Nanoparticles for Nasal Delivery of Vaccines

Monitoring Adaptive Immune Responses

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Nanopartikels voor nasale vaccins: Monitoren van adaptieve immuunresponsen
(met een samenvatting in het Nederlands)

Proefschrift

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General Introduction

NASAL VACCINATION

The continuous emergence of new pathogens and growing drug resistance of microorganisms asks for innovative vaccination strategies. An alternative to conventional multiple injection vaccines is the mucosal route of vaccine delivery. Mucosal vaccine development has grown extensively and a multitude of vaccine delivery systems have been developed for application via ocular[1,2], nasal[3], oral[4], rectal[5] and the vaginal route[6]. Of all the different routes of mucosal vaccination, the oral and nasal route are most accepted and easily accessible. Moreover, the nasal route of vaccination might be preferred over other mucosal delivery sites, due to the low proteolytic activity in the nasal mucosa that provides better antigen stability and because it requires a lower dose of antigen, which might also reduce the chance of producing negative side effects[7]. Similar to other forms of mucosal vaccination, nasal antigen administration can stimulate both antigen-specific systemic and mucosal adaptive immune responses[8,9]. Nasal administration of innocuous antigens like environmental allergens, food antigens or commensal bacteria derived antigens can induce a state of peripheral immunological tolerance[10]. In contrast, nasal delivery of pathogen-derived antigens favor the induction of an active inflammatory response directed against the pathogen that causes disease[11]. Since nasal antigen application can accomplish both, nasal vaccination is described for the prevention of infectious diseases such as influenza[12,13] and hepatitis B[14,15] and is also under development for treatment of autoimmune diseases and other chronic inflammatory disorders[16-18] and allergies[19]. Although nasal antigen delivery might be a cost effective innovative vaccination strategy, the physiology of the nasal cavity, including mucociliary clearance and the inefficient uptake of soluble antigens by nasal epithelial cells limits the efficacy of nasal vaccines. Therefore, nasal vaccines often require potent adjuvants and vaccine delivery systems that enhance their immunogenicity and delivery to the mucosal tissues.

NASAL MUCOSAL IMMUNE SYSTEM

Mucosal surfaces of the respiratory system, including the nasal cavity, are covered by epithelia of one cell-layer thickness. The nasopharynx-associated lymphoid tissue (NALT) consists of organized lymphoid follicles that occur directly beneath the mucosal follicle associated epithelial (FAE) cells and therefore do not have afferent lymphatics. However, the cervical lymph nodes (CLN) drain the nasopharyngeal mucosa and the activated adaptive immune cells of the NALT via the draining efferent lymphatics surrounding the NALT follicles[20]. The NALT is considered to be the equivalent of Waldeyer's ring in humans, which includes the nasopharyngeal tonsil, tubal tonsil, palatine tonsil and lingual tonsils[21]. The NALT represents not only a target tissue in strategies of local defense against invading pathogens but is also a site for induction of systemic antigen-specific B-cell and T-cell responses following nasal vaccination. Antigens delivered via the nasal mucosa can be transported to the NALT through the FAE that contains specialized villous microfold (M)-cells[20]. M-cells are easily accessible

for antigens because these cells do not secrete mucus or digestive enzymes like epithelial tissue[22]. In addition, M-cells lack a brush border and this facilitates the binding and delivery of pathogens and particles by transcytosis to the sub-epithelial lymphoid tissue of the NALT[23-25]. Although the exact route of nasal antigen uptake and presentation remains unclear it is most likely that once the antigen, either soluble or particulated, has entered the sub-epithelial region of the NALT, it will encounter professional antigen presenting cells (APCs) such as dendritic cells (DCs) that mediate adaptive immune responses[20].

MUCOSAL DENDRITIC CELLS

DCs play an important role in the regulation of the adaptive immune responses and were first described by Ralph Steinman[26]. DCs are derived from hematopoietic stem cells and can originate from both lymphoid and myeloid lineages. Tissue resident immature DCs continuously sample their environment for antigens. During the migration of antigen-loaded DCs to the draining lymph nodes, DCs undergo functional maturation as they process and present small peptide fragments on their cell surface via major histocompatibility complex (MHC) molecules along with appropriate costimulatory molecules to activate naive T-cells, in the T-cell areas of lymphoid tissues[27].
DCs at mucosal surfaces represent a first line of immune recognition between the body and environmental pathogens and antigens. Mucosal DCs can induce protective immunity to infectious antigens or they induce regulatory responses to innocuous antigens[28]. Several subsets of mucosal DCs have been described that display unique functions that are not shared by DCs from non-mucosal tissues, suggesting that the function of DCs depends also on the tissue microenvironment[29-31].

RETINOIC ACID AS A MUCOSA-ASSOCIATED CO-FACTOR FOR LYMPHOCYTE DIFFERENTIATION

Dietary vitamin A, in the form of retinyl esters, is constantly hydrolyzed into retinol and deployed from the liver into circulation. Mucosal APCs, epithelial cells and stromal cells can express alcohol dehydrogenases (ADH) that oxidize retinol into retinal. Retinal binds to retinaldehyde dehydrogenases (RALDH) for oxidation into retinoic acid (RA). The vitamin A metabolite RA has the potential to elicit a wide range of adaptive immune responses and plays a role in the induction of mucosal tolerance. In more detail, RA produced by mucosal DCs acts on naive T- and B-lymphocytes and induces the expression of mucosal homing receptors α4β7-integrin and CCR9. The function of RA depends largely on the microenvironment especially the cytokine milieu. For example, RA in the presence of TGF-β mediates the conversion of naive T-cells into forkhead box P3 (FoxP3) expressing regulatory T-cells (Tregs), while RA alone at high concentration inhibits the differentiation of Th17 cells. Moreover, RA together with IL-5 and IL-6 cytokines can promote class switching to IgA in B-cells[32,33], figure 1.

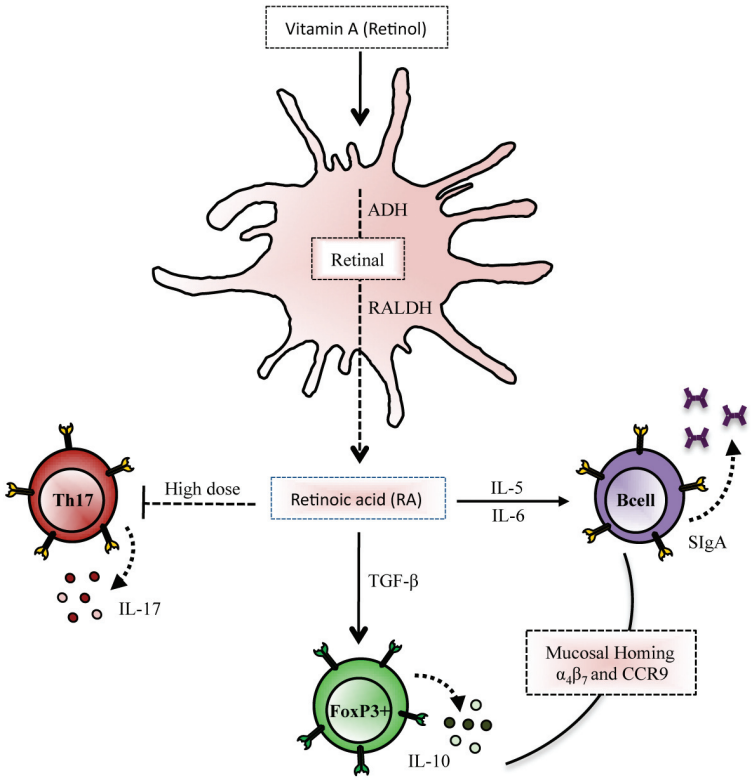

Figure 1 | Retinoic acid production and lymphocyte differentiation. Vitamin A (retinol) can be taken up by mucosal APCs, most likely dendritic cells, which express alcohol dehydrogenases (ADH) that oxidizes retinol into retinal. Retinal then binds to retinaldehyde dehydrogenases (RALDH) for oxidation into retinoic acid (RA). RA together with TGF-β mediates the differentiation of naive T-cells into FoxP3 expressing regulatory T-cells that often secrete the anti-inflammatory cytokine IL-10. RA in the presence of IL-5 and/or IL-6 can promote class switching of IgA in B-cells that differentiate into secretory IgA (SIgA) producing plasmacells, especially in mucosal tissues, including the NALT. RA also induces the expression of the mucosal homing receptors α4β7-integrin and CCR9 on both activated T-cells and B-cells. Finally, a relatively high dose of RA can suppress the differentiation of pro-inflammatory Th17 cells.

ADAPTIVE IMMUNE RESPONSES

$CD4^+$ T-helper cells

Naive $CD4^+$ T-cells express T-cell receptors (TCR) that recognize a specific antigenic peptide and undergo clonal expansion in the draining lymph nodes. Antigen-specific $CD4^+$ T-cells can differentiate into different subsets such as Th1, Th2, Th17 or Tregs depending on the cytokines present in the local microenvironment and direct interaction with the DCs[34]. Th1 cells express T-bet, a T-box transcription factor, that controls the expression of the Th1-cytokine IFN-γ[35] that mediates pro-inflammatory immune responses to protect the host from intracellular infection[36]. In contrast to T-bet, gata binding protein 3 transcription factor (GATA-3) is the transcription factor that induces Th2 cells that can

produce IL-4, IL-5, IL-10 and IL-13 cytokines[37,38]. Th2 cells are important for antibody-mediated immunity against extracellular pathogens. After nasal vaccination, Th2 cells can stimulate B-cells to produce systemic IgG antibodies and secretory IgA (SIgA) at mucosal sites[13,39]. Beside Th1 and Th2 cells there is another T helper cell subset termed Th17 cells that express the transcription factor orphan nuclear receptor RORγt transcription factor (RORγt)[40]. These Th17 cells produce IL-17a, IL-17f, IL-21 and IL-22 cytokines and protect the host from extracellular bacterial infection[41] and have been associated with nasal vaccination[42].

Regulatory T-cells (Tregs)

In general, the induction of Tregs after mucosal vaccination is the hallmark for mucosal tolerance induction. The forkhead transcription factor, FoxP3, can control both induced and natural Treg cell development and function and these $FoxP3^+$ Tregs can secrete the anti-inflammatory cytokine IL-10[43]. Natural $CD4^+$ Tregs arise in the thymus after ligation of high-affinity T-cell receptors and they account for approximately 5-10% of total peripheral $CD4^+$ T-cells. The cytokine TGF-β plays a crucial role in the induction of FoxP3 in developing thymocytes[44]. However, Tregs can also be induced or converted from effector T-cells during peripheral inflammation or by therapeutic interventions. Regulation of ongoing chronic inflammation in experimental arthritis[45] can be achieved by the induction of $FoxP3^+$ Tregs after nasal vaccination.

iTR35 Tregs are induced by the cytokine IL-35 and secrete IL-35, but not IL-10 or TGF-β. Moreover, iTR35 Tregs do not require FoxP3 expression for their development[46]. Whether IL-35 secreting Tregs are induced after nasal antigen application and can induce antigen-specific mucosal tolerance is unclear.

The differentiation of type 1 Tregs (Tr1) is induced by IFN-α and IL-10. Tr1 cells secrete TGF-β and are characterized by the secretion of high levels of IL-10. Tr1 cells can suppress naive and memory Th1 and Th2 responses[47]. Nasal administration of an anti-CD3 monoclonal antibody induces suppressive Tr1 cells that can control systemic autoimmunity[48].

T helper 3 (Th3) cells produce high levels of TGF-β that drives the induction of FoxP3 in activated T-cells to become induced Tregs[44] and promotes class switch to anti-inflammatory IgA and can inhibit Th1 and Th2 cells[49]. Th3 cells play a role in mucosal immunity. A role for Th3 cells in nasal tolerance induction against experimental myastenia gravis has been suggested by Shi *et al*[50].

γδ-T-cells, NKT cells and CTLs

Nasal mucosal γδ-T-cells that express the γδ-TCR play a role in airway diseases such as allergic and infectious rhinitis[51]. Natural Killer T (NKT) cells express NK cell markers such as CD161 and CD94 and unlike NK cells they also express an invariant TCR that can recognize glycolipids that are presented by the CD1d molecules. After activation, NKT cells rapidly secrete high amounts of IL-4 or IFN-γ. NKT cells play a role in autoimmune diseases, allergy, cancer immunity and in infectious diseases[52,53]. Although the NKT cell

frequency at the nasal mucosa is low, these cells have been shown to enhance protective immune responses against different strains of influenza virus[54].
$CD8^+$ T-cells also termed cytotoxic T lymphocytes (CTL) recognize infected cells that present virus-derived peptides on MHC class I molecules. CTLs produce IFN-γ that inhibits viral replication and they contain intracellular granulae that consist of perforin and granzyme that are released immediately after activation to rapidly induce apoptotic DNA fragmentation in the virus-infected cells and contribute to cross-protection against viruses of different subtypes[55]. $CD8^+$ T-cells have been described to protect the host in nasal vaccination studies via the induction of anti-viral immune responses[56]. In addition, $CD8^+$ T-cells can also have a regulatory phenotype and mediate mucosal tolerance induction after nasal antigen administration[57].

HUMORAL IMMUNE RESPONSES

The second type of immune cells that mediate adaptive immunity are the B-lymphocytes. B-cells develop from pluripotent stem cells in the bone marrow. Immature naive B-cells that express a mature B-cell receptor (BCR) leave the bone marrow and will continue to undergo maturation in peripheral secondary lymphoid tissues such as the spleen to form long-lived follicular B-cells, or marginal-zone B-cells. B-cells that express the mature BCR on their cell surface are able to bind a specific antigen. Like DCs, antigen-binding B-cells are located in the T-cell zone of the secondary lymphoid organs and can process and present antigens on MHC class II molecules to activate antigen-specific $CD4^+$ cells. Antigen recognition induces expression of the B-cell stimulatory molecule CD40 ligand (CD40L) on their cell surface and secretion of B-cell stimulatory cytokines, which drive the proliferation and differentiation of B-cells into antibody secreting plasmacells or into memory B-cells[58].
Antibodies contribute to immunity via neutralization, opsonization and complement activation. Neutralization by antibodies can limit tissue damage when they bind to a pathogen or a bacterial toxin to prevent them from entering target cells. Opsonization occurs when antibodies bind to bacteria that multiply outside cells. Opsonization can result in two different types of immune activation. The antigen:antibody immunecomplex can be recognized by phagocytes that express Fc receptors on their cellsurface, such as macrophages that engulf the antigen:antibody complex. Opsonization can also activate the classical pathway of the complement system that finally results in C3 conversion into C3a and C5a that are both peptide mediators of local inflammation and the recruitment of inflammatory cells and phagocytes[59].

