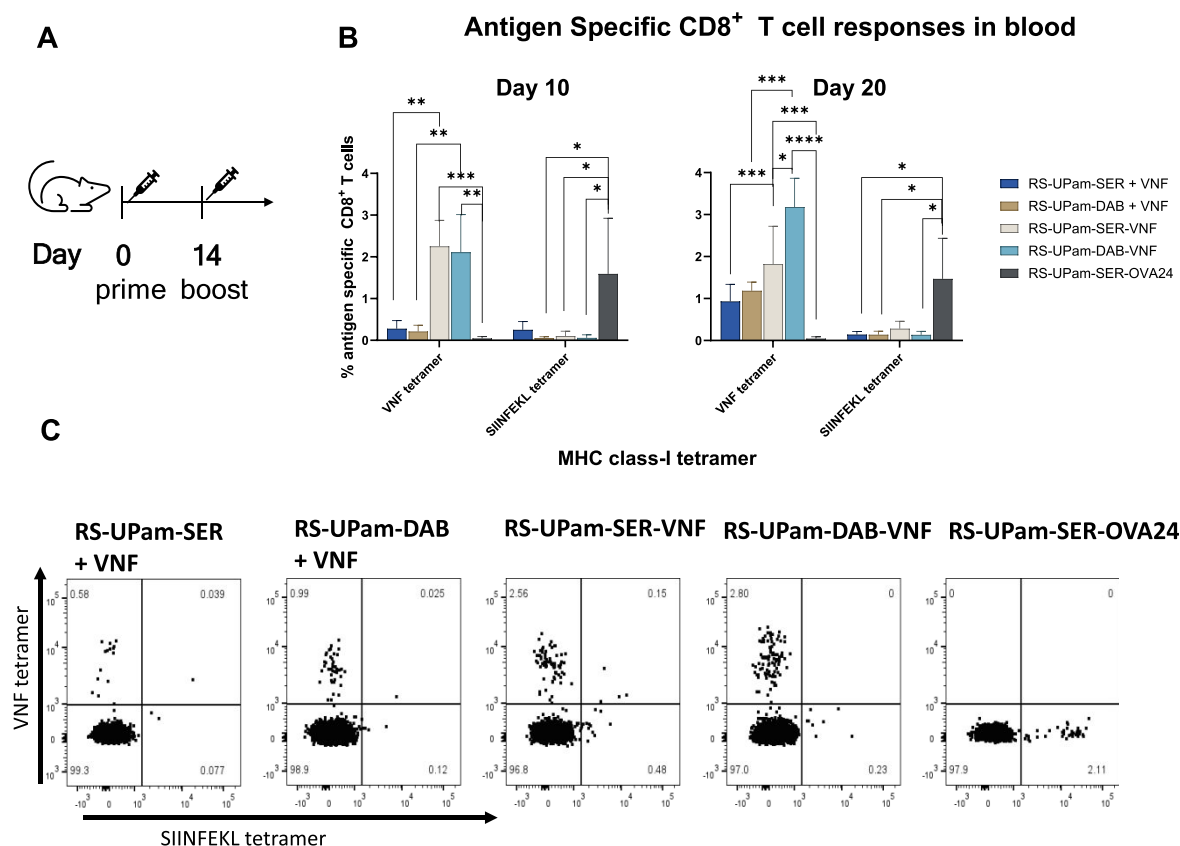


Figure 5. *In vivo* CD8⁺ T cell priming after UPam injection. (A) Naïve C57BL/6 receiving a prime boost vaccination at days 0 and 14 intradermally at the tail base with a final volume of 30 μ L with 2.5 nmol of either peptide VNF (10, 7.35 μ g), peptide mixed to 2.5 nmol of RS-UPam-Ser (1, 3.78 μ g) or 2.5 nmol of RS-UPam-Ser-VNF (14, 11.1 μ g), peptide mixed to 2.5 nmol of RS-UPam-Dab (2, 3.81 μ g) or 2.5 nmol of RS-UPam-Dab-VNF (16, 11.1 μ g), or RS-UPam-Ser conjugated to a control peptide (OVA24). (B) Levels of VNFNFGN- or SIINFEKL-specific CD8⁺ T cells in the blood of C57BL/6 mice determined at days 10 and 20 after initial injection using either an MHC class I-VNFNFGN or MHC class I-SIINFEKL tetramer, seen by flow cytometry. Data represented as mean $\pm$ SD ($n = 5$). (C) Representative flow cytometry plot representing MHC class I-VNFNFGN or MHC class I-SIINFEKL tetramer 20 days post initial injection.

