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

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Adjuvanted, antigen loaded N-trimethyl chitosan nanoparticles for nasal and intradermal vaccination: adjuvant- and site-dependent immunogenicity in mice

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Abstract

N-trimethyl chitosan (TMC) nanoparticles have been shown to increase the immunogenicity of subunit antigens after nasal and intradermal administration. This work describes a second generation of TMC nanoparticles containing ovalbumin as a model antigen (TMC/OVA nanoparticles) and an adjuvant (TMC/adjuvant/OVA nanoparticles). The selection of adjuvants included Toll-like receptor (TLR) ligands lipopolysaccharide (LPS), PAM₃CSK₄ (PAM), CpG DNA, the NOD-like receptor 2 ligand muramyl dipeptide (MDP) and the GM1 ganglioside receptor ligand, cholera toxin B (CTB) subunit. The TMC/adjuvant/OVA nanoparticles were characterised physico-chemically and their immunogenicity was assessed by determining the serum IgG, IgG1, IgG2a titres and secretory IgA levels in nasal washes after intradermal and nasal vaccination in mice.

After nasal vaccination, TMC/OVA nanoparticles containing LPS or MDP elicited higher IgG, IgG1 and sIgA levels than non adjuvanted TMC/OVA particles, whereas nanoparticles containing CTB, PAM or CpG did not. All nasally applied formulations induced only marginal IgG2a titres. After intradermal vaccination, the TMC/CpG/OVA and TMC/LPS/OVA nanoparticles provoked higher IgG titres than plain TMC/OVA particles. Additionally, the TMC/CpG/OVA nanoparticles were able to induce significant IgG2a levels. None of the intradermally applied vaccines induced measurable sIgA levels.

Altogether, our results show that co-encapsulation of an adjuvant with the antigen in TMC nanoparticles can significantly increase the immunogenicity of the antigen. However, the strength and quality of the response depends on the adjuvant as well as the route of administration.

Introduction

Most human vaccines are administered via injection into muscle or subcutaneous tissue. Notwithstanding the success of this approach, during the last decades it has also become apparent that muscle and subcutaneous tissue may not be the most ideal sites to induce an immune response. The skin and the mucosal linings for instance contain more immune cells capable of initiating an immune response [1, 2], which is most likely a consequence of the fact that pathogens generally invade the human body via these tissues. Various examples have shown that intradermal vaccination is more effective than intramuscular administration as the same level of protection is reached by injection of a smaller dose [3-5]. Moreover, applying the vaccine via the route through which the pathogen would normally invade could induce a type of immune response that provides better protection [6]. Nasal vaccination often induces the production of secretory IgA (sIgA) antibodies that can neutralise pathogens colonising the mucosal linings [7], whereas intramuscular administration does not induce sIgA.

Currently there are several vaccines on the market that use a different administration route (e.g., oral, intradermal and nasal) and they are well perceived by the vaccinee [8]. However, many of these vaccines are of live-attenuated nature, which makes them unsuitable for administration to young children, elderly or immune-compromised patients. Replacement of these vaccines by subunit vaccines would be a great improvement for safety reasons and would make them suitable for administration to these groups. However, such vaccines are difficult to develop as plain subunit antigens are poorly immunogenic. To enhance their immunogenicity, subunit antigens can be formulated into particulate vaccine delivery systems. This improves the uptake by antigen presenting cells (APCs) and when adjuvants are included it can also enhance the activation of these APCs [9]. Especially approaches that combine antigen and adjuvant into a particle have been shown to result in a strong immune response [10, 11]. We have recently shown that N-trimethyl chitosan (TMC) nanoparticles loaded with ovalbumin (OVA) as a model subunit antigen increased the immune response after nasal [12] as well intradermal administration [13]. Inclusion of an adjuvant may further improve the immunogenicity of TMC nanoparticles.

