CHALLENGES AND OPPORTUNITIES IN NASAL SUBUNIT VACCINE DELIVERY

mechanistic studies using ovalbumin as a model antigen

Proefschrift

Ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,
volgens het besluit van het College van Promoties
te verdedigen op 27 januari 2011
klokke 16:15

Door

Bernard Adam Slütter,
geboren te Warnsveld
in 1983

Promotoren:	Prof. W. Jiskoot
	Prof. J.A. Bouwstra

About the cover:
The application of a nasal vaccine could be accomplished using a spray, which is already common practice for several small molecular weight drugs. The formulation of vaccines however, still needs investigation. Should we formulate antigens in particles, perhaps?
Figure: Cislunar Aerospace, San Francisco, California.

The publication of thesis was financially supported by the J.E. Jurriaanse stichting.

Table of contents

1

General introduction and outline of this thesis

Vaccination

Since its application by Dr Edward Jenner in the 18th century, vaccination has revolutionized medicine. Large scale vaccination campaigns have resulted in the eradication of smallpox and the World Health Organization has set targets to eradicate polio, rubella and measles using a world wide vaccination strategy [1]. The goal of vaccination is to prime an individuals' immune system against a specific pathogen, so on second encounter the immune system is capable of quickly removing the threat. The classical approach is to use a non-pathogenic strain that closely resembles the pathogen (live-attenuated vaccines), or an inactivated pathogen. These vaccines generally provide good protection as they resemble the original pathogen the most. This approach, however, also brings safety risks. Vaccines based on live-attenuated or whole inactivated bacteria or viruses can contain a variety of biologically active compounds (e.g. toxins, bacterial cell membrane products) that can cause symptoms like fever and nausea. In the case of live-attenuated vaccines, reassortment with a wildtype virus could lead to regaining their pathogenicity. Moreover, in immuno-compromised patients these types of vaccines can cause disease symptoms, as these individuals are not capable of clearing the vaccines. Finally, most of these vaccines are injected intramuscularly or subcutaneously, as generally the large size and instability of the antigen does not allow application via mucosal routes. Injectable vaccines often cause pain/discomfort, local swelling (inflammation) and stiffness.

Vaccine coverage in the Western world is not optimal, as the turn up of the various state vaccination programs hardly ever passes 70% [2-5]. Although part of the vaccination refusals is of religious nature, an increasing population does not want to be immunized fearing the side effects of the vaccine and the discomfort upon injection [6]. This has prompted governments and health organizations to enlarge their funds for research and development of (safer) subunit and non-invasive vaccines (e.g. dermal, nasal, oral, pulmonary) [7-9] as better patient compliance may be expected.

Subunit vaccines

Adverse effects can be reduced by stripping the virulence factors from a pathogen, leaving only the part to which the immune system has to make antibodies or a T-cell response. Subunit vaccines only contain this antigenic part of the pathogen (often only a single protein) and are therefore safer and pharmaceutically better defined [10]. Although some subunit vaccines have been applied very successfully (e.g. diphtheria toxoid, pertussis toxoid and tetanus toxoid), most of them do not completely protect the vaccinated population [11]. Paradoxically, because of the lack of co-stimulatory factors, the immunogenicity of these types of vaccines is reduced. This is a direct consequence of the nature of the immune system; it will only develop a response if the encountered material is considered dangerous [12]. Antigen presenting cells (APCs; e.g. macrophages, dendritic cells) play a crucial role in the decision making by the immune system whether or not to respond and are therefore the key target in vaccination (Figure 1). On encounter with a pathogen APCs engulf it and break it down into small fragments (epitopes). Meanwhile various constituents of the pathogen contribute to activation of the APC (Figure 1a), making the cell capable of initiating an adaptive (T- and B-cell mediated) immune response [13]. These constituents are evolutionary conserved motives that are shared by many pathogens, so called pathogen associated molecular patterns (PAMPs), making it possible for the APC to distinguish between dangerous and innocuous antigens, via pathogen recognition receptors (PRR) [14, 15]. Plain subunit antigen will also be sampled by APCs, but because of the lack of PAMPs will be considered harmless and will not induce maturation of the APC (Figure 1b). It is therefore imperative to formulate the antigen in such a way that APCs do get activated e.g. by addition of PAMPs or the use of vaccine delivery systems [16].

Non-invasive vaccination

Currently, most vaccines are injected subcutaneously or intramuscularly as it is a simple procedure and allows accurate dosing. This does not mean however that muscle and subcutaneous tissue are sites that provide the best environment for inducing an immune response. Before the introduction of the hollow needle in the 19th century, vaccines were usually applied nasally or scratched into the skin [17]. As the exterior of the body is under constant attack by invading pathogens, the skin and the mucosal linings are densely equipped with APCs [18], whereas subcutaneous tissue and muscle contain very low numbers of APCs.

Not surprisingly, studies comparing the antibody response after intramuscular and intradermal vaccination clearly show superior antibody titers after intradermal injection [19-21]. Nonetheless intradermal vaccination did not establish itself as the standard administration method, because of poor protection in the elderly population but most of all because the intradermal injection technique is more difficult to master than the intramuscular one [22].

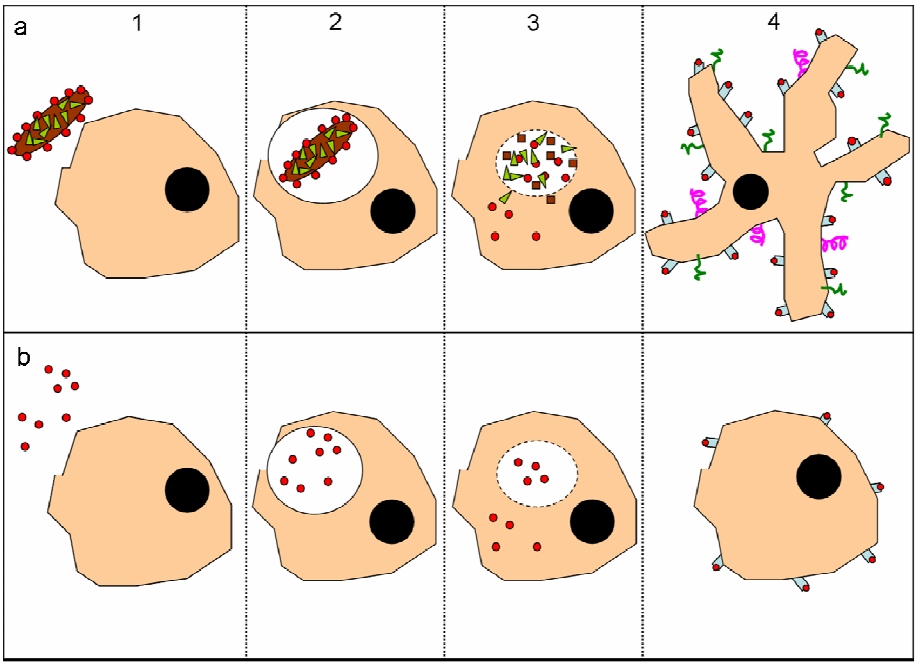


***Figure 1**: a) A bacterium is encountered by the APC (1) and subsequently engulfed (2). In the endosome the pathogen is degraded into epitopes (red) (3). Co-stimulatory factors (brown, green) on the bacterium activate the APC and make it express various co-stimulatory factors (4) enabling it to activate T- and B-cells. b) Plain subunit antigens encountered by a DC (1) are also taken up (2) and degraded into epitopes (3), but lack of virulence factors prohibits the activation of the APC (4).*

Similarly, very encouraging results were obtained by pulmonary vaccination against measles in Mexico [23], but the need for a delivery device (nebulizer) whereas intramuscular vaccination was just as effective, prohibited the widespread use of the pulmonary vaccine [24]. In this respect nasal and oral vaccination provide a more promising alternative. Oral application (e.g., as a tablet or capsule) may seem easy and convenient for the vaccinee, but the harsh gastro-intestinal conditions compromises the vaccines' stability. Currently, 1 inactivated and 4 live-

attenuated oral vaccines have been licensed (the polio vaccine already being administered over 1 billion times!), but no oral subunit vaccine has been marketed yet, illustrating the difficulty of making effective oral vaccines.

Table I. Advantages and disadvantages of different routes of immunization.

Immunization route	***Advantages***	***Disadvantages***
Intramuscular/ Subcutaneous	Powerful systemic immune response Accurate dosing	Invasive Limited mucosal immune response
Nasal	Non invasive Mucosal & systemic immune response Easily accessible Little degradation (compared to oral)	Mucociliary clearance Inefficient uptake of soluble antigens Application device needed
Oral	Non invasive Mucosal & systemic immune response Large surface area	Vaccine digestion in stomach and gut Inefficient uptake of soluble antigens Mucosal tolerance
Pulmonary	Non invasive Mucosal & systemic immune response Little degradation (compared to oral)	Delivery of antigen highly variable from person to person Dry powder inhaler or nebulizer needed Clearance from lungs
Dermal	Non or minimally invasive Large, easily accessible application area High density of immune cells in skin Mucosal immune response possible	May require (minimally) invasive technology (e.g. tattooing, microneedles) Patch or application device needed Less established technology

Nasal vaccination

The nasal cavity is easily accessible (e.g., nasal spray or nose drops) and the low enzymatic activity compared to the gastro-intestinal tract provides better antigen stability, making nasal administration very promising. Nonetheless, only 1 live attenuated flu vaccine (Flumist®) is on the market, showing that nasal vaccination is possible, but also challenging

Although the nasal flu vaccine has been well perceived by the public [25], because of the live attenuated nature of the vaccine, it only recommended for use in a population between the age of 2-50. Obviously this is not ideal as influenza is most threatening in young children

and the elderly. A subunit vaccine would be preferable, but until now only 1 nasal subunit vaccine had been licensed which had to be withdrawn from market because of presumed side effects of the adjuvant [26].

The poor efficacy of nasally administered vaccines is caused by the physiology of the nasal cavity. Compared to muscle or subcutaneous tissue, the nasal cavity has a different immunologic build up, as it is a mucosal site. Moreover, the antigen has to find its way through several barriers (mucus, epithelium), in a limited time frame (nose cleared every 20 minutes removing all constituents trapped in its mucus), before it is absorbed into the body. So, if nasal vaccination is to be successful, the vaccine's formulation should be adapted to the challenges the physiology of the nasal cavity provides. Indeed, in the literature a wide variety of vaccine formulations are described (mainly tested in mice) that increase the efficacy of nasally administered antigen [27-32]. Although these studies are very encouraging and provide valuable information on the use of absorption enhancers and adjuvants, an integral approach combining the positive characteristics of these various formulations is hardly described. Increasing knowledge on the pathways and bottlenecks involved in nasal vaccination will make it possible to optimize the formulations and rationally design nasal vaccines.

Aim and outline of this thesis

Nasal vaccination has the potential to provide protection combined with more patient comfort and a higher safety profile than classical injectable vaccines. However, the nasal physiology and immunological aspects of the nasal epithelium hamper the efficacy of nasally administered vaccines.

The aim of this thesis is therefore three-fold:

- to identify the principal hurdles to successful nasal vaccine delivery;
- to develop preclinical model systems to investigate these hurdles;
- to apply these principles to rationally design nasal subunit vaccine formulations in a preclinical setting.

In **Chapter 2** the main physiological hurdles that have to be overcome to render nasal vaccination successful are reviewed. The progress made in the field of nasal delivery of subunit vaccines is described and emerging opportunities for improving nasal vaccines are discussed.

Throughout this thesis ovalbumin (OVA), a 45-kDa protein purified from chicken eggs and widely used in immunology, was used as a model subunit antigen. As nasal administration of plain OVA does not result in effective seroconversion, the effectiveness of a nasal vaccine formulation can be easily assessed using this antigen. A model system to investigate one of the main physiological hurdles, penetration of the mucosal epithelium, is discussed in **Chapter 3**. A cell culture system based on an intestinal epithelial cell line (Caco-2) including M-cells is introduced as tool to assess the transport of vaccine delivery systems through the mucosal epithelium. Moreover, monocyte derived human dendritic cells (DC) are explored to investigate the role of DC uptake and maturation. The predictive value of these *in vitro* assays is studied by intraduodenal vaccination with OVA encapsulated in two potential mucosal vaccine delivery systems, chitosan and N-trimethyl chitosan (TMC) nanoparticles.

To address the mechanistics behind nasal vaccination, the characteristics of 3 nasal delivery systems, based on nanoparticles composed of PLGA, TMC, or both, are correlated to their capacity to induce antibody production (**Chapter 4**) and $CD4^+$ T-cell activation (**Chapter 5**) or tolerance (**Chapter 5**) after nasal administration in mice. Moreover, a new method to assess the residence time of an antigen in the nasal cavity is introduced (**Chapter 4**).

The effectiveness of cationic liposomes, a promising delivery system for injectable vaccines, as carriers for nasal vaccines is investigated in mice (**Chapter 6**) and compared to other application routes, i.e. epidermal, intradermal and intranodal administration. Furthermore, this chapter describes investigations on the usefulness of (1) encapsulating antigen in vesicles and (2) co-encapsulation or co-administration of an adjuvant.

In **Chapters 7-10** the knowledge gained from the first chapters on the mechanistics behind nasal vaccination is applied to improve the most promising delivery system tested, being nanoparticles based on TMC. In an attempt to make smaller TMC/antigen entities, TMC-antigen conjugates are developed and characterized physicochemically and immunologically in **Chapter 7**. Nasal application of these conjugates and their interaction with various components of the murine immune system are described in **Chapter 8**.

Chapter 9 concerns the replacement of tripolyphosphate, a physical crosslinker used to prepare TMC nanoparticles, with the adjuvant CpG, acting as a crosslinking agent as well as an immune modulator, and the effect on particle characteristic and immunogenicity. In **Chapter 10** a variety of other adjuvants described in the literature are encapsulated in TMC nanoparticles and their effectiveness as immune potentiators as are assessed in mice.

Chapter 11 summarizes the results and conclusions in this thesis. Moreover, future directions concerning the developed and rational design of nasal subunit vaccines are discussed.

References

1. WHO, *WHO preventable disease: Monotoring system*, W.H. Organization, Editor. 2009: Geneva.
2. Bitsori, M., et al., *Vaccination coverage among adolescents in certain provinces of Greece* Acta Paediatr, 2005. **94**(8): p. 1122-5.
3. Kalies, H., et al., *Immunisation status of children in Germany: temporal trends and regional differences* Eur J Pediatr, 2006. **165**(1): p. 30-6.
4. Rumke, H.C. and H.K. Visser, *Childhood vaccinations anno 2004. I. Effectiveness and acceptance of the Dutch National Vaccination Programme* Ned Tijdschr Geneeskd, 2004. **148**(8): p. 356-63.
5. Szucs, T.D. and D. Muller, *Influenza vaccination coverage rates in five European countries-a population-based cross-sectional analysis of two consecutive influenza seasons* Vaccine, 2005. **23**(43): p. 5055-63.
6. Crockett, M. and J. Keystone, *"I hate needles" and other factors impacting on travel vaccine uptake* J Travel Med, 2005. **12 Suppl 1**: p. S41-6.
7. *President Requests 2.6 Percent NIH Budget Increase in 2005* 2005; Available from: http://www.nih.gov/news/NIH-Record/03_02_2004/story03.htm.
8. Enserink, M. *Radical Steps Urged to Help Underserved* 2000; Available from: http://www.sciencemag.org/cgi/content/full/288/5471/1563a?ck=nck.
9. Harrington, M. *To Double, or Not to Double? Bioterror Buys a Budget Boost* 2002; Available from: http://www.aidsinfonyc.org/tag/activism/bioterror.html.
10. Favre, D. and J.F. Viret, *Biosafety evaluation of recombinant live oral bacterial vaccines in the context of European regulation* Vaccine, 2006. **24**(18): p. 3856-64.
11. Poland, G.A. and J.M. Neff, *Smallpox vaccine: problems and prospects* Immunol Allergy Clin North Am, 2003. **23**(4): p. 731-43.
12. Matzinger, P., *The danger model: a renewed sense of self.* Science, 2002. **296**(5566): p. 301-5.
13. Vance, R.E., R.R. Isberg, and D.A. Portnoy, *Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system.* Cell Host Microbe, 2009. **6**(1): p. 10-21.

14. Gordon, S., *Pattern recognition receptors: doubling up for the innate immune response* Cell, 2002. **111**(7): p. 927-30.
15. Kaisho, T. and S. Akira, *Toll-like receptors as adjuvant receptors* Biochim Biophys Acta, 2002. **1589**(1): p. 1-13.
16. Storni, T., et al., *Immunity in response to particulate antigen-delivery systems.* Adv Drug Deliv Rev, 2005. **57**(3): p. 333-55.
17. Brandtzaeg, P., *History of oral tolerance and mucosal immunity* Ann N Y Acad Sci, 1996. **778**: p. 1-27.
18. Randolph, G.J., V. Angeli, and M.A. Swartz, *Dendritic-cell trafficking to lymph nodes through lymphatic vessels.* Nat Rev Immunol, 2005. **5**(8): p. 617-28.
19. Chiu, S.S., et al., *Immunogenicity and safety of intradermal influenza immunization at a reduced dose in healthy children.* Pediatrics, 2007. **119**(6): p. 1076-82.
20. Kenney, R.T., et al., *Dose sparing with intradermal injection of influenza vaccine.* N Engl J Med, 2004. **351**(22): p. 2295-301.
21. Van Damme, P., et al., *Safety and efficacy of a novel microneedle device for dose sparing intradermal influenza vaccination in healthy adults.* Vaccine, 2009. **27**(3): p. 454-9.
22. Belshe, R.B., et al., *Serum antibody responses after intradermal vaccination against influenza.* N Engl J Med, 2004. **351**(22): p. 2286-94.
23. Sabin, A.B., et al., *Successful immunization of children with and without maternal antibody by aerosolized measles vaccine. I. Different results with undiluted human diploid cell and chick embryo fibroblast vaccines.* JAMA, 1983. **249**(19): p. 2651-62.
24. Laube, B.L., *The expanding role of aerosols in systemic drug delivery, gene therapy, and vaccination.* Respir Care, 2005. **50**(9): p. 1161-76.
25. Lin, C.J., et al., *Importance of vaccination habit and vaccine choice on influenza vaccination among healthy working adults.* Vaccine, 2010.
26. Mutsch, M., et al., *Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland.* N Engl J Med, 2004. **350**(9): p. 896-903.
27. Debin, A., et al., *Intranasal immunization with recombinant antigens associated with new cationic particles induces strong mucosal as well as systemic antibody and CTL responses.* Vaccine, 2002. **20**(21-22): p. 2752-63.
28. Hamdy, S., et al., *Enhanced antigen-specific primary CD4+ and CD8+ responses by codelivery of ovalbumin and toll-like receptor ligand monophosphoryl lipid A in poly(D,L-lactic-co-glycolic acid) nanoparticles.* J Biomed Mater Res A, 2007. **81**(3): p. 652-62.
29. Khatri, K., et al., *Plasmid DNA loaded chitosan nanoparticles for nasal mucosal immunization against hepatitis B.* Int J Pharm, 2008. **354**(1-2): p. 235-41.
30. Read, R.C., et al., *Effective nasal influenza vaccine delivery using chitosan.* Vaccine, 2005. **23**(35): p. 4367-74.
31. Revaz, V., et al., *Humoral and cellular immune responses to airway immunization of mice with human papillomavirus type 16 virus-like particles and mucosal adjuvants.* Antiviral Res, 2007. **76**(1): p. 75-85.
32. Sloat, B.R. and Z. Cui, *Strong mucosal and systemic immunities induced by nasal immunization with anthrax protective antigen protein incorporated in liposome-protamine-DNA particles.* Pharm Res, 2006. **23**(2): p. 262-9.

2

Rational design of nasal vaccines

Bram Slütter
Niels Hagenaars
Wim Jiskoot

Journal of Drug Targeting 2008 16(1):1-17

Abstract

Nasal vaccination is a promising alternative to classical parental vaccination, as it is non-invasive and, in principle, capable of eliciting strong systemic and local immune responses. However, the protective efficacy of nasally administered antigens is often impaired because of delivery problems: free antigens are readily cleared from the nasal cavity, poorly absorbed by nasal epithelial cells and generally have low intrinsic immunogenicity. In this review paper, we describe the main physiological hurdles to nasal vaccine delivery, survey the progress made in technological approaches to overcome these hurdles and discuss emerging opportunities for improving nasal vaccines. According to current insights, encapsulation of the antigen into bioadhesive (nano)particles is a promising approach towards successful nasal vaccine delivery. These antigen-loaded particles can be tailor made by supplying them with targeting ligands, adjuvants or endosomal escape mediators to form the desired vaccine that provides long-lasting protective immunity.

Introduction

Vaccination is the most cost effective way of fighting infectious diseases. Although some vaccination strategies have been very successful, novel approaches are needed to develop safe and effective vaccines against diseases like HIV/AIDS, malaria, influenza and cancer. Additionally, adverse reactions, like pain, fever, headaches, nausea and allergic reactions have led to declined patient compliance [1-6] and have prompted governments and health organizations to enlarge their funds for research and development of non-invasive vaccines [7-9]. Among the potential needle free routes, nasal vaccination is particularly attractive. The nasal cavity had been the preferred delivery site until the introduction of the hollow needle in the 19th century [10]. In the search for alternatives to the needle, the interest in nasal vaccination has reemerged. This paper will review the main physiological hurdles that have to be overcome to render nasal vaccination successful, describe the progress made in the field of nasal delivery of subunit vaccines, and discuss emerging opportunities for improving nasal vaccines.

Nasal vaccination

Nasal vaccination has several interesting advantages. The nose is easily accessible and the nasal cavity is equipped with a high density of dendritic cells (DC) that can mediate strong systemic and local immune responses against pathogens that invade the human body through the respiratory tract [11-13]. Mucosal immunity is mediated by secretory immunoglobulin A (sIgA) antibodies, which prevent pathogens from colonizing mucosal epithelia (e.g. respiratory tract, gastro intestinal tract) and hence clear the organisms before they invade the underlying tissue.

Local immunity in the upper airways, as well as systemic immunity, is mainly mediated by the lymphoid tissue referred to as nasal associated lymphoid tissue (NALT). NALT is comprised of agglomerates of cells involved in the initiation and execution of an immune response, like DC, T-cells and B-cells [14], situated underneath the nasal epithelium. NALT is most pronounced in the nasopharynx and the Waldeyer's ring, which includes the nasopharyngeal, tubal, palatine and lingual tonsils, making the adenoids an important part of the NALT. Indeed some studies have shown that sIgA excretion is dependent on these areas and tonsillectomy has been associated with decreased immunity [15, 16]. Moreover, Zuercher *et al* [17] showed the presence of germinal centers (places where plasma cells are located) in the NALT after

challenge with a reovirus, and Shimoda *et al* [18] showed that B-cells in the subepithelial region of the nose are prone to switch from IgM to IgA, indicating a role for the NALT as inductive site for immune responses. Mucosal immunity after nasal vaccination is, however, not restricted to the upper airways. Via a system called the common mucosal immune system, after nasal immunization sIgA antibodies also can be detected also in other mucosal secretions.

In spite of the large effort that has been directed to developing nasal vaccines, only one nasal vaccine is currently on the market (Table I). Furthermore, nasal vaccine delivery may be compromised in patients with respiratory infections and the need for an effective delivery device should not be overlooked. In attempts made to improve the immunogenicity of nasal subunit vaccines, the vaccine formulation plays a crucial role, as will be further discussed below.

Table I. Examples of nasal vaccines currently on the market or at different stages of development.[*]

Immunization route	Disease	Pathogen	Vaccine type	Phase
Nasal	Influenza	*Influenza virus*	Live attenuated	On market
	Hepatitis B	*Hepatitis B virus*	Subunit vaccine	Phase I
	Influenza	*Parainfluenza virus type 3*	Live attenuated	Preclinical
	Anthrax	*Bacillus anthracis*	Killed/inactivated	Preclinical
	Herpes	*Herpes simplex virus*	Killed/inactivated	Preclinical
	Bronchiolitis/ Pneumonia	*Respiratory syncytial virus*	Killed/inactivated	Preclinical
	Cervical cancer	*Human papillomavirus*	Subunit vaccine	Preclinical
	SARS	*SARS corona virus*	Subunit vaccine	Preclinical

[*]Based on [19] and [20]

Roadmap to successful nasal vaccine delivery

After nasal administration of a vaccine, a number of successive steps should lead to a protective immune response (Fig. 1). In this section we will describe these steps and discuss how a vaccine delivery system can enhance the immune response by promoting these steps.

Prolonging the nasal residence time

After intranasal (i.n.) administration, the first step in the trajectory towards an immune response is that the antigen reaches the NALT. In principle, there is no direct contact between DC in the subepithelial regions of the nose and the antigen in the lumen, although it has been suggested that DC can partially penetrate the epithelium making them capable of sampling the mucosal surface [21]. Therefore, during the limited nasal residence time of the vaccine, the antigen must cross the nasal epithelium. Increasing the residence time of the vaccine (normally ca. 20 minutes), with use of mucoadhesive substances, may therefore be a possible approach to improve the efficacy of a vaccine (Fig. 1).

M-cell targeting

The mechanism of antigen uptake through the epithelium is somewhat controversial. The epithelium is composed of only a thin layer of pseudostratified epithelial cells, connected by tight junctions. Since the diameter of tight junctions is only a few Ångstroms [22], it is very unlikely that (killed) bacteria or viruses, bulky antigens, or particulate vaccine delivery systems are able to penetrate this barrier by paracellular transport even if the tight junctions are widened up [23]. Transcellular transport is a more likely route by which (particulate) antigens reach the NALT. In particular, microfold cells (M-cells) serve as a portal for particulate antigens to enter the subepithelial region [12, 24-26]. M-cells are part of the NALT and cover the subepithelial dome containing DC, B-cells and T-cells. M-cells do not contain cilia and have relatively high concentrations of cytoskeleton protein vimentin [27, 28], making M-cells easily accessible and flexible, respectively, to be involved in transmembranous transport [29]. Indeed, after recognition and internalization, M-cells can transport particulate antigens to the NALT, by transcytosis [30]. Unlike epithelial cells, M-cells have been reported to efficiently take up antigens with a particulate nature and deliver them to a lymphatic environment rather than to the systemic circulation. This may explain why the increased efficiency of particulate antigens is undisputed, whereas increased drug transport using particles is still under discussion [31]. Hence, improving the uptake of a vaccine through M-cells would target the antigen to the underlying immune cells, and may thereby contribute to higher immune responses (Fig. 1).

DC signaling

After antigen uptake, DC mature and migrate to the nearby cervical lymph nodes, where they present the peptides on MHC class 2 (MHC II) molecules to helper T (Th) cells. Upon recognition of the MHC II-peptide complex and costimulation from APC, naïve Th cells

differentiate into effector Th cells, which can be divided in two major subtypes: Th1 and Th2 cells. Th1 cells are mainly involved in activation and proliferation of the cellular immune system, whereas Th2 cells are involved in stimulation and increase of the humoral immune responses. The DC signaling determines the fate of the naïve Th cell and can be influenced by the use of delivery systems and adjuvants. So, not only can delivery systems and adjuvants increase immune responses, they can also influence the Th1/Th2 balance, i.e. the type of immune response. Since the optimal balance of the immune reaction is dependent on the pathogen in question, induction of the desired type of immune response should be tailored for each specific vaccine, which can be achieved by the use of proper delivery systems and adjuvants.

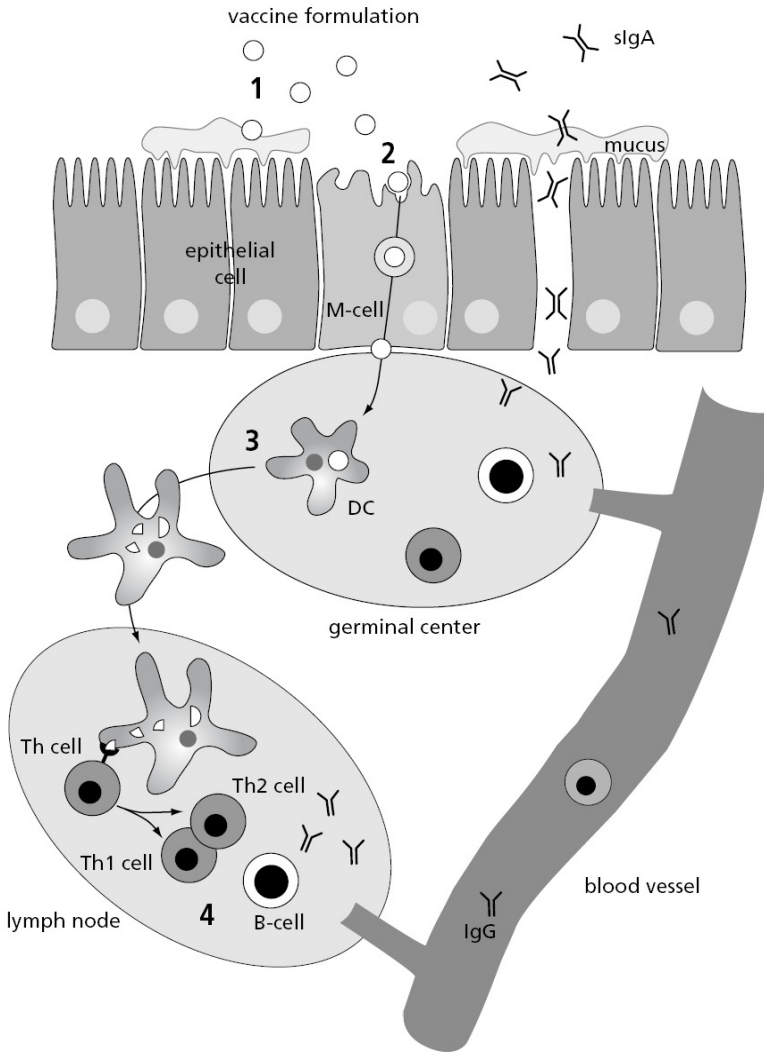


***Figure 1**: Schematic overview of the consecutive steps towards successful nasal vaccine delivery: 1) mucoadhesion; 2) antigen uptake, by M-cell transport; 3) delivery to and subsequent activation/maturation of DC; 4) induction of B-cell and T-cell responses. DC = dendritic cell, M-cell = microfold cell, Th cell = helper T cell.*

Induction of CTL immune responses.

Obtaining immunity against intracellular pathogens like intracellular bacteria and viruses often requires the induction of CTL responses. The induction of CTL with a vaccine can only be achieved when a number of requirements are fulfilled. Firstly, the vaccine should contain class 1 (MHC I) epitopes. Secondly, an MHC II epitope must be present in the vaccine, since a strong induction of CTL responses is only possible when Th cells are co-activated. Thirdly, the MHC I epitope should enter the MHC I presentation pathway.

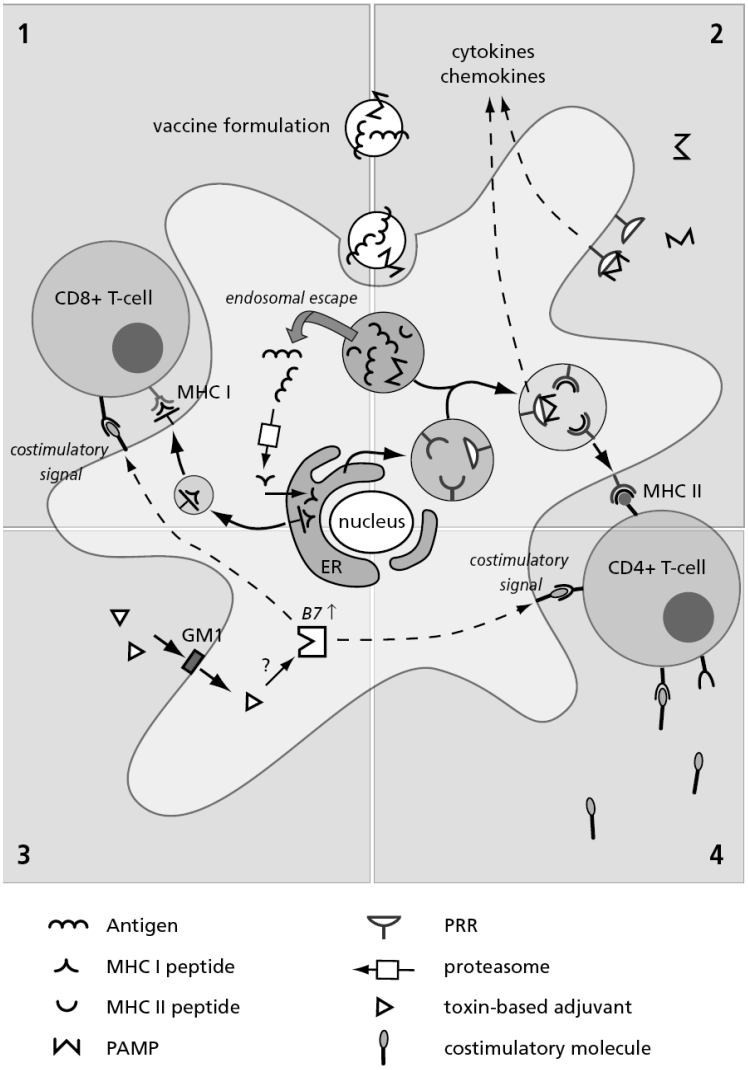


***Figure 2**:*
Various mechanisms a vaccine formulation can exploit to induce the desired immune response. 1) cytosolic delivery for targeting the antigen to MHC class I presentation; 2) targeting the innate immune system through pathogen recognition receptors (PRR); 3) the use of toxin-based adjuvants; 4) incorporation of cytokines or other costimulatory molecules. See text for further details.

DC copulsed with a Th1 and a Th2 inducing antigen were shown to direct these antigens to distinct compartments, leading to different, antigen dependent polarization of the immune response [32]. Presentation of exogenous antigens by MHC I molecules is called cross presentation [33, 34]. Recently, it was described that the mechanism of antigen uptake, which dictates the intracellular destination compartment, is not only involved in the activation and polarization of Th cells, but also determines whether the antigen is presented to either $CD4^+$ Th cells or $CD8^+$ CTL. This would suggest that a DC itself is not polarized upon ingestion of an antigen; rather, each intracellular compartment can prepare different instructions that can be presented to different T cells by one DC [35, 36]. Targeting mediators in the vaccine formulation could be employed to facilitate the delivery of endocytosed antigens to the desired intracellular compartments and thereby promote cross presentation (Figure 2).

Adjuvant targets. Adjuvants can be classified according to their mechanisms of action. One group of adjuvants acts through binding to pathogen recognition receptors (PRR) on cells. The binding of PAMP to PRR activates an intracellular signaling cascade in the innate immune cells, which eventually leads to DC maturation, cytokine production and costimulatory signaling to Th cells (Fig. 2).

The Toll-like receptor (TLR) family is a group of PRR that has been characterized in detail [37]. TLR are expressed by DC and recognize PAMP (Fig. 2) like lipopolysaccharide (LPS), dsRNA, CpG motifs and bacterial lipoproteins [38, 39]. Simultaneous stimulation of these innate immune receptors and antigen delivery to the DC generally leads to Th1 responses and Th1 dependent antibody isotypes [37], but some TLR ligands induce Th2 cytokines upon activation of the TLR [40]).

Another group of adjuvants are toxin based adjuvants. Enterotoxins like the cholera toxin (CT) and the *Escherichia coli* heat labile toxin (LT) are strong mucosal adjuvants that induce mucosal as well as serum antibody responses [41, 42]. LT and CT consist of a toxic A subunit with ADP-ribosyltransferase activity, which is linked non-covalently to a pentamer of B subunits that bind to ganglioside GM-1 receptors found on most cells [43]. Since the use of LT has been associated with neurological toxicity, efforts have been made to develop non-toxic mutants of LT and CT. The exact mechanism of adjuvanticity of toxin-based adjuvants is not fully understood, but the toxins CT and LT induce expression of B7 molecules on DC that can subsequently deliver costimulatory signals to Th cells (Fig. 2) [44].

Cytokines are probably the critical communication molecules of most classical adjuvants [45]. Therefore cytokines and other costimulatory molecules have been evaluated

as adjuvants to promote T-cell activation (Fig. 2). Similary, antibodies mimicking the binding of these molecules to receptors on the T-cells have been tested as adjuvants.

Approaches to improve nasal vaccine delivery

In this section we discuss, based on the roadmap described in the previous section, several approaches that have been described in the literature to improve the delivery and immunogenicity of nasally administered antigens.

Mucoadhesives

Subunit antigens, having little affinity for the nasal epithelium, are generally cleared within minutes. Prolonging the residence time is commonly accomplished by coadministering the antigen with mucoadhesives, usually polymers. The term mucoadhesive does not discriminate between the interaction with either the mucosal cell surface or the mucus covering this surface. If the adhesive also interacts with the antigen, both interactions can lead to a decreased mucociliary clearance of antigens.

Recently, Smart gave an overview of the basics and mechanisms of mucoadhesion [46]. Briefly, properties like hydrophilicity, crosslinking, charge, molecular weight and the presence of acidic or alkaline functional groups influence the mucoadhesion of a polymer. Mucoadhesive polymers can be divided in 3 categories according to their mechanism of interaction. The first category includes hydrophilic polymers that adsorb to the mucus by forming hydrogen bonds, like sodium alginate, sodium carboxymethylcellulose, hydroxypropyl methylcellulose and carbopol. The second class comprises cationic polymers, like chitosan-derived polymers interacting with the negatively charged mucin mainly by ionic interactions, although hydrogen bonds could also play a role [47, 48]. Additionally, chitosan derivatives can open tight junctions and thereby can increase the permeability of the epithelium [49], but its significance for improved antigen delivery is questionable. The third class of mucoadhesives involves thiolated polymers, thiomers, that can form covalent disulfide bonds with the cystein groups in mucin [50]. Recent studies show that thiomers are the strongest mucoadhesives [51].

Antigens coadministered with mucoadhesive polymers, like hyaluronic acid [52], chitosan [53] and carboxylmethylcellulose [54] have indeed shown increased antibody responses as compared to application of the antigen without any additives. However, serum antibody levels

reached by coadministration of *Bordetella pertussis* hemagglutinin vaccine [55], diphtheria toxoid [56], tetanus toxoid [57], anthrax protective protein [57], inactivated influenza virus [58] or herpesvirus 1 glycoprotein [59] with chitosan never exceeded levels reached by intramuscular (i.m.) injection, despite the capability of chitosan to increase the nasal residence time [60]. Clearly, a prolonged residence time is not the sole determinant for a successful vaccine.

Particulate antigen carriers

Uptake of antigens through the nasal epithelium can be increased by incorporation into particles [61]. For instance, i.n. administration in mice of antigens incorporated in nanoparticles composed of poly lactide-co-glycolide (PLGA), a biodegradable polymer, led to over 100 fold increased antibody responses in comparison with aqueous solution of parainfluenza virus proteins [62], hepatitis B soluble antigen [63], *Bordetella bronchiseptica* dermonecrotoxin [64] and recombinant HIV proteins [65]. Polystyrene beads loaded with hemagglutinin led to increased protection against influenza in mice [66], probably due to enhanced antigen uptake [67]. Nasal application of liposomes loaded with killed measles virus [68], formaldehyde-killed *Yersinia pestis* [69], or influenza A hemagglutinin [70] even elicited superior IgG antibody levels than i.m. administered alum adsorbed antigen.

Mucoadhesive particles. Particles composed of mucoadhesive polymers are even more effective antigen carriers, as they combine prolonged residence time in the nasal cavity with the beneficial properties of particulate systems. Chitosan particles are well-known mucoadhesive antigen carriers. Coadministration of soluble chitosan with cholera toxoid or ovalbumin (OVA) induced higher immune responses than administration of antigen alone, but incorporation of the CT or OVA antigen into chitosan nanoparticles resulted in superior serum antibody levels in rats [71]. Similarly, Amidi *et al* showed that nasally applied influenza antigens incorporated in trimethylated chitosan (TMC) nanoparticles elicited superior IgG and sIgA antibody response as compared to naked antigen or antigen coadministered with TMC [72].

Alternatively, particulate antigen carriers can be rendered mucoadhesive by coating them with mucoadhesive polymers. Intranasal vaccination with hepatitis B surface antigen (HBsAg) encapsulated in chitosan coated PLGA particles resulted in a 30 fold increase of serum IgG levels in comparison with uncoated HBsAg loaded PLGA particles [73]. Vila *et al* showed that chitosan coating of tetanus toxoid-containing PLA particles increases transport through the nasal epithelium in comparison with uncoated particles [74]. The increased transport was

accompanied by higher IgG responses against tetanus toxoid, indicating a positive effect of epithelial transport on vaccine efficacy.

Particle characteristics. The physicochemical properties of the particles most likely are critical to the effectiveness of the vaccine. For instance, particle size and zeta potential can impact the transport by M-cells as well as subsequent events, but the ideal particle characteristics are still under discussion.

The effect of particle size has not been thoroughly investigated for nasal vaccination. It has been determined that M-cells in Peyer's patches in the gut selectively take up particles with a diameter up to 10 μm [75] and that the particle size influences the type of immune response [76]. Xiang *et al* stated that particles resembling the size of viruses (20-200 nm) will be handled by the immune system as being a virus and elicit a cellular biased response, whereas particles with the size of a bacterium (between 0.5-5 μm) will favor a humoral response [77]. For nasal vaccination, several studies pointed to small (nano)particles being more rapidly absorbed by nasal M-cells [61, 71, 78-80], but no boundaries have been determined. Fujimura *et al* [81] showed that particles coated with the cationic polymers chitosan or poly-L-lysine were taken up by the NALT in a size range from 0.2 μm to 2 μm, with an increased uptake of smaller particles. Unfortunately, these particles did not carry an antigen, making it impossible to determine the effect of increased uptake on resulting immune responses.

As the cell membrane of M-cells is negatively charged, one can argue that a positive zeta potential is beneficial for M-cell transport. However, mucus and epithelial cells carry a negative charge as well, making electrostatic interactions very unspecific. Still, nasal application of a *Yersinia pestis* antigen in positively charged liposomes induced significantly stronger antibody responses than the same antigen in negatively charged liposomes [70, 82]. Likewise, nasal administration of HBsAg in positively charged PLGA microparticles resulted in significantly higher antibody levels than the same antigen in negatively charged PLGA microparticles (Jaganathan et al 2006). Although negatively charged or neutral particles have been reported to drastically increase antibody response after nasal immunisation [78, 83], positively charged particles seem to be superior to their negatively charged counterparts.

Improved mechanistic insight into the role of particle characteristics on antigen uptake will be necessary to resolve the ideal characteristics of a particulate carrier for uptake by the nasal epithelium.

M-cell targeting approaches

Specific M-cell targeting could further enhance vaccine efficacy. A variety of microorganisms, e.g. influenza viruses and group A streptococci, have been found to target

themselves to M-cells [12, 24, 25]. Complete bacteria can be used as vaccine carrier, exploiting their M-cell targeting mechanisms. Expression of *Streptococcus pneumoniae* antigens on live lactobacillus led to high IgG and sIgA titers in mice after i.n. administration [84]. Since live and inactivated lactobacilli induced similar protective immunity after nasal administration [85], the positive effect of lactobacillus is likely not due to prolonged residence time, but rather to increased bioadhesion or (M-cell mediated) uptake.

Virosomes are reconstituted virus envelopes, including a lipid bilayer and surface proteins. For instance, influenza virosomes (containing hemagglutinin and neuraminidase surface antigen) can be used as carriers to transport antigens to the cytosol of cells that overexpress sialic acid residues [86, 87] and might be exploited to target DC [88], but could target M-cells by the same mechanism. Virosomes have been shown to be excellent nasal carriers for several antigens like the F-protein of RSV [89] and DNA vaccines [90].

M-cells express several adhesion molecules on their cell surface that can bind pathogens. However, most work has been done on intestinal M-cells [91-93] and regional differences between M-cells exist [94, 95]. For instance, the plant lectin *Ulex europaeus* 1 lectin (UEA-1) [96] as well as lectins from other species [97-99] have been successfully used for targeting particles to intestinal M-cells in mice, but the specificity of UEA-1 for nasal M-cells is lower, as it also has affinity for nasal epithelial and goblet cells [100]. Despite this shortcoming, UEA-1 has been shown to increase M-cell transport and able to raise serum antibody levels when coadministered i.n. with DNA encoding HIV envelope protein [101].

Putative ligands that selectively target nasal M-cells include isolectin B_4 and *Maackia amurensis I* lectin [100], which recognize α-(1-3)-linked galactose and sialic acid, respectively [102]. Interestingly, sialic acid and galactose residues are involved in the initial binding of influenza virion to the host cell [103] and influenza A type viruses adhere efficiently to nasal M-cells *in vitro* [24]. Adherence of *Streptococcus pneumoniae* to the tracheal epithelium in chinchillas is dependent on the expression of sialic acid [104], showing the importance of these carbohydrate residues on the nasal epithelium for the entrance of these airborne pathogens. Nasal application of the model antigen HRP with isolectin B_4 significantly enhanced the antibody (IgG and sIgA) response to HRP in comparison with administration of HRP alone [102]. Recently it has been established that the Fc part of sIgA (and IgG alike) may also target M-cell, thereby creating a positive feedback loop [105]. Consequently coating particles with sIgA or IgG can increase M-cell transport [106] and have been show to increase the immunogenicity of liposomal HBsAg formulation after nasal administration [107].

Finally, several other receptors have recently been identified as potential M-cell targeting ligands, especially β_1-integrin [108]. Several pathogens use β_1-integrins to cross the intestinal

epithelium, such as *Yersinia pestis* [109] and *Escherichia coli* [110, 111]. Recently Gullberg *et al* showed that uptake of latex particles by human intestinal M-cells *in vitro* was increased when the particles were coated with a β_1-integrin ligand [112]. Hicks *et al* [113] showed that β_1-integrin is also readily expressed on nasal isolectin B_4 positive epithelial cells, i.e. most probably M-cells.

Intracellular targeting, induction of cytotoxic T cells

After antigen uptake through the nasal epithelium, their uptake and processing by DC are the next critical steps that determine the immune response. Antigen delivery systems that are capable of disrupting the DC's endosomal membrane and thereby promote endosomal escape can in principle be used to induce CTL responses. It has been shown that antigens incorporated in particulate antigen delivery systems are more effectively cross-presented than soluble antigens [114-116]. The efficiency of cross-presentation can vary between different types of particulate antigen delivery systems.

ISCOMs and ISCOMATRIX adjuvant are 40-nm cage-like structures composed of Quillaja saponins, cholesterol, and lipids that can incorporate or associate membrane antigens and DNA. ISCOMs are well-studied nasal and parenteral adjuvants that induce not only mucosal and systemic humoral responses, but also CTL responses [117-121]. It is thought that ISCOMs can deliver antigens to the APC's cytosol due to their membrane-disrupting properties [120], triggering endosomal escape. Additionally, it has been shown that CTL induction is markedly stronger when the antigen is physically attached to the ISCOMATRIX rather than administered unbound [118].

Virosomes can also induce strong CTL responses in addition to humoral and Th cell responses [86, 122] Influenza virosomes have been most extensively investigated. These virosomes contain influenza hemagglutinin, which binds to sialic acid residues on the cell surface and initiates receptor mediated endocytosis. Conformational changes in the influenza hemagglutinin due to acidification of the endosomes triggers the fusion of the endosomal membrane and the virosomal membrane, which enables release of the virosomal contents from the endosome into the cytosol. Subsequently the released antigens are degraded by the proteasome and presented through MHC I molecules [123]. Influenza virosomes have shown increased CTL responses against virosomal influenza [124-127], hepatitis C [128] and cancer antigens [129]. Virosomal influenza vaccines are the only virosomal vaccines that have been tested via the nasal route [130-133].

In addition to lipid based antigen delivery systems, polymeric biodegradable nanoparticles can enhance CTL induction *in vitro* [134] and *in vivo* after nasal vaccination

[135], as well as other routes [136-139]. Antigen loaded biodegradable PLGA nanoparticles are superior to nondegradable antigen adsorbed to latex nanoparticles [140], most likely due to hydrolysis of these polymeric nanoparticles in the acidic environment of endosomes. This facilitates endosomal escape [140-142] and antigen delivery into the cytosol, leading to enhanced MHC II presentation. The charge and structure of polymeric nanoparticles can also affect the uptake into DC. For instance, a positive charge has shown to increase phagocytic activity [141].

To summarize, vaccine delivery systems and endosomal escape mediators can be used for MHC II antigen presentation and thereby could increase CTL responses to an antigen.

Adjuvants

TLR ligands. CpG motifs in bacterial dsDNA are recognized by TLR 9. CpG oligodeoxynucleotides (ODN) have been tested in mice as adjuvant for nasal vaccines against several pathogens. Table III gives an overview of the results from studies in which TLR ligands have been tested as adjuvants for nasal vaccines. In general, the addition of CpG ODN to a nasal vaccine results in increased serum and mucosal antibody levels as well as increased cellular responses [143-145]. Generally, the addition of CpG ODN shifts the immune response from Th2 biased to a balanced Th1/Th2 response, i.e. it increases the production of Th1 cytokines and IgG2a.

Double stranded RNA and poly (I:C) are ligands for TLR 3. Poly (I:C) has been tested in mice as an adjuvant in a nasal influenza vaccine, resulting in protective immunity against influenza [146]. Poly (I:C) also increased humoral immune responses to two antigen formulations of *Bacillus anthracis*, inducing maturation and migration of DC and directing the immune response from mainly Th2 to a more balanced Th1/Th2 response. Moreover, sIgA was detected in broncheo alveolar lavage fluid [147, 148].

TLR 3 and TLR 9 are located in endosomal membranes. Storni *et al* suggested therefore that TLR 3 and 9 ligands should be taken up by the same DC as the antigen to exert their adjuvant effect [149]. Following this hypothesis, Joseph *et al* encapsulated CpG motifs in liposomes with influenza antigen, which on nasal administration in mice led to an increased anti-influenza IgG2a response, cellular responses (splenocyte proliferation, CTL response and IFN-γ production), and protection against influenza virus challenge [150]. This is likely due to enhanced liposomal delivery of CpG motifs to the endosomal compartment.

LPS, a major component of the outer membrane of Gram-negative bacteria, is a ligand for TLR 2 and TLR 4, and its adjuvant potential has been tested in various studies. Both Th1 [151, 152] and Th2 responses [153-156] have been found after nasal administration of LPS-

containing vaccines. These contrary results are not yet fully understood. Iwasaki and Metzhitov suggested that a lower dose of administered LPS corresponds to environmental antigens and induces Th2 responses and allergic inflammation, whereas a high dose corresponds to responses against infection and induces Th1 responses [37]. Monophosphoryl lipid A, a derivative of LPS and ligand for TLR 4, has similar adjuvant effects as LPS in nasal vaccines [151]. Increased mucosal sIgA and serum antibodies were found by using monophosphoryl lipid A as an adjuvant [151, 157] when compared to LPS [151].

Bacterial flagellins are ligands for TLR 5 and have been tested in nasal vaccines [158-160]. They induce mucosal and serum humoral responses. *Vibrio vulnificus* derived flagellin has thus far been the only flagellin tested as an adjuvant and induced mainly Th2 responses against model antigen tetanus toxoid [158]. Further research should clarify the potential of this group of TLR ligands as adjuvants for nasal vaccines.

Other PRR include the intracellular NOD1 and NOD2 proteins, scavenger receptors, macrophage mannose receptors and other C-type lectin receptors as well as type 3 complement receptors [37]. For instance, targeting of the C-type lectin, mannose receptor, on DC significantly increased antigen presentation on MHC II molecules [161]; [38].

Altogether, it seems that many TLR ligands, and possibly other PRR ligands, can act as adjuvant for nasal vaccines. However, the shift towards Th1 immune responses as observed with parenteral vaccinations seems to be less evident with nasal vaccination where balanced Th1/Th2 immune responses are mostly observed with these adjuvants. Further research in the immunological mechanisms involved in eliciting mucosal immune responses is necessary to understand the role these adjuvants can play in future (nasal) vaccines.

Toxin-based adjuvants. Enterotoxins like CT and LT are strong mucosal adjuvants that induce mucosal as well as serum antibody responses [41, 42]. In 1997 the first commercial nasal virosomal influenza vaccine adjuvanted with LT became available. Although the vaccine yielded a high percentage of protection, it was withdrawn from the market because its use was associated with an increased risk of developing Bell's palsy [162]. The cause of the Bell's palsy was linked to LT [163] and consequently LT and CT have no longer been used intensively in humans as an adjuvant for nasal vaccines. It has been reported that the coadministration of CT or LT redirects the antigen into the olfactory neuroepithelium, likely the cause of the neurological toxicity [164]. In an effort to make safe adjuvants based on CT and LT, several mutants of the toxins have been developed and tested [165]. Amino acid mutations in the ADP-ribosyltransferase domain in the A subunit from CT and LT resulted in effective nontoxic mutants [166-173].

Nasal application of vaccines adjuvanted with CT and its nontoxic mutants induces Th2 type immune responses as well as mucosal sIgA production, whereas the differentiation of Th1 cells is suppressed [174-177]. On the other hand, LT and some mutants like LT(K63) [42, 178] induce a more balanced Th1/Th2 response [179, 180]. Some mutants of LT, like LT(R72) induce a specific Th2 response [42, 178], while other LT mutants like induce a more Th1 polarized response [181]. A clear correlation between mutation and type of induced immune response has not been established. A construct of CT with a synthetic dimer of the D-fragment of *Staphylococcus aureus* protein A, which targets to B-cell Ig receptors, resulted in a strong nontoxic adjuvant that induced a balanced Th1/Th2 response against several tested antigens [182].

Cytokines and costimulatory molecules. Cytokines like IL-1 [183, 184], IL-12 [185-187], and type 1 IFN [188, 189] have been used as adjuvants for nasal vaccines to induce stronger and regulated Th1/Th2 immune responses [165]. Especially IFN type 1 and IL-12 are promising nasal adjuvants promoting Th1 type immune responses. Costimulatory signals are non-antigen specific signals delivered by activated APC to T-cells or by Th cells to B-cells. Several pathways can be exploited as target for adjuvants. CD28, CD40, CD134 and CD137 have been investigated as adjuvant targets [190]. Monoclonal antibodies that mimic the agonistic binding of costimulatory molecules have been tested as ligands for these CD molecules. Anti-CD40 monoclonal antibodies were coadministered as an adjuvant with a liposomal formulation of an influenza CTL epitope in subcutaneous and i.n. vaccination in mice. A decrease of lung viral titers after non-lethal challenge was observed after i.n. but not after subcutaneous vaccination or nasal vaccination without anti-CD40 [191].

Table III. Examples of nasal vaccination studies using Toll like receptor ligands.

Ligand	TLR	Formulation	Pathogen	Response*				Reference
				Mucosal sIgA	Serum Ab	CMI	Protection	
MALP-2	2-6	b-galactosidase with admixed MALP-2	none	Vaginal and bronchial IgA↑	IgG↑, IgG1^>IgG2a↑	IL-10↑ (Th2), IL-2 (Th1) only detectable with MALP-2	Not applicable	(Rharbaoui et al. 2002; Rharbaoui et al. 2004)
Poly (I:C)	3	Poly (I:C) + recombinant protective antigen (rPA), or capsular poly-γ-d-glutamic acid (PGA)–BSA conjugate	Anthrax *(Bacillus anthracis)*	Brochial sIgA ^	Balanced IgG1/IgG2a, but shift to Th1	Expression of CD80 and CD86 and TNF-α secretion in DC	Anthrax lethal toxin neutalizing activity *in vitro*	(Sloat and Cui 2006a,b)
Poly (I:C)	3	Poly (I:C) + Ethyl ether split virus	Influenza A virus	Nasal sIgA↑	IgG↑	Th1 and Th2 cytokines↑ (IFN-α,-β, -γ, IL-4, IL-6 and IL-12p40)	Cross-protection against nasal challenge	(Ichinohe et al. 2005)
LPS	4	Invaplex (LPS + Ipa proteins from *Shigella flexneri* 2a) admixed with OVA	none	Lung bronchial IgA↑	IgG1^ (Th2), no α-OVA IgG2a	Proliferation of CLN and spleen cells, Th2 cytokines IL-4, IL-5 and IL-10 produced	Not applicable	(Kaminski et al. 2006)
LPS	2-4	Proteoliposomes from *Neisseria meningitidis* B; cochleate structure with incorporated ovalbumin or *Leishmania* antigens	*Leishmania*, *Neisseria meningitidis*	IgA in saliva↑	α-PL IgG1^<IgG2a↑	MHCII ↑ in DC, IFNγ production in spleen cells	Smaller lesions after leishmania major infection	(Perez et al. 2004)
LPS	2-4	Liposomal OpaB and OpaJ formulations with admixed LPS	*Neisseria meningitidis*	Nasal sIgA↑	IgG2b↑>IgG2a^ (mixed Th1/Th2) LPS> Monophosphoryl lipid A	Not described	Bactericidal serum antibody titers	(de Jonge et al. 2004)
Monophosphoryl lipid A	4	Liposomal OpaB and OpaJ formulations with admixed LPS	*Neisseria meningitidis*	Nasal sIgA↑	IgG2b↑>IgG2a^ (mixed Th1/Th2)	Not described	Bactericidal serum antibody titers	(de Jonge et al. 2004)
flagellin from *Vibrio vulnificus*	5	Tetanus toxoid with admixed flagellin	*Clostridium tetani*	Nasal sIgA↑	IgG1>IgG2a	Increased TLR 5 expression in lymph nodes and spleen	Yes	(Lee et al. 2006)
CpG ODN	9	non-toxic CRM197 conjugated Hib–PRP with admixed CpG ODN	*Haemophilus influenzae* type b	A-PRP and α-CRM197 IgA in saliva and BAL	A-PRP and α-CRM179 IgG2a↑ and IgG3↑, shift to Th1	Not described	Not mentioned	(Mariotti et al. 2002)
CpG ODN	9	P6 outer membrane protein non-typable *Haemophilus influenzae (NTHI)* with admixed CpG ODN	Non-typeable *Haemophilus influenzae*	sIgA↑ in nasal wash (similar to CT)	IgG1^, IgG2a↑ balanced to Th1 (CT more IgG1)	IFNγ ↑in CD 8+ T-cells, IL-6↑ and IFNγ↑ in CD4+ T-cells	Increased clearance of NTHI nasal washes after challenge	(Abe et al. 2006; Kodama et al. 2006)
CpG ODN	9	Liposomal formulation of CpG ODN with admixed or co-encapsulated subunit vaccine	Influenza A virus	Nasal and bronchial IgA↑, coencapsulated >admixed	IgG1^ and IgG2a↑, more balanced IgG1/IgG2a than after i.m. (Th1)	IFNγ ↑(Th1), Spleen proliferation↑	Lung titers↓, lowest in co-encapsulated formulation	(Joseph et al. 2002)
CpG ODN	9	Formaldehyde inactivated influenza virus with admixed CpG 1826 or *E. coli* DNA	Influenza A virus	sIgA in saliva or vaginal washes ↔	Abs↑ at higher antigen dose, not significant for lower doses, *E. coli* DNA has stronger effect	Low proliferative responses from spleen of CLN isolated cells after *in vitro* restimulation	Not described	(Moldoveanu et al. 1998)
CpG ODN	9	Live Ankara virus vector with admixed CpG ODN	Vaccinia virus	Not described	No difference in virus neutralizing antibodies	CD8+ Tcell response ↑, CpG can substitute for CD4+ help	Protective dosage ↓, mediated primarily by CD8+ in lungs	(Belyakov et al. 2006)
CpG ODN	9	Whole cell sonicate with admixed CpG ODN	*Helicobacter pylori*	Not detected	IgG and IgG1 no difference, IgG2a↑(Th1)	IFNγ-producing CD4+ and CD8+ T-cells	Protection associated with Th1 and IFN-γ	(Shi et al. 2005)
CpG ODN	9	Recombinant M type 6 with admixed CPG ODN	*Streptococcus pyogenes*	IgA↑ in BAL	Total IgG↑, IgG1↑, IgG2a↑, IgG2b, Th1 type response CpG ODN< CT	Not described	Not described	(Teloni et al. 2004)
CpG ODN	9	Thelper-CTL epitope fusion peptides (pp65)	Cytomegalo-virus	Not described	Not described	CTL stimulation ↑	Not described	(La Rosa et al. 2002)

Conclusions

Although research and development of nasal vaccines has gained momentum over the last years, only one nasal vaccine is currently approved for human use, indicating that advances towards new effective vaccines have been slow, in particular for inactivated/subunit vaccines. However, the various attempts that have failed can teach us not to bet on one single horse. The opportunities in nasal vaccination are not in a single research field, but require the integration of immunology, biotechnology, microbiology and pharmaceutical sciences. Mechanistic insight into the hurdles that limit the efficacy of nasal vaccination will create opportunities for rationally designed nasal vaccines that can overcome these barriers. A concerted approach, combining various targeting techniques discussed in this paper, includes the use of particulate antigen carriers, which can be furnished with distinct functionalities such as mucoadhesive polymers, M-cell or DC targeting ligands, adjuvants and endosomal escape promoters. This could lead to “tailor made” vaccines that provide similar or even superior protection to diseases as provided by classical parental vaccines. The biggest challenge will be to combine these techniques in such a way that they do not interfere with one another, but synergistically enhance vaccine efficacy.

Acknowledgments

The authors thank Elly van Riet for critically reading the manuscript and her helpful suggestions, and Martijn Pieck for making the illustrations.

