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Challenges and opportunities in nasal subunit 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 subunit vaccine delivery : mechanistic studies using ovalbumin as a model antigen*. Retrieved from <https://hdl.handle.net/1887/16394>

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Antigen-adjuvant nanoconjugates for nasal vaccination, an improvement over the use of nanoparticles?

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Molecular Pharmaceutics, In Press

Abstract

Entrapment of antigens in mucoadhesive nanoparticles prepared from N-trimethyl chitosan (TMC) has been shown to increase their immunogenicity. However, because of their large size compared to soluble antigens, particles poorly diffuse through the nasal epithelium. The aim of this work was to study whether nasal vaccination with a much smaller TMC-antigen nanoconjugate would result in higher antibody responses as compared to TMC nanoparticles.

TMC was covalently linked to a model antigen, ovalbumin (OVA), using thiol chemistry. For comparison, TMC/OVA nanoparticles and solutions of OVA and a physical mixture of TMC and OVA were made. As shown previously for TMC-OVA nanoparticles, TMC-OVA conjugate prolonged the nasal residence time of the antigen. TMC-OVA conjugate diffused significantly better through a monolayer of lung carcinoma (Calu-3) cells than TMC/OVA nanoparticles did. Moreover, nasal immunization of mice with the conjugate resulted in significantly more OVA positive DCs in the cervical lymph nodes as compared to TMC/OVA nanoparticles. Mice nasally immunized with TMC-OVA conjugate produced high levels of secretory IgA in nasal washes and higher titers of OVA-specific IgG than mice immunized with any of the other formulations. Moreover, as compared to TMC/OVA nanoparticles, TMC-OVA conjugate induced a more balanced IgG1/IgG2a response.

In conclusion, the TMC-antigen nanoconjugate improves nasal delivery and immunogenicity of the antigen. This suggests that efficient co-delivery of antigen and adjuvant to DCs, rather than a particulate form of the antigen/adjuvant combination, is decisive for the immunogenicity of the antigen.

Introduction

The nasal mucosa is an attractive site for vaccination, as it is very accessible, low on proteolytic enzymes compared to the oral route, and presents a surface densely populated by immune cells, often referred to as the nasal associated lymphoid tissue (NALT). Various studies in rodents[1-4] and humans[5] have shown that the nasal epithelium not only can be the inductive site for the production of systemic (IgG) antibodies, but also can be the executive site for the secretion of local (sIgA) antibody responses. However, the amount of antigen that penetrates the nasal epithelium is limited and very large doses are necessary. Moreover, the tolerogenic nature of the nasal mucosa interferes with the induction of an adaptive immune response and makes the application of an adjuvant imperative for subunit vaccines[6].

Encapsulation of antigens into particulate systems is a popular method to increase the immunogenicity, as particles can facilitate the uptake of the antigen by dendritic cells (DCs) and the multimerization of epitopes on the particle surface can increase the immune recognition by B-cells[7, 8]. Not surprisingly, the nanoparticle approach has also been applied to nasal vaccines[9, 10]. Mucoadhesive particles can prolong the antigens' residence time in the nasal cavity[11] and can be supplied with adjuvants to break nasal tolerance. N-trimethyl chitosan (TMC) based nanoparticles combine mucoadhesiveness, adjuvant effect and even M-cell targeting[12, 13]. Nasal administration of ovalbumin, tetanus toxoid or hemagglutinin loaded TMC nanoparticles (TMC NP) resulted in strong antibody against the encapsulated antigen[14-16].

Although these advantages make the use of nanoparticles for nasal vaccination very appealing, a significant drawback is the increased size of the vaccine. Smaller entities have been associated with stronger immune responses[17, 18] as larger species evidently have more difficulties diffusing through the nasal epithelium[19]. M-cells present in the nasal epithelium have been reported to transport particulate structures from nano to micro scale, but the M-cell population is very small[20], probably making its contribution to the total amount of antigen reaching the subepithelium limited[18].

Recently we have reported on the synthesis and immunological properties of TMC-OVA conjugates[21]. After intramuscular administration, these nanoconjugates and TMC/OVA NP were equally effective at inducing systemic immune responses. We hypothesize that nasal vaccination with TMC-OVA conjugates results in higher antibody responses than administration of TMC/OVA NP as the conjugates may diffuse better through the nasal epithelium because of their smaller size, but still have mucoadhesive and immunostimulatory

characteristics, because of the co-localization of adjuvant and antigen. Therefore we investigated TMC-OVA's ability to diffuse through a mucosal epithelial monolayer *in vitro*, compared to TMC/OVA NP and plain OVA. Moreover, the nasal residence time of the nanoconjugates was studied in mice using a live imaging technique. To investigate the combined effect of nasal residence time, epithelial penetration capacity and ability of the nanoconjugate to be taken up by DC *in vivo*, the amount of OVA positive DCs in the draining lymph node was quantified 24 hour after nasal administration of the TMC-OVA conjugate. Finally, a nasal vaccination study in mice was undertaken to measure the immunogenicity.

