Antigen handling and cross-presentation by dendritic cells

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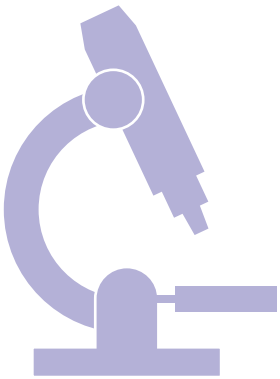


Synthesis and evaluation of fluorescent TLR ligand-peptide conjugates in dendritic cells

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Adjusted from the published version: Synthesis and evaluation of fluorescent Pam_3Cys peptide conjugates, Bioorganic & Medicinal Chemistry Letters 2016 Aug 1; 26(15): 3641-3645

ABSTRACT

Covalent conjugation of Toll-like receptors (TLRs) to synthetic antigen peptides have shown to be a promising tool for cancer vaccines. We have demonstrated before that TLR-2 ligand Pam_3CysSK_4 (Pam) linked to synthetic antigenic peptides was targeted into MHCI cross-presentation pathway and was able to strongly enhance the induction of specific $CD8^+$ T cells. Moreover, the configuration at the C-2 position of the glycerol moiety of the Pam linked to OVA peptide could affect its ability to activate dendritic cells (DCs). The *R*-Pam-peptide conjugate was superior in activating DCs and inducing specific $CD8^+$ T cells compared to *S*-Pam-peptide conjugate. In order to visualize and track these TLR ligand-peptide conjugates in DCs after uptake, we fluorescently labeled Pam conjugated to an OVA peptide with TAMRA or Cy5. Chirally pure *R*- and *S*-epimers of Pam were prepared and separately conjugated to an OVA model epitope, in which lysine was replaced by azidonorleucine. The azide function in the conjugate permitted labelling with different fluorophores by use of strain-promoted [3+2] cycloaddition. Combining the lipophilicity of Pam ligand with fluorophores influenced the solubility of the resulting conjugates in an unpredictable way and only the conjugates labeled with Cy5 were suitable for confocal fluorescence microscopy experiments. We show here that both epimers of the Cy5 labeled lipopeptides were internalized equally well. The presented results demonstrate the usefulness of strain-promoted azide-alkyne cycloaddition in the labelling of highly lipophilic lipopeptides without disturbing the *in vitro* activity of these conjugates with respect to activation of TLR-2. Further comparison between different types of fluorophores on CpG-peptide conjugates revealed that also these conjugates can activate DCs and efficiently be taken up in endosomal compartments.

INTRODUCTION

Conjugated cancer vaccines have attracted much attention as a promising lead for innovative therapeutic interventions (1–5). A particular flavor of conjugated vaccines, that has been extensively investigated through the years, comprises a structurally defined construct of a Toll-like receptor (TLR) agonist covalently attached to a synthetic peptide, that contains a T-cell epitope, either model or tumor associated (6). It has been discovered that a conjugate of this kind showed improved T-cell priming and tumor protection compared to a mixture of the individual antigenic peptide and TLR agonist (7, 8). The usefulness of such synthetic peptide-based conjugates in tumor vaccination has been demonstrated as well. A commonly used agonist in these studies is a lipopeptide known as Pam_3CysSK_4 (Pam) that binds to TLR-2/TLR-1 (9–11). This compound has been derived from the N-terminus of bacterial lipoprotein of, among others, Escherichia coli (12). Notably, when Pam was applied as a component of a vaccine candidate, either covalently attached to a longer peptide sequence or simply admixed with a peptide, it was often present as a mixture of *R*- and *S*-epimers at the glycerol moiety (10, 13–16, 8). However, it is known that the *R*-epimer is the biologically active one (9, 17). With the aid of non-labeled Pam conjugates it has been shown that *R*-epimer of Pam is indeed the one responsible for dendritic cell (DC) activation, which directly contributed to enhanced $CD8^+$ T cell responses, whereas the *S*-epimer did not activate DCs and therefore unable to prime $CD8^+$ T cells (17). Moreover, the uptake of both chirality of Pam was comparable, although in a TLR-2 independent manner, indicating that DC activation was mainly the cause of the observed differences in $CD8^+$ T cell priming.