to vaccination with a B cell epitope, coupled to a universal helper peptide PADRE (as in peptide PB1 13), was compared to its R-UPam-Ser conjugate 22. First, the conjugate was tested for its capacity to induce DC maturation *in vitro*, as measured by IL-12 production (Figure 7B). The R-UPam-Ser conjugate induced a significantly higher level of IL-12 than did the stand-alone peptide. At higher concentrations, conjugate 22 outperformed the stand-alone ligand 5. But, at lower concentrations, the ligand proved more potent than the conjugate, highlighting the importance of dosage for IL-12 production and DC maturation.

Subsequently, the efficacy of the UPam-PB1 conjugates in inducing antibody production was tested in the mice. Mice were injected at day 0 (prime) and received two boost vaccinations after 14 and 64 days (Figure 7A). The mice were vaccinated intradermally at the tail base with either R-UPam-Ser (5), PB1 epitope (13), or R-UPam-SER-PB1 conjugate (22) or subcutaneously in the right flank with a positive control for antibody production composed of a mixture of Pam₃CSK₄, incomplete Freund's adjuvant (IFA), and 13. The PADRE-specific CD4⁺ T cell response was observed in the blood 71 days after initial injection (Figure 7C), where the UPam conjugate 22 showed higher PADRE-specific T cell responses compared to both the R-UPam-Ser ligand 5 and the peptide 13 (latter not significant, $p = 0.069$). The IgG antibody serum levels were determined by performing an IgG ELISA

against a linear B cell epitope (B1) derived from the ACE-2 receptor-binding domain of the SARS-CoV-2 spike protein (Figure 7D). Sera from mice were collected 78 days after initial injection. In terms of B1-specific antibody production, the conjugate 22 results in dilution-dependent antibody responses. Antibody levels were similar to those of PADRE-B cell epitope peptide 13, indicating that the linear epitope is preserved in the UPam conjugate vaccine. Importantly, the antibody responses in sera were cross-reactive to the native spike SARS-CoV-2 protein (Figure 7E), and higher responses induced by the conjugate vaccine 22 were observed than by the PB1—we report that a linear B cell epitope from a SARS-CoV-2 spike protein could successfully be conjugated to UPam without loss of integrity of the epitope sequence.

CONCLUSIONS

This work describes the synthesis of several UPam conjugates (14–22) containing four different SLPs (VNF, REL, QQL, and PB1) using the solid-phase peptide synthesis. In addition, four chirally pure R-UPam derivatives (5–8) were synthesized, in which the AA flanking the core cysteine, serine (Ser), was replaced by 2,4-diaminobutyric acid (Dab), α -aminobutyric acid (Abu), or alanine (Ala). Evaluation of the conjugates demonstrates the potential of chirally pure R-UPam derivatives in enhancing dendritic cell (DC) maturation and stimulating specific immune responses. The chirally pure R-UPam-Ser 5

