Cancer vaccine strategies to improve immunotherapy: many roads lead to Rome

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DUAL PEPTIDE CONJUGATES SIMULTANEOUSLY TRIGGERING TLR2 AND TLR7 FOR CANCER VACCINATION

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ABSTRACT

Activation of Toll-like receptors (TLRs) can be exploited during vaccination to promote optimal T cell priming, and the stimulation of different TLRs can determine the outcome of the immune response induced. In this study, we investigate the possibility of simultaneously triggering TLR2 and TLR7 via a molecularly defined dual conjugate vaccine to induce antigen specific CD8 and CD4 T cell responses. We show that conjugation of a TLR2 or a TLR7 agonist to a peptide moiety enhances the efficacy of the vaccine compared to administration of unconjugated peptide and adjuvant. In addition, we report vaccine efficacy of dual conjugates bearing the two TLR agonists positioned on the N- and C-terminal ends of two synthetic long peptides. We show that these conjugates are immunologically active and can induce functional CD8 and CD4 responses, by analyzing cytokine profiles of specific T cells. These data show the potency of TLR2 and TLR7 dual conjugates and set the basis for their use for peptide-based T cell vaccination.

INTRODUCTION

Peptide-based vaccination necessitates of the combination of peptide antigen with strong adjuvants in order to activate the immune system and to generate an adequate antigen-specific response. The Toll-like receptor (TLR) family is a family of structurally related receptors that has evolved in vertebrate organisms to recognize different pathogen related-structures. Binding of a ligand to its TLR gives start to an inflammatory response and initiates the activation of innate and adaptive immune reactions. For this reason, TLR stimulation is also exploited for vaccination purposes.

While TLR agonists in vaccines are usually provided next to the antigen, we explored direct conjugation of defined TLR agonists to synthetic peptide antigens, to enhance vaccine efficacy via delivery of antigen and maturation signals to the same antigen-presenting cells (APCs). This was demonstrated for at least three different ligands: the TLR2 agonist Pam_3CysSK_4 [1, 2], the TLR4 agonist CRX-527 [3], and the TLR9 ligand CpG [1]. Conjugation of the TLR agonists to peptides resulted in enhanced uptake and antigen processing and presentation by DCs in vitro, and stronger induction of CD8 or CD4 T cell responses in vivo [1-3].

The TLR2 ligand Pam_3CysSK_4 has been successfully employed in cancer treatment. In pre-clinical studies, Pam conjugated vaccines demonstrated effective tumor eradication which was associated with the modulation of the tumor microenvironment towards a pro-inflammatory phenotype [4], as opposed to the suppressive micro-environment of untreated animals. The optimized version of this agonist UPam [5], also named Amplivant [4], has also been selected for the clinical testing of HPV-related malignancies as defined conjugates of six peptides derived from a E6 protein (Trial ID: NCT02821494). These conjugates demonstrated efficacy in activating human dendritic cells as well as enhanced ability to activate specific T cells derived from HPV16+ cervical cancer patients [6].

Recently, we synthetized and tested the conjugation of a TLR7 agonist derived from hydroxy-adenine which also demonstrated enhanced immunological activity in vitro compared to free peptide [7]. TLR7 and 8 represents an interesting TLR pathway for cancer immunotherapy. Both TLR7 and the structurally related TLR8 recognize viral ssRNA [8] and are strong type I interferon inducers, which promote Th1-skewed immunity aimed at clearing infected cells. Conjugation of a TLR7 agonist to protein antigen has been explored in HIV vaccines, where a broader Th1 and CTL response was observed both in mice and nonhuman primates [9]. TLR7 stimulation as local treatment has also been investigated for can-

cer immunotherapy. For example, treatment of tumors in mice with the synthetic agonist MEDI9197 was shown to induce pro-inflammatory cytokines such as IL-12, IFNα and IFNγ, and to activate local innate cells to promote Th1 polarization as well as activation of natural killer and CD8 T cells [10]. The same was reported for the hydroxy-adenine analogue R848 in pancreatic cancer [11].

