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Mechanistic studies on transcutaneous vaccine delivery : microneedles, nanoparticles and adjuvants

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Chapter 7

Small is beautiful: N-trimethyl chitosan-ovalbumin conjugates for microneedle-based transcutaneous immunisation

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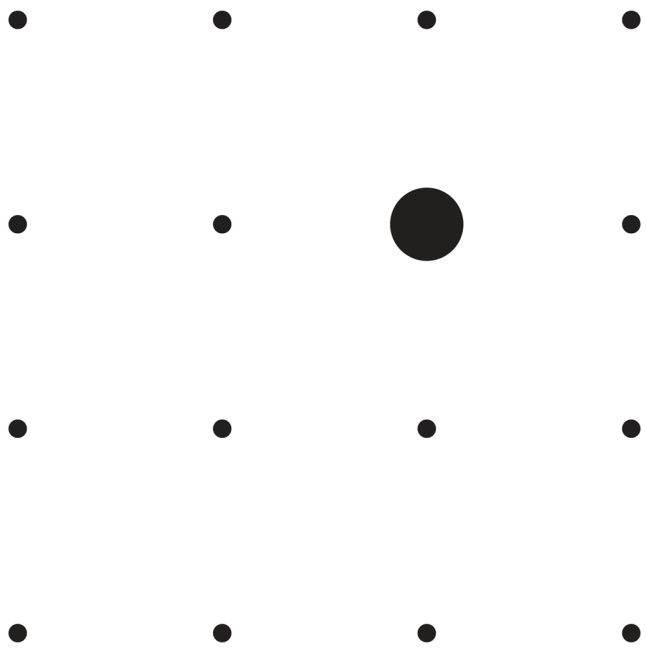


Submitted for publication

Abstract

For microneedle-based transcutaneous immunisation the formulation can greatly impact the transport of the antigen and adjuvant into the skin and subsequently to the lymph nodes. Therefore we immunised mice with ovalbumin (OVA) formulated in three different ways with N-trimethyl chitosan (TMC). TMC + OVA mixtures, TMC-OVA conjugates and TMC/OVA nanoparticles were applied transcutaneously, intradermally and intranodally, to explore the effect of the formulations' physical form on the number of OVA^+ dendritic cells (DCs) in the lymph node and the resultant immunogenicity (serum IgG titres).

Transcutaneously, the TMC-OVA conjugate induced the highest IgG levels and resulted in more OVA^+ DCs in the lymph nodes after 24 h than the other TMC formulations. Intradermally, all TMC-adjuvanted OVA formulations increased IgG titres compared to plain OVA. These formulations accumulated in the skin, prolonging OVA delivery to the lymph nodes. The prolonged delivery of TMC-adjuvanted OVA to lymph node resident DCs was also observed after intranodal immunisation, but in this case the higher uptake did not correspond with elevated antibody titres compared to plain OVA.

In conclusion, TMC-OVA conjugates are not more immunogenic, but are better taken up by DCs than TMC + OVA mixtures and penetrate the skin more efficiently than TMC/OVA nanoparticles.

Introduction

Transcutaneous immunisation as an alternative for the conventional intramuscular or subcutaneous vaccination routes has received a lot of attention in the past years [1]. Although the skin is an attractive vaccination site due to the presence of high amount of antigen presenting cells, the stratum corneum prevents efficient diffusion of vaccines into the skin. This barrier can be breached by using for instance microneedles [2, 3]. Different types of microneedles are available, each with their own advantages and disadvantages [2]. In our lab we have used solid microneedles to effectively pierce both human and mouse skin [4-6]. The microneedles have proven to be successful for transcutaneous immunisation [7-10]. Solid microneedles are more easily fabricated compared to hollow and biodegradable microneedles and lack the inconvenience of possible leakage associated with hollow microneedles. They were introduced by Henry et al. in 1998, who showed a 4 orders of magnitude increase in the transport of calcein through microneedle pre-treated skin *in vitro* [11]. Ding et al. showed that microneedle pre-treatment resulted in 1000-fold increase in antibody titres against diphtheria toxoid compared to application on intact skin [10]. However, still much higher doses are necessary to provoke comparable titres as after subcutaneous injection. This is probably a result of the low amount of antigen that reaches the dendritic cells (DCs) in the skin.