UPam Conjugates in Antibody Production

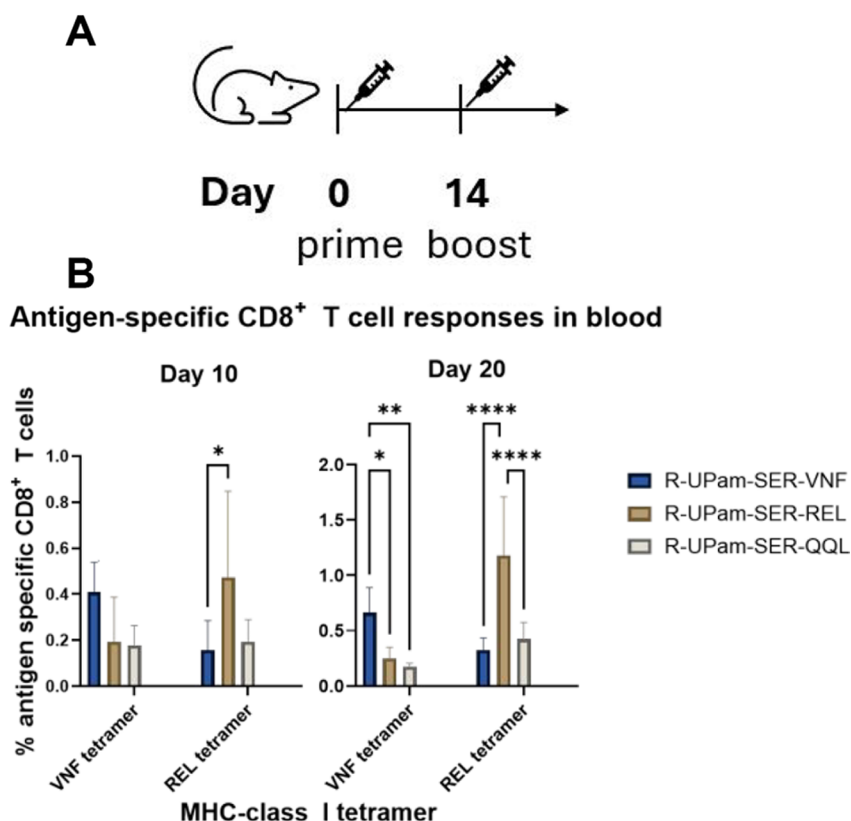


Figure 6. *In vivo* CD8⁺ T cell priming after the UPam injection of conjugates of two SARS-CoV-2 CD8 epitopes. (A) Naïve C57BL/6 receiving a prime boost intradermal vaccination at days 0 and 14 at the tail base with a final volume of 30 μ L with either 2.5 nmol chirally pure R-UPam conjugated to the VNF epitope (11.1 μ g), REL epitope (10.7 μ g), or control CD4 QQL epitope (11.8 μ g). (B) Levels of VNFNFGNL- or TGYHFREL-specific CD8⁺ T cells in the blood of C57BL/6 mice determined at days 10 and 20 after initial injection using either an MHC class I-VNFNFGNL or MHC class I-TGYHFREL tetramer, seen by flow cytometry. Data represented as mean $\pm$ SD ($n = 5$).

performed equally well as the corresponding diastereomeric mixture and outperformed S-UPam-Ser 9, indicating the superiority of the R-enantiomer. Optimization of the UPam ligand led to R-UPam-Dab 6 and R-UPam-Abu 7, which showed improved IL-12p40 production by DC. Conjugation of R-UPam ligands to the VNF epitope did not compromise DC maturation or TLR2 activation, and similar results were observed with the REL and QQL epitopes, highlighting the versatility of UPam conjugates. Conjugation of the UPam ligands to the VNF epitope delivered conjugates that significantly enhanced spike-specific CD8⁺ T cell responses in comparison to the mixture of the antigenic peptide and adjuvant. The R-UPam-Dab conjugate outperformed the R-UPam-Ser analog, confirming the enhanced efficacy of the optimized ligands. The R-UPam-Ser conjugates led to improved T cell responses. These conjugates were also capable of inducing antibody responses, showing that conjugation does not hamper the recognition of a linear B epitope. Overall, the findings described here underscore the potential of chirally pure R-UPam conjugates in vaccine development, particularly in inducing robust T cell responses while also highlighting their potential use to enhance humoral immunity. To this end, other adjuvants can be investigated, or mixtures of the conjugates with additional adjuvants (for example, IFA) may be explored.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.bioconjchem.5c00345>.

Detailed experimental procedures, supplementary figures and copies of NMR spectra of the synthesised compounds (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Ferry Ossendorp – Department of Immunology, Leiden University Medical Center, Leiden 2333 ZA, The Netherlands; Email: F.A.Ossendorp@lumc.nl

Dmitri V. Filippov – Leiden University, Leiden 2333 CC, The Netherlands; orcid.org/0000-0002-6978-7425; Email: filippov@chem.leidenuniv.nl

Authors

Giulia Castello – Department of Immunology, Leiden University Medical Center, Leiden 2333 ZA, The Netherlands