References

1. Rumke, H.C. and H.K. Visser, *[Childhood vaccinations anno 2004. I. Effectiveness and acceptance of the Dutch National Vaccination Programme]* Ned Tijdschr Geneeskd, 2004. **148**(8): p. 356-63.
2. Crockett, M. and J. Keystone, *"I hate needles" and other factors impacting on travel vaccine uptake* J Travel Med, 2005. **12 Suppl 1**: p. S41-6.
3. Bitsori, M., et al., *Vaccination coverage among adolescents in certain provinces of Greece* Acta Paediatr, 2005. **94**(8): p. 1122-5.
4. Kalies, H., et al., *Immunisation status of children in Germany: temporal trends and regional differences* Eur J Pediatr, 2006. **165**(1): p. 30-6.
5. Szucs, T.D. and D. Muller, *Influenza vaccination coverage rates in five European countries-a population-based cross-sectional analysis of two consecutive influenza seasons* Vaccine, 2005. **23**(43): p. 5055-63.
6. Hak, E., et al., *Negative attitude of highly educated parents and health care workers towards future vaccinations in the Dutch childhood vaccination program* Vaccine, 2005. **23**(24): p. 3103-7.
7. *President Requests 2.6 Percent NIH Budget Increase in 2005* 2005; Available from: http://www.nih.gov/news/NIH-Record/03_02_2004/story03.htm.
8. Enserink, M. *Radical Steps Urged to Help Underserved* 2000; Available from: http://www.sciencemag.org/cgi/content/full/288/5471/1563a?ck=nck.
9. Harrington, M. *To Double, or Not to Double? Bioterror Buys a Budget Boost* 2002; Available from: http://www.aidsinfonyc.org/tag/activism/bioterror.html.
10. Brandtzaeg, P., *History of oral tolerance and mucosal immunity* Ann N Y Acad Sci, 1996. **778**: p. 1-27.
11. Kyd, J.M., A.R. Foxwell, and A.W. Cripps, *Mucosal immunity in the lung and upper airway* Vaccine, 2001. **19**(17-19): p. 2527-33.
12. Neutra, M.R., A. Frey, and J.P. Kraehenbuhl, *Epithelial M cells: gateways for mucosal infection and immunization* Cell, 1996. **86**(3): p. 345-8.
13. van der Ven, I. and T. Sminia, *The development and structure of mouse nasal-associated lymphoid tissue: an immuno- and enzyme-histochemical study* Reg Immunol, 1993. **5**(2): p. 69-75.
14. Jeong, K.I., et al., *Ultrastructural study on the follicle-associated epithelium of nasal-associated lymphoid tissue in specific pathogen-free (SPF) and conventional environment-adapted (SPF-CV) rats* J Anat, 2000. **196 (Pt 3)**: p. 443-51.
15. Morag, A., et al., *In vitro correlates of cell-mediated immunity in human tonsils after natural or induced Rubella virus infection* J Infect Dis, 1975. **131**(4): p. 409-16.
16. Ogra, P.L., *Effect of tonsillectomy and adenoidectomy on nasopharyngeal antibody response to poliovirus* N Engl J Med, 1971. **284**(2): p. 59-64.
17. Zuercher, A.W., et al., *Nasal-associated lymphoid tissue is a mucosal inductive site for virus-specific humoral and cellular immune responses* J Immunol, 2002. **168**(4): p. 1796-803.
18. Shimoda, M., et al., *Isotype-specific selection of high affinity memory B cells in nasal-associated lymphoid tissue* J Exp Med, 2001. **194**(11): p. 1597-607.
19. Giudice, E.L. and J.D. Campbell, *Needle-free vaccine delivery* Adv Drug Deliv Rev, 2006. **58**(1): p. 68-89.
20. Diseases, I.f.V.R.N.V.a.I., *New Vaccines against Infectious Diseases: Research and Development Status* 2005.
21. Neutra, M.R., E. Pringault, and J.P. Kraehenbuhl, *Antigen sampling across epithelial barriers and induction of mucosal immune responses* Annu Rev Immunol, 1996. **14**: p. 275-300.

22. Watson, C.J., M. Rowland, and G. Warhurst, *Functional modeling of tight junctions in intestinal cell monolayers using polyethylene glycol oligomers* Am J Physiol Cell Physiol, 2001. **281**(2): p. C388-97.
23. Illum, L., *Nanoparticulate systems for nasal delivery of drugs: A real improvement over simple systems? .* J Pharm Sci, 2007. **96**(3): p. 473-83.
24. Fujimura, Y., et al., *The role of M cells of human nasopharyngeal lymphoid tissue in influenza virus sampling.* Virchows Arch, 2004. **444**(1): p. 36-42.
25. Fujimura, Y., *Evidence of M cells as portals of entry for antigens in the nasopharyngeal lymphoid tissue of humans* Virchows Arch, 2000. **436**(6): p. 560-6.
26. Park, H.S., et al., *Membranous cells in nasal-associated lymphoid tissue: a portal of entry for the respiratory mucosal pathogen group A streptococcus* J Immunol, 2003. **171**(5): p. 2532-7.
27. Gebert, A., *Identification of M-cells in the rabbit tonsil by vimentin immunohistochemistry and in vivo protein transport* Histochem Cell Biol, 1995. **104**(3): p. 211-20.
28. Gebert, A., B. Willfuhr, and R. Pabst, *The rabbit M-cell marker vimentin is present in epithelial cells of the tonsil crypt* Acta Otolaryngol, 1995. **115**(5): p. 697-700.
29. Styers, M.L., A.P. Kowalczyk, and V. Faundez, *Intermediate filaments and vesicular membrane traffic: the odd couple's first dance? .* Traffic, 2005. **6**(5): p. 359-65.
30. Brooking, J., S.S. Davis, and L. Illum, *Transport of nanoparticles across the rat nasal mucosa.* J Drug Target, 2001. **9**(4): p. 267-79.
31. Illum, L., *Nanoparticulate systems for nasal delivery of drugs: A real improvement over simple systems?* J Pharm Sci, 2006.
32. Cervi, L., et al., *Cutting edge: dendritic cells copulsed with microbial and helminth antigens undergo modified maturation, segregate the antigens to distinct intracellular compartments, and concurrently induce microbe-specific Th1 and helminth-specific Th2 responses.* J Immunol, 2004. **172**(4): p. 2016-20.
33. Ackerman, A.L. and P. Cresswell, *Cellular mechanisms governing cross-presentation of exogenous antigens.* Nat Immunol, 2004. **5**(7): p. 678-84.
34. Ackerman, A.L., et al., *Access of soluble antigens to the endoplasmic reticulum can explain cross-presentation by dendritic cells.* Nat Immunol, 2005. **6**(1): p. 107-13.
35. Burgdorf, S., et al., *Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation.* Science, 2007. **316**(5824): p. 612-6.
36. Blander, J.M. and R. Medzhitov, *On regulation of phagosome maturation and antigen presentation.* Nat Immunol, 2006. **7**(10): p. 1029-35.
37. Iwasaki, A. and R. Medzhitov, *Toll-like receptor control of the adaptive immune responses.* Nat Immunol, 2004. **5**(10): p. 987-95.
38. Pashine, A., N.M. Valiante, and J.B. Ulmer, *Targeting the innate immune response with improved vaccine adjuvants.* Nat Med, 2005. **11**(4 Suppl): p. S63-8.
39. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nat Rev Immunol, 2004. **4**(7): p. 499-511.
40. Wang, Q., et al., *A bacterial carbohydrate links innate and adaptive responses through Toll-like receptor 2.* J Exp Med, 2006. **203**(13): p. 2853-63.
41. Imaoka, K., et al., *Nasal immunization of nonhuman primates with simian immunodeficiency virus p55gag and cholera toxin adjuvant induces Th1/Th2 help for virus-specific immune responses in reproductive tissues.* J Immunol, 1998. **161**(11): p. 5952-8.

42. Ryan, E.J., et al., *Modulation of innate and acquired immune responses by Escherichia coli heat-labile toxin: distinct pro- and anti-inflammatory effects of the nontoxic AB complex and the enzyme activity.* J Immunol, 2000. **165**(10): p. 5750-9.
43. Arce, S., et al., *Differential binding of Escherichia coli enterotoxins LT-IIa and LT-IIb and of cholera toxin elicits differences in apoptosis, proliferation, and activation of lymphoid cells.* Infect Immun, 2005. **73**(5): p. 2718-27.
44. Yamamoto, M., et al., *Genetically manipulated bacterial toxin as a new generation mucosal adjuvant* Scand J Immunol, 2001. **53**(3): p. 211-7.
45. Schijns, V.E., *Immunological concepts of vaccine adjuvant activity.* Curr Opin Immunol, 2000. **12**(4): p. 456-63.
46. Smart, J.D., *The basics and underlying mechanisms of mucoadhesion.* Adv Drug Deliv Rev, 2005. **57**(11): p. 1556-68.
47. Bogataj, M., et al., *The correlation between zeta potential and mucoadhesion strength on pig vesical mucosa.* Biol Pharm Bull, 2003. **26**(5): p. 743-6.
48. Ugwoke, M.I., et al., *Nasal mucoadhesive drug delivery: background, applications, trends and future perspectives.* Adv Drug Deliv Rev, 2005. **57**(11): p. 1640-65.
49. Dodane, V., M. Amin Khan, and J.R. Merwin, *Effect of chitosan on epithelial permeability and structure* Int J Pharm, 1999. **182**(1): p. 21-32.
50. Bernkop-Schnurch, A., C.E. Kast, and M.F. Richter, *Improvement in the mucoadhesive properties of alginate by the covalent attachment of cysteine* J Control Release, 2001. **71**(3): p. 277-85.
51. Grabovac, V., D. Guggi, and A. Bernkop-Schnurch, *Comparison of the mucoadhesive properties of various polymers.* Adv Drug Deliv Rev, 2005. **57**(11): p. 1713-23.
52. Singh, M., M. Briones, and D.T. O'Hagan, *A novel bioadhesive intranasal delivery system for inactivated influenza vaccines* J Control Release, 2001. **70**(3): p. 267-76.
53. Jabbal-Gill, I., et al., *Stimulation of mucosal and systemic antibody responses against Bordetella pertussis filamentous haemagglutinin and recombinant pertussis toxin after nasal administration with chitosan in mice.* Vaccine, 1998. **16**(20): p. 2039-46.
54. Hilfenhaus, J., et al., *Herpes simplex virus subunit vaccine: characterization of the virus strain used and testing of the vaccine* Dev Biol Stand, 1982. **52**: p. 321-31.
55. Jabbal-Gill, I., et al., *Stimulation of mucosal and systemic antibody responses against Bordetella pertussis filamentous haemagglutinin and recombinant pertussis toxin after nasal administration with chitosan in mice* Vaccine, 1998. **16**(20): p. 2039-46.
56. McNeela, E.A., et al., *A mucosal vaccine against diphtheria: formulation of cross reacting material (CRM(197)) of diphtheria toxin with chitosan enhances local and systemic antibody and Th2 responses following nasal delivery* Vaccine, 2000. **19**(9-10): p. 1188-98.
57. Coeshott, C.M., et al., *Pluronic F127-based systemic vaccine delivery systems* Vaccine, 2004. **22**(19): p. 2396-405.
58. Read, R.C., et al., *Effective nasal influenza vaccine delivery using chitosan.* Vaccine, 2005. **23**(35): p. 4367-74.
59. Gogev, S., et al., *Glycol chitosan improves the efficacy of intranasally administrated replication defective human adenovirus type 5 expressing glycoprotein D of bovine herpesvirus 1* Vaccine, 2004. **22**(15-16): p. 1946-53.

60. Soane, R.J., et al., *Clearance characteristics of chitosan based formulations in the sheep nasal cavity* Int J Pharm, 2001. **217**(1-2): p. 183-91.
61. Koping-Hoggard, M., A. Sanchez, and M.J. Alonso, *Nanoparticles as carriers for nasal vaccine delivery* Expert Rev Vaccines, 2005. **4**(2): p. 185-96.
62. Shephard, M.J., et al., *Immunogenicity of bovine parainfluenza type 3 virus proteins encapsulated in nanoparticle vaccines, following intranasal administration to mice* Res Vet Sci, 2003. **74**(2): p. 187-90.
63. Feng, L., et al., *Pharmaceutical and immunological evaluation of a single-dose hepatitis B vaccine using PLGA microspheres* J Control Release, 2006. **112**(1): p. 35-42.
64. Kang, M.L., et al., *In vivo induction of mucosal immune responses by intranasal administration of chitosan microspheres containing Bordetella bronchiseptica DNT* Eur J Pharm Biopharm, 2006. **63**(2): p. 215-20.
65. Moore, A., et al., *Immunization with a soluble recombinant HIV protein entrapped in biodegradable microparticles induces HIV-specific CD8+ cytotoxic T lymphocytes and CD4+ Th1 cells* Vaccine, 1995. **13**(18): p. 1741-9.
66. Higaki, M., et al., *Enhancement of immune response to intranasal influenza HA vaccine by microparticle resin.* Vaccine, 1998. **16**(7): p. 741-5.
67. Brooking, J., S.S. Davis, and L. Illum, *Transport of nanoparticles across the rat nasal mucosa* J Drug Target, 2001. **9**(4): p. 267-79.
68. de Haan, A., et al., *Liposomes as an immunoadjuvant system for stimulation of mucosal and systemic antibody responses against inactivated measles virus administered intranasally to mice* Vaccine, 1995. **13**(14): p. 1320-4.
69. Baca-Estrada, M.E., et al., *Intranasal immunization with liposome-formulated Yersinia pestis vaccine enhances mucosal immune responses* Vaccine, 2000. **18**(21): p. 2203-11.
70. Joseph, A., et al., *A new intranasal influenza vaccine based on a novel polycationic lipid--ceramide carbamoyl-spermine (CCS) I. Immunogenicity and efficacy studies in mice*. Vaccine, 2006. **24**(18): p. 3990-4006.
71. Nagamoto, T., et al., *Novel chitosan particles and chitosan-coated emulsions inducing immune response via intranasal vaccine delivery* Pharm Res, 2004. **21**(4): p. 671-4.
72. Amidi, M., et al., *N-Trimethyl chitosan (TMC) nanoparticles loaded with influenza subunit antigen for intranasal vaccination: Biological properties and immunogenicity in a mouse model* Vaccine, 2007. **25**(1): p. 144-53.
73. Jaganathan, K.S. and S.P. Vyas, *Strong systemic and mucosal immune responses to surface-modified PLGA microspheres containing recombinant hepatitis B antigen administered intranasally* Vaccine, 2006. **24**(19): p. 4201-11.
74. Vila, A., et al., *Design of biodegradable particles for protein delivery* J Control Release, 2002. **78**(1-3): p. 15-24.
75. Shakweh, M., et al., *Poly (lactide-co-glycolide) particles of different physicochemical properties and their uptake by peyer's patches in mice* Eur J Pharm Biopharm, 2005. **61**(1-2): p. 1-13.
76. Matsunaga, Y., et al., *Oral immunization with size-purified microsphere beads as a vehicle selectively induces systemic tolerance and sensitization* Vaccine, 2000. **19**(4-5): p. 579-88.
77. Xiang, S.D., et al., *Pathogen recognition and development of particulate vaccines: does size matter? .* Methods, 2006. **40**(1): p. 1-9.

78. Vila, A., et al., *PLA-PEG particles as nasal protein carriers: the influence of the particle size* Int J Pharm, 2005. **292**(1-2): p. 43-52.
79. Jung, T., et al., *Tetanus toxoid loaded nanoparticles from sulfobutylated poly(vinyl alcohol)-graft-poly(lactide-co-glycolide): evaluation of antibody response after oral and nasal application in mice* Pharm Res, 2001. **18**(3): p. 352-60.
80. Sandri, G., et al., *Nanoparticles based on N-trimethylchitosan: evaluation of absorption properties using in vitro (Caco-2 cells) and ex vivo (excised rat jejunum) models* Eur J Pharm Biopharm, 2007. **65**(1): p. 68-77.
81. Fujimura, Y., et al., *Uptake of microparticles into the epithelium of human nasopharyngeal lymphoid tissue* Med Mol Morphol, 2006. **39**(4): p. 181-6.
82. Baca-Estrada, M.E., M. Foldvari, and M. Snider, *Induction of mucosal immune responses by administration of liposome-antigen formulations and interleukin-12* J Interferon Cytokine Res, 1999. **19**(5): p. 455-62.
83. Tafaghodi, M., S.A. Sajadi Tabassi, and M.R. Jaafari, *Induction of systemic and mucosal immune responses by intranasal administration of alginate microspheres encapsulated with tetanus toxoid and CpG-ODN* Int J Pharm, 2006. **319**(1-2): p. 37-43.
84. Oliveira, M.L., et al., *Induction of systemic and mucosal immune response and decrease in Streptococcus pneumoniae colonization by nasal inoculation of mice with recombinant lactic acid bacteria expressing pneumococcal surface antigen A* Microbes Infect, 2006. **8**(4): p. 1016-24.
85. Audouy, S.A., et al., *Development of lactococcal GEM-based pneumococcal vaccines* Vaccine, 2006.
86. Huckriede, A., et al., *The virosome concept for influenza vaccines.* Vaccine, 2005. **23 Suppl 1**: p. S26-38.
87. Bron, R., A. Ortiz, and J. Wilschut, *Cellular cytoplasmic delivery of a polypeptide toxin by reconstituted influenza virus envelopes (virosomes)* Biochemistry, 1994. **33**(31): p. 9110-7.
88. Cusi, M.G., *Applications of influenza virosomes as a delivery system.* Hum Vaccin, 2006. **2**(1): p. 1-7.
89. Cusi, M.G., et al., *Influenza virosomes are an efficient delivery system for respiratory syncytial virus-F antigen inducing humoral and cell-mediated immunity.* Vaccine, 2002. **20**(29-30): p. 3436-42.
90. Cusi, M.G., *The response to plasmid DNA-virosome vaccination: a role for circulating antibodies?* Trends Immunol, 2001. **22**(7): p. 355.
91. Owen, R.L. and D.K. Bhalla, *Cytochemical analysis of alkaline phosphatase and esterase activities and of lectin-binding and anionic sites in rat and mouse Peyer's patch M cells* Am J Anat, 1983. **168**(2): p. 199-212.
92. Hultgren, S.J., et al., *Pilus and nonpilus bacterial adhesins: assembly and function in cell recognition* Cell, 1993. **73**(5): p. 887-901.
93. Sharma, R., et al., *Lectin binding reveals divergent carbohydrate expression in human and mouse Peyer's patches* Histochem Cell Biol, 1996. **105**(6): p. 459-65.
94. Clark, M.A., M.A. Jepson, and B.H. Hirst, *Lectin binding defines and differentiates M-cells in mouse small intestine and caecum* Histochem Cell Biol, 1995. **104**(2): p. 161-8.
95. Lee, Y.S., *Lectin reactivity in human large bowel* Pathology, 1987. **19**(4): p. 397-401.
96. Foster, N., et al., *Ulex europaeus 1 lectin targets microspheres to mouse Peyer's patch M-cells in vivo* Vaccine, 1998. **16**(5): p. 536-41.
97. Carreno-Gomez, B., J.F. Woodley, and A.T. Florence, *Studies on the uptake of tomato lectin nanoparticles in everted gut sacs* Int J Pharm, 1999. **183**(1): p. 7-11.
98. Hussain, N., P.U. Jani, and A.T. Florence, *Enhanced oral uptake of tomato lectin-conjugated nanoparticles in the rat* Pharm Res, 1997. **14**(5): p. 613-8.

99. Russell-Jones, G.J., H. Veitch, and L. Arthur, *Lectin-mediated transport of nanoparticles across Caco-2 and OK cells* Int J Pharm, 1999. **190**(2): p. 165-74.
100. Takata, S., O. Ohtani, and Y. Watanabe, *Lectin binding patterns in rat nasal-associated lymphoid tissue (NALT) and the influence of various types of lectin on particle uptake in NALT* Arch Histol Cytol, 2000. **63**(4): p. 305-12.
101. Wang, X., et al., *Transgene vaccination using Ulex europaeus agglutinin I (UEA-1) for targeted mucosal immunization against HIV-1 envelope.* Vaccine, 2005. **23**(29): p. 3836-42.
102. Giannasca, P.J., J.A. Boden, and T.P. Monath, *Targeted delivery of antigen to hamster nasal lymphoid tissue with M-cell-directed lectins* Infect Immun, 1997. **65**(10): p. 4288-98.
103. Rogers, G.N. and J.C. Paulson, *Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin* Virology, 1983. **127**(2): p. 361-73.
104. Tong, H.H., et al., *Effect of neuraminidase on receptor-mediated adherence of Streptococcus pneumoniae to chinchilla tracheal epithelium* Acta Otolaryngol, 2002. **122**(4): p. 413-9.
105. Corthesy, B., *Roundtrip ticket for secretory IgA: role in mucosal homeostasis?* J Immunol, 2007. **178**(1): p. 27-32.
106. Favre, L., F. Spertini, and B. Corthesy, *Secretory IgA possesses intrinsic modulatory properties stimulating mucosal and systemic immune responses.* J Immunol, 2005. **175**(5): p. 2793-800.
107. Tiwari, B., et al., *Immunoglobulin immobilized liposomal constructs for transmucosal vaccination through nasal route.* J Liposome Res, 2010.
108. Tyrer, P., et al., *Microbial pattern recognition receptors mediate M-cell uptake of a gram-negative bacterium* Infect Immun, 2006. **74**(1): p. 625-31.
109. Clark, M.A., B.H. Hirst, and M.A. Jepson, *M-cell surface beta1 integrin expression and invasin-mediated targeting of Yersinia pseudotuberculosis to mouse Peyer's patch M cells* Infect Immun, 1998. **66**(3): p. 1237-43.
110. Frankel, G., et al., *The cell-binding domain of intimin from enteropathogenic Escherichia coli binds to beta1 integrins* J Biol Chem, 1996. **271**(34): p. 20359-64.
111. Cruz, N., et al., *Role of mucin, mannose, and beta-1 integrin receptors in Escherichia coli translocation across Caco-2 cell monolayers* Shock, 1994. **2**(2): p. 121-6.
112. Gullberg, E., et al., *Identification of cell adhesion molecules in the human follicle-associated epithelium that improve nanoparticle uptake into the Peyer's patches.* J Pharmacol Exp Ther, 2006. **319**(2): p. 632-9.
113. Hicks, W., Jr., et al., *Isolation and characterization of basal cells from human upper respiratory epithelium* Exp Cell Res, 1997. **237**(2): p. 357-63.
114. Foged, C., A. Sundblad, and L. Hovgaard, *Targeting vaccines to dendritic cells.* Pharm Res, 2002. **19**(3): p. 229-38.
115. Rock, K.L. and K. Clark, *Analysis of the role of MHC class II presentation in the stimulation of cytotoxic T lymphocytes by antigens targeted into the exogenous antigen-MHC class I presentation pathway.* J Immunol, 1996. **156**(10): p. 3721-6.
116. Harding, C.V. and R. Song, *Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class I MHC molecules.* J Immunol, 1994. **153**(11): p. 4925-33.
117. Lenarczyk, A., et al., *ISCOM based vaccines for cancer immunotherapy.* Vaccine, 2004. **22**(8): p. 963-74.

118. Malliaros, J., et al., *Association of antigens to ISCOMATRIX adjuvant using metal chelation leads to improved CTL responses.* Vaccine, 2004. **22**(29-30): p. 3968-75.
119. Stewart, T.J., et al., *ISCOMATRIX adjuvant: an adjuvant suitable for use in anticancer vaccines*, in *Vaccine*. 2004. p. 3738-43.
120. Pearse, M.J. and D. Drane, *ISCOMATRIX adjuvant for antigen delivery.* Adv Drug Deliv Rev, 2005. **57**(3): p. 465-74.
121. Sanders, M.T., et al., *ISCOM-based vaccines: the second decade.* Immunol Cell Biol, 2005. **83**(2): p. 119-28.
122. Daemen, T., et al., *Virosomes for antigen and DNA delivery.* Adv Drug Deliv Rev, 2005. **57**(3): p. 451-63.
123. Bungener, L., et al., *Virosome-mediated delivery of protein antigens in vivo: efficient induction of class I MHC-restricted cytotoxic T lymphocyte activity.* Vaccine, 2005. **23**(10): p. 1232-41.
124. Huckriede, A., et al., *Influenza virosomes in vaccine development.* Methods Enzymol, 2003. **373**: p. 74-91.
125. Lambkin, R., et al., *Strong local and systemic protective immunity induced in the ferret model by an intranasal virosome-formulated influenza subunit vaccine.* Vaccine, 2004. **22**(31-32): p. 4390-6.
126. Durrer, P., et al., *Mucosal antibody response induced with a nasal virosome-based influenza vaccine.* Vaccine, 2003. **21**(27-30): p. 4328-34.
127. Schumacher, R., et al., *Influenza virosomes enhance class I restricted CTL induction through CD4+ T cell activation.* Vaccine, 2004. **22**(5-6): p. 714-23.
128. Amacker, M., et al., *Peptide-loaded chimeric influenza virosomes for efficient in vivo induction of cytotoxic T cells.* Int Immunol, 2005. **17**(6): p. 695-704.
129. Schwaninger, R., et al., *Virosomes as new carrier system for cancer vaccines.* Cancer Immunol Immunother, 2004. **53**(11): p. 1005-17.
130. Glueck, R., *Review of intranasal influenza vaccine.* Adv Drug Deliv Rev, 2001. **51**(1-3): p. 203-11.
131. Gluck, U., J.O. Gebbers, and R. Gluck, *Phase 1 evaluation of intranasal virosomal influenza vaccine with and without Escherichia coli heat-labile toxin in adult volunteers.* J Virol, 1999. **73**(9): p. 7780-6.
132. de, B., et al., *An open-label comparison of the immunogenicity and tolerability of intranasal and intramuscular formulations of virosomal influenza vaccine in healthy adults.* Clin Ther, 2002. **24**(1): p. 100-11.
133. Cusi, M.G., et al., *Immunopotentiating of mucosal and systemic antibody responses in mice by intranasal immunization with HLT-combined influenza virosomal vaccine.* Vaccine, 2000. **18**(25): p. 2838-42.
134. Kwon, Y.J., et al., *Directed antigen presentation using polymeric microparticulate carriers degradable at lysosomal pH for controlled immune responses.* Mol Pharm, 2005. **2**(1): p. 83-91.
135. Partidos, C.D., P. Vohra, and M.W. Steward, *Induction of measles virus-specific cytotoxic T-cell responses after intranasal immunization with synthetic peptides.* Immunology, 1996. **87**(2): p. 179-85.
136. Partidos, C.D., et al., *CTL responses induced by a single immunization with peptide encapsulated in biodegradable microparticles.* J Immunol Methods, 1997. **206**(1-2): p. 143-51.
137. Partidos, C.D., et al., *Induction of cytotoxic T-cell responses following oral immunization with synthetic peptides encapsulated in PLG microparticles.* J Control Release, 1999. **62**(3): p. 325-32.
138. Partidos, C.D., et al., *Biodegradable microparticles as a delivery system for measles virus cytotoxic T cell epitopes.* Mol Immunol, 1996. **33**(6): p. 485-91.
139. Nixon, D.F., et al., *Synthetic peptides entrapped in microparticles can elicit cytotoxic T cell activity.* Vaccine, 1996. **14**(16): p. 1523-30.

140. Shen, H., et al., *Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles.* Immunology, 2006. **117**(1): p. 78-88.
141. Kwon, Y.J., et al., *Enhanced antigen presentation and immunostimulation of dendritic cells using acid-degradable cationic nanoparticles.* J Control Release, 2005. **105**(3): p. 199-212.
142. Kwon, Y.J., et al., *In vivo targeting of dendritic cells for activation of cellular immunity using vaccine carriers based on pH-responsive microparticles.* Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18264-8.
143. Abe, N., et al., *Nasal vaccination with CpG oligodeoxynucleotide induces protective immunity against non-typeable Haemophilus influenzae in the nasopharynx* Laryngoscope, 2006. **116**(3): p. 407-12.
144. Kodama, S., et al., *Safety and efficacy of nasal application of CpG oligodeoxynucleotide as a mucosal adjuvant* Laryngoscope, 2006. **116**(2): p. 331-5.
145. Joseph, A., et al., *Liposomal immunostimulatory DNA sequence (ISS-ODN): an efficient parenteral and mucosal adjuvant for influenza and hepatitis B vaccines* Vaccine, 2002. **20**(27-28): p. 3342-54.
146. Ichinohe, T., et al., *Synthetic double-stranded RNA poly(I:C) combined with mucosal vaccine protects against influenza virus infection.* J Virol, 2005. **79**(5): p. 2910-9.
147. Sloat, B.R. and Z. Cui, *Nasal immunization with a dual antigen anthrax vaccine induced strong mucosal and systemic immune responses against toxins and bacilli.* Vaccine, 2006. **24**(40-41): p. 6405-13.
148. Sloat, B.R. and Z. Cui, *Nasal immunization with anthrax protective antigen protein adjuvanted with polyriboinosinic-polyribocytidylic acid induced strong mucosal and systemic immunities.* Pharm Res, 2006. **23**(6): p. 1217-26.
149. Storni, T., et al., *Immunity in response to particulate antigen-delivery systems.* Adv Drug Deliv Rev, 2005. **57**(3): p. 333-55.
150. Joseph, A., et al., *Liposomal immunostimulatory DNA sequence (ISS-ODN): an efficient parenteral and mucosal adjuvant for influenza and hepatitis B vaccines.* Vaccine, 2002. **20**(27-28): p. 3342-54.
151. de Jonge, M.I., et al., *Intranasal immunisation of mice with liposomes containing recombinant meningococcal OpaB and OpaJ proteins.* Vaccine, 2004. **22**(29-30): p. 4021-8.
152. Perez, O., et al., *Novel adjuvant based on a proteoliposome-derived cochleate structure containing native lipopolysaccharide as a pathogen-associated molecular pattern.* Immunol Cell Biol, 2004. **82**(6): p. 603-10.
153. Redecke, V., et al., *Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma.* J Immunol, 2004. **172**(5): p. 2739-43.
154. Pulendran, B., et al., *Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo.* J Immunol, 2001. **167**(9): p. 5067-76.
155. Agrawal, S., et al., *Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos.* J Immunol, 2003. **171**(10): p. 4984-9.
156. Kaminski, R.W., K.R. Turbyfill, and E.V. Oaks, *Mucosal adjuvant properties of the Shigella invasin complex.* Infect Immun, 2006. **74**(5): p. 2856-66.
157. Childers, et al., *Adjuvant activity of monophosphoryl lipid A for nasal and oral immunization with soluble or liposome-associated antigen.* Infect Immun, 2000. **68**(10): p. 5509-16.
158. Lee, S.E., et al., *A bacterial flagellin, Vibrio vulnificus FlaB, has a strong mucosal adjuvant activity to induce protective immunity.* Infect Immun, 2006. **74**(1): p. 694-702.
159. Strindelius, L., M. Filler, and I. Sjoholm, *Mucosal immunization with purified flagellin from Salmonella induces systemic and mucosal immune responses in C3H/HeJ mice*. Vaccine, 2004. **22**(27-28): p. 3797-808.

160. Sbrogio-Almeida, M.E. and L.C. Ferreira, *Flagellin expressed by live Salmonella vaccine strains induces distinct antibody responses following delivery via systemic or mucosal immunization routes.* FEMS Immunol Med Microbiol, 2001. **30**(3): p. 203-8.
161. Lanzavecchia, A., *Mechanisms of antigen uptake for presentation* Curr Opin Immunol, 1996. **8**(3): p. 348-54.
162. Mutsch, M., et al., *Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland.* N Engl J Med, 2004. **350**(9): p. 896-903.
163. Couch, R.B., *Nasal vaccination, Escherichia coli enterotoxin, and Bell's palsy.* N Engl J Med, 2004. **350**(9): p. 860-1.
164. van Ginkel, F.W., et al., *Enterotoxin-based mucosal adjuvants alter antigen trafficking and induce inflammatory responses in the nasal tract.* Infect Immun, 2005. **73**(10): p. 6892-902.
165. Yuki, Y. and H. Kiyono, *New generation of mucosal adjuvants for the induction of protective immunity.* Rev Med Virol, 2003. **13**(5): p. 293-310.
166. Douce, G., et al., *Mucosal immunogenicity of genetically detoxified derivatives of heat labile toxin from Escherichia coli.* Vaccine, 1998. **16**(11-12): p. 1065-73.
167. Takahashi, H., et al., *Mutant Escherichia coli enterotoxin as a mucosal adjuvant induces specific Th1 responses of CD4+ and CD8+ T cells to nasal killed-bacillus calmette-guerin in mice.* Vaccine, 2006. **24**(17): p. 3591-8.
168. Kende, M., et al., *Enhancement of intranasal vaccination in mice with deglycosylated chain A ricin by LTR72, a novel mucosal adjuvant.* Vaccine, 2006. **24**(12): p. 2213-21.
169. Bowe, F., et al., *Mucosal vaccination against serogroup B meningococci: induction of bactericidal antibodies and cellular immunity following intranasal immunization with NadA of Neisseria meningitidis and mutants of Escherichia coli heat-labile enterotoxin.* Infect Immun, 2004. **72**(7): p. 4052-60.
170. Yamamoto, M., et al., *A nontoxic adjuvant for mucosal immunity to pneumococcal surface protein A.* J Immunol, 1998. **161**(8): p. 4115-21.
171. Yamamoto, M., et al., *Direct effects on antigen-presenting cells and T lymphocytes explain the adjuvanticity of a nontoxic cholera toxin mutant.* J Immunol, 1999. **162**(12): p. 7015-21.
172. Yamamoto, S., et al., *A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity.* Proc Natl Acad Sci U S A, 1997. **94**(10): p. 5267-72.
173. Pine, S., et al., *Intranasal immunization with influenza vaccine and a detoxified mutant of heat labile enterotoxin from Escherichia coli.* J Control Release, 2002. **85**(1-3): p. 263-70.
174. Ohmura, M., et al., *Highly purified mutant E112K of cholera toxin elicits protective lung mucosal immunity to diphtheria toxin.* Vaccine, 2001. **20**(5-6): p. 756-62.
175. Periwal, S.B., et al., *A modified cholera holotoxin CT-E29H enhances systemic and mucosal immune responses to recombinant Norwalk virus-virus like particle vaccine.* Vaccine, 2003. **21**(5-6): p. 376-85.
176. Yamamoto, M., et al., *Genetically manipulated bacterial toxin as a new generation mucosal adjuvant.* Scand J Immunol, 2001. **53**(3): p. 211-7.
177. Braun, M.C., et al., *Cholera toxin suppresses interleukin (IL)-12 production and IL-12 receptor beta1 and beta2 chain expression.* J Exp Med, 1999. **189**(3): p. 541-52.
178. Ryan, E.J., et al., *Mutants of Escherichia coli heat-labile toxin act as effective mucosal adjuvants for nasal delivery of an acellular pertussis vaccine: differential effects of the nontoxic AB complex and enzyme activity on Th1 and Th2 cells.* Infect Immun, 1999. **67**(12): p. 6270-80.

179. Takahashi, I., et al., *Mechanisms for mucosal immunogenicity and adjuvancy of Escherichia coli labile enterotoxin.* J Infect Dis, 1996. **173**(3): p. 627-35.
180. Yamamoto, M., et al., *Enterotoxin adjuvants have direct effects on T cells and antigen-presenting cells that result in either interleukin-4-dependent or -independent immune responses.* J Infect Dis, 2000. **182**(1): p. 180-90.
181. Takahashi, H., et al., *Mutant Escherichia coli enterotoxin as a mucosal adjuvant induces specific Th1 responses of CD4+ and CD8+ T cells to nasal killed-bacillus calmette-guerin in mice* Vaccine, 2006. **24**(17): p. 3591-8.
182. Lycke, N., *Targeted vaccine adjuvants based on modified cholera toxin.* Curr Mol Med, 2005. **5**(6): p. 591-7.
183. Egan, M.A., et al., *A comparative evaluation of nasal and parenteral vaccine adjuvants to elicit systemic and mucosal HIV-1 peptide-specific humoral immune responses in cynomolgus macaques.* Vaccine, 2004. **22**(27-28): p. 3774-88.
184. Staats, H.F. and F.A. Ennis, Jr., *IL-1 is an effective adjuvant for mucosal and systemic immune responses when coadministered with protein immunogens.* J Immunol, 1999. **162**(10): p. 6141-7.
185. Boyaka, P.N., et al., *IL-12 is an effective adjuvant for induction of mucosal immunity.* J Immunol, 1999. **162**(1): p. 122-8.
186. Marinaro, M., et al., *Use of intranasal IL-12 to target predominantly Th1 responses to nasal and Th2 responses to oral vaccines given with cholera toxin.* J Immunol, 1999. **162**(1): p. 114-21.
187. Boyaka, P.N., J.W. Lillard, Jr., and J. McGhee, *Interleukin 12 and innate molecules for enhanced mucosal immunity.* Immunol Res, 1999. **20**(3): p. 207-17.
188. Bracci, L., et al., *Type I IFN is a powerful mucosal adjuvant for a selective intranasal vaccination against influenza virus in mice and affects antigen capture at mucosal level.* Vaccine, 2005. **23**(23): p. 2994-3004.
189. Proietti, E., et al., *Type I IFN as a natural adjuvant for a protective immune response: lessons from the influenza vaccine model.* J Immunol, 2002. **169**(1): p. 375-83.
190. Barr, T.A., J. Carlring, and A.W. Heath, *Co-stimulatory agonists as immunological adjuvants.* Vaccine, 2006. **24**(17): p. 3399-407.
191. Ninomiya, A., et al., *Intranasal administration of a synthetic peptide vaccine encapsulated in liposome together with an anti-CD40 antibody induces protective immunity against influenza A virus in mice.* Vaccine, 2002. **20**(25-26): p. 3123-9.

3

Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination

Bram Slütter
Virgine Fievez
Anne des Rieux
Elly Van Riet
Véronique Préat
Laurence Plapied
Maria Alonso-Sandel
Yves-Jacques Schneider
Wim Jiskoot

Journal of Controlled Release 2009 138(2):113-21.

Abstract

For oral vaccination, incorporation of antigens into nanoparticles has been shown to protect the antigen from degradation, but may also increase its uptake through the intestinal epithelium via M-cells. The aim of this study was to understand the mechanisms by which oral administration of antigen-loaded nanoparticles induces an immune response and to analyze the effect of the nanoparticle composition on these mechanisms. Nanoparticles made from chitosan (CS) and its N-trimethylated derivate, TMC, loaded with a model antigen ovalbumin (OVA) were prepared by ionic gelation with tripolyphosphate. Intraduodenal vaccination with OVA-loaded nanoparticles led to significantly higher antibodies responses than immunization with OVA alone. TMC nanoparticles induced anti-OVA antibodies after only a priming dose. To explain these results, the interaction of nanoparticles with the intestinal epithelium was explored, *in vitro*, using a follicle associated epithelium model and visualized, *ex vivo*, using confocal laser scanning microscopy. The transport of OVA-FITC-loaded TMC nanoparticles by Caco-2 cells or FAE model was higher than OVA-FITC-loaded chitosan or PLGA nanoparticles. The association of nanoparticles with human monocyte derived dendritic cells and their effect on their maturation were determined with flow cytometry. TMC nanoparticles but not chitosan or PLGA nanoparticles had intrinsic adjuvant effect on DCs. In conclusion, depending on their composition, nanoparticles can increase the M-cell dependent uptake and enhance the association of the antigen with DC. In this respect, TMC nanoparticles are a promising strategy for oral vaccination.

Introduction

Whereas most pathogens gain access to their hosts via mucosal surfaces, most human vaccines currently available are licensed for non-mucosal administration e.g. via subcutaneous or intramuscular injections. However, mucosal vaccines have several attractive features compared with parenteral vaccines. Mucosal immune responses are most efficiently induced by administration of vaccines onto mucosal surfaces[1]. Moreover, mucosal immunization is needle-free, patient-friendly and reduces the risk of infection. Nonetheless, if mucosal vaccination is to become a feasible alternative for parenteral immunization, there are still hurdles to overcome before such an approach can be used widely. Oral vaccine delivery raises particular challenges: the bioavailability of orally delivered antigen is limited by possible degradation in the gastrointestinal environment. Moreover, most antigens are bulky substances and therefore not easily absorbed into the intestinal epithelium. Finally, the intestine is a fairly immuno-tolerant site, and the default response to an antigen will often be tolerance instead of immunity[2,3].

To facilitate effective mucosal immunization, the antigen must be protected from degradation, its uptake/absorption enhanced and immune cells activated. Therefore, oral vaccines should ideally be multimeric/particulate, adherent to the intestinal surface, effectively target M-cells and efficiently stimulate innate and adaptive immune responses [1]. Polymeric nanoparticles in which the antigen is encapsulated have been designed for oral immunization [4-6]. Various studies have shown increased antibody responses when antigens are orally administered in poly-(lactic-co-glycolic acid) (PLGA) particles [7-10]. Particles composed of bioadhesive material like chitosan (CS) and its soluble derivate N-trimethyl chitosan (TMC) characterised by its permanent positive charges irrespective of pH, can prolong the residence time of the antigen in the intestine, increase its permeation and enhance its immunogenicity [11,12,13,14,15]. Although most of the polymers used allow protection of the antigen from degradation, their effects on interaction with intestinal surface and on antigen uptake is less well recorded and depends on the type of polymer used [4]. What happens to an encapsulated antigen once it has reached the intestine is also not straightforward. M-cells in the follicle-associated epithelium (FAE) of intestinal Peyer's patches and isolated lymphoid follicles are gatekeepers of the mucosal immune system. They sample the gut lumen and transport antigens in the underlying mucosal lymphoid tissue for processing and initiation of an immune response [16]. Given their unique features to transcytose particles, M-cells are an interesting target in oral vaccine delivery. It has been shown that nanoparticles are actively taken up by the FAE through M-cells [5,12,16,17].

Moreover, M-cells have been suggested to transport antigen to underlying dendritic cells (DCs), without any degradation of the antigen, even without a protective carrier [18]. In vitro models of human FAE that have been recently developed are useful to study the contribution of M-cells to the transport of nanoparticles [5,17]

Interestingly, nanoparticles are also reported to increase the antigen uptake by DCs and to induce the maturation of DCs [19,20]. The use of a nanoparticulate delivery system might therefore work as a double edged sword as it increases the uptake into epithelium and subsequently the uptake into antigen presenting cells (APCs).

The aim of this study was to understand the mechanisms by which nanoparticles can enhance immune responses after oral administration apart from protection from degradation. Ovalbumin (OVA) was used as a model antigen and encapsulated with CS and TMC polymers to form nanoparticles. CS particles have been described as potential oral vaccine carrier [12] and transport of these particles by M-cells has been observed [21]. A drawback of CS is its water solubility. With CS' pK_a of 6.2, at physiological pH primary amines groups are protonated and consequently OVA/CS nanoparticles will loose their repulsive surface charge and show colloidal instability. The slightly acid environment of the jejunum will promote the stability of CS nanoparticles, but as soon as these particles are transported to the subepithelial space to interact with immune cells, the physiological pH will be deleterious for its stability. As TMC carries a permanent positive charge, OVA/TMC nanoparticles will not be affected by small pH shifts and may be a more suitable carrier for mucosal vaccination.

An intraduodenal immunization study with OVA/CS particles, OVA/TMC and unencapsulated OVA nanoparticles was performed to analyse the extent and the type of immune response elicited [22]. To explore whether transport into the FAE and interaction with DCs are indeed factors that contribute to the increased immune response caused by CS and TMC nanoparticles, first the interaction of these vaccine carriers with the enterocytes and FAE was investigated *in vivo* and *in vitro* [5,8], allowing a direct comparison and quantification of the transepithelial transport of CS and TMC nanoparticles. Secondly, the effect of nanoparticle uptake by DCs and on the maturation of DCs was assessed. Negatively charged PLGA nanoparticles of comparable size as OVA/CS and TMC/OVA were included to investigate the effect of nanoparticle composition on M-cell transport and DC interaction.

Materials and Methods

Materials

N-trimethyl chitosan with a degree of quaternisation of 15% was obtained from 92% deacetylated (MW 120 kDa) chitosan (Primex, Avaldsnes, N), by NaOH induced methylation as described by Sieval [6]. Poly(lactic-co-glycolic acid) 50:50 (PLGA) (L:G 50:50 average M_w 5,000-15,000) pentasodium tripolyphosphate (TPP), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), dichloromethane, dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ovalbumin (OVA) grade V and sodium cholate were obtained from Sigma-Aldrich (Steinheim, DE). FITC-ovalbumin (FITC-OVA) was purchased from Molecular Probes (Invitrogen Breda, NL). All culture media, including penicillin/streptomycin (PEST) and trypsin were obtained from Gibco (Invitrogen, Carlsbad, CA), unless indicated otherwise.

Nanoparticle preparation

CS and TMC nanoparticles were prepared by ionic complexation with pentasodium tripolyphosphate (TPP) [23]. CS and OVA were dissolved in a 0.1 M acetate buffer (pH 5) to a final concentration of respectively 1 mg/ml and 0.1 mg/ml. A TPP solution (1 mg/ml) was added under continuous stirring to weight ratio CS:TPP:OVA of 10:1.2:1. TMC and OVA were dissolved in a 5 mM Hepes buffer (pH 7.4) to a final concentration of 1 mg/ml and 0.1 mg/ml, respectively. TPP was added under continuous stirring to a weight ratio TMC:TPP:OVA of 10:1.8:1. Nanoparticles were collected by centrifugation (30 min 15000 g) on a glycerol bed, to avoid aggregation.

PLGA particles were prepared by a “water-in-oil-in-water” solvent evaporation method described by Garinot [8]. Briefly, 50 µL of 10 mg/mL OVA or FITC-OVA in 10mM phosphate buffer saline pH7.4 (PBS) was emulsified with 1 mL of dichloromethane containing 50 mg of PLGA with an ultrasonic processor for 15 s at 70 W (Branson Instruments, CT, USA). The secondary emulsion was prepared with 2 mL of 1% (w/v) sodium cholate in water. The double emulsion was then poured into 100 mL of a 0.3% sodium cholate aqueous solution, and stirred at 37°C for 45 min. The nanoparticle suspension was then washed twice in PBS by centrifugation at 22 000 g for 1 h.

Nanoparticle characterisation

Particle size distribution was determined by means of dynamic light scattering (DLS) using a NanoSizer ZS (Malvern Instruments, Malvern UK). The zeta potential of the particles was

measured with the NanoSizer ZS by laser Doppler velocimetry. Before the measurement, samples were diluted in 5 mM Hepes pH 7.4 or 50 mM acetate buffer pH 5.9 until a slight opalescent dispersion was obtained.

The OVA content of the particles was determined with a BCA protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.The encapsulation efficiency was expressed in percentage as the amount of OVA encapsulated compared to the amount of OVA used to form the nanoparticles. The process yield was expressed in percentage as the weight of nanoparticles compared to the amount of polymers used for the formulation.

Immunization with ovalbumin-loaded nanoparticles

Female Balb/c mice 6 weeks old at the beginning of the experiment were purchased from JANVIER (Le Genest-Saint-Isle, FR). The mice were kept in hanging wire cages and allowed access to food and drink *ad libitum*. Mice were fasted the day before their immunization. All experiments were approved by the ethical committee for animal care of the faculty of medicine of the Université Catholique de Louvain.

Mice received intramuscular injection of OVA (50 µg OVA/50 µL) as a positive control, or intraduodenal injections of OVA, OVA-loaded CS nanoparticles (OVA/CS) or TMC nanoparticles (OVA/TMC) (100 µl containing 50 µg of OVA). A boost was applied in similar fashion, 14 days after the priming. Blood samples were collected by retro-orbital punctures 14, 28, 42 days after priming. Sera isolated by centrifugation were stored at -20°C before analysis. OVA-specific IgG, IgG1 and IGg2a levels were determined by enzyme-linked immunosorbent assay (ELISA) [8]. Serum dilutions were made in OVA-coated plates (Nunc-Immnuno Plate F96 MAXISORP) and detection of anti-OVA antibodies was carried out using peroxidase-labelled rat anti-mouse immunoglobulin G, G1 and G2a (LO-IMEX, Brussels, BE). IgG titres were defined as the logarithm of the inverse of the sera dilution corresponding to an absorbance equal to 0.2.

Visualisation of OVA transport in vivo

Female Balb/c mice were administered 50 µg FITC-OVA encapsulated in TMC or CS nanoparticles by intraduodenal injection. After 1h mice were sacrificed, pieces of jejunum and Peyer's patches were harvested and washed with PBS. Tissues were formaldehyde fixed and incubated with PBS 0.2% Tween 100 and 2% Rhodamine-phalloïdin to stain membrane cells. Scanning laser confocal microscopy was used to visualise the luminal side.

Cell lines

Human colon carcinoma Caco-2 line (clone 1) was obtained from Dr. Maria Rescigno, University of Milano-Bicocca, Milan, IT [24] and maintained in supplemented Dulbecco's Modified Eagles Medium (DMEM) high glucose and L-glutamine, with 10% v/v foetal bovine serum (FBS) and 1% v/v non essential amino acids at 37^0C under a 5% CO_2 water saturated atmosphere. Human Burkitt's lymphoma Raji B-cell line (American Type Culture Collection, Manassas, VA, USA), was maintained in RPMI 1640, supplemented with 10% v/v FBS, 1% v/v L-glutamine and 1% v/v non essential amino acids.

Effect of polymers on cell viability

Toxicity of the formulations on Caco-2 cells was assessed using the MTT method. Caco-2 cells (10000/well) were seeded in a 96-well plate (Nunc, Roskilde, DK) and maintained for 2 days at 37°C and 5% CO_2. After 1 h exposure to 1 mg/ml of the various delivery systems, the cells were washed 3x with Hank's Balanced Salt Solution (HBSS) and incubated for 3 h with 0.5 mg/ml MTT in DMEM. Medium was removed and the purple formazan crystal was dissolved in 100 µl DMSO. Absorbance at 570 nm was measured using a µQuant ELISA plate reader (Biotek, Winooski, VT, USA).

In vitro human FAE culture

FAE cultures were performed according to the protocol improved by des Rieux [5]. Briefly, $5x10^5$ Caco-2 cells were seeded on Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA) coated 12-well Transwell inserts (Corning, Schiphol-Rijk, NL) and cultivated for 3 days in supplemented DMEM + 1% PEST. Inserts were inverted, a piece of silicon rubber (Labo-modern, Queveaucamps, BE) was placed around the basolateral side and transferred into a pre-filled Petri dish (VWR, Amsterdam, NL) with supplemented DMEM + 1% PEST. Inverted inserts were maintained for 10 days and the basolateral medium was refreshed every other day. $5x10^5$ Raji-B cells, resuspended in supplemented DMEM+ 1% PEST, were added to the basolateral compartment of the inserts. The co-cultures were maintained for 5 days. Mono-cultures were prepared in the same way, except that the Raji-B cells were left out.

Nanoparticle transport in vitro

For transport experiments mono- and co-culture inserts were reversed to their original orientation in 12-well plates (Corning) and washed with HBSS. After 20 minutes of equilibration at 37°C the trans-epithelial electrical resistance (TEER) was measured using a home made chop stick electrode couple to a MilliCellers® multimeter (Millipore, NL).

Nanoparticle formulations containing FITC-OVA were diluted to a final concentration of 1 mg/ml in HBSS (corresponding to approximately $0.9x10^8$ CS, $1.0x10^8$ TMC and $1.0x10^8$ PLGA nanoparticles per ml, as determined by flow cytometry) and 400 µl were applied to the apical compartment of the insert. At the basolateral side 1.2 ml HBSS was used as acceptor compartment. After incubation for 60 minutes at 37°C the amount of particles in the acceptor compartment was determined using flow cytometry (FACScalibur, Becton Dickinson) [5,8,17]. The transport of free FITC-OVA was evaluated by fluorimetry with an FS920 fluorimeter (Edinburgh Instruments, Campus Livingston, UK).

The Papp (cm/s) was calculated as follows:

$$Papp = dQ / dtAC_0$$

Where dQ/dt is the transport rate number of nanoparticles per ml or amount of FITC-OVA (mg) present in the basal compartment as function of time (s), A the area of Transwell (cm^2) and C_0 the initial concentration of nanoparticles (number/ml) or OVA (mg/ml) in the apical compartment.

The apical compartment was harvested and centrifuged (14000 g, 30 min). The supernatant was used to quantify the release of lactate dehydrogenase (LDH) according to the manufacturer's instructions (cytotoxicity detection kit, Roche, Woerden, NL).

Human monocytes derived dendritic cells culture

Monocytes were freshly isolated from human donor blood before each experiment by means of density gradients (Ficoll and Percoll) and depletion of platelets was performed by adherence of the monocytes in 24-well plate (Corning, Schiphol, NL) followed by washing. Monocytes ($5x10^5$ cells/well) were maintained for 6 days in RPMI 1640, supplemented with 10% v/v FBS, 1% glutamine, 1% v/v PEST, GM-CSF 250 U/ml (Biosource-Invitrogen, Breda, NL) and IL-4 100 U/ml (Biosource) at 37°C and 5% CO_2 to differentiate into immature DCs. Medium was refreshed after 3 days [25].

Interaction of nanoparticles with dendritic cells (DCs)

DCs were incubated for 4 h at 37°C in RPMI 1640 and 500 U/ml GMCSF with 2 µg/ml FITC-OVA either free or encapsulated in TMC, CS or PLGA nanoparticles (polymer concentration 20, 20 and 100 µg/ml respectively). Cells were washed 3 times with PBS containing 1% w/v bovine serum albumin and 2% v/v FBS before FITC-OVA association with DCs was quantified using

flow cytometry (FACScalibur, Becton Dickinson). Live cells were gated based on forward and side scatter. FITC-OVA association was expressed as the mean fluorescence intensity (MFI) in the FL-1 channel. Histogram overlay were created with WinMDI vs 2.9.

Effect of nanoparticles on DC maturation

DCs were incubated for 48 h at 37°C in RPMI 1640 and 500 U/ml GMCSF with 2 µg/ml OVA, either free or encapsulated in TMC, CS or PLGA nanoparticles and LPS (100 ng/ml) as a positive control. Cells were washed 3 times with PBS containing 1% w/v bovine serum albumin and 2% v/v FBS and incubated for 30 min with mixture of 50x diluted anti-HLADR-FITC and anti-CD86-APC (Becton Dickinson) on ice, to measure expression of MHCII and CD86 molecules on the DCs' cell surface, respectively. Cells were washed and expression of MHCII and CD86, both markers for mature DCs [26], was quantified using flow cytometry (FACScalibur). Live cells were gated based on forward and side scatter. The amount of MHCII and CD86 double positive cells was expressed as a percentage of the live cell population.

Statistics

Immunoglobulin levels were compared by Kruskal-Wallis non parametric tests (significance $p<0.05$). Two-way ANOVA with Bonferroni post-tests were used for the transport study. One-way ANOVA with Bonferroni post-tests was used for all the other in vitro studies.

Results and discussion

Nanoparticle formulations

All OVA-loaded nanoparticles showed a mean size distribution between 200 and 300 nm with comparable size distributions and a good process yield (Table I). With an ionic complexation method using TPP, adapted from Calvo [23], OVA was efficiently encapsulated into fairly monodisperse (PDI<0.25) CS and TMC nanoparticles with a mean hydrodynamic diameter of approximately 300 nm. For comparison, monodisperse (PDI< 0.15) PLGA nanoparticles with an average size of 240 nm were prepared by emulsification/solvent extraction [8].

TMC nanoparticles carried a positive surface charge at physiological pH. CS nanoparticles however lost their positive zeta potential at pH 7.4 due to deprotonation of the primary amine groups and showed major colloidal instability. Therefore, the characterisation and *in vitro*

experiments with CS nanoparticles were conducted at pH 5.9. PLGA nanoparticles carried a negative surface charged due to deprotonated carboxyl groups.

OVA was more efficiently encapsulated into CS or TMC nanoparticles (loading efficiency 65% and 67 % respectively) than into PLGA nanopartlices (42%). This may be due to the different preparation protocol as well as the hydrophobicity and negative charge of PLGA, disfavouring the encapsulation of the hydrophilic, negatively charged OVA (pI=4.8).

Table I: Characteristics of TMC, CS and PLGA nanoparticles

	Size (nm)	Polydispersity index	Zeta potential (mV)	Loading efficiency (%)	Yield (%)
CS/TPP/OVA*	290 ± 36	0.244 ± 0.011	43.3 ± 1.0	65 ± 4.9	72 ± 5.9
TMC/TPP/OVA**	291 ± 12	0.202 ± 0.045	27.8 ±1.5	66 ± 2.7	89 ± 1.5
PLGA/OVA**	240 ± 9.0	0.118 ± 0.014	-36.3 ±2.2	42 ± 0.9	91 ± 2.3

Data represent the mean of 4 independently prepared batches ± standard deviation.
** Measurements performed in 50 mM acetate buffer pH 5.9 ** Measurements performed in 5 mM Hepes pH 7.4*

Mucosal immunization with OVA-loaded TMC and CS nanoparticles

A mucosal immunization study was performed to compare the ability of OVA-loaded TMC and CS nanoparticles to elicit an immune response. Therefore, 50 µg of free OVA and 50 µg of encapsulated OVA in TMC and CS nanoparticles were administered by intraduodenal injection to mice.

Two weeks after priming, TMC nanoparticles showed an immune response in 5 out of 8 mice, whereas free OVA, OVA encapsulated in CS nanoparticles or intramuscular injection of OVA induced a low IgG response in 2 out of 8 mice (Fig. 1a). On day 42 (4 weeks after boosting), all mice vaccinated with OVA loaded nanoparticles showed a strong and significant enhancement (over 1000 fold) in IgG production compared to intraduodenal immunization with free OVA (Fig. 1b). No significant difference in IgG titres was observed between the 2 groups vaccinated with nanoparticles. Interestingly, OVA administered by intramuscular injection, while yielding a 100 fold higher IgG titre than free OVA given intraduodenally, resulted in a lower immune response than the intraduodenal delivery of OVA in nanoparticulate formulations. This could indicate that the increased immune response caused

by encapsulation of the antigen is not solely due to a more efficient delivery of the antigen to the target cells but also to an intrinsic immunopotentating capacity of the nanoparticles [19,20].

Compared to intraduodenal immunization with free OVA, TMC and CS encapsulated OVA induced higher IgG1 and IgG2a titres ($p<0.05$) (Fig. 2a). Mice vaccinated with free OVA by intramuscular injection and intraduodenal delivery showed a mean IgG2a/ IgG1 ratio of 0.5 and 0.1, respectively. Mice vaccinated with CS and TMC nanoparticles had a more balanced ratio of 1 and 0.8, respectively (Fig. 2b). It has been reported that soluble antigens usually elicit high levels of IgG1 antibodies, but very low levels of IgG2a [27]. Both polymers (TMC and CS) have been described in the literature as being mucosal adjuvants and their influence on the Th1/Th2 balance seems to be very dependent on the antigen and route of administration [28-30]. The shift in profile of the immune response towards a Th1 response by the nanoparticulate formulations could be due to proton scavenger properties of CS and TMC, which may facilitate endosomal escape of the antigen and thereby promote antigen presentation by the MHC class I pathway [31].

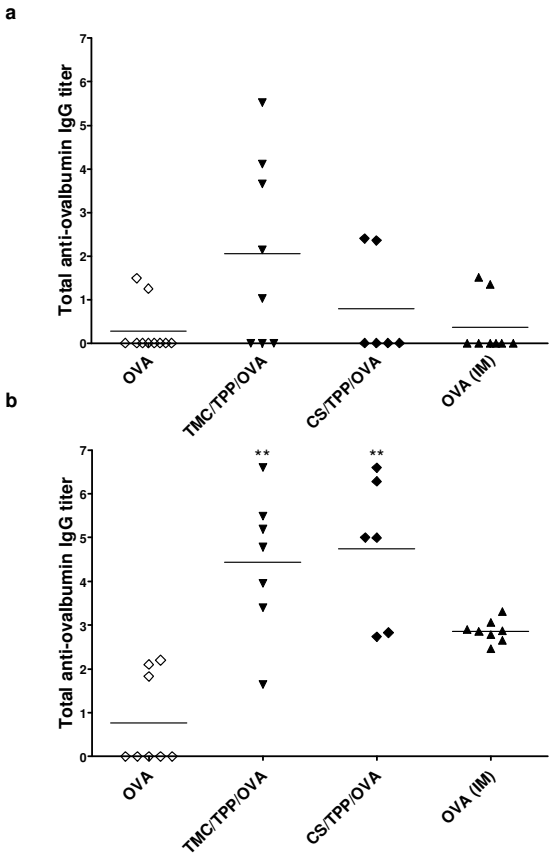


***Figure 1**. OVA-specific IgG titres in serum of Balb/c mice intraduodenally fed with OVA solution, OVA-loaded TMC nanoparticles or CS nanoparticles, as compared to intramuscular injection with OVA solution. a) Total IgG titres 14 days after priming. b) Total IgG titres 42 days after priming (28 days after boosting). **OVA-loaded TMC and CS nanoparticles induced higher IgG titres than OVA solution ($p<0.01$).*

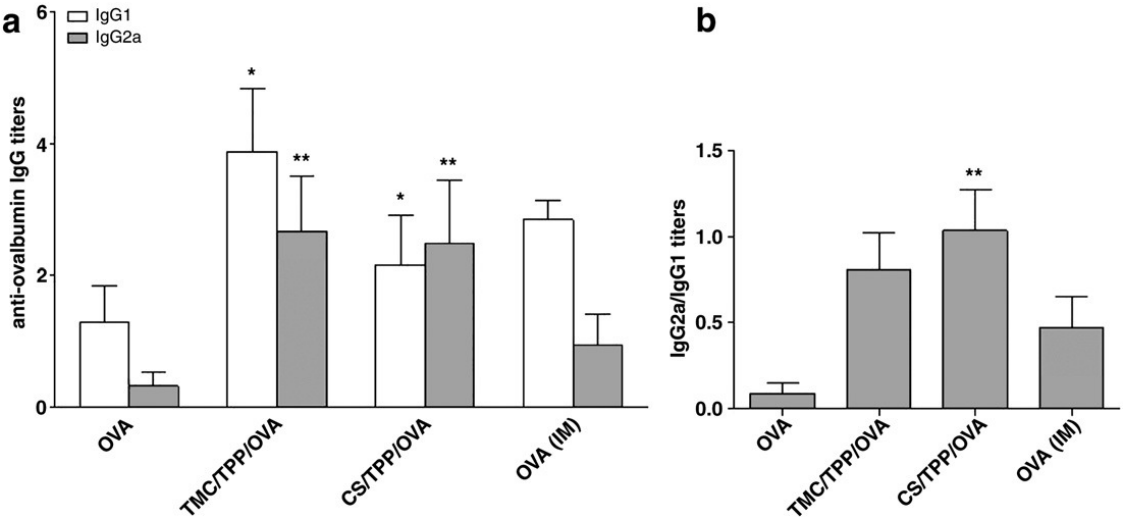


***Figure 2**. OVA-specific IgG1 and IgG2a titres in serum of Balb/c mice intraduodenally fed with OVA solution, OVA-loaded TMC and CS nanoparticles. Intramuscular injection was used as a positive control. a) IgG1 and IgG2a 28 days after boost. *IgG1 and **IgG2a induced by TMC/TPP/OVA and CS/TPP/OVA were significantly higher than those induced by free OVA (pb0.05). b) Ratio of IgG2a on IgG1 titres 28 days after boost. **Ratio induced by OVA-loaded CS nanoparticles immunization was significantly different from those induced by OVA solution (p<0.01).*

This study as well as other reports [12,32] clearly demonstrates that TMC and CS nanoparticles are potential candidates for oral delivery. It is generally accepted that nanoencapsulation can protect the antigen from degradation in the gastrointestinal tract; this however does not explain why encapsulation of OVA leads to a higher immune response than intramuscular injection or why a shift to Th1 response has been observed. Hence, the mechanisms by which TMC or CS nanoparticles could act as adjuvant, either as delivery systems and/or as immunomodulator were investigated. We hypothesized that the particulate form and the positive charge (unencapsulated OVA is negatively charged) account for the immuno-stimulatory effect. Therefore OVA-loaded PLGA nanoparticles with a similar size distribution as OVA/CS and OVA/TMC were included to serve as a negatively charged counterpart to the CS and TMC based particles. The effect of nanoparticle composition on their uptake by M-cells, was studied in vivo and in vitro. Moreover, their effect on DC uptake and maturation was assessed in vitro.