During infections, pathogens cause the simultaneous triggering of multiple TLR receptors which results in the fine-tuning of the most appropriate immune response. Therefore, the combination of different TLR ligands in cancer vaccines could synergize and potentiate the T cell responses by skewing them towards the most fitting response for tumor clearance. The previous pre-clinical studies with Pam demonstrated enhanced efficacy of the vaccine conjugate compared to the simple mixture of ligand and peptide, however a portion of the animals still failed to clear tumor [2]. To improve this effect, we synthetized a new conjugate bearing both Pam_3CysSK_4 and the TLR7 agonist 2-butoxy-8-hydroxyadenine, with the hypothesis that integration of the signaling from the two TLRs could further skew the immune response induced towards a more efficient anti-tumor T cell immune response.

RESULTS

We previously described that conjugation of either a CD8 or CD4 T cell epitope to the TLR2 ligand Pam_3CysSK_4 led to enhanced priming of T cell responses. We first investigated how the conjugation of the TLR7 agonist to synthetic long peptides containing either the CD8 and CD4 epitopes of ovalbumin (**Fig 1A**) affects the induction of CD8 and CD4 responses *in vivo*, and how they compare to the Pam_3CysSK_4 conjugates. The two sets of conjugates, or their respective components mixed, were injected at equimolar doses in a prime and boost setting (**Fig.1B**). The induction of CD8 responses specific for the ovalbumin epitope SIINFEKL was monitored over time in blood. The Pam_3CysSK_4 conjugates induce significantly higher SIINFEKL-specific CD8 responses compared to an equimolar mixture of the components (**Fig 1C**, left panel), as previously described [2]. Conjugation of the TLR7 agonist 2-butoxy-8-hydroxyadenine (hydroxy-adenine) to the peptides also results in higher induction of specific CD8 responses compared to the mixture (**Fig 1C**, right panel), confirming a beneficial effect of conjugation *in vivo* also for this ligand. At equimolar doses, the Pam conjugates induce a higher frequency of SIINFEKL-specific CD8 responses (around 1% of total CD8 T cells) compared to hydroxy-adenine (0.5% of total CD8). These responses were