Marjolein M.E. Isendoorn – Leiden University, Leiden 2333 CC, The Netherlands

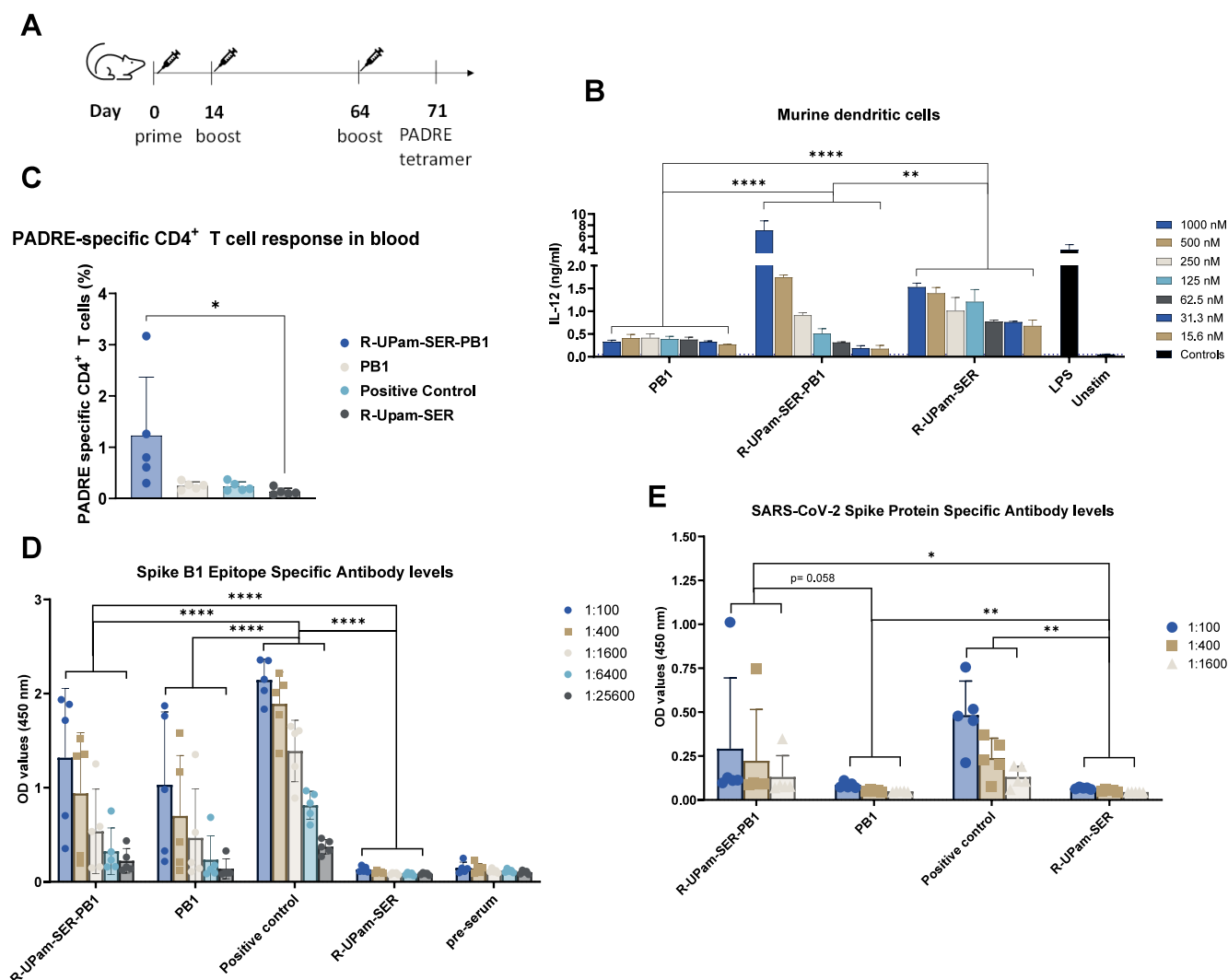


Figure 7. *In vivo* antibody production of SARS-CoV-2 spike protein. (A) Vaccination scheme. Naïve C57BL/6 received a prime boost vaccination at days 0, 14, and 64 with either the 20 nmol intradermally tail base of the PB1 epitope (13, 21.1 μ g), R-UPam-Ser-PB1 conjugate (22, 100 μ g), R-UPam-Ser (5, 30.2 μ g) alone, or subcutaneously in the right flank with a positive control composed of 10 nmol Pam₃CysSK₄ (15.1 μ g) mixed with 1 μ g poly(I:C) and 20 nmol 13 (21.1 μ g) in 50% incomplete Freund's adjuvant and 50% PBS. (B) Murine dendritic cell maturation *in vitro* by UPam-PB1 conjugates at indicated concentrations, as measured by IL-12p40 production for chirally pure R-epimer conjugated to a B cell epitope B1 (22). (C) Levels of PADRE-specific CD4⁺ T cells in the blood of C57BL/6 mice determined at day 71 after initial injection using an MHC class II-PADRE tetramer, as seen by flow cytometry. Data represented as mean $\pm$ SD ($n = 5$). Nonsignificant differences are not shown. One-way ANOVA with Tukey's multiple comparison is used. (D) Detection of antibody binding activities against the linear B1 epitope. The biotinylated B1 epitope was bound to streptavidin-coated plates for ELISA detection of specific serum IgG antibodies and (E) antibody reactivity to native SARS-CoV-2 spike protein. Antibody levels in same sera as panel (D) were measured by IgG ELISA in serum-dilution samples on spike protein-coated plates from mice at day 78 post initial injection. Intensities were measured at an absorbance of 450 nm with a microplate reader.