The aim of the present study was to co-encapsulate various adjuvants in OVA-loaded TMC (TMC/OVA) nanoparticles and to evaluate if these additional danger signals can further enhance the efficacy of the TMC/OVA nanoparticles when administered nasally or intradermally in mice. We selected 5 potential adjuvants based on their physical chemical properties and their reported adjuvant effect after intradermal and nasal administration: lipopolysaccharide (LPS) [14, 15], CpG [16, 17], PAM₃CSK₄ [17, 18], muramyl dipeptide (MDP)

[19, 20] and the non-toxic beta subunit of cholera toxin (CTB) [21, 22]. These adjuvants were co-complexed with OVA into TMC nanoparticles, rather than co-administered, as co-localization of antigen and adjuvant into one entity has been reported to be very beneficial for the resulting immune response [10, 11, 23]. The size and zeta potential was studied, to ensure that all particles had a similar physical form. The adjuvanted nanoparticles were administered nasally and intradermally to mice to assess the extent of the immune response (OVA specific IgG titres) and the type of immune response (IgG1/IgG2a, secretory IgA (sIgA)) that was elicited.

Materials and methods

Materials

TMC with a degree of quaternisation of 15% was synthesised from 92% deacetylated chitosan (MW 120 kDa, Primex, Siglufjörður, Iceland) as described previously [24]. Endotoxin free OVA grade VII was obtained from Merck (Darmstadt, Germany). Lipopolysaccharide (LPS) from E.Coli 0111:B4, Pam3Cys-Ser-(Lys)₄ (PAM), and CpG oligonucleotide 1826 were obtained from Invivogen (Toulouse, France). Horseradish peroxidase (HRP) conjugated goat anti-mouse IgA, 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 chromogen 3, 3', 5, 5'-tetramethylbenzidine (TMB) and the substrate buffer and all cell culture reagents. Nimatek[®] (100 mg/ml Ketamine, Eurovet Animal Health B.V., Bladel, The Netherlands), Oculentum Simplex (TEVA, 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). Cholera toxin B subunit (CTB), muramyl dipeptide (MDP) and all other salts/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.

Plain TMC/OVA nanoparticles

TMC/OVA nanoparticles were prepared as described before [25]. Briefly, 1 mg OVA was dissolved in 10 ml 0.1% (w/v) TMC in 5 mM Hepes pH 7.4. Under continuous stirring 1.7 ml 0.1% (w/v) TPP was added to obtain an opalescent dispersion. Nanoparticles were collected by centrifugation (10 min, 12000 g) and resuspended in water. For size and zeta potential measurements using a Nanosizer ZS apparatus (Malvern Instruments, Malvern, UK), nanoparticles were diluted in 5 mM Hepes pH 7.4 until slightly opalescent dispersions were obtained. Supernatants were stored to determine the loading efficiency with a BCA assay following the manufacturer's guidelines (Pierce, Perbio Science, Etten-Leur, The Netherlands).

Adjuvanted TMC/OVA nanoparticles

Adjuvanted nanoparticles were prepared in the same way as non-adjuvanted TMC nanoparticles, as the adjuvant was co-dissolved with OVA in the TMC solution. TMC/CpG nanoparticles were the only exception, and were prepared by replacing TPP with strongly negatively charged CpG (serving as physical crosslinker and adjuvant), as described previously [16]. To remove unencapsulated OVA or adjuvant, nanoparticles were collected by centrifugation (10 min, 12000 g) and resuspended in water. To determine the loading efficiencies of the adjuvants fluorescently labelled analogues were used and the amount of adjuvant in the supernatant was determined by fluorescence spectroscopy (FS920 fluorimeter, Edinburgh Instruments, Campus Livingston, UK).

Based on the pre-determined loading efficiencies of each adjuvant (table 2), the initial amount of adjuvant was chosen in such a way (table 1) that TMC nanoparticles carrying OVA and adjuvant in a 1:1 weight/weight ratio were prepared. This, to ensure that each formulation contained the same amount of TMC, OVA and adjuvant for the vaccination study.

