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

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Rational design of nasal vaccines

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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.

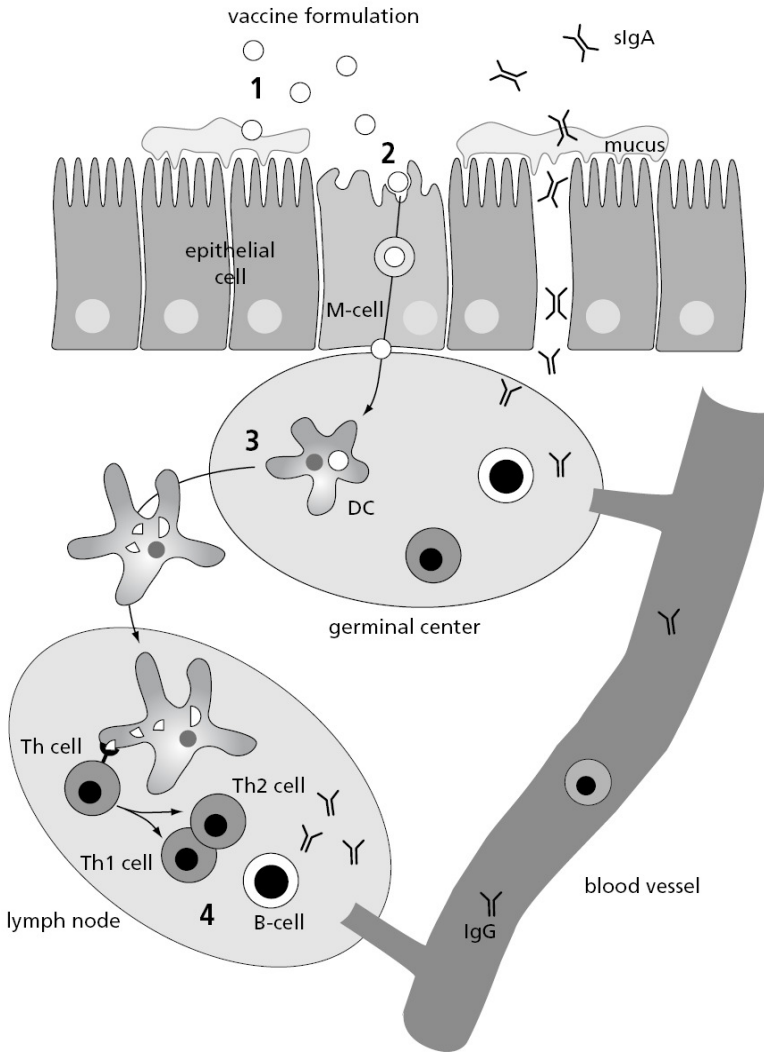

***Figure 1**: Schematic overview of the consecutive steps towards successful nasal vaccine delivery: 1) mucoadhesion; 2) antigen uptake, by M-cell transport; 3) delivery to and subsequent activation/maturation of DC; 4) induction of B-cell and T-cell responses. DC = dendritic cell, M-cell = microfold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

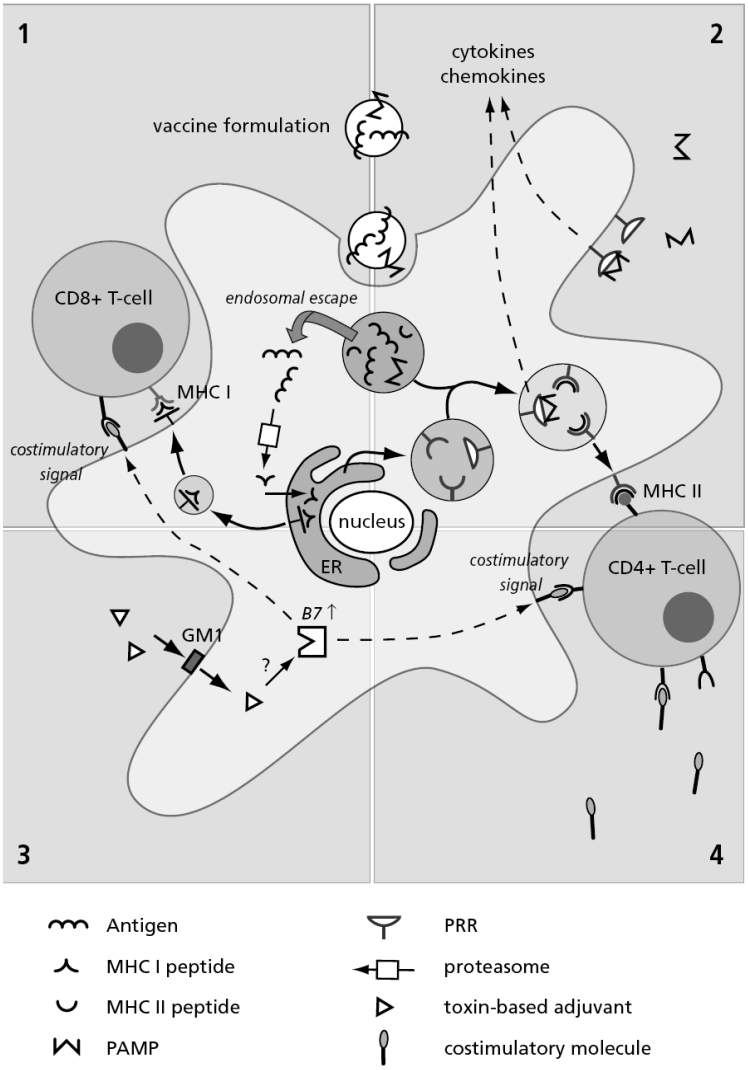

***Figure 2**:*
Various mechanisms a vaccine formulation can exploit to induce the desired immune response. 1) cytosolic delivery for targeting the antigen to MHC class I presentation; 2) targeting the innate immune system through pathogen recognition receptors (PRR); 3) the use of toxin-based adjuvants;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

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