Marcel Camps – Department of Immunology, Leiden University Medical Center, Leiden 2333 ZA, The Netherlands

Nico J. Meeuwenoord – Leiden University, Leiden 2333 CC, The Netherlands

Vincent F. H. Verhoeks – Leiden University, Leiden 2333 CC, The Netherlands; orcid.org/0009-0003-4144-0619

Thomas C. van den Ende – Leiden University, Leiden 2333 CC, The Netherlands

Jeroen D. C. Codée – Leiden University, Leiden 2333 CC, The Netherlands; orcid.org/0000-0003-3531-2138

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acs.bioconjchem.5c00345>

Author Contributions

[†]G.C. and M.M.E.I. contributed equally to this work.

Author Contributions

G.C. and M.C. performed all immunological experiments and formatted the data. M.M.E.I., V.F.H.V., T.C. v. d. E. and N.J.M. performed all chemical syntheses, including building block preparation and solid-phase syntheses. Experimental design and execution were overseen by D.V.F. and F.O. The manuscript was drafted by M.M.E.I., G.C., and D.V.F., with contributions and revisions from J.D.C.C. and F.O. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The work described in this paper is funded by ICI grant ICI00031 from the Institute of Chemical Immunology.

■ REFERENCES

- (1) Montero, D. A.; Vidal, R. M.; Velasco, J.; Carreño, L. J.; Torres, J. P.; Benachi, O. M. A.; Tovar-Rosero, Y. Y.; Oñate, A. A.; O’Ryan, M. Two Centuries of Vaccination: Historical and Conceptual Approach and Future Perspectives. *Front. Public Health* **2024**, *11*, No. 1326154.
- (2) Smith, C. C.; Olsen, K. S.; Gentry, K. M.; Sambade, M.; Beck, W.; Garness, J.; Entwistle, S.; Willis, C.; Vensko, S.; Woods, A.; Fini, M.; Carpenter, B.; Routh, E.; Kodysh, J.; O’Donnell, T.; Haber, C.; Heiss, K.; Stadler, V.; Garrison, E.; Sandor, A. M.; Ting, J. P. Y.; Weiss, J.; Krajewski, K.; Grant, O. C.; Woods, R. J.; Heise, M.; Vincent, B. G.; Rubinstein, A. Landscape and Selection of Vaccine Epitopes in SARS-CoV-2. *Genome Med.* **2021**, *13* (1), 1–23.
- (3) Palatnik-de-Sousa, I.; Wallace, Z. S.; Cavalcante, S. C.; Ribeiro, M. P. F.; Silva, J. A. B. M.; Cavalcante, R. C.; Scheuermann, R. H.; Palatnik-de-Sousa, C. B. A Novel Vaccine Based on SARS-CoV-2 CD4+ and CD8+ T Cell Conserved Epitopes from Variants Alpha to Omicron. *Sci. Rep.* **2022**, *12* (1), 1–21.
- (4) Magazine, N.; Zhang, T.; Bungwon, A. D.; McGee, M. C.; Wu, Y.; Veggiani, G.; Huang, W. Immune Epitopes of SARS-CoV-2 Spike Protein and Considerations for Universal Vaccine Development. *Immunohorizons* **2024**, *8* (3), 214–226.
- (5) Azkur, A. K.; Akdis, M.; Azkur, D.; Sokolowska, M.; van de Veen, W.; Brüggemann, M. C.; O’Mahony, L.; Gao, Y.; Nadeau, K.; Akdis, C. A. Immune Response to SARS-CoV-2 and Mechanisms of Immunopathological Changes in COVID-19. *Allergy: European Journal of Allergy and Clinical Immunology* **2020**, *75* (7), 1564–1581.
- (6) Moss, P. The T Cell Immune Response against SARS-CoV-2. *Nat. Immunol.* **2022**, *23* (2), 186–193.