To improve the uptake of antigen that does reach the DCs, antigens are often formulated into nanoparticles. This is a logical strategy as all pathogens are particulates. Nanoparticles are better taken up by DCs and can function as an antigen depot [12-14]. Additionally, co-localisation of antigen and adjuvant in a nanoparticle results in simultaneously delivery to the same DC, which is thought to be pivotal for induction of potent immune responses [15, 16]. In general, nanoparticles have proven to be successful [17, 18], but recent studies using liposomes or N-trimethyl chitosan (TMC) nanoparticles for intradermal and transcutaneous immunisation have questioned their benefit for these delivery routes [7, 8, 19]. TMC however is an interesting polymer since it has intrinsic adjuvant properties and induces DC maturation [19, 20]. Moreover, it has successfully been used for vaccination via various administration routes [7, 19-23]. In transcutaneous vaccination with microneedle arrays the diffusion of TMC nanoparticles, after application for 1 h on microneedle pre-treated skin, was shown to be significantly impaired compared to that of a TMC solution [7]. The delivery of nanoparticles into the skin can be optimised by prolonging the application time or by using smaller vaccine entities. The extended application of the formulations will allow more accumulation in and diffusion through the skin. By using conjugates between TMC and the model antigen ovabumin (OVA), we hypothesize that the diffusion through the conduits is improved, whereas the co-localisation of antigen and adjuvant is retained [24]. We have previously described the synthesis of this conjugate

[24], which links the antigen and the adjuvant by a disulfide bond, ensuring release once the conjugate is taken up by DCs [25, 26]. These conjugates enhanced DC uptake and maturation and were equally or more immunogenic compared to TMC nanoparticles after intramuscular vaccination [24] or nasal immunisation [27], respectively.
In this study we immunised mice with OVA-loaded TMC nanoparticles, a TMC-OVA conjugate, a mixture of a TMC and OVA solution and plain OVA by applying the formulations for 2 h on microneedle pre-treated skin. The immunogenicity of the formulations was assessed by measuring the antibody titres. The efficiency of the delivery of the antigen through the conduits to the lymph nodes was assessed by determining the amount of OVA positive (OVA^+) DCs in the draining lymph nodes. To discriminate between the different transport aspects: diffusion through the conduits into the skin; transport from the skin to the lymph nodes and DC uptake in the lymph nodes, the formulations were also administered by intradermal or intranodal injection.

Materials and Methods

Materials

TMC with a degree of quaternisation of 15% was synthesised from 92% deacetylated chitosan (MW 120 kDa, Primex, Siglufjordur, Iceland) as described previously [19]. Endotoxin free OVA grade VII was obtained from Merck (Darmstadt, Germany). Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (γ chain specific), IgG1 (γ1 chain specific) and IgG2a (γ2a chain specific) were purchased from Southern Biotech (Birmingham, USA). Invitrogen (Breda, The Netherlands) supplied AlexaFluor647 labelled OVA (OVA_{AF647}), chromogen 3,3',5,5'-tetramethylbenzidine (TMB) and the substrate buffer and all cell culture reagents. Anti CD11c-PE/Cy5 was acquired from Becton Dickinson (Breda, The Netherlands). Nimatek® (100 mg/ml Ketamine, Eurovet Animal Health B.V., Bladel, The Netherlands), Oculentum Simplex (Farmachemie, Haarlem, The Netherlands) and Rompun® (20 mg/ml Xylazine, Bayer B.V., Mijdrecht, The Netherlands) were obtained from a local pharmacy. Phosphate buffered saline (PBS) pH 7.4 was obtained from Braun (Oss, The Netherlands). N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), dithiothreitol (DTT), pentasodium tripolyphosphate (TPP), N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid) (HEPES) and all other chemicals were purchased at Sigma-Aldrich (Zwijndrecht, The Netherlands), unless stated otherwise.

Animals

Female BALB/c mice, 8 weeks old at the start of the vaccination study were purchased from Charles River (Maastricht, The Netherlands) and maintained under standardised

conditions in the animal facility of the Leiden/Amsterdam Center for Drug Research, Leiden University. The study was carried out under the guidelines compiled by the Animal Ethic Committee of the Netherlands.