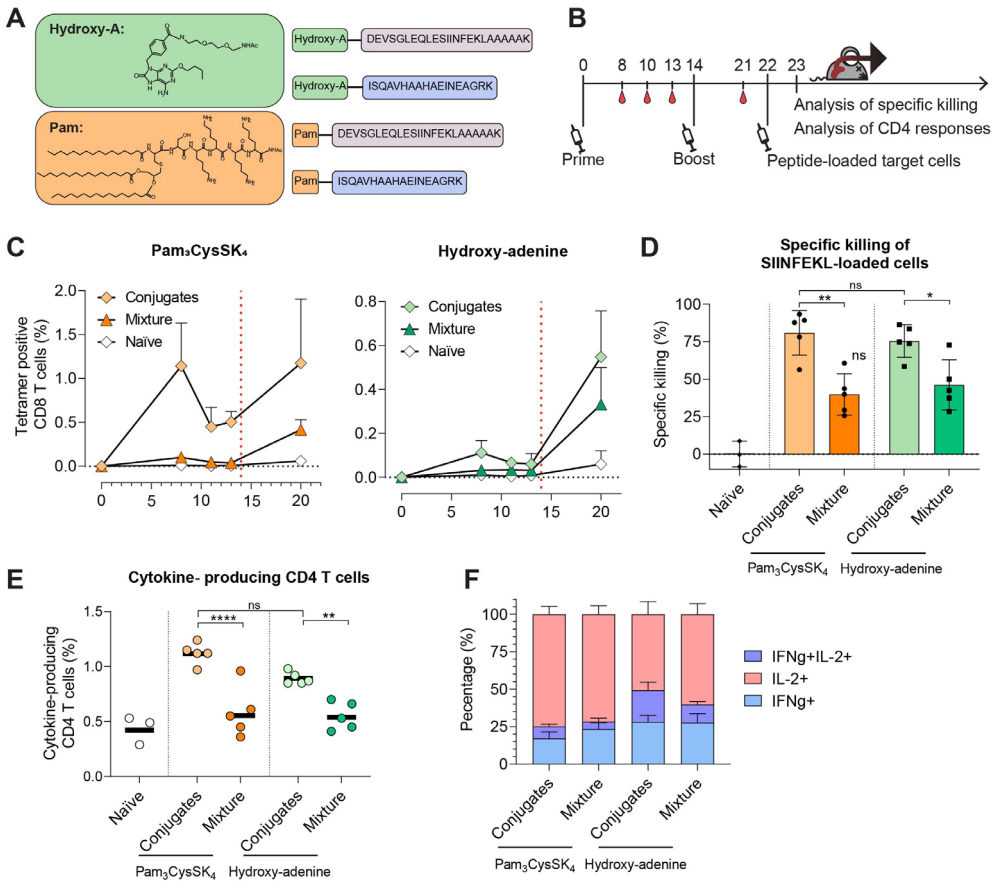

Figure 1: Enhanced CD8 and CD4 T cell priming by Pam_3CysSK_4 or hydroxy-adenine conjugated peptides A Schematic representation of the ligands and peptides used for conjugation and the four conjugates synthetized. **B** Schematic representation of the vaccination experiment. Mice (n=5) were injected with 5 nmol of each conjugates or an equimolar mixture of peptides and ligand at day 0 and 14. At different time points, blood was withdrawn for monitoring the induction of SIINFEKL-specific CD8 responses. At day 22, animals were injected with labelled cells loaded with either SIINFEKL or an irrelevant peptide. Next day mice were sacrificed for analysis of specific killing and CD4 responses via intracellular cytokine staining. **C** Frequency of SIINFEKL-specific cells as measured in blood by tetramer staining at the indicated days. The red line indicates the booster injection (day 14). **D** Calculated specific killing of SIINFEKL-loaded target cells. Statistical significance was determined by one-way ANOVA followed by multiple comparison and Tukey correction. * $p<0.05$, ** $p<0.01$. **E** Frequency of the CD4 responses induced upon vaccination as detected by cytokine staining. Represented are the fraction of total cells producing IL-2, IFNγ or both cytokines. **F** Production of IFNγ, IL-2 or both IFNγ and IL-2 by OVA Help-specific CD4 T cells.

tested for their functionality by analyzing specific killing in an *in vivo* cytotoxicity assay with SIINFEKL-loaded target cells. The groups that were vaccinated with the Pam or the hydroxy-adenine conjugates displayed strong killing capacity (**Fig 1D**). Even though the levels of SIINFEKL-specific cells measured one day before the essay was half compared to the Pam conjugates group (**Fig 1C**), the hydroxy-adenine conjugate performed as effectively as the Pam conjugates in killing capacity. Notably, for both ligands, mice vaccinated with the mixture of the components display significantly lower killing capacity compared to their respective conjugates (**Fig 1D**).

The induction of CD4 OVA-specific Helper responses was analyzed by intracellular cytokine staining. Both conjugates with Pam_3CysSK_4 or hydroxy-adenine ligands induced significantly higher frequency of specific CD4 T cells compared to their respective mixtures (**Fig 1E**). The two adjuvants displayed differences in cytokine production. Pam-adjuvanted vaccines induced a majority (75%) of IL-2 producing cells, and only 25% of these responses were positive for IFNγ. In contrast, cells primed with the TLR7 agonist hydroxy-adenine display a higher production of IFNγ. In particular, in the conjugated group, the cells that produced IFNγ reached 50% of the total response. In summary, conjugation of a TLR7 agonist to a peptide results in enhanced priming of both CD8 and CD4 responses, similarly to what previously reported with other conjugated vaccines. Moreover, stimulation of TLR2 or TLR7 during priming results in differential cytokine production by CD4 T cells.

To explore how dual TLR triggering could affect T cell priming and functionality, we designed dual Pam_3CysSK_4-hydroxy-adenine conjugates and investigated whether the two TLR2 and TLR7 agonists synergize on a single peptide. Two conjugates were synthetized bearing the TLR7 ligand at the N-terminus and Pam_3CysSK_4 at the C-terminus of two synthetic long peptides containing either the CD8 or the CD4 epitope of ovalbumin (**Fig 2A**). First, the effect of the dual conjugation on ligand activity was tested *in vitro*. Dendritic cells were incubated with the compounds and analyzed for IL-12 production. As can be seen in **Fig 2B**, the dual conjugates induce increased production of IL-12 compared to the single Pam or hydroxy-adenine conjugates. The dual conjugates were subsequently compared to the mixture of the free ligands in their ability to induce DC maturation. Conjugation of the two TLR2 and TLR7 agonists to the same peptide is necessary to induce enhanced IL-12 production (**Fig 2C**). In fact, the mixture of the two ligands was not as efficient in inducing IL-12.