- (7) Pardieck, I. N.; van der Sluis, T. C.; van der Gracht, E. T. I.; Veerkamp, D. M. B.; Behr, F. M.; van Duikeren, S.; Beyrend, G.; Rip, J.; Nadeau, R.; Beyranvand Nejad, E.; Mülling, N.; Brasem, D. J.; Camps, M. G. M.; Myeni, S. K.; Bredenbeek, P. J.; Kikkert, M.; Kim, Y.; Cicin-Sain, L.; Abdelaal, T.; van Gisbergen, K. P. J. M.; Franken, K. L. M. C.; Drijfhout, J. W.; Melief, C. J. M.; Zondag, G. C. M.; Ossendorp, F.; Arens, R. A Third Vaccination with a Dingle T Cell Epitope Confers Protection in a Murine Model of SARS-CoV-2 Infection. *Nat. Commun.* **2022**, *13* (1), 3966.
- (8) Bergamaschi, L.; Mescia, F.; Turner, L.; Hanson, A. L.; Kotagiri, P.; Dunmore, B. J.; Ruffieux, H.; De Sa, A.; Huhn, O.; Morgan, M. D.; Gerber, P. P.; Wills, M. R.; Baker, S.; Calero-Nieto, F. J.; Doffinger, R.; Dougan, G.; Elmer, A.; Goodfellow, I. G.; Gupta, R. K.; Hosmillo, M.; Hunter, K.; Kingston, N.; Lehner, P. J.; Matheson, N. J.; Nicholson, J. K.; Petrunkina, A. M.; Richardson, S.; Saunders, C.; Thaventhiran, J. E. D.; Toonen, E. J. M.; Weekes, M. P.; Göttgens, B.; Toshner, M.; Hess, C.; Bradley, J. R.; Lyons, P. A.; Smith, K. G. C. Longitudinal Analysis Reveals That Delayed Bystander CD8+ T Cell Activation and Early Immune Pathology Distinguish Severe COVID-19 From Mild Disease. *Immunity* **2021**, *54* (6), 1257–1275.
- (9) Notarbartolo, S.; Ranzani, V.; Bandera, A.; Guarini, P.; Bevilacqua, V.; Putignano, A. R.; Gobbi, A.; Galeota, E.; Manara, C.; Bombaci, M.; Pesce, E.; Zagato, E.; Favalli, A.; Sarnicola, M. L.; Curti, S.; Crosti, M.; Martinovic, M.; Fabbri, T.; Marini, F.; Donnici, L.; Lorenzo, M.; Mancino, M.; Ungaro, R.; Lombardi, A.; Mangioni, D.; Muscatello, A.; Aliberti, S.; Blasi, F.; De Feo, T.; Prati, D.; Manganaro, L.; Granucci, F.; Lanzavecchia, A.; Francesco, R. D.; Gori, A.; Grifantini, R.; Abrignani, S. Integrated Longitudinal Immunophenotypic, Transcriptional and Repertoire Analyses Delineate Immune Responses in COVID-19 Patients. *Sci. Immunol.* **2021**, *6* (62), No. eabg5021.
- (10) van Bergen, J.; Camps, M. G. M.; Pardieck, I. N.; Veerkamp, D.; Leung, W. Y.; Leijts, A. A.; Myeni, S. K.; Kikkert, M.; Arens, R.; Zondag, G. C.; Ossendorp, F. Multiantigen Pan-Sarbecovirus DNA Vaccines Generate Protective T Cell Immune Responses. *JCI Insight* **2023**, *8* (21), 1–15.
- (11) Zom, G. G.; Welters, M. J. P.; Loof, N. M.; Goedemans, R.; Loughheed, S.; Valentijn, R. R. P. M.; Zandvliet, M. L.; Meeuwenoord, N. J.; Melief, C. J. M.; de Gruijl, T. D.; Van der Marel, G. A.; Filippov, D. V.; Ossendorp, F.; Van der Burg, S. H. TLR2 Ligand-Synthetic Long Peptide Conjugates Effectively Stimulate Tumor-Draining Lymph Node T Cells of Cervical Cancer Patients. *Oncotarget* **2016**, *7* (41), 67087–67100.
- (12) Ashhurst, A. S.; Johansen, M. D.; Maxwell, J. W. C.; Stockdale, S.; Ashley, C. L.; Aggarwal, A.; Siddique, R.; Miemczyk, S.; Nguyen, D. H.; Mackay, J. P.; Counoupas, C.; Byrne, S. N.; Turville, S.; Steain, M.; Triccas, J. A.; Hansbro, P. M.; Payne, R. J.; Britton, W. J. Mucosal TLR2-Activating Protein-Based Vaccination Induces Potent Pulmonary Immunity and Protection against SARS-CoV-2 in Mice. *Nat. Commun.* **2022**, *13* (1), 1–18.
- (13) Jayakumar, A.; Castilho, T. M.; Park, E.; Goldsmith-Pestana, K.; Blackwell, J. M.; McMahon-Pratt, D.; Buxbaum, L. U. TLR1/2 Activation during Heterologous Prime-Boost Vaccination (DNA-MVA) Enhances CD8+ T Cell Responses Providing Protection against Leishmania (Viannia). *PLoS Negl Trop Dis* **2011**, *5* (6), No. e1204.