Materials and Methods

Materials

N-trimethyl chitosan (TMC) with a degree of quaternization of 20% was synthesized starting from 92% deacetylated chitosan (MW 120 kDa; Primex, Siglufjordur, Iceland), as earlier described [22]. Endotoxin free OVA was purchased at Merck (Darmstadt, Germany). Phycoerythrin (PE)-Cy5 labeled anti-CD11c- and Matrigel were acquired from Becton Dickinson (Franklin Lakes, NJ, USA). Microtiterplates were purchased at NUNC (Roskilde, Denmark). Phosphate buffered saline (pH 7.4) was obtained from Braun (Oss, NL). Normal 12-well plates as well as 12-well Transwell plates were obtained from Corning (Schiphol, NL), Invitrogen (Breda, NL) supplied fluorescein isothiocyanate labeled OVA (OVA_{FITC}), AlexaFluor647 labeled OVA (OVA_{AF647}), bovine serum albumin (BSA) and all cell culture products unless stated otherwise. LI-Cor (Lincoln, NE, USA) provided IRdye™ 800CW which was conjugated to OVA according to the manufacturer's instructions. N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), dithiothreitol (DTT), pentasodium tripolyphosphate (TPP) and all other salts/chemicals were purchased at Sigma-Aldrich (Zwijndrecht, NL), unless stated otherwise.

TMC-OVA nanoconjugate synthesis

TMC-OVA nanoconjugates were synthesized and characterized as described before [21]. Briefly, 10 mg TMC and 5 mg OVA were separately exposed to a 10 fold molar excess of SPDP for 1 h at room temperature, resulting in approximately 2 functionalized groups per TMC and per OVA molecule. Functionalized TMC was treated with DTT for 30 min at room temperature to obtain thiolated TMC. Thiolated TMC and functionalized OVA were mixed at a 1:1 molar ratio to allow disulfide bond formation overnight. The conjugate's hydrodynamic diameter

was obtained by dynamic light scattering (ZetaSizer Nano, Malvern Instruments, UK) and determined to be 28 nm $\pm$ 0.6. For determining the nasal residence time, transport over a Calu-3 monolayer and DC uptake in the lymph nodes, OVA was replaced by OVA-IR-dye 800CW, OVA_{FITC} and OVA_{AF647}, respectively.

TMC/OVA nanoparticles

TMC/OVA NP were obtained by ionic complexation with TPP 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 TMC/OVA NP as measured by dynamic light scattering was 312 ± 14 nm (polydispersity index 0.22) and the zeta potential, determined by laser Doppler electrophoresis, was 19.2 ± 3.5 mV. TMC/OVA_{FITC} and TMC/OVA_{AF647} NP with similar size and zeta potential were prepared by substituting OVA by its fluorescent counterpart.

Calu-3 cell culture

Calu-3 cells (ATCC, Washington, DC, USA) were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% v/v fetal bovine serum, 4.5 g/l glucose, 2 mM L-glutamine, 1% v/v non essential amino acids and 500 U/ml Penicilline/Streptomycine at 37°C and 5% CO₂.

For transport experiments the inserts of 12-well Transwell plates were coated with Matrigel according to the manufacturer's instructions. Calu-3 cells were seeded (5×10^5 per insert) and maintained for 14 days in supplemented DMEM. Medium at both the apical and the basolateral side was changed every other day. Integrity of the monolayer was assayed by measuring the transepithelial electrical resistance (TEER) using a home made dip stick electrode.

In vitro transport

Calu-3 monolayers were washed once with Hank's Balanced Salt Solution (HBSS) and allowed to equilibrate in HBSS for 30 min at 37°C. TEER was determined used a home made dip stick electrode. Subsequently the apical medium was removed and the insert were transferred to a 12-well plate containing 1.2 ml HBSS per well. Formulations were diluted in HBSS to a final concentration of 100 μ g/ml OVA_{FITC} and 300 μ l was added to the apical side. After a 60 min incubation period, inserts were transferred to a preheated (37°C) 12-well plate

containing 1.5 ml HBSS and TEER was assessed. Basolateral compartments were collected and total fluorescence determined using fluorescence spectroscopy (excitation at 495 nm emission at 520 nm, Infinite M1000, TECAN, Mechelen, Belgium).