Effector functions of IgG immunoglobulins
IgG is the main antibody isotype detected in blood and extracellular fluid and its main function is to control infection of body tissues. There are four IgG subclasses (IgG1, IgG2, IgG3, and IgG4) in humans. IgG1 and IgG2 are most abundantly present. Class switching

is induced or inhibited by different cytokines; however, much of the inhibitory effect might also be a result of directed switching to a different isotype. The Th2 cytokine IL-4 is responsible for class switching to IgG1, while it inhibits the production of IgG2, IgG3 and IgM[60]. In contrast, the Th1 cytokine IFN-γ mediates class switching to IgG2a and inhibits the production of IgG1, IgG2b, IgG3 and IgE[60,61]. The cytokine TGF-β1 can induce class switching to IgG2b in mice and blocks switching to most other IgG isotypes[62]. The induction of antigen-specific systemic IgG antibody responses has been described after nasal vaccination[12,63].

Effector function of secretory IgA (SIgA)

Mucosal effector sites provide local SIgA antibodies. Th2 cells that secrete IL-5 help mucosal B-cells to enhance class switching to IgA[39]. IgA-isotype class switching was found to occur in the NALT[64]. Most antigen-specific IgA^+ mucosal plasmacells produce dimeric IgA or polymeric IgA (PIgA). PIgA is released from the nearby activated mucosal plasmacells and binds to the polymeric immunoglobulin receptor (PIgR) that is expressed on the basolateral surface of mucosal epithelial cells. The PIgR carries PIgA to the apical surface of the mucosal epithelial cells via endocytosis and transcytosis. At the apical surface, the ligand-binding portion of the PIgR is proteolytically cleaved and released together with PIgA into apical secretions also known as SIgA[65]. SIgA released into the nasal passage is displayed on the monolayer of epithelial cells that line the NALT mucosa and form a first barrier of defence against invading antigens. Via a system called the common mucosal immune system, it becomes possible to induce SIgA antibody responses at peripheral mucosal inductive sites other than the original mucosal route of vaccination[20,66].

NANOPARTICLES AND ADJUVANTS FOR NASAL DELIVERY OF VACCINES

The immune response induced following nasal antigen delivery depends on many factors, such as the nature of the antigen (immune suppressive or immunogenic), the formulation (soluble versus particulate), antigen size, dose and frequency of administration[7]. Different kinds of vaccine delivery particles and adjuvants are available and under development to enhance the delivery and the immunogenicity of nasal vaccines.

Adjuvants

The discovery of new vaccine candidates against infectious, allergic and autoimmune diseases has emerged over the past years. However, these vaccines often require adjuvants to enhance the immunogenicity of the vaccine antigens. The adjuvant that is selected strongly depends on the immune response that is required to clear infection or enhance mucosal tolerance induction. Therefore, vaccine formulations should be constructed in such a way that optimal immune responses with the minimal side effects, are obtained. Adjuvants that are used for nasal delivery of vaccines can potentiate immune responses.

Depending on their nature, adjuvants can be divided into different categories, including the toxin-based adjuvants cholera toxin (CT) from *Vibrio cholera* and *Escherichia coli* heat-labile enterotoxin (LT)[67,68], pathogen-associated molecular patterns such as Toll-like receptor (TLR) ligands[69,70], cytokines including IL-1[71], IL-12[72] and type 1 IFN[73], the costimulatory molecules CD28, CD40, CD134 and CD137[74] and synthetic adjuvants like N-trimethyl chitosan (TMC).[12]. The type of immune response that adjuvants elicit depends on their specific physicochemical characteristics. They can for example enhance the uptake and presentation of the antigen by targeting specific immune cells such as M-cells and DCs and/or trigger specific immune receptors to elicit active inflammatory immune responses or induce a state of immunological tolerance.

Mucosal vaccine delivery particles

Since most infections and environmental allergies are acquired through the mucosal membranes, the interest in mucosal vaccine design has grown extensively. A multitude of vaccine delivery particles are currently available or under development and can be used to enhance the immunogenicity and the delivery of nasal vaccines including, bacterium-like particles (BLPs)[13,63,75], lipid-based vaccines including liposomes[76], immune stimulatory complexes (ISCOMs)[77] and virosomes[78], nanoparticles[11] and virus-like particles (VLPs)[79]. An advantage of vaccine delivery particles compared to soluble adjuvants is that the target antigen can be encapsulated by the particle and is protected against enzymatic degradation. The route of vaccination depends mainly on the location of the affected tissue, whereas the vaccine characteristics depend on the immune responses that are required. Immune responses can be divided into two categories, defence responses against invading pathogens and tolerance induction to innocuous antigens. Delivery particle systems can be modulated to become mucoadhesive to enhance the uptake of antigens by nasal epithelial cells and/or M-cells. In addition, targeting M-cells may improve the uptake and transport of a target antigen to the sub-epithelial region of the NALT and the subsequent secondary lymphoid organs including the deep and superficial located CLN[80]. Vaccine delivery particles that have a positive surface charge can enhance the interaction with the negatively charged cell membranes of epithelial cells and DCs, and thereby prolong the nasal retention time in the nasal cavity and enhance subsequent antigen uptake by DCs, due to favourable electrostatic interactions[81-82].

OBJECTIVE AND THESIS OUTLINE

The possibilities to modify vaccine delivery particles to enhance the immunogenicity of nasal vaccines seem tremendous. The research described in this thesis aims to unravel adaptive immune responses induced after nasal administration of several particle based vaccines that can either induce protective immunity or tolerance (figure 2). Previous studies showed that non-living BLPs that are derived from the food grade bacterium *Lactococcus lactis* are effective stimulators of local and systemic immune responses when administered

intranasally[13,63]. Moreover, *in vitro*, BLPs specifically interact with human TLR2, suggestive of a role for TLR2 dependent immune activation by BLPs[75].
In **Chapter 2**, we examined the role of TLR2 *in vivo* in BLP-dependent activation of local and systemic virus-specific immune responses after nasal administration of BLP mixed with split influenza vaccine in TLR2 knock-out mice.
In addition to BLP particles, we also investigated the effects of the synthetic poly-lactic-co-glycolic-acid (PLGA), poly-lactic-co-glycolic-acid N-trimethyl chitosan (PLGA-TMC) and N-trimethyl chitosan tripolyphosphate (TMC-TPP) nanoparticles. There is at the moment very limited knowledge about the capacity of nanoparticles to modulate the immunological outcome after nasal vaccination. **Chapter 3** describes the mechanism behind nasal nanoparticle vaccination, the characteristics of three nasal vaccine delivery particles composed of PLGA, PLGA-TMC and TMC-TPP are correlated to their capacity to induce antibody production.
Chapter 4 continues with the characteristics of the PLGA, PLGA-TMC and TMC-TPP nanoparticles and their capacity to activate $CD4^+$ T-cells locally and systemically. We also investigated the capacity of PLGA, PLGA-TMC and TMC-TPP nanoparticles to differentially regulate the immune response by characterizing $CD4^+$ T-cell differentiation and the ability to induce mucosal tolerance.
In **Chapter 5** we explored *in vitro* if PLGA nanoparticles compared to TMC-TPP nanoparticles trigger DCs to acquire RA producing capacity to induce $FoxP3^+$ $CD4^+$ T-cells via a TGF-β and RA dependent mechanism.
In **Chapter 6**, the role of heat shock proteins as therapeutic targets in autoimmune diseases and other chronic inflammatory conditions is reviewed.
Finally in **Chapter 7**, the findings described in this thesis are summarized and discussed in the context of recent developments in the research on mucosal applied vaccine delivery particles and adjuvants. Potential future research directions are indicated, which may resolve unanswered questions concerning the application of nasal vaccines. This will give further insight in the mechanism of how nanoparticles and adjuvants operate and how they can be used to modulate mucosal immune responses.

GRAPHICAL ABSTRACT

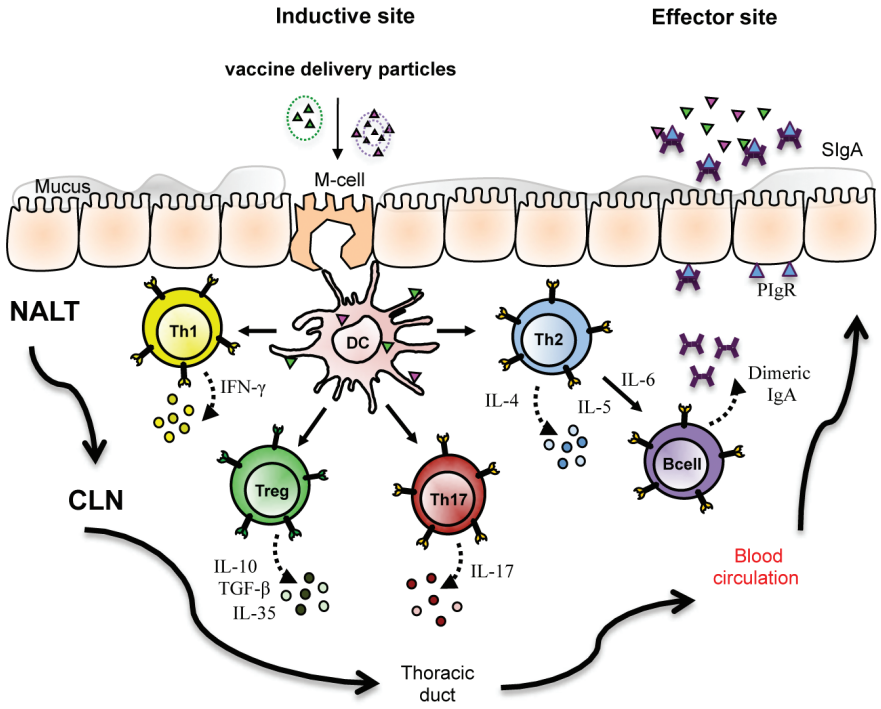

Figure 2 | Nanoparticles for nasal delivery of vaccines: induction of adaptive immune responses. After nasal administration, antigen-based delivery particles are transported to the nasopharynx-associated lymphoid tissue (NALT), through the follicular associated epithelial (FAE) cells that contain specialized villous microfold (M)-cells. Dendritic cells (DCs) process and present antigens to naive T-cells in the lymphoid tissues. T-cells that bear a T-cell receptor (TCR) that is specific for the presented peptide will undergo clonal expansion locally in the draining lymph nodes. Antigen-specific $CD4^+$ T-cells can differentiate into Th1, Th2, Th17 or regulatory T-cell subsets. Th2 cells activated at mucosal sites that secrete IL-5 help mucosal B-cells to enhance class switching to IgA. The IgA^+ B-cells rapidly migrate from the NALT to the draining cervical lymph nodes (CLN), through the efferent lymphatics. Finally, antigen-specific $CD4^+$ T-cells and IgA^+ B-cells migrate to effector sites, through the thoracic duct and blood circulation. IgA^+ B-cells then differentiate into IgA-producing plasmacells in the presence of cytokines such as IL-5 and IL-6 that are produced by Th2 cells and produce dimeric IgA or polymeric IgA (PIgA). PIgA is released from the nearby activated mucosal plasmacells and binds to the polymeric immunoglobulin receptor (PIgR) that is expressed on the basolateral surface of mucosal epithelial cells. The PIgR carries PIgA to the apical surface of the mucosal epithelial cells via endocytosis and transcytosis. SIgA is released into the nasal passage and displayed on the monolayer of epithelial cells that line the NALT mucosa and form a first barrier of defence against invading antigens.

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2

Inactivated influenza vaccine adjuvanted with bacterium-like particles induce systemic and mucosal influenza A virus specific T-cell and B-cell responses after nasal administration in a TLR2 dependent fashion

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ABSTRACT

Nasal vaccination is considered to be a promising alternative for parenteral vaccination against influenza virus as it is non-invasive and offers the opportunity to elicit strong antigen-specific responses both systemic and locally at the portal of entry of the pathogen. Previous studies showed that non-living bacterium-like particles (BLPs) from the food grade bacterium *Lactococcus lactis* are effective stimulators of local and systemic immune responses when administered intranasally. Moreover, *in vitro*, BLPs specifically interact with human Toll-like receptor 2 (TLR2), suggestive of a role for TLR2 dependent immune activation by BLPs. In the present study, we examined the role of TLR2 *in vivo* in immune activation after nasal administration of BLP mixed with split influenza vaccine (BLP-SV) of influenza A virus (IAV) using TLR2 knockout mice. The systemic Th1 cell and subsequent B-cell responses induced after intranasal BLP-SV vaccination depended on the interaction of BLPs with TLR2. Interestingly, the BLP-SV-induced class switch to IgG2c depended on the interaction of BLP with TLR2. Local induced IAV-specific Th1 cell responses and the mucosal B-cell responses also depended on interaction of BLP with TLR2. Strongly reduced SIgA levels were observed in TLR2 knockout mice both in the nasal and vaginal lavages. Moreover, detailed analysis of the T-cell response revealed that nasal BLP-SV vaccination promoted Th1/Th17 skewing that coincided with increased IAV-specific IgG2c antibody production. Altogether these results indicate that nasal BLP-SV vaccination induces IAV-specific T-cell and B-cell responses both systemically and at the site of virus entry in a TLR2-dependent fashion.

INTRODUCTION

Infection with influenza A virus (IAV) causes a contagious disease that affects mainly the upper respiratory tract and is still one of the leading causes of mortality and morbidity worldwide.[1,2] Most vaccines against influenza A and B in use today are administered via the parenteral route. Although these vaccines can induce virus-specific systemic immune responses, they barely activate the mucosal immune system, the port of entry of the influenza viruses.[3,4] Nasal vaccination therefore might be a promising alternative for parenteral vaccination against influenza virus, since this route of vaccination resembles more closely natural infection and it is known to elicit both systemic and mucosal immune responses.[4,5] In addition, nasal vaccination might enhance vaccine efficacy in contrast to parenteral vaccination since nasal vaccination is associated with secreted IgA (SIgA) antibody production at the mucosal surfaces.[5-7] Because SIgA forms a first line of defence against invading pathogens at the portal of entry,[8-10] it may help to prevent penetration and replication of influenza virus in the respiratory tract mucosa early after host cell invasion.