In the current study, we show that fluorescently labeled and chirally pure Pam-lipopeptides represent useful tools in the studies of antigen processing since these constructs allow a visual evaluation of the antigen uptake irrespective of the DC-maturation status. Towards this end, we synthesized Pam-peptide conjugates with the fluorescent label (TAMRA or Cy5) covalently attached to the modified side chain of a lysine residue in the commonly used model MHC-I epitope (SIINFEKL). This design of the labeled construct proved to be successful in our past studies that involved the monitoring of the intracellular trafficking of Pam-lipopeptides as mixtures of epimers at C-2 of the glycerol moiety (7). To be able to vary the type of fluorophore more readily, a convergent approach based on copper free click chemistry has been chosen in the present work (18–20). The fluorescent labels TAMRA and Cy5 were connected to the Pam-peptide conjugates. We showed that with the use of strain-promoted [3+2] cycloaddition, Pam-peptide conjugates could be successfully labeled with TAMRA and Cy5. The *R*-, but not *S*-epimer of fluorescently labeled Pam- peptide conjugates was able to activate DCs in a TLR-2-dependent manner, comparable to their unlabeled analogues. However, the uptake of the fluorescently labeled *R*- and *S*- Pam-peptide conjugates by DCs was comparable as shown by confocal microscopy. Combining TAMRA

with Pam-peptide conjugates influenced the solubility of the conjugates in such a manner that it was difficult to use for confocal experiments. To further investigate a different TLR-ligand and its properties when fluorescently labeled, we compared CpG- peptide conjugates labeled with Alexa Fluor 488 (A488) or Cy5. We have published before that CpG (a TLR-9 agonist) is taken up independently of TLR-9, but DC activation through TLR-9 is crucial for efficient $CD8^+$ T cell priming (7). We show here that both A488 and Cy5 labeled CpG-peptide conjugates induced comparable activation of DCs and are taken up efficiently in endosomal compartments.

RESULTS

Synthesis of fluorescently labeled TLR- peptide conjugates

In order to study the uptake and trafficking of TLR-peptide conjugates in DCs, TLR-2 ligand Pam_3CysSK_4 and TLR-9 ligand CpG were used. The *R*- and *S*- epimers of Pam_3CysSK_4(Pam)-OVA peptide conjugates were labeled with TAMRA or Cy5 and described in more detail in de published version of this study (21). In short, chirally pure *R*- and *S*- epimers of Pam were prepared and separately conjugated to an OVA model peptide (DEVSGLEQLESIINFEKL, $OVA_{247\text{-}264}$), in which lysine was replaced by azidonorleucine (Fig. 1). The azide function in the conjugate permitted labelling with different fluorophores by use of strain-promoted 3+2 cycloaddition. The synthesis of labeled CpG-OVA peptide conjugates was done as described before (7).

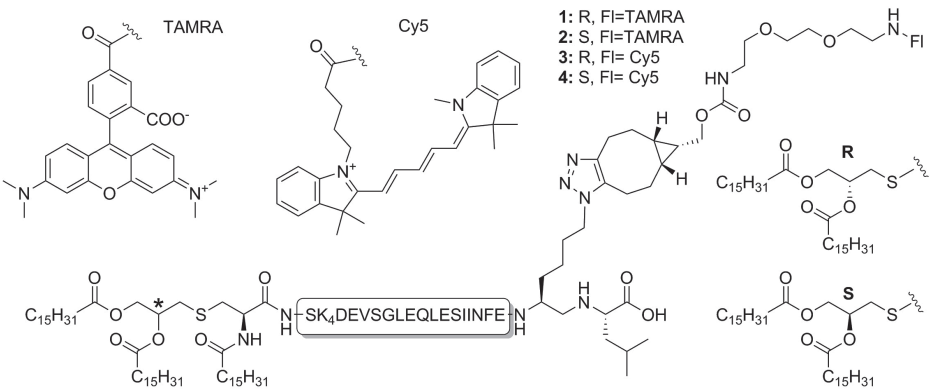

Figure 1. **Schematic overview of fluorescent labeling of TLR-ligand peptide conjugates.** Synthesis of TAMRA or Cy5 labeled *R*- and *S*- Pam_3CysSK_4 conjugated to OVA peptide (DEVSGLEQLESIINFEKL, $OVA_{247\text{-}264}$).