Nasal residence time

Nasal residence time measurements were performed in accordance to the protocol described by Hageraars et al.[23]. Female Balb/c (nu/nu) mice between 8 and 10 weeks old (Charles River, L'Arbresle, France) were lightly anesthetized using isoflurane prior to the administration of 3 µg (10 µl) OVA labeled with IRdye™ 800CW. The nose was wiped clean with a paper towel and immediately fluorescence intensity (excitation 710 nm, emission 760; 780; 800; 820 and 840 nm) was measured using an IVIS Spectrum® (Caliper Life Sciences (Hopkinton, MA, USA). Every 15 minutes, light anesthesia was applied again and fluorescence intensity was determined as described above. Between measurements, mice were conscious.

To calculate the mean fluorescence in the nasal cavity, the IR-dye 800CW specific signal was separated from the background fluorescence by spectral unmixing using Living Image 3.1 software (Caliper Life Sciences). Regions of interest (ROI) were set over the nasal cavity of the mice and the average pixel intensity within the ROI was quantified using the same software. Fluorescence intensity at t=0 was set as at 100%.

Antigen uptake by DCs in the lymph nodes

Eight weeks old female Balb/c mice were nasally administered 20 µg OVA_{AF647} in different formulations (in 10 µl PBS, 5 µl per nostril). After 24 h mice were sacrificed and cervical lymph nodes were collected. Single cell suspensions were obtained, by grinding the lymph nodes through 70 µm cell strainers. Lymphocytes were washed with PBS containing 1% w/v BSA and stained with 50x diluted anti-CD11c-PE-Cy7. Cells were analyzed with flow cytometry using a FACSCantoII (Becton Dickinson). DC population was determined based on the expression of CD11c and OVA⁺ cells in this population were quantified.

Vaccination

Eight week old female Balb/c mice nasally received formulations containing 20 µg OVA in a total volume of 10 µl PBS (5 µl per nostril). After 3 weeks, blood samples were drawn and mice received a similar nasal booster dose. After 6 weeks blood samples were taken from the femur artery and mice were sacrificed and nasal washes were performed.

Animal experiments were approved by the Ethical Committee of the Leiden University Medical Centre in accordance to the Dutch Animal Protection Act.

Determination of serum IgG, IgG1, IgG2a and secretory IgA

Microtiter plates (96 wells) were coated with 100 ng OVA in 100 mM sodium carbonate buffer pH 9.4 for 24 h at 4°C. To reduce non specific binding, well surfaces were blocked by incubation with 1% w/v BSA in PBS for 1 hour at 37°C. After washing, serial dilutions of serum, ranging from 20 to 2×10^6 , were applied for 1.5 hours at 37°C; nasal washes were added undiluted. After washing, OVA specific antibodies were detected by incubating HRP conjugated goat anti-mouse IgG, IgG1, IgG2a or IgA (1 h at 37°C) and, subsequently after extensive washing, with 50 µg tetramethylbenzidine (TMB)/ 1 µM H₂O₂ in sodium acetate buffer pH 5.5 for 15 min at room temperature. Reaction was stopped with 2 M H₂SO₄ and absorbance was determined at 450 nm with an EL808 micro plate reader (Bio-Tek Instruments, Bad Friedrichshall, Germany).

Statistics

All the data were analyzed with a one-way ANOVA with Bonferroni's post-test, with the exception of the antibody titers, which were analyzed with a Kruskal-Wallis test with Dunns post-test. Statistics were performed using GraphPad 5.0 for Windows.

Results

Nasal residence time

Increasing the nasal residence time of the antigen is presumed to be one of the key features by which TMC NP augment the immune response¹⁰[15]. In a recent study we showed that TMC NP indeed reduced the clearance rate of OVA from the nasal cavity with about 50%[13]. It is therefore imperative to know whether TMC-OVA nanoconjugates also possess this characteristic. Monitoring the decay of fluorescence in the nasal cavity allowed an assessment of the effect of TMC conjugation on the clearance of OVA (Figure 1). Conjugation of OVA to TMC prolonged the nasal residence time compared to plain OVA. Whereas OVA was practically cleared from the nasal cavity within 2 h, the clearance of TMC-OVA conjugates was strongly delayed, with a residence time in the nasal cavity exceeding 2.5 h. Interestingly, no significant difference between TMC-OVA conjugate and a TMC+OVA physical mixture was observed, indicating that the increased residence time is caused by the presence of TMC and does not rely on conjugation between TMC and OVA.