- (14) Lu, B. L.; Williams, G. M.; Verdon, D. J.; Dunbar, P. R.; Brimble, M. A. Synthesis and Evaluation of Novel TLR2 Agonists as Potential Adjuvants for Cancer Vaccines. *J. Med. Chem.* **2020**, *63* (5), 2282–2291.
- (15) Halliday, A.; Turner, J. D.; Guimarães, A.; Bates, P. A.; Taylor, M. J. The TLR2/6 Ligand PAM2CSK4 Is a Th2 Polarizing Adjuvant in Leishmania Major and Brugia Malayi Murine Vaccine Models. *Parasit Vectors* **2016**, *9* (1), 1–9.
- (16) Wiesmüller, K. H.; Bessler, W.; Jung, G. Synthesis of the Mitogenic S-[2, 3-Bis (Palmitoyloxy) Propyl]-N-Palmitoylpentapeptide from Escherichia Coli Lipoprotein. *Hoppe-Seyler’s Z. Physiol. Chem.* **1983**, *364* (1), 593–606.
- (17) Metzger, J.; Jung, G.; Bessler, W. G.; Hoffmann, P.; Strecker, M.; Lieberknecht, A.; Schmidt, U. Lipopeptides Containing 2-(Palmitoylamino)-6,7-Bis(Palmitoyloxy)Heptanoic Acid: Synthesis, Stereospecific Stimulation of B-Lymphocytes and Macrophages, and Adjuvanticity in Vivo and in Vitro. *J. Med. Chem.* **1991**, *34* (7), 1969–1974.
- (18) Makimura, Y.; Asai, Y.; Taiji, Y.; Sugiyama, A.; Tamai, R.; Ogawa, T. Correlation between Chemical Structure and Biological Activities of Porphyromonas Gingivalis Synthetic Lipopeptide Derivatives. *Clin. Exp. Immunol.* **2006**, *146* (1), 159–168.
- (19) Asai, Y.; Makimura, Y.; Ogawa, T. Toll-like Receptor 2-Mediated Dendritic Cell Activation by a Porphyromonas Gingivalis Synthetic Lipopeptide. *J. Med. Microbiol.* **2007**, *56* (4), 459–465.
- (20) Engel, J.; Mersmann, S.; Norrby, P. O.; Bolm, C. Mechanistic Insights into the Iridium-Catalyzed Hydrogenations of α,β -Unsaturated Ketones. *ChemCatChem* **2016**, *8* (19), 3099–3106.
- (21) Khan, S.; Weterings, J. J.; Britten, C. M.; de Jong, A. R.; Graafland, D.; Melief, C. J. M.; van der Burg, S. H.; van der Marel, G.; Overkleef, H. S.; Filippov, D. V.; Ossendorp, F. Chirality of TLR-2 Ligand Pam3CysSK4 in Fully Synthetic Peptide Conjugates Critically Influences the Induction of Specific CD8+ T-Cells. *Mol. Immunol.* **2009**, *46* (6), 1084–1091.
- (22) Buwitt-Beckmann, U.; Heine, H.; Wiesmüller, K.; Jung, G.; Brock, R.; Ulmer, A. J. Lipopeptide Structure Determines TLR2 Dependent Cell Activation Level. *FEBS J.* **2005**, *272*, 6354–6364.
- (23) Zähringer, U.; Lindner, B.; Inamura, S.; Heine, H.; Alexander, C. TLR2 - Promiscuous or Specific? A Critical Re-Evaluation of a Receptor Expressing Apparent Broad Specificity. *Immunobiology* **2008**, *213* (3–4), 205–224.
- (24) Willems, M. M. J. H. P.; Zom, G. G.; Khan, S.; Meeuwenoord, N.; Melief, C. J. M.; van der Stelt, M.; Overkleef, H. S.; Codee, J. D. C.; van der Marel, G. A.; Ossendorp, F.; Filippov, D. V. N-Tetradecylcarbamyl Lipopeptides as Novel Agonists for Toll-like Receptor 2. *J. Med. Chem.* **2014**, *57* (15), 6873–6878.
- (25) Speetjens, F. M.; Welters, M. J. P.; Slingerland, M.; van Poelgeest, M. I. E.; de Vos van Steenwijk, P. J.; Roozen, I.; Boekstijn, S.; Loof, N. M.; Zom, G. G.; Valentijn, A. R. P. M.; Krebber, W. J.;