Immunisation study

Groups of 8 mice (nasal) or 5 mice (intradermal) were vaccinated with the above mentioned formulations. Nasally the mice received 10 µg antigen and 10 µg adjuvant in a volume of 10 µl PBS (5 µl/nostril) and intradermally 2 µg of each in a volume of 30 µl PBS was applied. Intradermal immunisations were carried out under anaesthesia by intraperitoneal injection of 150 mg/kg ketamine and 10 mg/kg xylazine with a 30G needle as described before [26]. 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.

Table 1: Initial amounts of components used for formulation of adjuvants into TMC/OVA nanoparticles.

Nanoparticle	TMC (mg)	TPP (mg)	OVA (mg)	Adjuvant (mg)
TMC/OVA	10	1.8	1.0	-
TMC/CTB/OVA	10	2.0	1.0	0.83
TMC/LPS/OVA	10	2.0	1.0	1.7
TMC/PAM/OVA	10	2.0	1.0	5.0
TMC/MDP/OVA	10	2.0	1.0	1.3
TMC/CpG/OVA	10	-	1.0	0.5

Detection of serum IgG, IgG1, IgG2a and secretory IgA

OVA specific antibodies (IgG, IgG1 & IgG2a) in the sera and sIgA in the nasal washes were determined by sandwich ELISA as described previously [27]. Briefly, plates were coated overnight with 100 ng OVA. After blocking, two-fold serial dilutions of sera from individual mice were applied to the plates. HRP-conjugated antibodies against IgG, IgG1, IgG2a or IgA 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.

Statistics

Statistical analysis was performed with Prism 5 for Windows (Graphpad, San Diego, USA). Statistical significance was determined either by a one way or a two way analysis of variance (ANOVA) with a Bonferroni post-test, depending on the experiment set-up.

Results

Characterisation of the nanoparticles

Inclusion of adjuvants into TMC nanoparticles did not alter the physical nature of the particles substantially. All adjuvanted particles showed a similar average diameter (between 300-400 nm) and all were modestly positively charged (+13-21 mV). The capacity to encapsulate OVA was only marginally affected by the inclusion of any of the adjuvants (table 2). The loading efficiency of the adjuvant however, greatly differed depending on the characteristics of the adjuvant. The strongly negatively charged species CpG and CTB easily complexed with the nanoparticles, whereas the positively charged adjuvant PAM hardly associated with the positively charged TMC nanoparticles. The loading efficiency of the amphiphilic adjuvants LPS (weakly negatively charged) and MDP (neutral) was 35% and 42%, respectively. So, LPS and MDP were more efficiently encapsulated than the positively charged PAM, but less efficiently than the hydrophilic, negatively charged adjuvants CpG and CTB.

Table 2: Characteristics of adjuvanted TMC nanoparticles.

Formulation	Size (nm)	PDI*	ZP** (mV)	LE*** OVA (%)	LE Adjuvant (%)‡
TMC/OVA	314 +/- 31	0.12	18.2 +/- 1.8	63 +/- 6	-
TMC/CTB/OVA	323 +/- 39	0.29	14.7 +/- 2.4	56 +/- 4	68-74
TMC/LPS/OVA	365 +/- 46	0.33	13.3 +/- 2.9	52 +/- 0.1	32-37
TMC/PAM/OVA	375 +/- 99	0.11	15.5 +/- 0.2	59 +/- 7	8.1-9.4
TMC/MDP/OVA	418 +/- 89	0.15	13.6 +/- 1.7	60 +/- 1	41-43
TMC/CpG/OVA	304 +/- 22	0.20	20.9 +/- 2.0	52 +/- 7	52-62

*PDI = polydispersity index, **ZP = zeta potential and ***LE = loading efficiency. n=3 +/- SEM ‡n=2

Total serum IgG response after nasal and intradermal vaccination

The differently adjuvanted TMC/OVA formulations were administered intradermally and nasally to study their adjuvanticity and the site-dependency thereof. After nasal and intradermal vaccination TMC/OVA nanoparticles increased the IgG titres compared to OVA alone (figure 1A, B). In some cases the inclusion of an adjuvant into the TMC/OVA particle increased the immunogenicity even further.