Localization of antigen in Peyer's patches

A confocal microscopy of the gut of mice after intraduodenal delivery of free or encapsulated FITC-labelled OVA was performed to check if the TMC and CS particleswere specifically taken up by M-cells in Peyer's patch. Free FITC-OVAwas detected in neither regular (Fig. 3a) nor Peyer's patch epithelium (Fig. 3b), which could account for the poor immunogenicity of OVA observed after intraduodenal administration. Consistent with previous studies conducted by van der Lubben [12,21], who found M-cell specific uptake of CS microparticles, uptake of FITC-OVA encapsulated in CS (Fig. 3d) or TMC (Fig. 3f) nanoparticles was detected mainly in the Peyer's patches. In general, regular intestinal epithelium exhibited less intense OVA related fluorescence than the M-cell rich Peyer's patch area (Fig. 3c and e). x–z analysis clearly shows that CS and TMC nanoparticles were taken up by cells in the jejunum and Peyer's patches. This indicates that 1 h after of intraduodenal administration a significant part of the antigen has accumulated in the Peyer's patch, which is most probably due to M-cell specific uptake. The ability of M-cells to transport particulate structures is well established [33] and increasing the M-cell uptake has become an attractive strategy to improve current mucosal vaccines [34–36]. The particulate nature of the encapsulated FITC-OVA therefore seems to be one of the major reasons for the active uptake by the FAE. To support these findings and to assess whether encapsulation in CS or TMC nanoparticles is beneficial for M-cell mediated transport, FITC-OVA transport studies over an in vitro FAE was performed.

In vitro studies with FAE

Cell viability and monolayer integrity

As cell viability and monolayer integrity are vital for the interpretation of the transport experiments, the toxicity of the nanoparticles on Caco-2 cells was assessed with an MTT assay and a LDH release test, and monolayer integrity was evaluated by monitoring the TEER (Table 2). At 1.0 mg/ml, the same concentration as used in the transport experiments, no significant cytotoxicity of CS and PLGA nanoparticles on Caco-2 cells was observed by the MTT assay. However, TMC nanoparticles caused a 20% reduction of cell survival (pb0.05). No LDH release (b1%) was detected. MTT as well as LDH assay performed at pH 5.9 showed similar results (data not shown). The toxicity of TMC could be due to its higher positive charge density leading to a higher interaction with cell membrane and higher uptake [32]. CS and TMC have been reported as enhancers of oral absorption, due to the opening of tight junctions between

the epithelial cells [11-15,32]. The relatively small but significant decrease of electrical resistance across the monolayer of both Caco-2 monocultures and co-cultures induced by CS and TMC particles (pb0.05) indicates that nanoparticles composed of these cationic biopolymers modified the tight junctions. In contrast, no changes in TEER were observed after exposure to PLGA nanoparticles. Although tight junction opening has been described as mechanism promoting antigen uptake, it is unlikely that an antigen encapsulated in a 300 nm sphere, will diffuse through these tight junction openings as FITCdextran (4 kDa and 12 kDa) were not significantly transported by co-cultures [5]. Therefore the immune- enhancing effect of CS and TMC nanoparticles should not be attributed to their ability to modify tight junctions.

Nanoparticle transport across human FAE culture

M-cells present in the FAE are known for their transcytotic transport capacity and may be critical for effective antigen transport to subepithelial immune cells [1,16]. Therefore, nanoparticle transport across the intestinal epithelium was investigated in vitro by measuring the amount of FITC-OVA-loaded particles in the basolateral compartment of Caco-2 mono-cultures as well as Caco-2/Raji-B co-cultures. The Raji-B cells induce the generation of M-cells within the epithelial cell layer, permitting us to discriminate between M-cell dependent and M-cell independent transport (Fig. 4). All types of nanoparticles were more transported in the presence of M-cells, although only CS and TMC particle transport increased significantly ($p<0.01$).

Table II: Effect of nanoparticles on Caco-2 cell survival and monolayer integrity after 1 h of exposure to 1 mg/ml free or nanoparticulate OVA

	MTT test (%) [a]	Initial TEER (Ω/cm^2) [b]		Final TEER (% of initial value) [b]	
		Mono-culture	Co-culture	Mono-culture	Co-culture
OVA (aq)°	97.7 ± 10.5	240 ± 24	111 ± 11	99.4 ± 0.7	92.7 ± 2.7
CS/TPP/OVA*	93.3 ± 23.1	219 ± 15	101 ± 12	70.5 ± 5.0#	60.5 ± 3.1#
TMC/TPP/OVA°	78.3 ± 3.4#	222 ± 12	100 ± 7	74.8 ± 1.8#	70.7 ± 2.0#
PLGA/OVA°	103.0 ± 12.8	205 ± 12	117 ± 14	103.0 ± 1.6	96.3 ± 1.0

[a] Cell viability was assessed by an MTT test. Results are expressed as percentage (mean ± SEM; n=14) of the medium control (100%)

[b] Monolayer integrity was investigated by measuring the TEER before and after the experiment. Values represent mean ± SEM (n=14)

*# p<0.05, compared to OVA solution ° HBSS pH 7.4 * HBSS pH 5.9*

The increase in CS and TMC particle transport in the co-culture is consistent with the preferential localisation in Peyer's patches in vivo (Fig. 3). TMC particle transport by the mono-culture and co-culture was higher than CS and PLGA particle transport, independent of the presence of M-cells (pb0.01). Transport by co-cultures was equivalent for PLGA and CS particles. It could be related to the higher positive charge density which is a critical factor for bioadhesion and penetration enhancement towards intestinal epithelium. It could also be related to the increase of quaternization degree favouring the mucoadhesion or to the small toxic effect of TMC (Table 2), which may have negatively affected the integrity of the monolayer. For the lower transport of CS particles in comparison with TMC particles, a key element could be the instability of the CS particles. The colloidal stability of OVA/CS is fragile and very sensitive to pH shifts, ionic strength and medium composition [37,38]. Thus, it is well possible that many particles exposed to pHN6.5 had aggregated before they reached the basolateral compartment [39,37] or that CS could be less condensed because the amine groups will not be charged and so, be less transported [40,15].

In conclusion, nanoparticles seem to direct the antigen towards the Peyer's patches as their particulate nature favours M-cell specific uptake, compared to unencapsulated antigen. Hence, M-cell uptake seems to be the most likely route of CS or TMC nanoparticles, although active transport by epithelial cells [41] or uptake by extruding DCs [24] cannot be ruled out.

In vitro studies with DCs

Effect of nanoparticles on FITC-OVA association with DC

Immature human DCs were stimulated for 4 h with either FITCOVA alone or nanoparticles containing FITC-OVA to investigate if encapsulation of OVA increased its association with DCs. Treatment with 2 μg/ml FITC-OVA led to an increase in mean fluorescence intensity compared to unstimulated DCs, indicating association of the antigen with the DCs. The positively charged formulations (OVA/CS and OVA/TMC) showed significantly increased association with DCs (Fig. 5a). Interestingly, OVA incorporated in PLGA nanoparticles was not taken up by DCs more efficiently than free

OVA, probably because the DC-PLGA interaction was less pronounced due to the unfavourable electrostatic interactions between the negative surfaces of PLGA nanoparticles and the DC's cell membrane.

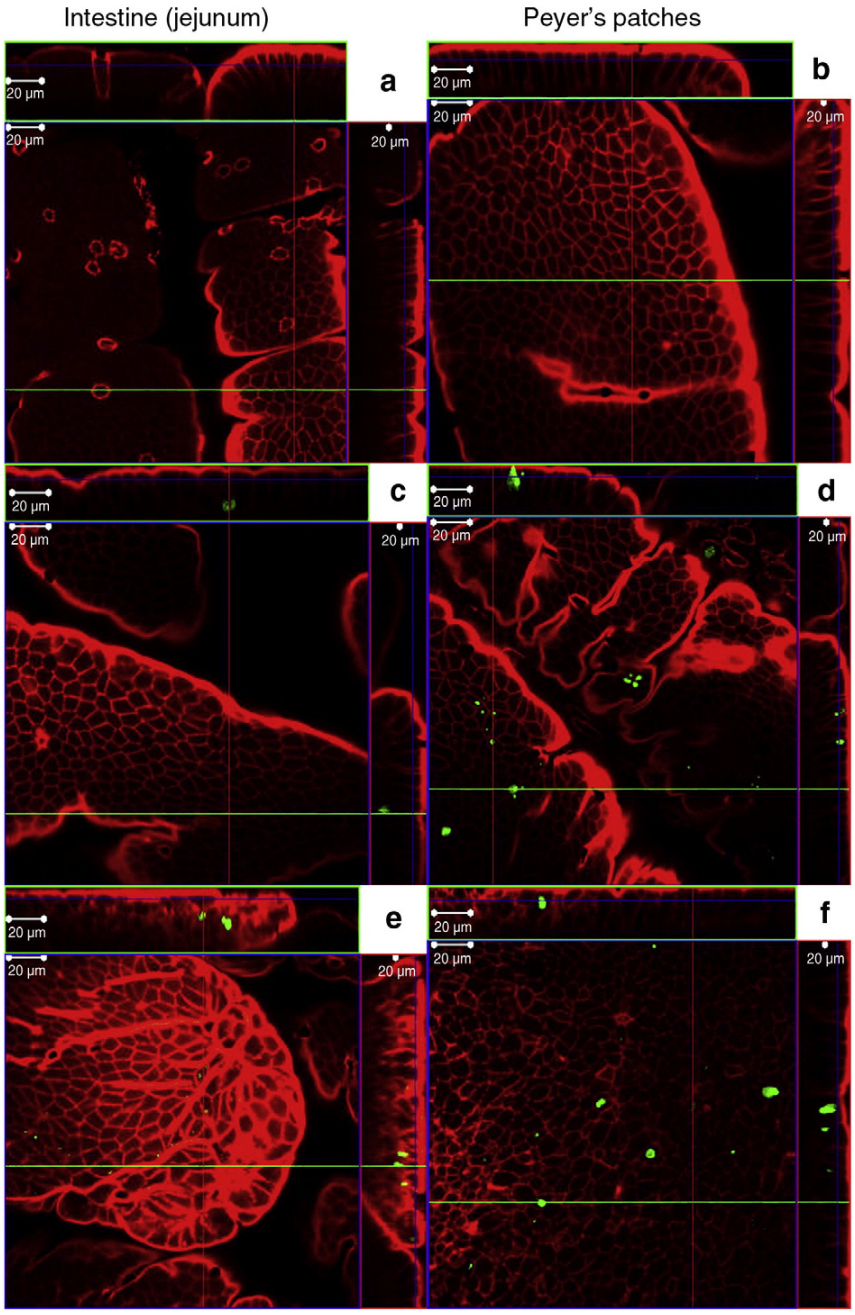


Figure 3. *Representative confocal microscopy images of murine jejunum (a, c, e) and a Peyer's patch (b, d, f) from murine jejunum, isolated 1 h after intraduodenal injection with 50 µg of FITC-OVA alone (a, b), incorporated in CS nanoparticles (c, d) or in TMC nanoparticles (e, f). Tissues were formaldehyde fixed for 90 min and incubated with PBS containing 0.2% Triton X-100 and Rhodamine-phalloïdin (1/50 v/v) to stain cell membranes. Scanning laser confocal microscopy was used to visualize the luminal side and to perform x–z analysis.*

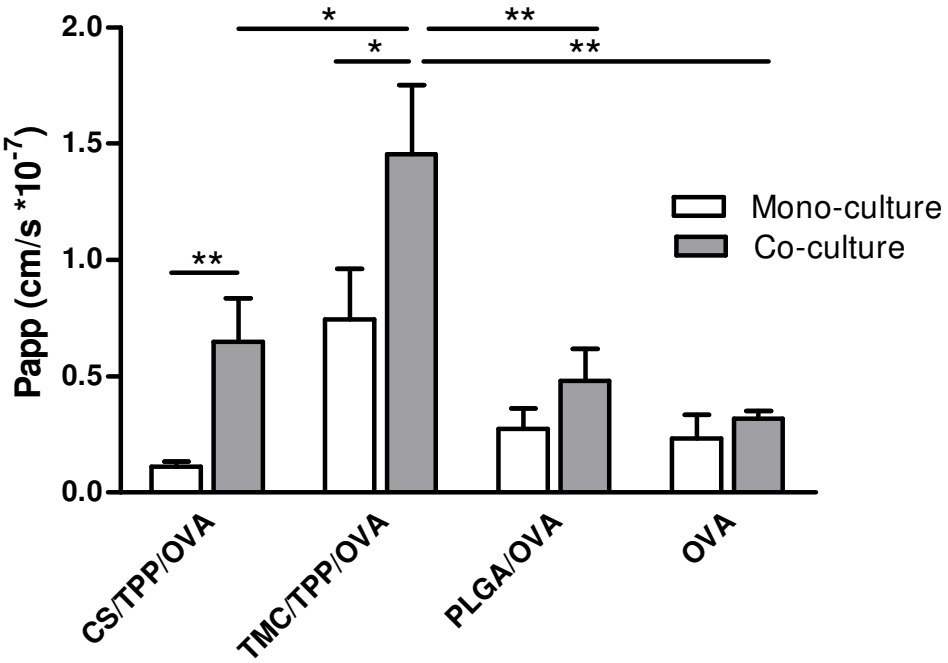


***Figure 4**: FITC-OVA-loaded nanoparticle transport over Caco-2 monolayers (white bars) and Caco-2/Raji-B co-cultures (grey bars) quantified by FACS analysis. Transport is expressed as apparent permeability (Papp). Error bars represent standard error of the mean (SEM). M-cell, only present in co-culture significantly enhance the transport of OVA inside CS and TMC particles, but not unformulated OVA (n=18). Level of significant differences in Papp values are indicated by * (p<0.05) and ** (p<0.01).*

Effect of nanoparticles on DC maturation

Uptake of antigen by DC does not necessarily mean that a successful immune response will be started. DCs sample their environment continuously, but only become activated if the antigen is recognized as dangerous. To measure whether nanoparticles supply a 'danger signal' next to increasing the delivery of the antigen, the maturation of human monocyte DC was studied. After 48 h of exposure to the different OVA formulations, clear differences in the maturation status of the DCs could be observed (Fig. 5b). The positive control, LPS-stimulated DCs expressed maturation markers to a very high extent, as 90% of the total DCs population was positive forMHCII as well as CD86. DCs treated with OVA showed no maturation, but OVA/TMC nanoparticles increased the number of MHCII/CD86 double positive DCs (pb0.01).

The type of biopolymer seems to be an important parameter determining DCs maturation, as for PLGA and CS nanoparticles no significant increase of mature DCs was observed.

Induction of DC maturation by CS and PLGA particles has been investigated by other groups with inconclusive results. Increased DC maturation by PLGA and CS biofilms in vitro [42] and by CS solutions in vivo [30] has been shown, but also studies claiming no stimulatory effect of these biopolymers exist [43,44]. Fisher et al. have recently addressed this discrepancy [45] and explained it by slight differences in preparation of the formulations and possible endotoxin contamination. To rule out LPS contamination in our experiments, TLR-4 transfected HEK cells were exposed to the formulations and we found the LPS content to be below the detection limit (<0.1 ng/ml, data not shown).

As only the (positively charged) TMC nanoparticles induced DC maturation, but no DC maturationwas found after exposure to soluble antigen, (negatively charged) PLGA particles and (positively charged) CS particles, the nanoparticles material rather than the zeta potential, is an important factor in the process of DCs maturation. Effect of TMC

on cell viability could also trigger DC maturation. As CS particles were incapable of activating DCs, but were effective in inducing an immune response, CS apparently acts as an adjuvant via a different mechanism. Possibly a depot is formed by OVA/CS particles that aggregate as soon as they are secreted into the subepithelial space and come into contact within a higher pH and medium constituents [39]. Why TMC has immunopotentiating capacity is unclear. To our best knowledge, this is the first time that the direct effect of TMC nanoparticles on DCs was assessed and TMC is a not a known ligand for pathogen recognition receptors. Further investigation into the adjuvant effect of TMC will therefore be necessary.

Conclusion

This study shows that if the right nanoparticle material is selected, the transport of the antigen into the FAE, association with APC, maturation of DCs and modulation of the immune response can be accomplished. The particulate nature of the delivery system increases the M-cell specific transport into the FAE and a positive surface charge seems to cause an enhanced association of antigen with DCs, increasing the probability of successful antigen uptake. Moreover, TMC nanoparticles exerted intrinsic adjuvant properties, as it induces maturation of DCs. These characteristics make TMC nanoparticles a promising delivery system for mucosal vaccination.

Acknowledgements

This work was performed within the framework of Top Institute Pharma project number D5-106 (NL) and Walloon region government with the project VACCINOR (WINNOMAT, BE). Laurence Plapied is FNRS Research Fellow (Fonds National de la Recherche Scientifique, BE). We thank the FNRS, the Région Bruxelloise and UCL for confocal equipments and Pr. Patrick Van Der Smissen for confocal training.

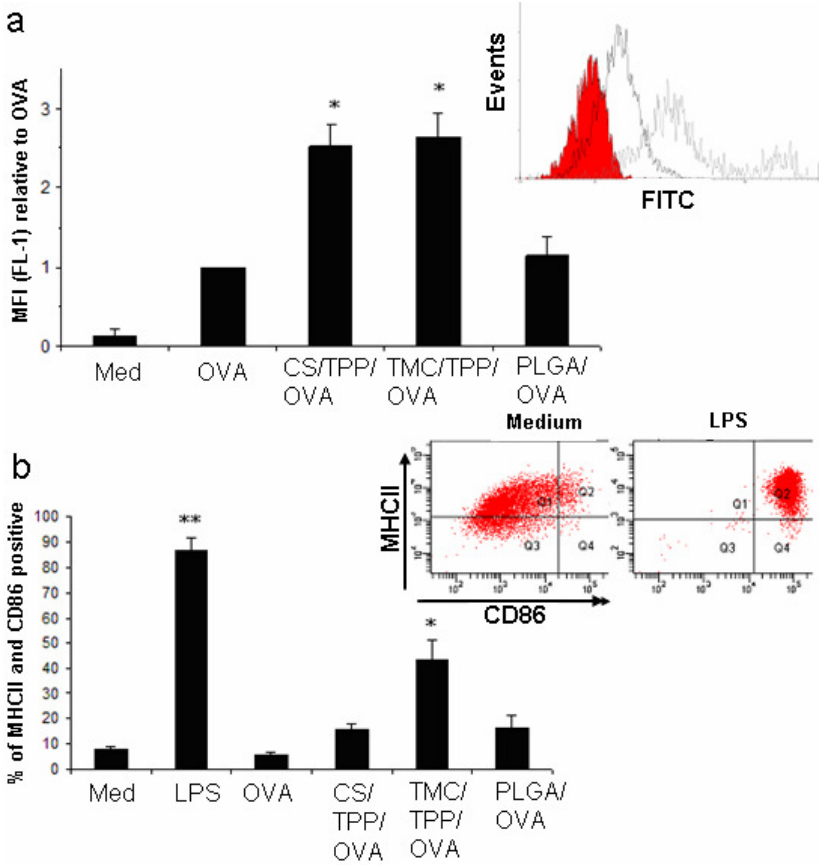


Figure 5. *Interaction of OVA with human monocyte derived DCs. DCs were exposed to 2 µg/ml soluble or encapsulated FITC-OVA for 4 h. a) OVA-FITC association with DCs measured by flow cytometry. Bars represent mean values of 5 experiments from 5 different donors. Insert: Representative overlay histogram of a single DC-association experiment: control DCs (filled), DCs pulsed with 2 µg/ml FITC-OVA (black line) or FITC-OVA loaded TMC nanoparticles (dashes line) analyzed after 4 h of incubation. b) Maturation of DCs exposed to 2 µg/ml soluble or encapsulated OVA, or 10 ng LPS, for 48 h. Cells were stained with anti-HLADR-FITC and anti-CD86-APC. Expression of surface molecules MHCII and CD86 was determined by flow cytometry. Double positive cells for MHCII and CD86 were considered matured (see insert). Bars represent mean values of 5 experiments from 5 different donors. Error bars represent SEM. *p<0.01; **p<0.001.*

References

[1] M.R.Neutra, P.A.Kozlowski, *Mucosal vaccines: the promise and the challenge*, Nature Reviews Immunology 6 (2) (2006) 148-158.

[2] B.Dubois et al., *Oral tolerance and regulation of mucosal immunity*, Cell Mol. Life Sci. 62 (12) (2005) 1322-1332.

[3] A.M.Mowat, *Anatomical basis of tolerance and immunity to intestinal antigens*, Nat. Rev. Immunol. 3 (4) (2003) 331-341.

[4] A.des Rieux et al. *Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach*, J. Control Release 116 (1) (2006) 1-27.

[5] A.des Rieux et al. *An improved in vitro model of human intestinal follicle-associated epithelium to study nanoparticle transport by M cells*, Eur. J. Pharm. Sci. 30 (5) (2007) 380-391.

[6] A.B.Sieval et al. *Preparation and NMR characterization of highly substituted N-trimethyl chitosan chloride,* Carbohydrate Polymers 36 (2-3) (1998) 157-165.

[7] A.Wierzbicki, et al. *Immunization strategies to augment oral vaccination with DNA and viral vectors expressing HIV envelope glycoprotein*, Vaccine 20 (9-10) (2002) 1295-1307.

[8] M.Garinot, et al. *PEGylated PLGA-based nanoparticles targeting M cells for oral vaccination,* J. Control Release 120 (3) (2007) 195-204.

[9] K.laoui-Attarki, et al. *Mucosal immunogenicity elicited in mice by oral vaccination with phosphorylcholine encapsulated in poly (D,L-lactide-co-glycolide) microspheres*, Vaccine 16 (7) (1998) 685-691.

[10] R.Rajkannan et al. *Development of hepatitis B oral vaccine using B-cell epitope loaded PLG microparticles,* Vaccine 24 (24) (2006) 5149-5157.

[11] S.M.van der Merwe et al. *Trimethylated chitosan as polymeric absorption enhancer for improved peroral delivery of peptide drugs*, European Journal of Pharmaceutics and Biopharmaceutics 58 (2) (2004) 225-235.

[12] I.van der Lubben et al. *Chitosan for mucosal vaccination*, Adv. Drug Deliv. Rev. 52 (2) (2001) 139-144.

[13] H.Y.Zhou, X.G.Chen, W.F.Zhang, *In vitro and in vivo evaluation of mucoadhensiveness of chitosan/cellulose acetate multimicrospheres*, J. Biomed. Mater. Res. A 83 (4) (2007) 1146-1153.

[14] F.Chen et al. *In vitro and in vivo study of N-trimethyl chitosan nanoparticles for oral protein delivery,* Int. J. Pharm. 349 (1-2) (2008) 226-233.

[15] G.Sandri et al. *Nanoparticles based on N-trimethylchitosan: evaluation of absorption properties using in vitro (Caco-2 cells) and ex vivo (excised rat jejunum) models*, Eur. J. Pharm. Biopharm. 65 (1) (2007) 68-77.

[16] D.J.Brayden, M.A.Jepson, A.W.Baird, *Keynote review: intestinal Peyer's patch M cells and oral vaccine targeting,* Drug Discov. Today 10 (17) (2005) 1145-1157.

[17] E.Gullberg et al. *Expression of specific markers and particle transport in a new human intestinal M-cell model*, Biochem. Biophys. Res. Commun. 279 (3) (2000) 808-813.

[18] S.Dahan et al. *Epithelia: lymphocyte interactions in the gut*, Immunol. Rev. 215 (2007) 243-253.

[19] S.T.Reddy, M.A.Swartz, J.A.Hubbell, *Targeting dendritic cells with biomaterials: developing the next generation of vaccines*, Trends Immunol. 27 (12) (2006) 573-579.

[20] Y.Zheng, et al. *Characterization of particles fabricated with poly(D, L-lactic-co-glycolic acid) and an ornithine-histidine peptide as carriers of oligodeoxynucleotide for delivery into primary dendritic cells*, J. Biomater. Sci. Polym. Ed 17 (12) (2006) 1389-1403.

[21] I.van der Lubben, et al. *In vivo uptake of chitosan microparticles by murine Peyer's patches: visualization studies using confocal laser scanning microscopy and immunohistochemistry*, J. Drug Target 9 (1) (2001) 39-47.

[22] M.Amidi et al. *Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system*, J. Control Release 111 (1-2) (2006) 107-116.

[23] P.Calvo et al. *Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers*, Journal of Applied Polymer Science 63 (1) (1997) 125-132.

[24] M.Rescigno et al. *Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria*, Nat. Immunol. 2 (4) (2001) 361-367.

[25] E.C.de Jong et al. *Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse Th cell-polarizing signals*, Journal of Immunology 168 (4) (2002) 1704-1709.

[26] P.L.Vieira et al. *Glucocorticoids inhibit bioactive IL-12p70 production by in vitro-generated human dendritic cells without affecting their T cell stimulatory potential*, J. Immunol. 161 (10) (1998) 5245-5251.

[27] I.Gutierro et al. *Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres*, Vaccine 21 (1-2) (2002) 67-77.

[28] G.P. Li et al. *Induction of Th1-type immune response by chitosan nanoparticles containing plasmid DNA encoding house dust mite allergen der p 2 for oral vaccination in mice*, Cell. Mol. Immunol. 6 (1) (2009) 45–50.

[29] E.A. McNeela et al. *Intranasal immunization with genetically detoxified diphtheria toxin induces T cell responses in humans: enhancement of Th2 responses and toxin-neutralizing antibodies by formulation with chitosan,* Vaccine 22 (8) (2004) 909–914.

[30] C. Porporatto, I.D. Bianco, S.G. Correa, *Local and systemic activity of the polysaccharide chitosan at lymphoid tissues after oral administration*, J. Leukoc. Biol. 78 (1) (2005) 62–69.

[31] P.Strong, H.Clark, K.Reid, *Intranasal application of chitin microparticles down-regulates symptoms of allergic hypersensitivity to Dermatophagoides pteronyssinus and Aspergillus fumigatus in murine models of allergy,* Clinical and Experimental Allergy 32 (12) (2002) 1794-1800.

[32] B.Sayin et al. *Mono-N-carboxymethyl chitosan (MCC) and N-trimethyl chitosan (TMC) nanoparticles for non-invasive vaccine delivery*, Int. J. Pharm. 363 (1-2) (2008) 139-148.

[33] L.J.Hathaway, J.P.Kraehenbuhl, *The role of M cells in mucosal immunity*, Cell Mol. Life Sci. 57 (2) (2000) 323-332.

[34] A.R.Foxwell, A.W.Cripps, J.M.Kyd, *Optimization of oral immunization through receptor-mediated targeting of M cells*, Hum. Vaccin. 3 (5) (2007) 220-223.

[35] F.Roth-Walter et al. *Targeting antigens to murine and human M-cells with Aleuria aurantia lectin-functionalized microparticles,* Immunol. Lett. 100 (2) (2005) 182-188.

[36] B.Slütter, N.Hagenaars, W.Jiskoot, *Rational design of nasal vaccines*, Journal of Drug Targeting 16 (1) (2008) 1-17.

[37] T.Lopez-Leon et al. *Physicochemical characterization of chitosan nanoparticles: electrokinetic and stability behavior*, Journal of Colloid and Interface Science 283 (2) (2005) 344-351.

[38] T. Sato, T. Ishii, Y. Okahata, *In vitro gene delivery mediated by chitosan. Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency*, Biomaterials 22 (15) (2001) 2075–2080.

[39] O. Germershaus et al. *Gene delivery using chitosan, trimethyl chitosan or polyethylenglycol-graft-trimethyl chitosan block copolymers: establishment of structure–activity relationships in vitro*, J. Controlled Release 125 (2) (2008) 145–154.

[40] A.F. Kotze et al. *Comparison of the effect of different chitosan salts and N-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2)*, J. Control Release 51 (1) (1998) 35–46.

[41] E.G. Ragnarsson et al. *Yersinia pseudotuberculosis induces transcytosis of nanoparticles across human intestinal villus epithelium via invasin dependent macropinocytosis*, Lab. Invest. 88 (11) (2008) 1215–1226.

[42] J.E. Babensee, A. Paranjpe, *Differential levels of dendritic cell maturation on different biomaterials used in combination products*, J. Biomed. Mater. Res. Part A 74A (4) (2005) 503–510.

[43] A. Luzardo-Alvarez et al. *Biodegradable microspheres alone do not stimulate murine macrophages in vitro, but prolong antigen presentation by macrophages in vitro and stimulate a solid immune response in mice,* J. Control. Release 109 (1–3) (2005) 62–76.

[44] C. Wischke et al. *Stable cationic microparticles for enhanced model antigen delivery to dendritic cells*, J. Control. Release 114 (3) (2006) 359–368.

[45] S. Fischer et al. T*he preservation of phenotype and functionality of dendritic cells upon phagocytosis of polyelectrolyte-coated PLGA microparticles,* Biomaterials28 (6) (2007) 994–1004.

4

Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: Nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen

Bram Slütter
Chantal Keijzer
Niels Hagenaars
Eric Kaijzel
Patrick Augustijns
Joke Bouwstra
Wim Jiskoot
Suzanne Bal
Roel Mallants
Ivo Que
Willem van Eden
Clemens Löwik
Femke Broere

Vaccine 2010 28(38):6282-91

Abstract

Nasal vaccination is a promising, needle-free alternative to classical vaccination. Nanoparticulate delivery systems have been reported to overcome the poor immunogenicity of nasally administered soluble antigens, but the characteristics of the ideal particle are unknown. This study correlates differences in physicochemical characteristics of nanoparticles to their adjuvant effect, using ovalbumin (OVA)-loaded poly(lactic-co-glycolic acid) nanoparticles (PLGA NP), N-trimethyl chitosan (TMC) based NP (TMC NP) and TMC-coated PLGA NP (PLGA/TMC NP).

PLGA NP and PLGA/TMC NP were prepared by emulsification/solvent extraction and TMC NP by ionic complexation. The NP were characterized physicochemically. Their toxicity and interaction with and stimulation of monocyte derived dendritic cells (DC) were tested *in vitro*. Furthermore, the residence time and the immunogenicity (serum IgG titers and secretory IgA levels in nasal washes) of the nasally applied OVA formulations were assessed in Balb/c mice.

All NP were similar in size, whereas only PLGA NP carried a negative zeta potential. The NP were non toxic to isolated nasal epithelium. Only TMC NP increased the nasal residence time of OVA compared to OVA administered in PBS and induced DC maturation. After i.m. administration all NP systems induced higher IgG titers than OVA alone, PLGA NP and TMC NP being superior to PLGA/TMC NP. Nasal immunization with the slow antigen-releasing particles, PLGA NP and PLGA/TMC NP, did not induce detectable antibody titers. In contrast, nasal immunization with the positively charged, fast antigen-releasing TMC NP led to high serum antibody titers and sIgA levels.

In conclusion, particle charge and antigen-release pattern of OVA-loaded NP has to be adapted to the intended route of administration. For nasal vaccination, TMC NP, releasing their content within several hours, being mucoadhesive and stimulating the maturation of DC, were superior to PLGA NP and PLGA/TMC NP which lacked some or all of these characteristics.

Introduction

The nasal cavity is one of the most promising administration sites for vaccines. The nose is easily accessible, low on proteolytic enzymes compared to the oral route and has interesting immunological characteristics. As the nasal cavity is a major route of entry for pathogens, the nasal epithelium is equipped with a large amount of immune cells to fight off infection and is capable of producing secretory IgA. Several studies have shown systemic as well as local antibody responses after nasal administration of an antigen [1-7]. Administration of subunit vaccines alone, however, seldom leads to a protective antibody response. The residence time of a soluble antigen in the nose is limited, which results in a very small dose reaching antigen-presenting cells (e.g. dendritic cells) in the sub epithelial region. Moreover, subunit vaccines are often poorly immunogenic as they lack the necessary danger signals to activate dendritic cells (DC) and subsequently, T-cells.

To overcome these obstacles, encapsulating antigen into particulate systems is a popular method [8]. Particles prepared with mucoadhesive substances can increase the antigens' residence time in the nasal cavity [9], increasing the chance of uptake by the epithelium. Obviously, a size reduction of the particle from micro to nano scale could be beneficial as nanoparticles (NP) penetrate the nasal epithelium more easily [10, 11]. Once particles have crossed the epithelium they can facilitate the uptake of the antigen by DC. Furthermore, the multimerization of epitopes on the particle surface and the possibility of co-encapsulation with adjuvants can increase the immune recognition by B-cells and other antigen-presenting cells [10, 11].

Evidently, a NP that has all of the above mentioned characteristics is preferred. How such a particle looks like in terms of its physical and chemical properties like material, size, surface charge, physical stability, antigen stability and antigen release profile is currently unknown [12]. In the literature a wide variety of particles, including liposomes, virosomes, oil-in-water emulsions, nanocomplexes and polymer based carriers are mentioned [13], all with a different immunological outcome. For instance, a particle capable of provoking a strong antibody response may fail to trigger the cellular arm of the immune system, or may not induce the production of mucosal, sIgA mediated immunity. This enigma stresses the importance of combining thorough characterization of the delivery system together with *in vitro* analysis of its interaction with immune cells and extensive evaluation of immunological effects *in vivo*.

Two of the most studied polymers for vaccine delivery are undoubtedly poly(lactic-co-glycolic acid) (PLGA) and chitosan (derivatives). Both polymers share the properties to be safe, biodegradable and suitable to prepare (nano)particles. PLGA has been used for controlled

drug release purposes for decades and is therefore an obvious choice for encapsulation of antigen. Because of PLGA's hydrophobic character, PLGA particles are generally prepared by oil-in-water emulsification or solvent evaporation techniques, generally resulting in negatively charged, smooth surfaced and spherical particles. These particles are relatively resistant to salt and pH induced instability, and slowly release their content, based on the hydrolysis rate of the polymer [14]. Promising results for nasal vaccination studies using PLGA particles have been reported [10, 15], but also unsuccessful results have been observed [16], which the authors attributed to the lack of mucoadhesiveness and immune-stimulating factors.

Chitosan (CS) is a (under acidic conditions) water soluble, positively charged polymer and therefore has completely different properties than PLGA. CS particles are often prepared by ionic complexation or spray drying techniques, resulting in positively charged, rather irregular shaped complexes [17, 18]. In contrast to PLGA, CS particles have been described as mucoadhesive and their ability to cross epithelial barriers has been widely accepted. However, they are susceptible to dissociation by exposure to salts and are very unstable at physiological pH [19]. An improvement over CS particles are particles prepared from N-trimethyl chitosan (TMC), a derivate of CS that carries a positive charge independent of the pH. Consequently, TMC particles are much more stable at neutral pH than CS particles. Nasal administration of tetanus toxoid or hemagglutinin loaded TMC nanoparticles (TMC NP) resulted in strong antibody- and hemagglutinin inhibition titers, respectively [20, 21]. Interestingly, recently TMC coated PLGA particles (PLGA/TMC) loaded with Hepatitis B surface antigen have been developed; nasal vaccination of mice with these particles resulted in a marked increase of antigen specific antibodies compared to nasal immunization with HBsAg loaded PLGA particles [22].

This study aims to characterize and compare the physical properties of PLGA NP, TMC NP and TMC-coated PLGA NP (PLGA/TMC NP) loaded with ovalbumin (OVA), a model antigen that elicits little response by itself. The impact of these characteristics on important aspects of nasal vaccination like local toxicity, DC uptake and DC maturation were investigated *in vitro* using a model for ciliary beat frequency (CBF) and human monocyte derived dendritic cells. *In vivo*, nasal residence was investigated using a live fluorescence imaging technique; immunogenicity in terms of systemic and secretory antibody (sub)class titers was investigated after nasal as well as i.m. administration to Balb/c mice. In parallel to these experiments extensive investigation into the T-cell responses resulting from nasal immunization with NP has been performed, the results of which will be presented elsewhere.

Materials and Methods

Materials

Ovalbumin (OVA) was purchased from Calbiochem (Merckbioscience, Beeston,UK). N-trimethyl chitosan with a degree of quaternization of 15% was obtained from 92% deacetylated (MW 120 kDa) chitosan (Primex, Alversham, Norway), by NaOH induced methylation as described by Bal [23]. KCl, NaCl, HNa_2PO_4 and KH_2PO_4 were purchased from Merck (VWR, Amsterdam, The Netherlands). Poly(lactic-co-glycolic acid) (PLGA) 50:50 Mw 5000-15000 Da, pentasodium tripolyphosphate (TPP), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), Tween 20, dichloromethane (DCM), dimethyl sulfoxide (DMSO), 2-mercapto ethanol and Protease Type XIV were obtained from Sigma-Aldrich (Steinheim, Germany). 10% polyacrylamide SDS-PAGE gels were acquired from Biorad (Veenendaal, The Netherlands). Poly-(ethylenimine) (PEI) was a generous gift from Wim Hennink (Utrecht Institute of Pharmaceutical Sciences, Utrecht, The Netherlands). Goat anti mouse IgG, IgG1, IgG2a or IgA conjugated with horseradish peroxidase was purchased at Southern Biotech (Birmingham, AL). DMEM-Ham's F12 (1:1) medium, Ultroser G and NU-serum were obtained from Life Technologies Ltd. (Paisley, UK). RPMI 1640, Foetal Bovine Serum (FBS), penicillin-streptomycin (P/S) solution, fluorescein isothiocyanate and Alexa fluor 647 conjugated ovalbumin (OVA-FITC and $OVA_{Alexa\ Fluor\ 647}$) and Lysotracker were acquired from Invitrogen (Breda, The Netherlands).

Nanoparticle preparation and characterization

Nanoparticle preparation

TMC NP were prepared by ionic complexation of TMC with TPP. To 5 ml of a 2 mg/ml TMC solution, 1 ml of 0.1 % w/v OVA solution was added under continuous stirring. Subsequently 0.3 ml water, 2 ml 25 mM Hepes pH 7.4 and 1.7 ml 0.1% w/v TPP solution were added. After 15 min of stirring, particles were collected by centrifugation (10000 g, 15 min) on a glycerol bed, washed once with water and finally resuspended in water and stored at 4°C.

PLGA NP were obtained with a double emulsion method. 50 µl of a 1% w/v OVA solution was dispersed in 1 ml 2.5% w/v PLGA in DCM by tip sonication (15 s 20 W). The obtained water-in-oil (w/o) emulsion was dispersed in 2 ml of 1% v/v Tween 20 by sonication (15 s, 20 W). This w/o/w emulsion was added drop wise to a 50 ml warm (40°C) 0.02% v/v Tween 20 aqueous solution (extraction medium) under continuous stirring to extract and evaporate the

DCM. After 1 hour, particles were collected by centrifugation (8000 g, 10 min), washed twice with water to remove free OVA and stored at 4°C until further analysis.

PLGA/TMC NP were prepared as described for the PLGA NP, with the difference that TMC was added to the extraction medium to a final concentration of 80 µg/ml. Using FITC-labelled TMC we estimated that ca. 90% of the added TMC was associated with the PLGA NP. Supernatants were stored at 4°C for determination of the loading efficiency.

NP containing OVA-FITC or $OVA_{Alexa\ Fluor\ 647}$ were prepared in exactly the same manner by replacing OVA with its fluorescent counterpart.

Physical characterization of nanoparticles

Size and morphology of the NP were visualized with scanning electron microscopy (SEM). 50 µl of 0.1% w/v particle suspension was air dried overnight on a metal stub. Particles were gold/palladium sputtered using a sputter coater device K650X (Emitech, Hailsham, UK) and analyzed with a JEOL JSM-6700F scanning electron microscope (Jeol, Tokyo, Japan).

Mean size distribution was determined with dynamic light scattering (DLS) using a NanoSizer ZS (Malvern Instruments, Malvern UK). The zeta potential of the particles was measured by laser Doppler velocimetry using the same apparatus. Before the measurement, samples were diluted in 5 mM Hepes pH 7.4 until a slightly opalescent dispersion was obtained.

Loading efficiency (LE) was calculated from the amount of OVA detected in the supernatant and expressed as percentage of the total amount of OVA added ($LE= 100-(OVA_{sup}/OVA_{tot}*100)$). OVA concentrations were determined with a BCA protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Assessment of antigen release and stability

To determine the release characteristics, NP containing OVA-FITC were diluted to a concentration of 1 mg/ml NP in 5 ml PBS containing 0.01% Tween 20. Dispersions were incubated at 37°C for 25 days under continuous stirring (200 rpm). At different time points, an aliquot (0.30 ml) was taken (and not replaced with fresh PBS). Aliquots were centrifuged for 20 min at 13000 g and supernatants were stored at 4°C until fluorescence intensity was assessed using an FS920 fluorimeter (Edinburgh Instruments, Campus Livingston, UK).

To determine the stability of the antigen, supernatants from OVA loaded particles were collected after 11 days. Residual OVA was extracted from the pellet according to a protocol by Ghassemi *et al* [24]. Briefly, the pellet was freeze dried overnight, and the lyophilized product

was reconstituted in 200 µl DMSO. Subsequently, 800 µl of 0.5 % w/v SDS and 0.05 M NaOH were added and the mixture was left at room temperature for 2 h.

OVA content was determined with BCA protein assay and a total of 0.6 µg OVA was loaded on a 10% poly(acrylamide) gel under reducing conditions. Protein bands were visualized with silver staining (Silver Stain Plus, Biorad) according to the manufacturer's instructions. Western blot analysis was performed as previously reported [25].

Ciliary beat frequency measurements

Cell isolation and culture

Human nasal epithelial cells were isolated from nasal biopsies according to a previously described method [26]. The cells were plated in 12-well plates pre-coated with 0.2% rat tail collagen at a density of $5*10^5$ cells/well in a final volume of 2 ml medium and incubated at 37°C and 5% CO_2. The medium was refreshed 24 h after plating and subsequently every other day. Ciliary beat frequency (CBF) measurements were performed on day eight to ten after plating. All experiments were performed in an air-conditioned room at a constant temperature of 22°C. Cell culture plates were removed from the incubator one hour prior to the experiment, in order to allow the medium to adapt to the environmental temperature. Cells were exposed for 45 minutes to 2.5 ml DMEM-Ham's F12 (1:1) medium (negative control), 0.5 mg/ml or 5 mg/ml of nanoparticle dispersion or 0.5 mg PEI (positive control), after which cells were washed twice with DMEM-Ham's F12 (1:1) medium. Cells were allowed to recover for 90 minutes after which CBF was assessed again.

Data acquisition

An inverted microscope (Olympus IX70) was used at a magnification of 600 times. A MotionScope high-speed digital camera and PCI application software, running in a Windows 2000 environment (Redlake MASD Inc., San Diego, CA), were used for image acquisition. The images were captured at a frame rate of 500 frames per second with a sampling interval of 2 ms, before, after and during exposure to the nanoparticles. A sequence of 1024 images was recorded for each area. Each sequence of frame-by-frame images was stored in a file folder containing 1024 TIF format files for later analysis. CBF was calculated as described before [27, 28].

Dendritic cells studies

Human monocyte derived dendritic cell culture

Monocytes were freshly isolated from human donor blood before each experiment by means of density gradients (Ficoll and Percoll) and depletion of platelets was performed by adherence of the monocytes in 24-well plate (Corning, Schiphol, The Netherlands) followed by washing. Monocytes (5*105 cells/well) were maintained for 6 days in RPMI 1640, supplemented with 10% v/v FBS, 1% v/v glutamine, 1% v/v P/S, GM-CSF 250 U/ml (Biosource-Invitrogen, Breda, The Netherlands,) and IL-4 100 U/ml (Biosource) at 37°C and 5% CO2 to differentiate into immature DC (imDC). Medium was refreshed after 3 days.

Interaction of nanoparticles with dendritic cells

ImDC were incubated for 4 h at 37°C in RPMI 1640 and 500 U/ml GMCSF with 2 µg/ml OVA-FITC either free or encapsulated in TMC, PLGA or PLGA/TMC NP. Cells were washed 3 times with PBS containing 1% w/v bovine serum albumin and 2% v/v FBS before OVA-FITC association with DC was quantified using flow cytometry (FACSCantoII, Becton Dickinson). Live cells were gated based on forward and side scatter. OVA-FITC association was expressed as the mean fluorescence intensity (MFI) in the FL-1 channel.

For confocal microscopy, 50,000 imDC were plated on a poly-lysine coated Petri dish (Corning) and incubated for 30 minutes at 37°C. Cells were washed with PBS, and exposed for 1 h to OVAAlexa Fluor 647 containing formulations. Cells were washed 3 times with PBS and exposed to 1 µM Lysotracker® for 15 minutes. Cells were washed 2 more times before visualization.

Effect of nanoparticles on DC maturation

DC were incubated for 48 h at 37°C in RPMI 1640 and 500 U/ml GMCSF with 2 µg/ml OVA, either free or encapsulated in PLGA, PLGA/TMC or TMC NP and LPS (100 ng/ml) as a positive control. Cells were washed 3 times with PBS containing 1% w/v bovine serum albumin and 2% v/v FBS and incubated for 30 min with a mixture of 50x diluted anti-HLA-DR-FITC, anti-CD83-phycoerythrin(PE) and anti-CD86-allophycocyanin(APC) (Becton Dickinson) on ice, to measure expression of MHCII, CD83 and CD86 molecules on the DC' cell surface, respectively. Cells were washed and expression of MHCII, CD83 and CD86 was quantified using flow cytometry (FACSCantoII, Becton Dickinson), assuming 100% maturation for LPS treated DC. Live cells were gated based on forward and side scatter.

***In vivo* studies**

Determination of nasal residence time

Nasal residence time measurements were performed in accordance to the protocol described by Hagenaars et al. [29]. In short, female Balb/c (nu/nu) mice (Charles River, L'Árbresle, France) mice were lightly anesthetized using isofluorane prior to the administration of 5 µg OVA conjugated to a near infrared dye (IRdye™ 800CW, LI-Cor, USA). Nose was cleaned with a paper towel and immediately fluorescence intensity in the nasal cavity was determined using an IVIS Spectrum® (CaliperLS, USA). Every 10 minutes, fluorescence intensity was determined. Between measurements, mice were conscious.

Administration of antigens, immunization and sampling schedules

Female Balb/c mice received 20 µg OVA per nasal or i.m. administration. One priming dose was followed by 2 nasal or i.m booster doses 3 and 6 weeks after priming. For nasal administration, formulations were applied in a volume of 10 µl PBS, 5 µl per nostril. For i.m. administration, 25 µl of formulation in PBS was injected in the thigh muscle. Blood samples were taken from the tail vein before every immunization and 2 weeks after the final booster dose.

Determination of serum IgG, IgG1, IgG2a and secretory IgA

Microtiter plates were coated with 100 ng OVA in carbonate buffer pH 9.4 for 24 hours at 4°C. To reduce non specific binding, wells were blocked with 1% BSA in PBS for 1 hour at 37°C. 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. OVA specific antibodies were detected using horseradish peroxidase (HRP) conjugated goat anti mouse IgG, IgG1, IgG2a or IgA (1 hour 37°C) and by incubating with tetramethylbenzidine (TMB)/H_2O_2 in acetate buffer pH 5.5 for 15 min at room temperature. Reaction was stopped with 2 M H_2SO_4 and absorbance was determined at 450 nm with an EL808 microplatereader (Bio-Tek Instruments, Bad Friedrichshall, Germany)

Statistics

All the data of the in vitro studies were analyzed with a one-way ANOVA with Bonneferoni's post-test, with the exception of size and zeta potential measurements, which were analyzed with a Students T-test. Antibody titers were analyzed with a Kruskal-Wallis test with Dunns post-test. Statistics were performed using GraphPad 5.0 for Windows.

Results

Physical characterization of nanoparticles

The NP characteristics are summarized in Table 1. Dynamic light scattering showed fairly monodisperse (PDI<0.25) NP with an average size of about 300 nm (PLGA and TMC NP) or 450 nm (PLGA/TMC NP). PLGA NP carried a negative charge at pH 7.4, whereas TMC NP and PLGA/TMC NP were slightly positively charged. Encapsulation efficiency of OVA (pI 4.8) was much higher in the positively charged particles (71.6% and 60.2%) compared to PLGA NP (34.2% $p<0.05$).

Table 1: Physical properties of OVA-loaded nanoparticles. Values represent mean +/- standard deviation of 5 independently prepared batches.

	Size (nm)	PDI	Zetapotential (mV)	Loading Efficiency (%)
PLGA/OVA	320 +/- 17.9	0.151 +/- 0.033	- 48.2 +/- 0.59	34.2 +/- 3.3
PLGA/TMC/OVA	448 +/- 55.9	0.234 +/- 0.031	24.5 +/- 0.90	71.6 +/- 6.2
TMC15/TPP/OVA	258 +/- 28.8	0.200 +/- 0.020	10.4 +/- 0.20	60.2 +/- 4.1

SEM reveals the size and the shape of the particles after air drying. The mean size of the PLGA and PLGA/TMC NP corresponded well to the size found with DLS. TMC NP appeared to be smaller than measured with the DLS, probably due to dehydration of the sample. PLGA and PLGA/TMC NP had a spherical appearance and a smooth surface (*Figure 1*). In contrast, TMC NP were irregularly shaped and had a ruffled surface area.

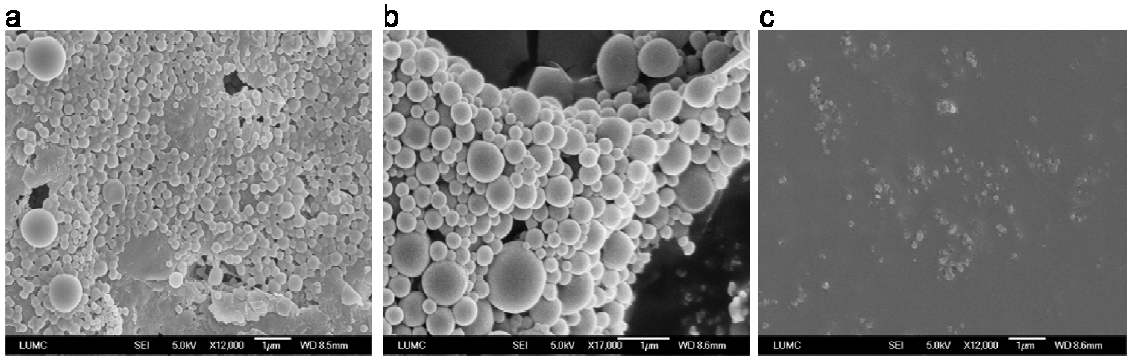


***Figure 1**: Scanning electron microscopy images of OVA-loaded nanoparticles: a) PLGA NP, b) PLGA/TMC NP and c) TMC NP.*

To simulate the stability of the NP after nasal administration, the size of the NP was assessed *in vitro* by incubation in PBS at 37°C (*Figure 2*). PLGA/TMC and TMC NP showed a small (<30%), but not significant ($p>0.05$) size increase, within 8 hours. TMC NP showed signs of aggregation, as the PDI increased ($p<0.05$). No changes in size and PDI values for the PLGA based nanoparticles were observed.

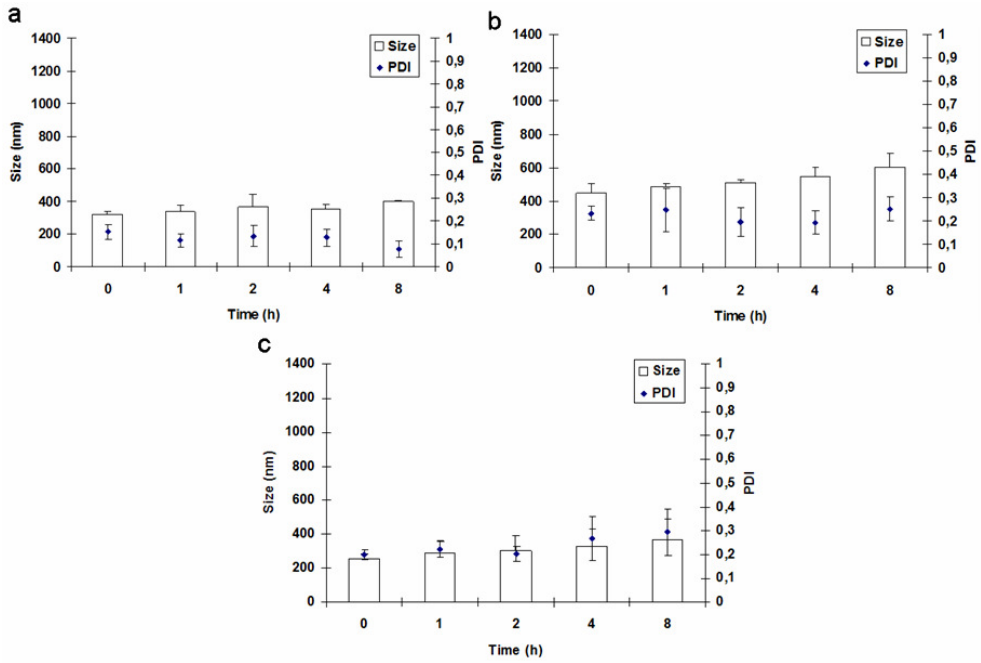


***Figure 2**: Short term (8 h) stability of OVA-loaded NP in PBS at 37°: a) PLGA NP, b) PLGA/TMC NP and c) TMC NP. Results are the average +/- SD of 3 independently prepared batches.*

Antigen release and stability

Release of OVA-FITC from the nanoparticles was monitored over 25 days in PBS pH7.4 at 37°C. PLGA NP showed no significant burst release (*figure 3b*) and up to 80% of their original content in 25 days (*figure 3a*). In contrast, TMC NP showed 20-30% release within the first 24 h, followed by no release over the remainder of 25 days. However, the release of OVA by these particles was enhanced by further dilution in PBS (*figure 3b*), showing that the disintegration of TMC NP is dependent on the concentration and thus is likely to occur very rapidly *in vivo*. This concentration dependent initial release was not observed for PLGA NP. PLGA/TMC NP showed release characteristics of TMC as well as PLGA NP, as a moderate concentration dependent OVA release over the first 24 h was observed (20% at 1 mg/ml, *figure 3b*), followed by progressive release up to 100% after 12 days (*figure 3a*).

As PLGA particles have been described as deleterious for the stability of incorporated biopharmaceuticals [30], the integrity of encapsulated (*Figure 4a*) OVA was investigated with SDS-PAGE and Western blotting. Sonication and contact with DCM did lead to some degradation and aggregation, however the majority of the OVA appeared to be intact with respect to preservation of size and epitopes (*Figure 4a lane 2,4,5*). Moreover, encapsulation in PLGA and TMC NP did not seem to adversely affect the integrity of OVA (*figure 4a lane 4-6*). However, OVA extracted from PLGA and PLGA/TMC was not recognized by anti-OVA IgG antibodies to a similar extent as native OVA or OVA extracted from TMC NP (*figure 4b*), indicating that some of the B-cell epitopes of OVA may have been damaged during the encapsulation process.

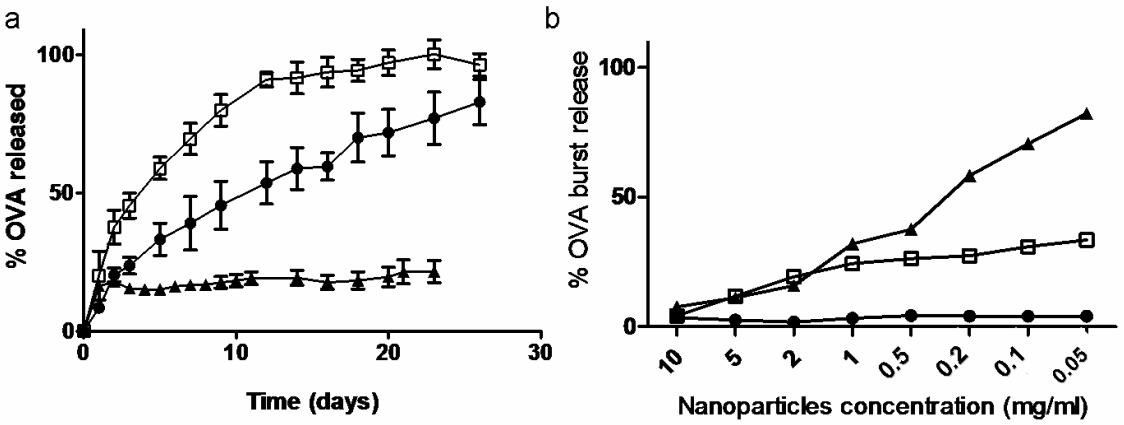


Figure 3: OVA release from PLGA NP (circles), PLGA/TMC NP (squares) and TMC NP (triangles). a) OVA release from 1 mg/ml particle dispersions was monitored over 25 days at 37°C in PBS. b) Burst release of OVA from these particles as function of NP concentration, assessed after 1 h incubation in PBS (pH7.4). Results are the average +/- SD of 3 independently prepared batches.

Toxicity

To explore the safety characteristics of the particles for nasal vaccination, the CBF was measured after 45 min exposure to the particle dispersions in PBS (*Figure 5*). Poly-(ethylenimine) (PEI) was used as positive (toxic) control. Application of a 0.5 mg/ml PEI solution resulted in the complete disappearance of the CBF, within 45 minutes. After removal of the polymer, no recovery of the CBF was recorded. All nanoparticulate formulations were less toxic than PEI solution and did not significantly decrease the CBF. Only at high concentrations (5 mg/ml) TMC/TPP slightly decreased the CBF by about 20% (*data not shown*).

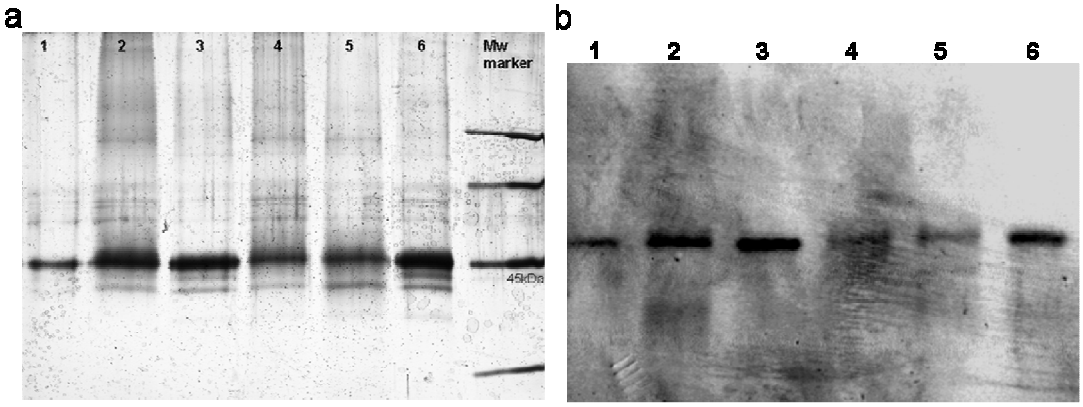


***Figure 4**: Stability of OVA after encapsulation into nanoparticles. a) Silver stained SDS-PAGE gel; b) Anti-OVA Western blot of a second gel run in parallel. 1 = OVA stock solution, 2 = OVA 2x15 s sonicated, 3 = OVA 2x15 s sonicated + DCM extracted, 4 = OVA extracted from PLGA NP, 5 = OVA extracted from PLGA/TMC NP and 6 = OVA extracted from TMC NP. Gel and blot are representative examples of 3 experiments.*

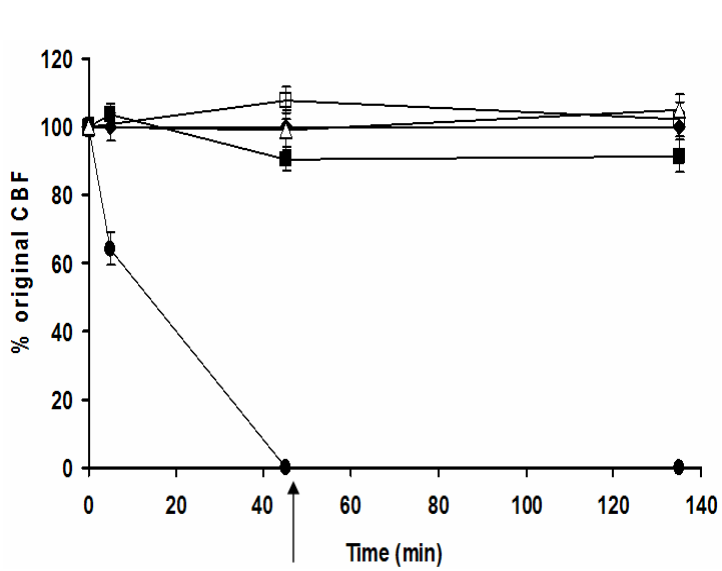


***Figure 5**: Ciliary beat frequency after exposure to 0.5 mg/ml OVA-loaded nanoparticles as a measure for nasal cilia toxicity. Nasal epithelium was exposed for 45 min to formulations, after which the epithelium was washed (arrow) and the CBF allowed to recover for 90 min. Closed diamond = non-exposed, closed circle= PEI solution (0.5 mg/ml), open square= PLGA, open triangle= PLGA/TMC and closed square= TMC/TPP. Data represent mean of 3 donors +/- SD.*

Nasal residence time

Prolonging the residence time of an antigen may be crucial for nasal delivery, as it increases the chance of absorption into the nasal epithelium. Using a fluorescent label we were able to study the clearance of OVA from the nasal cavity. An exponential decay in fluorescence intensity was observed for all formulations (*Figure 6*). The data could be reasonably fitted by an exponential decay function, from which apparent first-order clearance rate constants were determined (*Figure 6 insert*). Compared to an OVA solution, only TMC NP significantly decreased the clearance rate ($p<0.01$). The particulate structure of PLGA NP did not have an effect on the clearance rate of OVA, nor did a TMC coating around it.