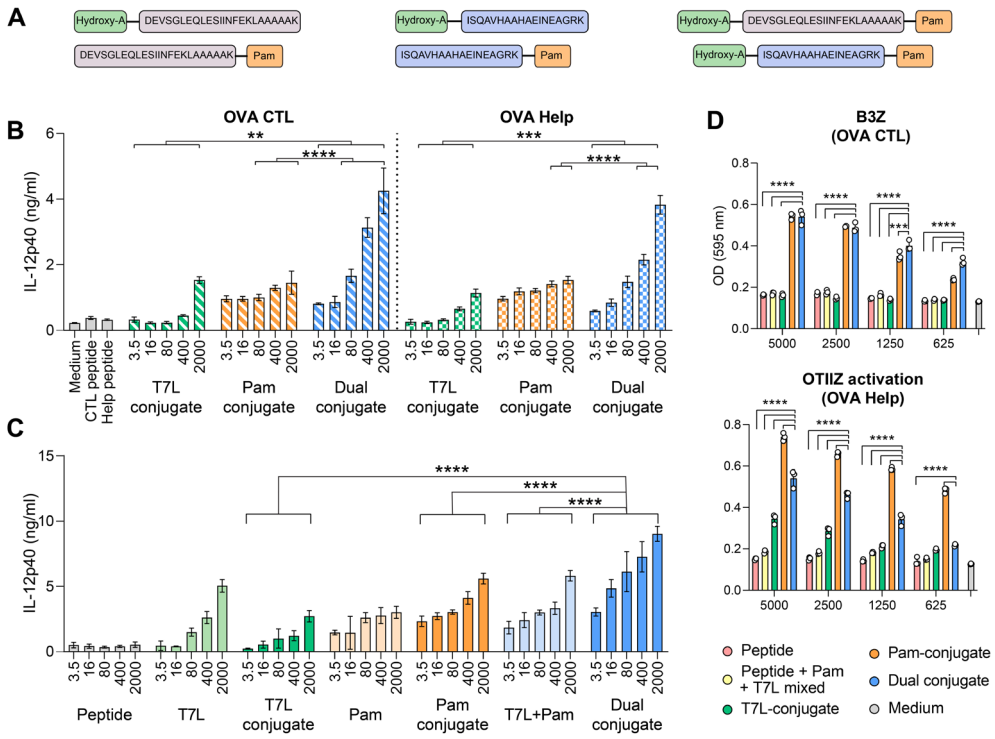

Figure 2: Immunological activity dual peptide conjugates bearing hydroxy-adenine and Pam3CysSk4 A Schematic representation of the conjugates synthetized. **B** IL-12 production upon overnight incubation of bone marrow-derived dendritic cells (BMDCs) with the indicated conjugates at varying concentrations. **C** Induction of IL-12 in BMDCs by the free TLR ligands or by ligands conjugated to the OVA CTL peptide. **D** Antigen presentation in MHC class I or class II of the conjugates to B3Z and OTIIZ hybridoma T cells, respectively. Activation of the two reported cell lines was detected by colorimetric reaction of CPRG by beta-galactosidase and measurement of absorbance at 595nm. Statistical significance was determined by two-way ANOVA followed by multiple comparison and Dunnet correction. *** $p<0.001$, **** $p<0.0001$

Secondly, MHC class I and II antigen presentation by the two dual conjugates were evaluated with the two reporter hybridoma T cell lines B3Z and OTIIZ, which are respectively specific for the CD8 and the CD4 epitopes of ovalbumin. Analysis of B3Z activation revealed that presentation of the CD8 epitope SIINFEKL was enhanced by the dual conjugate compared to free peptide and the hydroxy-adenine conjugate, in a similar fashion to the Pam_3CysSK_4 mono conjugate (**Fig 2D**).

MHC class II presentation of the Helper epitope and consequent OTIIZ activation were instead slightly lower in the dual conjugate compared to the Pam conjugate, while the hydroxy-adenine mono conjugate was not as efficient. Nevertheless, the Helper epitope in the dual conjugate was still presented more

efficiently compared to free peptide. Overall, these data indicate that the dual Pam_3CysSK_4-hydroxy-adenine conjugates exhibit robust immunological activity *in vitro*. In particular, the dual conjugates induce increased IL-12p40 cytokine production by DCs, as well as increased uptake and presentation of the epitopes compared to unconjugated peptide.