Nasally, the LPS- and MDP-loaded TMC/OVA nanoparticles elicited higher IgG titres compared to TMC/OVA nanoparticles ($p < 0.05$ figure 1A). Encapsulation of CTB, PAM or CpG into TMC/OVA nanoparticles did not affect the total serum IgG response compared to TMC/OVA nanoparticles.

After intradermal injection, TMC/LPS/OVA nanoparticles elicited higher IgG levels than plain TMC/OVA nanoparticles after both a priming ($p < 0.05$) and a booster dose ($p < 0.01$). In contrast to nasal administration, after a priming dose intradermal administration of TMC/CpG/OVA nanoparticles significantly increased IgG titres compared to plain TMC/OVA nanoparticles ($p < 0.05$ figure 1B) and co-encapsulation of MDP had no effect. Encapsulation of CTB and PAM into TMC/OVA nanoparticles did not lead to elevated IgG titres compared to non-adjuvanted TMC/OVA nanoparticles.

IgG subtyping of the immune response

Besides the IgG titres, the IgG1 and IgG2a antibody titres were measured to obtain insight into the type of immune response elicited by the different formulations. Compared to vaccination with OVA alone for both administration routes the main subtype produced after vaccination with TMC/OVA was IgG1, which followed a similar trend as the total IgG titres after the boost.

Nasally administered LPS- or MDP loaded TMC/OVA nanoparticles elicited higher IgG1 titres than plain TMC/OVA particles (figure 2A), whereas the other adjuvants did not show significant effects on the IgG1 response. None of the formulation induced substantial IgG2a levels. After intradermal immunisation, TMC/OVA nanoparticles induced the production of significantly more IgG1 compared to a solution of OVA, but no additional effect of the encapsulation of adjuvants was observed. However, TMC nanoparticles containing CpG significantly boosted the IgG2a production ($p < 0.001$), causing a decrease in the IgG1/IgG2a ratio compared to TMC/OVA nanoparticles (figure 2B).