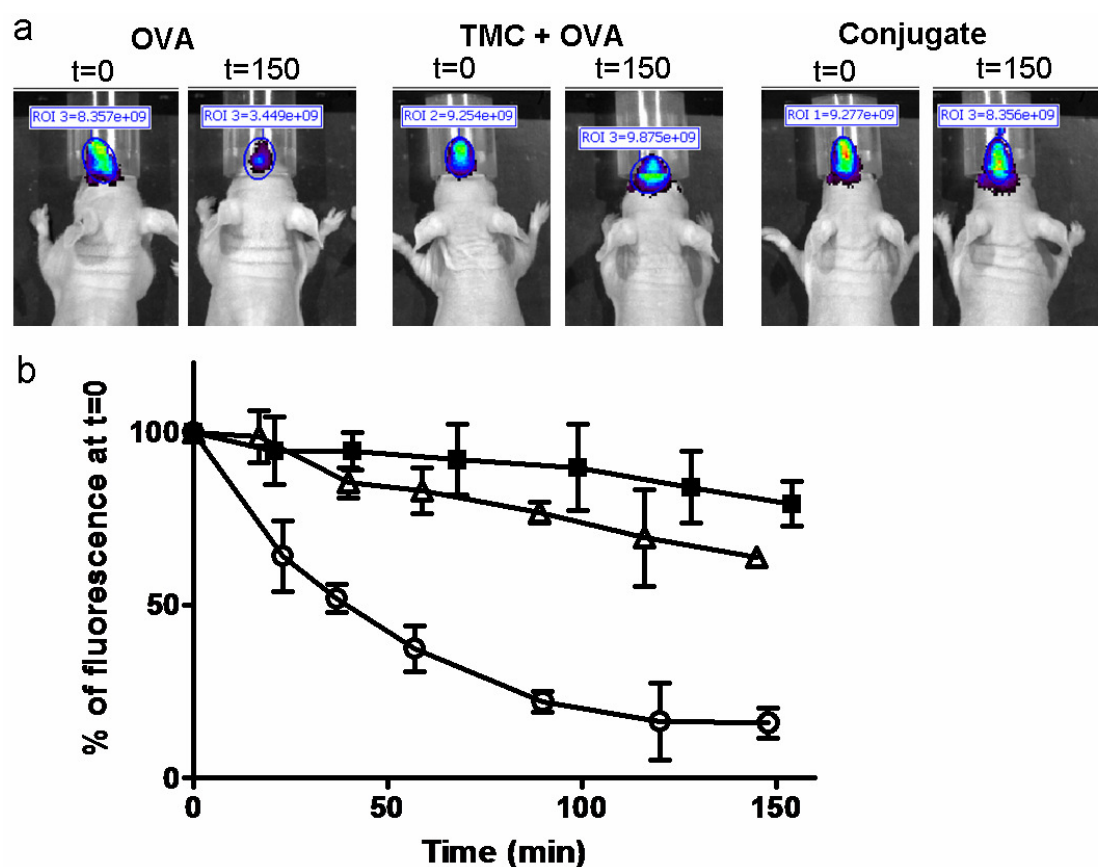


Figure 1: a) Nasal clearance of OVA after co-administration or conjugation with TMC. Emission spectra at 800 nm. b) Clearance derived from spectra. Circles OVA, squares OVA + TMC and triangles TMC-OVA conjugate. Error bars represent SD (n=3).

Antigen transport *in vitro*

Transport of the antigen through the nasal epithelium is a critical step in its delivery to antigen presenting cells (APCs). Calu-3 cells are lung cells that secrete mucus and form monolayers, making them an good *in vitro* model to study the transport of drugs and vaccines through respiratory epithelium[24][25]. Coadministration of OVA with TMC enhanced the transport of OVA through a Calu-3 monolayer ($p < 0.01$ Figure 2). This was accompanied by a decreased TEER, which was not observed for administration of OVA alone. Encapsulation of OVA into TMC/OVA NP resulted in a more than 10 fold reduction in the amount of transported OVA compared to plain OVA ($p < 0.001$). TMC-OVA conjugates showed a significantly higher transport rate than TMC/OVA NP ($p < 0.05$), although conjugation still reduced OVA transport through a Calu-3 monolayer compared to plain OVA ($p < 0.01$).