Fluorescently labeled Pam_3CysSK_4- peptide conjugates are efficiently taken up by DCs

To investigate the immunological function of the fluorescently labeled Pam_3CysSK_4 (Pam)-peptide conjugates, we compared the unlabeled *R*-and *S*- epimers of Pam-peptide conjugates with the TAMRA and Cy5 labeled variants on DC activation. We have shown before that the *R*-Pam is the active form which could efficiently activate DCs and promote $CD8^+$ T cell priming, whereas the *S*- Pam is the inactive form and failed to induce DC activation and specific T cell priming (17). DCs were stimulated with unlabeled or labeled *R*- or *S*- Pam-peptide conjugates for 48 hours and DC maturation was measured by IL-12 production. Similar to our previous findings, unlabeled *R*- Pam-peptide conjugate induced more IL-12 production by DCs than unlabeled *S*- Pam-peptide conjugate (Fig. 2A). Comparable results were found for the TAMRA or Cy5 labeled *R*- and *S*- Pam-peptide conjugates, showing intact immunogenicity of the fluorophore-labeled conjugates. However, TAMRA appeared to influence the solubility of the Pam-peptide conjugates in an unpredictable way, therefore the TAMRA variant of the conjugates will be excluded for the following experiments.

To corroborate the TLR-2 dependent activation of DCs by the fluorescent conjugates, the compounds were next assessed using HEK-cells transfected with TLR2. The level of IL-8 produced in the assay reflects the capacity of the conjugates to activate the receptor. Both unlabeled and Cy5 labeled *R*- Pam-peptide conjugates were able to trigger human TLR-2 and induced IL-8 production by HEK cells (Fig 2B). The *S*- Pam-peptide conjugate did not induce IL-8 production, regardless of labeled or unlabeled form, indicating the lack of triggering human TLR-2. To determine the receptor specificity of immunogenic lipopeptides for TLR-2, HEK cells expressing TLR-4 were stimulated with unlabeled or Cy5 labeled *R*- or *S*- Pam-peptide conjugates. None of the compounds were able to trigger human TLR-4 indicating not only the high specificity of the immunogenic lipopeptides for TLR-2, but also the absence of any inadvertent LPS contamination in the samples of the TLR-2 activating conjugates (Fig. 2C).

The uptake of Cy5 labeled *R*- and *S*- Pam-peptide conjugates by DCs was measured with confocal microscopy. After 15 min, both compounds were efficiently internalized by DCs (shown in red and overlay with DIC) and accumulated in hot spots surrounding the nucleus (Fig. 3). Similar as we have previously reported, no differences in localization or uptake were observed (7, 17).

