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Challenges and opportunities in nasal subunt vaccine delivery : mechanistic studies using ovalbumin as a model antigen

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Citation
Slütter, B. A. (2011, January 27). *Challenges and opportunities in nasal subunt vaccine delivery : mechanistic studies using ovalbumin as a model antigen*. Retrieved from https://hdl.handle.net/1887/16394

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Conjugation of ovalbumin to N-trimethyl chitosan improves immunogenicity of the antigen

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Journal of Controlled Release 2010 Apr 19;143(2):207-14

Abstract

Subunit vaccines are generally safer, but often less effective than live attenuated vaccines as they lack the necessary co-stimulatory factors. The formulation of an adjuvant like N-trimethyl chitosan (TMC) with an antigen can overcome its poor immunogenicity. Recent data suggest the importance of incorporating the antigen and the adjuvant into one entity for maximum immunostimulatory effect, e.g. by using (nano)particles.

In the present paper we introduce the conjugation of an antigen, ovalbumin (OVA), to TMC as an alternative to nanoparticles for subunit vaccination. OVA was covalently linked to TMC using thiol chemistry (SPDP method). The uptake of the resulting TMC-OVA conjugate by dendritic cells (DC) and its effect on DC maturation was assessed *in vitro* and its immunogenicity was investigated in mice. We found that with the SPDP method a reducible covalent bond between TMC and OVA could be introduced, without disrupting the protein's antigenicity and structure. Uptake of TMC-OVA conjugate by dendritic cells was similar to the uptake of TMC/OVA nanoparticles, over 5-fold increased compared to a solution of OVA and TMC. Mice immunized with TMC-OVA conjugate produced 1000-fold higher OVA specific IgG titers than mice immunized with either OVA or a physical mixture of TMC and OVA. Moreover, these antibody titers were slightly elevated compared to the titers obtained with TMC/OVA nanoparticles. Conjugation of the antigen to an adjuvant is therefore a viable strategy to increase the immunogenicity of subunit vaccines and may provide an alternative to the use of particles.

Introduction

Vaccination has been one of the most effective ways of preventing disease. However, development of new vaccines is increasingly complicated, in part due to the complex nature of the targeted diseases [1], but also because of regulatory concerns [2]. Safety issues, like local as well as systemic adverse effects and possible recombination of a weakened pathogen into a virulent species [3], have sparked the interest in subunit vaccines. Subunit vaccines contain only part of the pathogen (often only one single protein) and are stripped of any virulence factors. This makes them generally safer and pharmaceutically better defined. The lack of virulence factors, however, causes a dramatic decrease in the effectiveness of these subunit vaccines. Antigen presenting cells (APCs), like dendritic cells (DCs) and macrophages play a key role in effectively inducing an immune response. They continuously sample their environment for antigens and are capable of presenting epitopes of these antigens on MHC class I and/or MHC class II molecules. However, these APCs have to be stimulated by a danger signal, for them to mature and properly activate T-cells [4-6].

Recently, we and other groups have shown that co-administration of a chitosan derivate, N-trimethyl chitosan (TMC), with the antigen leads to increased antibody production and protection when compared to administration of an antigen alone [7-9]. Moreover, TMC was shown to be well tolerized by mice, biodegradable and (especially those with a low degree of quarternization) much less toxic than other cationic polymers [10, 11]. *In vitro* experiments showed that treatment of immature DCs with TMC induces upregulation of several maturation markers on DCs [12], indicating that TMC's immunopotentating effect is indeed mediated by DC activation. Simple co-administration of an antigen with an adjuvant may however not be the most effective way to administer a vaccine. For instance, particles have been associated with stronger immune responses compared to antigen solutions, as they allow multimeric antigen presentation and (depending on the type of delivery system) can create a depot effect [13]. Moreover, encouraging results using particles containing both the antigen and the adjuvant have been obtained [14]. Studies in which both components were combined in one delivery system have shown beneficial effects of the cointernalization of an antigen with a adjuvants like flagellin [15], CpG [16, 17] and LPS [18]. Similarly, TMC nanoparticles loaded with hemagluttin has been shown to be very immunogenic in mice [8]. It has been suggested that only an APC that has taken up the antigen and the adjuvant in significant amounts is able to activate T-cells, whereas an APC that has only taken up either of the two components does not stimulate T-cell proliferation [19, 20]. Therefore, combination of the antigen and the adjuvant in one entity may be a good strategy for future vaccine development. These studies

all used particulate systems to co-deliver antigen and adjuvant. We hypothesize that 'simple' covalent linkage of an antigen, ovalbumin (OVA), to an adjuvant, TMC, could insure that both reach the APC at the same time and therefore enhance the immune response as well (figure 1). By introducing a disulfide bond as the linker between the 2 molecules, both adjuvant and antigen should be released once the conjugate has reached the reducing environment of an APC endosome [21, 22]