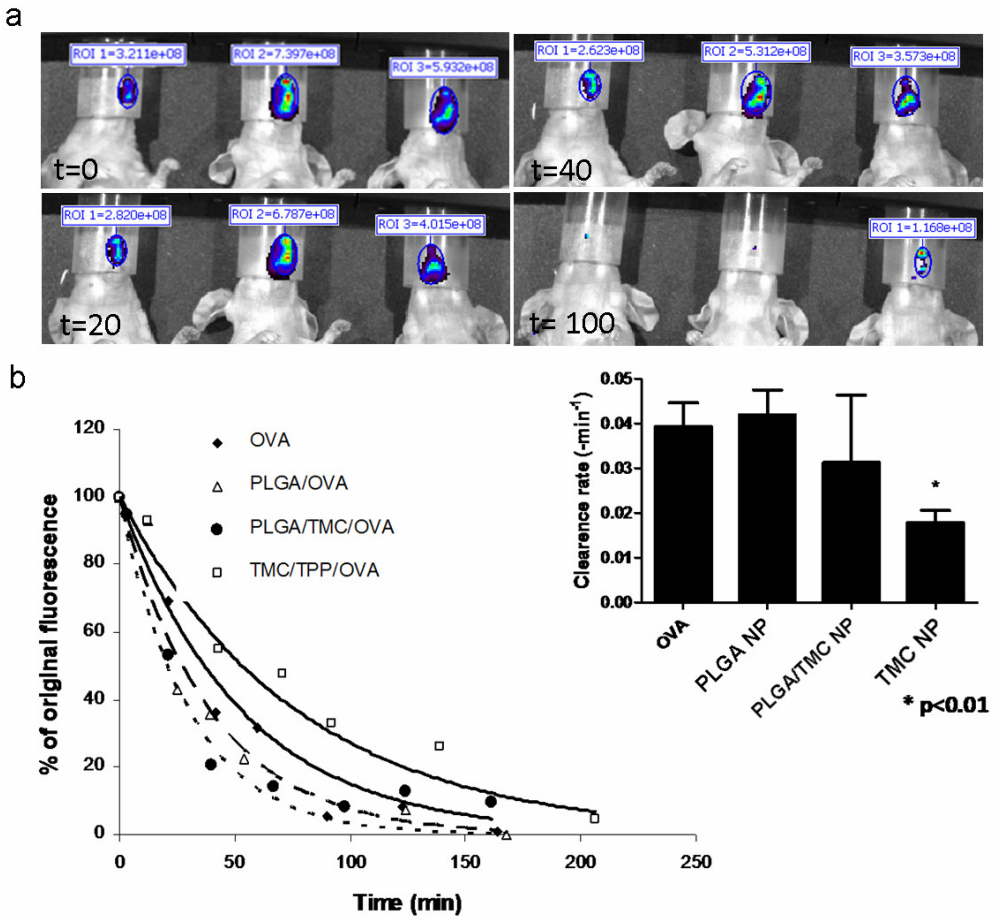


***Figure 6**: Nasal residence time of OVA determined using fluorescence detection of OVA-IRdye CW 800. a) Emission (λ=800 nm) 0, 20, 40 and 100 min after nasal administration of OVA-IRdye CW 800. b) Intensity of fluorescence signal from the nasal cavity normalized for time point 0. Individual time points were fitted using a model for exponential decay. b-insert) apparent first-order clearance rate constants of OVA from the nasal cavity were derived from exponential fits. Data represent mean +/- SD of n=3. * p<0.01*

Dendritic cells studies

Interaction and uptake of the nanoparticles by monocyte derived DC was studied using flow cytometry (*Figure 7a*) and confocal microscopy (F*igure 7b,c*). The positively charged particles (PLGA/TMC NP and TMC NP) interacted strongly with DC compared to PLGA NP and OVA alone ($p<0.05$). However, in the same experiment conducted at 4°C similar fluorescence levels for PLGA/TMC NP and TMC NP treated cells were observed, indicating that the fluorescence was mainly caused by association to the cell membrane rather than uptake by the DC. Indeed, confocal microscopy showed little evidence for TMC NP uptake (nor PLGA/TMC uptake, data not shown) by DC as the particles were almost exclusively detected on the outside of the cell membrane (*Figure 7c*). This is in contrast to a solution of OVA which accumulated in lysosomal compartments (*Figure 7b*).

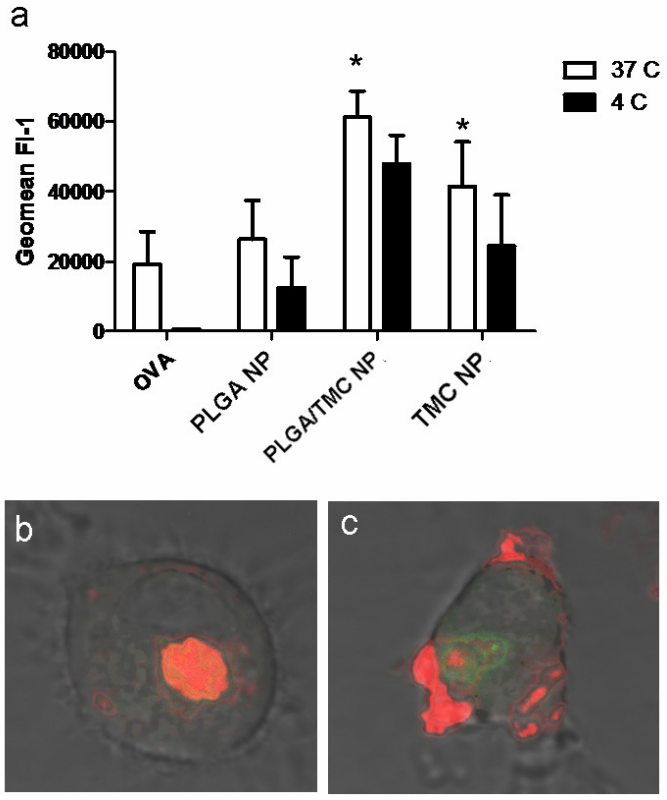


***Figure 7**: Interaction of OVA-loaded nanoparticles with DC. a) Association of particles with human DC quantified using flow cytometric analysis. Bars represent mean +/- SD of 6 different monocytes donors. *p<0.05 compared to OVA 37°C. Merged confocal microscopy image of DC exposed to b) $OVA_{alexafluor647}$ (Red) and Lysotracker® (Green) or c) $TMC/TPP/OVA_{alexafluor647}$ and Lysotracker. Orange corresponds to OVA colocalizing with lysosomes.*

Despite being poorly taken up by DC, TMC NP were able to induce DC maturation (*figure 8*). Although the expression of all measured maturation markers was not as extensive as after LPS exposure, it was significantly increased ($p<0.05$) compared to OVA or PLGA NP, both of

which did not result in increased DC maturation. Again TMC coated PLGA NP appeared to be the middle ground between PLGA and TMC NP, as all maturation markers seemed to be a bit upregulated, but only MHCII to a significant extent.

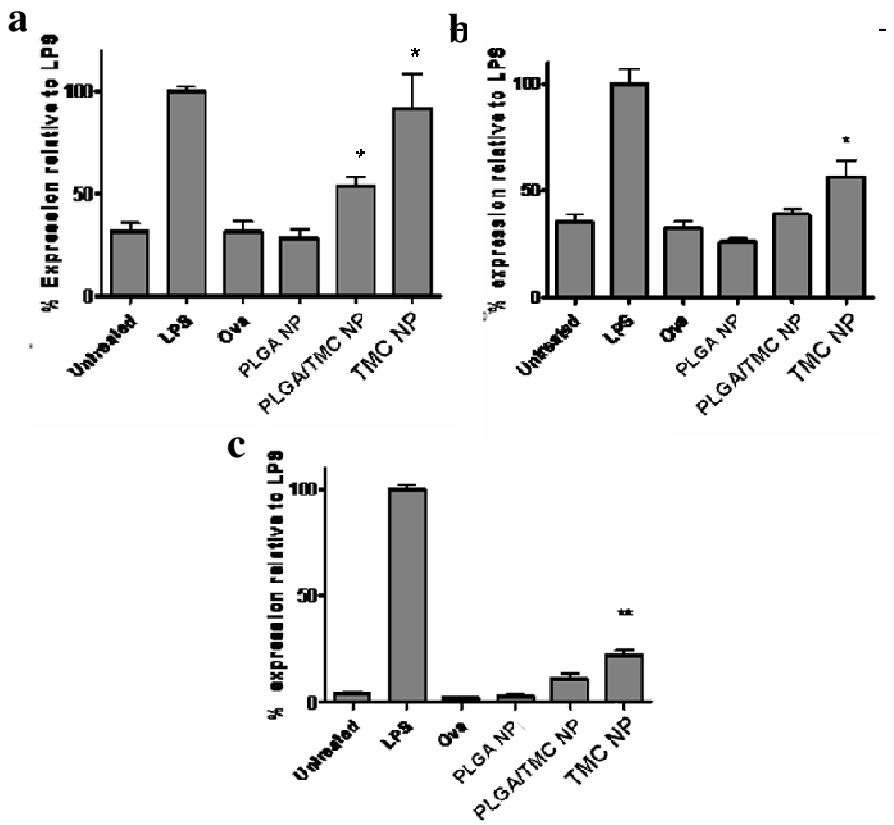


***Figure 8**: DC maturation after 48h stimulation with OVA-containing nanoparticles. Levels of a) MHCII, b) CD83 and c) CD86 were expressed as a percentage of LPS stimulated DC. Histogram represents the mean of 6 independent experiments. Error bars are SEM. * p<0.05 **p<0.01 vs. OVA-treated DC.*

Immunogenicity

Nasal vaccination revealed considerable differences between the NP. Negligible IgG titers were detected after nasal vaccination with PLGA NP and PLGA/TMC NP, whereas only a priming dose of TMC NP was necessary ($p<0.001$ compared to OVA) to induce detectable OVA specific IgG antibodies (*figure 9*). After the 3 nasal challenges, TMC NP immunized mice even

showed similar IgG titers as their i.m. vaccinated counterparts. Furthermore, only nasal washes from TMC NP nasally immunized mice contained OVA specific sIgA (*figure 10b*). All mice responded to i.m. immunization, irrespective of the formulation administered (*figure 9*). However, the NP formulations were more immunogenic than an OVA solution. Both PLGA NP and TMC NP elicited high IgG titers after a priming dose, whereas the titers induced by PLGA/TMC NP were only slightly higher than the OVA induced titers ($p<0.01$). Vaccination with PLGA NP caused a significant shift in the IgG1/IgG2a ratio towards IgG2a compared to i.m. OVA vaccination (*figure 10a*).

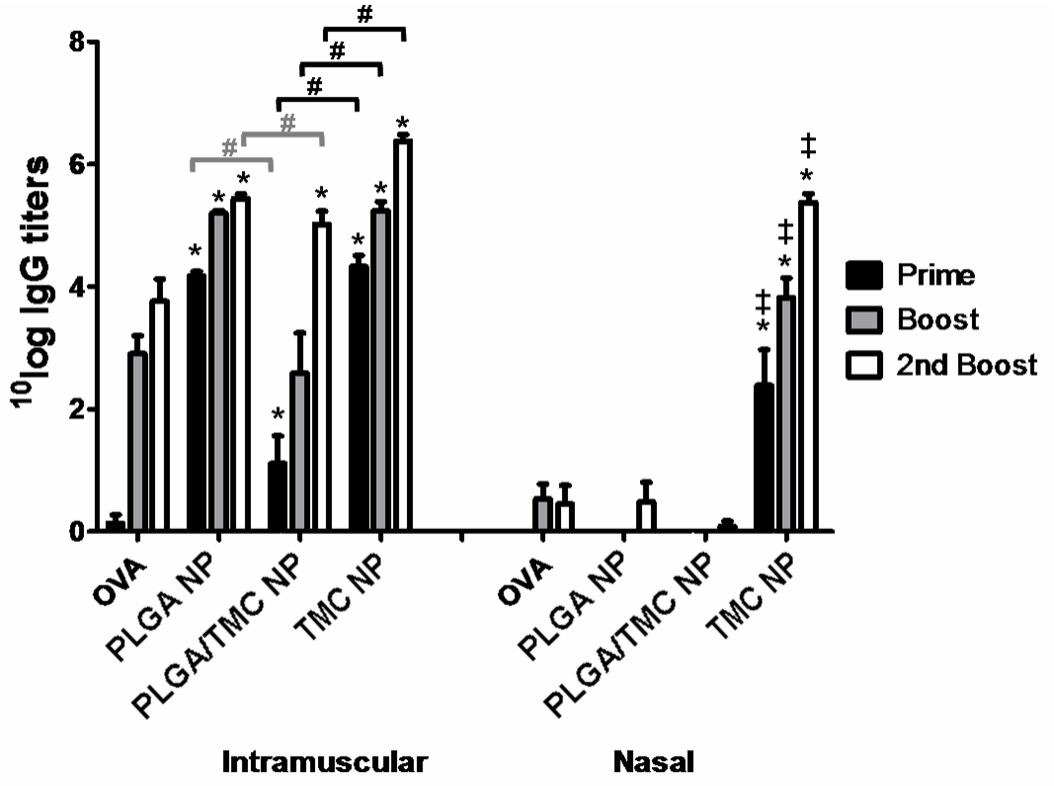


***Figure 9**: OVA specific IgG titers in serum of Balb/c mice 3 weeks after a priming, booster and 2nd booster dose of 20 µg OVA administered i.m. or nasally. Data represent mean +/- SEM, n=8. * p<0.05 compared to OVA i.m., ‡ p<0.05 compared to OVA nasal, # p<0.05.*

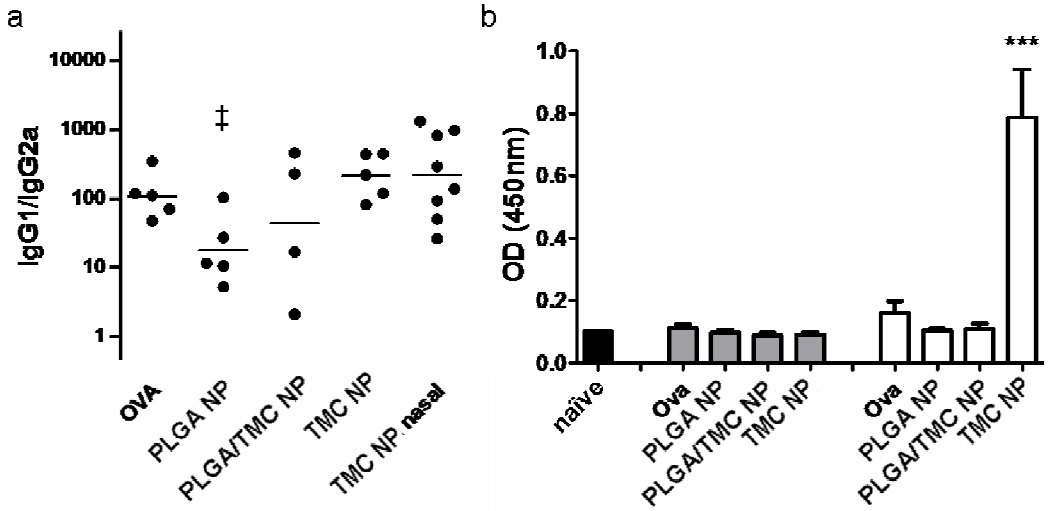


Figure 10**: a) IgG1/IgG2a balance after i.m. vaccination, and for TMC NP also after nasal vaccination. ‡ p<0.05 compared to OVA. Bar represents mean. b) OVA specific sIgA in nasal washes of mice after i.m. (grey bars) or nasal (white bars) administration. Bars represent mean (i.m. n=5, nasal n=8) +/- SEM . p<0.001 compared to naïve mice.*

Discussion

Although nanoparticles have been described as very promising nasal vaccine carriers [8, 13], surprisingly little is known about the physicochemical properties of nanoparticles in relation to the immune response they elicit. The particle size is probably one of the parameters which is most adequately described, as several studies using micro- and nanoparticles point to smaller particles being more immunogenic [8, 10, 11, 31-33]. Studies by Jung et al. and Gutierro et al. seem to indicate, however, that intranasally applied antigen-loaded particles of about 200 nm and 500 nm do not differ in immunogenicity [10, 32]. As the nanoparticles in our present study were all smaller than 500 nm and showed only minor differences in size compared to the above range, size variation between the particles is most probably not the factor that caused the differences in immunogenicity. To a somewhat lesser extent, the zeta potential of particles has been investigated, leading to the conclusion that a positive surface charge may be favourable in nasal vaccination [9, 21, 34, 35]. The result of our present study supports that conclusion, as the positively charged TMC NP outperformed the negatively charged PLGA NP after nasal administration. However, it is also clear that the zeta potential is not the sole determinant of the resulting antibody responses. TMC and PLGA/TMC

NP are both positively charged, but TMC NP induced superior IgG titers compared to PLGA/TMC NP.

Although measuring immune responses after nasal administration of antigen loaded nanoparticles is very useful, it restricts us to a mere trial-and-error based approach of nasal vaccine design. For instance, it does not answer the question why PLGA/TMC NP elicit a different immune response than TMC NP. Both particles are in the same size range and do not contain any known immunomodulatory substances other than TMC [20, 36, 37]. Focusing on the various aspects of nasal vaccination; like the clearance from the nasal cavity, the uptake by DC and the maturation of antigen-presenting cells as function of particle characteristics may answer this question and would allow us to improve nanoparticulate vaccine carriers in a rational way. Using this approach, we were able to explain differences in immunogenicity between PLGA, PLGA/TMC and TMC NP. Both PLGA and PLGA/TMC NP failed to elicit an antibody response after nasal vaccination. Nonetheless, after i.m. administration both particles induced higher IgG titers than OVA, implying that these particles can augment the immune response, but are not suitable for nasal administration. In contrast, TMC NP elicited strong antibody responses via both vaccination routes, indicating that TMC NP have certain characteristics which are profitable for nasal vaccination.

Like PLGA and PLGA/TMC NP, TMC NP were found not to be toxic to the cilia (*figure 5*) or mucosal epithelial cells [38-40] and are therefore likely do not damage the nasal epithelium. TMC NP do not promote the uptake by DC (*figure 7*), but do prolong the residence time of OVA in the nasal cavity (*figure 6*), compared to PLGA and PLGA/TMC NP. The nasal residence time of OVA encapsulated in the particles correlates with the IgG titers of mice nasally challenged with these particles, suggesting that the difference in immunogenicity between the particles is related to the delivery of antigen into the nasal epithelium.

PLGA's immunopotentiating effect after parenteral administration has been attributed to its slow release characteristics, leading to a depot formation and subsequently causing enhanced B-cell and T-cell proliferation [41-43]. Indeed, PLGA and PLGA/TMC NP released their content over a prolonged period of time (*figure 3*) and showed an increased antibody response when administered by i.m. injection. PLGA/TMC NP released OVA faster than PLGA *in vitro*, which may contribute to the slightly lower IgG titers in mice immunized i.m. with PLGA/TMC NP, as compared to the PLGA NP group. Although depot formation can be a mechanism to potentiate the immune response after parenteral injections, it is very unlikely that it is a driving force behind nasal vaccination, as the nasal residence time is limited. Moreover, in contrast to uptake by DC, antigen uptake by B-cells is a highly specific process mediated by the contact of the antigen with the B-cell receptor [44]. Therefore, uptake of OVA

by B-cells will be dependent on either surface coated or released OVA and encapsulated antigen is not easily taken up by B-cells [45]. As a consequence, for nasal vaccination a slow release rate may be detrimental as only little time for B-cell uptake is available. Keijzer *et al*. indeed showed that nasal immunization of mice with TMC NP and to a lesser extent PLGA/TMC, but not PLGA NP, results in OVA specific B-cells in the nasal associated lymphoid tissue and in cervical lymph nodes (manuscript in preparation). In the same study nasal immunization with PLGA NP or PLGA/TMC NP of mice which received an adoptive transfer of OVA specific $CD4^+$ T-cells showed effective T-cell activation and proliferation in the nasal associated lymphoid tissue and cervical lymph nodes. This indicates that PLGA and PLGA/TMC NP do cross the nasal epithelium and are taken up by DC (which in turn are capable of activating T-cells), but do not deliver their antigen to B-cells as effectively as TMC NP.

Finally, the nasal epithelium is renowned for being a tolerogenic site, making maturation of imDC into mature DC essential for an effective nasal vaccine. TMC NP were shown to stimulate the maturation of imDC, which may contribute to TMC NP's effectiveness as a nasal adjuvant. OVA-loaded PLGA particles have been reported before not to increase DC maturation [39, 46] and although TMC has been reported as an adjuvant [23], the addition of TMC to the PLGA particles only caused a small increase in expression of maturation markers (Figure 8). This could be explained by the substantially higher ratio TMC:OVA in the TMC NP compared to the PLGA/TMC particles (10:1 vs. 2:1), or to the different architecture of TMC NP versus PLGA/TMC particles.

The data presented in this study indicate that contributing factors to TMC NP being a good nasal vaccine carrier system may be that they (i) prolong the nasal residence time of its incorporated antigen, (ii) quickly release the antigen to promote the formation of OVA specific B-cells and (iii) effectively induce DC maturation breaking the nasal tolerance. However, TMC NP vaccinated mice showed little evidence of activation of the cellular arm of the immune system; the IgG1 titers far exceeded the IgG2a titers (*figure 10a*). This is in line with previous studies demonstrating that the use of TMC as an adjuvant induces Th2 type responses [20, 23, 47]. The addition of a Th1 skewing adjuvant (like CpG) to the TMC NP could make these carriers a more complete nasal vaccine formulation.

Conclusion

The composition and characteristics of nanoparticles greatly influence the extent and the type of immune response elicited after nasal vaccination. TMC NP were shown to be superior over PLGA NP and PLGA/TMC NP in the elicitation of antibody responses after nasal administration. This may be due to their mucoadhesiveness, the rapid release of the contained antigen, and immune stimulatory capacity, in order to respectively prolong the nasal residence time, promote uptake by B-cells and activate DCs.

Acknowledgements

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

References

1. Debin, A., et al., *Intranasal immunization with recombinant antigens associated with new cationic particles induces strong mucosal as well as systemic antibody and CTL responses.* Vaccine, 2002. **20**(21-22): p. 2752-63.
2. Hamdy, S., et al., *Enhanced antigen-specific primary CD4+ and CD8+ responses by codelivery of ovalbumin and toll-like receptor ligand monophosphoryl lipid A in poly(D,L-lactic-co-glycolic acid) nanoparticles.* J Biomed Mater Res A, 2007. **81**(3): p. 652-62.
3. Khatri, K., et al., *Plasmid DNA loaded chitosan nanoparticles for nasal mucosal immunization against hepatitis B.* Int J Pharm, 2008. **354**(1-2): p. 235-41.
4. Read, R.C., et al., *Effective nasal influenza vaccine delivery using chitosan.* Vaccine, 2005. **23**(35): p. 4367-74.
5. Revaz, V., et al., *Humoral and cellular immune responses to airway immunization of mice with human papillomavirus type 16 virus-like particles and mucosal adjuvants.* Antiviral Res, 2007. **76**(1): p. 75-85.
6. Sloat, B.R. and Z. Cui, *Strong mucosal and systemic immunities induced by nasal immunization with anthrax protective antigen protein incorporated in liposome-protamine-DNA particles.* Pharm Res, 2006. **23**(2): p. 262-9.
7. Sorichter, S., et al., *Immune responses in the airways by nasal vaccination with systemic boosting against Pseudomonas aeruginosa in chronic lung disease.* Vaccine, 2009. **27**(21): p. 2755-9.
8. Koping-Hoggard, M., A. Sanchez, and M.J. Alonso, *Nanoparticles as carriers for nasal vaccine delivery.* Expert Rev Vaccines, 2005. **4**(2): p. 185-96.
9. Jaganathan, K.S. and S.P. Vyas, *Strong systemic and mucosal immune responses to surface-modified PLGA microspheres containing recombinant hepatitis B antigen administered intranasally.* Vaccine, 2006. **24**(19): p. 4201-11.
10. Gutierro, I., et al., *Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres.* Vaccine, 2002. **21**(1-2): p. 67-77.
11. Vila, A., et al., *PLA-PEG particles as nasal protein carriers: the influence of the particle size.* Int J Pharm, 2005. **292**(1-2): p. 43-52.
12. Sharma, S., et al., *Pharmaceutical aspects of intranasal delivery of vaccines using particulate systems.* J Pharm Sci, 2009. **98**(3): p. 812-43.
13. Slütter, B., N. Hagenaars, and W. Jiskoot, *Rational design of nasal vaccines.* J Drug Target, 2008. **16**(1): p. 1-17.
14. Corrigan, O.I. and X. Li, *Quantifying drug release from PLGA nanoparticulates.* Eur J Pharm Sci, 2009. **37**(3-4): p. 477-85.
15. Gutierro, I., et al., *Influence of dose and immunization route on the serum Ig G antibody response to BSA loaded PLGA microspheres.* Vaccine, 2002. **20**(17-18): p. 2181-90.
16. Lemoine, D., et al., *Intranasal immunization against influenza virus using polymeric particles.* J Biomater Sci Polym Ed, 1999. **10**(8): p. 805-25.
17. Boddohi, S., et al., *Polysaccharide-Based Polyelectrolyte Complex Nanoparticles from Chitosan, Heparin, and Hyaluronan.* Biomacromolecules, 2009. **77**(1): p. 60-8.
18. Gan, Q., et al., *Modulation of surface charge, particle size and morphological properties of chitosan-TPP nanoparticles intended for gene delivery.* Colloids Surf B Biointerfaces, 2005. **44**(2-3): p. 65-73.
19. Amidi, M., et al., *Chitosan-based delivery systems for protein therapeutics and antigens.* Adv Drug Deliv Rev, 2010. **62**(1): p. 59-82.
20. Amidi, M., et al., *N-trimethyl chitosan (TMC) nanoparticles loaded with influenza subunit antigen for intranasal vaccination: biological properties and immunogenicity in a mouse model.* Vaccine, 2007. **25**(1): p. 144-53.

21. Sayin, B., et al., *Mono-N-carboxymethyl chitosan (MCC) and N-trimethyl chitosan (TMC) nanoparticles for non-invasive vaccine delivery.* Int J Pharm, 2008. **363**(1-2): p. 139-48.
22. Pawar, D., et al., *Evaluation of Mucoadhesive PLGA Microparticles for Nasal Immunization.* AAPS J, 2010. **12**(2): p. 130-7.
23. Bal, S.M., et al., *Efficient induction of immune responses through intradermal vaccination with N-trimethyl chitosan containing antigen formulations.* J Control Release, 2009. **142**(3): p. 374-83.
24. Ghassemi, A.H., et al., *Preparation and characterization of protein loaded microspheres based on a hydroxylated aliphatic polyester, poly(lactic-co-hydroxymethyl glycolic acid).* J Control Release, 2009. **138**(1): p. 57-63.
25. Slütter, B., et al., *Conjugation of ovalbumin to trimethyl chitosan improves immunogenicity of the antigen.* J Control Release, 2010. **143**(2): p. 207-14.
26. Jorissen, M., et al., *Ciliogenesis and coordinated ciliary beating in human nasal epithelial cells cultured in vitro.* Acta Otorhinolaryngol Belg, 1989. **43**(1): p. 67-73.
27. Mallants, R., M. Jorissen, and P. Augustijns, *Effect of preservatives on ciliary beat frequency in human nasal epithelial cell culture: single versus multiple exposure.* Int J Pharm, 2007. **338**(1-2): p. 64-9.
28. Dimova, S., et al., *High-speed digital imaging method for ciliary beat frequency measurement.* J Pharm Pharmacol, 2005. **57**(4): p. 521-6.
29. Hagenaars, N., et al., *Role of trimethylated chitosan (TMC) in nasal residence time, local distribution and toxicity of an intranasal influenza vaccine.* J Control Release, 2010. **144**(1): p. 17-24.
30. van de Weert, M., *Structural integrity of pharmaceutical proteins in polymeric matrices*, in *Biopharmacy and Pharmaceutical Technology*. 2001, Utrecht University: Utrecht. p. 168.
31. Fujimura, Y., et al., *Uptake of microparticles into the epithelium of human nasopharyngeal lymphoid tissue.* Med Mol Morphol, 2006. **39**(4): p. 181-6.
32. Jung, T., et al., *Tetanus toxoid loaded nanoparticles from sulfobutylated poly(vinyl alcohol)-graft-poly(lactide-co-glycolide): evaluation of antibody response after oral and nasal application in mice.* Pharm Res, 2001. **18**(3): p. 352-60.
33. Sandri, G., et al., *Nanoparticles based on N-trimethylchitosan: evaluation of absorption properties using in vitro (Caco-2 cells) and ex vivo (excised rat jejunum) models.* Eur J Pharm Biopharm, 2007. **65**(1): p. 68-77.
34. Baca-Estrada, M.E., M. Foldvari, and M. Snider, *Induction of mucosal immune responses by administration of liposome-antigen formulations and interleukin-12.* J Interferon Cytokine Res, 1999. **19**(5): p. 455-62.
35. Joseph, A., et al., *A new intranasal influenza vaccine based on a novel polycationic lipid--ceramide carbamoyl-spermine (CCS) I. Immunogenicity and efficacy studies in mice.* Vaccine, 2006. **24**(18): p. 3990-4006.
36. Hagenaars, N., et al., *Physicochemical and immunological characterization of N,N,N-trimethyl chitosan-coated whole inactivated influenza virus vaccine for intranasal administration.* Pharm Res, 2009. **26**(6): p. 1353-64.
37. Slütter, B., et al., *Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination.* J Control Release, 2009.
38. Amidi, M., et al., *Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system.* J Control Release, 2006. **111**(1-2): p. 107-16.
39. Slütter, B., et al., *Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination.* J Control Release, 2009. **138**(2): p. 113-21.
40. Verheul, R.J., et al., *Synthesis, characterization and in vitro biological properties of O-methyl free N,N,N-trimethylated chitosan.* Biomaterials, 2008. **29**(27): p. 3642-9.
41. Luzardo-Alvarez, A., et al., *Biodegradable microspheres alone do not stimulate murine macrophages in vitro, but prolong antigen presentation by macrophages in vitro and stimulate a solid immune response in mice.* J Control Release, 2005. **109**(1-3): p. 62-76.
42. Kanchan, V., Y.K. Katare, and A.K. Panda, *Memory antibody response from antigen loaded polymer particles and the effect of antigen release kinetics.* Biomaterials, 2009. **30**(27): p. 4763-76.

43. Waeckerle-Men, Y. and M. Groettrup, *PLGA microspheres for improved antigen delivery to dendritic cells as cellular vaccines.* Adv Drug Deliv Rev, 2005. **57**(3): p. 475-82.
44. Trombetta, E.S. and I. Mellman, *Cell biology of antigen processing in vitro and in vivo.* Annu Rev Immunol, 2005. **23**: p. 975-1028.
45. Dal Monte, P. and F.C. Szoka, Jr., *Effect of liposome encapsulation on antigen presentation in vitro. Comparison of presentation by peritoneal macrophages and B cell tumors.* J Immunol, 1989. **142**(5): p. 1437-43.
46. Fischer, S., et al., *The preservation of phenotype and functionality of dendritic cells upon phagocytosis of polyelectrolyte-coated PLGA microparticles.* Biomaterials, 2007. **28**(6): p. 994-1004.
47. Hagenaars, N., et al., *Relationship between structure and adjuvanticity of N,N,N-trimethyl chitosan (TMC) structural variants in a nasal influenza vaccine.* J Control Release, 2009. **140**(2): p. 126-33.

5

Nanoparticles differentially modulate the outcome of nasal vaccination by enhancing mucosal tolerance or inducing protective immunity

Chantal Keijzer
Wim Jiskoot
Willem van Eden
Bram Slütter
Ruurd van der Zee
Femke Broere

Manuscript in preparation

Abstract

In recent years, biocompatible and biodegradable polymeric nanoparticles have gained interest as antigen delivery systems. We investigated whether antigen-encapsulated PLGA (poly-Lactic-co-Glycolic Acid), PLGA-TMC (N-Trimethyl Chitosan) and TMC-TPP (Tri-Poly-Phosphate) nanoparticles can be used to modulate the immunological outcome towards active immunity or mucosal tolerance after nasal application.

The model protein ovalbumin (OVA) was encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles to explore induction of the antigen-specific B cell mediated humoral response and $CD4^+$ T cell mediated responses after nasal application in a BALB/c mouse model.

We have demonstrated that nanoparticles enhanced the antigen presentation capacity of dendritic cells as shown by increased in vitro $CD4^+$ T cell proliferation. We showed that nasal vaccination with low-dose OVA-encapsulated nanoparticles enhanced $CD4^+$ T cell proliferation in contrast to low-dose sOVA treatment and that this coincided with enhanced FoxP3 expression in the NALT and CLN only when PLGA encapsulated OVA was applied. In nasal prime boost vaccination studies we showed that only with TMC-TPP treatment a humoral immune response was induced, which coincided with the enhanced generation of OVA-specific B cells in the CLN. Finally, in an OVA-specific DTH-model, nasal vaccination with PLGA nanoparticles induced mucosal tolerance as revealed by decreased levels in ear swelling at 24 h post challenge.

We have uncovered a role for nanoparticles to differentially direct the nasal mucosal immune response, towards B cell mediated protective immunity or towards $CD4^+$ T cell mediated mucosal tolerance. The exploitation of this differential regulation capacity of nanoparticles to guide the immune response towards active or tolerogenic responses can lead to innovative vaccine development for prophylactic vaccination required in infectious diseases and for therapeutic vaccination in autoimmune diseases.

Introduction

Nasal vaccination is mainly described for the prevention of infectious diseases such as hepatitis B [1, 2] or influenza [3, 4]. However, in recent years, nasal application of antigen has become of interest in therapeutic interventions in the field of autoimmunity [5-8] and allergies [9]. Similar to other forms of mucosal immunization, such as oral immunization, nasal antigen application can stimulate antigen-specific responses locally and in the peripheral mucosal tissues [10-15]. Vaccination via the nasal mucosa might be preferred over oral vaccination due to the lower proteolytic activity at the nasal mucosa; this route of immunization requires a lower dose of antigen than for example oral immunization. Simultaneously, low antigen exposure might also reduce the chance of developing side-effects [16]. In general, mucosal antigen application can elicit protective immunity and/or a state of immunologic unresponsiveness, also termed antigen-specific mucosal tolerance [17-19]. Therefore, also nasal vaccination may divert immune responses to either activation of a protective antibody and/or T cell response desired during conventional vaccination or it may induce immunological tolerance desired as therapeutic treatment of autoimmune diseases.

The effectiveness of a nasal vaccine depends largely on the uptake of antigen by the nasopharynx-associated lymphoid tissue (NALT) [10, 11, 14, 15]. Since antigens are known to be more immunogenic in particulate form than in soluble form, advanced vaccine delivery systems are being developed, that specifically target NALT epithelium to enhance mucosal immunity [3, 13, 16, 20]. Nanoparticles are an example of such delivery systems and seem to be promising candidates for nasal vaccination due to their non-toxic characteristics [20-22].

In recent years, several *in vivo* studies have been conducted to investigate the additive role of nanoparticle mediated enhanced delivery of antigen at mucosal sites. The readout to evaluate the effectiveness of the applied vaccine relied mostly on induction of humoral responses as indicated by increased antigen-specific antibody titers [3, 23, 24]. However, it does not give insight in the underlying immunological mechanism that drives the response towards active immunity or tolerance induction. In addition, little is known about the role of $CD4^+$ T cells in nasal vaccination and how nanoparticle treatment might influence the activation of these cells, locally and in the peripheral tissues. Therefore, we set out to understand how we can direct the induced response towards active immunity or tolerance through nasal nanoparticle delivery. In general, it is accepted that the induced response following mucosal antigen application depends on many factors such as the nature of the antigen (soluble versus particulate), antigen dose, size and delivery to the mucosal tissues as shown by previously conducted studies. For example, nasal application of TMC-TPP particles is

known to elicit a more active immune response as described by Amidi *et al [3]* . In this study, mice received three successive intranasal treatments with 3 weeks interval of TMC-TPP containing monovalent H3N2 influenza antigen particles with an average size of 800 nm. Treated mice showed a significant increase in antigen-specific immune responses as shown by an increase in antigen-specific IgG1/IgG2a serum titers and increased IgA titers in nasal washes [3].

In contrast to induction of active immunity, a study conducted by Kim Wan-Uk *et al [23]* showed that mice fed with a single dose of 40 µg of type II collagen (CII)-containing PLGA particles, with an average size of 300 nm, had reduced severity of arthritis and reduced anti-CII-specific IgG antibody titers and CII-specific T cell responses.

As mentioned before, the nasal route of antigen delivery has some advantages compared to oral application. Since this route of immunization requires a lower dose of antigen due to the low proteolytic activity locally, it might also reduce the chance of developing side-effects [16]. This could be of great benefit in the treatment of ongoing chronic inflammatory diseases, such as rheumatoid arthritis.

To study the mechanisms behind active immunity or tolerance after nasal vaccination in more detail, we chose three polymeric nanoparticles that have previously shown to provoke active immunity or tolerance after nasal administration; PLGA (poly-lactic-co-glycolic acid) [25, 26], PLGA-TMC (N-trimethyl chitosan) [27] and TMC-TPP (tri-polyphosphate) [28, 29] nanoparticles that all contained the model antigen ovalbumin (OVA). These particles have a similar average diameter, but differ in their surface charge and antigen release kinetics [24]. We investigated whether these nanoparticles can shift the immunological outcome towards active immunity or tolerance after nasal antigen application. More specifically, we have explored the effect of nasal application of OVA-encapsulated nanoparticles compared to soluble OVA delivery. More insight in the mechanism by which nanoparticles drive the immune response towards tolerogenic or protective responses will assist future rational vaccine design not only to prevent infectious diseases but also for therapeutic vaccination in autoimmune diseases.

Materials and Methods

Mice

Male BALB/c mice (8-12 weeks) were purchased from Charles River Laboratories (Maastricht, The Netherlands). OVA-specific TCR transgenic (Tg) mice on BALB/c background (DO11.10 mice) [30], were bred at the Central Animal Laboratory (GDL), Utrecht University,

the Netherlands. All mice were kept in our animal facility under routine laboratory conditions. Experiments were approved by the Animal Experiment Committee of the Utrecht University (Utrecht, The Netherlands).

Antibodies, antigens and OVA encapsulated nanoparticles

In all *in vitro* and *in vivo* experiments, intact 98% pure OVA (either from Sigma Aldrich (Zwijndrecht, The Netherlands) or from Calbiochem (San Diego, CA) was used. OVA-encapsulated PLGA, PLGA-TMC and TMC-TPP nanoparticles were generated as described previously [24]. The anti-clonotypic mAb for the DO11.10 Tg TCR (KJ1.26) was purified from culture supernatant and biotinylated, according to the manufacturer's protocol (Molecular Probes, Leiden, The Netherlands). 7-Amino-actinomycin-D (7-AAD)-unconjugated, Anti-CD11c (HL3), anti-CD4 (RM4-5), anti-CD40 (3/23), anti-CD86 (GL1), anti-MHC class II (M5/114), anti-CD25 (PC61) and anti-CD69 (H1.2F3) antibodies were purchased from BD Pharmingen (Woerden, The Netherlands). Anti-FoxP3-PE (FJK-16s) and an appropriate isotype control were purchased from eBioscience (Breda, The Netherlands).

DC isolation and culture

Bone marrow-derived dendritic cells (BMDC) were cultured from BALB/c donor mice as previously described by (Lutz 1999) with minor modifications. Briefly, on day 0, femurs and tibia of adult BALB/c mice were flushed with Iscove's Modified Dulbecco's Medium (IMDM; Gibco, Invitrogen) that contained heat-inactivated 10% FCS (Bodinco). Single cell suspensions were seeded at 3 x 10^6 per petri dish in complete medium with 20 ng/ml murine rGM-CSF (Cytogen). On day 2 and 4, 10 ng/ml murine rGM-CSF was added. On day 7, the BMDCs were harvested and used for further experiments.

CD4+ T cell enrichment and CFSE labeling

Spleens were isolated from DO11.10 donor mice and were prepared into single cell suspensions. Erythrocytes were lysed and $CD4^+$ T cells were obtained by negative selection with sheep-anti-rat IgG Dynabeads (Dynal, Invitrogen, Breda, the Netherlands) using an excess amount of anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-MHC class II (M5/114), anti-CD8 (YTS169) mAbs. Enriched $CD4^+$ T cells were routinely pure between 85 and 90%. Labeling of cells with carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Leiden, The Netherlands) was performed as previously described by Broere et al. [31].

In vitro effect of nanoparticles on BMDC maturation, antigen uptake and T cell activation

To address direct maturation of BMDC by nanoparticles, BMDC were cultured in the presence of OVA containing PLGA, PLGA-TMC or TMC-TPP nanoparticles (25 ng/ml to 1 µg/ml), or 10 ng/ml LPS (Sigma) as a maturation control. After 24 h, DC maturation was determined by flow cytometry (FACS-Calibur; BD Pharmingen) and FlowJo Software V8.8.6.

BMDCs were incubated for 1.5 hours at either 4°C or 37°C with FITC-labeled OVA protein purchased from Molecular probes (Invitrogen, Breda, the Netherlands) solved in saline or incorporated into PLGA, PLGA-TMC or TMC-TPP. To quench external FITC, trypan blue stain (Gibco, Invitrogen) was added to each sample 5 minutes before FACS analysis at a final concentration of 0.02%. OVA-FITC uptake by BMDCs was analyzed by flow cytometry. BMDCs were pre-incubated at 37°C for 2 h in the presence of OVA protein solved in saline or OVA-nanoparticles; PLGA, PLGA-TMC or TMC-TPP at concentrations of 25 ng/ml, 0.5 µg/ml or 1 µg/ml. OVA-specific CD4+ T cells were added at a 1:10 ratio and T cell proliferation was assessed at 72 h post culture by CFSE dilution.

T cell activation in the local lymph nodes after nanoparticle vaccination

BALB/c acceptor mice were adoptively transferred with 1.10^7 CFSE-labeled $CD4^+KJ1.26^+$ cells in 100 µl saline, intravenously (i.v.) injected via the lateral tail vain. The next day mice received either a single application of 30 µg of OVA i.n. solved in 10 µl of saline or encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles, or a single immunization of 30 µg of OVA intramuscularly (i.m.) in the hind limbs solved in 50 µl of saline or encapsulated in nanoparticles. At 72 h post i.n. or i.m. OVA administration, the spleen, the nose-draining NALT [11, 32] and cervical lymph nodes (CLN) as well as the thigh-draining inguinal lymph nodes (ILN) were removed and single cell suspensions were analyzed to evaluate *in vivo* T cell division by flow cytometry as described earlier.

Nasal prime-boost vaccination

BALB/c mice received three nasal applications of 20 µg of OVA i.n. dissolved in 10 µl of saline or encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles, with a 3 week interval. Three weeks after third OVA vaccination, the spleen, NALT and CLN were removed and single cell suspensions were analyzed to evaluate OVA-specific T and B cell responses. Single cell suspensions were restimulated with OVA protein at final concentrations of 100 µg/ml for a 72 h period and 0.4 µCi ^{3}H-thymidine (Amersham Health, Little Chalfont, Buckinghamshire,UK) was added for an additional 18 h to address proliferation. Supernatants were analyzed for cytokine production.

The *in vivo* B cell response was assessed by detection of OVA-specific antibody titers in serum of immunized mice as described elsewere [24] by ELISPOT. Single cell suspensions from the NALT, CLN and spleen were cultured with OVA (1 µg/well) or control high protein binding filter plates (MultiScreen-IP, Millipore) for 48 hours. After incubation, SFC were detected with goat- anti mouse IgG-biotin (Sigma), Avidin-AP (Sigma). Plates were developed with NBT-BCIP (Roche) and analyzed by using the Aelvis spotreader and software. Data are shown as the net OVA-specific B cell count per $1*10^6$ cells calculated as background (spots medium coated plates) substracted from OVA-specific spots.

Mucosal tolerance induction and delayed-type hypersensitivity (DTH) reaction Balb/c mice received 20 µg of OVA i.n. three times at 24 h intervals either dissolved in saline or encapsulated in PLGA, PLGA-TMC or TMC-TPP nanoparticles. Control groups received saline alone or OVA at a final concentration of 300 µg in saline. Mice were sensitized for a DTH the next day with 100 µg of OVA in 25 µl of saline, mixed with 25 µl of IFA (Difco, BD. Alphen a/d Rijn, The Netherlands) subcutaneously (s.c.) administered in the tail base. Five days later, directly before challenge, the initial thickness of both ears was measured with an engineer's micrometer (Mitutoyo, Tokio, Japan). Subsequently, mice were challenged with 10 µg of OVA in 10 µl of saline given in the auricle of each ear and 24 h post-challenge, increase in ear thickness of both ears was determined. In all experiments the ear thickness was measured in a blinded fashion.

Luminex

The amount of cytokine secreted during a 72 h re-stimulation period was assessed by analyzing the culture supernatants. Briefly, fluoresceinated microbeads coated with capture antibodies for simultaneous detection of IFN-γ (AN18), IL-2 (JES6-1A12), IL-4 (BVD4-1D11), IL-5 (TRFK5), IL-6 (MP5-20F3), IL-10 (JES5-2A5), IL12p70 (9A5), IL-17A (TC11-18H10) and TNF-α (G281-2626)(BD Biosciences Pharmingen) were added to 50 µl of culture supernatant. Cytokines were detected by biotinylated antibodies IFN-γ (XMG1.2), IL-2 (JES6-5H4), IL-4 (BVD6-24G2), IL-5(TRFK5), IL-6 (MP5-32C11), IL-10 (SXC-1), IL12p70 (C17.8), IL-17(DuoSet ELISA kit, R&D systems Europe Ltd, Oxon, the U.K.) and TNF-α (MP6-XT3)(BD Biosciences Pharmingen) and PE-labeled streptavidin. Flurescence was measured using a Luminex model 100 XYP (Luminex, Austin, TX, USA).

Real-time PCR

Total mRNA was purified from single cell suspensions from NALT, CLN, ILN or spleen using the RNeasy kit (Qiagen Benelux B.V.) according to the manufacturer's protocol. RNA was reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, B.V) according to the manufacturer's protocol. Real-time quantitative PCR was performed using a MyiQ Single-Color Real-Time PCR detection system (Bio-Rad Laboratories B.V.) based on specific primers and general fluorescence detection with SYBR Green (iQ SYBR Green Supermix, Bio-Rad laboratories, Hercules, CA). Conditions for the Real-time quantitative reaction were (95°C for 3 min and 40 cycles of 95°C for 10 s and 59.5°C for 45 s). Expression was normalized to the detected Ct values of hypoxanthine-guanine phosphoribosyltransferase (HPRT) for each sample. The expression levels relative to HPRT were calculated according to the Real-Time PCR Bio-Rad manual by following the equation: relative expression level = $2^{-\Delta\Delta Ct}$ (Livak Method).

Specific primers were designed across different constant region exons resulting in the following primers:

HPRT sense 5'-CTGGTGAAAAGGACCTCTCG-3', antisense 5'- TGAAGTACTCATTATAGTC AAGGGCA-3'. **IL-10** sense 5'- GGTTGCCAAGCCTTATCGGA-3', antisense 5'- ACCTGCTCC ACTGCCTTGCT-3'. **FoxP3** sense 5'- CCCAGGAAAGACAGCAACCTT-3', antisense 5'-TTCT CACAACCAGGCCACTTG-3'. **IL-4** sense 5'- GGTCTCAACCCCCAGCTAGT-3', antisense 5'- GCCGATGATCTCTCTCAAGTGAT-3'. **IL-17** sense 5'- GCTCCAGAAGGCCCTCAGA-3', anti sense 5'- AGCTTTCCCTCCGCATTGA-3'. **IFN-γ** sense 5'- TCAAGTGGCATAGATGTGGAAG AA-3', antisense 5'- TGGCTCTGCAGGATTTTCATG-3'. **T-BET** sense 5'- CAACAACCCCTTT GCCAAAG-3', antisense 5'- TCCCCCAAGCAGTTGACAGT-3'. **GATA-3** sense 5'- AGAACCG GCCCCTTATCAA-3', antisense 5'- AGTTCGCGCAGGATGTCC-3'.

Statistics

Statistical analysis was performed with Prism software (Graphpad Software Inc., San Diego, version 4.00) using an unpaired two-tailed Student's *t* test, a one-way ANOVA followed by Tukey's multiple comparison test or by Dunn's multiple comparison test or Bonferroni's multiple comparison test. Error bars represent the SEM as indicated. Statistical differences for the mean values are indicated as follows: *, $P< 0.05$; **, $P< 0.01$; ***, $P<0.001$.

Results

Differential uptake of OVA labeled FITC by BMDCs after in vitro nanoparticle treatment

From previous studies we know that nanoparticles show differences in localization after DC encounter as visualized by tracing uptake of OVA [20]. Based on these results we hypothesized that these differences in localization might modulate the subsequent antigen presentation capacity of DCs.

To investigate this hypothesis, we treated DCs *in vitro* with OVA encapsulated PLGA, PLGA-TMC or TMC-TPP nanoparticles or soluble OVA (sOVA) as a control and studied phenotypic and functional differences. First we studied whether nanoparticle treatment has an effect on DC maturation, viability and differentiation. The cells were stained for $CD11c^{+}$, MHC-class-II^{+}, and 7-AAD^{-} and analyzed for their expression of CD40 and CD86. We did not observe differences in DC viability or maturation after nanoparticle treatment at OVA concentrations varying from 1 ng/ml to 1 µg/ml. Furthermore, we could not detect significant differences in culture supernatants that were analyzed for cytokine secretion of TNF-α, IL-12p70, IL-4, IL-5 (**data not shown**).

Next, we studied the uptake of the PLGA, PLGA-TMC and TMC-TPP nanoparticles with OVA labeled FITC. Then the cells were stained for CD11c and MHC-class-II and uptake of OVA-FITC was assessed by flow cytometric analysis after silencing extracellular FITC signaling with trypan blue. OVA-FITC association with DCs is shown as the FITC ΔMFI expression (figure **1A**) or percentage of OVA-FITC positive cells (figure **1B**).

OVA-FITC uptake by DCs treated with TMC-TPP was lower compared to soluble OVA (sOVA)-FITC treatment as shown by a low FITC ΔMFI expression and decreased percentage of OVA-FITC positive cells (figure **1A-B**). Furthermore, compared to sOVA, PLGA-TMC treatment enhanced the antigen uptake by DCs even at low (25 ng/ml) OVA concentrations. Both PLGA and PLGA-TMC treatment enhanced antigen uptake was observed with OVA at 0.25 µg/ml. We could not detect differences in antigen uptake at 1.00 µg/ml sOVA, PLGA or PLGA-TMC treatment suggesting a maximum antigen uptake after 1.5 h of incubation (figure **1B**).

In summary, nanoparticle characteristics affected the antigen uptake by DCs *in vitro* as shown by a lower number of OVA-FITC positive cells when DCs encounter TMC-TPP particles compared to PLGA *and PLGA-TMC*.

OVA-encapsulated nanoparticles enhance OVA-specific $CD4^+$ T cell proliferation in vitro

To investigate whether enhanced antigen uptake by DCs also affects the antigen presentation capacity of DCs, nanoparticle treated cells were studied *in vitro* by co-culture assay. DCs treated with OVA encapsulated PLGA, PLGA-TMC or TMC-TPP particles were cultured in the presence of OVA-specific CFSE-labeled $CD4^+$ T cells isolated from spleen of DO11.10 mice. T cell proliferation as measured by CFSE dilution served as readout for antigen presentation efficiency of DCs.

Antigen presentation capacity of DCs was significantly enhanced after nanoparticle treatment in contrast to sOVA since T cells stimulated by particle treated DCs showed enhanced T cell proliferation compared to T cells cultured in the presence of sOVA treated DCs. Especially, PLGA and PLGA-TMC particles potently enhanced CD4+ T cell proliferation even at a low OVA concentration of 25 ng/ml (figure **1C**). Additionally, in the culture supernatants of T cells stimulated in the presence of 1.0 µg/ml OVA containing PLGA or PLGA-TMC particles more IL-2, IFN-γ and IL-10 was detected compared to cultures with TMC-TPP particles or sOVA (figure **1D**).

In conclusion, all three OVA loaded nanoparticles enhanced the antigen presentation by DCs, as shown by increased $CD4^+$ T cell proliferation profiles as compared to sOVA.

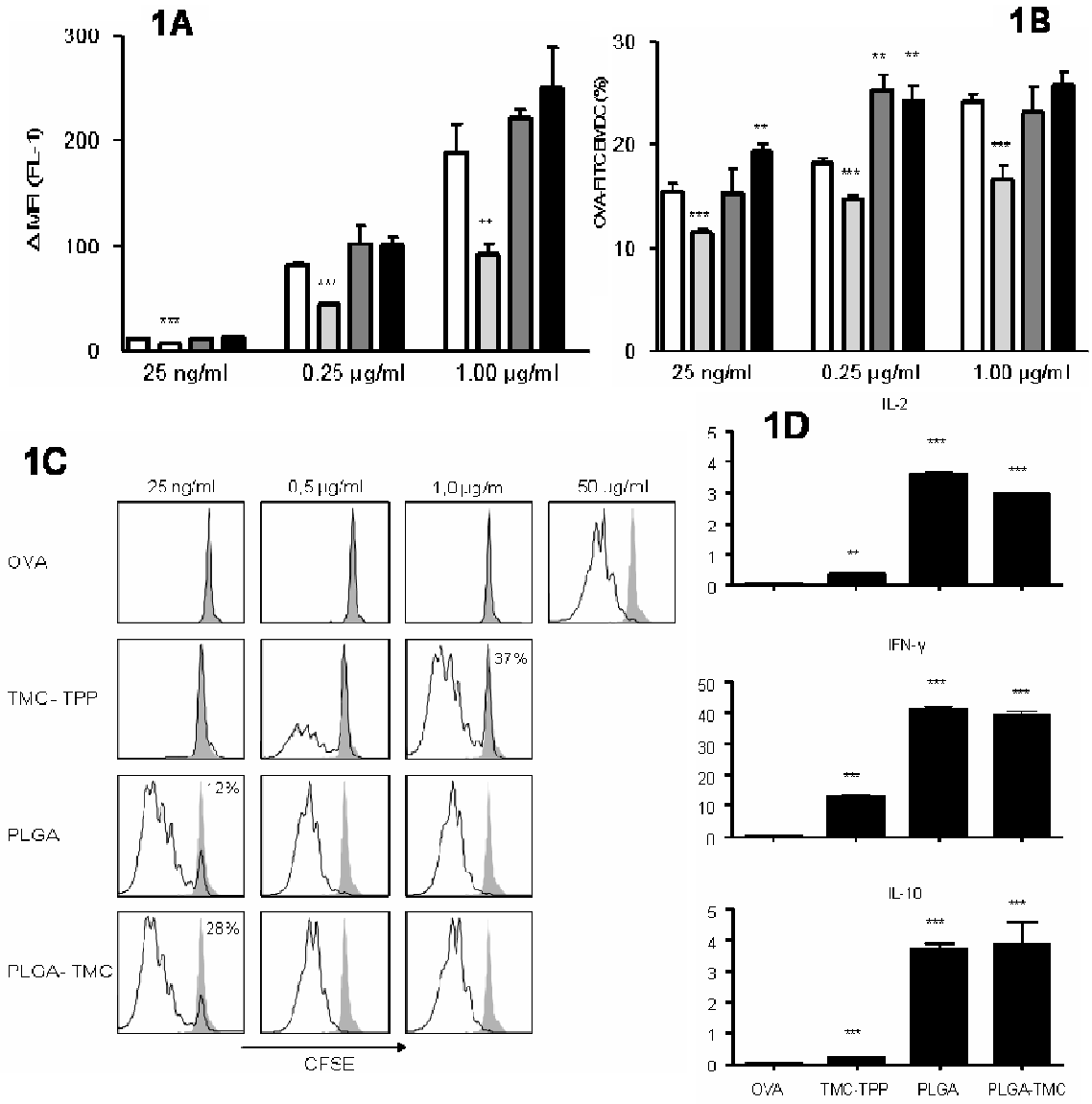


***Figure 1**: Nanoparticle mediated enhanced antigen presentation capacity of BMDCs in vitro. BMDC were incubated in the presence of sOVA-FITC or OVA-FITC encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles at different concentrations. External FITC signaling was silenced by trypan blue. The ΔMFI of OVA-FITC was assessed by subtraction of FITC signaling at 4°C from 37°C (**1A**). OVA-FITC uptake by BMDC shown as the percentage of OVA-FITC positive cells 1.5 h post-culture were calculated as the percentage at 4°C subtracted from the percentages at 37°C (**1B**). Data are representative for 3 independent experiments. mean ± standard error of the mean (s.e.m.).BMDCs were cultured in the presence of sOVA or OVA encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles at different concentrations and OVA-specific CFSE-labeled $CD4^+$ T cells Gray filled histograms; unstimulated $CD4^+$ T cells, Black overlays; $CD4^+$ T cell division patterns at different OVA concentrations after 72 hours (**1C**). Data are are representative for at least 3 independent experiments. Cytokine concentrations of IL-2, IFN-γ and IL-10 (ng/ml) were determined in culture supernatants, after 72 h of culture (**1D**). Data are representative for 3 independent experiments. mean ± s.e.m. Statistically significant ** (P< 0.01), *** (P< 0.001).*

Enhanced OVA-specific CD4$^+$ T cell proliferation at mucosal (nasal) and non-mucosal (intramuscular) sites and enhanced local mRNA expression of FoxP3 after nanoparticle vaccination

Next, we questioned whether nanoparticle treatment also affects T cell response *in vivo*. We know from former studies that especially TMC-TPP nanoparticles induced increased generation of antigen-specific IgG1 and IgG2a antibody titers after both i.n. and i.m. vaccination, whereas PLGA and PLGA-TMC only resulted in higher IgG titers after i.m vaccination and had little effect on the humoral immune response after i.n. treatment [24]. Here, we explored whether nanoparticle treatment affects the CD4$^+$ T cell response and how this depends on nanoparticle type. First we studied the short-term CD4$^+$ T cell response in mice that were either treated i.n. or i.m. with 30 µg of sOVA or OVA encapsulated particles. Proliferation of OVA-specific CFSE-labeled CD4$^+$ T cells was addressed locally in the draining lymph nodes as well as systemic in the spleen at 72 h post treatment (figure **2A**).

Nasal vaccination enhanced local CD4$^+$ T cell proliferation in the NALT and CLN compared to sOVA treatment, irrespective of the type of nanoparticles. However, none of the formulations induced measurable CD4$^+$ T cell activation in the spleen at 72 hours after vaccination upon i.n. immunization (figure **2A; left**). In contrast, non-mucosal vaccination resulted in proliferation both in the draining ILN and spleen at this time point (figure **2A; right**).

We could not detect significant differences in cytokine profiles in culture supernatants of the isolated draining CLN and ILN organs after particle vaccination (**data not shown**). However, we observed a significant increase in the relative FoxP3 mRNA expression in the CLN and a slightly increased expression in the NALT of mice that had received a single i.n. PLGA vaccination (figure **2B**). Mice that were vaccinated i.m. with TMC-TPP particles showed less expression of FoxP3 mRNA compared to PLGA and PLGA-TMC treated mice.

These data show that i.n. vaccination with low-dose OVA encapsulated nanoparticles enhanced CD4$^+$ T cell proliferation in contrast to low-dose sOVA treatment (**2A**) and coincided with enhanced FoxP3 in the NALT and CLN only when PLGA encapsulated OVA was applied. This effect was lacking in the i.m. treated mice in all treatment groups (figure **2B**) showing that both particle and route of application determine the outcome of the CD4$^+$T cell response. Although induced CD4$^+$ T cell proliferation mediated by TMC-TPP was less efficient compared to PLGA and PLGA-TMC treatment *in vitro* (figure **1C**), we were not able to detect such significant differences in CD4$^+$ T cell proliferation profiles *in vivo* (figure **2A**).