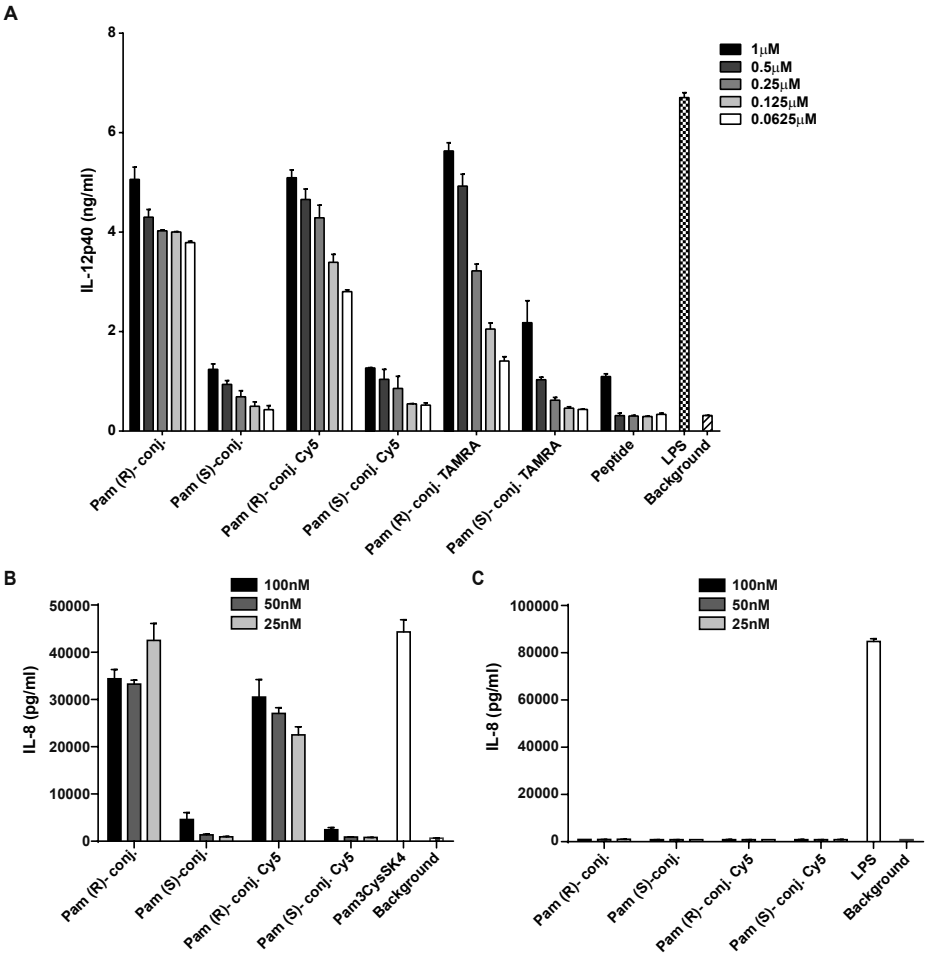

Figure 2. Activation of dendritic cells by Pam-peptide conjugates. (A) DCs were stimulated with titrated amounts of either unlabeled, TAMRA or Cy5 labeled, *R*- or *S*-Pam for 48 hours. LPS (1.25 μg/ml) and peptide (DEVSGLEQLESIINFEKL) were used as positive and negative control, respectively. Untreated cells were depicted as background signal. Supernatants were harvested and analyzed for IL-12 cytokine secretion by ELISA. One representative from three independent experiments is shown. Error bars represent SD**. (B)** Ability of immunogenic lipopeptides in triggering human IL-8 production via TLR-2. HEK TLR-2 cells were incubated with titrated amounts of unlabeled or Cy5 labeled, *R*- or *S*-Pam for 24 hours. Untreated cells and cells treated with Pam3CysSK4 (100 ng/ml) were used as negative and positive control, respectively. Error bars represent SD. **(C)** HEK TLR-4 cells were incubated with titrated amounts of unlabeled or Cy5 labeled, *R*- or *S*-Pam for 24 hours. Untreated cells and LPS (10 ng/ml) treated cells were used as negative and positive control, respectively. Supernatants were subsequently analyzed for IL-8 production by ELISA. The graphs are representative of two different independent experiments performed in duplicate. Error bars represent SD.

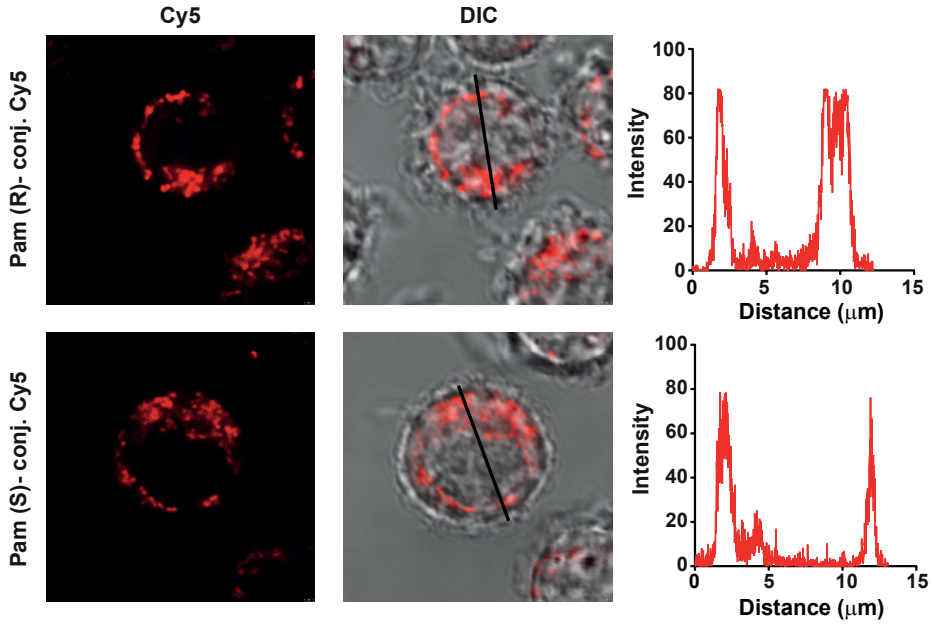

Figure 3. Uptake of *R*- and *S*- Pam-peptide conjugates by dendritic cells. DCs were incubated for 15 min with Cy5 labeled *R*- or *S*- Pam-peptide conjugates (1 μM). The uptake and localization of the compounds were analyzed with confocal laser scanning microscopy. Differential interference contrast (DIC) was used to image cell contrast. Intensity histograms were created for a selected area (indicated by a line on the image) with the ImageJ software. The images are representative for multiple cells from at least 3 experiments.