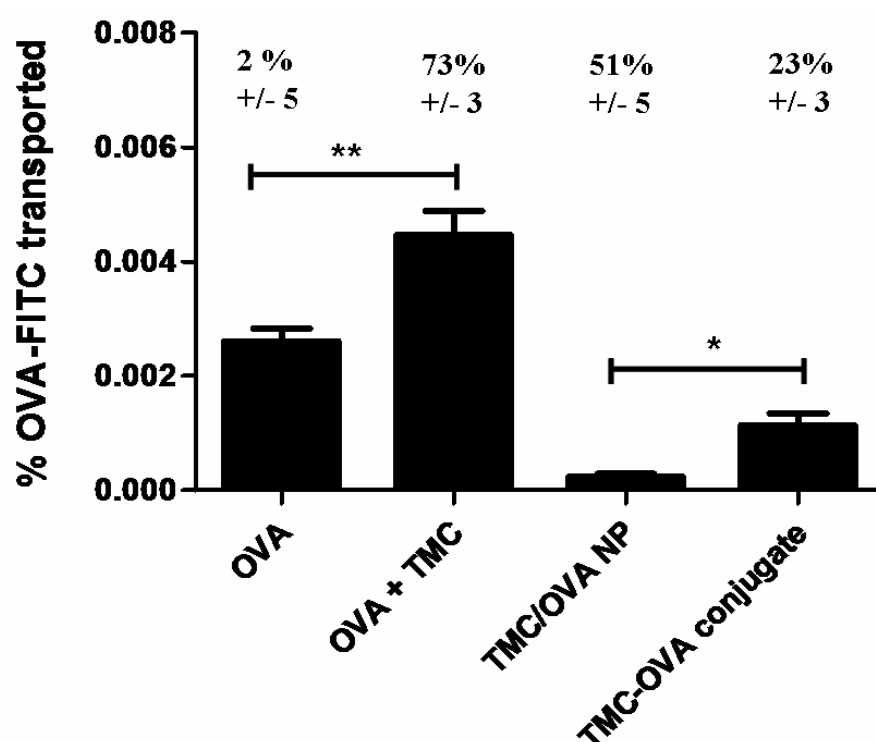


Figure 2: Diffusion of OVA-FITC through a Calu-3 cell monolayer as a measure for mucosal epithelial permeability. Bars represent mean +/- SD (n=9). * $p < 0.05$, ** $p < 0.01$. TEER decrease (% +/- SD) after 1 h exposure to the formulation is indicated above.

Antigen delivery to DCs in vivo

After passing the epithelium, the antigen can either drain through the interstitium to the nearby cervical lymph nodes where it can be taken up by DCs, or it can first be taken up by local DCs that will subsequently transport the antigen to the lymph node[26]. One day after nasal administration the cumulative effect (direct or DC mediated antigen delivery) should be visible in the cervical lymph nodes. Analysis of the DCs isolated from the cervical lymph nodes showed that the delivery of OVA from the nasal cavity to the lymph nodes was significantly enhanced by conjugation of the antigen to or co-administration with TMC, compared to immunization with plain OVA or TMC/OVA NP ($p < 0.05$, Figure 3). No significant differences between TMC/OVA and TMC-OVA conjugate were observed.