Clearly, the nasal route of vaccination is attractive in the protection against respiratory infections, however, effective stimulation of the nasal mucosal immune system with non-replicating vaccine antigens usually requires the use of an antigen carrier system and/or adjuvant.[11,12] However, this route of immunization is associated with the occurrence of facial nerve paralysis (Bell's Palsy) as a result of the use of *Escherichia coli* heat-labile toxin (LT) or mutants thereof, as adjuvant. Clearly, the use of toxins or toxoids should be avoided as nasal adjuvant. An example of a recently developed nasal immunostimulatory system is the bacterium-like particle (BLP) derived from the food-grade bacterium *Lactococcus lactis*.[13,14] BLPs are obtained by an acid pre-treatment, which degrades all cellular components, including DNA and proteins but leaves the peptidoglycan shell intact. The result is a non-living particle that still has the shape and size of an untreated bacterium. The procedure is applicable to all Gram-positives, hence the name that was formerly used: Gram-positive Enhancer Matrix (GEM).[13,14] Because of their safe use and adjuvant activity,[15,16] BLPs are an attractive adjuvant candidate for the development of nasal influenza vaccines.

Previously, we showed that intranasal (i.n.) immunization with influenza monovalent subunit vaccine of strain A/Wisconsin (H3N2) mixed with BLPs strongly potentiate immunogenicity of influenza subunit vaccine resulting in both local and systemic immune responses.[15,16] *In vitro* studies using a panel of human Toll-like receptors (TLRs) expressed in HEK293 cells suggests that BLPs have the capacity to mediate TLR2 signaling. Also, TLR2-specific blocking antibodies reduced the BLP-induced IL6 production by murine $CD11c^+$ DCs *in vitro*.[17] However, it is currently unclear if TLR2 activation via BLPs is fully responsible for the enhanced activation of the adaptive immune system *in vivo* as measured by T-cell and B-cell activation. First of all, TLR2 can form heterodimers with other TLRs, specifically TLR1 and TLR6.[18,19] Especially TLR2/TLR1 dimers were shown important in the induction of a protective mucosal Th17 immune response *in vivo*, whereas TLR2/TLR6 heterodimers were not.[20] In addition, TLR2 is expressed on the surface of a large number of immune cells including macrophages,[21] monocytes and dendritic cells,[22]

M cells,[23] B cells,[24] and T cells,[25] including regulatory T-cells [26] capable of differentially regulating the immune response. Although there is ample evidence that vaccination with BLP adjuvanted vaccines induces protective immunity, it remains to be proven whether TLR2 mediated effects are responsible for the observed activation of the adaptive immune response *in vivo*.

To address the proposed role of TLR2 *in vivo* in the BLP-dependent activation of the adaptive immune system we explored the local and systemic influenza A virus specific T-cell and B-cell responses in TLR2 knockout (TLR2KO) and wild-type (wt) control mice after i.n. administration of BLPs supplemented with inactivated influenza vaccine.

MATERIALS AND METHODS

Mice

Female BALB/c wild-type mice (6-8 weeks) were purchased from Harlan Laboratories, Zeist, The Netherlands. C57BL6/J and B6.129-Tlr2$^{tm1Kir/J}$ mice (6-8 weeks) were purchased from Jackson Laboratories, France. All mice were kept under standard housing conditions at the University of Groningen, The Netherlands. Animal experiments were evaluated and approved by the Committee for Animal Experimentation of the University of Groningen, The Netherlands, according to the guidelines provided by Dutch Animal Protection Act.

Preparation of BLP-SV

Influenza monovalent split vaccines of strain A/Beijing/262/95 (H1N1) and A/Sydney/5/97 (H3N2) were purchased from AdImmune Corp, Taiwan (egg derived, formalin inactivated). The concentration of the haemagglutinin (HA) in the vaccine was determined using the single radial immunodiffusion assay. The standard BLP-SV vaccines consisted of influenza monovalent SV containing 5 µg HA antigen mixed with BLPs (0.15 mg dry-weight). BLPs were prepared as described before.[13,14] BLPs were stored at -80°C until use. BLPs and SV, were mixed just prior to i.n. administration. All i.n. vaccine doses were delivered in a final volume of 10 µl of PBS.

Immunization

Mice to be i.n. immunized were lightly anaesthetised with 2.5% v/v isofluorane over oxygen (0.8 L/min). Once anaesthetised, the mice were vaccinated i.n. every 10 days with 10 µl of sterile PBS containing BLP-SV (BLPs mixed with the influenza A strain (A/Beijing/262/95 (H1N1)) or SV alone and sacrificed at day 34 of the experiment. Mice were vaccinated i.n. 3 times on day 0, 14 and 28 with 10 µl of sterile PBS containing BLP-SV (BLPs mixed with the influenza A strain (A/Sydney/5/97(H3N2)) or SV alone and sacrificed at day 42 of the experiment. SV without BLPs was administered i.m. in 50 µl of PBS as a positive control for the immunogenicity of the antigenic materials.

Serum collection, nasal, lung and vaginal lavages

Blood was collected via punction from the orbital plexus for antibody measurements and the mice were sacrificed on day 34 or 42 via exsanguination by heart puncture under O_2/isofluran anaesthesia. Subsequently, nasal, lung and vaginal washes were conducted for SIgA antibody measurements. For nasal and lung lavages, 1 ml PBS that contained Roche "complete" protease inhibitor (according to manufacturer's description) was used. The tube containing the lavage fluid was placed on ice and centrifuged at 300-400 x g for 5 min at 4°C and supernatants were collected. Vaginal lavages were conducted by repeated pipetting of 0.2 mL of PBS supplemented with Roche "complete" protease inhibitor. All lavage samples were stored at -20°C.

IAV-specific IgG, IgG1, IgG2c and SIgA ELISA

ELISA was performed as previously described.[27] Briefly, ELISA plates (Greiner, The Netherlands) were coated overnight at 4°C with influenza monovalent split vaccines of strain (A/Sidney/5/97 H3N2, AdImmune). The plates were washed twice and blocked in 200 µl of a 2.5% solution of Protifar Plus (Nutricia™) in coating buffer (0.05 M carbonate-bicarbonate pH 9.6-9.8) for 45 min at 4°C and washed four times before samples were applied. Sera were applied in serial two-fold or triple-fold dilutions and a mouse control serum sample positive for A/Sidney/5/97 was included on each plate. For detection of SIgA, 100 µl of the lavage was used undiluted in the first well and subsequently serial two-fold diluted. The plates were incubated for 1.5 h at 4°C, washed 3 times and incubated for 1 h at 4°C with anti-mouse Ig-HRP conjugates (Southern Biotech). After incubation, the plates were washed 3-4 times and incubated for 30 min with 100 µl staining solution (1 tablet of OPD (o-Phenylenediamine dihydrochloride) dissolved in 100 ml 0.05 M phosphate-citrate buffer pH 5.0 and 40 µl H_2O_2). After incubation the reaction was stopped by adding 50 µl 2 M H_2SO_4 per sample and the absorbance was determined at 492 nm.

IAV-specific IFN-γ T-cell and B-cell ELISPOT

The IAV-specific IFN-γ T-cell and IAV-specific B-cell response in the spleen and local draining cervical lymph nodes (CLN) or inguinal lymph nodes (ILN) lymph nodes after i.n. BLP-SV or i.m. SV vaccination was assessed by ELISPOT. For detection of IAV-specific B-cells, cells were directly cultured in high protein binding filter plates (MultiScreen-IP, Millipore) that were pre-coated with Vaxigrip® suspension for injection: strains 2009/2010, Sanofi Pasteur MSD, lot: E7068 at 1 µg per well dissolved in 50 µl of PBS. For detection of IAV-specific IFN-γ T-cells, cells were cultured in the presence of HA antigen or IMDM (Gibco, Invitrogen) as a control that was supplemented with heat-inactivated 5% FCS (Bodinco, The Netherlands), $5x10^{-5}$ M 2-mercaptoethanol, penicillin (100 units/ml) and streptomycin (100 µg/ml) (Gibco, Germany) for 72 h at 37°C in high protein binding filter plates (MultiScreen-IP, Millipore) that were pre-coated with a rat-anti-mouse IFN-γ monoclonal antibody (clone AN-18, purchased at BD Biosciences, Pharmingen) at 0.1 µg per well dissolved in 50 µl of PBS for 48 h at 37°C.

After incubation, spot forming units of IAV-specific B- and T-cells were detected with goat-anti-mouse IgG-biotin (Sigma) and Avidin-AP (Sigma). Plates were developed with NBT-BCIP (Roche) and analyzed by using the Aelvis spotreader and software. Data are shown as IAV-specific IFN-γ T-cell or the IAV-specific B-cell count per 10^6 cells above background.

Luminex

Single cell suspensions were prepared from spleen and draining lymph nodes and cells were cultured for 72 h in the presence of ConA at 2.5 µg/ml or IMDM (Gibco, Invitrogen) at 37°C. Analyzing the culture supernatants assessed the amount of cytokine secreted during a 72 h T-cell re-stimulation. Briefly, fluoresceinated microbeads coated with capture antibodies for simultaneous detection of IL-17A (TC11-18H10) and IL-5 (TRFK5) were added to 50 µl of culture supernatant. Cytokines were detected by biotinylated antibodies IL17 (DuoSet ELISA kit, R&D systems Europe Ltd, the U.K.), IL-5 (TRFK5) and PE-labeled streptavidin (BD Biosciences Pharmingen). Fluorescence was measured using a Luminex model 100 XYP (Luminex, USA). Data are shown as the cytokine concentration above background in pg/ml.

Statistics

Statistical analysis was performed with Prism software (Graphpad Software Inc., San Diego, version 4.00). An unpaired two-tailed t-test was used in Figure 2. One-way ANOVA followed by a Bonferroni's multiple comparisons test was used in Figure 4C. One-way ANOVA followed by a Kruskal-Wallis test and Dunn's multiple comparison test was used in all other experiments.

RESULTS

To investigate the role of TLR2 in BLP-mediated local and systemic IAV-specific T-cell and B-cell activation, B6.129-Tlr2^{tm1Kir}/J mice (TLR2KO) and C57BL6/J (wt controls) were immunized i.n. with BLP-SV (A/Sidney/5/97, H3N2). As a control, wt mice were i.m. immunized with SV alone. Fourteen days after the last immunization, cells from the draining lymph nodes (dLN) and spleen were isolated and analyzed for IAV-specific IFN-γ producing cells and IAV-specific B-cells.

In the local dLN significantly reduced numbers of IAV-specific IFN-γ producing T-cells (Figure 1A) and lower numbers of IAV-specific B-cells (Figure 1B) were observed in TLR2KO mice compared to the number of cells in wt control mice. Similar to the observations made in the local dLN, also significantly lower numbers of IAV-specific IFN-γ producing T-cells (Figure 1C) and a slight reduction in IAV-specific B-cell numbers (Figure 1D) were observed in the spleen of TLR2KO mice compared to vaccinated wt mice. These data indicate that induction of IAV-specific IFN-γ T-cell and B-cell responses both

Table 1 | Local and systemic T-cell responses induced after vaccination

		IFN-γ producing cells (per 10^6 cells)	
Vaccine formulation	Route of administration	Spleen	dLN
BLP-SV	Intranasal	93 ± 12	70 ± 30
SV	Intramuscular	114 ± 30	20 ± 10

BALB/c mice were vaccinated three times with 10 µl of BLP-SV (A/Beijing/262/95) or SV (A/Beijing/262/95) in PBS, i.n. or i.m, respectively. dLNs and spleens were isolated and single cells were re-stimulated *ex vivo* with HA antigen for 48 h at 37°C in anti-IFN-γ pre-coated ELISPOT plates for IAV-specific T-cell detection. Data are shown as the IAV-specific IFN-γ T-cell count per 10^6 cells above background (spots counted on medium coated plates). Data are shown as the mean ± SEM with n=5 mice per group.

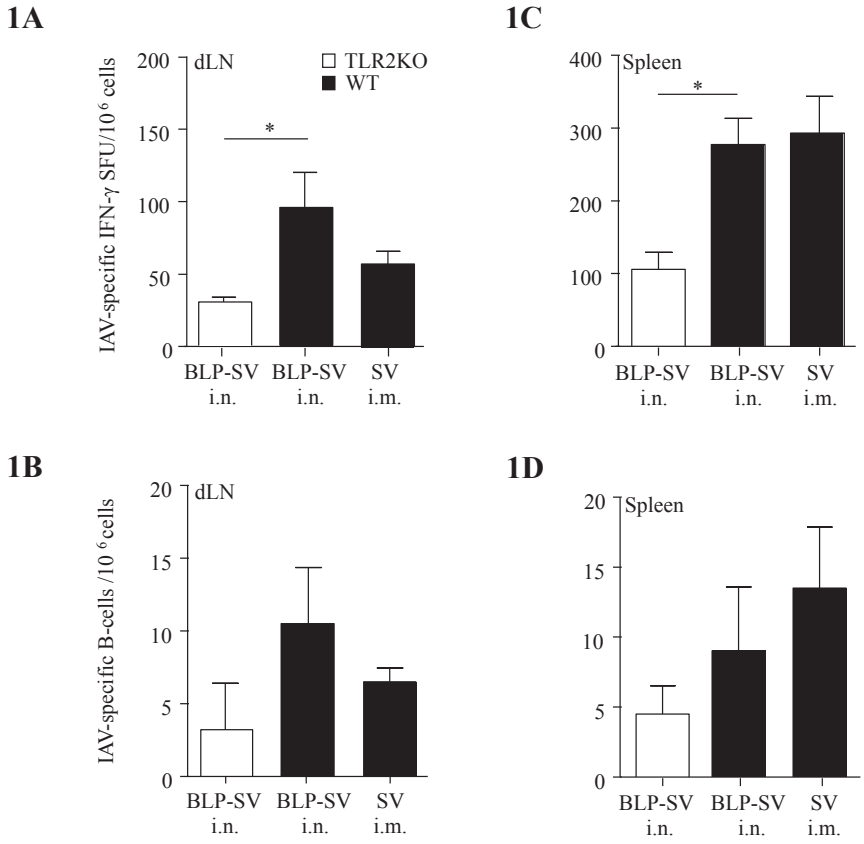

Figure 1 | Local and systemic T-cell and B-cell responses induced after i.n. BLP-SV vaccination are TLR2 dependent. C57BL6/J wt mice and TLR2KO mice were vaccinated three times with 10 µl of BLP-SV (A/Sydney/5/97) or SV (A/Sydney/5/97) in PBS, i.n. or i.m, respectively. Draining lymph nodes and spleens were isolated and single cells were re-stimulated *ex vivo* with HA antigen for 48 h at 37°C in anti-IFN-γ pre-coated ELISPOT plates for IAV-specific T-cell detection or directly cultured in Vaxigrip® pre-coated plates to detect IAV-specific B-cells. Data are shown as the IAV-specific IFN-γ T-cell count in dLN (**1A**) and spleen (**1C**) or as the IAV-specific B-cell count in dLN (**1B**) and spleen (**1D**) per 10^6 cells above background (spots counted on medium coated plates). Data are shown as the mean ± SEM with n=5 mice per group. Statistical significance; **P*<0.05.

in the local dLN and spleen requires interaction of BLP with TLR2.
The IAV-specific IFN-γ T-cell responses in the dLN of wt controls were slightly higher after i.n. BLP-SV immunization compared to the responses after i.m. immunization with SV alone although this did not reach statistical significance. The systemic IFN-γ T-cell response observed in spleen was similar after i.n. and i.m. immunization (Figure 1). Similar observations were made when BALB/c mice were immunized i.n. and i.m. with BLP-SV and SV, respectively (Table 1).