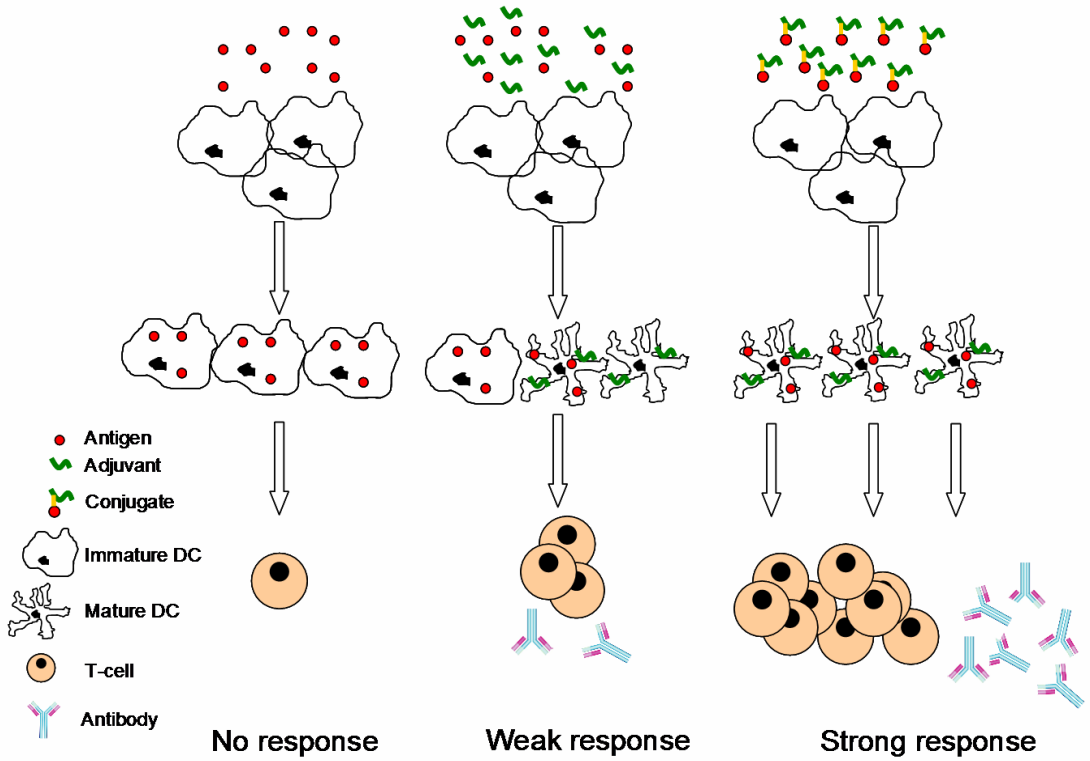

***Figure 1**: Subunit antigens are taken up by DCs but lack the necessary danger signals to induce DC maturation. An antigen-adjuvant mixture stimulates the activation of immature DCs, but antigen and adjuvant should be taken up simultaneously by a DC to effectively induce T-cell activation and antibody production. An antigen-adjuvant conjugate increases the chance of simultaneous uptake of both adjuvant and antigen, resulting in many mature antigen-carrying DCs, strong T-cell proliferation and high antibody levels.*

Chitosan, as a polymer of interest in pharmaceutical applications, has been conjugated to various chemical entities, mainly through its abundant primary amine groups [23, 24]. Similar conjugation strategies may be used for TMC [25], especially TMC with a low degree of quaternization (20% in this study) which still carries residual primary amines. The aim of the

present paper was to conjugate OVA to TMC and evaluate the immunogenicity of these conjugate compared to TMC/OVA nanoparticles, a mixture of OVA and TMC, and plain OVA. Using a method earlier described by Dijk-Wolthuis *et al* [26], both the protein and the polymer were thiolated by treatment with N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), followed by the formation of a disulfide bond. To assess its immunogenicity, uptake of the conjugate by DCs and subsequent DC activation was investigated *in vitro* and its ability to induce antibodies was determined *in vivo*.

Materials and Methods

Materials

N-trimethyl chitosan (TMC) with a degree of quaternization of 20% was synthesized starting from 92% deacetylated (MW 120 kDa) chitosan (Primex, Siglufjordur, IC), by NaOH induced methylation as earlier described [27]. The average molecular weight of TMC was 90 kDa (determined by gel permeation chromatography (GPC) with low angle light scattering detection [11]). The number of primary amines present on the synthesized TMC was determined with a ninhydrin assay [28], to be 55 NH_2/mol TMC. Antibodies, polyclonal rabbit anti-OVA IgG and goat anti-rabbit IgG-HRP conjugate, were acquired from Millipore (Amsterdam, NL) and anti-CD86-APC from Becton Dickinson (Franklin Lakes, NJ USA). Invitrogen (Breda, NL) supplied fluorescein isothiocyanate (FITC) labeled OVA and all cell culture products. Endotoxin free OVA was purchased at Calbiochem (Merck, Darmstad, DE). N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), dithiothreitol (DTT), Carboxymethyl Sepharose gel, pentasodium tripolyphosphate (TPP) and all other salts/chemicals were acquired from Sigma-Aldrich (Zwijndrecht, NL), unless stated otherwise.

TMC-OVA conjugate synthesis

TMC was functionalized with pyridyldithiol using the heterobifunctional crosslinker N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), to accommodate disulfide bond formation. TMC was dissolved in 10 mM phosphate buffered saline (0.9% w/v NaCl, pH 7.4) to a final concentration of 2.5 mg/ml. SPDP dissolved in acetonitrile (35 mg/ml) was added to the TMC solution, in a TMC:SPDP molar ratio ranging from 1:1 till 1:40. These ratios correspond to an NH_2/SPDP ratio of 55:1 to 1.4:1. After 1 h of shaking at room temperature, the reaction was stopped by removing the unreacted crosslinker and reaction intermediates with a PD-10 desalting column (GE Healthcare, Eindhoven, NL).

OVA was similarly functionalized with SPDP (figure 2a). OVA was dissolved in PBS pH 7.4 to a final concentration of 1 mg/ml. SPDP in acetonitrile (35 mg/ml) was added to the OVA solution, in molar ratio OVA:SPDP ranging from 1:1 till 1:15. After 1 h of shaking at room temperature, the reaction was stopped by removing unreacted SPDP and reaction intermediates with a PD-10 desalting column.

Prior to the final conjugation step, functionalized TMC-PDP was reduced to remove the protective pyridine-2-thione group and obtain sulfhydryl activated polymer (figure 2b). This was done by adding a 1 mg excess of 1% w/v dithiothreitol (DTT) dissolved in water to the polymer solution. After 30 min of mild shaking, the excess DTT was removed with a PD-10 desalting column. Subsequently, the activated TMC (TMC-SH) was mixed with the OVA-PDP and incubated for 16 h at 40°C in PBS (figure 2c). The average number of –SH moieties per TMC molecule was kept constant at 1 or 2 moles –SH per mol TMC, while the average number of PDP groups on OVA was varied from 1 to 7 moles PDP per mol OVA. Molar ratio TMC:OVA before conjugation was set at 1:1.