Vaccine formulations

TMC nanoparticles were prepared by ionic complexation with TPP and OVA as described before [19]. Briefly, OVA followed by TPP were added to a 0.2% (w/v) of TMC in 5 mM HEPES (pH 7.4) in a 10:1.0:1.7 TMC:OVA:TPP ratio under continuous stirring. The nanoparticles suspension was centrifuged for 15 min at 10,000 g on a glycerol bed and resuspended in 5 mM HEPES (pH 7.4). A mixture of TMC and OVA was prepared by adding solutions of both components together in a 2.5:1 (w/w) ratio (TMC + OVA). TMC-OVA conjugates were synthesised and purified as described previously [24]. 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. Functionalised TMC was treated with DTT for 30 min at room temperature to obtain thiolated TMC. Thiolated TMC and functionalised OVA were mixed a 1:1 molar ratio to allow disulfide bond formation overnight. The size of the nanoparticles and the conjugate was measured with dynamic light scattering and the zetapotential was determined by laser Doppler velocimetry using a Zetasizer$^{(R)}$ Nano ZS (Malvern, Instruments, United Kingdom). Formulations containing OVA_{AF647} with similar size and zetapotential were produced by substituting OVA by its fluorescent counterpart.

Microneedles

Assembled metal microneedle arrays with a length of 300 μm (300A) were manufactured from commercially available 30G hypodermic needles [4]. These microneedles were positioned in a 4x4 pattern in a polymer mould (diameter 5 mm) with a pitch of 1.25 mm. An electrical applicator was used to apply the microneedles with a speed of 3 m/s to ensure reproducible piercing of the skin.

Immunisation study

Groups of 8 mice (transcutaneous immunisation) or 5 mice (intradermal and intranodal immunisation) were vaccinated with the above mentioned formulations. All immunisations were applied under anaesthesia by intraperitoneal injection of 150 mg/kg ketamine and 10 mg/kg xylazine. Transcutaneous immunisation with the microneedles was performed as described previously [10]. However, whereas in previous studies the formulations were applied occlusively for 1 h, in the present study in most cases the formulation application was extended to 2 h (~2 cm^2 area restricted by a metal ring) before they were washed off.

A dose of 100 µg was applied in a volume of 70 µl. To circumvent the skin barrier, the formulations (2 µg/30 µl) were also injected intradermally with a 30G needle as described before [19]. To circumvent transport to the lymph nodes, the formulations (0.2 µg/10 µl) were also injected directly into the inguinal lymph node as described by Johansen *et al* [28]. After 3 weeks blood samples were drawn from the tail vein and the mice received a similar booster vaccination. After 6 weeks total blood was collected from the femur artery and the mice were sacrificed. Blood samples were collected in MiniCollect® tubes (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) till clot formation and centrifuged for 10 minutes at 10,000 g to obtain cell-free sera. The sera were stored at −80°C until further use.

Antigen uptake by DCs in the lymph nodes

Mice were vaccinated as described above, but with OVA_{AF647}-containing formulations. After 4 or 24 h mice were sacrificed and inguinal lymph nodes were collected. Single cell suspensions were obtained in RPMI with 10% foetal calf serum, 50 µM β-mercaptoethanol, 2 mM glutamine, 1 mM sodium pyruvate and 500 U/L penicillin/streptomycin, by grinding the lymph nodes through 70 µm cell strainers. Lymphocytes were washed with PBS containing 1% w/v bovine serum albumin and stained with CD11c-PE-Cy7. Cells were analysed with flow cytometry using a FACSCanto II (Becton Dickinson). DC population was determined based on the expression of CD11c and the number of OVA^+ cells in this population was quantified.

Detection of serum IgG, IgG1 and IgG2a

OVA specific antibodies (IgG, IgG1 & IgG2a) in the sera were determined by sandwich ELISA as described previously [19]. Briefly, plates (NUNC, Roskilde, Denmark) were coated overnight with 100 ng OVA. After blocking, two-fold serial dilutions of sera from individual mice were applied into the wells. HRP-conjugated antibodies against IgG, IgG1 and IgG2a were added and detected by TMB. Absorbance was determined at 450 nm with an EL808 micro plate reader (Bio-Tek Instruments, Bad Friedrichshall, Germany). Antibody titres were expressed as the reciprocal of the sample dilution that corresponds to half of the maximum absorbance at 450 nm of a complete s-shaped absorbance-log dilution curve.

Statistical analysis

Statistical analysis was performed with Prism 5 for Windows (Graphpad, San Diego, USA). Statistical significance was determined by a two-way analysis of variance (ANOVA) with a Bonferroni post-test.