To investigate how i.n. BLP-SV vaccination affects systemic T-cell differentiation we analysed IL-5 and IL-17 production of activated splenocytes. After i.n. BLP-SV vaccination the enhanced IAV-specific IFN-γ T-cell responses coincided with a slightly increased production of IL-17A cytokine (Figure 2A) and significantly decreased secretion of IL-5 cytokine (Figure 2B) compared to SV i.m. vaccinated mice. Together these results indicate that the IAV-specific T-cell and B-cell responses induced after i.n. BLP-SV administration are TLR2 dependent and results in Th1/Th17 skewing.

Activation of B-cells in mucosa-associated lymphoid tissues is associated with production of SIgA at the mucosal surfaces.[8,9] To investigate whether the reduced IAV-specific B-cells in the dLN in TLR2KO mice affect the production of SIgA, nasal and peripheral vaginal lavages were analyzed for SIgA antibodies. In wt mice significant levels of SIgA were observed locally in the nasal and lung lavages, but also in the peripheral vaginal lavages after i.n. BLP-SV administration, while mice vaccinated i.m. with SV alone showed decreased or absent SIgA levels (Figure 3A). In contrast to the levels observed in wt mice, low to absent SIgA levels were measured in nasal (Figure 3B) and vaginal (Figure 3C) lavages in TLR2KO mice. In addition, very low levels of SIgA antibodies were measured in mucosal lavages when SV alone was administered either i.n. or i.m. The data show that local and peripheral SIgA production after i.n. BLP-SV administration depends on the interaction of BLP with TLR2.

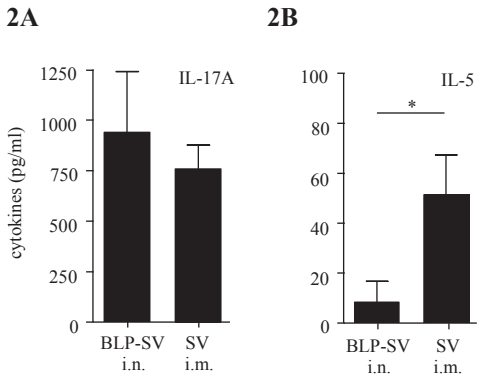

Figure 2 | Intranasal BLP-SV vaccination results in Th1/Th17 skewing. C57BL6/J wt mice and TLR2 KO mice were vaccinated three times with 10 µl of BLP-SV (A/Sydney/5/97) or SV (A/Sydney/5/97) in PBS i.n. or i.m, respectively. Spleens were isolated and single cells were re-stimulated *ex vivo* with ConA for 72 h at 37°C. Cytokine concentrations in pg/ml of IL17A (**2A**) and IL-5 (**2B**) were determined in culture supernatants. Data are shown as the mean ± SEM with n=5 mice per group. Statistical significance; *P< 0.05.

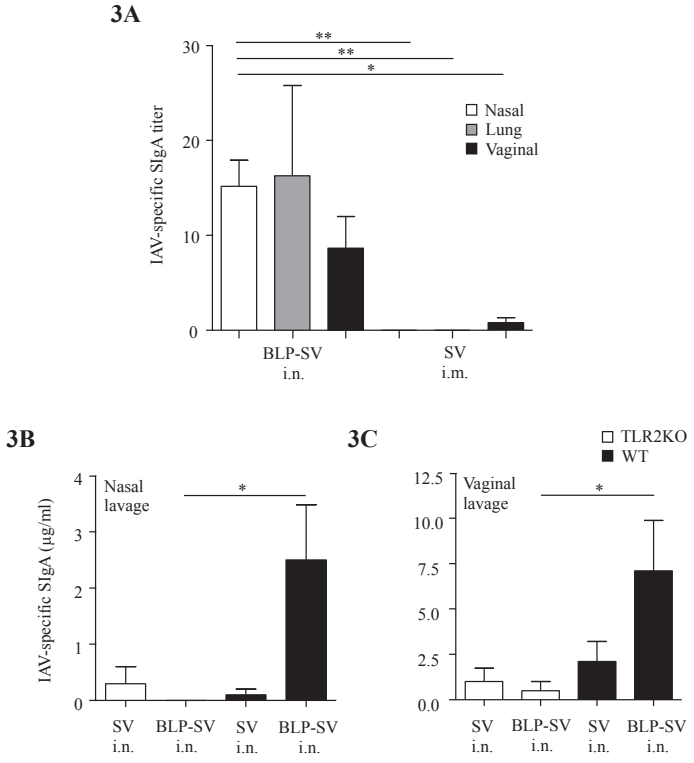

Figure 3 | Local and peripheral IAV-specific SIgA responses induced after i.n. BLP-SV vaccination are TLR2 dependent. BALB/c wt mice were vaccinated three times with 10 µl of BLP-SV (A/Beijing/262/95) or SV (A/Beijing/262/95) in PBS, i.n. or i.m, respectively. Nasal, lung and vaginal lavages were collected and IAV-specific SIgA production was detected with ELISA (**3A**). C57BL6/J wt mice and TLR2KO mice were vaccinated three times i.n. with 10 µl of BLP-SV (A/Sydney/5/97) or SV (A/Sydney/5/97) in PBS. For the detection of IAV-specific SIgA titers, nasal (**3B**) and vaginal (**3C**) lavages were collected and IAV-specific SIgA antibody production was detected with ELISA. Data are shown as the mean ± SEM. Figure 3A with n=14 or n=5 mice per group, respectively, for i.n. or i.m. treatment and figures 3B-3C with n=10 mice per group. Statistical significance; **P*<0.05, ***P*<0.01.

Next, we explored if the observed enhanced IAV-specific B-cell response after i.n. BLP-SV vaccination in wt mice compared to TLR2KO mice as shown in Figure 1B and 1D also affected IAV-specific systemic antibody production.

We observed an enhanced IAV-specific IgG response in serum of wt mice after booster vaccination with i.n. BLP-SV in contrast to vaccinated TLR2KO mice, which resembles the IgG response of the SV vaccine in wt mice (Figure 4A and 4B). Then, we investigated if IgG class switch to IgG1 or IgG2c after i.n. BLP-SV vaccination also depended on TLR2 interaction. Here, we showed that BLP-SV-induced class switch to IgG2c depended on the interaction of BLP with TLR2 (Figure 4C). In contrast the IAV specific IgG1 response was not reduced in TLR2KO mice compared to wt control mice (Figure 4D). We therefore

suggest that the increase in IgG1 in the TLR2KO mice after both i.n. BLP-SV and SV immunization might indicate an inhibitory role for TLR2 on class switch to IgG1.
Thus, both IAV-specific systemic Th1 cell and subsequent B-cell responses that were associated with enhanced IgG2c antibody production induced after i.n. BLP-SV vaccination depended on interaction of BLP with TLR2.

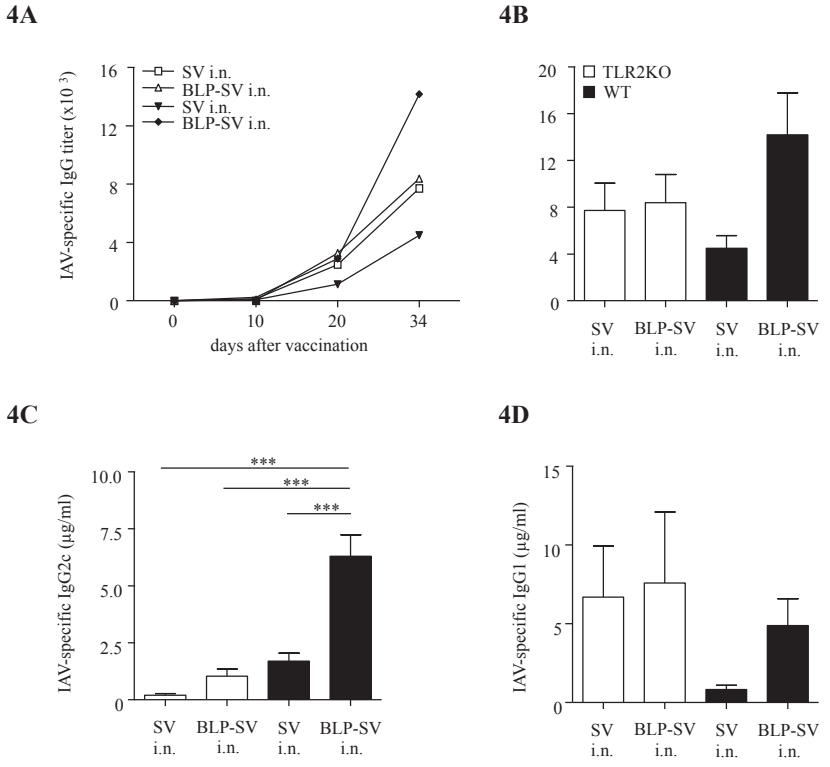

Figure 4 | Intranasal BLP-SV vaccination induced B-cell responses result in TLR2 dependent IgG2c antibody production. C57BL6/J wt mice and TLR2 KO mice were vaccinated three times i.n. with 10 µl of BLP-SV (A/Sydney/5/97) or SV (A/Sydney/5/97) in PBS. For IAV-specific IgG responses, serum was collected and IAV-specific IgG production was detected with ELISA. Data are shown as IAV-specific IgG titers in serum after a priming, booster and second booster vaccination (**4A**). Data are shown as the IAV-specific IgG titer after the second booster vaccination (**4B**). For IgG class switch, serum was collected after the second booster vaccination and IAV-specific IgG2c (**4C**) and IAV-specific IgG1 (**4D**) responses were detected with ELISA. Data are shown as the mean ± SEM with n=10 mice per group. Statistical significance; ***P< 0.001.

DISCUSSION

Earlier studies have demonstrated *in vitro* that BLPs can activate TLR2 signaling in human TLR-transfected HEK cells and mouse dendritic cells.[17] This implies that TLR2 activation by BLP could be responsible for enhancing adaptive immune responses *in vivo*, but formal proof for this was lacking. Previous studies showed that the effect of TLR2 triggering on the outcome of the immune response *in vivo* is variable and depends on several unknown factors: TLR2 can form heterodimers with other TLRs, specifically TLR1 and TLR6[18,19] and TLR2 is expressed by a plethora of immune cells. [21-26] Furthermore, the immunostimulatory activity of BLPs *in vivo* could be the result of activation of innate receptors different from TLR, for example, NOD receptors. Here, we provided clear evidence for an essential role of TLR2 in the BLP-dependent activation of the IAV-specific adaptive immune responses *in vivo* upon nasal vaccination.

Here, we showed that both local and systemic IAV-specific IFN-γ T-cell (Figure 1A and 1C) and B-cell responses (Figure 1B and 1D) required the activation of TLR2 by BLPs given that the number of cells was significantly reduced in TLR2KO mice after i.n. BLP-SV vaccination compared to wt control mice. Since IFN-γ producing Th1 cells are known to promote IgG2c production by B-cells [28] we explored if the IgG class switch to IgG2c also depended on the interaction of BLPs with TLR2. The data showed a significantly reduced IAV-specific IgG2c antibody production in TLR2KO mice after i.n. BLP-SV vaccination compared to wt control mice (Figure 4C) that correlated with reduced numbers of IFN-γ producing T-cells. Therefore, we suggest that the enhanced IgG class switch to IgG2c was mediated by IAV-specific IFN-γ producing T-cells and this required the interaction of BLPs with TLR2. Since interaction of BLPs with TLR2 skewed the responses towards Th1 type, i.n. BLP-SV vaccination, as expected, did not affect IgG class switch to IgG1 (Figure 4D). In addition, we found that i.n. BLP-SV vaccination also modestly enhanced the response towards Th17 type (Figure 2A). The role of Th17 and other IL-17 producing cells in protection against influenza infections is still not completely clear.[29] However, IL-17 producing cells might be beneficial in protection against severe influenza infections, since enhanced numbers of IL-17 producing influenza specific T cells can protect the host against an, otherwise lethal, influenza infection.[30] Surprisingly, the influenza A virus itself has been described to inhibit Th17-mediated immunity thereby enhancing the risk of complicating secondary *S. aureus* infections.[31]

TLR ligands have been studied previously in influenza virus studies and i.n. pre-treatments with especially TLR2 and TLR4 ligands were found to protect mice against lethal influenza pneumonia in an antigen independent manner.[32] Moreover, i.n. immunization with influenza-derived peptides coupled to bacterial-derived lipids induced DC maturation via TLR2 binding and enhanced activation of IFN-γ secreting $CD8^+$ T-cells at the site of infection after i.n. exposure to influenza virus.[33] Earlier it was shown that nasal immunization with BLP activated and enhanced the maturation of DCs that enhanced the activation of IFN-γ producing $CD4^+$ T-cells.[17] However, the BLP interaction with TLR2 *in vivo* might involve

other cell types since TLR2 is expressed on many immune cells, including B-cells.[24] For example, B-cell intrinsic MyD88 signals can also drive IFN-γ production from T-cells and result in enhanced T-cell dependent IgG2c antibody responses.[34] Therefore, we suggest that the interaction of BLPs with TLR2 expressed by antigen presenting cells, such as DCs but also B cells, requires further investigation to understand the mechanism that drives the immunological outcome after nasal vaccination.