Next, the ability of the dual conjugates to prime CD8 or CD4 T cells *in vivo* was evaluated. The dual conjugates, or the corresponding mono conjugates, were injected in mice intradermally at the tail base. The lymph nodes were collected 7 days later for *ex vivo* expansion followed by analysis of the primed CD8 and CD4 responses by intracellular cytokine staining (**Fig 3A**). As previously observed, the conjugate bearing the TLR7 agonist hydroxy-adenine was less strong than the Pam3CysSk4 conjugate at inducing antigen-specific CTL responses (**Fig 3A**). The dual CTL conjugate induced high levels of CD8 specific T cell responses (**Fig. 3B**), which was higher than the hydroxy-adenine mono conjugate, and comparable to the Pam_3CysSK_4 mono conjugate. In addition, the mixture of the two ligands and the peptide also displayed high levels of specific CD8 responses. When looking at the polyfunctionality of these responses, all groups display a majority of IFNγ/TNFα double producing CD8 T cells (**Fig. 3C**). Of notice, compared to both mono conjugates and the mixture, the dual conjugate displays the highest proportion of double positive cells. As for the CD4 responses, the Pam_3CysSK_4 mono conjugate induces the highest amounts of CD4 T cell responses (**Fig. 3D**), followed by the dual conjugate, the hydroxy-adenine conjugate and the mixture. When characterizing the different cytokines produced by these responses, the hydroxy-adenine conjugate promotes, as a classical Th1 inducer, a majority of $CD40L^+/IFN\gamma^+$ and $CD40L^+/IFN\gamma^+/IL\text{-}2^+$ cells, while production of IFNγ was lower in cells primed by the Pam conjugate. Overall, the dual conjugate induces a higher portion of $CD40L^+/IL\text{-}2^+$ CD4 T cells compared to the two mono conjugates. Moreover, a significant portion of cells in the different vaccination groups is $CD40L^+$ but does not produce any of the cytokines analyzed.

To summarize, these results indicate that the dual conjugates synthetized are immunologically active and capable of inducing T cell responses *in vivo*. We re port optimal CD8 T cell induction by the dual conjugates, with enhanced IFNγ/TNFα double production by the dual conjugate compared to the two mono conjugates. As for CD4 induction, we report a differential cytokine profile based on the presence of the TLR7 or TLR2 agonists.