Results

In a pilot study, the effect of prolonging the application time of a vaccine formulation on microneedle pre-treated skin was studied in the absence of TMC (figure 1). A two-fold increase of the application time resulted in a nine-fold and thirty-fold amplification of IgG titres after the prime and boost immunisation, respectively ($p<0.001$). Even for a relatively small soluble antigen like OVA (9 nm) the delivery through the much larger conduits is a slow process. This emphasises the importance of studying the delivery parameters for microneedle-based transcutaneous vaccination and provides rationale for extending the application time in the studies with the TMC formulations. Based on these results it was decided to use 2 h application for transcutaneous immunisation.

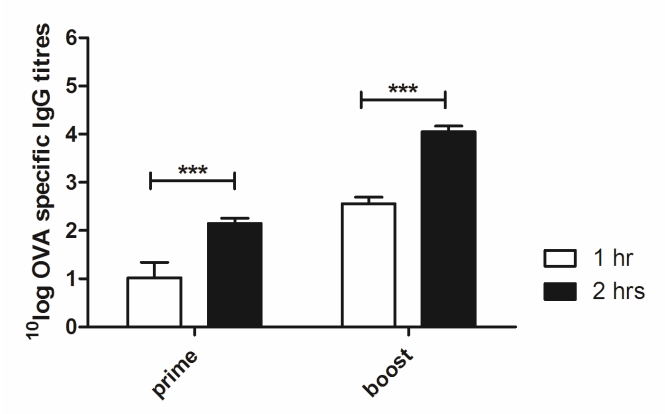


Figure 1. OVA specific IgG titres after transcutaneous immunisation. After microneedle pre-treatment an OVA solution was applied for 1 or 2 h on the skin. Mean + SEM of 8 mice. *** $p<0.001$

Immunisation studies with TMC-based formulations

We immunised mice with OVA, a mixture of TMC + OVA, TMC nanoparticles and TMC-OVA conjugates via the transcutaneous, intradermal and intranodal route. The formulations used for vaccination had a similar TMC:OVA ratio and were of a broad size range (table 1). Transcutaneously, the TMC-OVA conjugate, the smallest co-localised entity in this study, outperformed the other formulations (figure 2A). After the prime vaccination the conjugate induced significantly higher IgG titres than the other three formulations ($p<0.001$). Also after the boost the TMC-OVA conjugate proved to be significantly better than OVA alone ($p<0.01$), although also the TMC nanoparticles significantly elevated the IgG titres compared to plain OVA ($p<0.05$). A physical mixture of TMC + OVA elicited IgG levels that were not significantly higher than plain OVA ($p=0.25$).

Table 1. Characteristics of formulations.

Formulation	TMC:OVA (w/w)	Size [nm]
OVA	n.a.	8 ± 1
TMC + OVA	2.5:1	n.a.*
TMC-OVA conjugate	2:1	28 ± 1
TMC/OVA nanoparticles	2.5:1	276 ± 6

*= n.a. not applicable

When the formulations were administered by intradermal injection, circumventing transport along the conduits, all TMC-based formulations were equally potent in enhancing the IgG titres compared to non-adjuvanted OVA (figure 2B). Both after prime and boost vaccination the titres were elevated ($p<0.001$) by using TMC in the formulations. After intranodal vaccination, where the antigen is directly injected at the site where the immune response is initiated, all four formulations induced similarly high IgG titres (figure 2C).
Besides the total IgG titres, the IgG1 and IgG2a titres after the boost were measured as well. Almost exclusively IgG1 was produced after immunisation with all formulations via the three different delivery routes, indicative of a Th2 biased response as reported before [7, 29, 30]. Only after intradermal immunisation with the TMC nanoparticles four out of five mice also had measurable IgG2a titres (values around 100, data not shown).