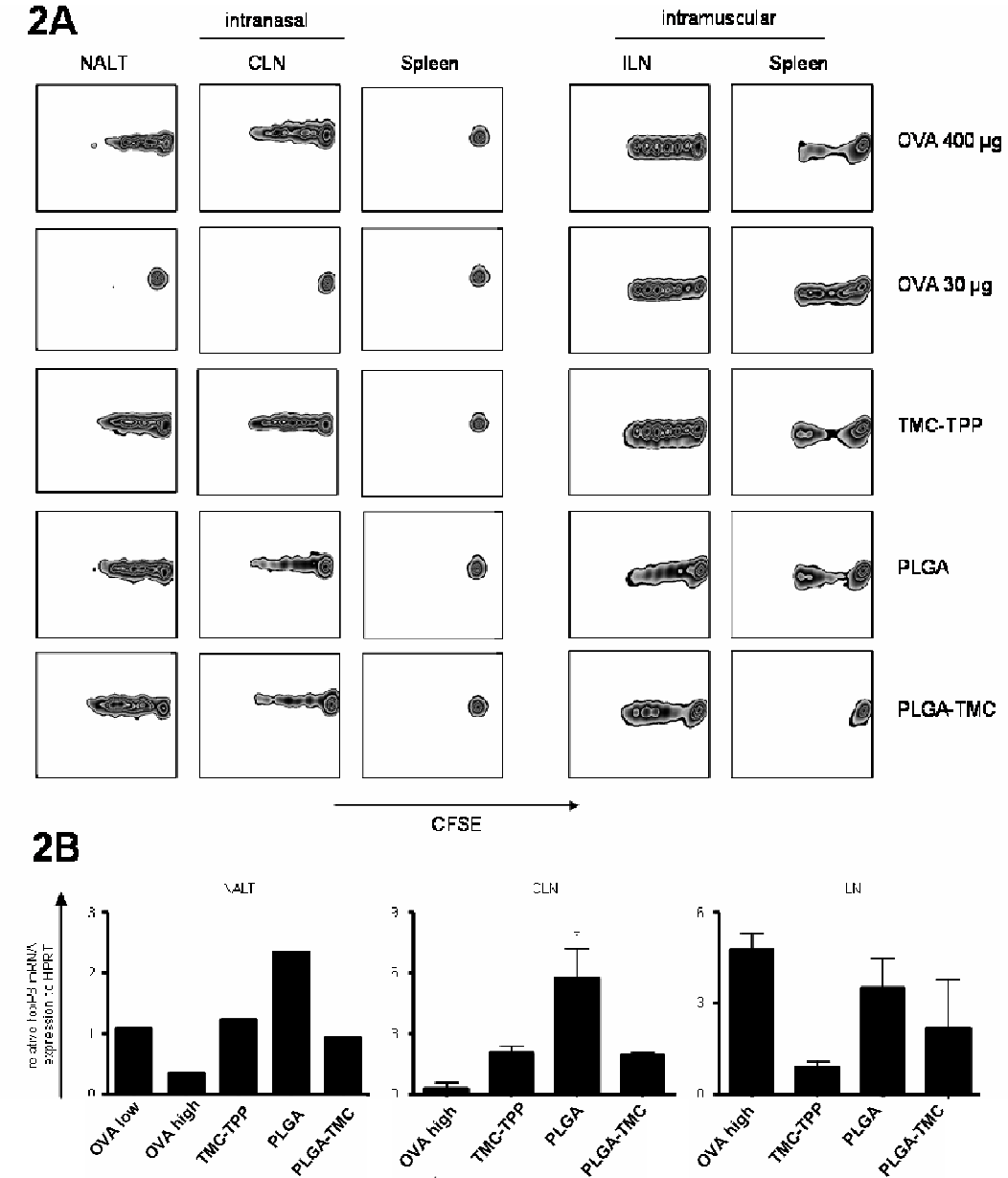


Figure 2: *Enhanced OVA-specific $CD4^+$ T cell proliferation at mucosal and non-mucosal sites, after nanoparticle treatment. OVA-specific CFSE labeled CD4+ T cells were transferred to BALB/c acceptor mice one day prior to vaccination. Mice received a single i.n. application of 30 µg of sOVA or OVA encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles. For induction of a non-mucosal response, mice received a single i.m. immunization in the hind limbs. At 72 h post i.n. or i.m. OVA administration, in vivo T cell division was addressed in spleen, nose-draining NALT and CLN as well as the thigh-draining ILN (**2A**). Data are representative for at least 3 (intranasal) and 2 (intramuscular) independent transfer studies.*
*Total mRNA was purified from single cell suspensions from *NALT, CLN, and ILN. Relative mRNA expression to HPRT of FoxP3 was determined 72 h post i.n. or i.m. OVA application (**2B**).*
*NALT: intranasal treatment (cells isolated from NALT were pooled per group). CLN: PLGA (n=5); PLGA-TMC (n=3); TMC-TPP (n=4) and ILN (n =3 mice per group); mean ± s.e.m. Statistically significant: * (P< 0.05).*

OVA-specific CD4$^+$ T cell and B cell response after nasal prime-boost vaccination

In the previous study we explored the differences in the induction of an OVA-specific CD4+ T cell response in a T cell transfer study. The effect of differences in antigen delivery (soluble versus particulate), antigen dose (low or high) and route of administration (i.n. or i.m.) was assessed by comparing the induced CD4+ T cell response 72 h post nasal vaccination. We were able to detect a significant difference in the enhanced CD4$^+$ T cell proliferation profile 72 h post nasal vaccination of mice that received a single low-dose of OVA encapsulated PLGA, PLGA-TMC or TMC-TPP nanoparticles in contrast to sOVA treatment (figure **2A**). In addition, the local CD4$^+$ T cell response seemed to be specifically shifted to a regulatory response upon i.n. PLGA treatment as shown by enhanced expression of the relative mRNA FoxP3 expression in these mice (figure **2B**). As mentioned before, in a previous study we described that especially i.n. TMC-TPP vaccination induced enhanced generation of antigen-specific IgG1 and IgG2a antibody titers while PLGA and PLGA-TMC had hardly any effect on the induced humoral immune response [24].

We performed an additional vaccination study to further assess the effect of i.n. nanoparticle treatment on the induction of an OVA-specific T cell or B cell response. Briefly, mice received 20 µg of OVA i.n. three times at three week intervals. Three weeks after the final OVA application single cell suspensions from the spleen, NALT and CLN were analyzed to evaluate OVA-specific T and B cell responses by ^{3}H-thymidine incorporation or ELISPOT, respectively. We could not detect significant differences in the T cell proliferation profiles of CLN and spleen after i.n. treatment. However, a slightly enhanced T cell proliferation profile was observed after PLGA vaccination in contrast to PLGA-TMC and TMC-TPP as indicated by the SI and cpm (figure **3A-B**).

Beside the T cell response we investigated if nanoparticle treatment had a differential effect on B cell stimulation. Here, results showed that after i.n. vaccination, both sOVA and TMC-TPP treatment slightly enhanced the generation of OVA specific B cells locally in the draining CLN as shown in figure (figure **3C**). Moreover, increased numbers of OVA-specific B cells were detected in the spleens of mice after TMC-TPP and PLGA-TMC vaccination (**data not shown).** These results correlate with the findings of increased generation of antigen-specific antibodies after nasal TMC-TPP vaccination as described by Slütter *et al* [24].

In summary, we were able to detect significant differences in the induced type of immune response after nanoparticle treatment. Nasal treatment with low-dose OVA encapsulated PLGA nanoparticles enhanced the CD4+ T cell response and relative FoxP3 mRNA expression locally in the NALT and CLN. Although TMC-TPP nanoparticles showed to be superior in the activation of the humoral arm of the nasal mucosal immune system as shown by increased

generation of OVA-specific B cells and OVA-specific antibody titers in serum and nasal washes, this effect was not observed for PLGA treated mice. From these data we hypothesized that nasal application of slow release PLGA nanoparticles induced T cell mediated mucosal tolerance whereas TMC-TPP showed to induce protective immunity upon i.n. vaccination. To further study if these differences are of functional importance, the nanoparticles we tested in an OVA-specific delayed-type hypersensitivity (DTH) model.

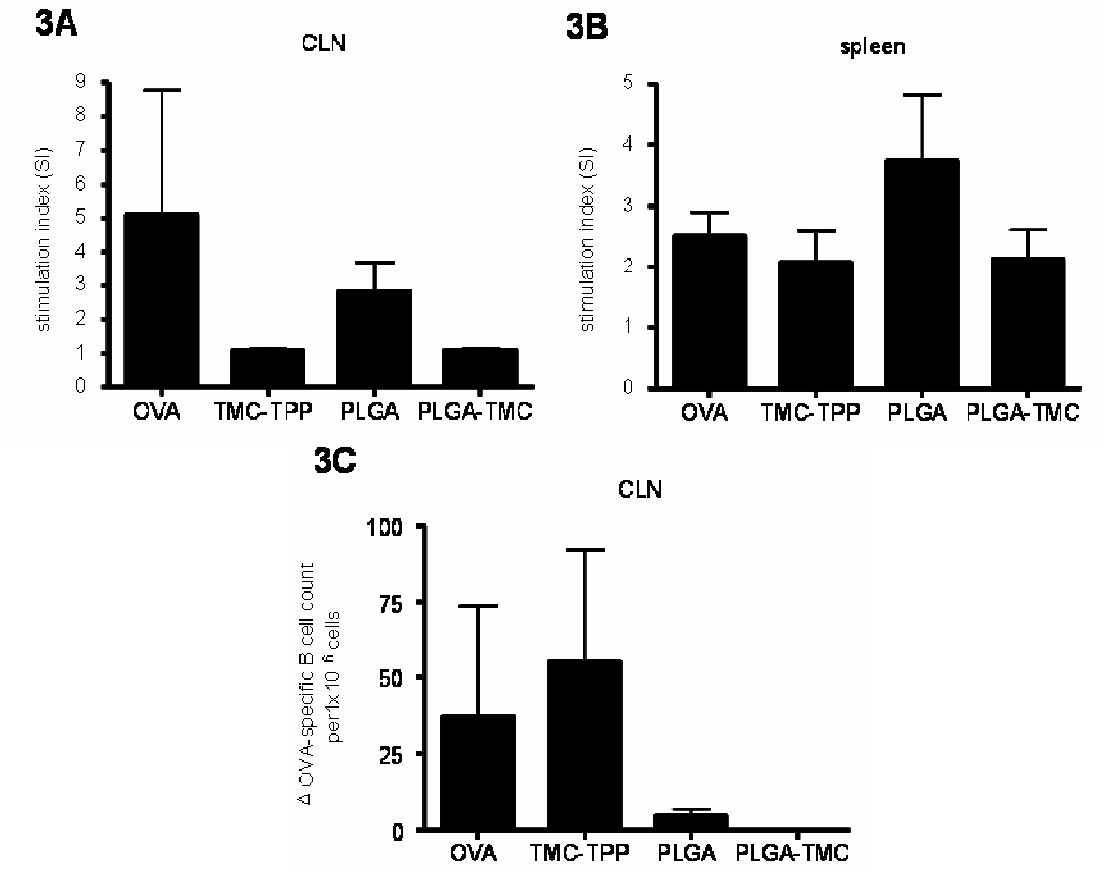


Figure 3: *OVA-specific CD4$^+$ T cell and B cell response after nasal prime-boost vaccination. Effect of nanoparticles after nasal vaccination on OVA-specific T cells* ***(A-B).*** *Mice received three times 20 µg of OVA solved in PBS or encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles. Three weeks post the final OVA administration, the OVA-specific T cell proliferation was assessed ex vivo. Single cell suspensions were re-stimulated for 72 h in the presence of OVA prior to ^{3}H-thymidine incorporation. Data are shown as the stimulation index (SI) of cells isolated from the CLN (**3A**) or spleen (**3B**). Effect of nanoparticles after nasal vaccination on OVA-specific B cells (**C**). Mice were treated as described above. Three weeks post the final OVA administration, the OVA-specific B cell response was assessed ex vivo 48 h post re-stimulation of single cell suspensions by ELISPOT. Data are shown as cells isolated from CLN (**3C**). Data are shown as the Δ OVA-specific B cell count per 1*10^6 cells calculated as background (spots counted on medium coated plates) subtract from OVA-specific spots. n=5 mice per group; mean ± standard error of the mean (s.e.m.).*

Nasal application of slow-release PLGA particles suppressed a Th-1-mediated hypersensitivity reaction, while TMC-TPP enhanced humoral immunity, in an OVA-specific delayed-type hypersensitivity (DTH)-model

In general we showed that after nasal vaccination PLGA treatment induced a more T cell mediated immune response, whereas TMC-TPP particle treatment resulted in increased numbers of OVA-specific B cells. To see if these differences are of functional importance, the nanoparticles were tested in a DTH-model. After 24 h, changes in ear swelling were determined and compared with values prior to challenge. Clearly, PLGA nanoparticle treatment suppressed the OVA specific DTH response, whereas PLGA-TMC and TMC-TPP nanoparticle treated mice failed to suppress the DTH response 24 h post challenge (figure **4A**). In contrast to PLGA and PLGA-TMC, nasal application of TMC-TPP significantly increased the generation of OVA-specific B cells locally in the draining CLN (figure **4B**) and to a lower extent in the spleen (figure **4C**). This suggests a main role for TMC-TPP in the activation of the humoral immune response and agrees with earlier findings [24]. In addition, a significant increase in the relative IL-10 mRNA expression was detected in the CLN (figure **4D**), but not in the spleen (**data not shown**) of mice that were tolerized by PLGA vaccination. Although we could detect increased expression of relative FoxP3 mRNA in the T cell transfer study (figure **2B**) we were not able to detect such significant differences in the DTH model, probably due to experimental differences in timing and presence of OVA-specific T cells in draining lymph nodes (**data not shown**).

To summarize, nasal treatment with PLGA nanoparticles induced mucosal tolerance but did not induce protective immunity as shown by the absence of antigen-specific responses (figure **4A-C**). In contrast to PLGA and PLGA-TMC immunization, only TMC-TPP treatment led to activation of the humoral immune response as shown by local increased generation of antigen-specific B cells (figure **3C, 4B-C**) and increased levels of antigen-specific antibody titers systemically. We were not able to detect a clear T or B cell induced response upon nasal PLGA-TMC treatment (figure **3 and 4**). However, we can conclude that PLGA-TMC immune regulation was somewhere intermediate, since PLGA-TMC treatment only partially suppressed the DTH response (figure **4A**).

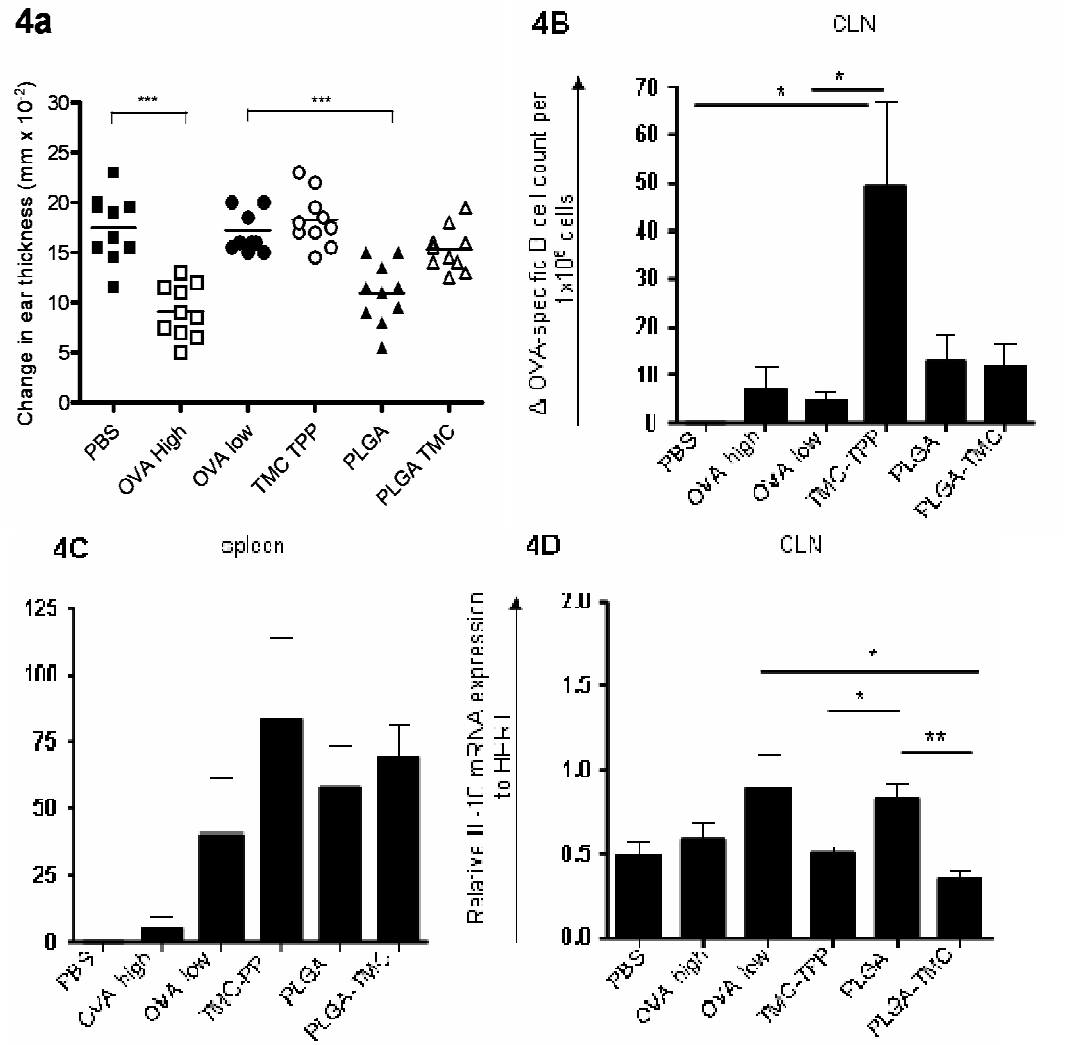


***Figure 4**: Low Nasal application of slow-release PLGA particles suppressed a Th-1-mediated hypersensitivity reaction, while TMC-TPP enhanced humoral immunity, in an OVA-specific DTH model. Effect of nanoparticles on nasally induced suppression of a DTH response in BALB/c mice **(4A)**. Mice received 20 µg of OVA i.n. either solved in PBS (black circles) or encapsulated in TMC-TPP (white circles), PLGA (black triangles) or PLGA-TMC (white triangles) nanoparticles for three successive days. Control mice were treated with PBS only (black squares) or with OVA at a concentration of 100 µg (white squares). Mice were sensitized subcutaneously at the tail base with 100 µg of OVA in IFA 1 day post the final nasal OVA administration. Five days after sensitization, mice were challenged with 10 µg of OVA in 10 µl of PBS in the auricle of both ears. After 24 h, changes in ear thickness were determined and compared with values before challenge. Enhanced OVA-specific B cell response induced after nasal nanoparticle treatment (**4B-C**). OVA-specific B cell response was assessed ex vivo 48 h post re-stimulation of single cell suspensions by ELISPOT. Data are shown as cells isolated from CLN (**4B**) and spleen (**4C**) Data are shown as the Δ OVA-specific B cell count per $1*10^6$ cells calculated as background (spots counted on medium coated plates) subtract from OVA-specific spots. OVA-encapsulated PLGA nanoparticles enhance local mRNA expression of IL-10 after nasal vaccination (4D). Total mRNA was purified from single cell suspensions from the CLN and subsequently reverse transcribed into cDNA. Relative mRNA expression to HPRT of and IL-10 was determined 48 h post OVA challenge in the CLN (**4D**) of mice. n=5 mice per group; mean ± standard error of the mean (s.e.m.). One representative experiment of two is shown.* (P< 0.05), ** (P< 0.01), *** (P< 0.001).*

Discussion

The nasal route is an attractive alternative for classical vaccination based on several specific characteristics of the mucosal tissue [16, 33]. Former studies already showed that upon nasal nanoparticle treatment the humoral immune response can be enhanced and that autoimmunity could be suppressed by mucosal tolerance induction [3, 23, 24]. Since there is not much known about the role of $CD4^+$ T cells upon nasal nanoparticle treatment, we explored how nanoparticle treatment affected $CD4^+$ T cell activation both *in vitro* and *in vivo*. In this study we investigated whether OVA-encapsulated nanoparticles can modulate the immunological outcome towards active immunity or tolerance after nasal antigen application.

We showed *in vitro* that particle characteristics influenced OVA-specific $CD4^+$ T cell proliferation as shown in **figure 1C** by the absence of activated T cell after sOVA treatment. While PLGA and PLGA-TMC nanoparticles enhanced $CD4^+$ T cell proliferation to a degree that all T cells had divided at least one time, we observed a relative high peak of undivided T cells when DCs were pulsed with TMC-TPP nanoparticles at similar concentrations, at 72 h post culture. The amount of antigen uptake does not necessarily correlate with the percentage of cell division since we observed a relative lower uptake of OVA-FITC in TMC-TPP pulsed DCs, whereas T cell proliferation was enhanced. One possible explanation can be that the differential $CD4^+$ T cell proliferation profiles are caused by the diversity in how the nanoparticles encounter DCs. Previous studies have described significant differences in how nanoparticles interact with DCs *in vitro* [20]. TMC-TPP particles release the antigen by a mechanism of rapid content release. Since these particles easily associate with the outer cell wall [24, 34], antigen may be efficiently taken up by DCs in contrast to sOVA that is scattered throughout the entire culture supernatant. Although these particles are less efficiently taken up compared to PLGA and PLGA-TMC they may in time increase the antigen uptake by DCs as shown by enhanced $CD4^+$ T cell proliferation *in vitro* in comparison with sOVA at 72 h post culture. We were not able to detect differences in DC maturation or phenotype suggesting that particle treatment mainly affects the antigen presentation capacity of DCs. Since direct evidence is lacking, the functional interaction of nanoparticles with DCs may receive further attention.

We showed that low-dose OVA encapsulated nanoparticles enhanced OVA-specific $CD4^+$ T cell proliferation locally in the NALT and CLN after a single nasal application, which was not seen with low-dose sOVA. This showed the superiority of nanoparticle mediated OVA delivery versus sOVA delivery. Interestingly, due to the absence of activated OVA-specific T cells in the spleen following nasal treatment we can conclude that more time is required to elicit systemic

responses in comparison to non-mucosal antigen immunization (figure **2A**). Our data also confirm that the route of antigen delivery, mucosal versus non-mucosal, differentially activated the immune system as previously described by Unger *et al* [19].

It was already described that both protective immunity and mucosal tolerance induction could be enhanced after nasal nanoparticle treatment [10]. The differential role of nasal nanoparticle application in the activation of fundamental T and B cells function however had not been investigated. In a prime-boost vaccination study, we showed that nasal vaccination with low-dose OVA-encapsulated PLGA nanoparticles enhanced primarily T cell activation both in the local CLN and systemically in the spleen. Such enhanced T cell proliferation was not detected after TMC-TPP or PLGA-TMC application. In contrast to PLGA and PLGA-TMC, treatment with TMC-TPP led to increased generation of OVA-specific B cells locally in the CLN and this correlates with increased levels of antigen-specific antibody titers. Altogether, these data clearly show that nanoparticles differentially modulate the activation of T and B cells after nasal delivery.

From the literature it is known that CD4+CD25+FoxP3+ regulatory T cells play an important role in the induction of mucosal tolerance [35, 36]. Therefore, we decided to explore the expression of FoxP3 by T cells after nasal nanoparticle treatment. We were capable of detecting a significant difference in the mRNA FoxP3 expression levels between the nanoparticle treatment groups locally in the CLN. Interestingly, we observed an increased FoxP3 expression only for PLGA treatment (figure **2B**).

Finally, we studied the functional significance of these basic findings by testing the nanoparticles in a DTH-model. We found that when mice were tolerized for OVA by nasal treatment with low-dose OVA-encapsulated PLGA nanoparticles, a T cell mediated tolerogenic response was induced. This type of tolerance induction was lacking in TMC-TPP treated mice that again showed a significant increased generation of OVA-specific B cells in the CLN in contrast to the other treatment groups. Although we observed a significant increased expression of FoxP3 mRNA in mice treated with PLGA nanoparticles in the OVA-specific CD4+ T cell transfer studies, we were not able to detect such differences in FoxP3 expression levels in this experiment. However, in the T cell transfer studies, the number of OVA-specific T cells was enhanced as compared to the DTH model, making it less likely to detect these cells. In addition, transferred T cells in the transfer model were analyzed 72 h post transfer when OVA-specific T cells were still present locally in the draining lymph nodes as shown earlier in figure 2A. Compared to the transfer model, in the DTH experiment, T cells were analyzed 9 days after the last nasal immunization giving T cells enough time to enter the circulation and making it difficult to detect them locally. Although the subtype of T cell that mediated

tolerance remains unclear, we were able to observe a significant increased expression of IL-10 mRNA after PLGA treatment locally in the CLN (figure **4D**). Therefore we suggest that tolerance after nasal PLGA treatment may be IL-10 mediated.

In conclusion, our results indicate that nasal PLGA or TMC-TPP nanoparticles can shift the antigen-specific immune response to tolerance or active immunity, respectively. These findings may increase the possibility to use nanoparticles to drive the immune response towards tolerance or protective immunity and enable future rational vaccine design not only to prevent infectious diseases but also for therapeutic vaccination in autoimmune diseases.

Acknowledgment

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

References

1. Jaganathan, K.S. and S.P. Vyas, *Strong systemic and mucosal immune responses to surface-modified PLGA microspheres containing recombinant Hepatitis B antigen administered intranasally.* Vaccine, 2006. **24**(19): p. 4201.
2. Makidon, P.E., et al., *Pre-clinical evaluation of a novel nanoemulsion-based hepatitis B mucosal vaccine.* PloS one, 2008. **3**(8): p. e2954.
3. Amidi, M., et al., *N-trimethyl chitosan (TMC) nanoparticles loaded with influenza subunit antigen for intranasal vaccination: biological properties and immunogenicity in a mouse model.* Vaccine, 2007. **25**(1): p. 144.
4. Hagenaars, N., et al., *Role of trimethylated chitosan (TMC) in nasal residence time, local distribution and toxicity of an intranasal influenza vaccine.* Journal of controlled release : official journal of the Controlled Release Society, 2010. **144**(1): p. 17.
5. Holmgren, J. and C. Czerkinsky, *Mucosal immunity and vaccines.* Nature medicine, 2005. **11**(4 Suppl): p. S45.
6. Liang, J., et al., *HSP65 serves as an immunogenic carrier for a diabetogenic peptide P277 inducing anti-inflammatory immune response in NOD mice by nasal administration.* Vaccine, 2010. **28**(19): p. 3312.
7. Shi, F.-D., et al., *Nasal administration of multiple antigens suppresses experimental autoimmune myasthenia gravis, encephalomyelitis and neuritis.* Journal of the neurological sciences, 1998. **155**(1): p. 1.
8. Tarkowski, A., et al., *Treatment of experimental autoimmune arthritis by nasal administration of a type II collagen-cholera toxoid conjugate vaccine.* Arthritis and Rheumatism, 1999. **42**(8): p. 1628.
9. Liu, Z., et al., *Local nasal immunotherapy: efficacy of Dermatophagoides farinae-chitosan vaccine in murine asthma.* International archives of allergy and immunology, 2009. **150**(3): p. 221.
10. Harmsen, A., et al., *Cutting Edge: Organogenesis of Nasal-Associated Lymphoid Tissue (NALT) Occurs Independently of Lymphotoxin-{alpha} (LT{alpha}) and Retinoic Acid Receptor-Related Orphan Receptor-{gamma}, but the Organization of NALT Is LT{alpha} Dependent.* The Journal of Immunology, 2002. **168**(3): p. 986.
11. Heritage, P.L., et al., *Comparison of Murine Nasal-associated Lymphoid Tissue and Peyer's Patches.* American Journal of Respiratory and Critical Care Medicine, 1997. **156**(4): p. 1256.
12. Hiroi, T., et al., *Nasal immune system: distinctive Th0 and Th1/Th2 type environments in murine nasal-associated lymphoid tissues and nasal passage, respectively.* European journal of immunology, 1998. **28**(10): p. 3346.
13. Kiyono, H. and S. Fukuyama, *NALT- versus Peyer's-patch-mediated mucosal immunity.* Nature reviews.Immunology, 2004. **4**(9): p. 699.
14. Rodriguez-Monroy, M.A., S. Rojas-Hernandez, and L. Moreno-Fierros, *Phenotypic and functional differences between lymphocytes from NALT and nasal passages of mice.* Scandinavian journal of immunology, 2007. **65**(3): p. 276.
15. Wu, H.Y., et al., *Induction of antibody-secreting cells and T-helper and memory cells in murine nasal lymphoid tissue.* Immunology, 1996. **88**(4): p. 493.
16. Davis, S.S., *Nasal vaccines.* Advanced Drug Delivery Reviews, 2001. **51**(1-3): p. 21.
17. Faria, A.M. and H.L. Weiner, *Oral tolerance.* Immunological reviews, 2005. **206**: p. 232.
18. Mestecky, J., Z. Moldoveanu, and C.O. Elson, *Immune response versus mucosal tolerance to mucosally administered antigens.* Vaccine, 2005. **23**(15): p. 1800.
19. Unger, W.W.J., et al., *Early Events in Peripheral Regulatory T Cell Induction via the Nasal Mucosa.* The Journal of Immunology, 2003. **171**(9): p. 4592.
20. Slutter, B., et al., *Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination.* Journal of controlled release : official journal of the Controlled Release Society, 2009. **138**(2): p. 113.

21. Csaba, N., M. Garcia-Fuentes, and M.J. Alonso, *Nanoparticles for nasal vaccination.* Advanced Drug Delivery Reviews, 2009. **61**(2): p. 140.
22. Dobrovolskaia, M.A., D.R. Germolec, and J.L. Weaver, *Evaluation of nanoparticle immunotoxicity.* Nature nanotechnology, 2009. **4**(7): p. 411.
23. Kim, W.U., et al., *Suppression of collagen-induced arthritis by single administration of poly(lactic-co-glycolic acid) nanoparticles entrapping type II collagen: a novel treatment strategy for induction of oral tolerance.* Arthritis and Rheumatism, 2002. **46**(4): p. 1109.
24. Slutter, B., et al., *Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: Nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen.* Vaccine, 2010. **28**(38): p. 6282.
25. Gutierro, I., et al., *Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres.* Vaccine, 2002. **21**(1-2): p. 67-77.
26. Gutierro, I., et al., *Influence of dose and immunization route on the serum Ig G antibody response to BSA loaded PLGA microspheres.* Vaccine, 2002. **20**(17-18): p. 2181-90.
27. Pawar, D., et al., *Evaluation of Mucoadhesive PLGA Microparticles for Nasal Immunization.* AAPS J, 2010. **12**(2): p. 130-7.
28. Amidi, M., et al., *Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system.* J Control Release, 2006. **111**(1-2): p. 107-16.
29. Slutter, B., N. Hagenaars, and W. Jiskoot, *Rational design of nasal vaccines.* Journal of drug targeting, 2008. **16**(1): p. 1.
30. Robertson, J.M., P.E. Jensen, and B.D. Evavold, *DO11.10 and OT-II T Cells Recognize a C-Terminal Ovalbumin 323-339 Epitope.* The Journal of Immunology, 2000. **164**(9): p. 4706.
31. Broere, F., et al., *Cyclooxygenase-2 in mucosal DC mediates induction of regulatory T cells in the intestine through suppression of IL-4.* Mucosal immunology, 2009. **2**(3): p. 254.
32. Wu, H.Y., H.H. Nguyen, and M.W. Russell, *Nasal lymphoid tissue (NALT) as a mucosal immune inductive site.* Scandinavian journal of immunology, 1997. **46**(5): p. 506.
33. Mitragotri, S., *Immunization without needles.* Nature reviews.Immunology, 2005. **5**(12): p. 905.
34. Slutter, B., et al., *Conjugation of ovalbumin to trimethyl chitosan improves immunogenicity of the antigen.* Journal of controlled release : official journal of the Controlled Release Society, 2010. **143**(2): p. 207.
35. Hori, S., T. Nomura, and S. Sakaguchi, *Control of Regulatory T Cell Development by the Transcription Factor Foxp3.* Science, 2003. **299**(5609): p. 1057.
36. Iliev, I.D., et al., *Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning.* Mucosal immunology, 2009. **2**(4): p. 340.

6

Adjuvant effect of cationic liposomes and CpG depends on administration route.

Bram Slütter
Ding Zhi
Joke A. Bouwstra
Suzanne Bal
Wim Jiskoot

Manuscript submitted

Abstract

In this study we explored the immunization route-dependent adjuvanticity of cationic liposomes loaded with an antigen (ovalbumin; OVA) and an immune potentiator (CpG). Mice were immunized intranodally, intradermally, transcutaneously (with microneedle pre-treatment) and nasally with liposomal OVA/CpG or OVA/CpG solution.

In vitro, OVA/CpG liposomes showed enhanced uptake by DCs of both OVA and CpG compared to OVA+CpG solution. A similar enhanced uptake by DCs was observed *in vivo* when fluorescent OVA/CpG liposomes were administered intranodally. However, after transcutaneous and nasal application a lower uptake of OVA/CpG liposomes compared to an OVA+CpG solution was observed. Moreover, the IgG titers after nasal and transcutaneous administration of OVA/CpG liposomes were reduced compared to administration of an OVA+CpG solution. Although serum IgG titers may suggest limited added value of liposomes to the immunogenicity, for all routes, OVA/CpG liposomes resulted in elevated IgG2a levels, whereas administration of OVA+CpG solutions did not.

These data show that encapsulation of antigen and adjuvant into a cationic liposome has a beneficial effect on the quality of the antibody response in mice after intranodal or intradermal immunization, but impairs proper delivery of antigen and adjuvant to the lymph nodes when the formulations are administered transcutaneously or nasally.

Introduction

Vaccine development has shifted focus from the classical live-attenuated and inactivated vaccines to the development of subunit vaccines. Subunit vaccines, consisting of purified proteins, are safer than live-attenuated and inactivated vaccines, but lack strong immunogenicity. A common strategy to improve their immunogenicity is encapsulation of the antigen into nanoparticles. Antigen encapsulation offers the advantages of i) protection from enzymatic breakdown, ii) sustained antigen release over time [1, 2], iii) enhanced uptake by professional antigen presenting cells such as DCs [3] and iv) possibility of co-encapsulation of adjuvants [4, 5]. Liposomes are a type of nanoparticles that have been widely studied as antigen carriers and their usage in vaccination studies dates back to 1974 [6]. Although liposomes themselves are not very immunogenic, they have been described to enhance the immune response because of the above mentioned advantages [6-11]. Cationic liposomes are considered the most effective vaccine delivery systems for administration via injection [12-14]. To improve the immunogenicity of liposomes, adjuvants can be co-encapsulated together with the antigen [15, 16]. Recently we showed that intradermal vaccination in mice with 250-nm sized cationic liposomes containing ovalbumin (OVA) and CpG, a Toll-like receptor 9 (TLR9) ligand, induced strongly elevated IgG2a titers and IFN-γ production by restimulated splenocytes [17].

Besides the attention given to vaccine formulation, interest is aroused for vaccine delivery via non-invasive routes, such as the nose and the skin [18, 19]. Both the nose and the skin are in direct contact with the environment and therefore densely populated with immune cells to protect the body against pathogens. The nasal epithelium is equipped with the nasal associated lymphoid tissue (NALT) and the skin is densely populated with epidermal Langerhans cells (LCs) and dermal dendritic cells (DCs). The presence of high numbers of DCs at these delivery sites could facilitate the liposome's full potential to enhance antigen uptake by DCs and induce a potent, protective immune response. Moreover, liposomes can protect the antigen from enzymatic activity, which is especially an issue for nasal vaccination. Nonetheless, the beneficial effect of liposomes and nanoparticles in general for these delivery routes is under debate [20-23]. For instance, (nano)particulate matter could have more difficulties crossing the skin barrier [23, 24] and to a lesser extent (because of possible M-cell transport [3, 4]) the nasal epithelium [25] (figure 1).

The aim of the present study was to investigate the impact of encapsulating the antigen and the adjuvant in cationic liposomes on the (antibody mediated) immune response after administration via several immunization routes. We explored the influence of liposomal

antigen and adjuvant co-encapsulation on both the transport through the epithelium and transport of the vaccine to the lymph nodes, by quantifying the amount of OVA and CpG positive DCs (CpG^+ or OVA^+) in the draining lymph nodes after intranodal, intradermal, transcutaneous (in combination with microneedle pre-treatment) and nasal administration. The added value of OVA- and CpG-containing cationic liposomes on immunogenicity was evaluated, by determining the total serum IgG levels and secretory IgA levels in nasal washes. Finally, the quality of the immune response was addressed by measuring IgG subtypes (IgG1 and IgG2a).

Material and Methods

Materials

Soybean phosphatidylcholine (PC), 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) and 1,2-dioleoyl-sn-glycero-3-ghosphoethanolamine (DOPE) were kindly provided by Lipoid GmbH (Ludwigshafen, Germany). Endotoxin free OVA was purchased at Merck (Darmstadt, Germany). Anti CD11c-PE/Cy7, CD86-FITC was acquired from Becton Dickinson (Franklin Lakes, NJ USA). Invitrogen (Breda, The Netherlands) supplied fluorescein isothiocyanate AlexaFluor647 labeled OVA (OVA_{AF647}), bovine serum albumin (BSA), chromogen 3, 3', 5, 5'-tetramethylbenzidine (TMB) and the substrate buffer. Polyclonal rabbit anti-OVA IgG and goat anti-rabbit IgG-HRP conjugate were acquired from Southern Biotech (Birmingham, AL, USA). CpG-ODN 2006 and its fluorescein isothiocyanate labelled equivalent (CpG_{FITC}) were purchased at Invivogen (Toulouse, France). Nimatek® (100 mg/ml Ketamine, Eurovet Animal Health B.V., Bladel, The Netherlands), Oculentum Simplex (Farmachemie, Haarlem, The Netherlands), Rompun® (20 mg/ml Xylazine, Bayer B.V., Mijdrecht, The Netherlands) and phosphate buffered saline (PBS 0.9% NaCl) were obtained from a local pharmacy. All other chemicals were purchased at Sigma-Aldrich (Zwijndrecht, The Netherlands), unless stated otherwise.

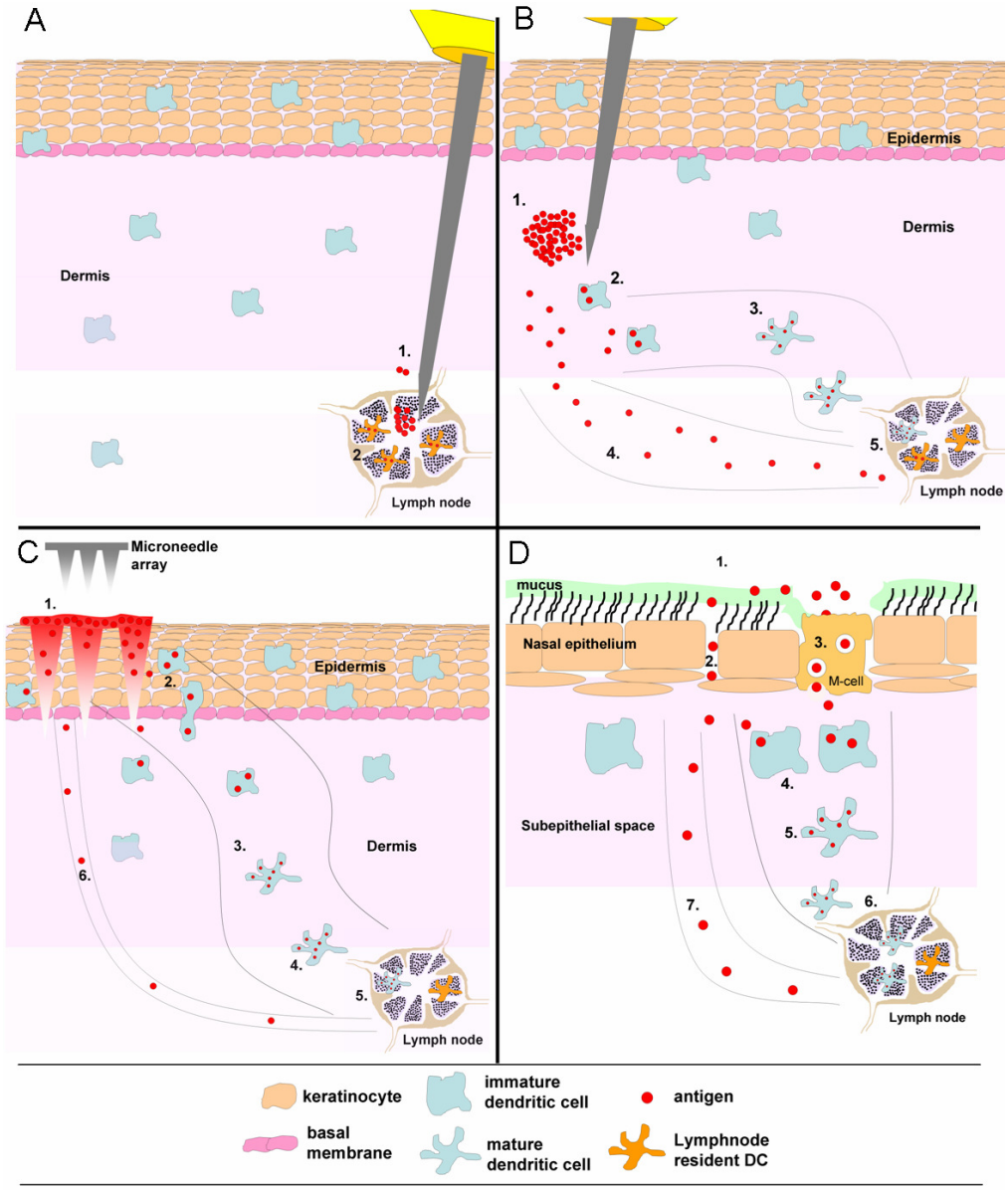


***Figure 1**: Schematic illustration (relative size of organs and cells not on scale) of antigen transport mechanisms after immunization via different administration routes. A) After intranodal injection (1) the antigen will directly be taken up by lymphnode resident DCs (2). B) After intradermal injection (1) the antigen can be taken up by an immature DC (2) which matures (3) or drains directly to the lymph node (4). This will result in both peripheral as well as lymph node resident DCs that are antigen positive (5). C) Transcutaneous immunization: microneedle application creates small conduits through which the antigen can diffuse (1). Immature DCs are abundantly present in the epidermis and dermis and will take up the antigen (2,) and will subsequently cross the basal membrane (3), mature (4) and reach the lymph node (5). Because the long route the antigen has to take, direct drainage of the antigen to the lymph node (6) may be limited. D) After nasal administration (1) the antigen can pass the epithelium either by paracellular diffusion between the epithelial cells(2) or through active transport by M-cells(3) and be taken up by immature mucosal DCs (4) which mature (5) and drain to the lymph node (6). Direct drainage to the lymphnode is an option (7), but might be limited.*

Preparation and characterization of liposomes

Cationic liposomes were prepared using the film rehydration method [26], followed by extrusion as described previously [17]. Briefly, a thin lipid film was made of PC, DOTAP and DOPE (9:1:1 molar ratio) by evaporating the chloroform using a rotary evaporator followed by flushing with nitrogen. To prepare empty liposomes, the film was rehydrated in a 10 mM phosphate buffer pH 5 (0.1 mM Na_2HPO_4 and 9.9 mM NaH_2PO_4), whereas OVA loaded liposome were prepared by rehydration in a 10 mM phosphate buffer

pH 7.4 (7.7 mM Na_2HPO_4 and 2.3 mM NaH_2PO_4), containing 1.5 mg/ml OVA. The final concentration of lipids was 5% (w/v).

To obtain liposomes of an equal size the solution was extruded (LIPEX^{TM} extruder, Northern Lipids Inc., Canada) 4 times through a carbonate filter with a pore size of 400 nm and 4 times through a filter with a pore size of 200 nm (Nucleopore Millipore, Amsterdam, The Netherlands). For adjuvanted liposomes, after rehydration CpG (final concentration of 1.5 mg/ml) was added and the dispersions were freeze-dried followed by rehydration and extrusion as described above.

After monodisperse, unilamellar liposomes were obtained, unencapsulated antigen and adjuvant were separated from the liposomes by a Vivaspin 2 centrifugal concentrator (PES membrane, MWCO 300 kDa, Sartorius Stedim, Nieuwegein, The Netherlands) and (by using their fluorescently labelled analogues) quantified with a FS920 fluorimeter (Edinburgh Instruments, Campus Livingston, UK). The same technique was applied to investigate the association of OVA with empty liposomes. The size of the liposomes was determined by dynamic light scattering (DLS) and the zeta potential was measured by laser Doppler velocimetry using a Zetasizer® Nano ZS (Malvern Instruments,UK).

Vaccination

8 week old female Balb/c mice (Charles River, Maastricht, The Netherlands) received OVA or OVA- and CpG-containing formulations via transcutaneous, nasal, intradermal or intranodal administration. Based on literature and earlier studies from our group [27-29] the antigen dose and volume was adjusted to the administration route. The dose was chosen is such a way that the IgG response after immunization with a solution of OVA would be minimal and allow optimal discrimination between administration of OVA alone and liposomal OVA formulations.

For transcutaneous vaccination mice were shaved before pre-treatment with microneedles as described by Ding et al. [30]. Assembled metal microneedle arrays (4x4) with a length of 300 µm were used and applied with an electrical applicator as described before [24]. The formulations were applied occlusively on the skin (~2 cm^2 area restricted by a metal ring).

After 2 h the abdominal skin of the mice was washed extensively with lukewarm water. Intranodal injections in the inguinal lymph nodes were performed as described by Johansen et al [31]. Intradermal and nasal immunizations were carried out as described previously [27, 28]. After 3 weeks, blood samples were drawn from the tail vein and mice received a booster dose. After 6 weeks blood samples were drawn from the femur artery and mice were sacrificed.

Determination of serum IgG, IgG1, IgG2a and secretory IgA

Microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 ng OVA per well in a 100 mM carbonate buffer pH 9.4. Wells were blocked with 1% BSA in PBS for 1 hour at 37°C. Serial dilutions were applied for 1.5 hours after which OVA-specific antibodies were detected using HRP conjugated goat anti-mouse IgG, IgG1, IgG2a or IgA. Enzyme activity was determined by incubating with TMB/H_2O_2 in 100 mM acetate buffer pH 5.5 for 15 min at room temperature. Reaction was stopped with 2 M H_2SO_4 and absorbance was determined at 450 nm with an EL808 microplatereader (Bio-Tek Instruments, Bad Friedrichshall, Germany).

In vitro uptake by dendritic cells

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh human blood using a Ficoll gradient as previously described [32]. Subsequently, monocytes were isolated from the PBMCs using a Percoll gradient as previously described [33]. After isolation, monocytes were adhered on 24-well plates by incubation for 1 hr 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, 2 mM glutamine, 1 mM sodium pyruvate, 500 U/L penicillin/streptomycin, 250 U/ml GM-CSF and 100 U/ml IL-4.

Immature DCs were exposed for 4 h at 37°C to 0.5 µg/ml CpG_{FITC} and/or 0.5 µg/ml OVA_{AF647} in free or encapsulated form. Cells were washed three times with FACS buffer (1% w/v BSA in PBS with 2% v/v fetal bovine serum), and the number of FITC or AF647 positive DCs (CpG^+ or OVA^+) was quantified with a flow cytometer (FACSCanto II, Becton Dickinson) using quadrant analysis.

Determination of antigen specific DCs in lymph node

Mice were vaccinated with the same formulations as described in Table I, but OVA was substituted with OVA_{AF647} and CpG with CpG_{FITC}. After 4 or 24 h mice were sacrificed, the draining lymph nodes (for transcutaneous, intradermal and intranodal administration the inguinal and for nasal the cervical lymph nodes) were removed and single cell suspensions obtained. Cells were washed with FACS buffer and stained with anti-CD11c-PE-Cy7 to allow

detection of DCs. Using flow cytometry (FACSCantoII, Becton Dickinson) the amount of OVA_{AF647}^{+} and CpG_{FITC}^{+} DCs was determined.

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 processed with a Kruskal-Wallis test with Dunn's post-test. Statistics were performed using GraphPad 5.0 for Windows.

Results

Cationic liposomes improve uptake of OVA and CpG by DCs in vitro

To assess whether our cationic liposomes increase the uptake of OVA and CpG, the uptake by human monocyte derived DCs was measured *in vitro*. Consistent with our previous study [17], OVA-containing liposomes with an average size of 130 nm bearing a positive zeta potential (23 mV) and OVA/CpG liposomes with a size of 263 nm and a zeta potential of 18 mV were obtained. The cationic liposomes significantly enhanced the uptake of their encapsulated cargo by DCs (figure 1). Plain OVA was readily taken up by DCs, as approximately 20% of the DCs was OVA^{+} after 4 h of incubation with OVA (figure 2A), but when encapsulated into liposomes, the number of OVA^{+} DC was significantly increased ($p<0.001$), reaching a value of approximately 50% OVA^{+} DCs. In contrast to OVA, non encapsulated CpG was hardly taken up by DCs in its plain form as only 6% CpG^{+} DCs were observed (figure 2B). Encapsulation of CpG in cationic liposomes however increased the number of CpG^{+} DCs with an order of magnitude. Co-encapsulation of OVA and CpG^{+} in cationic liposomes caused a major improvement in the number of $OVA/CpG^{+/+}$ DCs (figure 2C), compared to addition of a solution of OVA and CpG. In conclusion, co-encapsulation of OVA and CpG in cationic liposomes enhanced their concomitant uptake by DCs *in vitro*.

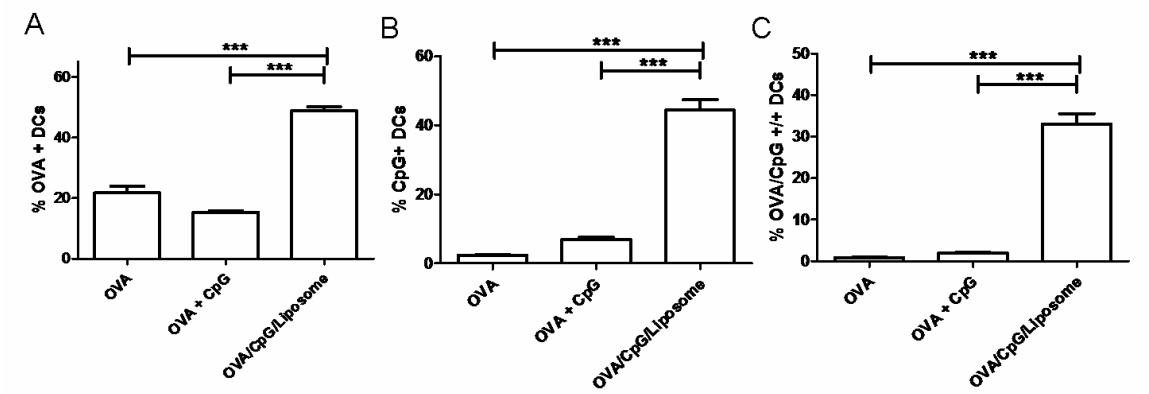


***Figure 2**: Uptake of a) OVA and b) CpG by human monocyte derived DCs determined by FACS analysis. c) number of OVA and CpG double positive DCs after 4 h exposure. n=3 Average + SEM. *** p<0.001*

Intranodal vaccination

Liposomes enhance the uptake of OVA and CpG by lymph node resident DCs.

Because of the large numbers of T cells present in the lymph nodes, activated DCs that have taken up an antigen have a good chance of finding antigen specific T cells there. Therefore the draining lymph node is the most prominent site of activation for T cells after immunization and can be considered the actual target of vaccines. As a consequence, direct injection of a vaccine into the lymph nodes could be a very efficient method of administration, requiring only very low amounts of antigen to result in an effective immune response, as processes like peripheral uptake of the antigen by DCs and drainage to the lymphnodes are circumvented (figure 1A). Indeed after intranodal injection a rapid uptake of fluorescently labeled OVA and CpG by DCs was observed as can be inferred from the high percentage of OVA^+ and CpG^+ DCs already after 4 h (figure 3). Liposomal encapsulation of OVA and CpG led to significantly elevated numbers of OVA^+, CpG^+ and $OVA/CpG^{+/+}$ DCs compared to the administration of a soluble mixture of OVA and CpG. The percentage of DCs that had taken up both OVA and CpG increased by 4-fold compared to injection of a physical mixture of CpG and OVA. After 24 h the levels had decreased drastically, suggesting that OVA and CpG had been processed. Alas, next to in vitro, also in vivo cationic liposomes have the potential to increase the delivery of the antigen and the adjuvant.

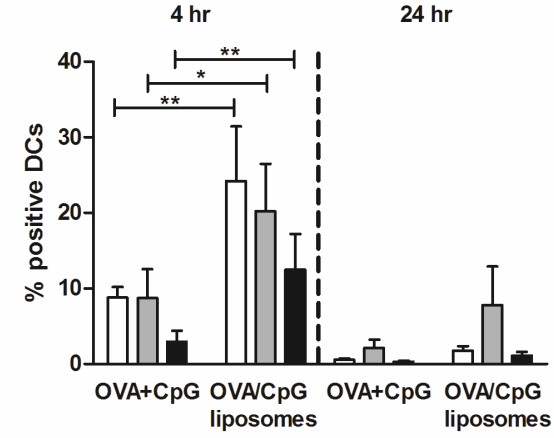


***Figure 3**: Quantification of OVA$^+$ (white bars), CpG$^+$ (grey bars) and OVA/CpG$^{+/+}$ (black bars) DCs in the draining lymph nodes 4 and 24 h after intranodal vaccination, Bars represent the mean n=3 + SEM. *p<0.05, **p<0.01.*

High IgG2a titers after intranodal injection of OVA/CpG liposomes

In spite of the favorable effect of liposomal co-encapsulation of OVA and CpG on DC uptake, intranodal vaccination showed similar total serum IgG titers for all formulations (figure 4A). Both after the prime and booster immunization no effect of either liposome encapsulation or CpG was observed, indicating that an antigen injected directly into the lymph node does not need a delivery vehicle or an adjuvant to induce a humoral immune response. However, whereas IgG titers provide information about the extent of the antibody response, subtyping of the IgG response can give insight into the type of immune response elicited. Therefore, IgG1 levels, indicative of a Th2 type (humoral) response and IgG2a titers, indicative of a Th1 type (cellular) response [34, 35], were quantified to investigate the quality of the immune response after the boost immunization (figure 4B). IgG1 titers appeared to be virtually in synchronicity with the IgG titers, again indicating that all formulations triggered the humoral immune response. However, co-encapsulation of CpG and OVA in liposomes drastically increased IgG2a levels compared to OVA ($p<0.001$) as well as non encapsulated OVA + CpG ($p<0.001$). As such OVA/CpG liposomes caused a significant decrease in IgG1/IgG2a ratio, compared to all other formulations ($p<0.05$, figure 3C). This shows that intrinsically, OVA/CpG liposomes are a very immunogenic and effectively delivery system that can induce a mixed Th1/Th2 type immune response.

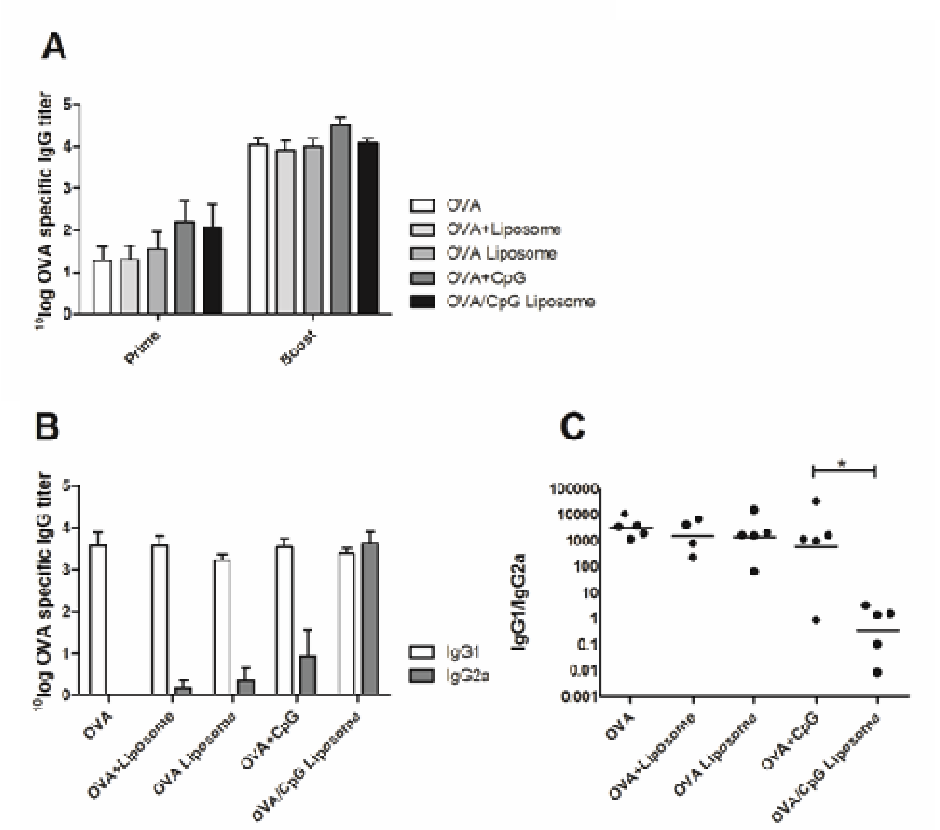


***Figure 4**: OVA-specific serum IgG, IgG1 and IgG2a titers after intranodal vaccination. A: IgG titers after prime and boost. B: IgG1 and IgG2a titers after booster immunization. Bars represent SEM of n=5 (A and B). C: Corresponding IgG1/IgG2a ratio for each individual mouse. Mice non-respondent for either IgG1 or IgG2a were removed from the data set. Bar represents geomean * p<0.05.*

Intradermal vaccination

Cationic liposomes reduce direct drainage to the lymph node

In contrast to intranodal injection, antigen that is administered intradermally can reach the lymph nodes in two ways, it can either directly drain in a quick manner to nearest lymph node via the interstitial fluid and lymphoid vessels or it can be taken up by local DCs and transported to the draining lymph nodes in a process that takes longer (figure 1). Intradermal injection of fluorescent OVA and CpG showed the presence of both these routes; already 4 h after administration, OVA^+ DCs could be detected in the lymph nodes, but these cells had not

taken up CpG (figure 5). Liposomal delivery to the lymph nodes after intradermal administration was a slower process, as not 4 h but only 24 h after injection OVA^+ and CpG^+ DCs were found in the draining lymph nodes. These suggest both OVA and CpG were retained at the injection side. Indeed the formation of an antigen depot that was visible by eye at the injection site, even 24h after injection. Liposomal encapsulation did not further increase the number of OVA^+ and CpG^+ DCs after 24 h compared to intradermal administration of non encapsulated OVA and CpG.

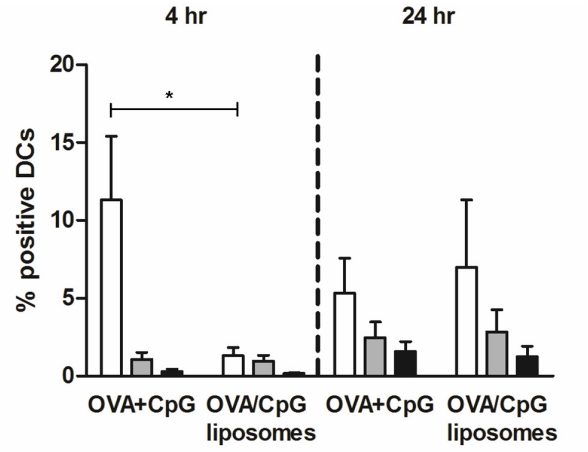


***Figure 5**: Quantification of OVA^+ (white bars), CpG^+ (grey bars) and $OVA/CpG^{+/+}$ (black bars) DCs in the draining lymph nodes 4 and 24 h after intradermal vaccination, Bars represent the mean n=3 + SEM. *p<0.05*

Cationic liposomes have an adjuvant effect and increase IgG2a levels significantly

Vaccination via the intradermal route showed that cationic liposomes can have an adjuvant effect when mixed with OVA ($p<0.01$), whereas encapsulated liposomal OVA did not lead to a significant increase in IgG levels (figure 6A). Addition of CpG clearly increased the antibody levels compared to OVA after the first ($p<0.001$), as well as the second immunization ($p<0.001$) and also compared to OVA + liposomes after the first vaccination ($p<0.05$). Intradermal application of OVA/CpG liposomes increased the IgG levels to a similar extent as a solution of OVA and CpG.

After intradermal administration IgG1 titers mimicked the IgG titers and IgG2a levels were very low with the exception those of the mice immunized with OVA/CpG liposomes (figure 6B). Just like after intranodal vaccination, these mice had significantly higher IgG2a titers compared to those receiving plain OVA ($p<0.001$), resulting in a significant shift in the IgG1/IgG2a ratio (figure 6C). OVA + CpG did not provoke significantly higher IgG2a titers.