DC handling of fluorophore labeled CPG-conjugates

To further investigate a different TLR-ligand and its properties when fluorescently labeled, we used Alexa Fluor 488 (A488) or Cy5 labeled CpG-peptide conjugates. DC activation was measured by stimulating DCs with unlabeled or labeled CpG-peptide conjugates for 48 hours. All CpG-peptide conjugates significantly induced IL-12 production by DCs, regardless whether the conjugates were labeled or unlabeled (Fig. 4A). Moreover, both A488 and Cy5 labeled CpG-peptide conjugates were able to induce CD40 and CD86 expression on DCs (Fig. 4B). The uptake of the labeled CpG-peptide conjugates by DCs was visualized by confocal microscopy. DCs were stimulated with either A488 or Cy5 labeled CpG-peptide conjugates, or incubated simultaneously with A488 and Cy5 CpG-peptide conjugates for 24 hours. Both A488 and Cy5 labeled compounds were efficiently taken up by DCs and accumulated in hot spots in the cell cytosol (Fig. 5, first and second panel), which is in line with our previous work (7).

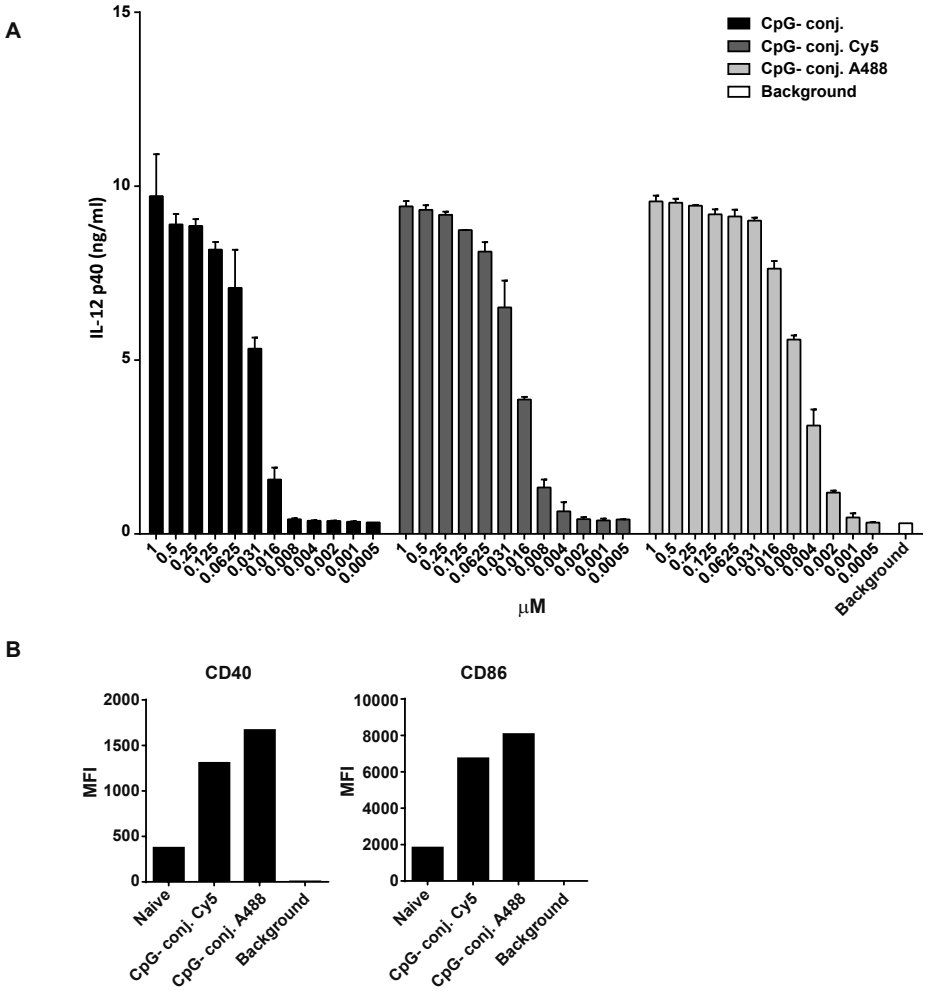

Figure 4. Activation of dendritic cells by CpG-peptide conjugates. (A) DCs were stimulated with titrated amounts of unlabeled, A488 or Cy5 labeled CpG-peptide conjugates for 48 hours. Untreated cells were depicted as background signal. Supernatants were harvested and analyzed for IL-12 cytokine secretion by ELISA. Error bars represent SD**. (B)** DCs were stimulated with A488 or Cy5 labeled CpG-peptide conjugates (1μM) for 48 hours. Naïve DCs were not treated with any compounds, and cells not stained with antibodies were used as background. CD40 and CD86 expression on DCs was measured by flow cytometry and depicted in mean fluorescence intensity (MFI).

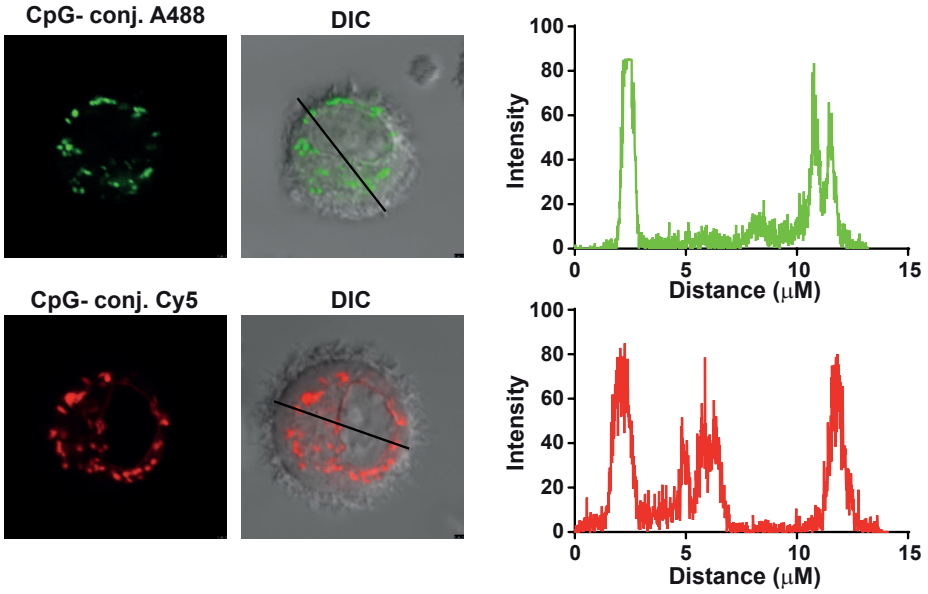