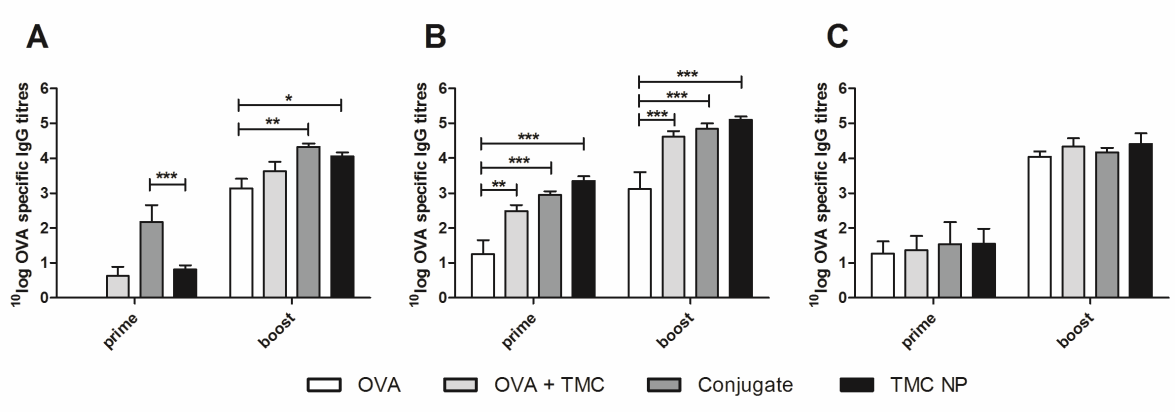


Figure 2. OVA specific IgG titres after a prime and a booster vaccination by the transcutaneous (2 h application, A), intradermal (B) and intranodal route (C). Data are expressed as the mean ± SEM of 8 (A) or 5 mice (B/C). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

DC uptake in the lymph nodes

To elucidate the influence of transport on the observed antibody titres, the number of DCs in the lymph nodes that had taken up OVA was quantified after application of fluorescently labelled OVA via the different immunisation routes. Transcutaneous application of the

formulations resulted in a very low number of OVA^+ DCs in the lymph nodes (figure 3A and D); after 4 h no OVA^+ DCs could be detected, but after 24 h application of OVA or the TMC-OVA conjugate did result in OVA uptake by DCs. For the other formulations the levels were barely higher than the background fluorescence.

Intradermally, after 4 h the highest amount of OVA could be found in the lymph nodes if a solution of OVA was administered, whereas the TMC-based formulations reduced the direct lymph node drainage in a size dependent manner (figure 3B). After 24 h TMC + OVA mixture and the TMC nanoparticles were able to elevate the OVA uptake, whereas the TMC-OVA conjugate did not have a significant effect (figure 3E). This extended delivery of OVA could be ascribed to a depot effect as both 4 and 24 h after intradermal injection of all OVA_{AF647}-containing TMC formulations a depot was visible at the injection site (figure 4). This depot was not present if a solution of OVA was used. It is known that for instance liposomal formulations can form a depot in the skin [31] and here we show that this is also the case for TMC. Since TMC is a positively charged polymer it likely will interact with (negatively charged) cells and collagen matrix present in the dermis. Also the linear structure of the polymer could promote entanglement in the collagen matrix.

Intranodal injection resulted in the largest population of OVA^+ DCs; up to 40% of the DCs in the lymph nodes had taken up OVA (figure 3C and F). The IgG titres correspond well with the DC uptake in the lymph nodes, as after 4 h the different formulations induced comparable OVA uptake. For plain OVA the uptake was a fast process: whereas after 4 h 40% of the DCs were OVA^+, after 24 h the amount of OVA^+ DCs had already decreased by 10-fold. The TMC-based formulations were able to prolong the exposure to OVA after 24 h ($p=0.08$), just as was the case after intradermal immunisation.