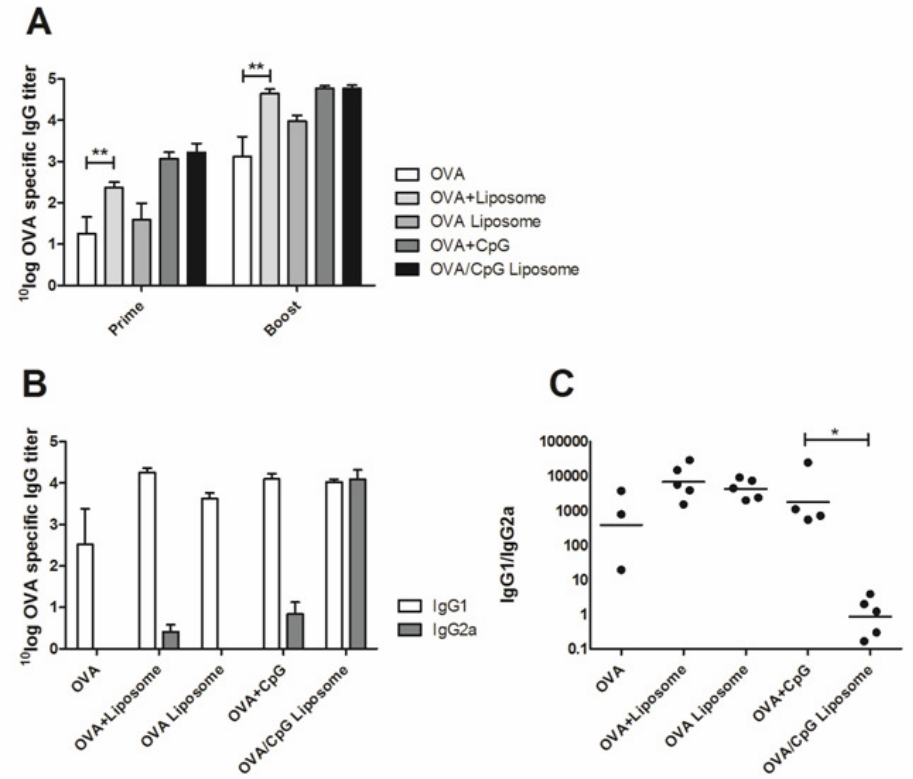


***Figure 6**: OVA-specific serum IgG, IgG1 and IgG2a titers after intradermal vaccination. A: IgG titers after prime and boost. B: IgG1 and IgG2a titers after booster immunization. Bars represent SEM of n=5 (A and B). C: Corresponding IgG1/IgG2a ratio for each individual mouse. Mice non-respondent for either IgG1 or IgG2a were removed from the data set. Bar represents geomean * p<0.05, ** p<0.01.*

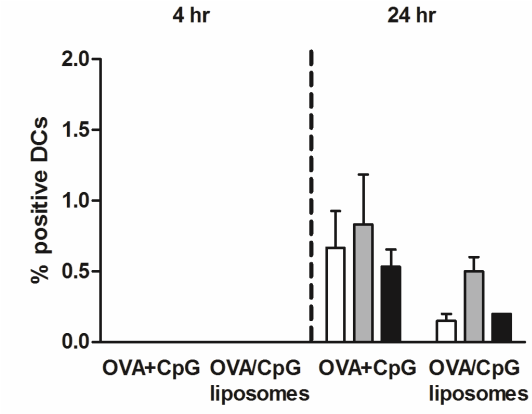


***Figure 7**: Quantification of OVA^{+} (white bars), CpG^{+} (grey bars) and OVA/CpG$^{+/+}$ (black bars) DCs in the draining lymph nodes 4 and 24 h after transcutaneous vaccination with microneedle pretreatment, Bars represent the mean n=3 + SEM.*

Transcutaneous immunization

Liposomes reduce transport of OVA and CpG through the skin

Transcutaneous administration involves an extra transport step across the epidermis compared to intranodal and intradermal injection (figure 1). Consequently, there was no trafficking to the lymph node resident DCs after 4 h. Only after 24 h measurable OVA and CpG levels were observed (figure 7). A clear detrimental effect of liposomal encapsulation on the amount of OVA and CpG reaching the lymph nodes is shown, as application of non encapsulated OVA and CpG resulted in significantly higher numbers of OVA^+ and CpG^+ DCs than administration of OVA/CpG liposomes.

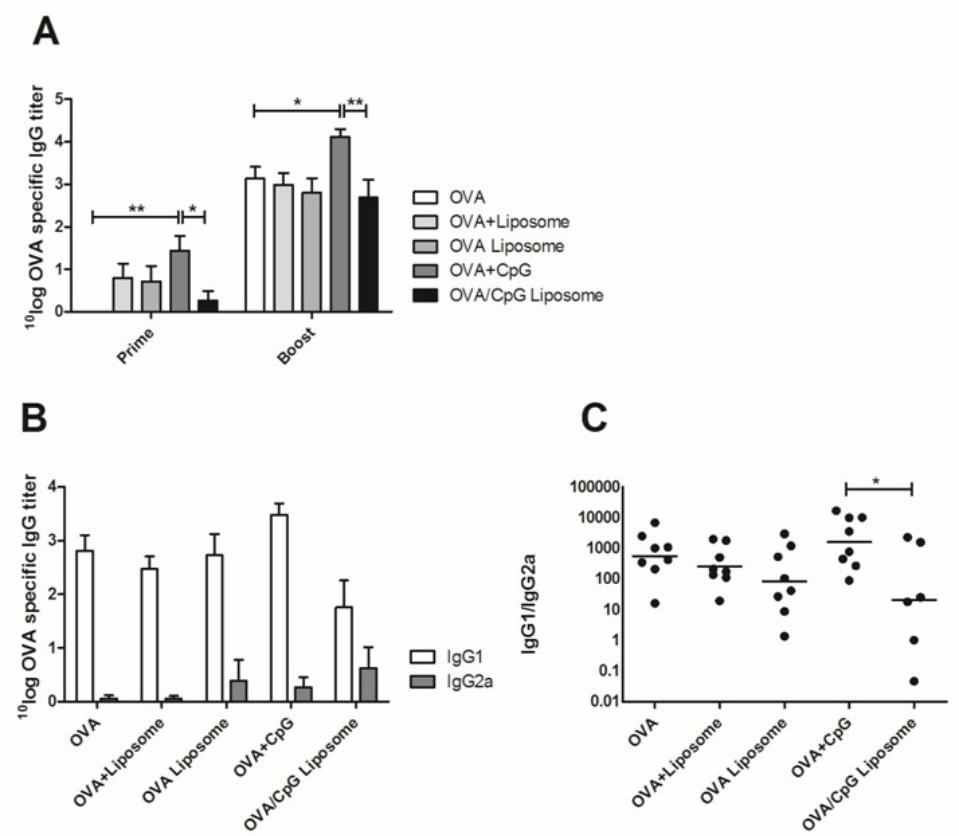


***Figure 8**: OVA-specific serum IgG, IgG1 and IgG2a titers after transcutaneous vaccination. A: IgG titers after prime and boost. B: IgG1 and IgG2a titers after booster immunization. Bars represent SEM of n=8 (A and B). C: Corresponding IgG1/IgG2a ratio for each individual mouse. Mice non-respondent for either IgG1 or IgG2a were removed from the data set. Bar represents geomean * $p<0.05$, ** $p<0.01$.*

Liposomal co-encapsulation of OVA and CpG reduce IgG titers, but enhance IgG2a levels

The immune enhancing effect of cationic liposome, as observed after intradermal administration was not apparent after transcutaneous administration. Regardless whether OVA was encapsulated or not encapsulated in liposomes no increased IgG titers compared to plain OVA after transcutaneous immunization on microneedle pre-treated skin were observed (figure 8A). Contrarily, administration of a OVA + CpG solution resulted in strongly enhanced IgG titers both after the prime and subsequent booster vaccination compared to an OVA solution ($p<0.05$). This effect was abolished by encapsulation of antigen and adjuvant into liposomes ($p<0.01$). As far as the subtiters are concerned, unlike after intranodal and intradermal vaccination, transcutaneous immunization with encapsulated and non-encapsulated CpG did not significantly elevate OVA-specific IgG2a titers (figure 8B), but encapsulated CpG reduced the IgG1 levels, thereby causing a significant decrease in the IgG1/IgG2a ratio ($p<0.05$).

Nasal vaccination

Liposomes reduce transport of OVA and CpG through the nasal epithelium

Comparable to transcutaneous vaccination, nasal administration involves an extra transport step across the epithelium (figure 1). Ergo, 4 h after nasal application of fluorescent OVA and CpG no OVA^+ and CpG^+ DCs were detected in the cervical lymph nodes (figure 9). After 24 h DCs had taken up OVA and CpG, but similarly as after transcutaneous administration; the numbers of OVA and CpG positive DCs in the draining (cervical) lymph node were reduced when a liposomal formulation was used.

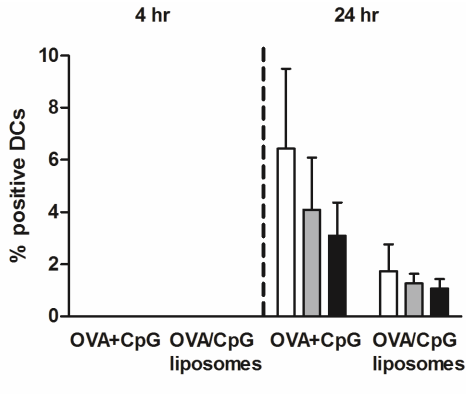


***Figure 9**: Quantification of OVA^+ (white bars), CpG^+ (grey bars) and $OVA/CpG^{+/+}$ (black bars) DCs in the draining lymph nodes 4 and 24 h after transcutaneous vaccination with microneedle pretreatment, Bars represent the mean n=3 + SEM.*

Liposomal co-encapsulation of OVA and CpG not necessary to induce IgG2a titers

Mice receiving the formulations nasally showed a similar IgG pattern compared to transcutaneous vaccination (figure 10A): liposomes did not stimulate the anti-OVA IgG response and soluble OVA adjuvanted with CpG induced the strongest response already after a priming dose ($p<0.001$ compared to OVA). When CpG was co-encapsulated with OVA in liposomes the effect of CpG was reduced, altough the serum antibody levels were higher as compared to OVA alone. This was not observed in contrast to transcutaneous vaccination. Nasal co-administration of OVA + CpG, did significantly increase the IgG2a levels compared to OVA (figure 10B, $p<0.001$). Co-encapsulation of CpG and OVA in liposomes also increased the IgG2a titers compared to encapsulation of OVA alone ($p<0.01$), but did not result in a significant shift in the IgG1/IgG2a ratio compared to a solution of OVA and CpG (figure 9C), as observed for intradermally and intranodally vaccinated mice.

Finally, only nasal administration resulted in detectable levels of secretory IgA (sIgA) in the nasal washes of the mice. Nasal immunization with a both encapsulated as well free OVA and CpG induce significantly higher levels of sIgA than vaccination with OVA alone (figure 11).

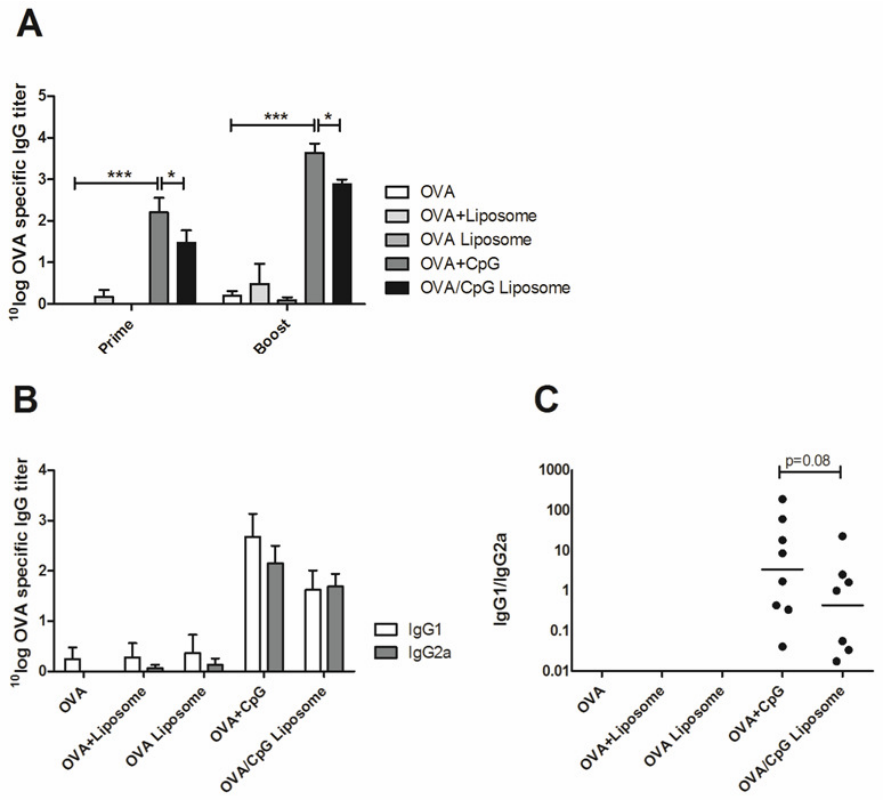


***Figure 10**: OVA-specific serum IgG, IgG1 and IgG2a titers after nasal vaccination. A: IgG titers after prime and boost. B: IgG1 and IgG2a titers after booster immunization. Bars represent SEM of n=8 (A and B). C: Corresponding IgG1/IgG2a ratio for each individual mouse. Mice non-respondent for either IgG1 or IgG2a were removed from the data set. Bar represents geomean * p<0.05, *** p<0.001.*

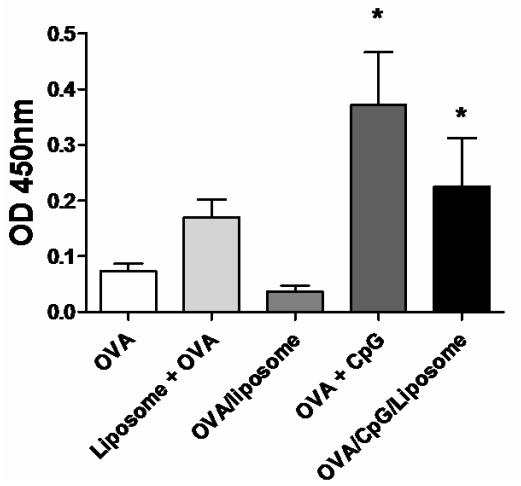


***Figure 11**: OVA-specific secretory IgA in nasal washes after nasal vaccination. Bars represent SEM of n=8 * p<0.05 compared to OVA.*

Discussion

Nasal and microneedle-based transcutaneous vaccination potentially provides a safe and patient friendly alternative to classical vaccine administration via the needle. However, vaccination via non-invasive routes is challenging as the antigen will first have to pass a barrier (nasal epithelium or the skin), which limits the amount of antigen that reaches the DCs. To provoke a strong immune response with a limited amount of antigen, high immunogenicity of vaccine is very important. The immunogenicity of subunit vaccines can be enhanced if the antigen is properly formulated. Therefore the use of cationic liposomes as a carrier system makes sense; they provide efficient antigen encapsulation and their particulate nature makes them a natural target for DCs, which can enhance the uptake of their cargo by DCs. Co-encapsulation of an adjuvant will result in the concomitant delivery of antigen and adjuvant to DCs, which has been described as crucial for a potent immune response [36, 37]. However, compared to solutions of antigens and adjuvants, liposomes might have difficulties passing the epithelial barriers.

Co-encapsulation of antigen and adjuvant into liposomes had a positive effect when the formulations were injected: intranodal or intradermal injection of OVA/CpG liposomes strongly boosted IgG2a titers (indicative for a Th1 response), whereas administration of non-

encapsulated OVA + CpG did not. This effect may be related to the site of action of the adjuvant used. CpG is a ligand for TLR9, which is localized in the endosomal compartment of APCs. It is therefore imperative for CpG to be endocytosed by the APC, in order to be able to interact with TLR9. Here we show that *in vitro*, cationic liposomes can facilitate CpG uptake by DCs, adding to the increase in IgG2a titers after intradermal and intranodal injection. *In vivo* the DC uptake after intranodal injection corresponds well with the *in vitro* data, as in both cases a clear benefit of co-encapsulation on DC uptake was observed.

Interestingly, after intranodal administration OVA/CpG liposomes was the only formulation to have a beneficial effect on the IgG2a titer, whereas all formulations induced similar IgG and IgG1 titers via this route. It is likely that the injection itself will already induce a danger signal to the residing DCs, thereby inducing DC activation and maturation [38], sufficient to induce a humeral (IgG1) response [29]. The additional benefit of adjuvanted liposomes on the total immune response via this route, using this amount of antigen, is therefore negligible, but the effect on the immune bias is substantial. Since high IgG2a levels were obtained with the OVA/CpG liposomes, this indicates that liposomal co-encapsulation of antigen and adjuvant is essential for induction of a Th1 type immune response.

Intradermal administration of OVA with empty liposomes significantly increased antibody titers compared to administration of OVA alone. As empty liposomes do not activate immature DCs, the adjuvanticity of these vesicles can most probably be attributed to the antigen depot it forms upon injection, likely through interaction of liposomes and antigen with the extracellular matrix. According to Henriksen-Lacey et al. this depot could be detected up to 14 days post intramuscular or subcutaneous injection and promoted the immunogenicity of the antigen [10]. However, the current study also shows that depot formation prohibits rapid drainage of the antigen to the lymph node. Antigens in solution can directly drain to the lymph node and be taken up by a large population of immature lymph node resident DCs [39], as reflected in our study by the high number of OVA^+ DCs found in the lymph nodes 4 h after injection of OVA. Liposomal administration and the resulting antigen depot reduced the amount of antigen that directly drains to the lymph nodes, but induced prolonged OVA delivery compared to a mixture of OVA and CpG. These two processes can induce two distinct waves of antigen reaching the lymph nodes, and may be imperative for provoking a good (memory) immune response [40]. This could explain why intradermal application of OVA with empty liposomes induced a better response than OVA loaded liposomes. The latter does not allow direct drainage of the antigen to the lymph nodes, but only a prolonged release, whereas the first approach might have resulted in both direct and prolonged release.

Nasally and especially transcutaneously, liposomes were found to be a suboptimal vaccine delivery system. Although nasal administration of OVA/CpG loaded liposomes did show an increase in IgG titers compared to administration of OVA alone, this was due to the presence of CpG rather than its formulation into liposomes, as nasal administration of OVA + CpG as a solution induced superior antibody titers. After transcutaneous vaccination with microneedles, encapsulation of CpG into liposomes even completely inhibited the positive effect of the adjuvant. We showed that liposomes dramatically decreased the amount of antigen and adjuvant reaching the DCs. Significantly less OVA^+ or CpG^+ DCs were detected in the draining lymph nodes 24 h after nasal or transcutaneous administration of OVA/CpG liposomes compared to administration of OVA + CpG. Possibly the concomitant size increase caused by the encapsulation into the liposome and the positive charge of the delivery system obstructed the transport of the antigen and adjuvant to the lymph node.

An interesting difference between the the nasal and the transcutaneous route was observed; whereas after transcutaneous immunization a solution of OVA or non-adjuvanted liposomes were capable of inducing seroconversion in all mice, nasal vaccination required the addition CpG to induce measurable antibody titers. This may be related to the nasal epithelium being a rather tolerogenic immunization site [41], making the activation of DCs with an adjuvant an important requirement for the induction of antibodies. The skin DCs, however, are known to not only playing a role in tissue homeostasis, but also having a strong pro-inflammatory function [42, 43]. This may have an evolutionary purpose as a micro-organism that has breached the skin barrier is more dangerous that an organism that has ended up in the nasal epithelium.

Although after nasal and transcutaneous vaccination the total humoral immune response did not benefit from co-encapsulation of OVA and CpG into liposomes, liposomal co-encapsulation may have a pronounced effect on the induction of a cellular response. Nasal and transcutaneous administration of OVA/CpG liposomes induced relatively more IgG2a compared to IgG1 than the administration of a physical mixture of these 2 components. Co-localization of antigen and adjuvant therefore still remains an important mechanism to enhance the immunogenicity of a non-injectable subunit vaccine, but should not be established by using a particulate delivery system. Antigen-adjuvant conjugates or Fc-receptor immune complexes have been reported to very efficiently target DCs [44, 45] and due to their smaller size, may be more suitable for nasal and transcutaneous administration.

Conclusion

Despite the advantages of using cationic liposomes as a vaccine adjuvant, careful consideration should be given when such systems are designed for transcutaneous and nasal vaccination. These data show that, intrinsically, liposomes containing both the antigen as well as the adjuvant enhance the immunogenicity of the antigen and promote the induction of both IgG1 and IgG2a type antibodies. However, likely due to poor penetration of the microneedle pre-treated skin and nasal mucosa, they are unsuitable for application via the transcutaneous or nasal route.

Acknowledgements

The authors thank Pål Johansen for his support in acquiring the technique of intranodal vaccination. This research was performed under the framework of TI Pharma project number D5-106 “vaccine delivery: alternatives for conventional multiple injection vaccines”.

References

1. HogenEsch, H., *Mechanisms of stimulation of the immune response by aluminum adjuvants.* Vaccine, 2002. **20**: p. S34-S39.
2. Eldridge, J.H., et al., *Biodegradable and Biocompatible Poly(Dl-Lactide-Co-Glycolide) Microspheres as an Adjuvant for Staphylococcal Enterotoxin-B Toxoid Which Enhances the Level of Toxin-Neutralizing Antibodies.* Infection and Immunity, 1991. **59**(9): p. 2978-2986.
3. Trombetta, E.S. and I. Mellman, *Cell biology of antigen processing in vitro and in vivo.* Annu Rev Immunol, 2005. **23**: p. 975-1028.
4. Singh, M., A. Chakrapani, and D. O'Hagan, *Nanoparticles and microparticles as vaccine-delivery systems.* Expert Rev Vaccines, 2007. **6**(5): p. 797-808.
5. Schlosser, E., et al., *TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses.* Vaccine, 2008. **26**(13): p. 1626-37.
6. Allison, A.G. and G. Gregoriadis, *Liposomes as immunological adjuvants.* Nature, 1974. **252**(5480): p. 252.
7. Nakanishi, T., et al., *Positively charged liposome functions as an efficient immunoadjuvant in inducing cell-mediated immune response to soluble proteins.* Journal of Controlled Release, 1999. **61**(1-2): p. 233-240.
8. Nakanishi, T., et al., *Positively charged liposome functions as an efficient immunoadjuvant in inducing immune responses to soluble proteins.* Biochemical and Biophysical Research Communications, 1997. **240**(3): p. 793-797.
9. Brgles, M., et al., *Liposome fusogenicity and entrapment efficiency of antigen determine the Th1/Th2 bias of antigen-specific immune response.* Vaccine, 2009. **27**(40): p. 5435-42.
10. Henriksen-Lacey, M., et al., *Liposomes based on dimethyldioctadecylammonium promote a depot effect and enhance immunogenicity of soluble antigen.* Journal of Controlled Release, 2010. **142**(2): p. 180-6.
11. Singh, M. and D. O'Hagan, *Advances in vaccine adjuvants.* Nature Biotechnology, 1999. **17**(11): p. 1075-81.
12. Yan, W., W. Chen, and L. Huang, *Mechanism of adjuvant activity of cationic liposome: phosphorylation of a MAP kinase, ERK and induction of chemokines.* Mol Immunol, 2007. **44**(15): p. 3672-81.
13. Christensen, D., et al., *Liposome-based cationic adjuvant formulations (CAF): past, present, and future.* J Liposome Res, 2009. **19**(1): p. 2-11.
14. Arigita, C., et al., *Liposomal meningococcal B vaccination: role of dendritic cell targeting in the development of a protective immune response.* Infect Immun, 2003. **71**(9): p. 5210-8.
15. Gursel, I., et al., *Sterically stabilized cationic liposomes improve the uptake and immunostimulatory activity of CpG oligonucleotides.* Journal of Immunology, 2001. **167**(6): p. 3324-3328.
16. Holten-Andersen, L., et al., *Combination of the cationic surfactant dimethyl dioctadecyl ammonium bromide and synthetic mycobacterial cord factor as an efficient adjuvant for tuberculosis subunit vaccines.* Infection and Immunity, 2004. **72**(3): p. 1608-17.
17. Bal, S.M., et al., *Co-encapsulation of antigen and adjuvant in cationic liposomes affects the quality of the immune response in mice after intradermal vaccination.* submitted, 2010.
18. Slütter, B., N. Hagenaars, and W. Jiskoot, *Rational design of nasal vaccines.* Journal of Drug Targeting, 2008. **16**(1): p. 1-17.
19. Nicolas, J.F. and B. Guy, *Intradermal, epidermal and transcutaneous vaccination: from immunology to clinical practice.* Expert Review of Vaccines, 2008. **7**(8): p. 1201-14.
20. Koping-Hoggard, M., A. Sanchez, and M.J. Alonso, *Nanoparticles as carriers for nasal vaccine delivery.* Expert Review of Vaccines, 2005. **4**(2): p. 185-196.

21. Illum, L., *Nanoparticulate systems for nasal delivery of drugs: A real improvement over simple systems?* Journal of Pharmaceutical Sciences, 2007. **96**(3): p. 473-483.
22. Combadiere, B. and B. Mahe, *Particle-based vaccines for transcutaneous vaccination.* Comparative Immunology Microbiology and Infectious Diseases, 2008. **31**(2-3): p. 293-315.
23. Ding, Z., et al., *Transcutaneous Immunization Studies in Mice Using Diphtheria Toxoid-Loaded Vesicle Formulations and a Microneedle Array.* Pharm Res, 2010.
24. Bal, S.M., et al., *Microneedle-Based Transcutaneous Immunisation in Mice with N-Trimethyl Chitosan Adjuvanted Diphtheria Toxoid Formulations.* Pharm Res, 2010.
25. Slütter, B., et al., *Antigen-adjuvant nanoconjugates for nasal vaccination, an improvement over the use of nanoparticles?* Molecular Pharmaceutics, 2010. **Submitted**.
26. Bangham, A.D., M.M. Standish, and J.C. Watkins, *Diffusion of univalent ions across the lamellae of swollen phospholipids.* J Mol Biol, 1965. **13**(1): p. 238-52.
27. Bal, S.M., et al., *Efficient induction of immune responses through intradermal vaccination with N-trimethyl chitosan containing antigen formulations.* Journal of Controlled Release, 2010. **142**(3): p. 374-83.
28. Slütter, B., et al., *Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: Nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen.* Vaccine, 2010. **28** p. 6282-6291.
29. Mohanan, D., et al., *Administration routes affect the quality of immune responses: a cross-sectional evaluation of particulate antigen-delivery systems.* Journal of Controlled Release, 2010. **In press**.
30. Ding, Z., et al., *Microneedle arrays for the transcutaneous immunization of diphtheria and influenza in BALB/c mice.* Journal of Controlled Release, 2009. **136**(1): p. 71-78.
31. Johansen, P., et al., *Direct intralymphatic injection of peptide vaccines enhances immunogenicity.* Eur J Immunol, 2005. **35**(2): p. 568-74.
32. de Jong, E.C., et al., *Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse th cell-polarizing signals.* Journal of Immunology, 2002. **168**(4): p. 1704-9.
33. Vieira, P.L., et al., *Glucocorticoids inhibit bioactive IL-12p70 production by in vitro-generated human dendritic cells without affecting their T cell stimulatory potential.* Journal of Immunology, 1998. **161**(10): p. 5245-51.
34. Collins, J.T. and W.A. Dunnick, *Germline transcripts of the murine immunoglobulin gamma 2a gene: structure and induction by IFN-gamma.* Int Immunol, 1993. **5**(8): p. 885-91.
35. Severinson, E., C. Fernandez, and J. Stavnezer, *Induction of germ-line immunoglobulin heavy chain transcripts by mitogens and interleukins prior to switch recombination.* Eur J Immunol, 1990. **20**(5): p. 1079-84.
36. Fischer, S., et al., *Concomitant delivery of a CTL-restricted peptide antigen and CpG ODN by PLGA microparticles induces cellular immune response.* Journal of Drug Targeting, 2009. **17**(8): p. 652-61.
37. O'Hagan, D.T., M. Singh, and J.B. Ulmer, *Microparticle-based technologies for vaccines.* Methods, 2006. **40**(1): p. 10-9.
38. Matzinger, P., *The danger model: a renewed sense of self.* Science, 2002. **296**(5566): p. 301-5.
39. Wilson, N.S., et al., *Most lymphoid organ dendritic cell types are phenotypically and functionally immature.* Blood, 2003. **102**(6): p. 2187-94.
40. Itano, A.A., et al., *Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity.* Immunity, 2003. **19**(1): p. 47-57.
41. Vajdy, M. and D.T. O'Hagan, *Microparticles for intranasal immunization.* Adv Drug Deliv Rev, 2001. **51**(1-3): p. 127-41.
42. Loser, K. and S. Beissert, *Dendritic cells and T cells in the regulation of cutaneous immunity.* Adv Dermatol, 2007. **23**: p. 307-33.

43. Kautz-Neu, K., et al., *Leishmaniasis, contact hypersensitivity and graft-versus-host disease: understanding the role of dendritic cell subsets in balancing skin immunity and tolerance.* Exp Dermatol, 2010.
44. Hervouet, C., et al., *Langerhans cells prime IL-17-producing T cells and dampen genital cytotoxic responses following mucosal immunization.* Journal of Immunology, 2010. **184**(9): p. 4842-51.
45. Schuurhuis, D.H., et al., *Immune complex-loaded dendritic cells are superior to soluble immune complexes as antitumor vaccine.* Journal of Immunology, 2006. **176**(8): p. 4573-80.

7

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

Bram Slütter
Zhi Ding
Wim Hennink
Peter Soema
Rolf Verheul
Wim Jiskoot

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]

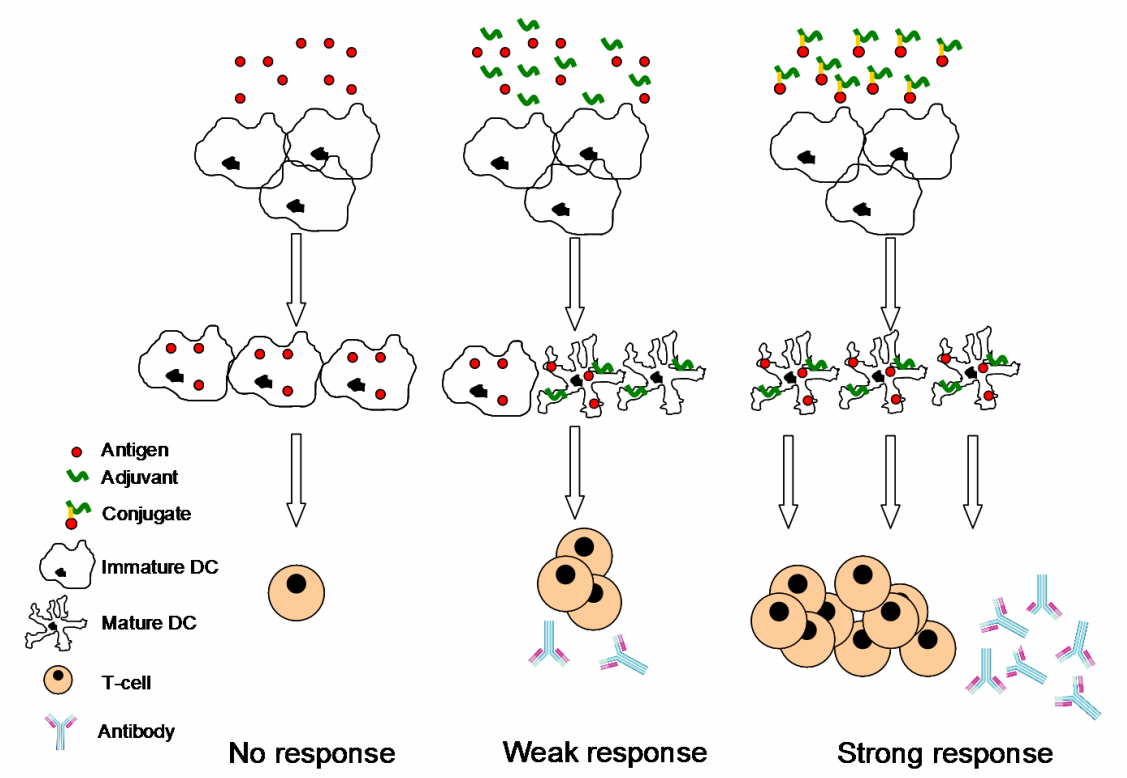


***Figure 1**: Subunit antigens are taken up by DCs but lack the necessary danger signals to induce DC maturation. An antigen-adjuvant mixture stimulates the activation of immature DCs, but antigen and adjuvant should be taken up simultaneously by a DC to effectively induce T-cell activation and antibody 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.

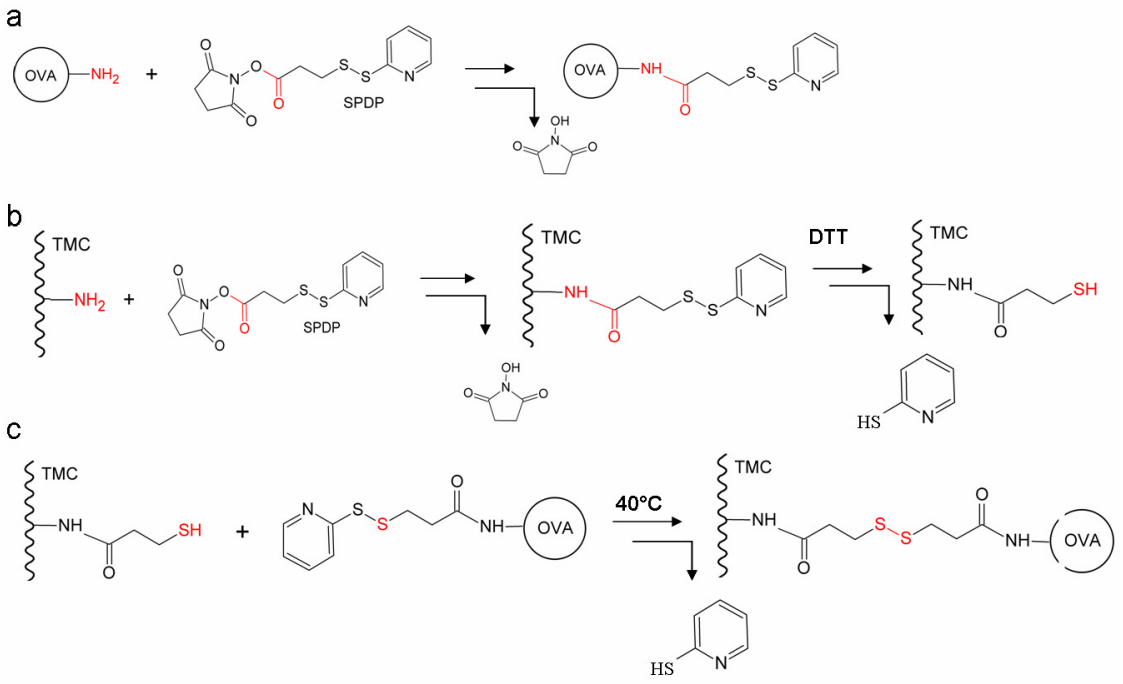


***Figure 2**: Reaction scheme of TMC-OVA conjugation. (a) Primary amine groups on OVA are functionalized with SPDP. (b) Primary amine groups on TMC are similarly functionalized and subsequently reduced with DTT, yielding TMC containing thiol groups. (c) An activated thiol group on TMC reacts with a 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.

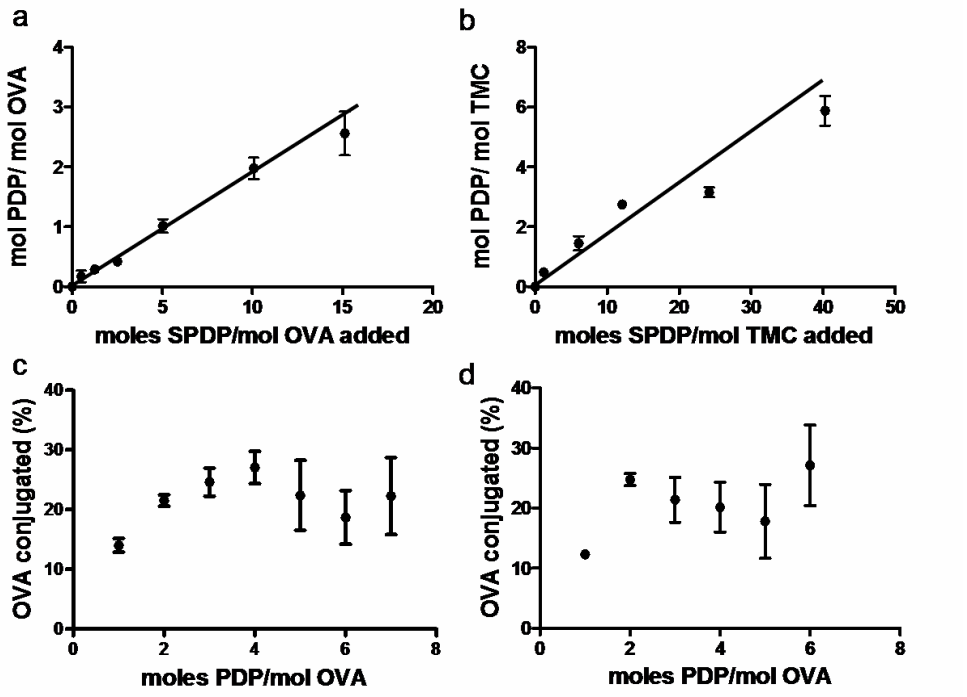


***Figure 3**: Functionalization and conjugation of TMC and OVA. Effect of SPDP feed on the amount of functionalized group on OVA (a) and TMC (b). PDP incorporation was monitored by UV-Vis detection at 343 nm. Efficiency of OVA conjugation to TMC-SH containing 1 mol SH/mol TMC (c) or 2 mol SH/mol TMC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

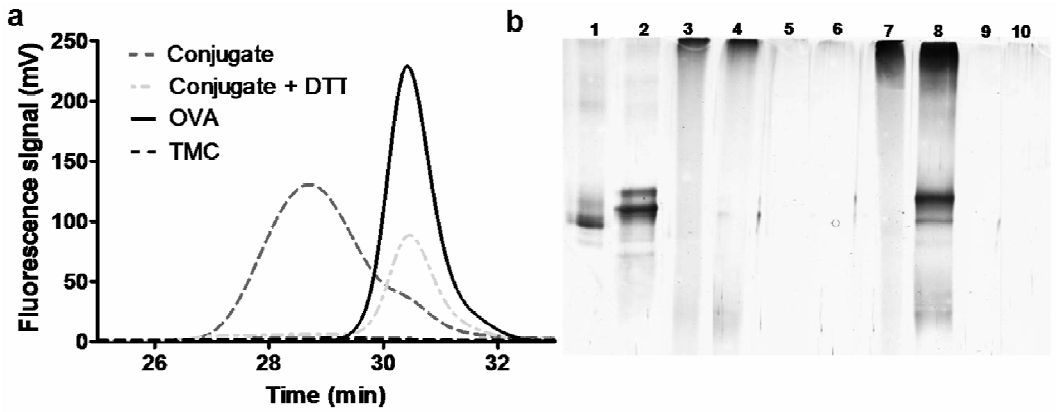


Figure 4: *(a) GPC chromatograms based on fluorescence detection (ex 295 nm, em 340 nm) of TMC-OVA conjugate (Conjugate), reduced TMC-OVA conjugate (Conjugate + DDT), OVA, and TMC. (b) SDS-PAGE under reducing (even lanes) and non-reducing (odd lanes) conditions of OVA (lane 1,2), TMC (lane 3,4), TMC-OVA 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).

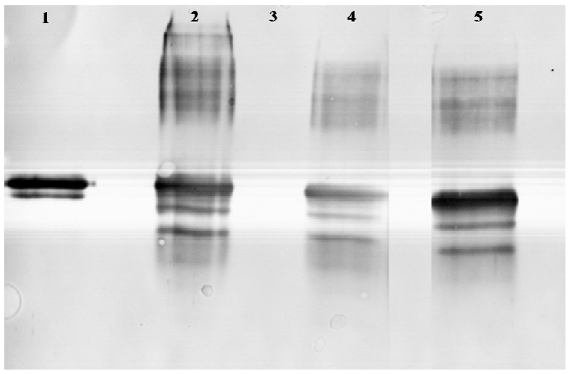


Figure 5*: Western blot of SDS-PAGE run under reducing conditions, using polyclonal anti-OVA IgG to detect the presence of intact epitopes. Lane 1: OVA; lane 2: OVA incubated for 16h at 40°C (equivalent conditions used for conjugation); lane 3: TMC; lane 4; TMC + OVA; lane 5: TMC-OVA.*

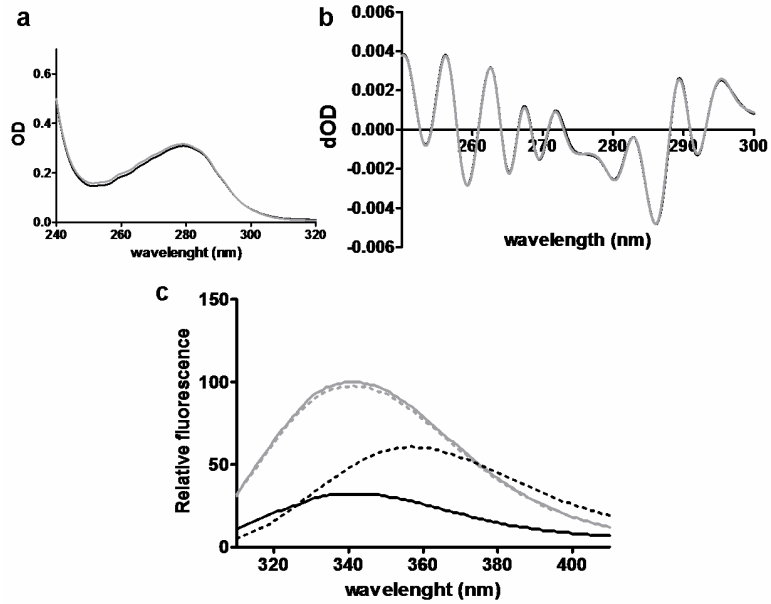


Figure 6: *(a) UV absorption spectrum and (b) 2nd derivate spectrum of 0.5 mg/ml OVA (grey line) and 2.5 mg/ml TMC-OVA (corresponding to 0.5 mg/ml OVA) conjugate (black line). c) Fluorescence emission spectra of OVA and TMC-OVA conjugate. Spectra of a mixture of OVA and TMC (dashed grey line) and unfolded 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 ($p=0.08$).

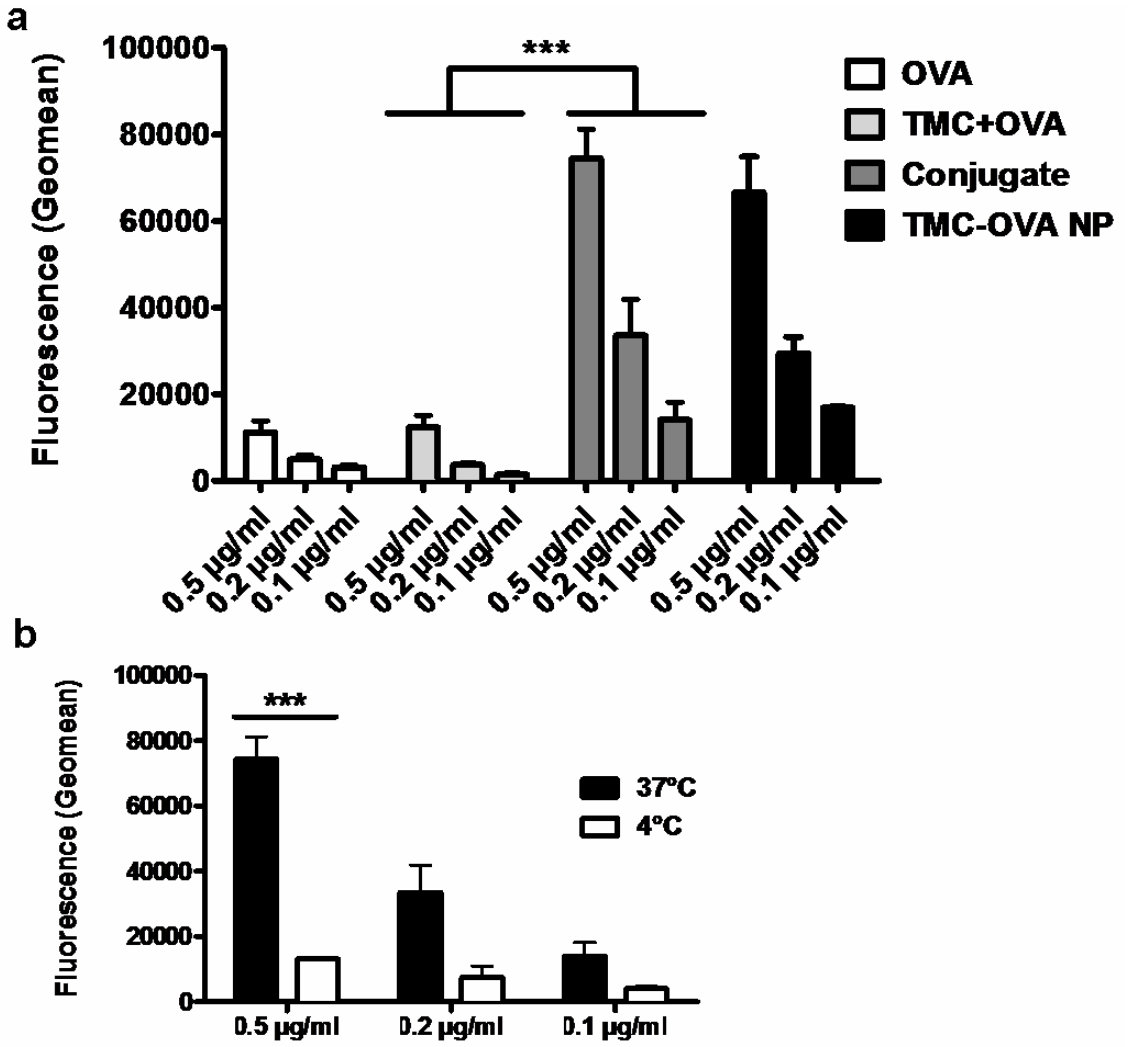


Figure 7**: (a) Dose dependent association of OVA, a mixture of OVA and TMC, TMC-OVA conjugate and TMC/OVA nanoparticles by DCs incubated at 37°C for 4 h. Conjugate as well nanoparticles showed a significant increase in uptake compared to OVA and TMC+OVA (= p<0.0001). (b) Active uptake versus passive 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).*

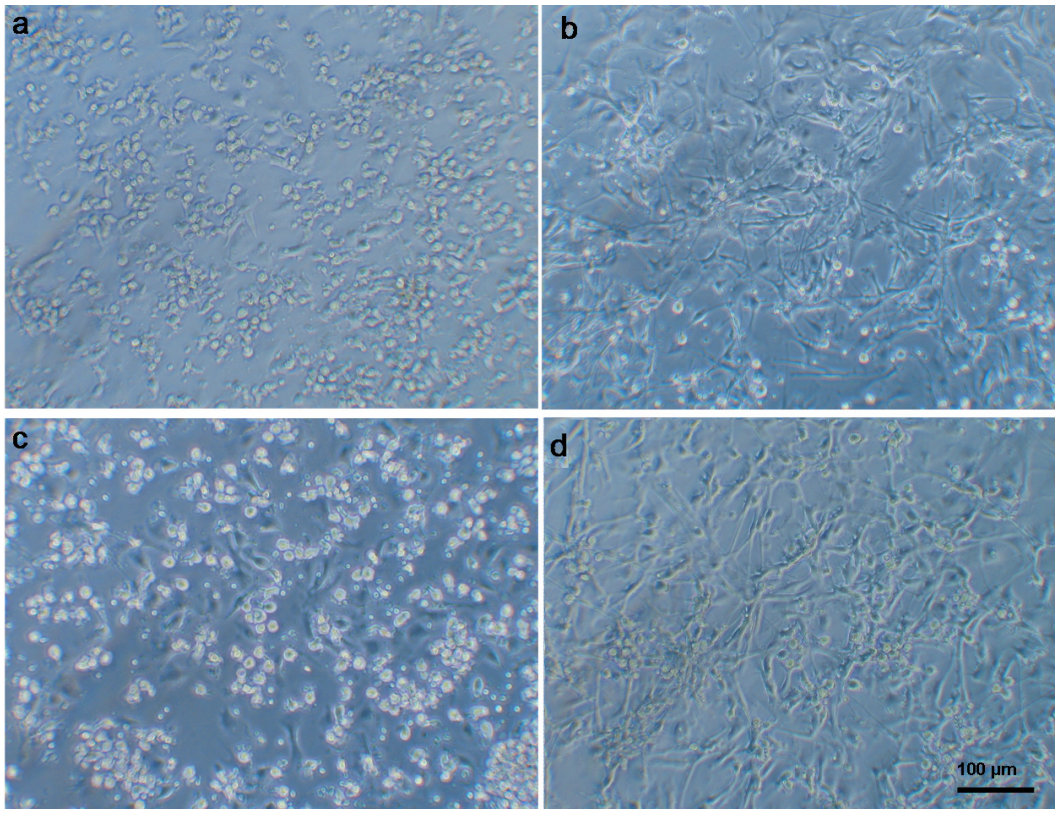


***Figure 8**: Representative example of micrographs of DCs treated for 4 h with (a) 0.2 µg/ml OVA, (b) 100 ng/ml LPS, (c) 0.2 µg/ml OVA + 1 µg/ml TMC and (d) 1 µg/ml TMC-OVA conjugate (corresponding to 0.2 µg/ml OVA). Magnification 20x.*

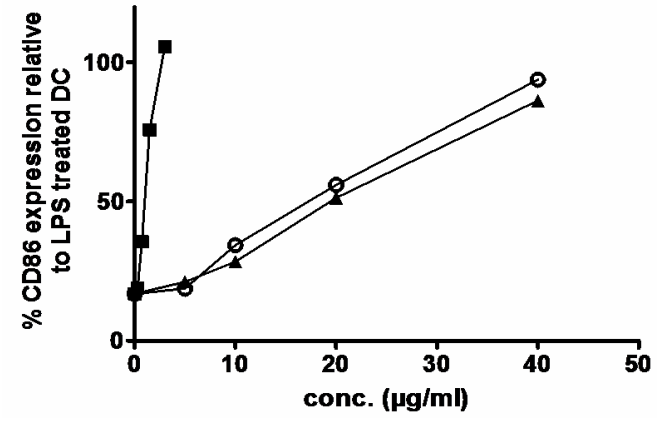


***Figure 9**: CD86 expression as a measure for DC maturation. Immature DCs were pulsed with increasing amounts of TMC/OVA mixture (open circles), conjugate (closed squares), or TMC/OVA nanoparticles (closed triangles) for 4 h, after which medium was replaced and CD86 expression was quantified after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

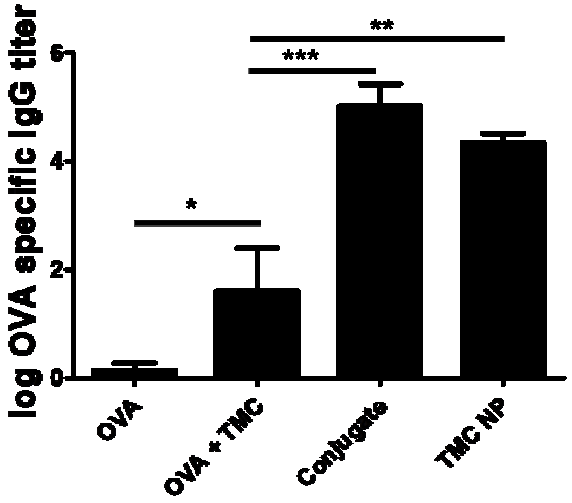


Figure 10: *OVA specific serum IgG titers after a single dose of 20 µg OVA, OVA mixed with TMC, TMC-OVA conjugate (conjugate) and TMC/OVA nanoparticles (TMC NP). * = p<0.05; ** = p<0.01; ***= p<0.001 Data represent mean +/- SD (n=5).*

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).

References

1. Wack, A. and R. Rappuoli, *Vaccinology at the beginning of the 21st century.* Curr Opin Immunol, 2005. **17**(4): p. 411-8.
2. Metz, B., et al., *Quality-control issues and approaches in vaccine development.* Expert Review of Vaccines, 2009. **8**(2): p. 227-238.
3. Huang, D.B., J.J. Wu, and S.K. Tyring, *A review of licensed viral vaccines, some of their safety concerns, and the advances in the development of investigational viral vaccines (vol 49, pg 179, 2004).* Journal of Infection, 2005. **51**(2): p. 174-174.
4. Steinman, R.M., *Dendritic cells and the control of immunity: Enhancing the efficiency of antigen presentation.* Mount Sinai Journal of Medicine, 2001. **68**(3): p. 160-166.
5. Wilson, N.S. and J.A. Villadangos, *Regulation of antigen presentation and cross-presentation in the dendritic cell network: Facts, hypothesis, and immunological implications.* Advances in Immunology, Vol 86, 2005. **86**: p. 241-305.
6. Steinman, R.M., et al., *Dendritic cell function in vivo during the steady state: a role in peripheral tolerance.* Ann N Y Acad Sci, 2003. **987**: p. 15-25.
7. Hagenaars, N., et al., *Physicochemical and immunological characterization of N,N,N-trimethyl chitosan-coated whole inactivated influenza virus vaccine for intranasal administration.* Pharm Res, 2009. **26**(6): p. 1353-64.
8. Amidi, M., et al., *N-trimethyl chitosan (TMC) nanoparticles loaded with influenza subunit antigen for intranasal vaccination: biological properties and immunogenicity in a mouse model.* Vaccine, 2007. **25**(1): p. 144-53.
9. Sayin, B., et al., *Mono-N-carboxymethyl chitosan (MCC) and N-trimethyl chitosan (TMC) nanoparticles for non-invasive vaccine delivery.* Int J Pharm, 2008. **363**(1-2): p. 139-48.
10. Amidi, M., et al., *Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system.* Journal of Controlled Release, 2006. **111**(1-2): p. 107-116.
11. Verheul, R.J., et al., *Synthesis, characterization and in vitro biological properties of O-methyl free N,N,N-trimethylated chitosan.* Biomaterials, 2008. **29**(27): p. 3642-3649.
12. Slutter, B., et al., *Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination.* J Control Release, 2009. **138**(2): p. 113-21.
13. Peek, L.J., C.R. Middaugh, and C. Berkland, *Nanotechnology in vaccine delivery.* Adv Drug Deliv Rev, 2008. **60**(8): p. 915-28.
14. O'Hagan, D.T., M. Singh, and J.B. Ulmer, *Microparticle-based technologies for vaccines.* Methods, 2006. **40**(1): p. 10-9.
15. Salman, H.H., J.M. Irache, and C. Gamazo, *Immunoadjuvant capacity of flagellin and mannosamine-coated poly(anhydride) nanoparticles in oral vaccination.* Vaccine, 2009. **27**(35): p. 4784-90.
16. Standley, S.M., et al., *Incorporation of CpG oligonucleotide ligand into protein-loaded particle vaccines promotes antigen-specific CD8 T-cell immunity.* Bioconjug Chem, 2007. **18**(1): p. 77-83.
17. Li, W.M., M.B. Bally, and M.P. Schutze-Redelmeier, *Enhanced immune response to T-independent antigen by using CpG oligodeoxynucleotides encapsulated in liposomes.* Vaccine, 2001. **20**(1-2): p. 148-57.
18. Demento, S.L., et al., *Inflammasome-activating nanoparticles as modular systems for optimizing vaccine efficacy.* Vaccine, 2009. **27**(23): p. 3013-21.
19. Blander, J.M. and R. Medzhitov, *Toll-dependent selection of microbial antigens for presentation by dendritic cells.* Nature, 2006. **440**(7085): p. 808-12.
20. Schlosser, E., et al., *TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses.* Vaccine, 2008. **26**(13): p. 1626-37.

21. West, K.R. and S. Otto, *Reversible covalent chemistry in drug delivery.* Curr Drug Discov Technol, 2005. **2**(3): p. 123-60.
22. Meng, F., W.E. Hennink, and Z. Zhong, *Reduction-sensitive polymers and bioconjugates for biomedical applications.* Biomaterials, 2009. **30**(12): p. 2180-98.
23. Masuko, T., et al., *Thiolation of chitosan. Attachment of proteins via thioether formation.* Biomacromolecules, 2005. **6**(2): p. 880-4.
24. Yang, L., et al., *Novel synthesis and in vitro drug release of polymeric prodrug: Chitosan-O-isopropyl-5'-O-d4T monophosphate conjugate.* Bioorg Med Chem Lett, 2009. **19**(9): p. 2566-9.
25. Yin, L., et al., *Drug permeability and mucoadhesion properties of thiolated trimethyl chitosan nanoparticles in oral insulin delivery.* Biomaterials, 2009. **30**(29): p. 5691-700.
26. van Dijk-Wolthuis, W.N.E., et al., *A versatile method for the conjugation of proteins and peptides to poly[2-(dimethylamino)ethyl methacrylate].* Bioconjugate Chemistry, 1999. **10**(4): p. 687-692.
27. Sieval AB, T.M., Kotze AF, Verhoef JE, Brussee J, Junginger HE, *Preparation and NMR characterization of highly substituted N-trimethyl chitosan chloride.* Carbohydrate Polymers, 1998. **36**(2-3): p. 8.
28. Prochazkova, S., K.M. Varum, and K. Ostgaard, *Quantitative determination of chitosans by ninhydrin.* Carbohydrate Polymers, 1999. **38**(2): p. 115-122.
29. Verheul, R.J., et al., *Synthesis, characterization and in vitro biological properties of O-methyl free N,N,N-trimethylated chitosan.* Biomaterials, 2008. **29**(27): p. 3642-9.
30. Kueltzo, L.a.M., CR, *Ultraviolet Absorbtion Spectroscopy*, in *Methods for Structural Analysis of Protein Pharmaceuticals*, W. Jiskoot, Editor. 2005, American Association of Pharmaceutical Scientists: Arlington. p. 1-25.
31. de Jong, E.C., et al., *Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse Th cell-polarizing signals.* Journal of Immunology, 2002. **168**(4): p. 1704-1709.
32. Vieira, P.L., et al., *Glucocorticoids inhibit bioactive IL-12p70 production by in vitro-generated human dendritic cells without affecting their T cell stimulatory potential.* Journal of Immunology, 1998. **161**(10): p. 5245-5251.
33. Ding, Z., et al., *Immune modulation by adjuvants combined with diphtheria toxoid administered topically in BALB/c mice after microneedle array pretreatment.* Pharm Res, 2009. **26**(7): p. 1635-43.
34. Qiu, W.H., et al., *Ultrafast quenching of tryptophan fluorescence in proteins: Interresidue and intrahelical electron transfer.* Chemical Physics, 2008. **350**(1-3): p. 154-164.
35. Nordly, P., et al., *Status and future prospects of lipid-based particulate delivery systems as vaccine adjuvants and their combination with immunostimulators.* Expert Opin Drug Deliv, 2009. **6**(7): p. 657-72.
36. Singh, M. and D. O'Hagan, *Advances in vaccine adjuvants.* Nature Biotechnology, 1999. **17**(11): p. 1075-1081.
37. Carlsson, J., H. Drevin, and R. Axen, *Protein thiolation and reversible protein-protein conjugation - N-succinimidyl 3-(2-pyridyldithio)propionate, a new heterobifunctional reagent.* Biochemical Journal, 1978. **173**(3): p. 723-737.
38. Kircheis, R., et al., *Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery.* Gene Ther, 1997. **4**(5): p. 409-18.
39. Wagner, E., et al., *DNA-binding transferrin conjugates as functional gene-delivery agents: synthesis by linkage of polylysine or ethidium homodimer to the transferrin carbohydrate moiety.* Bioconjug Chem, 1991. **2**(4): p. 226-31.
40. Dormitzer, P.R., J.B. Ulmer, and R. Rappuoli, *Structure-based antigen design: a strategy for next generation vaccines.* Trends Biotechnol, 2008. **26**(12): p. 659-67.
41. Martinez Gomez, J.M., et al., *Surface coating of PLGA microparticles with protamine enhances their immunological performance through facilitated phagocytosis.* J Control Release, 2008. **130**(2): p. 161-7.
42. Ali, O.A. and D.J. Mooney, *Sustained GM-CSF and PEI condensed pDNA presentation increases the level and duration of gene expression in dendritic cells.* J Control Release, 2008. **132**(3): p. 273-8.

43. Davies, O.R., et al., *Surface modification of microspheres with steric stabilizing and cationic polymers for gene delivery.* Langmuir, 2008. **24**(14): p. 7138-46.
44. Khan, S., et al., *Distinct uptake mechanisms but similar intracellular processing of two different Toll-like receptor ligand-peptide conjugates in dendritic cells.* Journal of Biological Chemistry, 2007. **282**(29): p. 21145-21159.
45. Adams, E.W., et al., *Carbohydrate-mediated targeting of antigen to dendritic cells leads to enhanced presentation of antigen to T cells.* Chembiochem, 2008. **9**(2): p. 294-303.
46. Paoletti, L.C. and R.C. Kennedy, *Neutralizing antibody induced in mice by novel glycoconjugates of human immunodeficiency virus type 1 gp120 and env2-3.* J Infect Dis, 2002. **186**(11): p. 1597-602.
47. Theilacker, C., et al., *Construction and characterization of a Pseudomonas aeruginosa mucoid exopolysaccharide-alginate conjugate vaccine*. Infect Immun, 2003. **71**(7): p. 3875-84.
48. Kashef, N., et al., *Synthesis and characterization of Pseudomonas aeruginosa alginate-tetanus toxoid conjugate*. J Med Microbiol, 2006. **55**(Pt 10): p. 1441-6.
49. Chan, M., et al., *Synthesis and immunological characterization of toll-like receptor 7 agonistic conjugates.* Bioconjug Chem, 2009. **20**(6): p. 1194-200.
50. Napolitani, G., et al., *Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells.* Nat Immunol, 2005. **6**(8): p. 769-76.

8

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

Bram Slütter
Ivo Que
Clemens Löwik
Wim Jiskoot
Suzanne M. Bal
Eric Kaijzel
Joke Bouwstra

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 vacation 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 +/- 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 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 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% CO_2.

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 µg/ml OVA_{FITC} and 300 µl 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 Hagenaars et al.[23]. Female Balb/c (nu/nu) mice between 8 and 10 weeks old (Charles River, L'Arbresle, France) were lightly anesthetized using isofluorane 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 H_2O_2 in sodium acetate buffer pH 5.5 for 15 min at room temperature. Reaction was stopped with 2 M H_2SO_4 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 response[10,][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.