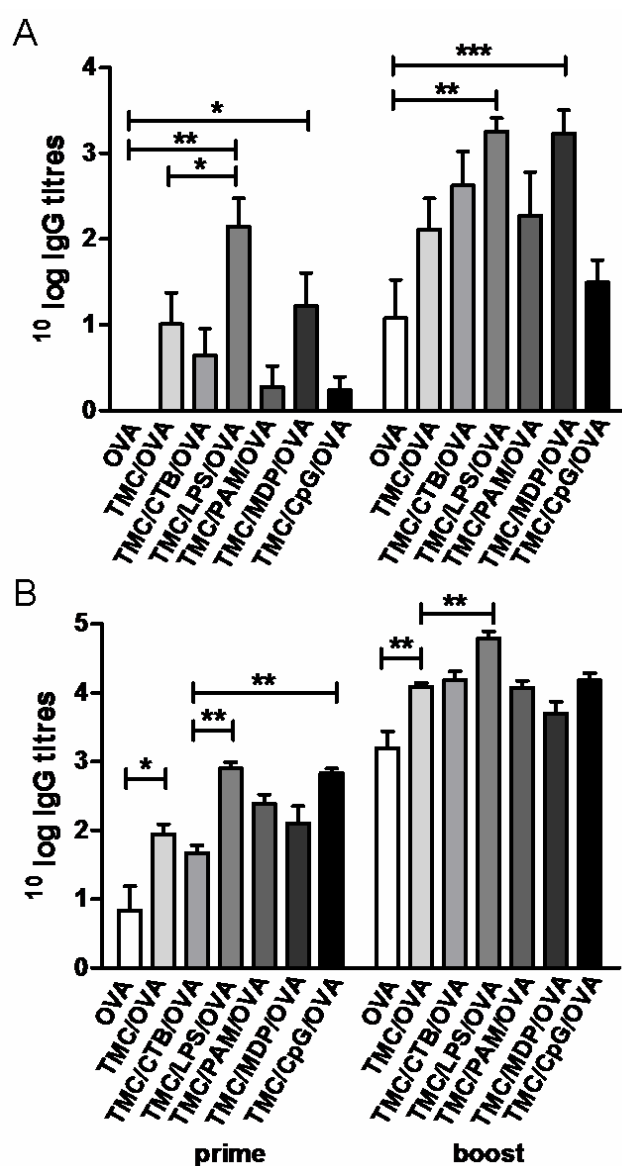


Figure 1. OVA specific serum IgG titres after nasal (A) and intradermal (B) immunisation. Data are presented as mean $\pm$ SEM of 8 (A) or 5 (B) mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Production of sIgA

Secretory IgA is an important mediator of mucosal immunity and can therefore provide protection against respiratory pathogens. Intradermal administration did not induce detectable sIgA levels in the nasal washes (data not shown). In contrast, nasal vaccination with TMC/OVA nanoparticles containing LPS, MDP or CpG did result in increased levels of sIgA in some mice (figure 3). The nasal application of plain TMC nanoparticles or nanoparticles adjuvanted with CTB or PAM did not trigger sIgA production.

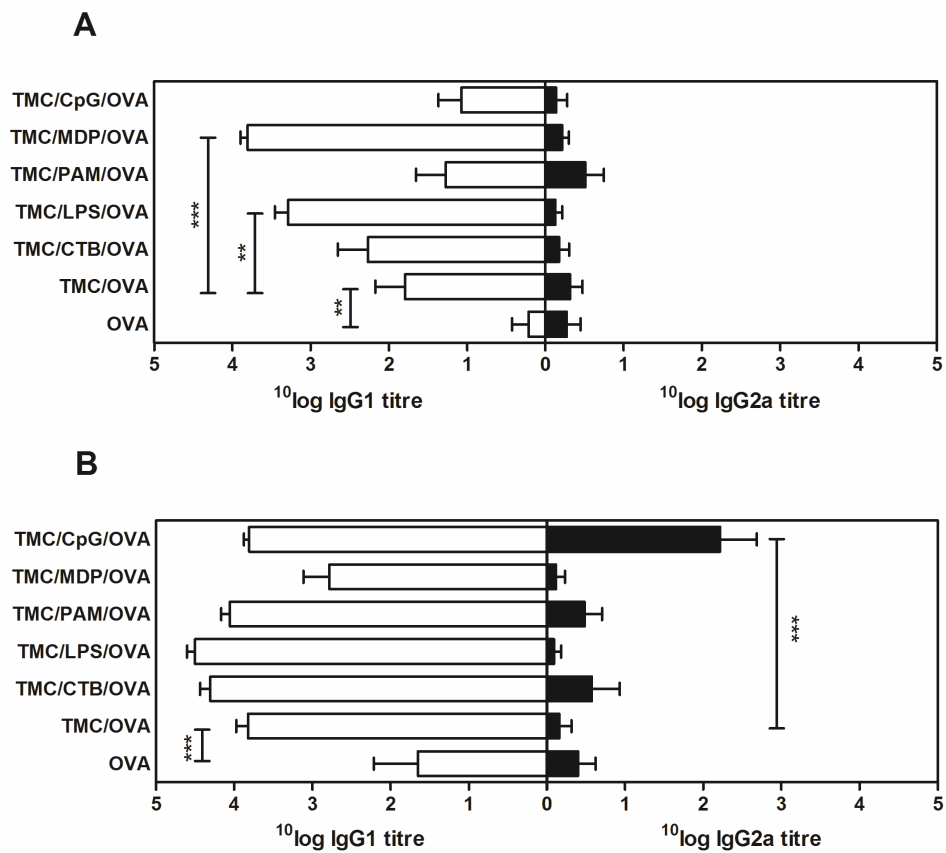


Figure 2. OVA specific serum IgG1 (white bars) and IgG2a (black bars) titres 3 weeks after a booster dose nasally (A) and intradermally (B). Data are presented as mean $\pm$ SEM, ** $p < 0.01$ *** $p < 0.001$.