SIgA at mucosal tissues forms a first line of defence against invading pathogens at the portal of entry,[8-10] therefore, we explored if the induction of mucosal SIgA responses after i.n. BLP-SV vaccination required BLP interaction with TLR2. Indeed, the data showed that SIgA responses measured in nasal (Figure 3B) and vaginal lavages (Figure 3C) were TLR2 dependent. Previously, it was shown that i.n. vaccination with BLP vaccines induced enhanced SIgA at mucosal tissue in BALB/c mice compared to parenteral vaccination.[15,35] The potency to induce a mucosal SIgA response was independent of the mouse strain tested, as both C57BL6/J and BALB/c mice induced strong responses (Figure 3). Similar to the local immune response induced by BLP adjuvanted vaccination, also systemically induced immune responses in BALB/c and C57BL6/J are comparable as shown by enhanced IFN-γ producing cells and IAV-specific IgG titers.[17,35]

Although the IL-5 cytokine is a differentiation marker for B-cells that produce IgA[36] we did not detect significant IL-5 cytokine secretion after i.n. BLP-SV vaccination (Figure 2B). Since TLR2 signalling can also trigger IgA production by human B-cells directly,[37] we suggested that the SIgA responses are at least partly enhanced due to the interaction of BLP with TLR2 on B cells (Figure 3B and 3C).

Previously, it has been shown that BLP adjuvanted vaccines induce protective immunity to subsequent infection.[15,17] Moreover, recent data showed that i.n. vaccination with a BLP adjuvanted influenza vaccine results in improved protection against both homologous and heterologous influenza challenge infections as compared to protection levels observed after conventional parenteral influenza vaccination.[35] These data underline that enhanced systemic and mucosal B-cell responses induced by i.n. vaccination with BLPs result in a strong protective and broad immune response.

In conclusion, the interaction of BLPs with TLR2 *in vivo* is required for the enhanced activation of systemic and local IAV-specific adaptive immune responses as observed after i.n. BLP-SV vaccination. Especially the ability to induce local IAV-specific immune responses, in particular elevated levels of IAV-specific IFN-γ producing T-cells and IgA antibody secreting B-cells, make BLPs an attractive immune stimulator to be used in nasal vaccination against influenza infection.

ACKNOWLEDGEMENTS

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3

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

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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 DCs. *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 in chapter 4.

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 et al[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

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 CELL 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 ($5*10^5$ 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% CO_2 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 GM-CSF 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 $OVA_{\text{Alexa Fluor 647}}$ 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 GM-CSF 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) 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 at 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 Bonferroni 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

OVA-loaded nanoparticle type	Size (nm)	PDI	Zeta potential (mV)	Loading efficiency (%)
PLGA	320 +/- 17.9	0.151 +/- 0.033	- 48.2 +/- 0.59	34.2 +/- 3.3
PLGA/TMC	448 +/- 55.9	0.234 +/- 0.031	24.5 +/- 0.90	71.6 +/- 6.2
TMC	278 +/- 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.

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.

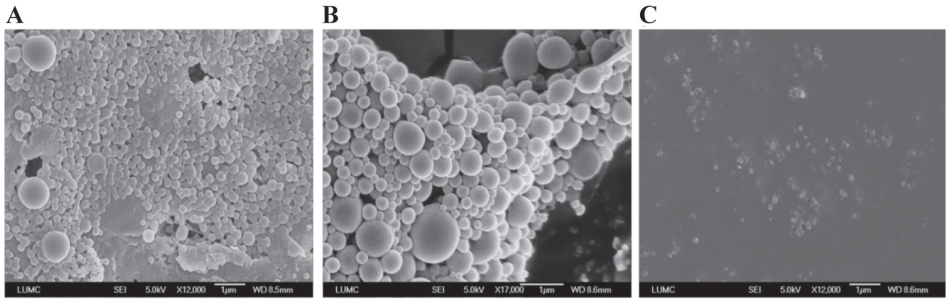

Figure 1 | Scanning electron microscopy images of OVA-loaded nanoparticles: A) PLGA NP, B) PLGA/TMC NP and C) TMC NP.

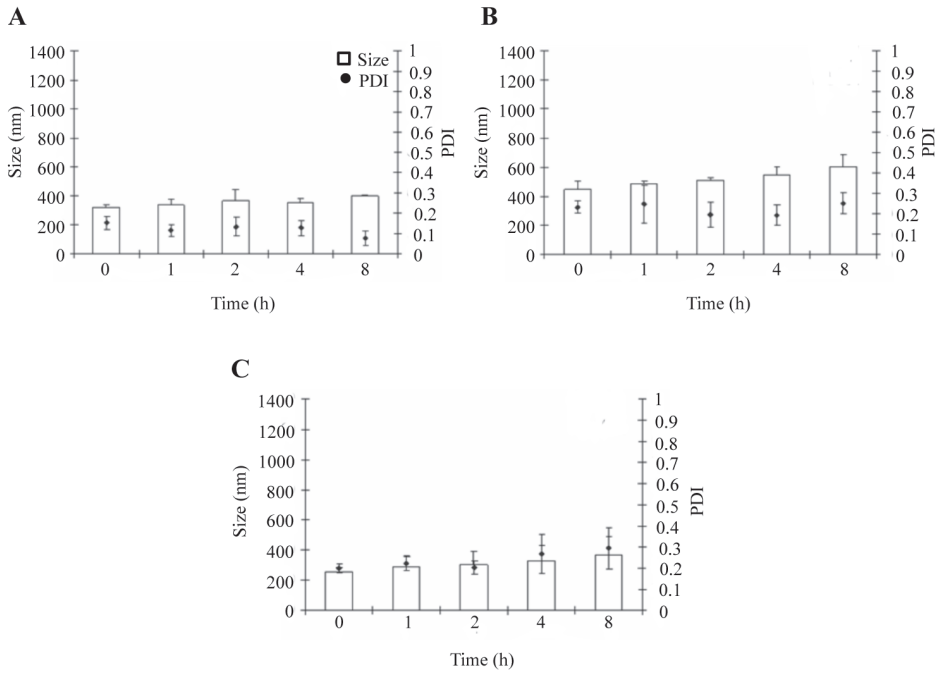

Figure 2 | Short term (8 h) stability of OVA-loaded NP in PBS at 37˚C: A) PLGA NP, B) PLGA/TMC NP and C) TMC NP. Results are the average +/- SD of 3 independently prepared batches.

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.

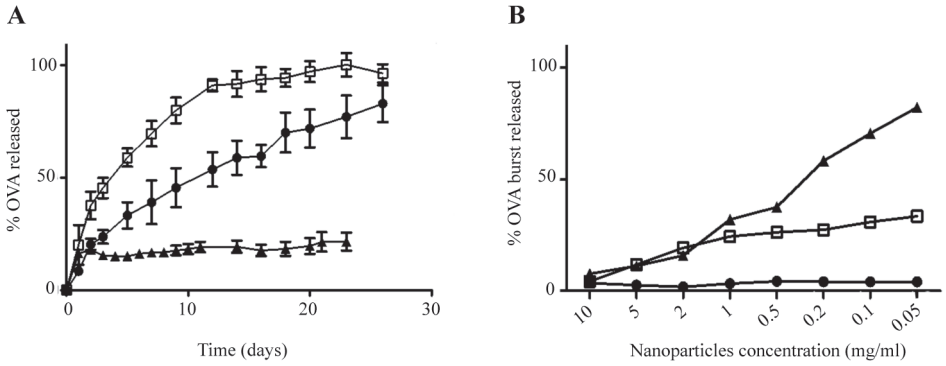

Figure 3 | OVA release from PLGA NP (circles), PLGA/TMC NP (squares) and TMC NP (triangles. A) OVA release from 1 mg/ml particle dispersions was monitored over 25 days at 37°C in PBS. B) Burst release of OVA from these particles as function of NP concentration, assessed after 1 h incubation in PBS (pH7.4).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

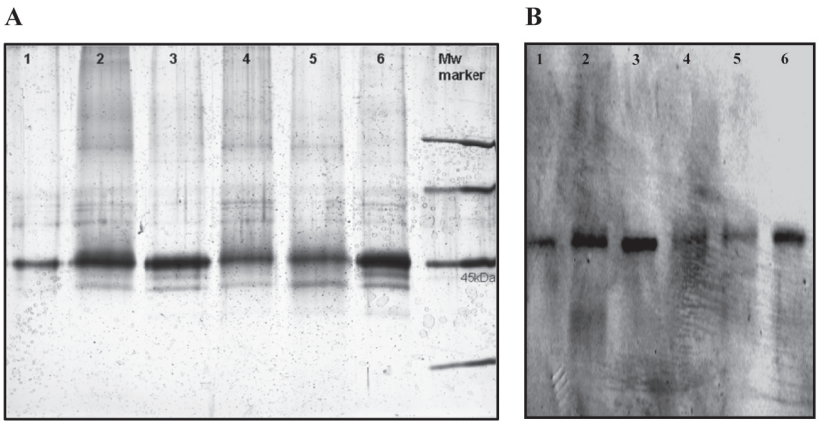

Figure 4 | Stability of OVA after encapsulation into nanoparticles. A) Silver stained SDS-PAGE gel; B) Anti-OVA Western blot of a second gel run in parallel. 1=OVA stock solution, 2= OVA 2x15 s sonicated, 3= OVA 2x15 s sonicated+ DCM extracted, 4= OVA extracted from PLGA NP, 5= OVA extracted from PLGA/TMC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

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

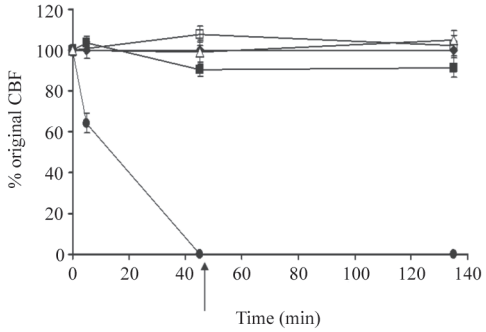

Figure 5 | Ciliary beat frequency after exposure to 0.5 mg/ml OVA-loaded nanoparticles as a measure for nasal cilia toxicity. Nasal epithelium was exposed for 45 min to formulations, after which the epithelium was washed (arrow) and the CBF allowed to recover for 90 min. Closed diamond = non-exposed, 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.

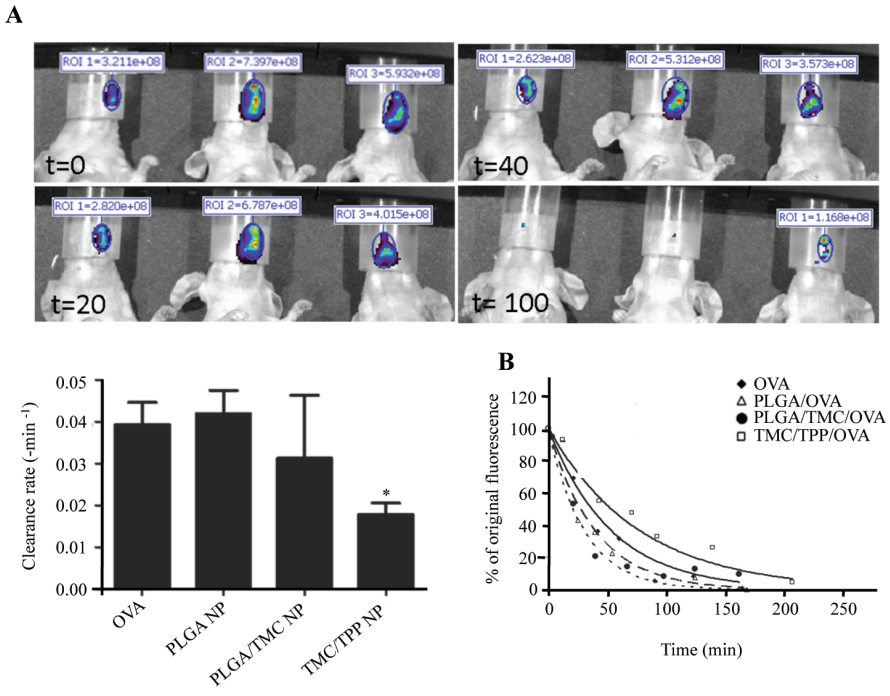

Figure 6 | Nasal residence time of OVA determined using fluorescence detection of OVA-IRdye CW 800. A) Emission (λ=800 nm) 0, 20, 40 and 100 min after nasal administration of OVA-IRdye CW 800. B) Intensity of fluorescence signal from the nasal cavity normalized for time point 0. Individual time points 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. * $p<0.01$

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*).

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.

Dendritic cell studies

Interaction and uptake of the nanoparticles by monocyte derived DC was studied using flow cytometry (Figure 7A) and confocal microscopy (Figure 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).

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.

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.