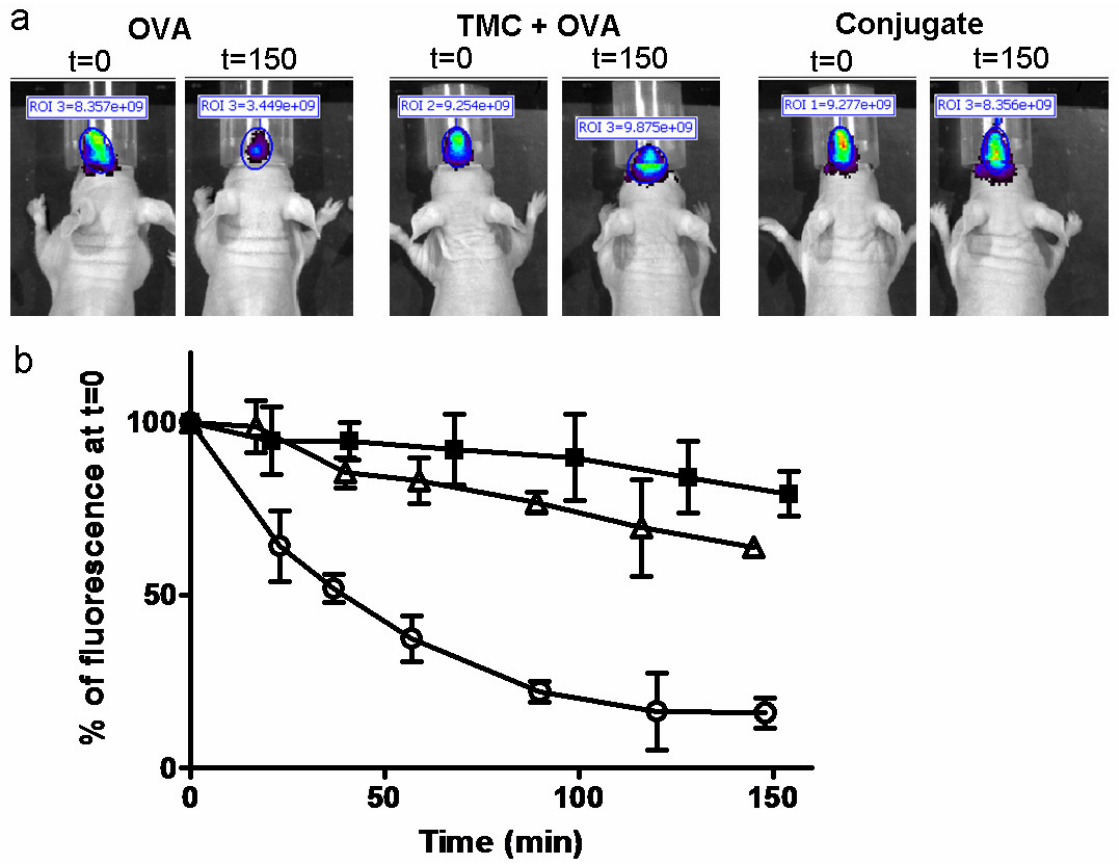


Figure 1: a) Nasal clearance of OVA after co-administration or conjugation with TMC. Emission spectra at 800 nm. b) Clearance derived from spectra. Circles OVA, squares OVA + TMC and triangles TMC-OVA conjugate. Error bars represent SD (n=3).

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 ($p<0.01$).

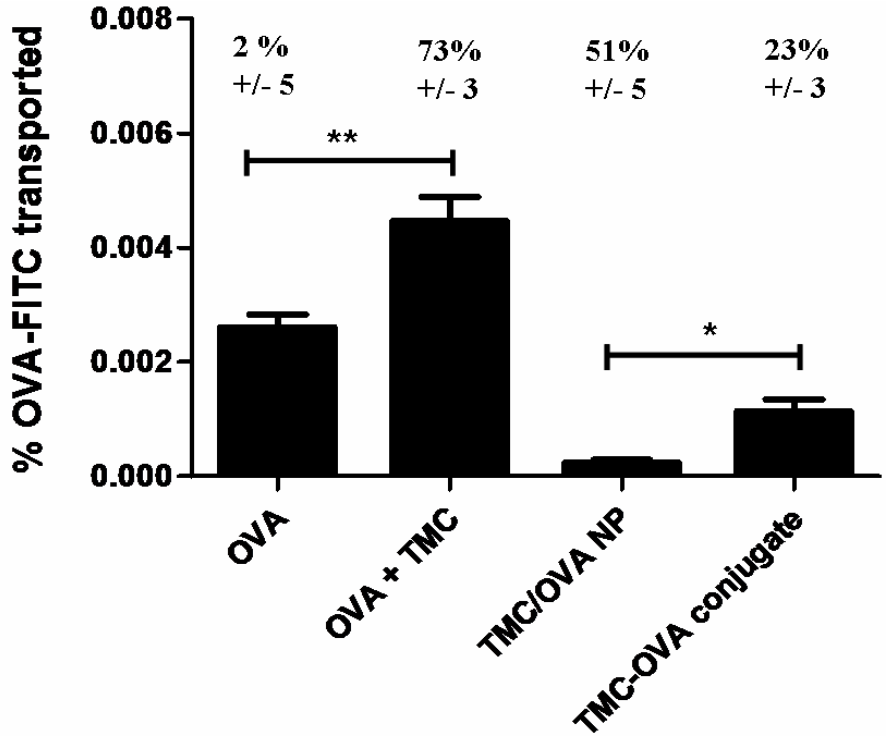


*Figure 2: Diffusion of OVA-FITC through a Calu-3 cell monolayer as a measure for mucosal epithelial permeability. Bars represent mean +/- SD (n=9). * p<0.05, ** p<0.01. TEER decrease (% +/- SD) after 1 h exposure to the formulation is indicated above.*

Antigen delivery to DCs in vivo

After passing the epithelium, the antigen can either drain through the interstitum 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.

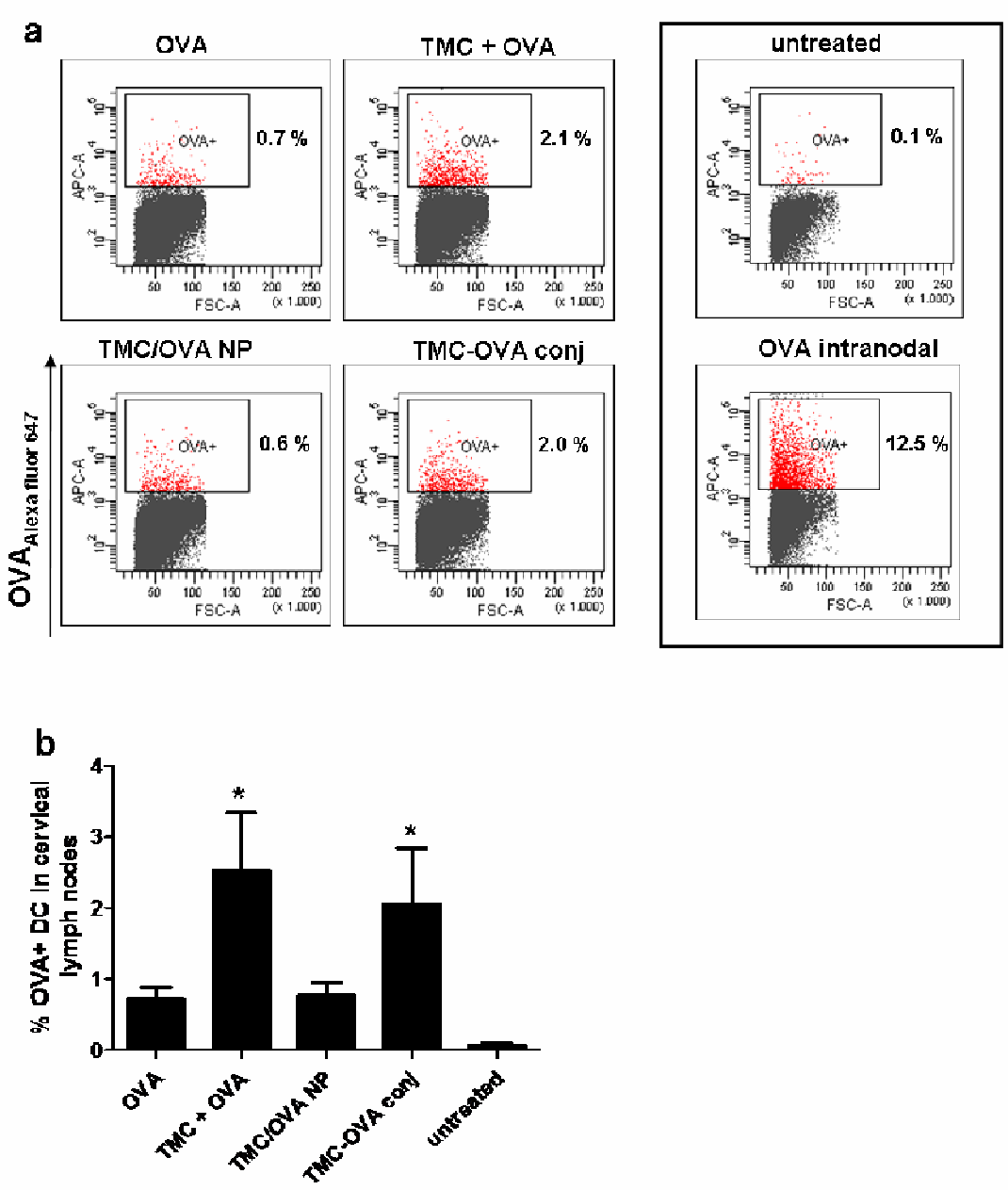


*Figure 3: a) Representative flow cytometry histograms of single cell suspensions of cervical lymph nodes. 24 h after application of 20 µg OVA_{AF647}. Cell were gated for $CD11c^+$. Lymph nodes from non vaccinated mice were used as negative control (untreated), and those of mice vaccinated intranodally 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+/- 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.

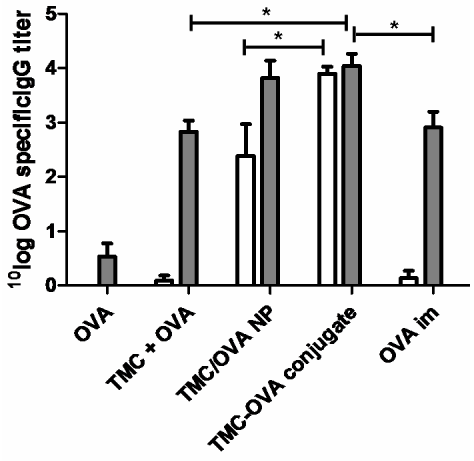


*Figure 4: OVA specific serum IgG titers after nasal vaccination with a priming dose (white bars) or a booster dose (gray bars) of OVA. Mean +/- SD (n=8). *p<0.05*

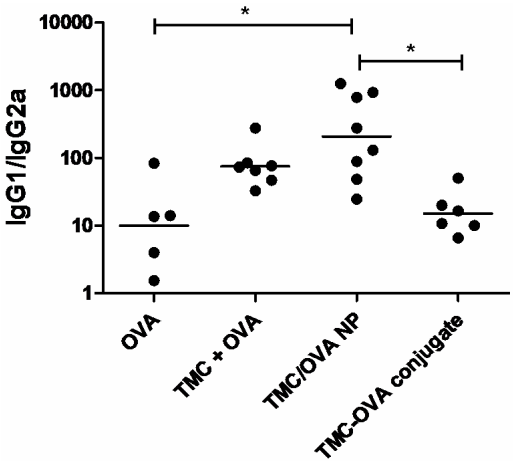


*Figure 5: IgG1/IgG2a ratio indicative of the quality of the immune response. Bar represent geometric mean. * p<0.05*

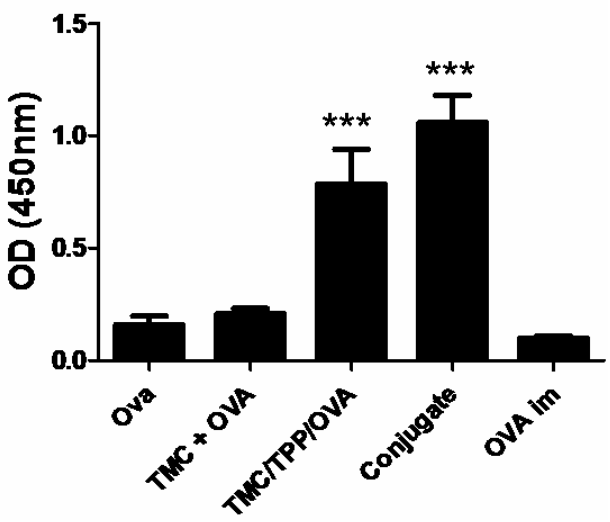


*Figure 6: OD450 values reflecting sIgA levels in nasal washes retrieved from mice after having received two nasal doses. Bars represent mean +/- SEM (n=8). ***p<0.001 compared to nasal administration of OVA.*

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 I: 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	

References

1. Debin, A., et al., *Intranasal immunization with recombinant antigens associated with new cationic particles induces strong mucosal as well as systemic antibody and CTL responses.* Vaccine, 2002. **20**(21-22): p. 2752-63.
2. Revaz, V., et al., *Humoral and cellular immune responses to airway immunization of mice with human papillomavirus type 16 virus-like particles and mucosal adjuvants.* Antiviral Res, 2007. **76**(1): p. 75-85.
3. Sloat, B.R. and Z. Cui, *Strong mucosal and systemic immunities induced by nasal immunization with anthrax protective antigen protein incorporated in liposome-protamine-DNA particles.* Pharm Res, 2006. **23**(2): p. 262-9.
4. Sorichter, S., et al., *Immune responses in the airways by nasal vaccination with systemic boosting against Pseudomonas aeruginosa in chronic lung disease.* Vaccine, 2009.
5. Brandtzaeg, P., *Immunology of tonsils and adenoids: everything the ENT surgeon needs to know.* Int J Pediatr Otorhinolaryngol, 2003. **67 Suppl 1**: p. S69-76.
6. Vajdy, M. and D.T. O'Hagan, *Microparticles for intranasal immunization.* Adv Drug Deliv Rev, 2001. **51**(1-3): p. 127-41.
7. Trombetta, E.S. and I. Mellman, *Cell biology of antigen processing in vitro and in vivo.* Annu Rev Immunol, 2005. **23**: p. 975-1028.
8. Dal Monte, P. and F.C. Szoka, Jr., *Effect of liposome encapsulation on antigen presentation in vitro. Comparison of presentation by peritoneal macrophages and B cell tumors.* J Immunol, 1989. **142**(5): p. 1437-43.
9. Koping-Hoggard, M., A. Sanchez, and M.J. Alonso, *Nanoparticles as carriers for nasal vaccine delivery.* Expert Rev Vaccines, 2005. **4**(2): p. 185-96.
10. Slütter, B., N. Hagenaars, and W. Jiskoot, *Rational design of nasal vaccines.* J Drug Target, 2008. **16**(1): p. 1-17.
11. Jaganathan, K.S. and S.P. Vyas, *Strong systemic and mucosal immune responses to surface-modified PLGA microspheres containing recombinant hepatitis B antigen administered intranasally.* Vaccine, 2006. **24**(19): p. 4201-11.
12. Slütter, B., et al., *Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination.* J Control Release, 2009. **138**(2): p. 113-21.
13. Slütter, B., et al., *Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: Nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen.* Vaccine, 2010. **28**(38): p. 6282-91.
14. Slütter, B. and W. Jiskoot, *Dual role of CpG as immune modulator and physical crosslinker in ovalbumin loaded N-trimethyl chitosan (TMC) nanoparticles for nasal vaccination.* J Control Release, 2010. **In press**.
15. Amidi, M., et al., *N-trimethyl chitosan (TMC) nanoparticles loaded with influenza subunit antigen for intranasal vaccination: biological properties and immunogenicity in a mouse model.* Vaccine, 2007. **25**(1): p. 144-53.
16. Sayin, B., et al., *Mono-N-carboxymethyl chitosan (MCC) and N-trimethyl chitosan (TMC) nanoparticles for non-invasive vaccine delivery.* Int J Pharm, 2008. **363**(1-2): p. 139-48.
17. Jung, T., et al., *Tetanus toxoid loaded nanoparticles from sulfobutylated poly(vinyl alcohol)-graft-poly(lactide-co-glycolide): evaluation of antibody response after oral and nasal application in mice.* Pharm Res, 2001. **18**(3): p. 352-60.
18. Almeida, A.J., H.O. Alpar, and M.R. Brown, *Immune response to nasal delivery of antigenically intact tetanus toxoid associated with poly(L-lactic acid) microspheres in rats, rabbits and guinea-pigs.* J Pharm Pharmacol, 1993. **45**(3): p. 198-203.

19. Brooking, J., S.S. Davis, and L. Illum, *Transport of nanoparticles across the rat nasal mucosa.* J Drug Target, 2001. **9**(4): p. 267-79.
20. Kraehenbuhl, J.P. and M.R. Neutra, *Epithelial M cells: differentiation and function.* Annu Rev Cell Dev Biol, 2000. **16**: p. 301-32.
21. Slütter, B., et al., *Conjugation of ovalbumin to trimethyl chitosan improves immunogenicity of the antigen.* J Control Release, 2010. **143**(2): p. 207-14.
22. Sieval AB, T.M., Kotze AF, Verhoef JE, Brussee J, Junginger HE, *Preparation and NMR characterization of highly substituted N-trimethyl chitosan chloride.* Carbohydrate Polymers, 1998. **36**(2-3): p. 8.
23. Hagenaars, N., et al., *Role of trimethylated chitosan (TMC) in nasal residence time, local distribution and toxicity of an intranasal influenza vaccine.* J Control Release, 2010.
24. Foster, K.A., et al., *Characterization of the Calu-3 cell line as a tool to screen pulmonary drug delivery.* International Journal of Pharmaceutics, 2000. **208**(1-2): p. 1-11.
25. Christensen, D., et al., *CAF01 liposomes as a mucosal vaccine adjuvant: In vitro and in vivo investigations.* International Journal of Pharmaceutics, 2010. **390**(1): p. 19-24.
26. Itano, A.A., et al., *Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity.* Immunity, 2003. **19**(1): p. 47-57.
27. Singh, M., A. Chakrapani, and D. O'Hagan, *Nanoparticles and microparticles as vaccine-delivery systems.* Expert Rev Vaccines, 2007. **6**(5): p. 797-808.
28. Blander, J.M. and R. Medzhitov, *Toll-dependent selection of microbial antigens for presentation by dendritic cells.* Nature, 2006. **440**(7085): p. 808-12.
29. Hamdy, S., et al., *Enhanced antigen-specific primary CD4+ and CD8+ responses by codelivery of ovalbumin and toll-like receptor ligand monophosphoryl lipid A in poly(D,L-lactic-co-glycolic acid) nanoparticles.* J Biomed Mater Res A, 2007. **81**(3): p. 652-62.
30. Schlosser, E., et al., *TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses.* Vaccine, 2008. **26**(13): p. 1626-37.
31. Illum, L., *Nanoparticulate systems for nasal delivery of drugs: a real improvement over simple systems?* J Pharm Sci, 2007. **96**(3): p. 473-83.
32. Sharma, S., et al., *Pharmaceutical aspects of intranasal delivery of vaccines using particulate systems.* J Pharm Sci, 2009. **98**(3): p. 812-43.
33. van der Merwe, S.M., et al., *Trimethylated chitosan as polymeric absorption enhancer for improved peroral delivery of peptide drugs.* Eur J Pharm Biopharm, 2004. **58**(2): p. 225-35.
34. Kotze, A.F., et al., *Enhancement of paracellular drug transport with highly quaternized N-trimethyl chitosan chloride in neutral environments: in vitro evaluation in intestinal epithelial cells (Caco-2).* J Pharm Sci, 1999. **88**(2): p. 253-7.
35. Hamman, J.H., M. Stander, and A.F. Kotze, *Effect of the degree of quaternisation of N-trimethyl chitosan chloride on absorption enhancement: in vivo evaluation in rat nasal epithelia.* Int J Pharm, 2002. **232**(1-2): p. 235-42.
36. Verheul, R.J., et al., *Influence of the degree of acetylation on the enzymatic degradation and in vitro biological properties of trimethylated chitosans.* Biomaterials, 2009. **30**(18): p. 3129-35.
37. Amidi, M., et al., *Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system.* J Control Release, 2006. **111**(1-2): p. 107-16.
38. Verheul, R.J., S. van der Wal, and W.E. Hennink, *Tailorable thiolated trimethyl chitosans for covalently stabilized nanoparticles.* Biomacromolecules, 2010. **11**(8): p. 1965-71.
39. Tobio, M., et al., *Stealth PLA-PEG nanoparticles as protein carriers for nasal administration.* Pharm Res, 1998. **15**(2): p. 270-5.
40. Vila, A., et al., *PLA-PEG particles as nasal protein carriers: the influence of the particle size.* Int J Pharm, 2005. **292**(1-2): p. 43-52.

41. Hagenaars, N., et al., *Physicochemical and immunological characterization of N,N,N-trimethyl chitosan-coated whole inactivated influenza virus vaccine for intranasal administration.* Pharm Res, 2009. **26**(6): p. 1353-64.
42. Bal, S.M., et al., *Efficient induction of immune responses through intradermal vaccination with N-trimethyl chitosan containing antigen formulations.* J Control Release, 2010. **142**(3): p. 374-83.
43. Bal, S.M., et al., *Microneedle-based transcutaneous immunisation in mice with N-trimethyl chitosan adjuvanted diphteria toxoid formulations.* Pharm Res, 2010. **In press**.
44. Manolova, V., et al., *Nanoparticles target distinct dendritic cell populations according to their size.* Eur J Immunol, 2008. **38**(5): p. 1404-13.
45. Brgles, M., et al., *Liposome fusogenicity and entrapment efficiency of antigen determine the Th1/Th2 bias of antigen-specific immune response.* Vaccine, 2009. **27**(40): p. 5435-42.
46. Xiang, S.D., et al., *Pathogen recognition and development of particulate vaccines: does size matter?* Methods, 2006. **40**(1): p. 1-9.

9

Dual role of CpG as immune modulator and physical crosslinker in ovalbumin loaded N-trimethyl chitosan (TMC) nanoparticles for nasal vaccination

Bram Slütter
Wim Jiskoot

Journal of Controlled Release 2010 148: 117-121

Abstract

Nasal vaccination is a promising, but challenging vaccination strategy. Poor absorption by the nasal epithelium and failure to break nasal tolerance are regarded as important reasons for poor efficacy of nasally applied vaccines. Formulation of the antigen into mucoadhesive nanoparticles, made of N-trimethyl chitosan (TMC) crosslinked with tripolyphosphate (TPP), has been shown to overcome these obstacles. However, although nasally administered antigen loaded TMC/TPP nanoparticles induce a strong humoral response, antibody subtyping indicates a Th2 bias. To design a nasal antigen delivery system capable of inducing stronger Th1 type responses, TPP as a crosslinking agent was replaced by unmethylated CpG DNA, a TLR-9 ligand and a potent inducer of Th1 responses, to prepare ovalbumin (OVA) loaded TMC nanoparticles (TMC/CpG/OVA). Several physicochemical characteristics of TMC/CpG/OVA (size, zetapotential, loading efficiency and antigen release profile) were assessed and compared to TMC nanoparticles prepared by crosslinking with TPP (TMC/TPP/OVA). Mice were nasally administered TMC/TPP/OVA and TMC/CpG/OVA after which antibody responses in serum and nasal washes were assessed and T-cell activation in the spleens determined.

TMC/CpG/OVA showed similar physical properties as TMC/TPP/OVA in terms of particle size (380 nm), zetapotential (+21 mV) and antigen release characteristics. Nasal administration of TMC/CpG/OVA and TMC/TPP/OVA to mice resulted in comparable serum IgG levels (ca. 1000 fold higher than those induced by unadjuvanted OVA) and local secretory IgA levels. Moreover, TMC/CpG/OVA induced a 10 fold higher IgG2a response than TMC/TPP/OVA and enhanced the number of OVA specific IFN-gamma-producing T-cells in the spleen.

In conclusion, OVA loaded TMC nanoparticles, containing CpG as adjuvant and crosslinker, are capable of provoking strong humoral as well as Th1 type cellular immune responses after nasal vaccination.

Introduction

Nasal vaccination has gained much interest over the past decades as it is non-invasive and thereby expected to increase patient compliance. Additionally, vaccination via the nose has been shown to induce, besides systemic humoral (IgG mediated) and cellular responses, local as well as distal secretory immune responses (secretory IgA (sIgA) mediated) [1-3], making the mucosal linings less vulnerable to infection. Moreover, the cross reactivity of sIgA is relatively high compared to IgG antibodies [4, 5], making the induction of local immune responses a promising strategy to target highly variable pathogens, like influenza viruses [6].

Nonetheless, nasal immunization with subunit vaccines is challenging, as residence time in the nasal cavity is limited and therefore the uptake by the nasal epithelium is low. Moreover, the nasal epithelium is renowned for being a rather tolerogenic site [7, 8], making it difficult for subunit antigens to provoke an immune response. Vaccine formulation may be instrumental to successful nasal vaccination. Encapsulation of the antigen into particulate carrier systems has been explored extensively in recent years [9] and holds great promise as particles can be specifically designed to meet the challenges nasal vaccination provide [10]. Among the large variety of particles that can be found in the literature, chitosan based particles are among the most studied ones [11]. Chitosan is a cheap, biodegradable, mucoadhesive polymer. In rodents, particles prepared from chitosan have been shown to effectively induce systemic antibody responses against ovalbumin (OVA) and cholera toxin [12], Hepatitis B surface antigen [13], and Meningococcal C oligosaccharides [14]. More recently chitosan derivatives have been developed, like thiolated chitosans [15] to enhance its mucoadhesiveness and trimethylated chitosans (TMC) [16] to improve its solubility at physiological pH. Especially TMC has been shown to be a very promising nasal vaccine carrier. Nanoparticles prepared from TMC by ionic crosslinking with tripolyphosphate (TPP) increase the nasal residence time of the encapsulated antigen [17], improve the uptake of the antigen by M-cells [18] and additionally promote maturation of dendritic cells (DCs) [9-11]. Consequently, TMC particles loaded with antigens, e.g. tetanus toxoid [12], meningococcal C oligosaccharides [19] or hemagglutinin [20] induce strong systemic as well local antibody responses. Moreover, intranasally administered TMC-coated whole inactivated influenza virus resulted in protection of mice against a challenge with a lethal dose of influenza virus [21]. Nonetheless, a significant drawback of TMC is its tendency to promote a humoral (Th2 type) rather than a Th1 type immune response [20, 22]. Strong Th1 type responses are important for many vaccines that we do not have [23], such as HIV/AIDS and tuberculosis vaccines, underscoring the importance of developing vaccine carrier systems capable of inducing these

responses. The bias of TMC's adjuvant effect toward a Th2 response is not restricted to the nasal administration route, as it is also observed after intradermal [24] and intramuscular administration of TMC-adjuvanted antigen (unpublished data). However, different types of immune responses have been reported after nasal vaccination [25-28], depending on the adjuvant used. As TPP does not act as an adjuvant but solely services as a crosslinking agent to promote TMC nanoparticle formation, we propose it should be possible to substitute TPP with a crosslinking agent that does have an adjuvant effect. Unmethylated CpG DNA is a Toll like receptor 9 ligand and described as a Th1 response-inducing adjuvant, also after nasal administration [23]. Furthermore, phosphate groups on CpG render it negatively charged, which could make CpG a possible crosslinking agent to prepare TMC nanoparticles.

The aim of this paper was to study whether CpG can replace TPP as a crosslinker to prepare ovalbumin (OVA)-containing TMC nanoparticles and whether these new carrier systems are capable of redirecting the TMC-induced Th2 type response towards a more Th1 type response, while maintaining strong systemic and local antibody responses. The TMC/CpG/OVA nanoparticles were compared to TMC/TPP/OVA nanoparticles with respect to their physicochemical characteristics and immunogenicity after nasal administration in mice.

Material and Methods

Materials

Ovalbumin (OVA) was purchased from Calbiochem (Beeston, UK) and CpG DNA (ODN 2006) as well as fluorescein isothiocyanate coupled CpG (CpG-FITC) from InvivoGen (Toulouse, France). N-trimethyl chitosan with a degree of quaternization of 15% was synthesized from 92% deacetylated (MW 120 kDa) chitosan (Primex, Avaldsnes, Norway) and characterized by NMR, as described by Bal et al. [29]. KCl, NaCl, HNa_2PO_4, KH_2PO_4 and bovine serum albumin (BSA) were purchased from Merck (Amsterdam, The Netherlands). Pentasodium tripolyphosphate (TPP), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), Tween 20 and 2-mercapto ethanol were obtained from Sigma-Aldrich (Steinheim, Germany). Goat anti-mouse IgG, IgG1, IgG2a and IgA conjugated with horseradish peroxidase was purchased from Southern Biotech (Birmingham, AL). BDOpteia IFN-γ ELISA kit was bought from Becton Dickinson (Breda, The Netherlands). RPMI 1640, fetal bovine serum (FBS), penicillin-streptomycin (P/S) solution, L-glutamine, sodium pyruvate and fluorescein isothiocyanate coupled OVA (OVA-FITC) were acquired from Invitrogen (Breda, The Netherlands), and 70-µm cell strainers from VWR (Amsterdam, The Netherlands).

Nanoparticle preparation

OVA loaded TMC/TPP (TMC/TPP/OVA) nanoparticles were prepared as described before [18]. Briefly, 20 mg TMC and 1 mg OVA were dissolved in 8.3 ml 5 mM Hepes pH 7.4. Under continuous stirring 3.4 ml 0.1% w/v TPP was added to induce ionic complexation into nanoparticles. Particles were collected by centrifugation (10 min, 12000 g), resuspended and washed once with water. OVA loaded TMC/CpG (TMC/CpG/OVA) nanoparticles were prepared in the same way as TMC/TPP/OVA, replacing TPP by CpG. A total amount of 0.9 mg CpG was added to 20 mg TMC and 1 mg of OVA; the addition of more CpG caused aggregation and a dramatic increase of the polydispersity index (PDI), whereas the addition of less CpG reduced the number of particles formed (data not shown). Supernatants were stored for determining the loading efficiency and nanoparticles were stored at 4°C until further analysis. OVA-FITC loaded nanoparticles were prepared by substituting OVA-FITC for OVA.

Size and zetapotential

Particle suspensions were diluted in 5 mM Hepes pH 7.4 until a slightly opalescent dispersion was achieved. Hydrodynamic diameter (average and PDI) and zetapotential were determined with a Nanosizer (Malvern Instruments, Malvern, UK) by dynamic light scattering and laser Doppler electrophoresis, respectively.

Loading efficiency

To determine the loading efficiency, the OVA content of the nanoparticles as well supernatants, was determined using micro bicinchoninic acid (mBCA) protein assay (Pierce, Etten Leur, The Netherlands) according to the manufacturer's instructions. To determine the encapsulation efficiency of CpG, FITC-labeled CpG was included and the amount of CpG-FITC was determined in the supernatant as well as in the particle formulation using fluorescence spectroscopy (FS920 fluorimeter, Edinburgh Instruments, UK; excitation 495 nm, emission 520 nm; band widths 5 nm).

Particle stability and antigen release in vitro

TMC/TPP/OVA-FITC and TMC/CpG/OVA-FITC were diluted to a final particle concentration of 1 mg/ml in 10 mM phosphate buffered saline (PBS) pH 7.4 containing 0.01% Tween 20 and stored in several aliquots at 37°C. At different time points nanoparticle size was determined with DLS after which the dispersions were centrifuged (10 min 14000 g) and supernatants were collected allowing quantification of the released OVA-FITC with fluorescence spectroscopy (excitation 495 nm, emission 520 nm; band widths 5 nm).

Nasal vaccination

Female Balb/c mice (Harlan, Boxmeer, The Netherlands), 6-8 weeks old, received 3 nasal doses of 20 µg OVA or an equivalent dose encapsulated OVA with intervals of 3 weeks. Mice receiving CpG were nasally administered 20 µg (3.1 nmol) of the adjuvant, either as a CpG solution with OVA or as a suspension of TMC/CpG/OVA nanoparticles. Three OVA injections of 20 µg OVA were administered intramuscularly as control. For nasal administration, formulations were applied in a volume of 10 µl PBS, 5 µl per nostril. For i.m. administration, 25 µl of formulation in PBS was injected in the thigh muscle. Blood samples were taken 2 weeks after the final booster dose. After sacrificing the animals, spleens were harvested and nasal washes collected.

Determination of serum IgG, IgG1, IgG2a and secretory IgA

Micro titer plates (Nunc, Roskilde, Denmark) were coated with OVA, by incubation of 1 µg/ml OVA in 40 mM sodium carbonate buffer pH 9.4 for 24 hours at 4°C. To reduce aspecific binding, wells were blocked with 1% (w/v) BSA in PBS for 1 hour at 37°C. After extensive washing with PBS serial dilutions of serum ranging from 20 to $2*10^6$ were applied, whereas nasal washes were added undiluted. After incubation for 1.5 hours at 37°C and extensive washing, OVA specific antibodies were detected using HRP conjugated goat anti mouse IgG, IgG1, IgG2a or IgA (1 hour 37°C) and by incubating with 0.1 mg/ml TMB and 30 µg/ml H_2O_2 in 110 mM sodium acetate buffer pH 5.5 for 15 min at room temperature. Reaction was stopped with 2 M H_2SO_4 and absorbance was determined at 450 nm with an EL808 microplate reader (Bio-Tek Instruments, Bad Friedrichshall, Germany).

T-cell activation study

T-cell activation was studied using a protocol described by Christensen et al. [30]. Single cell suspensions were prepared, by grinding spleens over 70 µm cell strainers and rinsing with spleen medium (RPMI 1640 supplemented with 10% v/v FBS, 1% v/v glutamine, 1% v/v P/S and 0.05 mM 2-mercaptoethanol). Splenocytes were restimulated with 20 µg/ml OVA and maintained for 5 days at 37 °C and 5% CO_2. IFN-γ levels in culture supernatant were determined using a BDOpteia IFN-γ ELISA according to the manufacturer's instructions.

Statistics

Serum antibody titers were analyzed with a Kruskal-Wallis test with Dunn's post-test. Antibody levels in nasal washes as well as splenocyte responses were analyzed with a one-way ANOVA with Bonferroni post-test. Statistics were performed using GraphPad 5.0 for Windows.

Table 1: Particle characteristics

Nanoparticles	Size (nm)	PDI	Zetapotential (mV)	Loading efficiency OVA (%)	Loading efficiency CpG (%)	Burst release (%)
TMC/TPP/OVA	314 +/- 31	0.12 +/- 0.09	18.2 +/- 1.8	63 +/- 6	-	25 +/- 2
TMC/CpG/OVA	304 +/- 22	0.20 +/- 0.11	20.9 +/- 2.0	52 +/- 7	56 +/- 5	46 +/- 1*

*Values represent mean of 3 individually prepared batches ± standard deviation. Burst release was defined as the percentage of OVA release after 1 h in PBS. * p<0.001 compared to TMC/TPP/OVA.*

Results and discussion

Nanoparticle characterization

The characteristics of the TMC/CpG/OVA nanoparticles and the TMC/TPP/OVA nanoparticles were comparable in size and zetapotential (Table 1). Both particle types showed an average hydrodynamic diameter of ca. 300 nm, were fairly monodisperse (PDI 0.1-0.2) and had a positive zetapotential of about +20 mV. Moreover, changing the crosslinker did not alter the loading efficiency (Table 1) and the release pattern (data not shown), as both particle showed a burst release followed by no release over 48 hours. TMC/CpG/OVA did show a significantly higher burst under physiological conditions ($p<0.001$ Student's t-test). This may be related to the higher amount and charge density of TPP compared to CpG, allowing a stronger interaction with TMC. Similar large burst releases (>50%) have been observed for even less densely negatively charged polymers like dextran sulfate and hyaluronic acid (Verheul *et al.* unpublished results).

These results indicate that ionic crosslinking of TMC is just as easily achieved with other phosphate group-bearing entities as with TPP and TMC/CpG/OVA nanoparticles appear to be physically very similar to TMC/TPP/OVA nanoparticles.

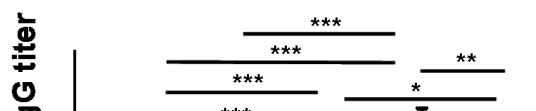
Nasal vaccination

Nasal vaccination with subunit antigen is challenging as only a very limited amount of soluble antigen will be taken up by the nasal epithelium and subsequently be processed by DCs. This is reflected in the observation that nasal administration of a solution of plain OVA

resulted in negligible antibody titers, whereas intramuscular injection of the same dose of OVA induced high IgG titers after a booster dose (Figure 1). Coadministration of OVA with TMC, positively affected the IgG response ($p<0.001$ compared to OVA after prime as well as booster dose), as reported before for TMC mixed with other antigens [20, 21]. TMC can enhance the uptake of antigens through the nasal epithelium [31, 32] as it opens tight junction [33, 34] and can prolong the disposition of antigen in the nasal cavity [35]. The addition of CpG as an adjuvant resulted in enhanced antibody titers ($p<0.05$), but to a significantly lesser extent as TMC ($p<0.001$). Possible reasons for the weaker adjuvant effect of soluble CpG as compared to TMC are: CpG probably does not prolong the nasal residence time of the antigen, the adjuvant itself resides in the nasal cavity for only a short period of time and it may not be taken up by the nasal epithelium as efficiently as TMC.

An even better approach than the application of solutions seems a particulate delivery system comprising the antigen and TMC, as both TMC/TPP/OVA and TMC/CpG/OVA vaccinated mice showed significantly enhanced IgG titers compared to OVA alone or a mixture of soluble TMC and OVA ($p<0.05$ after a priming dose). As the size of these nanoparticles would inhibit rather than induce intercellular transport through the tight junctions between nasal epithelial cells, nanoparticles promote the immunogenicity of the antigen in a different way. The mere particulate structure could favor uptake by M-cells [18, 36-38], allowing antigen access to the subepithelial space. There, multimerization of epitopes of the particle's surface could contribute to an improved uptake by DCs and B-cells [39, 40].

Besides a systemic antibody response, both TMC nanoparticles also induced a potent mucosal immune response, indicating effective uptake of OVA by local B-cells. Nasal washes of both TMC/TPP/OVA and TMC/CpG/OVA vaccinated mice contained comparable elevated levels of sIgA ($p<0.05$) (Figure 2), whereas no significant sIgA elevation was detected in nasal washes after vaccination with plain OVA solution. Solutions of OVA with adjuvant (TMC or CpG) also showed an increase in sIgA levels but significantly lower than the sIgA levels induced by TMC/CpG/OVA ($p<0.05$). Although local antibodies are not often used as a correlate of protection, the interest in sIgA is increasing. sIgA is recognized as an important factor in mucosal homeostasis [41] and is capable of inducing M-cell transport of neutralized antigen [42], thereby delivering the antigen to local DCs [43]. Antigen specific sIgA at mucosal surfaces could therefore protect the host from future infection by directly neutralizing the pathogen, but also by acting as an early warning signal for the immune system. Furthermore, sIgA production after nasal vaccination is not restricted to the upper airways, as via a system called the common mucosal immune system [44], sIgA antibodies can be detected also in other mucosal secretions.



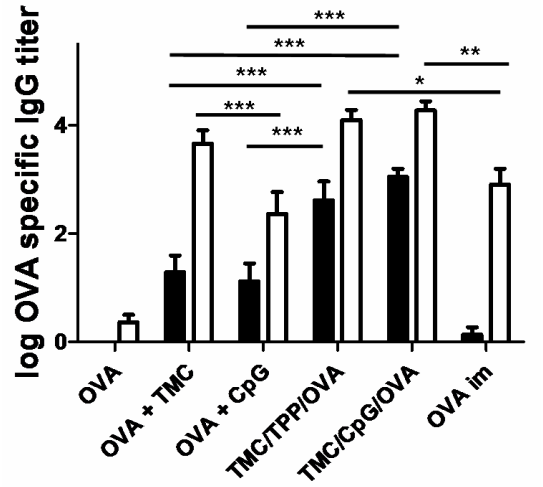


***Figure 1**: OVA specific serum IgG titers in serum after a priming (black bars) and a booster dose (white bars). Mice received 3 doses of 20 µg OVA nasally or intramuscularly (OVA im). The 2nd boost did not further increase IgG levels and is not shown for reasons of clarity. All formulations except for OVA im after priming were significantly higher than OVA (p<0.01). Bars represent mean n=8 ± SEM. * p<0.05, **p<0.01, ***p<0.001*

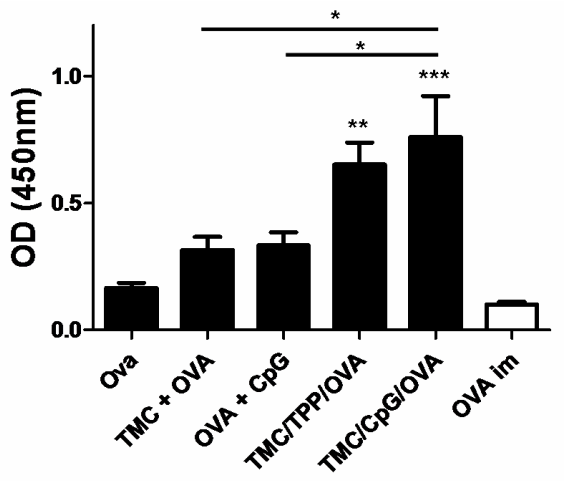


***Figure 2**: OVA specific IgA levels in nasal washes of nasally immunized Balb/c mice. Bars represent mean n=8 ± SEM. *p<0.05 compared to TMC/CpG/OVA, **p<0.01 compared to OVA ***p<0.001 compared to OVA.*

The major important difference between the effects of TMC/TPP/OVA nanoparticles and TMC/CpG/OVA nanoparticles appeared to be the type of response elicited (Figure 3). TMC/TPP/OVA caused a predominant IgG1 response ($p<0.05$), whereas TMC/CpG/OVA vaccinated mice showed a decreased IgG1/IgG2a ratio, indicating that the inclusion of CpG into TMC nanoparticle promoted a Th1 response. Similarly, coadministration of TMC led to an increased IgG1/IgG2a ratio indicating a shift towards Th2, whereas the addition of CpG to OVA decreased the IgG1/IgG2a ratio. The Th1-inducing effect of nasally administered CpG has been observed before [45, 46] and TMC/CpG/OVA nanoparticles seem to exert a similar effect. This was confirmed by the T-cell activation study, showing that splenocytes from TMC/CpG/OVA immunized mice produced large quantities of IFN-γ after restimulation with OVA (Figure 4), even more than mice immunized with a solution of OVA and CpG. Splenocytes from mice vaccinated with a solution of OVA and TMC or TMC/TPP/OVA did not produce more IFN-γ than

splenocytes from naïve mice. So, changing the crosslinker from TPP to CpG strongly shifted the T-cell polarization towards the Th1 direction.

Overall, TMC/CpG particles seem to be capable of eliciting strong humoral responses, both local (sIgA) and systemic (IgG, IgG1, IgG2a), as well as a Th1 type response, making them a promising vaccine carrier for nasally applied OVA and, most likely, a wide variety of other antigens for which a Th1 type immune response is needed.

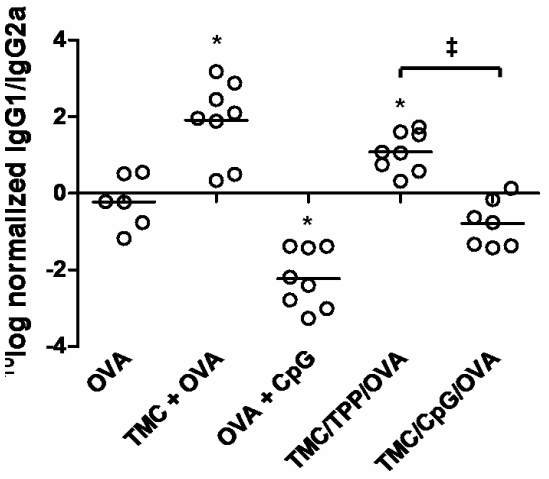


***Figure 3**: Serum IgG1/IgG2a levels normalized for the average OVA IgG1/IgG2a ratio. * p<0.05 compared to OVA. ‡ p<0.05.*

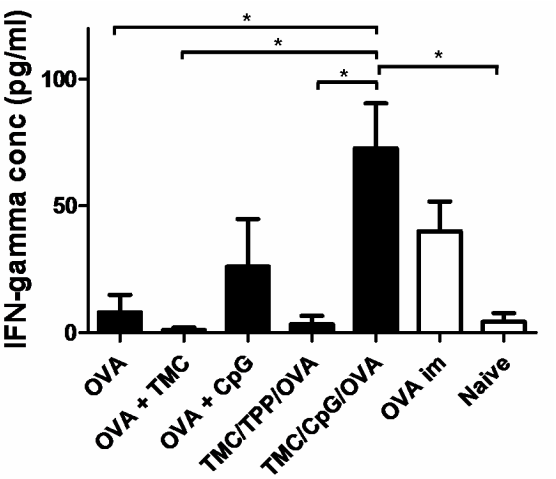


***Figure** 4: IFN-γ production by splenocytes restimulated with OVA. Values represent mean n=5 ± SEM. * p<0.05*

Conclusion

TMC/TPP/OVA nanoparticles have previously been shown to be very effective nasal vaccine carriers. Replacing TPP by CpG as a crosslinking agent to obtain TMC/CpG/OVA nanoparticles modulated the immune response towards a Th1 response after nasal vaccination, while maintaining the strong systemic and local antibody responses observed with TMC/TPP nanoparticles. TMC/CpG nanoparticles therefore are an interesting nasal delivery system for vaccines requiring broad humoral as well as strong Th1 type cellular immune responses.

Acknowledgements

This research was performed under the framework of TI Pharma project number D5-106 “vaccine delivery: alternatives for conventional multiple injection vaccines”. The authors thank Dr. Elly van Riet for reading the manuscript and her valuable suggestions.

References

1. Hagenaars, N., et al., *Head-to-head comparison of four nonadjuvanted inactivated cell culture-derived influenza vaccines: effect of composition, spatial organization and immunization route on the immunogenicity in a murine challenge model.* Vaccine, 2008. **26**(51): p. 6555-63.
2. Minne, A., et al., *The delivery site of a monovalent influenza vaccine within the respiratory tract impacts on the immune response.* Immunology, 2007. **122**(3): p. 316-25.
3. Tacket, C.O., et al., *Humoral, mucosal, and cellular immune responses to oral Norwalk virus-like particles in volunteers.* Clin Immunol, 2003. **108**(3): p. 241-7.
4. Brandtzaeg, P., *Induction of secretory immunity and memory at mucosal surfaces.* Vaccine, 2007. **25**(30): p. 5467-84.
5. Tamura, S.I., et al., *Superior cross-protective effect of nasal vaccination to subcutaneous inoculation with influenza hemagglutinin vaccine.* Eur J Immunol, 1992. **22**(2): p. 477-81.
6. Ichinohe, T., et al., *Cross-protection against H5N1 influenza virus infection is afforded by intranasal inoculation with seasonal trivalent inactivated influenza vaccine.* J Infect Dis, 2007. **196**(9): p. 1313-20.
7. Czerkinsky, C., et al., *Mucosal immunity and tolerance: relevance to vaccine development.* Immunol Rev, 1999. **170**: p. 197-222.
8. Samsom, J.N., *Regulation of antigen-specific regulatory T-cell induction via nasal and oral mucosa.* Crit Rev Immunol, 2004. **24**(3): p. 157-77.
9. Koping-Hoggard, M., A. Sanchez, and M.J. Alonso, *Nanoparticles as carriers for nasal vaccine delivery.* Expert Rev Vaccines, 2005. **4**(2): p. 185-96.
10. Slutter, B., N. Hagenaars, and W. Jiskoot, *Rational design of nasal vaccines.* J Drug Target, 2008. **16**(1): p. 1-17.
11. Amidi, M., et al., *Chitosan-based delivery systems for protein therapeutics and antigens.* Adv Drug Deliv Rev, 2010. **62**(1): p. 59-82.
12. Nagamoto, T., et al., *Novel chitosan particles and chitosan-coated emulsions inducing immune response via intranasal vaccine delivery* Pharm Res, 2004. **21**(4): p. 671-4.
13. Khatri, K., et al., *Plasmid DNA loaded chitosan nanoparticles for nasal mucosal immunization against hepatitis B.* Int J Pharm, 2008. **354**(1-2): p. 235-41.
14. Baudner, B.C., et al., *The concomitant use of the LTK63 mucosal adjuvant and of chitosan-based delivery system enhances the immunogenicity and efficacy of intranasally administered vaccines.* Vaccine, 2003. **21**(25-26): p. 3837-44.
15. Bernkop-Schnurch, A., et al., *Thiomers: preparation and in vitro evaluation of a mucoadhesive nanoparticulate drug delivery system.* Int J Pharm, 2006. **317**(1): p. 76-81.
16. Verheul, R.J., et al., *Synthesis, characterization and in vitro biological properties of O-methyl free N,N,N-trimethylated chitosan.* Biomaterials, 2008. **29**(27): p. 3642-9.
17. Amidi, M., et al., *Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system.* J Control Release, 2006. **111**(1-2): p. 107-16.
18. Slutter, B., et al., *Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination.* J Control Release, 2009. **138**(2): p. 113-21.
19. Baudner, B.C., et al., *Protective immune responses to meningococcal C conjugate vaccine after intranasal immunization of mice with the LTK63 mutant plus chitosan or trimethyl chitosan chloride as novel delivery platform.* J Drug Target, 2005. **13**(8-9): p. 489-98.
20. Amidi, M., et al., *N-trimethyl chitosan (TMC) nanoparticles loaded with influenza subunit antigen for intranasal vaccination: biological properties and immunogenicity in a mouse model.* Vaccine, 2007. **25**(1): p. 144-53.

21. Hagenaars, N., et al., *Physicochemical and immunological characterization of N,N,N-trimethyl chitosan-coated whole inactivated influenza virus vaccine for intranasal administration.* Pharm Res, 2009. **26**(6): p. 1353-64.
22. Hagenaars, N., et al., *Relationship between structure and adjuvanticity of N,N,N-trimethyl chitosan (TMC) structural variants in a nasal influenza vaccine.* J Control Release, 2009. **140**(2): p. 126-33.
23. Friede, M. and M.T. Aguado, *Need for new vaccine formulations and potential of particulate antigen and DNA delivery systems.* Adv Drug Deliv Rev, 2005. **57**(3): p. 325-31.
24. Bal, S.M., et al., *Efficient induction of immune responses through intradermal vaccination with N-trimethyl chitosan containing antigen formulations.* J Control Release, 2010. **142**(3): p. 374-83.
25. Zygmunt, B.M., et al., *Intranasal immunization promotes th17 immune responses.* J Immunol, 2009. **183**(11): p. 6933-8.
26. Bielinska, A.U., et al., *Nasal immunization with a recombinant HIV gp120 and nanoemulsion adjuvant produces Th1 polarized responses and neutralizing antibodies to primary HIV type 1 isolates.* AIDS Res Hum Retroviruses, 2008. **24**(2): p. 271-81.
27. Tanaka, N., et al., *Intranasal immunization with phosphorylcholine induces antigen specific mucosal and systemic immune responses in mice.* Vaccine, 2007. **25**(14): p. 2680-7.
28. Saluja, V., et al., *Intranasal Delivery of Influenza Subunit Vaccine Formulated with GEM Particles as an Adjuvant.* AAPS J.
29. Bal, S.M., et al., *Efficient induction of immune responses through intradermal vaccination with N-trimethyl chitosan containing antigen formulations.* J Control Release, 2009 **142**(3):374-83.
30. Christensen, D., et al., *CAF01 liposomes as a mucosal vaccine adjuvant: In vitro and in vivo investigations.* Int J Pharm, 2009. **390**(1): p.19-24.
31. Hamman, J.H., M. Stander, and A.F. Kotze, *Effect of the degree of quaternisation of N-trimethyl chitosan chloride on absorption enhancement: in vivo evaluation in rat nasal epithelia.* Int J Pharm, 2002. **232**(1-2): p. 235-42.
32. Florea, B.I., et al., *Enhancement of bronchial octreotide absorption by chitosan and N-trimethyl chitosan shows linear in vitro/in vivo correlation.* J Control Release, 2006. **110**(2): p. 353-61.
33. Verheul, R.J., et al., *Influence of the degree of acetylation on the enzymatic degradation and in vitro biological properties of trimethylated chitosans.* Biomaterials, 2009. **30**(18): p. 3129-35.
34. Kotze, A.F., et al., *Enhancement of paracellular drug transport with highly quaternized N-trimethyl chitosan chloride in neutral environments: in vitro evaluation in intestinal epithelial cells (Caco-2).* J Pharm Sci, 1999. **88**(2): p. 253-7.
35. Hagenaars, N., et al., *Role of trimethylated chitosan (TMC) in nasal residence time, local distribution and toxicity of an intranasal influenza vaccine.* J Control Release.
36. des Rieux, A., et al., *An improved in vitro model of human intestinal follicle-associated epithelium to study nanoparticle transport by M cells.* Eur J Pharm Sci, 2007. **30**(5): p. 380-91.
37. van der Lubben, I.M., et al., *Chitosan for mucosal vaccination.* Adv Drug Deliv Rev, 2001. **52**(2): p. 139-44.
38. Brayden, D.J., M.A. Jepson, and A.W. Baird, *Keynote review: intestinal Peyer's patch M cells and oral vaccine targeting.* Drug Discov Today, 2005. **10**(17): p. 1145-57.
39. Dal Monte, P. and F.C. Szoka, Jr., *Effect of liposome encapsulation on antigen presentation in vitro. Comparison of presentation by peritoneal macrophages and B cell tumors.* J Immunol, 1989. **142**(5): p. 1437-43.
40. Jiskoot, W., et al., *Immunological risk of injectable drug delivery systems.* Pharm Res, 2009. **26**(6): p. 1303-14.
41. Fagarasan, S. and T. Honjo, *Regulation of IgA synthesis at mucosal surfaces.* Curr Opin Immunol, 2004. **16**(3): p. 277-83.
42. Corthesy, B., *Roundtrip ticket for secretory IgA: role in mucosal homeostasis?* J Immunol, 2007. **178**(1): p. 27-32.

43. Favre, L., F. Spertini, and B. Corthesy, *Secretory IgA possesses intrinsic modulatory properties stimulating mucosal and systemic immune responses.* J Immunol, 2005. **175**(5): p. 2793-800.
44. Bienenstock, J. and M.R. McDermott, *Bronchus- and nasal-associated lymphoid tissues.* Immunol Rev, 2005. **206**: p. 22-31.
45. Abe, N., et al., *Nasal vaccination with CpG oligodeoxynucleotide induces protective immunity against non-typeable Haemophilus influenzae in the nasopharynx.* Laryngoscope, 2006. **116**(3): p. 407-12.
46. Joseph, A., et al., *Liposomal immunostimulatory DNA sequence (ISS-ODN): an efficient parenteral and mucosal adjuvant for influenza and hepatitis B vaccines.* Vaccine, 2002. **20**(27-28): p. 3342-54.

10

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

Bram Slütter
Rolf Verheul
Wim Jiskoot
Suzanne Bal
Joke A. Bouwstra

Manuscript submitted

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), PAM_3CSK_4 (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], PAM_3CSK_4 [17, 18], muramyldipeptide (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 zetapotential 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, Siglufjordur, 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 zetapotential measurements using a Nanosizer ZS apparatus (Malvern Instruments, Malvern, UK), nanoparticles were diluted in 5 mM Hepes pH7.4 until slightly opalescent dispersions was 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 ensured 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 Ig***A**

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 particle 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 = zetapotential 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).

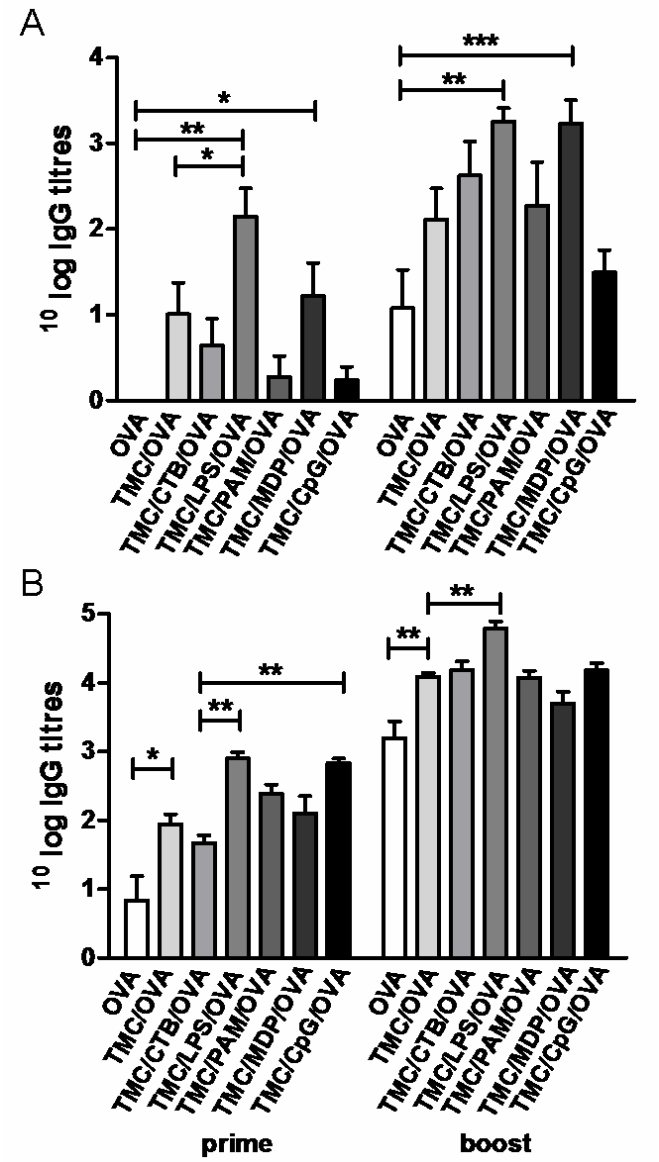


***Figure 1**.OVA specific serum IgG titres after nasal (A) and intradermal (B) immunisation. Data are presented as mean ± SEM of 8 (A) or 5(B) mice. * p<0.05, ** p<0.01, *** p<0.001.*

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.

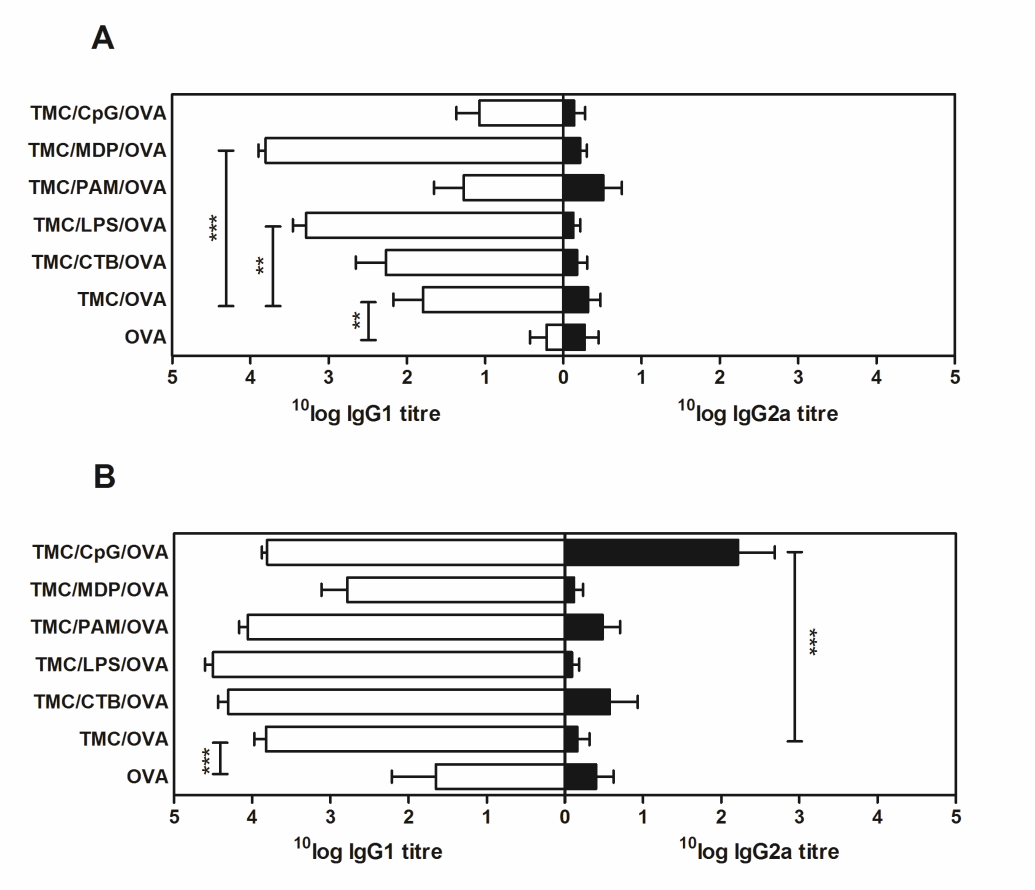


***Figure 2**.OVA specific serum IgG1 (white bars) and IgG2a (black bars) titres 3 weeks after a booster dose nasally (A) and intradermally (B). Data are presented as mean ± SEM, ** p<0.01 ***p<0.001.*

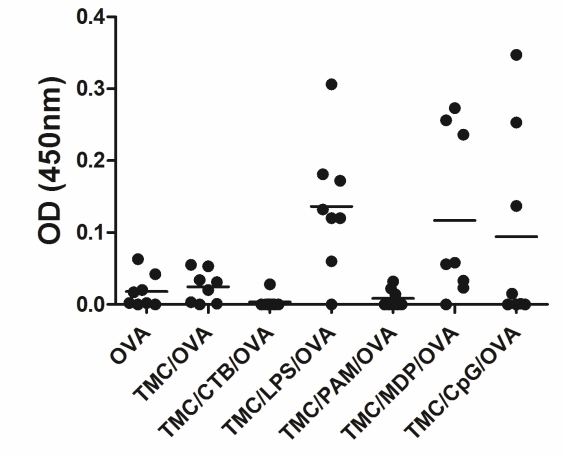


***Figure 3**: Secretory IgA levels in nasal washes of individual mice 3 weeks after a nasal booster dose. Bar represents mean.*

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, PAM_3CSK_4, 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 case 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.