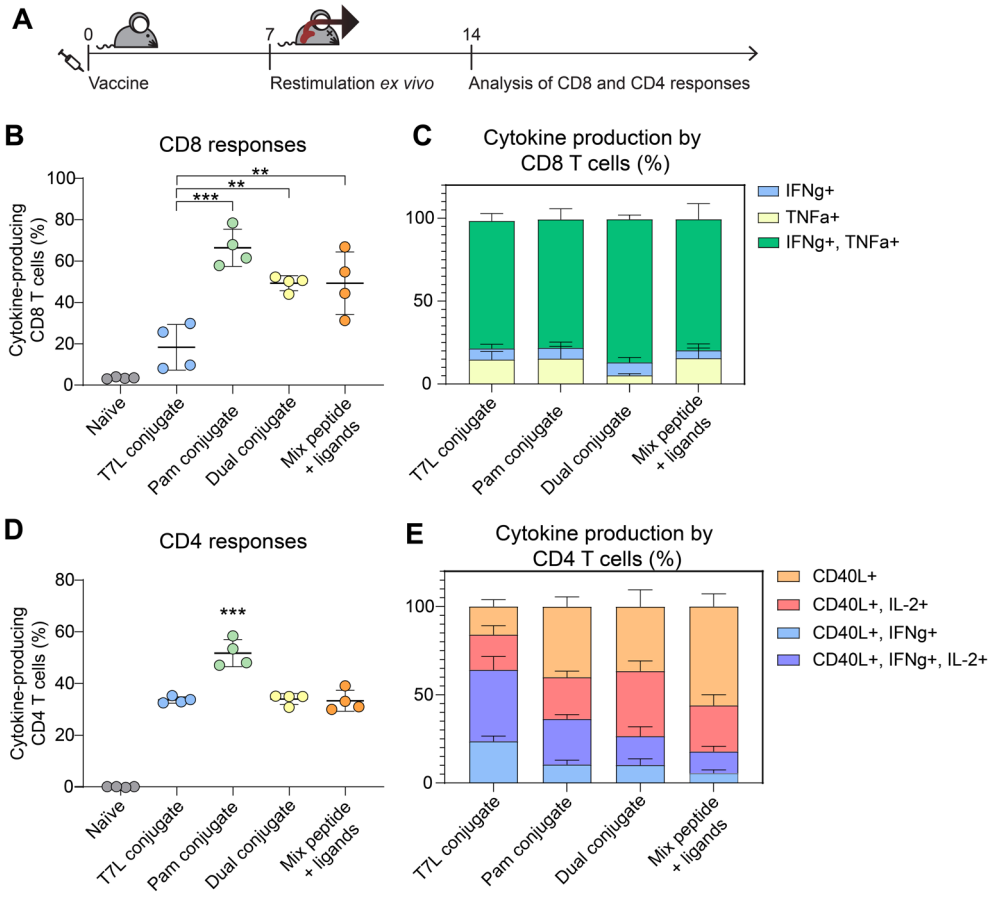

Figure 3: Effective priming of antigen-specific CD8 and CD4 T cells by the dual conjugates A Schematic representation of the experiment. Mice (n=4) were injected with 5 nmol of either the mono conjugates, the dual conjugates or a mix of peptide and ligands bearing either the OVA CTL or OVA Help peptide. One week later, splenocytes were isolated and expanded *ex vivo* with peptide-loaded dendritic cells (with no adjuvant included). After one week, CD8 and CD4 responses specific for the OVA peptides were analyzed via intracellular cytokine staining. **B** Frequency of CD8 T cell vaccine-specific responses represented as the total of cytokine producing cells in response to OVA CTL peptide. **C** Fraction of CD8 T cells producing IL-2, IFNγ, or both cytokines within the cytokine-producing CD8 T cells. **D** Frequency of CD4 T cell vaccine-specific responses represented as the total of cytokine producing cells in response to OVA Help peptide. **E** Fraction of CD4 T cells producing CD40L, IL-2, IFNγ, or more than one of these cytokines within the cytokine-producing CD4 T cells. Statistical significance was determined by two-way ANOVA followed by multiple comparison and Dunnet correction. *** $p<0.001$, **** $p<0.0001$

DISCUSSION

In this work, we explore the design of a peptide conjugate that combines two distinct TLR agonists in a single vaccine molecule. Differential TLR signaling can influence both the innate and the adaptive system towards the most optimal type of response to clear a given pathogen. The analysis of the mono conjugates shows that the two ligands have different effects. On one side, the amplitude of the CD8 responses is larger for the TLR2 ligand, (**Fig 1**) suggesting that Pam_3CysSK_4 is more potent than hydroxy-adenine. On the other side, despite the lower frequency of CD8 responses induced by hydroxy-adenine, both conjugates achieve high killing of antigen-specific cells, showing the strength of the TLR7 pathway. As for CD4 responses, both conjugates enhance the amount of specific T cells induced, but the two ligands induce a differential cytokine skewing. IFNγ induction by the TLR7 agonist is in line with the anti-viral role that this receptor has, while Pam_3CysSK_4 promotes higher IL-2 production. Besides the different cytokine skewing, these data evidence how conjugation represents a superior vaccine formulation compared to the classical delivery of adjuvant next to the antigen. The mechanism by which this happens may vary based on the adjuvant. For example, conjugation of Pam was described to enhance *in vitro* and *in vivo* uptake by DCs [1]. This effect was not dependent on TLR2 but on unidentified endocytosis pathways, most likely mediated by a scavenger receptor present on DCs. The same was observed for a TLR9 ligand. Instead, conjugation of a TLR7/8 agonist to protein antigen did not mediate increased uptake, however the conjugated vaccine was shown to activate and enter the cross-presentation pathway within DCs [12]. As reported by Oh and colleagues, unconjugated antigen was endocytosed but was unable of being presented. Antigen presentation could be induced by the TLR7/8 conjugate in multiple DC subsets and was directly dependent on type I interferons, which also promoted DC recruitment and accumulation in the lymph nodes [13, 14]. IL-12 was also found to be indispensable for T cell priming for this conjugate, however this cytokine acted on T cells and did not regulate any DC function [13].