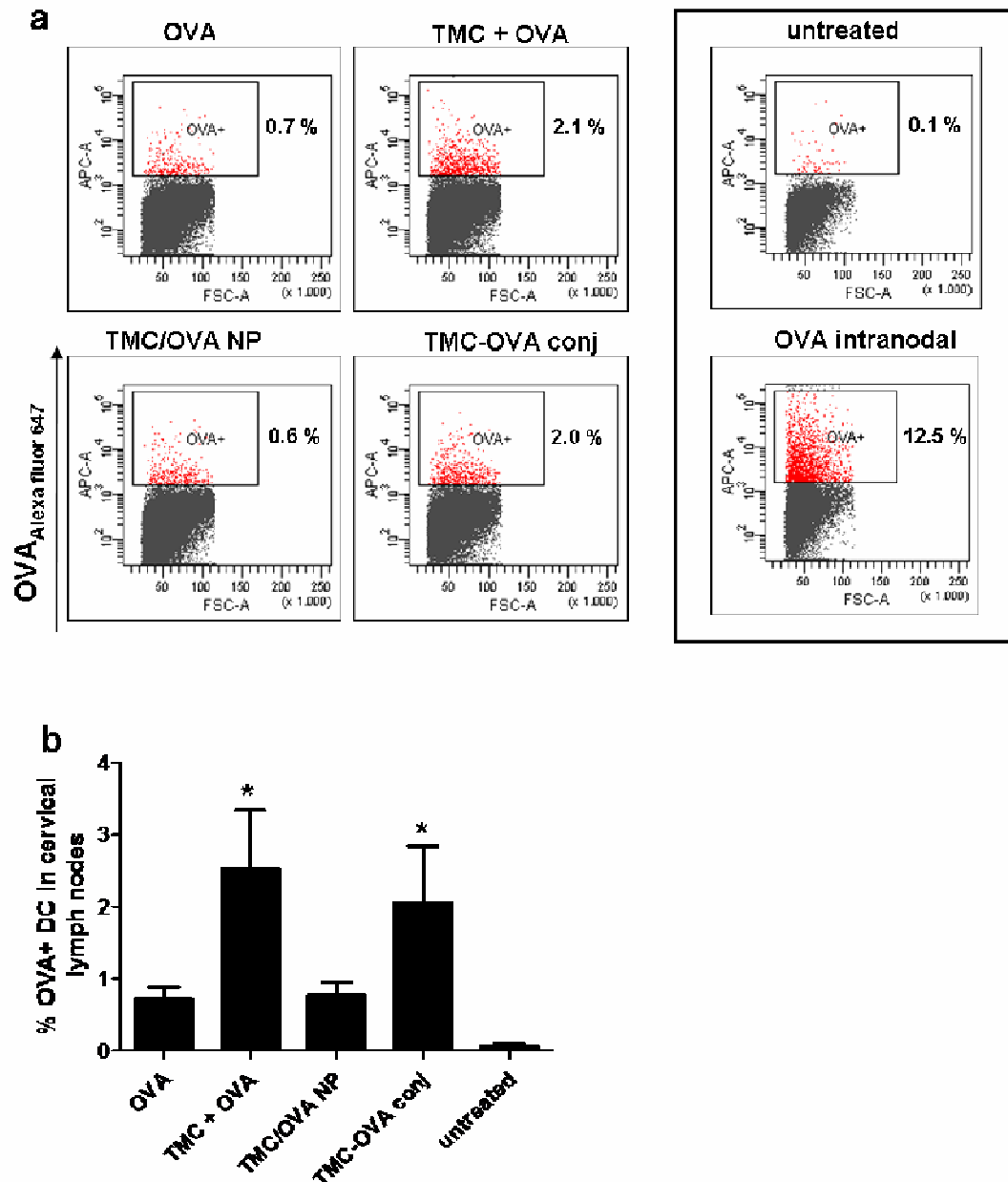


Figure 3: a) Representative flow cytometry histograms of single cell suspensions of cervical lymph nodes. 24 h after application of 20 μg OVA_{AF647}. Cells were gated for CD11c⁺. Lymph nodes from non vaccinated mice were used as negative control (untreated), and those of mice vaccinated intranodally with 0.2 μg OVA_{AF647} as a positive control. Percentages indicate the number of DC within the OVA⁺ region b) Number of OVA⁺ DC in cervical lymph node 24 h after application of 20 μg OVA_{AF647}. n=4 $\pm$ SEM * p<0.05 compared to OVA.

Immunogenicity

To investigate whether the observed differences in transport and delivery to DCs were reflected in the immunogenicity of the formulations, a nasal vaccination study was performed. To assess the effect on the systemic antibody response, OVA specific IgG titers were determined. As the delivery system has can also influence the quality of the immune response, IgG subclasses (IgG1 and IgG2a) were quantified and sIgA levels were measured in nasal washes.

TMC-OVA nanoconjugates led to substantial OVA specific IgG titers already after a single nasal immunization (Figure 4), being significantly higher than titers of mice nasally vaccinated once with OVA alone, OVA/TMC mixture ($p<0.001$), TMC/OVA NP ($p<0.05$), or intramuscularly vaccinated with OVA ($p<0.01$). After a booster only TMC/OVA NP vaccination resulted in similar high titers as TMC-OVA nanoconjugates ($p=0.29$). Besides the more rapid onset of an immune response induced by the nanoconjugates than by TMC/OVA NP, the two formulations differed in the type of immune response elicited (Figure 5). Whereas vaccination with TMC-OVA conjugate resulted in a rather balanced IgG1/IgG2a profile, immunization with a physical mixture of TMC and OVA or TMC/OVA NP resulted in antibody profile towards an IgG1 (indicative of a Th2 type) response ($p<0.05$).

TMC-OVA conjugates and TMC/OVA NP induced high levels of OVA-specific sIgA compared to a physical mixture of TMC and OVA or OVA alone (Figure 6), illustrative of a mucosal immune response.

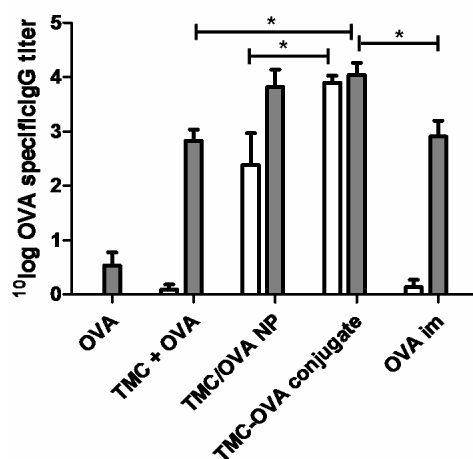


Figure 4: OVA specific serum IgG titers after nasal vaccination with a priming dose (white bars) or a booster dose (gray bars) of OVA. Mean $\pm$ SD ($n=8$). * $p<0.05$