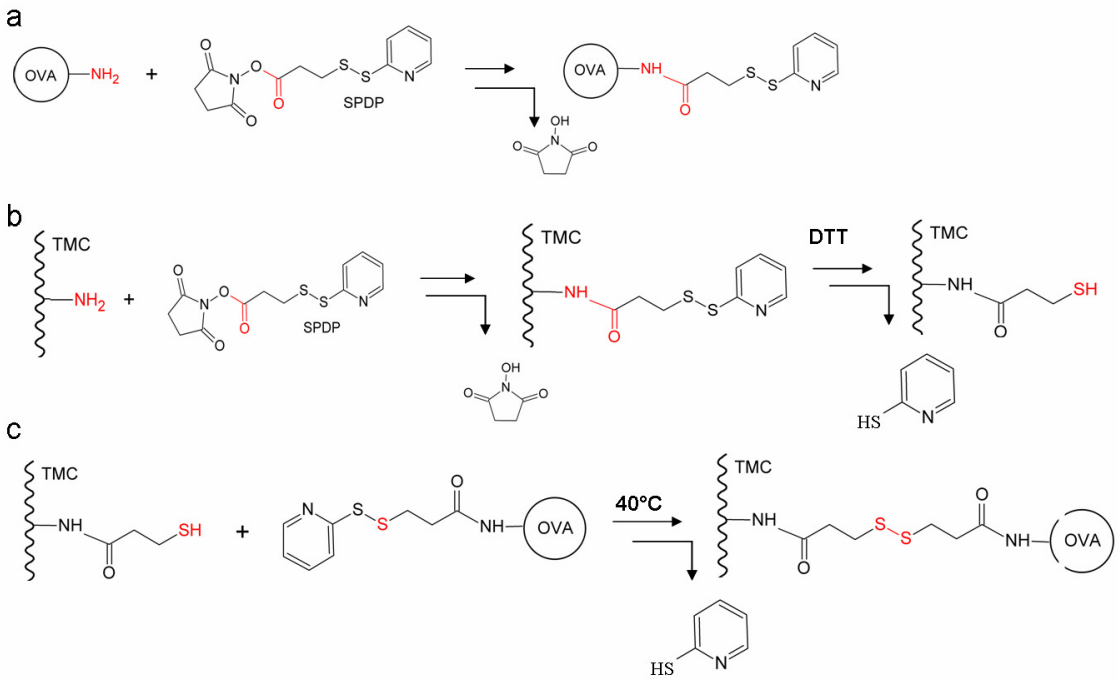

***Figure 2**: Reaction scheme of TMC-OVA conjugation. (a) Primary amine groups on OVA are functionalized with SPDP. (b) Primary amine groups on TMC are similarly functionalized and subsequently reduced with DTT, yielding TMC containing thiol groups. (c) An activated thiol group on TMC reacts with a disulfide bond of functionalized OVA, creating a disulfide bond between the two molecules. Reaction was performed at 40°C and stopped after 16 h.*

Purification of TMC-OVA conjugates

Free OVA was removed from the reaction mixture by solid phase extraction. Carboxymethyl Sepharose gel (Sigma-Aldrich), a cation exchange sorbent, was packed into an empty syringe with a final column volume of 4 ml. The column was equilibrated at room temperature with PBS pH 7.4, after which 5 ml reaction mixture was applied onto the column. Free OVA and other impurities were eluted with PBS, after which the TMC-OVA conjugate as well as free TMC was eluted using a 20 mM citrate buffer containing 1 M NaCl, pH 3.3. Fractions were collected and analyzed for protein content with a bicinchoninic acid (BCA) protein assay (Pierce, Etten Leur, NL) according to the micro plate procedure provided by the manufacturer. The conjugate-containing fractions were pooled and dialyzed against distilled water for 1 day, and subsequently freeze-dried for 48 h at -60°C and 0.8 mbar. Prior to use or analysis, the conjugate was reconstituted in PBS.

GPC

The formation of conjugates was determined by gel permeation chromatography (GPC) by adapting a method described by Verheul et al [29]. In short, a Shodex OHPak SB-806 column (15 cm) was used with 0.3 M sodium acetate, adjusted to pH 4.4 with acetic acid, as running buffer to minimize interaction between free OVA and TMC. An online 2475 Multi-Wavelength Fluorescence Detector (Waters, Milford MA, excitation 295 nm, emission 340 nm) was used to measure Trp fluorescence intensity. Fractions were collected for SDS-PAGE analysis.

SDS-PAGE & Western blotting

SDS-PAGE was performed to detect covalently bound OVA and to check for residual free OVA. Samples were run at 120 V under reducing and non-reducing conditions, in a 10% SDS-polyacrylamide gel. Samples were prepared in electrophoresis loading buffer (60 mM Tris-HCl, pH 6.8, with 25% glycerol and 2% SDS, 0.1% bromophenol blue solution and 5% v/v β-mercaptoethanol) and heated for 2 min at 90°C. After electrophoresis, bands were stained using a Silver Stain Plus kit (Bio-Rad, Veenendaal, NL), according to the manufacturer's instructions. To perform Western blot analysis bands were transferred to a nitrocellulose membrane (Whatman, Maidstone, UK) overnight at 30 V. Blot was blocked with 8% non fat milk (Campina, Amersfoort, The Netherlands) and incubated with polyclonal rabbit anti-OVA IgG for 24 h. Subsequently, the blot was treated with goat anti-rabbit IgG-HRP conjugate, followed by staining with 4-chloro-1-naphtol/peroxide.

UV spectroscopy

UV-VIS spectra were recorded using an Agilent 8354 spectrophotometer (Agilent Technologies, CA, USA). TMC, OVA and TMC-OVA conjugate were diluted to a final concentration equivalent to 0.5 mg/ml OVA and 1 mg/ml TMC in a 50 mM acetate buffer pH 3.0 with 0.9% NaCl. Tertiary protein structure was investigated by taking the second derivative of the zero order spectra [30].

Steady-state fluorescence

To study protein conformation, the intrinsic fluorescence of OVA (diluted to 0.05 mg/ml) was measured. Steady-state fluorescence was performed with an FS920 fluorimeter (Edinburgh Instruments, UK) at 25°C using a quartz cuvette with a path length of 10 mm. OVA's tryptophan residues were excited at λ=295 nm. Emission spectra were recorded between 305 and 400 nm, with steps of 1 nm and a cumulative addition of 2 scans per spectrum. All acquired spectra were corrected by subtracting the background spectrum of the buffer (PBS).