Combination of the two ligands onto a single molecule enhances the maturating properties of the vaccine while not hampering the processing and presentation properties of the conjugated peptides (**Fig.2**). Vaccination with the dual conjugate *in vivo* induces effective priming of CD8 and CD4 T cell responses (**Fig. 3**). Given the limited number of cytokines analyzed, it was not possible to in detail characterize the functional phenotype of the CD8 and CD4 T cells induced by the dual conjugate versus the mono conjugates, however inclusion of more

cytokines could further elucidate the type of response promoted. In a study using human monocyte-derived DCs, TLR2 signaling was combined with TLR7/8 agonists [15]. Interestingly, it was reported that TLR2 interfered with the type I interferon amplification loop initiated by TLR7/8 stimulation, which resulted in the inhibition of the production of the Th1-promoting cytokine IL-12p70, formed by the heterodimer of the p40 and p35 subunits. The result was the skewing towards a Th2 and Th17 phenotype. We detected increased production of IL-12p40, which is a subunit of both IL-12p70 and IL-23. The latter one is involved in Th17 skewing; therefore, it should be determined whether in our setting the synergy between TLR2 and TLR7 also results in Th17 promotion. In contrast, an adjuvant incorporating covalently linked TLR2 and TLR7 agonists have been reported to synergize in promoting poly-functional CTL responses and balanced Th1/Th2 responses [16].

TLR synergism may be influenced by the type of cells targeted and their activation status. For example, it was reported that TLR2 stimulation induced both pro-inflammatory cytokines and type I interferons in monocytes, however the production of type I interferons was abolished upon differentiation into macrophages [17]. This underlines that responsiveness to the ligands themselves may vary from cell type and differentiation status. Therefore, it will be useful to determine in our immune system which cells are the recipients of the conjugates and how the two pathways interact within these cells. Finally, it is yet to be established how these responses will deal with different type of pathogens or malignancies.

In conclusion, we have developed a novel dual TLR ligand-peptide conjugate that simultaneously triggers TLR2 and TLR7. The dual conjugates display immunological activity and proper CD8 and CD4 T cell priming. Further characterization of the conjugates and their performance in immune control will determine whether this approach will potentiate peptide-based vaccines.

MATERIAL AND METHODS

Peptide synthesis. Ovalbumin-derived antigenic peptides DEVSGLEQLESIINFEKLAAAAAK and ISQAVHAAHAEINEAGRK were assembled using solid-phase peptide synthesis that was performed on a TRIBUTE® Peptide Synthesiser (Gyros Protein Technologies AB, Arizona, USA) starting with Tentagel S-RAM resin (0.22-0.25 mmol/g) on a 100 µmol scale using established Fmoc protocols [7]. The C-terminal lysine residue of the peptides was side-chain protected with a very TFA-sensitive 4-monomethoxytrityl (MMT) protective group to enable the selective installation of a second ligand. Using established protocols1-2, the first

TLR-ligand, either TLR-2 or TLR-7, was attached to the N-terminus of the peptides via an amide bond. Subsequently, the MMT protecting group was selectively removed by exposing the resin to a solution of 1% TFA in DCM 10 times for 30 seconds and 2 times for 5 minutes. The resin was six times washed with DCM, dried under a N2 flow, and the second TLR-ligand (either for TLR-7 or TLR-2) was coupled to the thus liberated amine of the lysine side chain using an amide bond forming reaction. Conjugates and peptides were deprotected and cleaved by shaking the resin with a mixture of 95:2.5:2.5 TFA:H2O:TIS for 105 minutes in a plastic syringe fitted with a filter and purified with RP HPLC.

Cell culture. Bone marrow-derived dendritic cells were differentiated from bone marrow stem cells that were harvested from the femurus and tibia of C57BL/6 mice and cultured in IMDM (Lonza) supplemented with FCS (Greiner) , Glutamax (Gibco), penicillin (Gibco), β-mercaptoethanol (Merck) and R1 supernatant. R1 supernatant was obtained by culturing NIH3T3 fibroblasts transfected with GM-CSF. The B3Z and OTIIZ cell lines were cultured in IMDM medium (Lonza supplemented with 8% FCS (Greiner), penicillin and streptomycin, glutamine (Gibco) β-mercaptoethanol (Merck), and hygromycin B (AG Scientific Inc, San Diego, CA, USA) to maintain expression of the beta-galactosidase reporter gene.