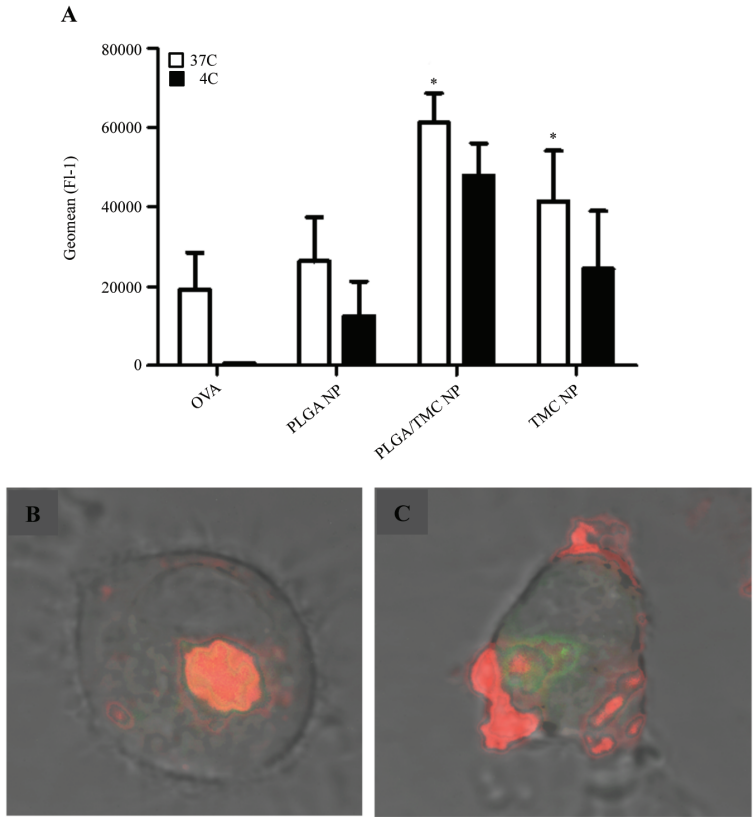

Figure 7 | Interaction of OVA-loaded nanoparticles with DC. A) Association of particles with human DC quantified using flow cytometric analysis. Bars represent mean +/- SD of 6 different monocytes donors. *p<0.05 compared to OVA 37°C. Merged confocal microscopy image of DC exposed to B) $OVA_{alexafluor647}$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

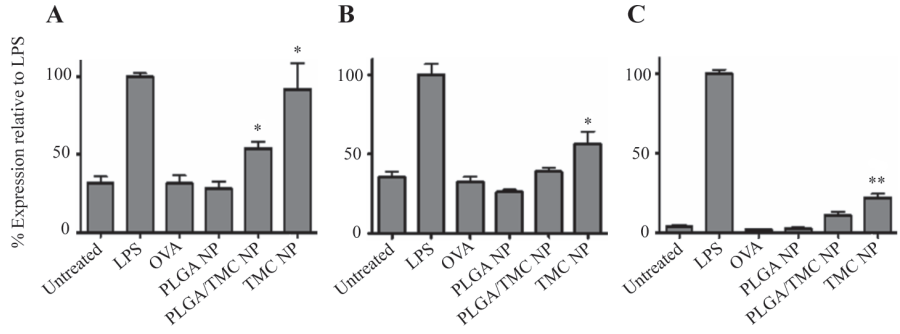

Figure 8 | DC maturation after 48h stimulation with OVA-containing nanoparticles Levels of A) MHCII, B) CD83 and C) CD86 were expressed as a percentage of LPS stimulated DC. Histogram represents the mean of 6 independent experiments. Error bars are SEM. * p<0.05 **p<0.01 vs. OVA-treated DC.

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).

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

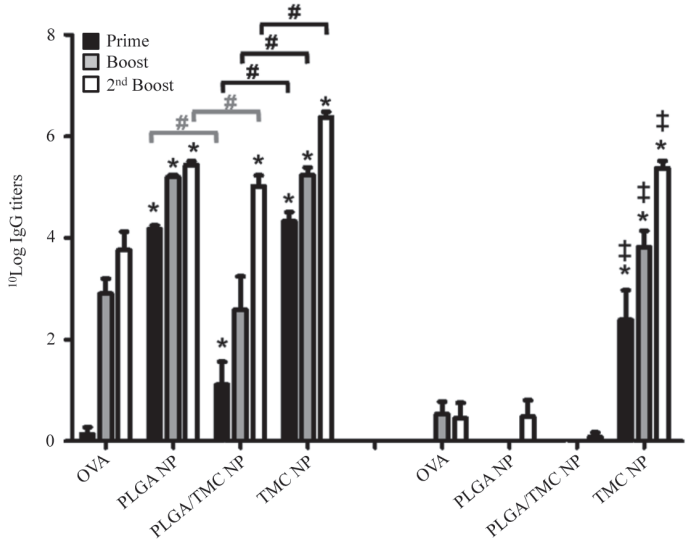

Figure 9 | OVA specific IgG titers in serum of Balb/c mice 3 weeks after a priming, booster and 2nd booster dose of 20 µg OVA administered i.m. or nasally. Data represent mean +/- SEM, n=8. * $p<0.05$ compared to OVA i.m., ‡ $p<0.05$ compared to OVA nasal, # $p<0.05$.

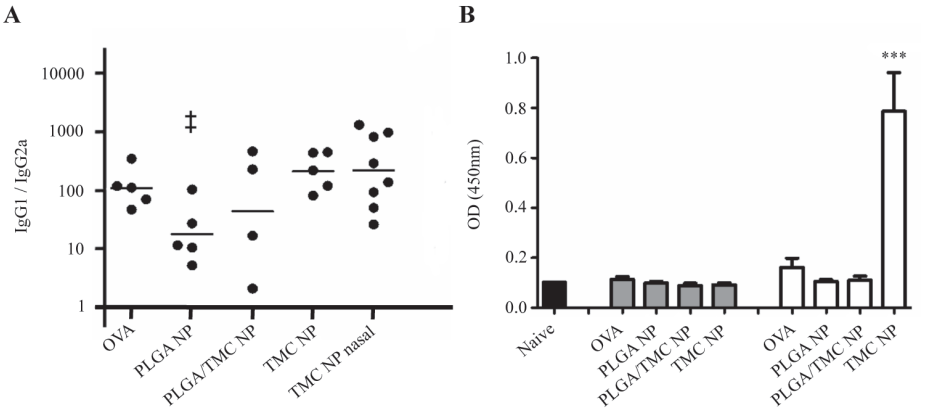

Figure 10 | OVA-specific IgG1/IgG2a balance after i.m. vaccination and sIgA responses in nasal washes. A) IgG1/IgG2a balance after i.m. vaccination, and for TMC NP also after nasal vaccination. ‡ $p<0.05$ compared to OVA. Bar represents mean. B) OVA specific sIgA in nasal washes of mice after i.m. (grey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

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 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 (chapter 4). 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 to break 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.

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4

PLGA, PLGA-TMC and TMC-TPP nanoparticles differentially modulate the outcome of nasal vaccination by inducing tolerance or enhancing humoral immunity

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ABSTRACT

Development of vaccines in autoimmune diseases has received wide attention over the last decade. However, many vaccines showed limited clinical efficacy. To enhance vaccine efficacy in infectious diseases, biocompatible and biodegradable polymeric nanoparticles have gained interest as antigen delivery systems.

We investigated in mice whether antigen-encapsulated PLGA (poly-lactic-co-glycolic acid), PLGA-TMC (N-trimethyl chitosan) or TMC-TPP (tri-polyphosphate) nanoparticles can also be used to modulate the immunological outcome after nasal vaccination.

These three nanoparticles enhanced the antigen presentation by dendritic cells, as shown by increased *in vitro* and *in vivo* $CD4^+$ T-cell proliferation. However, only nasal PLGA nanoparticles were found to induce an immunoregulatory response as shown by enhanced Foxp3 expression in the nasopharynx associated lymphoid tissue and cervical lymph nodes. Nasal administration of OVA-containing PLGA particle resulted in functional suppression of an OVA-specific Th-1 mediated delayed-type hypersensitivity reaction, while TMC-TPP nanoparticles induced humoral immunity, which coincided with the enhanced generation of OVA-specific B-cells in the cervical lymph nodes. Intranasal treatment with Hsp70-mB29a peptide-loaded PLGA nanoparticles suppressed proteoglycan-induced arthritis, leading to a significant reduction of disease.

We have uncovered a role for PLGA nanoparticles to enhance $CD4^+$ T-cell mediated immunomodulation after nasal application. The exploitation of this differential regulation of nanoparticles to modulate nasal immune responses can lead to innovative vaccine development for prophylactic or therapeutic vaccination in infectious or autoimmune diseases.

INTRODUCTION

Nasal vaccination is described for the prevention of infectious diseases such as hepatitis B[1,2] or influenza[3,4]. However, recently, nasal antigen application has also become of interest as a route of vaccination in the field of autoimmunity[5-9] and allergy[10,11]. Similar to other forms of mucosal immunization, nasal antigen application can stimulate antigen-specific responses locally and in the peripheral mucosal tissues[12-15]. Vaccination via the nasal mucosa might be preferred over oral vaccination given the low proteolytic activity in the nasal mucosa; this route of immunization requires a lower dose of antigen than that of oral immunization, which might also reduce the chance of producing negative side-effects[16]. The immune response induced 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[16]. Although the immune response that is induced following mucosal antigen application depends on the antigen that is used, nanoparticle characteristics might also play an important role. For tolerance induction to self antigens used for vaccination in autoimmune diseases one would prefer to combine a self antigen with a vaccine that favors tolerance induction. On the other hand, in the case of prevention of infectious diseases an immunogenic antigen with a vaccine that enhances humoral immunity is preferred. Therefore, rational future vaccine design might benefit from knowledge of immunomodulatory characteristics of nanoparticles. In recent years, several *in vivo* studies have been conducted to investigate nanoparticle-mediated delivery of antigen at mucosal sites. Nanoparticles are available as non-toxic delivery systems with promise for nasal vaccination[17-20]. Since mucosal antigen application elicits different immune responses such as T-helper 2 (Th2)-mediated humoral immunity or T-helper 1 (Th1)-mediated Delayed-Type Hypersensitivity (DTH)[21-23], we explored if nanoparticles can differentially modulate the outcome of nasal vaccination. The readout to evaluate the efficacy of the applied vaccine relies mostly on induction of humoral immune responses as indicated by increased antigen-specific antibody titers[4,18,24]. However, this does not give insight into the underlying immunological mechanisms that drive the response towards humoral immunity or DTH. Marazuela *et al* previously showed that intranasal administration of PLGA particles that contained a peptide with the major T-cell epitope of Ole e 1 induced a modified Th2 response and prevented mice from allergic sensitization of the whole protein[25,26]. However, little is still known about the role of $CD4^+$ T-cells in nasal vaccination and how different nanoparticle treatment might influence the activation of these cells, locally and in the peripheral tissues.

To study the mechanisms behind humoral immunity or DTH after nasal vaccination, three polymeric nanoparticles were analyzed that are efficient antigen delivery systems; PLGA (poly-lactic-co-glycolic acid), PLGA-TMC (N-trimethyl chitosan) and TMC-TPP (tri-polyphosphate) nanoparticles that all contained the model antigen ovalbumin (OVA). These particles have a similar average diameter of 250-500 nm, but differ in their surface charge and antigen release kinetics[17,18].

For example, nasal application of TMC-TPP particles has been described in the field of

influenza vaccination and elicits humoral immune responses as shown by a significant increase in antigen-specific IgG1/IgG2a serum titers and increased sIgA titers in nasal washes[4]. In contrast to induction of humoral immunity, mice fed a single dose of 40 μg of type II collagen (CII)-containing PLGA particles had reduced severity of arthritis and reduced anti-CII-specific IgG antibody titers and CII-specific T-cell responses[24].
We investigated in an OVA-specific DTH model whether nanoparticles itself cause a shift in the immunological outcome after nasal antigen application as shown by the activation and differentiation of $CD4^+$ T-cell responses both at the site of vaccination and systemically. In addition, we investigated the relevance of nanoparticle mediated mucosal tolerance after nasal application in a proteoglycan induced arthritis (PGIA) model[27] with heat shock protein 70 (Hsp70) peptide mB29a encapsulated nanoparticles. Hsp70 is known to be one of the most conserved Hsps and has been shown to have disease suppressive properties[28-30]. Mouse Hsp70-peptide mB29a has been shown to be immunosuppressive after nasal application in the PGIA model[31]. Therefore, we explored the additional immunosuppressive effect of nanoparticle treatment by encapsulating the mB29a peptide.
This study was performed to obtain more insight into the mechanism by which nanoparticles drive the immune response towards immunomodulatory responses. The data may assist future rational vaccine design for prophylactic or therapeutic vaccination in infectious and autoimmune diseases, respectively.

MATERIALS AND METHODS

Ethics statement
All mice were kept in our animal facility at the Central Animal Laboratory (GDL), University Utrecht, The Netherlands under standard housing conditions. Experiments were approved by the Animal Experiment Committee of the University Utrecht, Utrecht, The Netherlands (Permit Number: 2007.II.03.072 and 2009.II.08.075).

Mice
Male BALB/c mice (8-12 weeks) and female BALB/c mice (retired breeders aged between 16-26 weeks) were purchased from Charles River Laboratories (Maastricht, The Netherlands). OVA-specific TCR transgenic (Tg) mice on BALB/c background (DO11.10 mice), were bred at the Central Animal Laboratory (GDL), University Utrecht, The Netherlands.

Antibodies and antigen encapsulated nanoparticles
In all *in vitro* and *in vivo* experiments, endotoxin-low OVA was purchased at Calbiochem (San Diego, CA). Murine Hsp70 peptide mB29a (HspA9-derived sequence VLRVINEPTAAALAY) was encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles and OVA-encapsulated PLGA, PLGA-TMC and TMC-TPP nanoparticles were generated as described previously[17,18]. For detailed information about the particle

characteristics such as size, zeta potential, loading efficiency and the polydispersity index, we refer to the paper published by Slütter *et al*[18]. Anti-DO11.10 TCR (KJ1.26) was purchased from Molecular Probes (Invitrogen, Breda, 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), 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 culture

Bone marrow-derived dendritic cells (BMDC) were cultured from BALB/c donor mice as previously described with minor modifications[42]. Briefly, femurs and tibia of adult BALB/c mice were flushed with culture medium. Single cell suspensions were seeded in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with $5x10^{-5}$ M 2-mercaptoethanol, penicillin (100 units/ml) and streptomycin (100 µg/ml) (Gibco, Karlsrule, Germany) and 20 ng/ml murine rGM-CSF (Cytogen, The Netherlands). On day 2 and 4, 10 ng/ml murine rGM-CSF was added. The cells were cultured in a humidified 5% CO_2 atmosphere at 37ºC. On day 7, the BMDCs were routinely pure between 70% and 80% based on CD11c and MHC class II expression 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. $CD4^+$ 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) mAb. 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[43].

***In vitro* effect of nanoparticles on DC**

To address maturation of BMDC by nanoparticles, BMDC were cultured in the presence of PLGA, PLGA-TMC or TMC-TPP nanoparticles containing 25 ng/ml to 1.0 µg/ml OVA 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) dissolved in PBS or with antigen 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% and uptake was analyzed by flow cytometry.

BMDCs were pre-incubated at 37°C for 2 h in the presence of OVA protein dissolved in PBS or incorporated in nanoparticles (PLGA, PLGA-TMC or TMC-TPP) at concentrations of 25 ng/ml, 0.5 µg/ml or 1.0 µg/ml. OVA-specific $CD4^+$ T-cells were added at an 1:10 DC:T-cell ratio and T-cell proliferation was assessed after 72 h by CFSE dilution.