TMC/OVA nanoparticles

TMC/OVA nanoparticles were obtained by ionic complexation with TTP and OVA, as described before [12]. In short, OVA was added to a 0.2% w/v TMC solution in 5 mM Hepes (pH 7.4). Under continuous stirring (300 rpm) TPP was added to a weight ratio TMC:OVA:TPP of 10:1.0:1.7. Particles were washed and collected by centrifugation on a glycerol bed for 15 min at 12000 g and resuspended in 5 mM Hepes (pH 7.4). The particle size of the obtained particles was measured by dynamic light scattering (ZetaSizer Nano, Malvern Instruments, UK) and the zeta potential was determined by laser Doppler electrophoresis using the same apparatus

Dendritic cell studies

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh human blood using a Ficoll gradient as previously described [31]. Subsequently, monocytes were isolated from the PBMCs using a Percoll gradient as previously described [32]. After isolation, monocytes were adhered on 24-wells plates by incubation for 1 h at 37°C and 5% CO_2, and depleted of platelets by washing. Monocytes were differentiated into immature DCs by incubation for 6 days with RPMI 1640 medium supplemented with 10% fetal bovine serum, 250 U/ml GM-CSF and 100 U/ml IL-4.

For uptake of the TMC-OVA conjugate by DCs, FITC labeled OVA was covalently linked to TMC following the same method as described above. Immature DCs were exposed to TMC-OVA-FITC conjugates for 4 h at either 37°C or 4°C. Cells were washed three times with FACS buffer (10 mg/ml bovine serum albumin in PBS with 2% v/v fetal calf serum), and the fluorescence of the OVA-FITC-containing DCs was quantified with a flow cytometer (FACSCanto II, Becton Dickinson).

Dendritic cell maturation was determined by pulsing immature DCs with OVA, TMC and OVA, TMC-OVA conjugate, TMC/OVA nanoparticles or LPS for 4 h. Cells were washed with culture medium and plated in a 24-wells plate in the presence of GM-CSF. After 48 h, the DCs were washed twice with FACS buffer and stained with anti-CD86-APC for 30 min on ice. CD86 expression was determined with flow cytometry.

Immunogenicity

Eight week old female BALB/c mice received one intramuscular dose of 20 µg OVA in either free (with or without TMC), conjugated (TMC-OVA conjugate) or particulate form (TMC-OVA nanoparticles). Blood samples were taken one day before and 3 weeks after immunization. IgG titers were determined using a similar ELISA procedure as for anti-diphtheria toxoid (DT) [33], replacing the DT coating with an OVA coating (100 ng/well).

Statistics

The immunization data were analyzed with a Kruskal-Wallis test. DC uptake studies were analyzed using a two-way ANOVA with Bonferroni's posttest. All analyses were performed with Graphpad Prism 5 software for Windows.

Results

Synthesis of TMC-OVA conjugate

To establish a disulfide bond between OVA and TMC both molecules were functionalized with protected thiol groups, by reaction with SPDP. Reduction with DTT (figure 2) enabled us to monitor with UV spectroscopy the number of protected thiol groups (PDP) introduced per protein/TMC molecule, as the resulting leaving group, pyridine-2-thione, has an extinction maximum at 343 nm. We found that the number of PDP groups introduced can be controlled by the feed of SPDP: a linear relationship between PDP incorporation and SPDP feed was observed, up to 3 moles PDP per mol OVA or TMC (figure 3a,b), after which the reaction

became unpredictable due to precipitation. This allows tailoring the average number of PDP groups on both OVA and TMC. For the conjugation step, the number of functional groups on OVA was varied, while the average number of functional groups on TMC was either 1 or 2 moles per mol TMC, as a higher substitution degree induced precipitation of the polymer. An increasing number of PDP groups on OVA led to an increase in a higher conjugation efficiency, up to a maximum efficiency of ≈ 25% (figure 3c,d). Increasing the number of thiol groups on TMC from 1 to 2, decreased the number of required PDP groups on OVA from 4 to 2 to reach an efficiency of 25%, but did not lead a higher maximum efficiency.

Based on the results described above, a TMC-OVA conjugate made from TMC-SH molecules containing 2 thiol groups on average and OVA-PDP containing an average of 2 PDP moieties per molecule was selected for further studies. Protein analysis showed 17% w/v OVA in the freeze dried product, corresponding to a molar ratio OVA:TMC of about 1:3 in the conjugate, indicating the presence of residual free TMC.

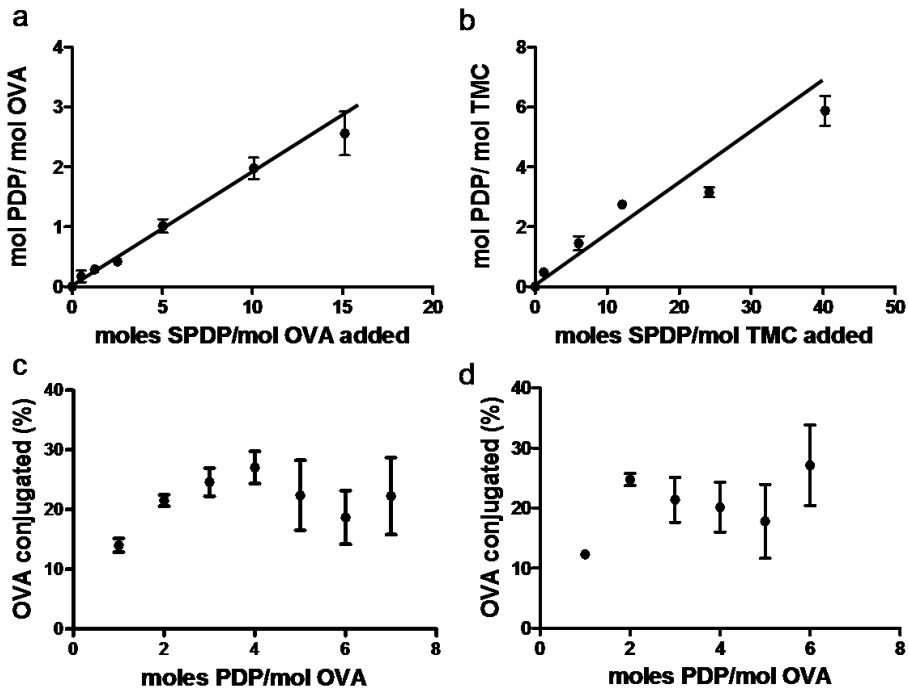

***Figure 3**: Functionalization and conjugation of TMC and OVA. Effect of SPDP feed on the amount of functionalized group on OVA (a) and TMC (b). PDP incorporation was monitored by UV-Vis detection at 343 nm. Efficiency of OVA conjugation to TMC-SH containing 1 mol SH/mol TMC (c) or 2 mol SH/mol TMC (d).*