Figure 5. Uptake of CpG-peptide conjugates. DCs were incubated with A488 (green) or Cy5 (red) labeled CpG-peptide conjugates (1μM) for 2 hours and chased for 24 hours. The uptake and localization of the compounds were analyzed with confocal laser scanning microscopy. Differential interference contrast (DIC) was used to image cell contrast. Intensity histograms and overlays were created for a selected area (indicated by a line on the image) with the ImageJ software.

DISCUSSION

TLR-ligands covalently conjugated to synthetic peptides have been an attractive target for cancer vaccination strategies, especially when the peptide antigen and a defined adjuvant are combined in one compound. We have demonstrated before that TLR-2 ligand Pam_3CysSK_4 (Pam) linked to synthetic antigenic peptides was targeted into MHCI cross-presentation pathway, strongly enhancing the induction of specific $CD8^+$ T cells, and efficiently inducing antitumor immunity (17, 8). In the current study we investigated the possibilities in coupling different fluorescent dyes to the *R* (active)- and *S* (inactive)- Pam conjugated to an OVA peptide. Using strain-promoted [3+2] cycloaddition, a small set of TAMRA and Cy5 labeled Pam-lipopeptides was successfully synthesized. The *R*- but not *S*-epimer of Pam in the prepared fluorescent lipopeptides triggered DC maturation in a TLR-2-dependent manner and at comparable levels as their unlabeled analogues. However, the poor aqueous solubility of the conjugates containing TAMRA precluded the use of those for microscopy studies. This reminds that attaining sufficient solubility remains a major challenge in the synthesis of Pam-based constructs labeled with fluorophores. Nevertheless, Cy5 labeled *R*- and *S*-Pam-peptide conjugates could successfully be used for confocal microscopy and were both