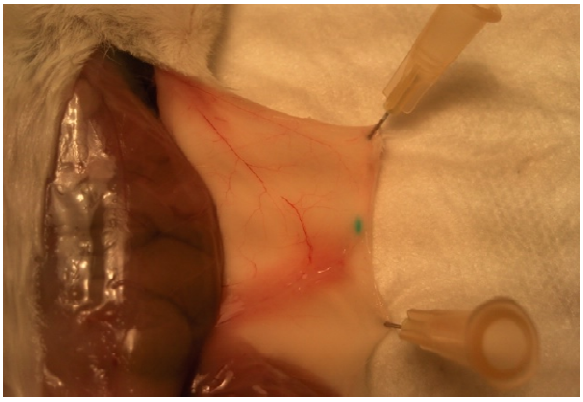
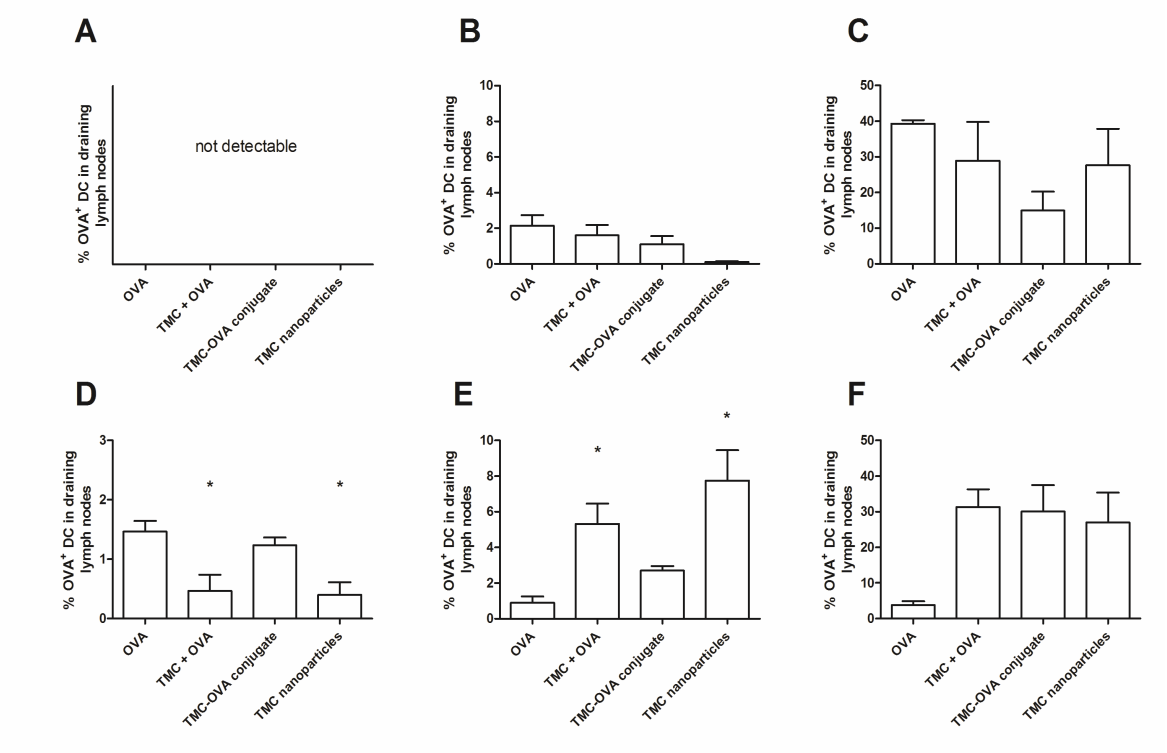


Figure 4. Picture of injection site 24 h after intradermal injection of TMC nanoparticles.

Figure 3. Quantification of the amount of OVA^+ DCs in the lymph nodes 4 (A-C) and 24 h (D-F) after A/D: transcutaneous vaccination with microneedle pre-treatment, B/E: intradermal immunisation and C/F: intranodal vaccination. Data are expressed as mean ± SEM of at least 3 mice. * significantly different compared to OVA ($p<0.05$).



Discussion

Vaccines are administered via a variety of routes, but knowledge on the different requirements of vaccine formulations for the various delivery routes is sparse [32]. In this study we compared four different formulations for transcutaneous, intradermal and intranodal vaccination. Non-adjuvanted OVA and three TMC-containing OVA formulations were selected that differ with respect to size (OVA conjugated with TMC, or encapsulated in larger TMC nanoparticles) and co-localisation of adjuvant and antigen (nanoparticles and conjugate versus TMC + OVA in solution). In this study we did not only compared the formulations with respect to the antibody response they induced, but also quantified the antigen uptake in the lymph nodes. This was the first time that the antigen uptake in the lymph nodes after transcutaneous immunisation with microneedles was quantified. Guebre-Xabier et al. could detect OVA^+ DCs in the draining lymph nodes 24 h after application of OVA together with heath labile enterotoxin (LT) on abraded mice skin [33]. In a similar manner Belyakov et al. measured the uptake of LT applied on abraded skin and could measure LT^+ DCs after 48 h [34]. However, the effect of formulating antigens into nanoparticles or conjugates on the DC uptake in the lymph nodes after transcutaneous immunisation has not been measured before. It provides an elegant method of comparing the efficiency of different vaccine formulations. Furthermore, it makes it possible to compare different immunisation routes.