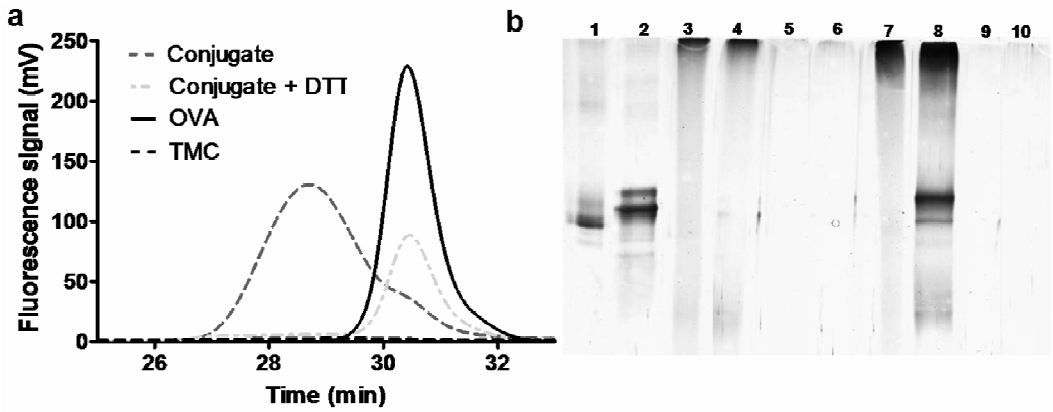

Figure 4: *(a) GPC chromatograms based on fluorescence detection (ex 295 nm, em 340 nm) of TMC-OVA conjugate (Conjugate), reduced TMC-OVA conjugate (Conjugate + DDT), OVA, and TMC. (b) SDS-PAGE under reducing (even lanes) and non-reducing (odd lanes) conditions of OVA (lane 1,2), TMC (lane 3,4), TMC-OVA conjugate fractions 25-27 min (lane 5,6), 27-29.5 min (lane 7,8) and 29.5-32 min (lane 9,10).*

Characterization of TMC-OVA conjugate

Formation of the conjugate was confirmed with GPC, using fluorescence detection (ex 295 nm, em 340 nm). TMC is not detected with this method, making it specific for OVA. Conjugates contained larger species than native OVA as the main conjugate peak shifted from 30-31 min to 28-29 min (figure 4a), whereas a mixture of TMC and OVA did not cause such as shift (data not shown). Reduction of the conjugate with DTT restored the native OVA peak at 30-31 min (figure 4a). SDS-PAGE on collected fractions confirmed that the peak at 28-29 min contained TMC-OVA conjugate (figure 4b lane 7,8), as only under reducing conditions a band at 45 kDa was visible. Although the shoulder in the TMC-OMV conjugate peak in GPC indicates the presence of a small fraction of free OVA in the conjugate sample (figure 4a), SDS-PAGE was unable to detect OVA in this fraction (figure 4b lane 9,10).

As a change in protein structure could adversely affect the immunogenicity of the antigen, the protein structure was investigated. Western blotting revealed that antigenic epitopes on OVA conjugated to TMC were still intact (figure 5), but does not give information on the overall conformation of OVA. Intrinsic fluorescence can be used to detect changes in the local environment of Trp residues inside a protein and UV spectroscopy gives information on Phe, Tyr and Trp. Therefore these techniques can give insight into the tertiary structure of the protein. As the individual UV spectra of Phe, Tyr and Trp strongly overlap, the 2nd derivative was used to enhance the resolution. The 2nd derivative spectra of native OVA and conjugated

OVA practically overlapped (figure 6b), indicating no change in tertiary structure. Similarly, no shift in fluorescence emission maximum of the Trp residues was detected after excitation at 295 nm (figure 6c), indicating that the polarity of the direct environment of the Trp residues had not changed after conjugation. The reduced fluorescence signal in conjugated OVA (figure 6c) is likely due to the introduction of the S-S bonds, since both S-S and S-H groups have been reported to quench Trp fluorescence [34]. Indeed, the addition of TMC-PDP or TMC-SH to native OVA also reduced the fluorescence signal to a similar extent (data not shown).

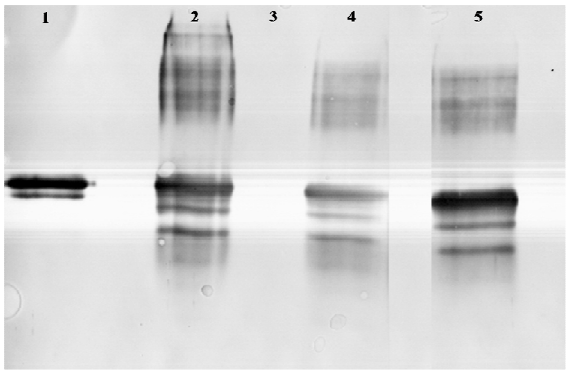

Figure 5*: Western blot of SDS-PAGE run under reducing conditions, using polyclonal anti-OVA IgG to detect the presence of intact epitopes. Lane 1: OVA; lane 2: OVA incubated for 16h at 40°C (equivalent conditions used for conjugation); lane 3: TMC; lane 4; TMC + OVA; lane 5: TMC-OVA.*