taken up by dendritic cells to the same extent. These results corroborate previous findings that suggested a TLR-independent uptake of the peptides conjugated to a TLR-ligand (7). Furthermore, we used A488 and Cy5 to label CpG-peptide conjugates and showed similar DC activation and uptake in endosomal compartments. However, interpreting data by using fluorescently labeled compounds could sometimes be challenging and new approaches to track chemically defined synthetic peptide vaccines should be investigated. Not only can fluorophores affect the uptake and routing of the compounds of interest, but it can also interrupt with antigen processing due to their bulky and hydrophobic structures compared to the relatively small peptides. One of the new possibilities to overcome these problems is the use of click chemistry (reviewed in (22)). By using biorthogonal peptides and ligation of a complementary fluorophore to the biorthogonal amino acid side chain at the end of the experiment, surface labeling of MHCI loaded minimal epitopes was quantified on APCs (23). One can imagine the possibilities with this new technique for accurate tracking of the compound of interest, even *in vivo*, bypassing solubility problems or characteristic changes caused by the type of fluorophore. However, the click chemistry technique is still limited by poor signal-to-noise ratios and further optimization is needed for future use.

MATERIALS AND METHODS

IL-12p40 ELISA

D1 dendritic cells (immature splenic dendritic cell line derived from B6 (H-2b) mice) were plated in a 96-wells plate and incubated with a titration of the compounds for 48 hours. Supernatants were collected and tested with ELISA for IL-12p40 using a standard sandwich ELISA. Coating Ab: rat anti-mouse IL-12p40 mAb (clone C15.6, Biolegend). Detection Ab: biotinylated rat anti-mouse IL-12p40 mAb (clone C17.8, Biolegend). Streptavidin-Poly-HRP (Sanquin) and 3,3',5,5' Tetramethylbenzidine (Sigma-Aldrich) were used as enzyme and substrate, respectively.

Confocal microscopy

D1 dendritic cells were incubated with Cy5 labeled *R*- or S- Pam-peptide conjugates (1 μM) for 15 min at 37 ˚C and washed with culture medium. Alexa 488 or Cy5 labeled CpG-peptide conjugates (1μM) were added to dendritic cells for 24 hours. The cells were plated out into glass-bottom Petri dishes (MatTek) and imaged using the Leica SP5-STED with a 63x objective lens. Differential interference contrast (DIC) was used to image cell contrast. Images were acquired in 10x magnification and processed with Leica LAS AF Lite software.

Activity assay on transfected TLR-2/4 HEK cells assay

Human TLR-expressing HEK cells were cultured in DMEM medium enriched with Penicillin/ Streptomycin/ Glutamine and 1% FCS. HEK TLR-2 and HEK TLR-4 cells were cultured in the presence of G418 (Geneticin, 0.5 mg/mL). Suspensions of 100 µL cells (1.106 cells/mL) were stimulated for 24 h with compounds unlabeled or Cy5 labeled *R*- or S- Pam-peptide conjugates. Pam3CysSK4 (100ng/mL) and LPS (10 ng/mL) were used as a positive control for TLR-2, and TLR-4, respectively. Supernatants were subsequently analyzed for IL-8 production by ELISA.

Flow cytometry

D1 DCs were incubated with A488 or Cy5 labeled CpG-peptide conjugates (1 µM) for 48 hours. Cells were the harvested and incubated for 20 min with CD40 (clone 3/23) and CD86 (clone GL-1) antibodies. Cells were washed twice and measured immediately with flow cytometry.

ACKNOWLEDGEMENTS

This work is part of the research program TOP with project number 91211011, which is financed in part by the Netherlands Organization for Scientific Research (NOW-ZonMw). We thank Dr. Gerbrand J. van der Heden van Noort for his contribution in the optimization of the cyclopropanation reaction. F.C. acknowledges financial support though an NOW-CW Veni grant number 722.014.008.

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