In vitro DC maturation assay. The test compounds were dissolved in DMSO at a concentration of 1 mM and sonicated in water bath for 15 minutes. Murine bone marrow-derived dendritic cells were seeded in 96-well plates at a density of 50.000 cells/well and incubated with titrated amounts of compounds. After 3 hours of incubation, the cells were washed once and incubated with fresh medium. After 16 hours, supernatant was harvested for ELISA analysis (Biolegend) to measure the amount of produced IL-12p40.

In vitro antigen presentation assay. The test compounds were dissolved in DMSO at a concentration of 1 mM and sonicated in water bath for 15 minutes. 50.000 D1 cells were seeded in 96-well flat bottom plates and incubated with the indicated test compounds. After 2 hours 50.000 B3Z or OTIIZ were added per well and incubated with the D1 cells overnight. The following day, B3Z or OTIIZ activation was detected by measurement of absorbance at 595 nm upon color conversion of chlorophenol red-β-D-galactopyranoside (Calbiochem) the reporter enzyme.

Mice. Female C57BL/6 mice were purchased from Charles River Laboratories. Congenic CD45.1^{+} C57BL/6 mice were bred at the Leiden University medical Centre animal facility. All animal experiments were in accordance with the Dutch

national regulations and received ethical and technical approval by the local Animal Welfare Body.

Immunization of mice and in vivo specific killing. 6-8 weeks old C57BL/6 female mice were injected intradermally at the tail base with 5 nmol of Pam_3CysSK_4 or hydroxy-adenine conjugates or an equimolar mix of peptides and ligands. Fourteen days later, the animals were boosted with the same vaccine formulations. At different time points during the experiments, 20 µl of blood were collected from the tail vein for detection of SIINFEKL-specific T cell responses via SIINFEKL-H2-Kb tetramer staining. At the end of the experiments, spleens were removed and processed in single cell suspensions for *ex vivo* analysis. Splenocytes were incubated for 5 hours with peptide-loaded DCs in the presence of Brefeldin A, to block cytokine exocytosis. Afterwards, samples cells were stained for surface markers and intracellularly for IL-2, IFNγ and TNFα. For analysis of *in vivo* cytotoxicity, splenocytes were harvested from $CD45.1^+$ C57BL/6 naïve mice, processed into single cell suspension and labelled with either 5 or 0.005 µM CFSE for 10 min at 37°C. Cells that were labelled with 0.005 µM CFSE (CFSE low) were loaded for 1 hour at 37°C with 1µM SIINFEKL peptide, while cells that were labelled with 5 µM CFSE (CFSE high) were loaded in the same conditions with an irrelevant epitope derived from the E6 protein of Human Papilloma Virus (sequence: RAHYNIVTF). 1'000'000 splenocytes per peptide-loaded group were injected intravenously in vaccinated or naïve mice. One day after transfer, mice were sacrificed and the spleens were harvested and processed into single cell suspensions. Splenocytes were subsequently stained with eFluor®450 anti-CD45.1 antibody (eBioscience, San Diego, USA) and analyzed by flow cytometry to detect $CD45.1^+/CFSE^+$ target cells. Specific killing was calculated according to the following equation: Specific killing = 100 - [100*((CFSE target peptide)/(CFSE irrelevant) immunized mice)/((CFSE target peptide)/(CFSE irrelevant) naïve mice)]

Immunization of mice and ex vivo expansion. 6-8 weeks old C57BL/6 female mice were injected intradermally at the tail base with 5 nmol of Pam_3CysSK_4 or hydroxy-adenine conjugates or the dual conjugates, or an equimolar mixture of peptide and ligand. Seven days later, lymph nodes were removed and processed in single cell suspensions and incubated with peptide-loaded BMDCs for 7 days. After 7 days, cells were incubated for 5 hours with peptide-loaded DCs in the presence of Brefeldin A, to block cytokine exocytosis. Afterwards, samples cells were stained for surface markers and intracellularly for IL-2, IFNγ and CD40L or TNFα.

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