T-cell activation in the local lymph nodes after nanoparticle vaccination
BALB/c recipient mice were adoptively transferred with 10^7 CFSE-labeled $CD4^+KJ1.26^+$ cells in 100 µl PBS, intravenously. The next day mice received a single application of 30 µg of OVA dissolved in 10 µl of PBS or encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles i.n. or intramuscular i.m. in the hind limbs. 72 h after i.n. or i.m. OVA administration, the spleen, NALT and CLN as well as the thigh-draining ILN were harvested and single cell suspensions were analyzed.

Delayed-type hypersensitivity (DTH) reaction
BALB/c mice received 20 µg of OVA i.n. three times at 24 h intervals either dissolved in PBS or encapsulated in PLGA, PLGA-TMC or TMC-TPP nanoparticles. Control groups received PBS alone or OVA at a final concentration of 100 µg in PBS. Mice were sensitized for a DTH the next day with 100 µg of OVA in 25 µl of PBS, mixed with 25 µl of Incomplete Freund's Adjuvant (IFA) (Difco, BD. Alphen a/d Rijn, The Netherlands) subcutaneously (s.c.) administered in the tail base. Five days later, ear-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 PBS given in the auricle of each ear and 24 h post-challenge, the increase in ear thickness of both ears was determined.
The early B-cell response was assessed by detection of OVA-specific B-cells of immunized mice by ELISPOT. Single cell suspensions from the NALT, CLN and spleen were cultured with OVA (1 µg/well) or control on high protein binding filter plates (MultiScreen-IP, Millipore) for 48 hours. After incubation, spot forming units were detected with goat-anti mouse IgG-biotin (Sigma) and Avidin-AP (Sigma). Plates were developed with NBT-BCIP (Roche) and analyzed by using the Aelvis spotreader and software. Data are shown as the OVA-specific B-cell count per 10^6 cells (antigen-induced-background).

Nasal tolerance induction and assessment of arthritis
Female retired breeder BALB/c mice were treated 3 times i.n. with 10 µg of mouse Hsp70-peptide mB29a in PBS or encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles dissolved in 10 µl PBS on days -7, -5, -3. Arthritis was induced by intraperitoneal (i.p.) injections of 300 µg proteoglycan (PG) protein with 2 mg of the synthetic adjuvant dimethyl-dioctadecyl-ammoniumbromide (DDA) (Sigma) emulsified in PBS (total volume of 200 µl) on day 0 and day 21 as described[44,45]. After the second PG immunization, the onset and severity of arthritis were determined using a standard visual scoring system based on swelling and redness of the paws as described[44].

Luminex
$CD4^+$ T-cells isolated from the spleen of DO11.10 mice were incubated with BMDCs isolated from BALB/c wt mice at an 1:10 DC:T-cell ratio and stimulated with sOVA or OVA encapsulated in nanoparticles in IMDM supplemented with $5x10^{-5}$ M 2-mercaptoethanol, penicillin (100 units/ml) and streptomycin (100 µg/ml) (Gibco, Karlsrule, Germany) at 37ºC. The amount of cytokine secreted after a 72 h T-cell re-

stimulation 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-10 (JES5-2A5), (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-10 (SXC-1), and PE-labeled streptavidin (BD Biosciences Pharmingen). Fluorescence was measured using a Luminex model 100 XYP (Luminex, Austin, TX, USA).

RT-PCR analysis

Total mRNA was purified from single cell suspensions from NALT, CLN, ILN 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. RT-PCR was performed using a MyiQ Single-Color RT-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 by 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'-TGAAGTACTCATTATAGTCAAGGGCA-3'
IL-10
sense 5'- GGTTGCCAAGCCTTATCGGA-3'
antisense 5'-ACCTGCTCCACTGCCTTGCT-3'
Foxp3
sense 5'-CCCAGGAAAGACAGCAACCTT-3'
antisense 5'-TTCT CACAACCAGGCCACTTG-3'

Statistics

Statistical analysis was performed with Prism software (Graphpad Software Inc., San Diego, version 4.00) using a one-way ANOVA followed by a Kruskal-Wallis test and a Dunn's multiple comparison test for the *in vitro* cytokine and FoxP3 mRNA assay. One-way ANOVA followed by Bonferroni's multiple comparison test was used for statistical analysis of the Delayed-type hypersensitivity reaction. An unpaired two-tailed Student's T-test was used for statistical analysis in all other experiments. Error bars represent the S.D. or S.E.M. 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 FITC labeled OVA by BMDCs after nanoparticle treatment

We previously showed nanoparticle differences in association with and uptake by DCs as visualized by tracing uptake of OVA *in vitro*[17,18]. These differences in association might modulate the subsequent antigen presenting capacity of DCs.

To investigate this, we treated DCs *in vitro* with OVA encapsulated in PLGA, PLGA-TMC or TMC-TPP nanoparticles or soluble OVA (sOVA) as a control and studied phenotypic and functional differences between treated DCs. No differences in DC maturation or viability were observed as analyzed by CD11c, MHC-class-II, CD40, CD86 and 7-AAD staining after nanoparticle treatment at OVA concentrations varying from 1 ng/ml to 1 µg/ml. We did not detect significant differences in cytokine profiles in culture supernatants (data not shown).

Next, we studied the uptake of OVA-FITC encapsulated in PLGA, PLGA-TMC and TMC-TPP nanoparticles by DCs silencing extracellular attached FITC signaling with trypan blue. OVA-FITC association with DCs is shown as the FITC expression (ΔMFI) (Figure 1A) or percentage of OVA-FITC positive cells (Figure 1B).

OVA-FITC uptake by DCs treated with TMC-TPP was lower compared to sOVA-FITC treatment as shown by a low FITC ΔMFI expression and decreased percentage of OVA-FITC positive cells (Figures 1A and 1B). 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 with OVA at 0.25 µg/ml. Apart from a reduced uptake of TMC-TPP particles we could not detect differences in antigen uptake at 1 µg/ml between sOVA, PLGA or PLGA-TMC treatment. The latter is being suggestive of saturated antigen uptake after 1.5 h of antigen incubation (Figure 1B).

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

Nanoparticles enhance OVA-specific $CD4^+$ T-cell proliferation *in vitro*

To investigate whether the small differences in antigen uptake by DCs could affect the antigen presenting capacity of DCs, nanoparticle treated DCs were studied *in vitro* by co-culture with OVA-specific T-cells. DCs treated with OVA encapsulated PLGA, PLGA-TMC or TMC-TPP particles were cultured for 72 h in the presence of OVA-specific CFSE-labeled $CD4^+$ T-cells.

The antigen presenting capacity of DCs was enhanced after nanoparticle treatment 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 strongly 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 µg/ml of OVA containing PLGA or PLGA-TMC particles more IL-2

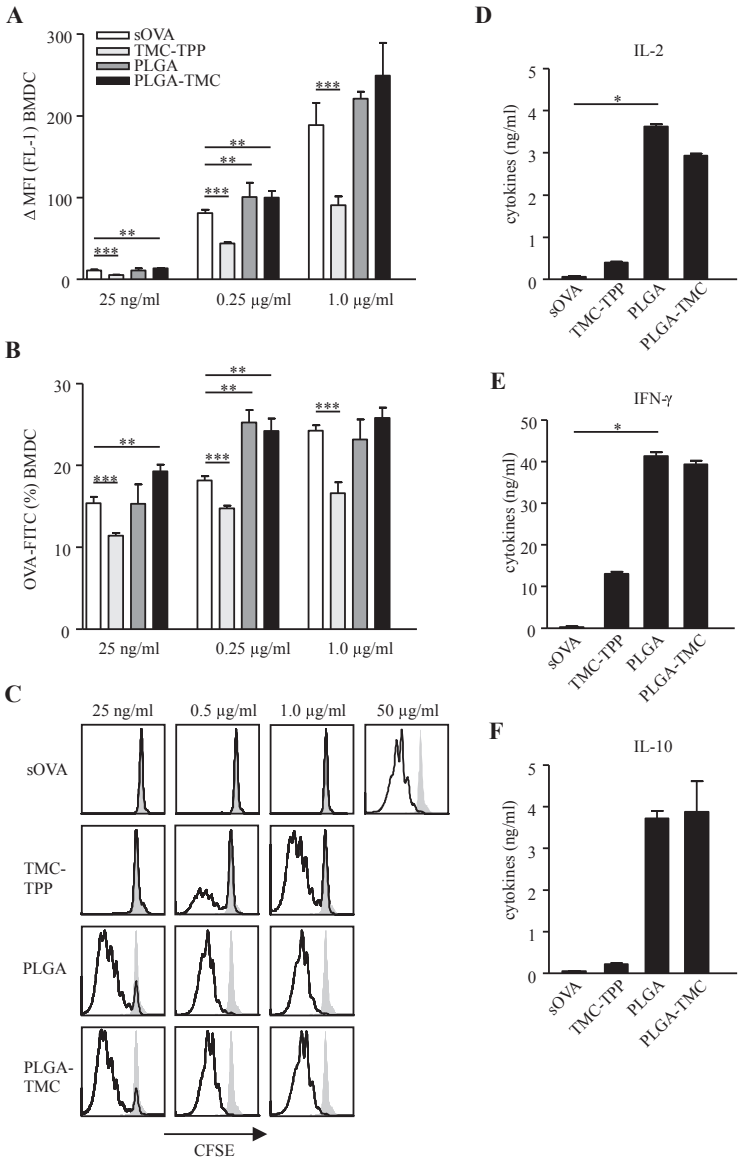

Figure 1 | Nanoparticle mediated enhanced antigen presentation capacity of BMDCs *in vitro*. BMDC were incubated in the presence of sOVA-FITC or OVA-FITC encapsulated into PLGA, PLGA-TMC or TMC-TPP nanoparticles at different concentrations. External FITC signaling was silenced by trypan blue. A. The ΔMFI of OVA-FITC was assessed by subtraction of FITC signaling at 4°C from 37°C. B. OVA-FITC uptake by BMDC shown as the net percentage of OVA-FITC positive cells. C. CFSE-labeled $CD4^+$ T-cells were incubated with BMDC stimulated with sOVA or OVA encapsulated in nanoparticles. Gray filled histograms; unstimulated $CD4^+$ T-cells, Black overlays; $CD4^+$ T-cell division profiles at different OVA concentrations after 72 hours. D-F. Cytokine concentrations of IL-2, IFN-γ and IL-10 (ng/ml) were determined in culture supernatants, after 72 h of culture. Data are representative for 3 independent experiments; mean ± SEM. Statistically significant: *, $P< 0.05$; **, $P< 0.01$ and ***, $P<0.001$.

(Figure 1D), IFN-γ (Figure 1E) and IL-10 (Figure 1F) was detected compared to cultures with TMC-TPP particles or sOVA. No T-cell proliferation or cytokine secretion was induced by empty nanoparticles (data not shown).
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.

Nasal vaccination enhances *in vivo* $CD4^+$ T-cell activation and differentially induces Foxp3 expression

Next, we questioned whether nanoparticle treatment also affected $CD4^+$ T-cell responses *in vivo*. Previously, we showed that especially TMC-TPP nanoparticles enhanced 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[18].
First we studied the short-term $CD4^+$ T-cell response in mice that were 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 systemically in the spleen 72 h after treatment (Figures 2A and 2D).
Nasal vaccination induced strong local $CD4^+$ T-cell proliferation in the nasopharynx-associated lymphoid tissue (NALT) and cervical lymph nodes (CLN), irrespective of the type of nanoparticle, whereas low-dose sOVA did not. None of the formulations induced measurable $CD4^+$ T-cell activation in the spleen at 72 hours after vaccination upon i.n. immunization (Figure 2A). In contrast, non-mucosal vaccination resulted in proliferation both in the draining inguinal lymph nodes (ILN) and spleen at this time point (Figure 2D).
We did 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 an increased expression in the relative Foxp3 mRNA expression in the CLN (Figure 2C) and a slightly increased expression in the NALT (Figure 2B) of mice that had received a single i.n. PLGA vaccination. Mice that were vaccinated i.m. with TMC-TPP particles showed less expression of Foxp3 mRNA compared to PLGA and PLGA-TMC treated mice in the ILN (Figure 2E).
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 (Figure 2A) and coincided with enhanced Foxp3 mRNA expression in the NALT and CLN only when PLGA encapsulated OVA was applied (Figures 2B and 2C). This effect was lacking in the i.m. treated mice of all treatment groups (Figure 2E) showing that, both particle and route of application determine the outcome of the $CD4^+$ T-cell response.

PLGA nanoparticle vaccination suppressed DTH response

To see if the differences in T-cell response induced after nasal treatment are of functional importance, the nanoparticles were tested for immunomodulation in a DTH-model. Mice received 20 µg of OVA i.n. three times at 24 h intervals either dissolved in PBS or

encapsulated in PLGA, PLGA-TMC or TMC-TPP nanoparticles. Subsequently, mice were sensitized by OVA/IFA and challenged with OVA in the auricle of the ear. Ear-thickness was determined 24 h after challenge and compared with measures before challenge. PLGA nanoparticle treatment suppressed the OVA-specific DTH response, whereas PLGA-TMC and TMC-TPP nanoparticles did not (Figure 3A). Non-tolerized mice showed a strong ear-thickness response comparable to low dose sOVA (17.45 ± 1.44), whereas mice tolerized by high dose sOVA significantly reduced a DTH response (9.1 ± 1.21).