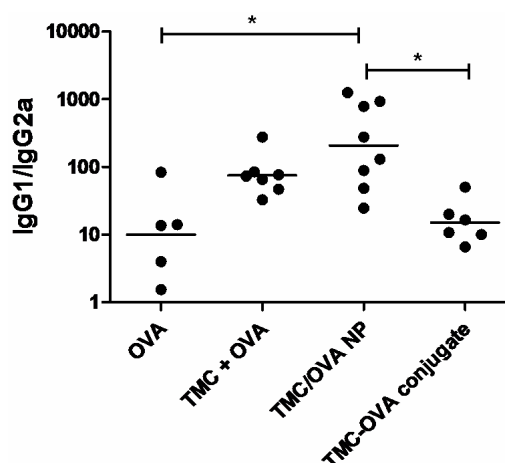
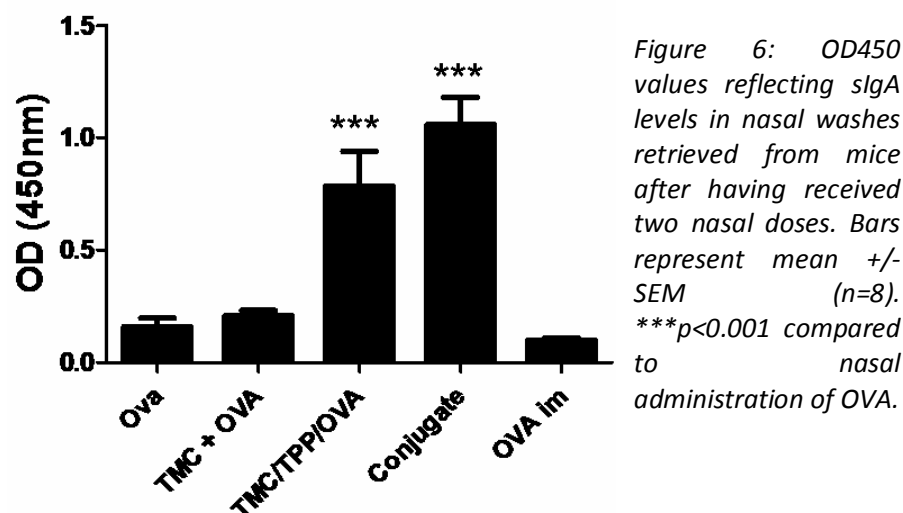


Figure 5: IgG1/IgG2a ratio indicative of the quality of the immune response. Bar represent geometric mean. * $p<0.05$



Discussion

A wide range of (nano)particulate systems have been shown to increase the immunogenicity of the encapsulated antigen when administered by injection[27]. Although these systems greatly differ in size, shape, charge and release profiles, the explanations for their immune potentiation are remarkably homogeneous. The adjuvant effect of particles has often been attributed to their ability to form slow release depots, to enhance antigen uptake and presentation by APCs or to enhance the activation of APCs. Moreover, the necessity of co-localizing antigen and adjuvant in one entity to induce proper T-cell proliferation has been clearly shown[28] and may be the most important explanation why particles have an immunostimulatory effect[27, 29, 30].

The benefit of using nanoparticulate formulations for nasal vaccination has become evident in the last decades[10, 31], as many studies have shown higher antibody responses towards encapsulated antigen than to soluble antigen[32]. Although nasal and parenteral vaccination share the fact that antigens have to be taken up by APCs and these APCs have to be activated, we recently demonstrated that the nasal delivery route requires a different nanoparticle design compared to the parenteral route[13]. Firstly, the residence time in the nasal cavity is limited due to mucociliary clearance, making a beneficial effect of a depot highly unlikely and favoring the use of mucoadhesive particles. Secondly, the need to pass the nasal epithelium may require an antigen-adjuvant construct to be as small as possible[17, 18]. TMC/OVA NP (diameter ca. 300 nm) are mucoadhesive and prolong the nasal residence time,

but are relatively large entities compared to soluble OVA (diameter ca. 5 nm). In this respect, TMC-OVA nanoconjugates (diameter ca. 28 nm) seem a logical design. We have shown before that TMC-OVA conjugates induce the uptake of OVA by DCs to a similar extent as TMC/OVA NP and also activate these DCs[21]. Here we show that they also prolong the nasal residence time (Figure 1), as earlier shown also for TMC/OVA NP[13], and seem to have an advantage over TMC/OVA NP as their transport over epithelial cells was higher (Figure 2). TMC is a known absorption enhancer[33, 34] by opening tight junctions between epithelial cells[35-37] and is not toxic for Calu-3 cells at concentrations similar to the ones used in the current study [37, 38]. Indeed, the addition of TMC decreased the TEER of a Calu-3 cell monolayer and increased OVA transport. Encapsulation of OVA in TMC NP, however, dramatically decreased its transport, although the tight junctions were opened judging from a decrease in TEER (data not shown). This indicates that TMC/OVA NP are indeed too big for intercellular transport and would have to rely on transcellular transport (e.g. M-cell transport).