Acknowledgement

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

References

1. Kupper, T.S. and R.C. Fuhlbrigge, *Immune surveillance in the skin: mechanisms and clinical consequences.* Nat Rev Immunol, 2004. **4**(3): p. 211-22.
2. Neutra, M.R., E. Pringault, and J.P. Kraehenbuhl, *Antigen sampling across epithelial barriers and induction of mucosal immune responses.* Annu Rev Immunol, 1996. **14**: p. 275-300.
3. Chiu, S.S., et al., *Immunogenicity and safety of intradermal influenza immunization at a reduced dose in healthy children.* Pediatrics, 2007. **119**(6): p. 1076-82.
4. Kenney, R.T., et al., *Dose sparing with intradermal injection of influenza vaccine.* N Engl J Med, 2004. **351**(22): p. 2295-301.
5. Van Damme, P., et al., *Safety and efficacy of a novel microneedle device for dose sparing intradermal influenza vaccination in healthy adults.* Vaccine, 2009. **27**(3): p. 454-9.
6. Zuercher, A.W., et al., *Nasal-associated lymphoid tissue is a mucosal inductive site for virus-specific humoral and cellular immune responses.* J Immunol, 2002. **168**(4): p. 1796-803.
7. Shimoda, M., et al., *Isotype-specific selection of high affinity memory B cells in nasal-associated lymphoid tissue.* J Exp Med, 2001. **194**(11): p. 1597-607.
8. Flood, E., et al., *Children's Perceptions of Influenza Illness and Preferences for Influenza Vaccine.* Journal of Pediatric Health Care, 2010.
9. O'Hagan, D.T., M. Singh, and J.B. Ulmer, *Microparticle-based technologies for vaccines.* Methods, 2006. **40**(1): p. 10-9.
10. Fischer, S., et al., *Concomitant delivery of a CTL-restricted peptide antigen and CpG ODN by PLGA microparticles induces cellular immune response.* J Drug Target, 2009. **17**(8): p. 652-61.
11. Blander, J.M. and R. Medzhitov, *Toll-dependent selection of microbial antigens for presentation by dendritic cells.* Nature, 2006. **440**(7085): p. 808-12.
12. Slütter, B., et al., *Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: Nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen.* Vaccine, 2010. **28** p. 6282-6291.
13. Bal, S.M., et al., *Microneedle-Based Transcutaneous Immunisation in Mice with N-Trimethyl Chitosan Adjuvanted Diphtheria Toxoid Formulations.* Pharm Res, 2010.
14. Conlan, J.W., et al., *Mice intradermally-inoculated with the intact lipopolysaccharide, but not the lipid A or O-chain, from Francisella tularensis LVS rapidly acquire varying degrees of enhanced resistance against systemic or aerogenic challenge with virulent strains of the pathogen.* Microb Pathog, 2003. **34**(1): p. 39-45.
15. de Jonge, M.I., et al., *Intranasal immunisation of mice with liposomes containing recombinant meningococcal OpaB and OpaJ proteins.* Vaccine, 2004. **22**(29-30): p. 4021-8.
16. Slütter, B. and W. Jiskoot, *Dual role of CpG as immune modulator and physical crosslinker in ovalbumin loaded N-trimethyl chitosan (TMC) nanoparticles for nasal vaccination.* J Control Release, 2010.
17. Bal, S.M., et al., *Co-encapsulation of antigen and adjuvant in cationic liposomes affects the quality of the immune response in mice after intradermal vaccination.* submitted, 2010.
18. Zhou, C., X.D. Kang, and Z. Chen, *A synthetic Toll-like receptor 2 ligand decreases allergic immune responses in a mouse rhinitis model sensitized to mite allergen.* J Zhejiang Univ Sci B, 2008. **9**(4): p. 279-85.
19. Puri, N., et al., *An investigation of the intradermal route as an effective means of immunization for microparticulate vaccine delivery systems.* Vaccine, 2000. **18**(23): p. 2600-12.
20. Moschos, S.A., et al., *Adjuvant synergy: the effects of nasal coadministration of adjuvants.* Immunol Cell Biol, 2004. **82**(6): p. 628-37.
21. Chen, D.X., et al., *Epidermal powder immunization using non-toxic bacterial enterotoxin adjuvants with influenza vaccine augments protective immunity.* Vaccine, 2002. **20**(21-22): p. 2671-2679.

22. Matsuo, K., et al., *Induction of innate immunity by nasal influenza vaccine administered in combination with an adjuvant (cholera toxin).* Vaccine, 2000. **18**(24): p. 2713-22.
23. Schlosser, E., et al., *TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses.* Vaccine, 2008. **26**(13): p. 1626-37.
24. Sieval, A.B., et al., *Preparation and NMR characterization of highly substituted N-trimethyl chitosan chloride.* Carbohydrate Polymers, 1998. **36**(2-3): p. 157-165.
25. Slütter, B., et al., *Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination.* J Control Release, 2009. **138**(2): p. 113-21.
26. Bal, S.M., et al., *Efficient induction of immune responses through intradermal vaccination with N-trimethyl chitosan containing antigen formulations.* J Control Release, 2010. **142**(3): p. 374-83.
27. Bal, S.M., et al., *Efficient induction of immune responses through intradermal vaccination with N-trimethyl chitosan containing antigen formulations.* Journal of Controlled Release, 2010. **142**(3): p. 374-83.
28. Pasare, C. and R. Medzhitov, *Toll-like receptors: linking innate and adaptive immunity.* Adv Exp Med Biol, 2005. **560**: p. 11-8.
29. Miller, L.S. and R.L. Modlin, *Toll-like receptors in the skin.* Semin Immunopathol, 2007. **29**(1): p. 15-26.
30. Ueno, H., et al., *Dendritic cell subsets in health and disease.* Immunol Rev, 2007. **219**: p. 118-42.
31. Bogefors, J., et al., *Nod1, Nod2 and Nalp3 receptors, new potential targets in treatment of allergic rhinitis?* Allergy, 2010.
32. Hubert, F.X., et al., *Differential pattern recognition receptor expression but stereotyped responsiveness in rat spleen dendritic cell subsets.* J Immunol, 2006. **177**(2): p. 1007-16.
33. Hugot, J.P., et al., *Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease.* Nature, 2001. **411**(6837): p. 599-603.
34. Ogura, Y., et al., *A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease.* Nature, 2001. **411**(6837): p. 603-6.
35. Kobayashi, K.S., et al., *Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract.* Science, 2005. **307**(5710): p. 731-4.
36. Loving, C.L., et al., *Nod1/Nod2-mediated recognition plays a critical role in induction of adaptive immunity to anthrax after aerosol exposure.* Infect Immun, 2009. **77**(10): p. 4529-37.
37. Mitsui, H., et al., *Differential expression and function of Toll-like receptors in Langerhans cells: comparison with splenic dendritic cells.* J Invest Dermatol, 2004. **122**(1): p. 95-102.
38. Ding, Z., et al., *Immune modulation by adjuvants combined with diphtheria toxoid administered topically in BALB/c mice after microneedle array pretreatment.* Pharm Res, 2009. **26**(7): p. 1635-43.
39. Scharton-Kersten, T., et al., *Transcutaneous immunization with bacterial ADP-ribosylating exotoxins, subunits, and unrelated adjuvants.* Infect Immun, 2000. **68**(9): p. 5306-13.
40. Ban, E., et al., *CpG motifs induce Langerhans cell migration in vivo.* Int Immunol, 2000. **12**(6): p. 737-45.
41. Jakob, T., et al., *Bacterial DNA and CpG-containing oligodeoxynucleotides activate cutaneous dendritic cells and induce IL-12 production: implications for the augmentation of Th1 responses.* Int Arch Allergy Immunol, 1999. **118**(2-4): p. 457-61.
42. Imaoka, K., et al., *Nasal immunization of nonhuman primates with simian immunodeficiency virus p55gag and cholera toxin adjuvant induces Th1/Th2 help for virus-specific immune responses in reproductive tissues.* J Immunol, 1998. **161**(11): p. 5952-8.
43. Mutsch, M., et al., *Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland.* N Engl J Med, 2004. **350**(9): p. 896-903.
44. Pizza, M., et al., *Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants.* Vaccine, 2001. **19**(17-19): p. 2534-41.

45. Ayalew, S., et al., *Intranasal vaccination of calves with Mannheimia haemolytica chimeric protein containing the major surface epitope of outer membrane lipoprotein PlpE, the neutralizing epitope of leukotoxin, and cholera toxin subunit B.* Vet Immunol Immunopathol, 2009. **132**(2-4): p. 295-302.
46. Rask, C., et al., *Mucosal and systemic antibody responses after peroral or intranasal immunization: effects of conjugation to enterotoxin B subunits and/or of co-administration with free toxin as adjuvant.* APMIS, 2000. **108**(3): p. 178-86.
47. Sun, J.B., C. Czerkinsky, and J. Holmgren, *Mucosally induced immunological tolerance, regulatory T cells and the adjuvant effect by cholera toxin B subunit.* Scand J Immunol, 2010. **71**(1): p. 1-11.
48. Aspord, C. and C. Thivolet, *Nasal administration of CTB-insulin induces active tolerance against autoimmune diabetes in non-obese diabetic (NOD) mice.* Clin Exp Immunol, 2002. **130**(2): p. 204-11.
49. Lycke, N., *Targeted vaccine adjuvants based on modified cholera toxin.* Curr Mol Med, 2005. **5**(6): p. 591-7.
50. Anjuere, F., et al., *Transcutaneous immunization with cholera toxin B subunit adjuvant suppresses IgE antibody responses via selective induction of Th1 immune responses.* J Immunol, 2003. **170**(3): p. 1586-92.
51. Amidi, M., et al., *N-trimethyl chitosan (TMC) nanoparticles loaded with influenza subunit antigen for intranasal vaccination: biological properties and immunogenicity in a mouse model.* Vaccine, 2007. **25**(1): p. 144-53.
52. McAleer, J.P. and A.T. Vella, *Understanding how lipopolysaccharide impacts CD4 T-cell immunity.* Crit Rev Immunol, 2008. **28**(4): p. 281-99.
53. Patel, M., et al., *TLR2 agonist ameliorates established allergic airway inflammation by promoting Th1 response and not via regulatory T cells.* J Immunol, 2005. **174**(12): p. 7558-63.

11

Summary and perspectives

Summary

Nasal vaccination is a promising alternative to classical vaccination via needle injections. The nasal epithelium is equipped with a large number of immune cells, capable of both initiating and providing a protective immune response. Moreover, the nasal cavity is very accessible, allowing simple administration (nasal spray or nose drops), and the proteolytic environment is relatively low (compared to oral) providing a less hostile environment for the administered vaccines. The licensing of the first nasal vaccine (Flumist®) has shown the possibilities nasal vaccination provides. However, the inability so far to develop an effective subunit vaccine that would, unlike Flumist®, also be suitable for young children and elderly has also shown the challenge this administration route comprises (**Chapter 1**).

Vaccine formulation could be the key to successful nasal immunization as one can equip the antigen with the necessary tools to meet the challenges the nasal cavity offers. In order to rationally design nasal vaccine formulations, we will need to know the challenges the antigen will meet. Therefore this thesis has set out 3 aims:

1. To identify the major physiological hurdles subunit antigens have to overcome to elicit an immune response after nasal administration.
2. To develop methods *in vivo* or *in vitro* that allow these hurdles to be studied.
3. To use the obtained knowledge to rationally design nasal vaccine formulations.

The first aim is addressed in **Chapter 2** where the nasal physiology is reviewed and a road map to successful nasal vaccination is presented.

First of all, the nasal cavity has primarily evolved to keep substances out. A mucus layer covering the entire epithelium is replaced every 20 min, thereby removing all its constituents and thereby greatly limiting the time for the antigen to be taken up by epithelium. Formulation of the antigen with muco-adhesive substances like sodium alginate, carbopol, chitosan and N-trimethyl chitosan (TMC) can prolong the nasal residence time of antigen. A second hurdle identified is the passage through the nasal epithelium. Epithelial cells are closely stacked together by tight junctions, which leave little space for intercellular transport of large proteins. The inclusion of a tight junction opener like chitosan in a vaccine formulation can temporarily increase the permeability of the epithelium. The presence of M-cells in the epithelium offers the possibility of transcellular transport. As M-cells preferably transport particulate matter, the use of micro- or nanoparticle is advocated. Finally, when the antigen has passed the epithelium it has to be taken up by dendritic cells (DCs) and processed to elicit

the proper response. Besides the use of particulate systems, the use DC targeting ligands can facilitate endocytosis by DCs. In order to be able to activate T-cells, DCs will have to mature. This can be promoted by the addition of an adjuvant in the formulation. The choice of adjuvant can greatly influence the extent and type of immune response elicited.

The optimal nasal formulation will therefore be multifactorial and can be furnished with distinct functionalities such as mucoadhesive polymers, M-cell or DC targeting ligands and adjuvants. The opportunities in nasal vaccination ask for a concerted approach combining various targeting techniques is advocated.

Methods to investigate the nasal residence time of the antigen, transport by M-cells, the uptake by DCs and the maturation of DCs are discussed in **Chapters 3 and 4**.

An *in vitro* model for M-cells, based on intestinal epithelial cells co-cultured with a B-cell line, was assessed for its predictive value in the studies described in **Chapter 3.** Transport by M-cells of ovalbumin (OVA) loaded into TMC nanoparticles was higher than that of unencapsulated OVA or OVA loaded into chitosan nanoparticles. This was confirmed by *ex vivo* confocal fluorescent microscopic inspection of murine jejunum, showing that the M-cell model has predictive value. Moreover, an *in vitro* model for studying antigen uptake by DCs was introduced. Monocytes isolated from human volunteers were cultured into immature DCs, with which uptake of OVA in nanoparticles could be studied. TMC nanoparticles improved the association of OVA with DCs and induced activation of DCs. This correlated with the immunogenicity of OVA-loaded TMC nanoparticles after intraduodenal administration, which was significantly better than that of chitosan particles or a solution of OVA.

In **Chapter 4** a novel method of determining the nasal residence time of antigen using live imaging techniques is introduced. Three different types of nasal vaccine carriers PLGA, PLGA/TMC and TMC nanoparticles were investigated for their ability to decrease the clearance of OVA from the nasal cavity. Only TMC nanoparticles significantly prolonged the nasal residence time. Mice were nasally vaccinated with TMC nanoparticles and compared to the 2 other classes of nanoparticles, which did not prolong the nasal residence time. Interestingly, only the TMC nanoparticles elicited high anti-OVA antibody responses, whereas after intramuscular administration all classes of particles enhanced the immune response.

In contrast, nasal administration of OVA loaded PLGA nanoparticles appeared to result in tolerance rather than immunity (**Chapter 5**), as PLGA vaccinated mice showed a reduction in delayed type hypersensitivity against OVA. Vaccination with PLGA nanoparticles promoted the upregulation of the tolerogenic transcription factor FoxP3 in $CD4^+$ T-cell and did not increase the number OVA specific B-cells, which is necessary for an antibody response. Nasal

immunization with OVA loaded TMC nanoparticles resulted in the opposite result, i.e. a large number of OVA specific B-cells was detected in the cervical lymph nodes and spleen.

From the results presented in **Chapters 4 and 5** it can be concluded that not only the ability to prolong the nasal residence time but also other characteristics of nanoparticles greatly influence (the quality of) the immune response elicited after nasal vaccination. Next to their mucoadhesiveness, TMC nanoparticles have other characteristics that make them a more suitable carrier system for nasal vaccination compared to PLGA or PLGA/TMC nanoparticles. Also the rapid release of the contained antigen (promotes uptake by B-cells) and immune stimulatory capacity (counteracting tolerance) are designated as the key characteristics that make TMC nanoparticles potentially successful nasal carrier systems when protective immunity is aimed for.

In **Chapter 6** studies are described in which the formulation of OVA with a delivery system and an adjuvant was investigated. A promising nasal delivery system, cationic liposomes, was used to investigate whether the antigen and adjuvant should be combined in one carrier. Mice were nasally immunized with solutions of OVA and the adjuvant CpG, or with OVA and CpG encapsulated in liposomes. The resulting immune response was measured by determination of anti-OVA antibodies in serum and compared to immunization via other administration routes (transcutaneous with microneedle pre-treatment, intradermal and intranodal). Encapsulation of the CpG and OVA in liposomes had a detrimental effect on the IgG titers after nasal (and transcutaenous) administration compared to co-administration of soluble OVA and CpG, whereas after intradermal or intranodal injection of OVA/CpG-liposomes the immune response was improved compared to soluble OVA and CpG. To gain more insight into the mechanism behind the differences between the responses elicited, the uptake of OVA and CpG by DCs in the draining lymph nodes was investigated after administration of the formulation via the different administration routes. This showed that encapsulation of OVA and CpG in liposomes reduced the amount of antigen and adjuvant reaching the DCs after nasal and transcutaneous administration, whereas after intranodal injection encapsulation had a positive effect on the number of OVA and CpG positive DCs.

These data imply that co-encapsulation of the antigen and adjuvant into a cationic liposome can have a beneficial effect on the antibody response against OVA after parenteral injection. However, the concomitant size increment impairs proper transport of antigen and adjuvant to the lymph node when administered via the nasal or the transcutaneous route.

Whereas **Chapters 3-5** describe TMC nanoparticles as a very promising nasal delivery system for subunit antigen, **Chapter 6** indicates that liposomal carriers may have difficulties

penetrating the nasal epithelium, because of their relatively large size. This would imply that a smaller entity that still has the same characteristics as TMC nanoparticles would be an even better choice for nasal delivery. In **Chapter 7** studies are presented in which such a possibility was investigated, as it introduces the conjugation of an antigen, OVA, to TMC as an alternative to nanoparticles for subunit vaccination. The size of these constructs was significantly smaller than that of TMC nanoparticles (30 nm vs. 300 nm). OVA was covalently linked to TMC using thiol chemistry (SPDP method [1]). It was 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. Moreover, TMC-OVA conjugates were shown to be very comparable to TMC nanoparticles regarding their co-localization of OVA and TMC and their interaction with DCs. Uptake of TMC-OVA conjugate by DCs was similar to the uptake of TMC/OVA nanoparticles, i.e. over 5-fold increase compared to a solution of OVA and TMC. Mice intramuscularly immunized with TMC-OVA conjugate produced about 1000-fold higher OVA specific IgG titers than mice immunized with OVA and about 100-fold higher than mice receiving a physical mixture of TMC and OVA. These antibody titers were even slightly elevated compared to the titers obtained with TMC/OVA nanoparticles.

Just like TMC/TPP/OVA nanoparticles, TMC-OVA nanoconjugate prolonged the nasal residence time of the antigen (**Chapter 8**)**.** The immunogenicity of TMC-OVA nanoconjugate was assessed after nasal vaccination and compared with that of TMC/TPP/OVA nanoparticles, solutions of OVA and a physical mixture of TMC and OVA. 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. The improved performance of TMC-OVA conjugates might be attributed to better penetration of the nasal epithelium. *In vitro* the conjugates diffused significantly better through a monolayer of lung carcinoma (Calu-3) cells than TMC/TPP/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/TPP/OVA nanoparticles. 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.

A second way to improve TMC nanoparticles as nasal delivery system is to combine it with an adjuvant, which is investigated in the studies described in **Chapter 9 and 10.** In previous chapters it has been shown that TMC/TPP nanoparticles effectively induce antibody responses. However, in some cases a strong cellular response is highly desirable, e.g. for

vaccination against intracellular bacteria (e.g. M. tuberculosis) or viruses (e.g. HIV, Influenza A). Therefore in **Chapter 9** the composition of TMC nanoparticles has been altered. Whereas TMC was physically crosslinked with the strongly negatively charged molecule tripolyphosphate (TPP) in earlier chapters, here TMC was crosslinked with CpG DNA, an adjuvant known to provoke a cell mediated immune response. TMC/CpG/OVA nanoparticles showed similar physicochemical characteristics as TMC/TPP/OVA nanoparticles in terms of particle size (ca. 380 nm), zeta potential (+21 mV) and antigen release characteristics. Nasal administration of TMC/CpG/OVA and TMC/TPP/OVA nanoparticles to mice resulted in comparable serum IgG levels (ca. 1000 fold higher than those induced by unadjuvanted OVA) and local secretory IgA levels. Moreover, TMC/CpG/OVA nanoparticles induced a 10 fold higher IgG2a response than TMC/TPP/OVA nanoparticles and increased the number of OVA specific IFN-gamma-producing T-cells in the spleen. This shows that nasally administered OVA loaded TMC nanoparticles, containing CpG as adjuvant and crosslinker, are capable of provoking strong humoral and mucosal responses as well as Th1 type cellular immune responses and are therefore an all-round vaccine delivery system.

Finally in **Chapter 10,** next to CpG various other adjuvants are described for inclusion in TMC nanoparticles. Toll like Receptor (TLR) ligands (including lipopolysaccharide (LPS), PAM_3CSK_4 and CpG DNA), a NOD-like receptor-2 ligand (muramyl dipeptide (MDP)) and a GM1 ganglioside receptor ligand (cholera toxin B subunit) were encapsulated with OVA in TMC nanoparticles by ionic crosslinking with TPP. Physical characteristics like the nanoparticles' size, zeta potential and loading efficiency were determined to ensure that these parameters were similar between all particles. The effectiveness of the adjuvant loaded OVA-containing TMC particles was assessed *in vivo* by nasal vaccination of Balb/c mice using intradermal vaccination as a control. LPS loaded nanoparticles elicited the strongest IgG titers after nasal as well as intradermal vaccination. Moreover, LPS loaded nanoparticles induced higher sIgA levels than unadjuvanted TMC nanoparticles. Distinct differences between administration routes were observed: IgG titers after nasal immunization with MDP loaded particles were increased; nanoparticles with CpG showed decreased IgG levels compared to plain TMC particles; CpG loaded TMC particles after intradermal administration induced higher IgG and IgG2a titers; and MDP did not have an addition effect at all. This study shows that the inclusion of an adjuvant in OVA loaded TMC nanoparticles can significantly enhance the immune response. The selection of the adjuvant is not arbitrary and depends on the route of administration and the type of response required.

Perspectives

This thesis has set out to identify the limiting steps in nasal vaccination, study those limitations and find possible solutions to tackle the hurdles associated with these issues. In the next paragraphs several important aspects of nasal vaccine design are discussed and recommendations for future development are provided.

The antigen determines the formulation

The core of each vaccine formulation is the antigen. It is therefore not surprising that the rational design of a nasal vaccine should be based on the physicochemical and biological/immunological characteristics of the antigen, as well as the source (e.g. bacterium, tumor cell) from which it is derived. The model antigen in this thesis (OVA) is a water soluble negatively charged protein, with little mucoadhesive and immune stimulating properties. For instance, TMC nanoparticles were shown to be excellent nasal carriers for this antigen as the mucoadhesive and immunostimulatory characteristics of TMC compensated for the poor characteristics of OVA in this respect. Moreover, OVA easily complexes with the positively charged TMC polymers into nanoparticles, leading to a high encapsulation efficiency and, as shown in **Chapter 10**, adjuvants can be co-encapsulated to further increase and/or modulate the immune response. Notwithstanding these favorable characteristics of TMC as a nasal adjuvant for OVA, TMC may not be the ideal adjuvant for every antigen. Positively charged antigens are difficult to associate with TMC nanoparticles, leading to a lower encapsulation efficiency (unpublished results) and thus loss of costly antigen. Similarly, antigens with large lipophilic domains, such as hepatitis B surface antigen or hemagglutinin and neuraminidase, may profit more from formulation in liposomes as these membrane proteins can be incorporated in the liposomal bilayer, thereby mimicking more closely the natural way these antigens are presented to the immune system.

In some cases the antigen itself already has mucoadhesive or immune stimulating properties. For instance, Hagenaars et al. [2] used whole inactivated influenza virus (WIV) and showed that co-administration with TMC did not prolong the nasal residence time of the antigen, as the plain antigen already resided in the nasal cavity for more than 4 hours. Although formulation of WIV with TMC did improve the immune response, one could argue that focusing on immune potentiation rather than mucoadhesion may be a better approach to improve the immune response to this antigen.

Finally, each vaccine should elicit a tailored immune response that is strongly dependent on the pathogen (or disease) to be combated. To repel pathogens that reside in the bodies' interstitial spaces, antibodies can be instrumental, making a humoral response desirable.

When intracellular bacteria or viruses are concerned, the help of cytotoxic T-lymphocytes (CTLs) and other leukocytes involved in the cellular immune response is required. A mucosal (sIgA mediated) response can prohibit pathogens that invade our bodies via mucosal surfaces from colonizing these epithelia and may therefore be very useful for the protective efficacy of a vaccine as well. This should be kept in mind when formulating an antigen for nasal administration. As can be concluded from this thesis, TMC increases sIgA production and causes a Th2 type response after nasal administration and may therefore be a good choice if a humoral immune response against respiratory or intestinal pathogens is required. This would make TMC based formulations potentially useful for future nasal vaccination against for instance diphtheria, influenza and polio.

TMC is however not likely to be the "weapon of choice" if a cellular response is required, for instance against herpes, HIV, malaria or RSV infection. The use of a different antigen delivery system that is more capable of eliciting cellular response, like ISCOMs or the addition of an adjuvant that can enhance the T-cell mediated immunity should be considered.

Nasal residence time, a critical parameter

Nasally applying an antigen that is cleared from the nasal cavity within minutes seems a waste of vaccine if the antigen does not get a chance to be absorbed into the nasal epithelium. The pivotal role of a prolonged residence in the nasal cavity is supported by various studies that have shown increased antibody response after nasal administration of antigen with mucoadhesive substances. A recent study by Nochi et al. [3] even shows that when the antigen is present up to days after administration (using a mucoadhesive nanogel), no additional adjuvant is needed to boost the immune response, emphasizing the potential of a long nasal residence time. In this thesis the importance of delaying nasal clearance is underlined (**Chapter 4**). Moreover, it is not hard to accomplish, as simple co-administration of TMC already caused a significant increase in the nasal residence time of OVA (**Chapter 8**). Similarly, studies in which mucoadhesives like chitosan or carbopol were co-administered with the antigen describe a similar mode of action [4, 5]. Therefore, the inclusion of a mucoadhesive polymer in a vaccine formulation seems one of the simplest ways to improve the efficacy of a nasal vaccine.

Small is beautiful

Particulate antigens have been associated with higher immune responses as compared to soluble antigens [6]. Particles offer the distinct advantage of being efficiently phagocytosed by DCs and transcytosed by M-cells. Moreover, multimeric antigen presentation can improve the

antigen specific uptake by B-cells. Indeed, particles can have a positive effect on the immune response after vaccination. However, careful consideration to the particle characteristics is recommended. As can be concluded from **Chapter 6** the size of the particle is an important point to consider. Nanoparticles are generally more effective than microparticles, but small nanoparticles (100 nm) were not more effective than 500 nm particles. As the particles in this size range all greatly exceed the maximum diameter of a tight junction in the nasal epithelial, we can assume their transport to the subepithelium to be mainly dependent on active transport by M-cells. Therefore equipping particles with an M-cell targeting ligand or selection of particles that naturally have a strong affinity for M-cells is more likely to improve M-cell transport than further reduction in particle diameter. Alternatively, a drastic size reduction might improve the passive, intercellular uptake by the nasal epithelium. In **Chapter 8** it is shown that TMC-OVA conjugates (size ca. 30 nm) cross the nasal epithelium more effectively than TMC/OVA nanoparticles and consequently induce a higher immune response. In conclusion, nasal vaccination could benefit from particulate formulations, however with respect to passing the epithelial barrier a diameter as small as possible is preferred to facilitate passive .

Adjuvants

The arsenal of adjuvants at our disposal is steadily growing. Whereas for more than a century alum was the only approved adjuvant for human use, recently new adjuvants like squalene emulsions (e.g., MF-59) and non toxic variants of LPS (e.g., MPL) were licensed for the European market. Increasing knowledge on the activation of the immune system (specifically the activation of APCs) has speeded up this process, as it has explained the mode action of several adjuvants from which the mechanism was unknown until recently (e.g., alum, MDP and LPS), which is a perquisite for approval by the American Food and Drug Administration. Moreover, the observation that APC maturation can be triggered via specific pathogen recognition receptors (PRRs, e.g. Toll-like receptors and NOD-like receptors) has led to the identification of new ligands that could act as adjuvants. As the signaling cascade resulting from activation of PRRs is becoming more clear, in the near future it may be possible to select the proper adjuvant according to the nature of the vaccine and the type of immune response required .

As has been pointed out in **Chapter 10**, the use of adjuvants can be very beneficial for nasal vaccination, but not every adjuvant is a good nasal adjuvant. Whether or not an adjuvant is a good choice for nasal use, will depend on the type of response required (e.g. antibodies or cytotoxic T-lymphocytes) and on the dose of adjuvant required. In concurrence with the

"danger model" adjuvants are supposed to be dangerous goods and consequently there is only a small margin between immune potentiation and toxicity. Therefore not all adjuvants are suitable because their effective dose is a toxic one. For instance, heat labile enterotoxin (LT) was successfully used as an adjuvant with virosomes, but after nasal administration LT was presumably taken up by olfactory neurons, eventually leading to Bell's palsy [7]. Whether an adjuvant will have a strong positive effect on the immune response raised by a nasal vaccine likely depends on how easily the adjuvant is absorbed into the nasal epithelium and on the expression of its complementary receptor on nasal DCs. For instance, in **Chapter 9**, CpG was a more effective as an adjuvant when formulated in the TMC nanoparticles as a delivery system. Interestingly, mice that received 2x 20 µg CpG in TMC nanoparticles elicited strong IgG and sIgA titers, whereas mice that received 2x 10 µg CpG (**Chapter 10**) in the same particle formulation did not develop an antibody response. This suggests that activation of the receptor for CpG (TLR-9) on DCs in the nose may not be that easy to establish. In this respect TLR-4 ligand (MPL) or MDP (a NOD-like receptor 2 ligand) in combination with TMC particles may be better candidates for nasal vaccination. Literature on PPR expression on nasal DCs is scarce, making it very difficult to select the right adjuvant *a priori*. However, the high dose of CpG was well tolerated by the mice, making CpG still a promising candidate adjuvant for nasal vaccination. Nonetheless, increasing knowledge on the expression pattern of PRR on nasal DCs would be very helpful for the rational design of nasal vaccination including the choice of 'the right' adjuvant.

Combine and conquer

The major challenge in formulation of nasal vaccine formulation is to manage the interplay between antigen, delivery vehicle and adjuvant in such a way that the optimal response is obtained. The research described in this thesis addressed this challenge and has identified some "must do's", if formulation with soluble antigens such as OVA is concerned.

Must do's:

- Add a mucoadhesive to overcome the short residence time.
- Use small entities, to improve penetration through the nasal epithelium.
- Add an adjuvant to overcome nasal tolerance.
- Co-localize antigen and adjuvant to achieve better DC uptake/maturation.

In accordance with these 4 “must do’s”, also a certain “degree of freedom” was observed when OVA was formulated with TMC. This could be important, as it may simplify the design of the formulation.

Degrees of freedom:

- The mucoadhesive can be administered in conjunction with the delivery system or in a free form.
- Co-localization of adjuvant and antigen does not necessarily have to be achieved through the use of particles but can also be effectuated by conjugation of antigen and adjuvant.

Combining these rules of thumb, it seems that a small, co-localized antigen-adjuvant entity (like a conjugate or a nanoparticle <50nm) formulated in a solution with a mucoadhesive (mucoadhesive in a free form) could be a very promising approach. Based on the results in this thesis, in general a conjugate between an antigen with MPL, would be an interesting choice as LPS (of which MPL is a derivate) turned out to be the best adjuvant for OVA in **Chapter 10.** MPL is a less toxic variant of LPS and already licensed for human use. With the addition of TMC (as a mucoadhesive and additional adjuvant) antigen-LPS conjugates may provide effective future nasal subunit vaccines.

Into the clinic, bears on the road

Even if such nasal vaccine formulation with a relevant antigen is successful in a laboratory setting transferring it to the clinic successfully, will be large effort. The lack of correlation between mice and man is one of the first hurdles to take. Mice have been instrumental in the mechanistical aspects of nasal vaccination in this thesis, but will never be able to fully predict the immune response in humans. For instance, a very obvious difference between mice and man is the size of the nasal epithelium. Relatively the nasal epithelium of mice 4 times larger than the human epithelium, which could cause an overestimation of the absorbance and residence time of the antigen in mice compared to humans. Furthermore, the murine immune system is different than the human immune system, like the expression patterns of PRRs and the secretion of different antibody subtypes. Finally, many pathogens do not cause disease in mice. Mouse strains susceptible for these pathogens are being developed, but still these models have their limitations. Mice can be useful to test for local toxicity of the vaccine. TMC for instance was well tolerated by all mice in this study and has recently been applied to pigs with no resulting damage to the nasal epithelium (unpublished data). This, in combination

with the high antibody titers in mice, may be convincing enough to start a first small clinical trial.

Furthermore it seems imperative to keep the formulation simple. From the experience with the one nasal vaccine on the market (Flumist) we know nasal administration is well perceived by the public and especially by children [8]. However, this positive perception is only sustained if the costs are low. The first year Flumist was introduced only 500,000 vaccines were sold, whereas a year later when the price was drastically lowered the sales quadrupled [9]. A formulation can therefore only be commercially interesting if the antigen and adjuvant are readily available and the formulation is cheap, reliable and scalable. Also in this respect, the addition of TMC to the formulation is very feasible. The material from which TMC is derived, chitin, is the second most abundant polymer on earth and therefore readily available. Although it is a very heterogeneous substance, synthesis routes to standardize the production of TMC have already been established [10]. TMC is therefore also in this respect a very promising vaccine adjuvant.

Finally, the pharmaceutical form will be very important. A nasal spray seems an obvious choice, however this would require the vaccine to be in solution. Vaccine solutions are generally unstable and require cold storage and imply a short shelf life. A product in dry powder form is much more stable and could therefore be more easily distributed; also to countries were maintaining the cold chain is not self-evident. Lyophilization of OVA without damaging its antigenic epitopes has been shown in this thesis and for various other antigens this technique has also been successfully applied. If a simple and cheap delivery device can be developed to apply the vaccine as a powder or to reconstitute the vaccine just before application, nasal vaccination may become the new standard in vaccination.

References:

1. van Dijk-Wolthuis, W.N., et al., *A versatile method for the conjugation of proteins and peptides to poly[2-(dimethylamino)ethyl methacrylate].* Bioconjug Chem, 1999. **10**(4): p. 687-92.
2. Hagenaars, N., et al., *Role of trimethylated chitosan (TMC) in nasal residence time, local distribution and toxicity of an intranasal influenza vaccine*. J Control Release, 2010. **144**(1): p. 17-24.
3. Nochi, T., et al., *Nanogel antigenic protein-delivery system for adjuvant-free intranasal vaccines.* Nat Mater, 2010. **9**(7): p. 572-8.
4. Soane, R.J., et al., *Clearance characteristics of chitosan based formulations in the sheep nasal cavity.* Int J Pharm, 2001. **217**(1-2): p. 183-91.
5. Bromberg, L.E., *Enhanced nasal retention of hydrophobically modified polyelectrolytes.* J Pharm Pharmacol, 2001. **53**(1): p. 109-14.
6. O'Hagan, D.T. and R. Rappuoli, *Novel approaches to vaccine delivery.* Pharm Res, 2004. **21**(9): p. 1519-30.
7. Mutsch, M., et al., *Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland.* N Engl J Med, 2004. **350**(9): p. 896-903.
8. Flood, E.M., et al., *Children's Perceptions of Influenza Illness and Preferences for Influenza Vaccine.* Journal of Pediatric Health Care. **In Press, Corrected Proof**.
9. Wikipedia.org. *FluMist*. 2010; Available from: http://en.wikipedia.org/wiki/Nasal-spray_flu_vaccine.
10. Verheul, R.J., et al., *Synthesis, characterization and in vitro biological properties of O-methyl free N,N,N-trimethylated chitosan.* Biomaterials, 2008. **29**(27): p. 3642-9.

Appendix

Nederlandse Samenvatting

List of abbreviations

List of publications

Curriculum vitae

Nawoord

Nederlandse Samenvatting

Vaccinatie via de neus, verder te noemen nasale vaccinatie, is een veelbelovend alternatief voor de normale injecteerbare vaccines. De neusholte is heel toegankelijk, waardoor eenvoudige toediening van een vaccin, bijvoorbeeld als neusspray of neusdruppels, mogelijk is. Het neusepitheel is bovendien uitgerust met een groot aantal immuuncellen, waardoor het in staat is een beschermende immuunreactie te bewerkstelligen. Ook de enzymatische activiteit er relatief laag (in vergelijking met bijvoorbeeld de darmen) waardoor een vaccin in de neus minder snel afgebroken wordt.

Er is inmiddels al een nasaal vaccin op de markt (griepvaccin, Flumist®), wat aantoont dat nasale vaccinatie zeker mogelijk is. Een nadeel van dit vaccin is echter dat het berust op een verzwakt griepvirus. Dit is weliswaar geen probleem voor gezonde mensen, maar bij ouderen, baby's en mensen met een verzwakte afweer kan dit vaccin vervelende bijwerkingen hebben. Tot dusver is het nog niet gelukt om een veilig en effectief nasaal vaccin te ontwikkelen dat slechts uit een deel (een antigeen) van een virus bestaat (subunit vaccin). In tegenstelling tot Flumist® zou zo'n subunit vaccin ook geschikt kunnen zijn voor jonge kinderen en ouderen (**Hoofdstuk 1**). Dit proefschrift stelt zich dan ook ten doel de mogelijkheid van nasale vaccinatie met subunit antigenen te onderzoeken.

De vaccinformulering kan de sleutel zijn tot succesvolle nasale vaccinatie. Het antigeen moet met de nodige hulpstoffen worden voorzien om de uitdagingen die de neusholte biedt aan te kunnen gaan. Voor het ontwerpen van nasale vaccinformuleringen, zullen we eerst moeten weten welke hindernissen het antigeen zal tegenkomen vanaf het moment van toedienen tot aan het bereiken van een beschermende immuunsrepons. Daarom zijn in dit proefschrift drie doelstellingen beschreven:

1. Het identificeren van de belangrijkste barrières die subunit antigenen moeten overwinnen om een immuunreactie op te wekken na nasale toediening.
2. Het ontwikkelen van methoden die het mogelijk maken deze hindernissen te onderzoeken.
3. Het gebruiken van de verkregen kennis om nasale vaccinformuleringen te ontwerpen en te testen.

Het eerste doel komt aan de orde in **Hoofdstuk 2**, waar de fysiologie van de neus wordt beschreven en een stappenplan voor een succesvolle nasale vaccin wordt geïntroduceerd. De neusholte heeft zich in de eerste plaats ontwikkeld om stoffen buiten te houden. Een

mucuslaag (snotlaag) welke het gehele epitheel overdekt wordt elke 20 minuten vervangen, waarmee alles wat in en op de mucus terechtgekomen is verwijderd wordt. Dit betekent dus dat een antigeen slechts kort in de neusholte zal verblijven en dus weinig kans krijgt om goed opgenomen te worden door het neusepitheel. Formuleren van het antigeen met plakkerige "muco-adhesieve" stoffen, zoals natriumalginaat, carbopol, chitosaan en N-trimethylchitosaan (TMC), zou de nasale verblijftijd van het antigeen kunnen verlengen.

Een tweede hindernis is de doorgang door het neusepitheel. Epitheelcellen zijn nauw met elkaar verbonden door zogenaamde "tight junctions", die weinig ruimte laten voor transport van grote eiwitten tussen de cellen door. Het toevoegen van "tight junction openers", stoffen die deze nauwe doorgangen tussen de cellen verwijden, zoals het bio-polymeer chitosaan, kan een tijdelijke verhoging van de doorlaatbaarheid van het epitheel veroorzaken. De aanwezigheid van gespecialiseerde "transportcellen", zogenaamde M-cellen, in het epitheel biedt de mogelijkheid voor transport door de cel zelf heen. Zulke M-cellen transporteren bij voorkeur hele kleine deeltjes (nano- of microdeeltjes). Van dit feit zou men gebruik kunnen maken, door het antigeen in zo'n deeltje te verpakken.

Ten slotte, wanneer het antigeen het epitheel is gepasseerd, moet het worden opgenomen door dendritische cellen (DCs). Dit zijn de cellen die uiteindelijk de immuunreactie initiëren. Net als M-cellen zijn DCs in staat nanodeeltjes op te nemen. Door aan de met antigeen beladen deeltjes moleculen te koppelen die zich speciaal aan DCs binden (DC-liganden), kan de opname door DCs vergemakkelijkt worden. Om ervoor te zorgen dat de DCs vervolgens in staat zijn de uiteindelijke uitvoerders van de immuunreactie, T-cellen en B-cellen, aan te sturen, zullen de DCs geactiveerd moeten worden. Dit kan worden bevorderd door de toevoeging van een hulpstof (adjuvans) aan de formulering. De keuze van het adjuvans is belangrijk, want het kan sterke invloed hebben op de omvang en de aard van de immuunreactie.

De optimale nasale vaccinformulering zal daarom vermoedelijk bestaan uit verschillende componenten, zoals mucoadhesieve polymeren, M-cel- of DC-liganden en adjuvantia. Een effectief nasaal vaccin vraagt dus om een gecoördineerde aanpak, gericht op het vernuftig combineren van de benodigde componenten.

Methoden om de nasale verblijftijd van het antigeen, het transport door M-cellen, de opname door DCs en de activering van DCs te onderzoeken, worden besproken in de **Hoofdstukken 3-4**. Een celkweekmodel voor de M-cellen, gebaseerd op darmepitheelcellen samen gekweekt met een B-cellijn, werd beoordeeld op zijn voorspellende waarde voor het transport van antigenen door het darmepitheel in levende muizen (**Hoofdstuk 3**). Uit een studie met deze

M-cellen bleek dat wanneer het subunit antigeen ovalbumine (OVA) werd verpakt in nanodeeltjes gemaakt van het polymeer TMC, OVA gemakkelijker werd getransporteerd dan wanneer OVA niet in nanodeeltjes was verpakt. Dit zelfde effect werd waargenomen in levende muizen, wat liet zien dat het M-cel-model voorspellende waarde heeft. Tevens is in dit hoofdstuk een kweekmodel van DCs beschreven. Witte bloedcellen geïsoleerd uit menselijke vrijwilligers werden gekweekt tot DCs, waarmee de opname van een model subunit antigen, OVA, kon worden onderzocht. Ook hier verbeterden TMC-nanodeeltjes de associatie van OVA met DCs en stimuleerden ook nog eens de activering van de DCs. Dit alles bleek te correleren met een beduidend sterkere immuunreactie na het toedienen van met OVA beladen TMC-deeltjes dan na het toedienen van een oplossing van OVA.

In **Hoofdstuk 4** wordt een nieuwe methode voor het bepalen van de nasale verblijftijd van het antigeen met behulp van een beeldvormende techniek in levende muizen geïntroduceerd. Drie verschillende soorten potentiële nasale vaccinsystemen, PLGA-, PLGA/TMC- en TMC-nanodeeltjes, werden onderzocht op hun vermogen om de verblijftijd van OVA in de neusholte te verlengen. Van de onderzochte deeltjes bleken alleen TMC-nanodeeltjes dit te doen. Muizen werden daarop nasaal gevaccineerd met de drie verschillende nanodeeltjes. Hierbij bleek dat alleen de TMC-nanodeeltjes hoge OVA antilichaamtiters opleverden, terwijl na intramusculaire toediening alle deeltjes een versterking van de immuunreactie gaven. Hieruit kan men concluderen dat het verlengen van de nasale verblijftijd een belangrijke factor voor succes is en TMC-nanodeeltjes daarom interessante vaccinformuleringen zijn. Dat laatste blijkt eens te meer uit **Hoofdstuk 5**. Hier wordt de aard van de immuunreactie die TMC en PLGA oproepen verder onderzocht. Analyse van de T-cellen en de B-cellen laat zien dat TMC-deeltjes zorgen voor een actieve immuunrespons met productie van antistoffen tot gevolg, terwijl nasale vaccinatie met PLGA-deeltjes juist leidt tot tolerantie. Dit laatste kan echter heel erg interessant zijn, omdat in het geval van auto-immuunziekten (bijvoorbeeld reuma, multiple sclerose en de ziekte van Crohn), het induceren van tolerantie wellicht een goede therapie zou kunnen zijn.

Zoals in het stappenplan aangegeven, is het toevoegen van een adjuvans ook een mogelijkheid om nasale vaccins te verbeteren. In **Hoofdstuk 6** wordt onderzocht hoe een adjuvans dan moeten worden geformuleerd. Een antigeen en een adjuvans kunnen immers samen of apart worden toegediend en wellicht is het zelfs een goed idee (met de studies in **Hoofdstuk 3-5** in het achterhoofd) om het antigeen met het adjuvans samen in een nanodeeltje te stoppen. Een veelbelovende nasale antigeendrager zijn positief geladen liposomen, welke werden gebruikt om te onderzoeken of het antigeen en het adjuvans

moeten worden gecombineerd in één transportsysteem. Liposomen zijn waterbolletjes met een buitenlaag van fosfolipiden (vetachtige moleculen die opgebouwd zijn uit o.a. verzuren en glycerol). In de liposomen kunnen moleculen zoals antigenen en adjuvantia ingebouwd worden. Muizen werden nasaal gevaccineerd met liposomen waar OVA en het bekende adjuvans CpG ingekapseld waren of met oplossingen van OVA en het adjuvans CpG (zonder liposomen). De resulterende immuunrespons werd vergeleken met vaccinatie via andere toedieningwijzen (op de huid, in de huid en rechtstreeks in een lymfeklier). Inkapseling van CpG in liposomen bleek een nadelig effect op de immuunreactie na toediening via de neus of op de huid ten opzichte van toediening van een mengsel van opgelost OVA en CpG. Interessant is overigens dat na injectie in de huid of in een lymfeknoop van in liposomen ingekapseld OVA en CpG de immuunreactie wel werd verbeterd. Uit verdere bestudering van de lymfeklieren na nasale toediening, bleek dat het inkapselen van OVA en CpG in liposomen leidde tot een verminderde hoeveelheid antigeen en adjuvant in de lymfeklieren. Deze gegevens duiden erop dat het inkapselen in een liposoom het vervoer van antigen en adjuvant door het neusepitheel nadelig beïnvloedt en daardoor bij nasale vaccinatie tot een verminderde immuunreactie leidt.

Hoofdstukken 3-5 beschrijven TMC-nanodeeltjes als een veelbelovend systeem voor nasale toediening van subunit antigenen. **Hoofdstuk 6** laat echter zien dat nanodeeltjes ook moeilijkheden kunnen ondervinden bij met het passeren van het neusepitheel, vanwege hun relatief grote omvang. Bij elkaar opgeteld, zou men kunnen veronderstellen dat een kleiner construct dat nog steeds dezelfde eigenschappen als TMC-nanodeeltjes heeft, een nog betere keuze voor nasale toediening zou zijn. In **Hoofdstuk 7** wordt deze veronderstelling getoetst. OVA werd chemisch, via een disulfidebinding, aan een TMC-polymeer vastgekoppeld. De omvang van de verkregen TMC-OVA constructen (ca. 30 nm) is aanzienlijk kleiner dan die vanTMC-nanodeeltjes (ca. 300 nm). Er werd vastgesteld dat op deze manier een omkeerbare covalente binding tussen TMC en OVA kon worden ingevoerd, onder behoud van de structuur van het antigeen. Opname van TMC-OVA conjugaat door DCs was vergelijkbaar met die van TMC/OVA-nanodeeltjes, en ruim 5 maal hoger in vergelijking met een oplossing van OVA en TMC. Na intramusculaire (d.w.z. in een spier) vaccinatie met TMC-OVA conjugaat produceerden muizen ongeveer 1000- en 100-voudig hogere OVA specifieke IgG-titers dan muizen gevaccineerd met respectievelijk alleen OVA en een mengsel van TMC en OVA. Het antilichaamniveau was zelfs iets hoger dan de niveaus verkregen na intramusculaire injectie van TMC/OVA-nanodeeltjes.

Net als TMC/OVA nanodeeltjes, verlengen TMC-OVA conjugaten de nasale verblijftijd van het antigeen (**Hoofdstuk 8**). De immunogeniciteit van nasaal toegediende TMC-OVA conjugaten werd vergeleken met die van TMC/OVA-nanodeeltjes, een oplossing van OVA en een mengsel van TMC en OVA. Muizen die nasaal waren vaccineerd met TMC-OVA conjugaat produceerden antilichamen in hun long- en neusvocht en bovendien induceerde het conjugaat hogere antilichaamniveaus in het bloed dan de andere formuleringen. Deze uitstekende resultaten met de TMC-OVA conjugaten kunnen worden toegeschreven aan een betere penetratie door het neusepitheel. In een kweekmodel diffundeerden de conjugaten in vergelijking met TMC/OVA-nanodeeltjes beter door een laag van longepitheelcellen. Bovendien leidde nasale toediening van conjugaten aan muizen tot hogere opname van OVA in de lymfeklieren in vergelijking met nasale toediening van TMC/OVA-nanodeeltjes. Kortom, de TMC-OVA nanoconjugaten verbeteren de penetratie van OVA door het nasale epitheel en verhogen zo immunogeniciteit van het antigeen. Bovendien valt hieruit af te leiden dat het tegelijkertijd afleveren van antigeen en adjuvant aan DCs belangrijk is voor het uitlokken van een immuunreactie; wellicht belangrijker zelfs dan zorgen dat het antigeen in een deeltje verpakt zit.

Een andere manier om TMC-nanodeeltjes als nasaal transportsysteem te verbeteren is ze te combineren met een adjuvans. Deze benadering wordt bestudeerd in de studies beschreven in **Hoofdstuk 9 en 10**. In de vorige hoofdstukken is aangetoond dat nasaal toegediende TMC-nanodeeltjes de antilichaamrespons tegen het ingekapselde antigeen bevorderen. Echter, in sommige gevallen is de aanmaak van antilichamen niet voldoende om een pathogeen te bestrijden en is er ook een sterke T-celreactie nodig, bijvoorbeeld bij vaccinatie tegen intracellulaire bacteriën (bijv. tuberculosebacillen) of virussen (bijv. HIV of griepvirus). In **Hoofdstuk 9** is daarom de bereiding van de TMC-nanodeeltjes gewijzigd. Terwijl in eerdere hoofdstukken de positief geladen TMC-polymeren fysisch verknoopt werden door gebruikmaking van het negatief geladen molecuul tripolyfosfaat (TPP), werd hier TMC verknoopt met het eerder genoemde adjuvans CpG. Dit is een negatief geladen adjuvans dat bekend staat om het uitlokken van een T-celreactie. TMC/CPG/OVA-deeltjes hebben vergelijkbare fysisch-chemische eigenschappen als TMC/TPP/OVA-deeltjes; bijv. de deeltjesgrootte (ca. 350 nm) en de elektrische lading (+21 mV). Nasale toediening van TMC/CPG/OVA- en TMC/TPP/OVA-deeltjes aan muizen resulteerde in vergelijkbare antistofwaarden (ca. 1000 maal hoger dan die na nasale toediening van een oplossing van OVA). Echter, nasale toediening van TMC/CPG/OVA-deeltjes, in vergelijking met TMC/TPP/OVA-deeltjes, resulteerde in een 10 maal hoger niveau aan OVA-specifieke IgG2a-

antistoffen en een toename van het aantal IFN-γ producerende T-cellen. Dit toont aan dat TMC/CpG/OVA-deeltjes veel beter de T-cel gemedieerde immuunreactie kunnen bevorderen dan TMC/TPP/OVA-deeltjes. Kortom, TMC/OVA nanodeeltjes, met CpG als adjuvans en crosslinker, is een "all-round" vaccindragersysteem, omdat het in staat is zowel antistofproductie als T-celactivering te bewerkstelligen.

Uiteindelijk worden in **Hoofdstuk 10** naast CpG diverse andere adjuvantia beschreven in combinatie met TMC-deeltjes. Verschillende receptoren op DCs kunnen worden aangezet om de DC te activeren. Zo zijn er de zogenaamde Toll like receptoren (TLR) en NOD-like receptoren die kunnen worden geactiveerd door interactie met respectievelijk TLR- of NOD-liganden. De onderzochte TLR-liganden, lipopolysaccharide (LPS), PAM3CSK4 en CpG, en de NOD-2-ligand muramyl dipeptide (MDP) hebben daarom de potentie om DCs te activeren. Deze liganden werden samen met OVA ingekapseld in TMC-nanodeeltjes door verknoping met TPP. De bereiding van de deeltjes was zodanig geoptimaliseerd dat hun fysieke kenmerken zoals grootte en lading vergelijkbaar waren. De effectiviteit van adjuvant geladen TMC deeltjes werd beoordeeld door de nasale vaccinatie in muizen, in vergelijking met vaccinatie in de huid. TMC/OVA-nanodeeltjes met LPS of MDP ontlokten de hoogste antistof titers na nasale vaccinatie. Niet alle adjuvantia waren echter even effectief en hun effectiviteit bleek afhankelijk te zijn van de toedieningsroute. Deze studie toont aan dat de combinatie van TMC-nanodeeltjes als dragersysteem met een adjuvans, de immuunreactie aanzienlijk kan verbeteren. De selectie van het adjuvant is echter niet willekeurig en hangt af van de toedieningsroute.

Met behulp van deze bevindingen kunnen we een aantal conclusies trekken (**Hoofdstuk 11**). De belangrijkste obstakels voor nasale subunit vaccins zijn, (i) de korte verblijftijd van het antigeen in de neus, (ii) de beperkte opname van het antigeen door het neusepitheel, (iii) de gelimiteerde opname van het antigeen door DCs en (iv) inductie van tolerantie.

Deze vier punten kunnen verholpen worden door het antigeen te combineren met hulpstoffen of te verpakken in nanodeeltjes, maar we hebben ook kunnen concluderen dat de ene maatregel de ander soms tegenwerkt. Zo kan het samen inpakken van antigeen en adjuvans in een nanodeeltje de opname door DCs verbeteren en tolerantie voorkomen, maar zorgt het voor een verminderde opname door het neusepitheel. Gelukkig geldt dat niet voor alle obstakels. Zo lijkt het toevoegen van een plakkerige "muco-adhesieve" stof aan de formulering om de nasale verblijftijd van het antigeen te verlengen een eenvoudige, effectieve maatregel. Het lijkt niet uit te maken of de muco-adhesieve stof los wordt toe gevoegd of in

de vorm van een nanodeeltje. Een adjuvans daarentegen lijkt bij voorkeur wel degelijk (fysisch of chemisch) aan het antigeen gekoppeld te moeten zijn. Op basis van hiervan zou een ideale nasale formulering er dus als volgt uit kunnen zien:

Een antigeen gekoppeld aan een adjuvans, met daaraan een muco-adhesieve stof toegevoegd.

De mucoadhesieve stof (bijvoorbeeld TMC) verlengt de nasale verblijftijd (i). Een antigeen gekoppeld aan een adjuvans kan de opname door DCs verbeteren (iii) en tolerantie voorkomen (iv) omdat het adjuvans de DCs activeert. Omdat zo'n antigeen-adjuvansconstruct vrij klein is, is het ook nog eens aannemelijk dat het (in vergelijking met nanodeeltjes) beter wordt opgenomen door het nasale epitheel (ii).

Natuurlijk is dit nu nog slechts toekomstmuziek en betreffen de bevindingen die in dit proefschrift beschreven zijn slechts muizenstudies, die in de praktijk wel eens afwijken van studies in mensen. Verder onderzoek omtrent de werking en eventuele bijeffecten van bijvoorbeeld TMC en adjuvantia zal moeten uitwijzen in hoeverre deze voor bruikbaar zijn voor de ontwikkeling van veilige en effectieve nasale vaccins voor toepassing bij de mens. Ook zal het productieproces en de wijze van toediening zo goedkoop mogelijk gehouden moeten worden om nasale vaccins concurrerend met injecteerbare vaccins te maken.

List of abbreviations

ANOVA	Analysis of variance
APC	Antigen presenting cell
Caco-2	Colon carcinoma cell
CBF	Ciliary beat frequency
CpG	Unmethylated C-G motif
CS	Chitosan
CT	Cholera toxin
CTB	Cholera toxin beta subunit
CTL	Cytotoxic T-lymphocyte
DC	Dendritic cells
DCM	Dichloromethane
DLS	Dynamic light scattering
FAE	Follicle-associated epithelium
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
HbsAg	Hepatitis B surface antigen
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IgG1	Immunoglobulin G subtype 1
IgG2a	Immunoglobulin G subtype 2a
IgM	Immunoglobulin G
IL	Interleukin
i.n.	intranasal
ISCOM	Immune stimulatory complex
LC	Langerhans cells
LE	Loading efficiency
LN	Lymphnode
LPS	Lipopolysaccharide
LT	*Escherichia coli* heat labile toxin
M-cell	Microfold cell
MHC	Major histocompatibility complex
MDP	muramyl dipeptide
NALT	Nasal associated lymphoid tissue

NLR	NOD like recepor
NOD	Nucleotide-binding oligomerization domain containing
NP	Nanoparticles
OVA	Ovalbumin
PAM	Palmitol
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PDI	Polydispersity index
PLGA	poly(lactic-co-glycolic acid)
PRR	Pathogen recognition receptor
sIgA	secretory Immunoglobulin A
SD	Standard deviation
SEM	Standard error of the mean
SPDP	N-succinimidyl 3-(2-pyridyldithio) propionate
TEER	Transepithelial electrical resistance
Th1	T helper 1
Th2	T helper 2
TMC	N-trimethyl chitosan
TLR	Toll-like receptor
TPP	Tripolyphosphate
UEA-1	*Ulex europaeus* 1 lectin
ZP	Zetapotential

List of Publications

1. Slütter B, Bal S, Verheul R, Bouwstra J, Jiskoot W. **Adjuvant selection and site dependant effects for nasal and intradermal subunit vaccination using N-trimethyl chitosan nanoparticles as a delivery system.** (Submitted)
2. Slütter B, Bal S, Zhi D, Jiskoot W, Bouwstra J. **Adjuvant effect of cationic liposomes and CpG depends on administration route.** (Submitted)
3. Slütter B, Bal S, Que I, Kaijzel E, Löwik C, Bouwstra J, Jiskoot W. **Antigen-adjuvant conjugates for nasal vaccination, an improvement over the use of nanoparticles?** Mol Pharm (2010), in press
4. Mohanan, D., Slütter, B., Jiskoot, W., Bouwstra, J.A., Henriksen-Lacey, M., Perrie, Y., Kündig, T.M., Gander, B., and Johansen, P. **Administration routes affect the quality of immune responses: a cross-sectional evaluation of particulate antigen-delivery systems**. *J. Control. Release* (2010), in press.
5. Slütter B and Jiskoot W. **Dual role of CpG as immune modulator and physical crosslinker in ovalbumin loaded N-trimethyl chitosan (TMC) nanoparticles for nasal vaccination.** J Contr Release J. Control. Release 2010 148: 117-121
6. Slütter B, Keijzer C, Bal S, Que I, Kaijzel E, Löwik, Mallants R, Augustijns P, Broere F, van Eden W, Jiskoot W. **Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: Nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen**. (Vaccine, 2010 28:6282–91
7. Hagenaars N, Mania M, de Jong P, Que I, Nieuwland R, Slütter B, Glansbeek H, Heldens J, van den Bosch H, Löwik C, Kaijzel E, Mastrobattista E, Jiskoot W. **Role of trimethylated chitosan (TMC) in nasal residence time, local distribution and toxicity of an intranasal influenza vaccine.** J Control Release. 2010 144(1):17-24
8. Slütter B, Soema PC, Ding Z, Verheul R, Hennink W, Jiskoot W. **Conjugation of ovalbumin to trimethyl chitosan improves immunogenicity of the antigen.** J Control Release. 2010 Apr 19;143(2):207-14.
9. Bal SM, Slütter B, van Riet E, Kruithof AC, Ding Z, Kersten GF, Jiskoot W, Bouwstra JA. **Efficient induction of immune responses through intradermal vaccination with N-trimethyl chitosan containing antigen formulations.** J Control Release. 2010 Mar 19;142(3):374-83.
10. Slütter B*, Plapied L*, Fievez V, des Rieux A, Schneider Y, van Riet E, Jiskoot W, Préat V. **Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination.** J Control Release. 2009 Sep 1;138(2):113-21

11. Slütter B*, Hagenaars N*, Jiskoot W. **Rational design of nasal vaccines.** J Drug Target. 2008 Jan;16(1):1-17.
12. Kel JM, Slütter B, Drijfhout JW, Koning F, Nagelkerken L. **Mannosylated self-peptide inhibits the development of experimental autoimmune encephalomyelitis via expansion of nonencephalitogenic T cells**. J Leukoc Biol. 2008 Jul;84(1):182-90.

Curriculum Vitae

Bram Slütter was born on February 8th 1983 in Warnsveld, The Netherlands. After his graduation from high school in 2001 (Staring College, Lochem), he studied Bio-Pharmaceutical Science at the University of Leiden, The Netherlands. Before receiving his master degree in 2006, he did 2 research internships; in 2005 at the Division of Biopharmaceutics of the Leiden Amsterdam Center for Drug Research (LACDR) on mast cell degranulation and blood coagulation under the supervision of Saskia de Jager and Eric Biessen, and in 2006 at TNO Leiden on a project concerning T-cell responses in experimental autoimmune encephalitis, under supervision of Junda Kel and Lex Nagelkerke. In October 2006 Bram started his PhD project on "Nasal vaccine delivery: alternatives for conventional multiple injection vaccines" led by Prof. Jiskoot and Prof. Bouwstra at the Division of Drug Delivery Technology of the LACDR. This PhD project was performed within the framework of Top Institute Pharma project number D5-106, a collaboration between Leiden University, Utrecht University, Vrije Universiteit Amsterdam and the Netherlands Vaccine Institute. In 2007 he worked as visiting scientist at the lab of Prof Veronique Préat in Brussels for 4 months. From November 2010 he works as postdoctoral reseacher with Prof. Harty at the University of Iowa (Iowa City, IA).