Meeuwenoord, N. J.; Janssen, C. A. H.; Melief, C. J. M.; van der Marel, G. A.; Filippov, D. V.; van der Burg, S. H.; Gelderblom, H.; Ossendorp, F. Intradermal Vaccination of HPV-16 E6 Synthetic Peptides Conjugated to an Optimized Toll-like Receptor 2 Ligand Shows Safety and Potent T Cell Immunogenicity in Patients with HPV-16 Positive (Pre-)Malignant Lesions. *J. Immunother Cancer* **2022**, *10* (10), No. e005016.

(26) van den Ende, T. C. *Design, Synthesis and Evaluation of Antigenic Peptide Conjugates Containing Toll-like Receptor Agonists*; Leiden University, 2023. <https://hdl.handle.net/1887/3564186>.

(27) Li, C.; Lee, A.; Grigoryan, L.; Arunachalam, P. S.; Scott, M. K. D.; Trisal, M.; Wimmers, F.; Sanyal, M.; Weidenbacher, P. A.; Feng, Y.; Adamska, J. Z.; Valore, E.; Wang, Y.; Verma, R.; Reis, N.; Dunham, D.; O'Hara, R.; Park, H.; Luo, W.; Gitlin, A. D.; Kim, P.; Khatri, P.; Nadeau, K. C.; Pulendran, B. Mechanisms of Innate and Adaptive Immunity to the Pfizer-BioNTech BNT162b2 Vaccine. *Nat. Immunol* **2022**, *23* (4), 543–555.

(28) Lan, J.; Ge, J.; Yu, J.; Shan, S.; Zhou, H.; Fan, S.; Zhang, Q.; Shi, X.; Wang, Q.; Zhang, L.; Wang, X. Structure of the SARS-CoV-2 Spike Receptor-Binding Domain Bound to the ACE2 Receptor. *Nature* **2020**, *581* (7807), 215–220.

(29) Alexander, J.; del Guercio, M.-F.; Maewal, A.; Qiao, L.; Fikes, J.; Chesnut, R. W.; Paulson, J.; Bundle, D. R.; DeFrees, S.; Sette, A. Linear PADRE T Helper Epitope and Carbohydrate B Cell Epitope Conjugates Induce Specific High Titer IgG Antibody Responses. *J. Immunol.* **2000**, *164* (3), 1625–1633.

(30) Khan, S.; Bijker, M. S.; Weterings, J. J.; Tanke, H. J.; Adema, G. J.; Van Hall, T.; Drijfhout, J. W.; Melief, C. J. M.; Overkleef, H. S.; Van Der Marel, G. A.; Filippov, D. V.; Van Der Burg, S. H.; Ossendorp, F. Distinct Uptake Mechanisms But Similar Intracellular Processing of Two Different Toll-Like Receptor Ligand-Peptide Conjugates in Dendritic Cells. *J. Biol. Chem.* **2007**, *282* (29), 21145–21159.

(31) Zom, G. G.; Willems, M. M. J. H. P.; Khan, S.; Van Der Sluis, T. C.; Kleinovink, J. W.; Camps, M. G. M.; Van Der Marel, G. A.; Filippov, D. V.; Melief, C. J. M.; Ossendorp, F. Novel TLR2-Binding Adjuvant Induces Enhanced T Cell Responses and Tumor Eradication. *J. Immunother Cancer* **2018**, *6* (1), 1–13.



CAS BIOFINDER DISCOVERY PLATFORM™

**PRECISION DATA
FOR FASTER
DRUG
DISCOVERY**

CAS BioFinder helps you identify
targets, biomarkers, and pathways

Unlock insights

CAS
A division of the
American Chemical Society