TMC-OVA conjugates, being more bulky than OVA, did not penetrate the Calu-3 monolayer as efficiently as a physical mixture of TMC and OVA, but the conjugate's transport was much better than that of TMC/OVA NP (Figure 2). This is likely the reason why more OVA⁺ DCs were detected in the cervical lymph nodes 24 h after nasal administration of TMC-OVA conjugates than after TMC/OVA NP (Figure 3). These results are in accordance with a study by Brooking et al.[19] who investigated the size dependent penetration of particles through the nasal epithelium and found that the smallest particles (20 nm) reached the highest peak concentration in the bloodstream. Unfortunately the particle disposition in the lymph nodes was not reported. However, in an earlier report, encapsulation of tetanus toxoid (TT) into poly-lactic acid (PLA) nanoparticles did not increase the TT concentration in cervical lymph nodes compared to the nasal application of TT solution[39]. Only when PLA particles were coated with poly-(ethylene glycol) the transport of encapsulated TT was enhanced[39, 40]. This indicates that particles can experience difficulties passing the nasal epithelium and their physicochemical characteristics affect the delivery of the encapsulated antigen to the draining lymph nodes.

Interestingly, a similar number of OVA⁺ DCs was found after application of TMC-OVA conjugate and TMC/OVA mixture, whereas based on the *in vitro* transport one would expect more OVA⁺ DCs after administration of TMC/OVA mixture. This could be explained by the improved uptake of TMC-OVA by DCs due to the TMC-OVA co-localization as observed earlier[21], which might compensate for the inferior transport of the nanoconjugate compared to TMC/OVA mixture. A similar explanation could be applied to the difference between TMC/OVA NP and soluble OVA. Although OVA diffuses through the epithelium with

more ease (Figure 2), the prolonged nasal residence time and superior delivery of TMC/OVA NP to cervical DCs compared to plain OVA make the total number of OVA⁺ DCs comparable (Figure 3).

The nasal vaccination study reveals the cumulative effect of the formulation parameters (Table I). Only mice that received an adjuvanted (TMC-containing) formulation, developed OVA specific IgG titers after nasal administration (Figure 4). Secondly, mice that received TMC and OVA in co-localized form developed higher IgG titers as well as sIgA levels than mice that received TMC and OVA as a mixture, most likely because of improved antigen uptake by DCs in conjunction with improved DC maturation. Finally, from the two co-localized formulations TMC-OVA nanoconjugates outperformed TMC/OVA NP after the priming dose, probably because of superior uptake of the conjugates through the nasal epithelium. Although this explanation is very tempting and straightforward, a significant Th1 shift after vaccination with TMC-OVA conjugates compared TMC/OVA NP (Figure 5) could also indicate a more complex answer. TMC is generally associated with a strong IgG1 response[15, 41, 42], indicative of a Th2 bias, but also occasionally has been described to elicit substantial IgG2a antibody titers[12, 43]. Endotoxin determination with LAL-test showed no evidence of contamination of the nanoconjugate with endotoxin (<0.1EU/mg), suggesting the absence of immune stimulatory compounds other than TMC. However, parameters like the antigen dose, exposure time, interaction with pathogen recognition receptor and the mode of uptake by APCs can all influence the Th1/Th2 balance[44, 45]. Interestingly, it has been suggested that smaller particles may induce a more Th1 biased response, compared to larger particles with the same make up, as smaller particles resemble the dimensions of viruses [46], which could explain the Th1 shift observed here with the TMC-OVA nanoconjugate as compared to TMC/OVA NP. A Th1 shift could be beneficial in case of vaccination against intracellular bacteria or viruses. This, combined with the strong total antibody level makes it worthwhile to explore the mechanism of nasal vaccination with antigen-adjuvant nanoconjugates more closely.

Conclusion

The co-localization of antigen and adjuvant seems to be the driving force behind the immune potentiating effect of TMC based nanoparticles after nasal administration, rather than the particulate antigen design. This makes nasal vaccination with TMC-antigen nanoconjugates a very promising strategy, as these conjugates are more easily take up by the nasal epithelium than larger nanostructures, while preserving the property of co-delivering the adjuvant (TMC) and antigen to APCs.

Acknowledgment

This research was performed under the framework of TI Pharma project number D5-106 “vaccine delivery: alternatives for conventional multiple injection vaccines”.

Table 1: Summary of findings in this study. The extent of the immune response against OVA, in relation to the formulation parameters.

	Immune response	Advantage	Disadvantage
OVA	Negligible	• Good epithelial penetration	• Short residence time • Poor antigen uptake by DCs • Poor DC maturation
TMC + OVA	Moderate	• Good epithelial penetration • Prolonged residence time • Adjuvant effect	• Poor antigen uptake by DCs • No co-localization of adjuvant and antigen • Th2 biased response
TMC/OVA NP	Good	• Prolonged residence time • Adjuvant effect • Good antigen uptake by DCs • Co-localization of adjuvant and antigen	• Poor epithelial penetration • Th2 biased response
TMC-OVA conjugate	Very Good	• Prolonged residence time • Adjuvant effect • Good antigen uptake by DCs • Co-localization of adjuvant and antigen	

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