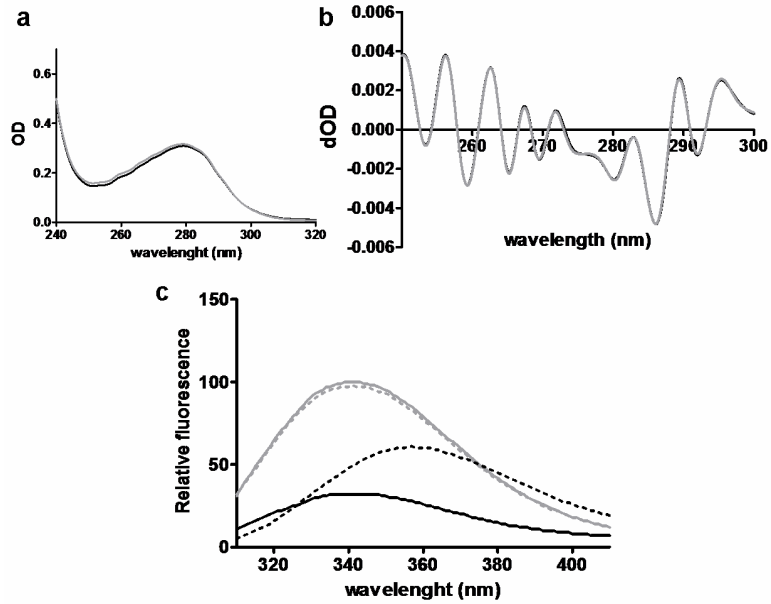

Figure 6: *(a) UV absorption spectrum and (b) 2nd derivate spectrum of 0.5 mg/ml OVA (grey line) and 2.5 mg/ml TMC-OVA (corresponding to 0.5 mg/ml OVA) conjugate (black line). c) Fluorescence emission spectra of OVA and TMC-OVA conjugate. Spectra of a mixture of OVA and TMC (dashed grey line) and unfolded OVA (OVA + 6 M guanidine, dashed black line) are shown for*

TMC/OVA nanoparticles

Relatively monodisperse (polydispersity index 0.23) nano sized (280 ± 32 nm) TMC nanoparticles were produced. Particles carried a positive charge as indicated by their positive zeta potential, 21 ± 4.3 mV.

Dendritic cell studies

As uptake of the antigen by DCs is a critical step in the initiation of an adaptive immune response [4, 5], the extent to which the TMC-OVA conjugate was internalized by DCs was quantified *in vitro*. Concentration dependent association of OVA with DCs was observed, which was significantly enhanced by conjugation to TMC as well as encapsulation in TMC nanoparticles ($p<0.001$), but not by coadministration of TMC (figure 7a). Moreover, TMC-OVA conjugate was taken up actively, as at 4°C DC association was limited ($p<0.001$, figure 7b).

TMC-OVA conjugate induced maturation of DC from antigen capturing to an antigen presenting (dendritic) phenotype (figure 8). Untreated immature DCs have a more or less round appearance, which was not notably changed after incubation with OVA (figure 8a) or OVA mixed with TMC (figure 8c), whereas DCs treated with LPS (figure 8b) or the conjugate (figure 8d) showed a dendritic phenotype. Expression of maturation marker CD86 was markedly increased after exposure of DCs to TMC-OVA conjugate as compared to a mixture of TMC and OVA or TMC/OVA nanoparticles (figure 9). Endotoxin levels were determined with a LAL assay (GenScript, Piscataway, NJ) and found to be similarly low for TMC and TMC-OVA conjugate (« 10 EU/mg).

Immunogenicity

An immunization study in mice was performed to investigate the immunogenicity of the TMC-OVA conjugate compared to a solution of OVA, a mixture of OVA and TMC, and TMC/OVA nanoparticles. The addition of TMC to OVA caused a significantly increased antibody production, compared to administration of OVA alone ($p<0.05$, figure 10). Conjugation of TMC and OVA, however drastically improves IgG production compared to both of these groups ($p<0.001$). The average IgG titer in mice immunized with TMC-OVA conjugate was slightly higher than that of mice immunized with TMC/OVA nanoparticles, although the difference was not statistically significant ($p=0.08$).