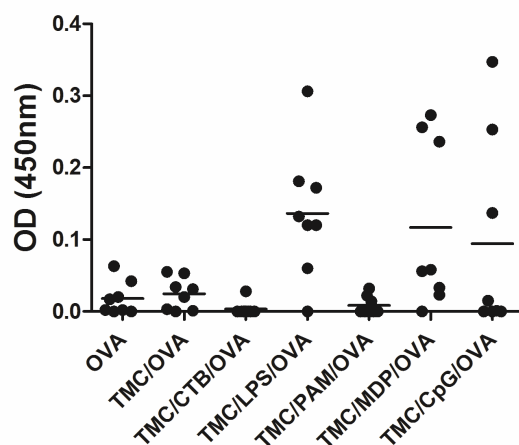


Figure 3: Secretory IgA levels in nasal washes of individual mice 3 weeks after a nasal booster dose. Bar represents mean.

Discussion

The field of adjuvants is rapidly evolving. Whereas alum had been the only approved adjuvant for many years, recently squalene emulsions (MF-59) and monophosphoryl lipid A (a LPS derivate) have been licensed for use in Europe. Increased knowledge on the activation of the innate immune system has led to the identification of new adjuvants that activate APCs specifically via Toll-like receptors (TLRs) [28] or NOD-like receptors (NLRs). Moreover, detoxification of known adjuvants (MPL instead of LPS; CTB instead of cholera toxin) and the development of new adjuvants exploiting the increased knowledge on activation of the innate immune system (CpG, PAM₃CSK₄, MDP), will probably increase the arsenal of adjuvants for commercial human vaccines in the future. This progress is crucial for the development of subunit vaccines as the addition of an adjuvant seems inevitable in order to yield a strong immune response. The large number of DCs in the dermis and nasal epithelium potentially makes application of adjuvanted vaccines at these sites very attractive, as it can directly result in activation of DCs. Nonetheless, a delivery system to enhance the uptake of both the antigen and the adjuvant will be an important utensil, as generally physical mixtures of adjuvant and antigen are inferior to systems where both components are co-localised. TMC nanoparticles are excellent antigen carriers as they associate with DCs and, because of their intrinsic adjuvanticity, activate DCs [25, 26]. As a consequence, in direct comparison with other vaccine delivery systems TMC nanoparticle have shown to be a more effective carrier for mucosal or dermal administration than PLGA nanoparticles [12], positively charged liposomes (unpublished data) and chitosan nanoparticles [25]. The beneficial effect of TMC nanoparticles as a carrier system was clearly observed in this study. Even though the OVA dose chosen was twofold lower than in the previous studies [12, 16, 26] (to better detect the effect of the encapsulated adjuvant), OVA-loaded TMC nanoparticles enhanced the IgG1 titres compared to nasal or intradermal administration of OVA alone.