Interestingly all three delivery routes showed a different effect of the formulations. For vaccination via the transcutaneous route the importance of the size of the adjuvant/antigen combination as well as the co-localisation of antigen and adjuvant was evident. The smaller conjugate was superior in inducing a fast serum IgG response and in enhancing the OVA uptake in the lymph nodes compared to the TMC nanoparticles and a TMC + OVA mixture. Even though plain OVA was also taken up by DCs in the lymph nodes, the lack of co-stimulatory components in this formulation resulted in a less effective antibody response.

The DC uptake studies in the lymph nodes make it evident that despite the much higher dose applied transcutaneously compared to intradermal and intranodal injection, the amount of OVA that is taken up by DCs in the lymph nodes is significantly lower. This matches the previously published data comparing the OVA^+ DCs after transcutaneous application, intradermal and subcutaneous injection of OVA [33]. They showed that 1 h of transcutaneous application of a much higher antigen dose resulted in lower numbers of OVA^+ DCs compared to intradermal administration. We observed similar results after quantifying the OVA uptake from OVA/CpG liposomes administered via the transcutaneous and intradermal route (unpublished data).

Prolongation of the application time resulted in significantly elevated IgG levels after administration of OVA. Moreover, whereas in a previous study applying the TMC nanoparticles for 1 h on microneedle pre-treated skin did not result in elevated IgG titres [7], in the current study after application for 2 h significantly higher titres compared to an OVA solution were obtained. For a mixture of TMC + OVA the opposite was observed: even though adding TMC to an antigen (diphtheria toxoid) improved the antibody titres in a previous after 1 h of application [7], this effect was not observed after 2 h of application. A logical next step would be to further prolong the application time. This has not been done so far, as it is difficult to anesthetise the animals for a longer period. However, if the microneedle approach would be applied to humans instead of mice, patches could easily be worn for up to 24 h or even longer. This is expected to lead to lower doses required for successful transcutaneous immunisation.

Whereas for transcutaneous vaccination the entity size is the most important parameter, for intradermal vaccination this apparently plays a minor role. Transport to the lymph nodes from the dermis is a relatively fast process, as the lymphatic vessels are present just below the epidermis and have a diameter of 10-80 µm [35]. Other factors, such as the depot formation are of considerable importance. It is known that the retention of the antigen and its slow release from the depot can stimulate the immune response, by the attraction of antigen presenting cells [31, 36]. However, if the antigen passage to the lymph nodes is impaired because of electrostatic interactions with the extracellular matrix in the skin, this can have a detrimental effect on the immune response. This was illustrated

by the TMC-OVA conjugate, where the antigen and adjuvant are covalently linked. The disulfide bond linkage will only be degraded once the conjugate is taken up into the DCs reducing cytoplasm, thereby prohibiting direct drainage of OVA from the injection site to the lymph nodes. The necessary uptake by and subsequent migration of DCs to the lymph nodes means that conjugated OVA will reach the lymph nodes over a longer time span. After subcutaneous injection of cationic liposomes that also formed a depot, the maximum amount of antigen could be detected in the lymph nodes 5 days past injection [37]. A kinetic study of lymph node trafficking might reveal that the OVA from the conjugate will reach the lymph nodes at a later time point, as is expected from the antibody titres where the three TMC-containing formulations were equally potent. It remains to be studied whether the adjuvant effect of TMC can be further improved by shielding its positive charge, for instance by PEGylation to reduce the depot formation [38].
Intranodally, it was surprising that the controlled antigen release in the lymph nodes with the TMC-containing formulations did not correlate with elevated IgG titres. It was expected that the *in vivo* DC uptake after intranodal injection would correlate with the *in vitro* DC uptake, where the TMC-OVA conjugate and the nanoparticles increased the OVA uptake after 4 h and an TMC + OVA solution did not [19, 24]. Apparently in the present study the *in vitro* model is not representative for the *in vivo* situation. Bachmann et al. stated that in the lymphoid tissue there are abundant co-stimulation signals present and as long as there is a sufficient load of antigen, no adjuvant is necessary [39]. This may explain why no beneficial effect of the TMC was observed. Moreover, damage to the surrounding tissue as a result of the intranodal injection could provide a danger signal similar to that of an adjuvant [40].

Conclusion

The optimal vaccine formulation differs for each administration site as optimum delivery parameters for one route of administration can not simply be extrapolated to other routes. Focusing on transcutaneous vaccination, the size of the vaccine entity and co-localisation of antigen and adjuvant are crucial parameters when designing formulations to effectively enhance the immune response. Conjugates of an antigen and an adjuvant offer in this respect better perspectives than (nano)particles.

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