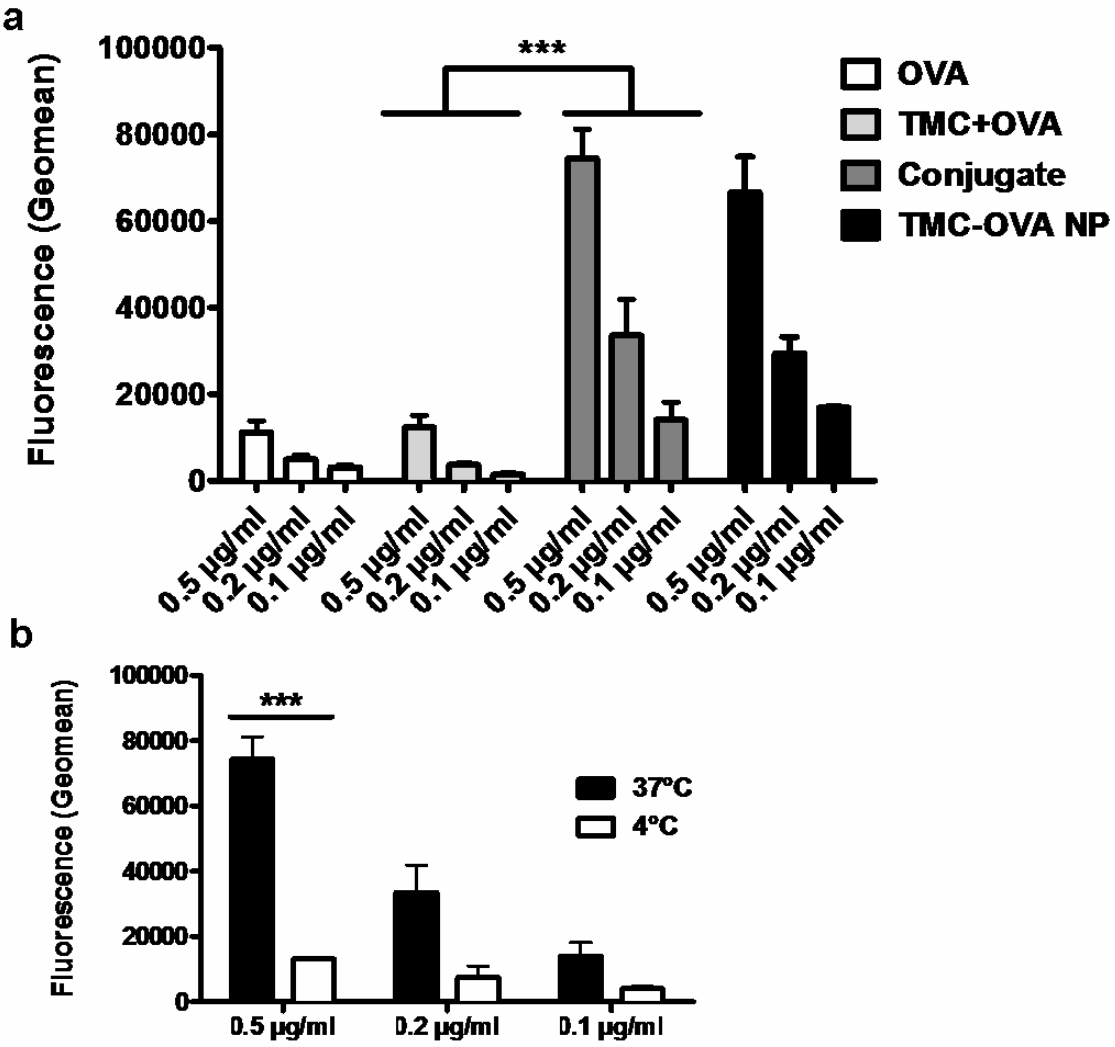

Figure 7**: (a) Dose dependent association of OVA, a mixture of OVA and TMC, TMC-OVA conjugate and TMC/OVA nanoparticles by DCs incubated at 37°C for 4 h. Conjugate as well nanoparticles showed a significant increase in uptake compared to OVA and TMC+OVA (= p<0.0001). (b) Active uptake versus passive association was investigated by exposing DC to TMC-OVA conjugate at 37°C and 4°C. Data are a representative example of 4 different monocyte donors. Bars represent mean +/- SD (n=3).*

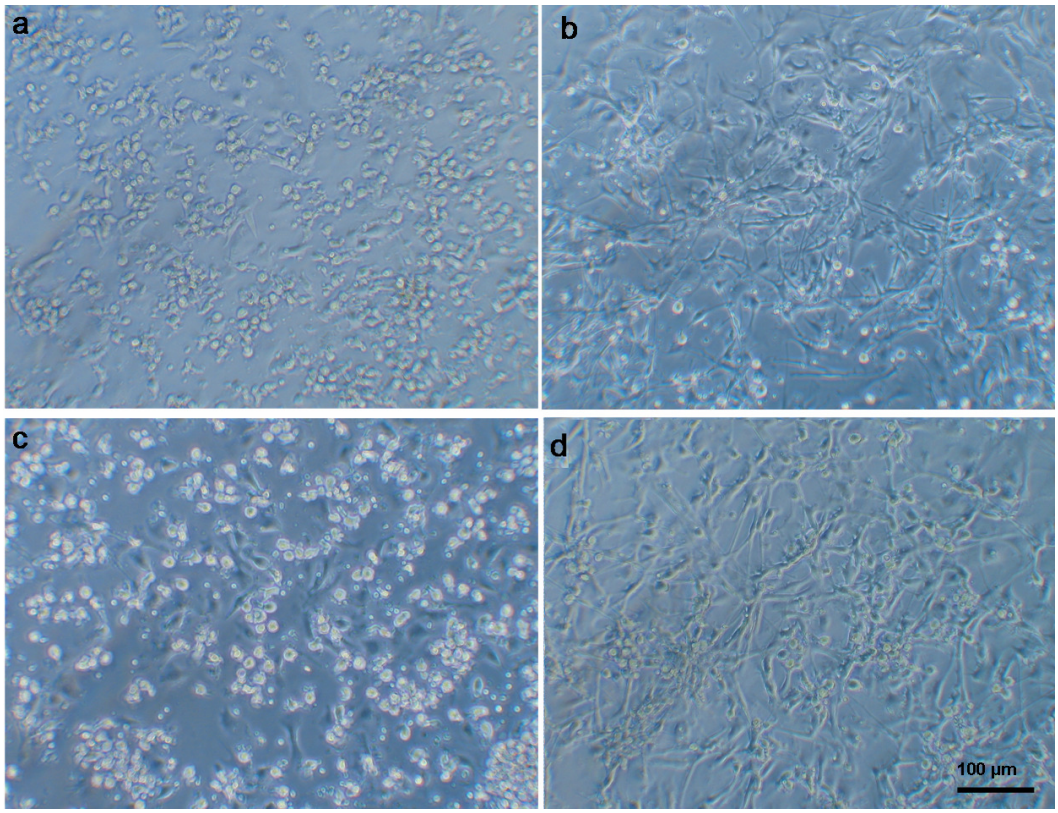

***Figure 8**: Representative example of micrographs of DCs treated for 4 h with (a) 0.2 µg/ml OVA, (b) 100 ng/ml LPS, (c) 0.2 µg/ml OVA + 1 µg/ml TMC and (d) 1 µg/ml TMC-OVA conjugate (corresponding to 0.2 µg/ml OVA). Magnification 20x.*

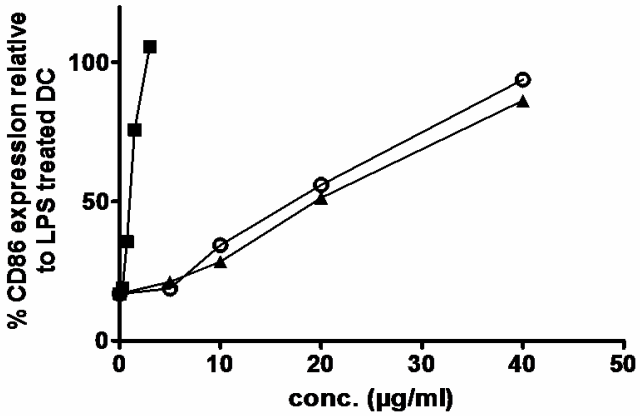

***Figure 9**: CD86 expression as a measure for DC maturation. Immature DCs were pulsed with increasing amounts of TMC/OVA mixture (open circles), conjugate (closed squares), or TMC/OVA nanoparticles (closed triangles) for 4 h, after which medium was replaced and CD86 expression was quantified after 48 h by using flow cytometry.*

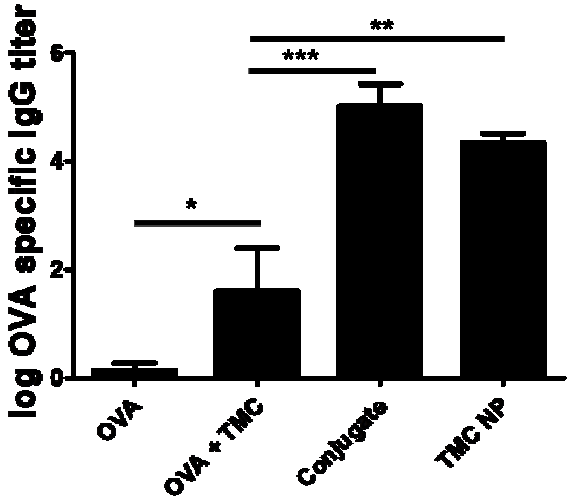

Figure 10: *OVA specific serum IgG titers after a single dose of 20 µg OVA, OVA mixed with TMC, TMC-OVA conjugate (conjugate) and TMC/OVA nanoparticles (TMC NP). * = p<0.05; ** = p<0.01; ***= p<0.001 Data represent mean +/- SD (n=5).*