Co-encapsulation of adjuvants have been reported to further increased the immunogenicity of the carrier system [10, 11, 23], however the activity of the adjuvants appeared to be administration site specific. Nasally, co-encapsulation of LPS and MDP in TMC/OVA nanoparticles elicited higher IgG titres than TMC/OVA nanoparticles, whereas intradermally LPS and CpG were the most effective adjuvants. It has been reported that the expression of TLRs and NLRs on DCs is dependent on the micro-environment of the DC and the DC subset [29-32], which may explain the differential effects of adjuvants when comparing the nasal and intradermal route. For instance, the effect of the NOD2 ligand MDP could be explained in this manner. NOD2 plays an important role in Crohn's disease [33, 34] and NOD2-

deficient mice are more susceptible to *Listeria monocytogenes* and *Bacillus anthracis*, two bacteria that cause infection via a mucosal site [35, 36]. Moreover, Bogefors et al. recently reported that different NLRs, including NOD2, are present in the nose [31]. This implicates an important role for the NOD2 receptor in mucosal immunity, which concurs with the positive effect found for MDP after nasal administration. Regarding intradermal vaccination, the receptors for LPS (TLR4) and CpG (TLR9), the two adjuvants that showed a strong effect after intradermal administration, are readily expressed on murine keratinocytes, Langerhans cells and DCs [30, 37]. Previous murine vaccination studies via the skin have shown the adjuvanticity of CpG [38, 39], which induced migration of Langerhans cells and DCs from the skin to the lymph nodes [40, 41].

In a previous nasal vaccination study the TMC/CpG/OVA nanoparticles were equally potent as non-adjuvanted TMC nanoparticles and particularly stimulated the IgG2a response [16]. However, since the applied dose in the present study was two times lower, this effect may have been masked. These results together with the elevated sIgA levels for 3 out of 8 mice indicate that whereas CpG can function as an adjuvant for nasal vaccination, the adjuvant dose may be crucial.

For both the intradermal and nasal route, CTB was unable to further promote the antibody titres compared to non-adjuvanted TMC/OVA nanoparticles. We have shown before that CT is able to boost the immune response after intradermal administration [13] and for both vaccination via the skin and nose CT is a well known adjuvant [42]. However, the toxicity of CT is a concern for nasal and intradermal administration. Especially after a nasal vaccine containing heat-labile enterotoxin (LT, a potent mucosal adjuvant with ADP-ribosylating activity like CT) was withdrawn from the market [43], CT is not considered a promising adjuvant for human nasal use anymore. CTB is a less toxic CT analogue [44] and successful nasal administration of CTB as an adjuvant has been reported [20, 22, 45, 46]. However, in a few cases it has also been linked to the induction of tolerance [47-49], the opposite of what is desired in the current vaccination study. Anjuère et al. compared the ability of CT and CTB to provoke an immune response after transcutaneous immunisation [50]. They reported CTB to be poorly efficient in inducing anti-OVA IgG levels, whereas CT provoked a strong humoral immune response. This shows that the adjuvant effect of CTB depends on the antigen, the formulation and the administration route.

Besides the extent of the immune response, also the type of immune response is an important parameter to consider, when selecting an adjuvant. TMC nanoparticles appear to be a Th2-biasing carrier system, as described before [13, 16, 51], regardless of the administration route. Encapsulation of most of the adjuvants did not significantly change the

Th1/Th2 ratio. LPS and PAM have been reported to augment the Th1 response after intramuscular and intraperitoneal administration [52, 53], but do not appear to elicit this effect after nasal or intradermal immunisation when co-encapsulated with the antigen in TMC nanoparticles. Only intradermal administration of TMC/CpG/OVA nanoparticles was able to counter the Th2 bias and increase the IgG2a levels (indicative of a Th1 response). Nasally, this effect of CpG was not observed, whereas an earlier study using a CpG dose that was twice as high, reported a clear Th1 biasing effect of TMC/CpG/OVA nanoparticles [16], also indicating an important role for the adjuvant dose. Overall, the effect of an adjuvant seems to be greatly dependent the dose, the type of antigen, the way it is formulated and –last but not least– the site of administration.

Conclusion

Inclusion of an adjuvant into antigen loaded TMC nanoparticles for nasal and intradermal vaccine delivery can be good strategy to improve the immunogenicity of the antigen. The success of this approach strongly depends on the selection of the adjuvant in conjunction with the site of administration.

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