Discussion

Subunit vaccines are notorious for the fact that they are safer, but less immunogenic than live attenuated or whole inactivated vaccines. One way of overcoming this decreased immunogenicity is the use of adjuvants. Although the practice of using adjuvants has been known for more than a century (e.g. the use of alum), only recently a few new adjuvants have been approved for human administration [35]. The main pitfall for an adjuvant still remains the potential health risk associated with its use as an immune stimulating compound [36]. Here we report on a method to increase the efficiency of the adjuvant, which could permit lowering the adjuvant dose.

The proteinaceous antigen, OVA, was coupled to polymeric adjuvant, TMC, using the SPDP method. This method, first described by Carlsson et al [37], has been developed to covalently link proteins with each other, but has also been used to synthesize protein-polymer conjugates [26, 38, 39] with the important advantage of introducing a disulfide bond, which is reversible under reducing conditions [22]. Detachment of the polymer from the protein, once the conjugate has been taken up by a DC, is a requisite for unaltered processing of the antigen, making the SPDP method an interesting approach. Free amine moieties on OVA and TMC were functionalized with protected thiol groups. The functionalization of TMC and OVA could be controlled, but reaction efficiencies were low compared to other reports [26]. Moreover, the total conjugation yield (25%) was lower than expected, as previous studies

reported yields ranging from 50-90% [26, 38, 39]. This could be explained by aggregation of protein and polymer, which was getting more pronounced with higher degrees of PDP functionalization on TMC and OVA (data not shown). It is likely that the aggregation of the molecules interfered with the disulfide bond formation between the protein and the polymer, resulting in a loss of coupling efficiency.

Nevertheless, the SPDP method proved to be a very useful way of covalently linking TMC and OVA, as the conjugation was reversible under reducing conditions and the epitopes on OVA were still intact. Moreover, we did not detect any changes in the structure of OVA using 2^{nd} derivative UV spectroscopy and intrinsic fluorescence spectroscopy. Protein conformation has not been regarded as essential with respect to the immunogenicity of its T-cell epitopes as these are mostly continuous or linear. B-cells epitopes however, have been reported to be discontinuous (conformational) [40]. The preservation of the protein structure makes the SPDP method an interesting strategy also to couple other protein-based antigens to an adjuvant carrying primary amine groups or free –SH groups.

The TMC-OVA conjugate exerts interesting immunological properties. Besides the expected enhanced immune response by the TMC-OVA conjugate due to the simultaneous uptake by and maturation of APC, uptake studies with DCs also showed an increased antigen uptake compared to a mixture of TMC and OVA, similar to that observed for TMC/OVA nanoparticles. This suggests that TMC directly facilitates antigen uptake by DCs and not indirectly, for instance via upregulation of receptors on the DC's surface, or by disrupting the cell membrane. Uptake-enhancing effects of cationic polymers have been reported before [12, 41-43], which was attributed to non-specific interactions between the positively charged polymers and the negatively charged cell surfaces, followed by active uptake. A similar effect was observed here, as at 4°C a fraction of conjugated OVA was associated with DCs, indicating interaction on the cell surface or passive diffusion into the DCs. However, the amount of engulfed TMC-OVA conjugate by DC at 37°C greatly surpassed the amount engulfed at 4°C, pointing to an important role for active uptake of the conjugate and the TMC particles after adsorption to the cell membrane. The possibility of increasing antigen uptake via conjugation with a DC specific targeting ligand has been reported previously [44], however, TMC has not been described as a specific ligand for receptors on the DC cell surface. C-type lectins play a role in the recognition of carbohydrate residues of bacterial surfaces, and have been suggested to recognize chitosan via non-deacetylated units (N-acetylglucosamine residues) [45]. Due to the treatment with NaOH, the number of N-acetylglucosamine residues in TMC is low (an average of 6.5 N-acetyl residues per TMC molecule), but this may still be sufficient to contribute to the effective uptake of the conjugate.

Exposure of DCs to low concentrations of TMC-OVA conjugate was accompanied with a strong induction of DC maturation (Figure 8). Surprisingly, an equivalent amount of soluble TMC or TMC nanoparticles was unable to activate DCs. Only a >20 fold more concentrated TMC solution was capable of inducing similar CD83 (data not shown) and CD86 expression (figure 9), indicating that the conjugate had a more potent adjuvant effect compared to unconjugated TMC or TMC particles. Importantly, endotoxin levels of TMC and TMC-OVA conjugate were comparably low, indicating that the effect on DC maturation was not caused by LPS contamination.

The *in vitro* findings were reflected *in vivo* as, in line with earlier reports about antigen-adjuvant conjugates [44, 46-49], the overall immunogenicity of TMC-OVA conjugate was shown to exceed that of soluble OVA/TMC mixture. Moreover, immunization with the conjugate induced at least similar IgG titers as immunization with TMC-OVA nanoparticles, showing that concomitant delivery of antigen and adjuvant, without the need for a particulate carrier, is sufficient to obtain a potent immune response. These findings suggest that conjugation may be a sound strategy in the design of subunit vaccines. Here we focused on conjugation of a single adjuvant, but the SPDP method also allows the ligation of multiple adjuvants, which could be even more effective [50] and may in future even allow manipulation of the type of immune response.

Conclusion

Conjugation of an antigen to an adjuvant is a promising strategy to enhance the immunogenicity of subunit vaccines. The SPDP crosslinker is well suited to covalently couple OVA to the polymeric adjuvant TMC, as the resulting linkage is reversible and protein conformation unchanged. TMC-OVA conjugates are efficiently taken up by DCs and the immunogenicity is superior to that of unconjugated OVA, even matching the immunogenicity of TMC nanoparticles.

Acknowledgement

This work was performed within the framework of Top Institute Pharma project number D5-106 (NL).

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