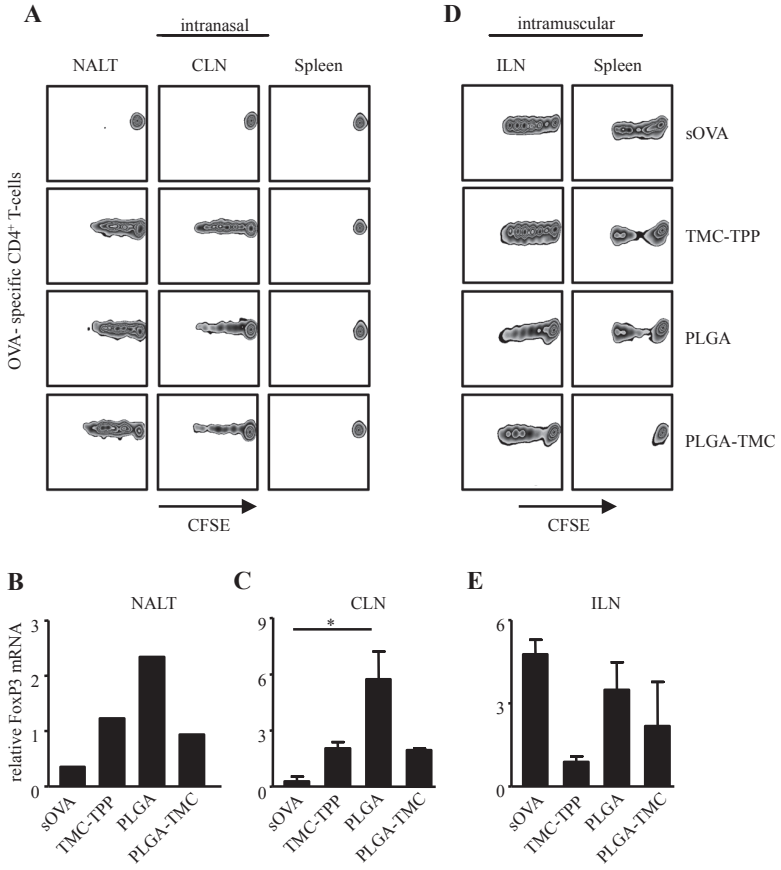

Figure 2 | Enhanced OVA-specific CD4⁺ T-cell proliferation, after nanoparticle administration. A and D. OVA-specific CFSE labeled CD4⁺ T-cells were transferred to BALB/c recipient mice one day prior to vaccination. Mice received a single i.n. application of 30 µg of sOVA or OVA encapsulated into PLGA, 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 OVA administration, *in vivo* T-cell division was addressed in spleen, nose-draining NALT and CLN as well as the thigh-draining ILN. Data are representative for at least 3 i.n. and 2 i.m. independent transfer studies. B, C and E. 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 OVA application. Cells isolated from NALT were pooled per group. LN data are representative for at least 3 to 5 mice per group; mean ± SEM. Statistically significant: *, $P < 0.05$.

In contrast to PLGA and PLGA-TMC, nasal application of TMC-TPP led to a systemic OVA-specific B-cell response (Figure 3C) and significantly increased humoral immunity locally in the draining CLN (Figure 3B). In agreement with earlier studies, this suggested a role for TMC-TPP in the activation of the humoral immune response after nasal treatment[18]. In addition, PLGA-TMC and TMC-TPP nanoparticles seemed to have an inhibitory effect on IL-10 mRNA expression locally in the draining CLN, whereas PLGA induced IL-10 mRNA expression did not differ from sOVA (Figure 3D). Although we

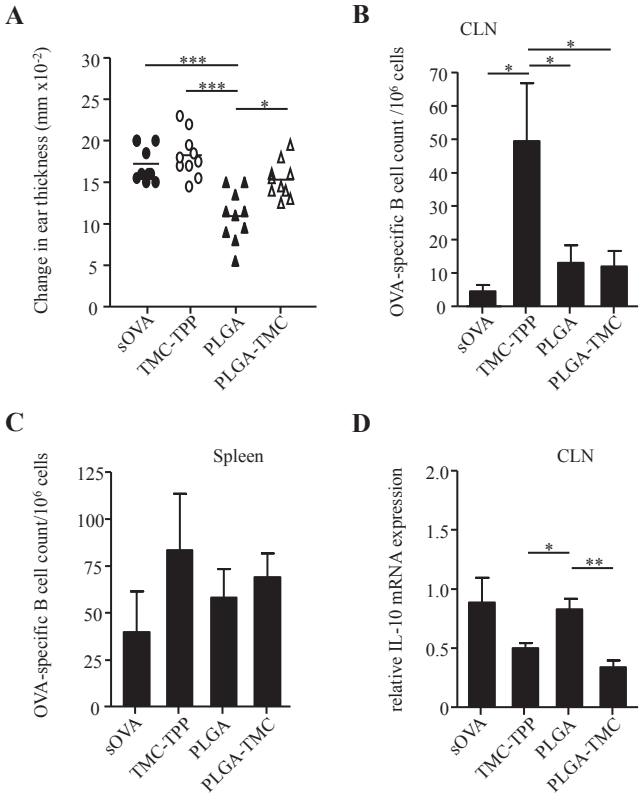

Figure 3 | Nasal application of PLGA particles suppressed a Th-1-mediated hypersensitivity reaction, while TMC-TPP enhanced humoral immunity. A. Mice received 20 µg of OVA i.n. either dissolved in PBS (black circles) or encapsulated in TMC-TPP (white circles), PLGA (black triangles) or PLGA-TMC (white triangles) nanoparticles for three successive days. Mice were sensitized subcutaneously after nasal OVA administration and subsequently challenged in the auricle of both ears. Changes in ear-thickness were determined and compared with values before challenge. B and C. OVA-specific B-cell response induced after nasal nanoparticle treatment. OVA-specific B-cell response was assessed by ELISPOT. Data are shown as the OVA-specific B-cell count per 10^6 cells from CLN and spleen above background (spots counted on medium coated plates). D. Relative mRNA expression to HPRT of IL-10 was determined in the CLN of mice. Data are shown as the mean ± SEM. of n=5 mice per group. Statistically significant: *, $P< 0.05$; **, $P< 0.01$; ***, $P< 0.001$.

detected increased expression of relative Foxp3 mRNA in the T-cell transfer study (Figures 2B and 2C) we were not able to detect such differences in the DTH model, probably due to experimental differences in timing and the presence of OVA-specific T-cells in the transfer model.

To summarize, only nasal treatment with PLGA nanoparticles induced nasal tolerance to a low dose antigen but did not enhance humoral immunity as shown by the absence of antigen-specific B-cell responses (Figures 3A and 3B). In contrast to PLGA and PLGA-TMC immunization, only TMC-TPP treatment led to activation of humoral immunity as shown by local increased generation of antigen-specific B-cells (Figure 3B) and increased antigen-specific antibody titers systemically (see[18]).

Enhanced protection against arthritis after nasal application of mB29a PLGA nanoparticles

We demonstrated that upon nasal application of OVA-PLGA nanoparticles, mice were able to suppress a local induced Th-1 mediated inflammatory response in an OVA-specific DTH-model. Additionally, we investigated whether enhanced nasal tolerance induction mediated by PLGA treatment was sufficient to suppress a chronic inflammatory response. Hsp70-peptide mB29a was encapsulated into each of the nanoparticles and we tested the capacity of the nanoparticles to modulate the response towards mucosal tolerance in the PGIA mouse model. Mice were tolerized by three times i.n. hsp70-peptide treatment either dissolved in PBS or encapsulated in nanoparticles prior to arthritis induction by two i.p. PG/DDA immunizations with a three week interval. These initial data showed proof of principle that treatment with PLGA nanoparticles reduced mean arthritis scores that lasted up to 30 days after disease development (Figures 4A and 4B), which correlated with earlier antigen release kinetics studies[18]. As expected, we did not observe an immunosuppressive function for TMC-TPP, as the mean arthritis scores (1.2 ± 0.2) in these mice were not significantly lower than that of control mice (data not shown). Furthermore, the lower mean arthritis scores observed after PLGA or PLGA-TMC treatment coincided with a later onset of disease and lower maximum arthritis scores (Table 1).

Table 1 | Onset of disease and maximum arthritis scores

Treatment group	Onset of disease	Max. arthritis scores
PBS	15.0 ± 3.6	5.2 ± 2.8
PLGA	23.0 ± 8.2	1.2 ± 0.6
PLGA-TMC	27.7 ± 9.9	1.2 ±1.3

Hsp70-mB29a peptide loaded PLGA, PLGA-TMC nanoparticles or PBS control (10 µg) were given i.n. on day -7, -5 and -3 and arthritis was induced by PG/DDA immunization on day 0 and 21. Arthritis symptoms were scored as described in materials and methods. Day of onset and maximum arthritis scores were depicted as mean ± SEM. of n=3 mice per group of one experiment.

DISCUSSION

In the area of vaccine development, nasal delivery is an attractive route also given the non-invasive needle-free administration[16,32]. Earlier studies already showed that nasal nanoparticle treatment enhanced humoral immunity[4,18] or suppressed this by mucosal tolerance induction[24], depending on the antigen-particle combination. Since there was 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*.

Particle characteristics modulated DC-induced OVA-specific $CD4^+$ T-cell proliferation *in vitro* (Figure 1C) since low-dose sOVA was not able to activate T-cells, whereas particle incorporated OVA had a differential capacity to do so. The proliferative response was not a result of antigen-independent particle induced activation since empty nanoparticles were not able to activate T-cells (data not shown). Although the uptake of sOVA-FITC in Figure 1A and 1B was more efficient in contrast to TMC-TPP nanoparticle treatment at various OVA concentrations, the amount of sOVA was insufficient to activate T-cells *in vitro* as shown in Figure 1C. In addition, sOVA-FITC uptake was similar to that of PLGA and PLGA-TMC at 1 µg/ml concentrations (Figure 1A and 1B) however it was not sufficient for sOVA to induce T-cell activation (Figure 1C). Although sOVA treated cells will also present their antigen via MHC class II we suggested that the expression of MHC molecules will be maintained for a shorter period of time in contrast to nanoparticle induced expression as previously described[33,34]. Here, the authors showed that PLGA-microspheres *in vitro* induced prolongation of antigen presentation by the MHC class I molecule[33,34] and MHC class II molecule[34] by antigen presenting cells.

Therefore, we suggest that nanoparticle mediated T-cell activation at similar low OVA concentrations was enhanced due to differences in MHC class II expression and antigen presentation in contrast to sOVA and that antigen uptake does not necessarily need to correlate with T-cell activation (Figure 1).

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 of sOVA. This showed the superiority of nanoparticle mediated OVA delivery versus sOVA delivery (Figure 2A). In addition, systemic $CD4^+$ T-cell activation following nasal treatment requires a longer time frame as compared to non-mucosal antigen immunization (Figure 2). These data confirm that also the route of antigen delivery activates the immune system, as previously described[23].

Since $CD4^+CD25^+Foxp3^+$ regulatory T cells play an important role in the induction of mucosal tolerance[35,36], we explored Foxp3 expression after nasal nanoparticle treatment. Interestingly, Foxp3 mRNA expression was increased locally in the CLN, only after PLGA treatment (Figure 2C) although proliferation profiles were comparable for all particles (Figure 2A). This suggests that different nanoparticles induced a differential T-cell response and only T-cells activated in presence of PLGA nanoparticles obtained a tolerogenic phenotype as compared to nasal treatment with PLGA-TMC or TMC-TPP (Figures 2B and 2C).

And indeed nasal treatment with low-dose OVA-encapsulated PLGA nanoparticles induced a functional immunomodulatory T-cell response as shown by a reduced DTH response (Figure 3A). We did not observe a tolerogenic effect in the DTH model when mice were treated with low-dose sOVA alone. We suggest that this is due to the low-dose sOVA concentration (3x20 µg) that was used for tolerization of the mice since high-dose sOVA (3x100 µg) was sufficient to induce tolerance (data not shown) and[23,37]. The absence of stimulatory effects of the low-dose sOVA or OVA incorporated in the nanoparticles on dendritic cells argues against LPS contamination. Moreover, if our OVA contained high doses of LPS contamination, high-dose sOVA would not be expected to suppress a DTH.

Nevertheless, low-dose sOVA administration might still favor tolerance induction as shown by the IL-10 mRNA expression in the draining CLN (Figure 3D) however it might not be strong enough to actually suppress the inflammatory response.

Interestingly enough, PLGA particles that were capable of reducing DTH reactivity also had the capacity to induce IL-10 (Figure 3D), suggesting that the characteristics of the PLGA nanoparticle enhanced tolerance induction in contrast to low-dose sOVA.

No immunosuppressive reaction was seen in TMC-TPP treated mice that showed significantly increased OVA-specific B-cells in the CLN (Figure 3B) but not in spleen (Figure 3C). Although we were able to detect low titers of total IgG OVA-specific antibodies in the serum of treated mice, these differences were not significant (data not shown). We therefore concluded that at this time point no systemic but only a local OVA-specific B-cell response was induced in the CLN. In a previous study we explored the effect of nanoparticle treatment on B-cell activation during a 10-week vaccination study[18]. Here, nasal treatment with TMC-TPP nanoparticles resulted in significantly enhanced OVA-specific IgG titers in serum, in contrast to PLGA and PLGA-TMC treatment. Moreover, only after nasal vaccination with TMC-TPP nanoparticles, the antibody titers were comparable to those obtained after i.m. TMC-TPP treatment.

Taken together, these data suggest that upon nasal nanoparticle application, specifically TMC-TPP nanoparticles activate the humoral arm of the mucosal immune system (Figure 3B and[18]).

Additionally, we looked at nanoparticle-mediated immunomodulation after nasal application in a chronic inflammatory condition by using the PGIA model. In various experimental arthritis models, Hsp (-peptides) were shown to have a capacity to down modulate arthritis, which seems to be mediated by the induction of Hsp-specific regulatory $CD4^+$ T-cells[38-41]. Recently, we uncovered a therapeutic potential for mouse Hsp70-peptide mB29a to suppress arthritis after nasal application[31].

Here, we show that mB29a peptide encapsulation into PLGA and PLGA-TMC nanoparticles enhanced the tolerogenic capacity of the peptide. Intranasal treatment with 30 µg of peptide dissolved in PBS did not reduce the severity of arthritis compared to encapsulated peptide (Tabel 1). We have seen that nasal application of encapsulated antigen can induce a tolerogenic response not only in a typical Th1 mediated DTH response, but also in principle in a model of chronic and relapsing arthritis. A difference in tolerogenic capacity was

observed for the PLGA-TMC particles that suppress PGIA completely (Figure 4), but only partially suppressed the OVA DTH response (Figure 3A). This difference can be the result of the nature of the antigen and the chronic nature of the model itself. Hsp70 in the PGIA model is a self antigen that is expressed also by immune cells of the host, thereby enhancing the regulatory T cells induced by the intranasal treatment. In addition, arthritis is a chronic inflammation in contrast to the DTH response prolonging the effective window of the tolerogenic effect of PLGA-TMC treatment.

In conclusion, our results indicate that nasal administration of antigen by PLGA-containing nanoparticles can enhance an immunosuppressive response even at a low antigen dose, while TMC-TPP nanoparticles enhance humoral immunity. As mentioned in the introduction both antigen and vaccine characteristic affect the induced type of the immune response. Our results confirm that particle and antigen combinations need to be carefully constructed to design successful vaccines that induce the preferred type of immune response. These findings may help the development of nanoparticle-based interventions to drive an antigen-specific immunomodulatory response and will enable future rational vaccine design for prophylactic and therapeutic vaccination, respectively in infectious diseases and autoimmune diseases.

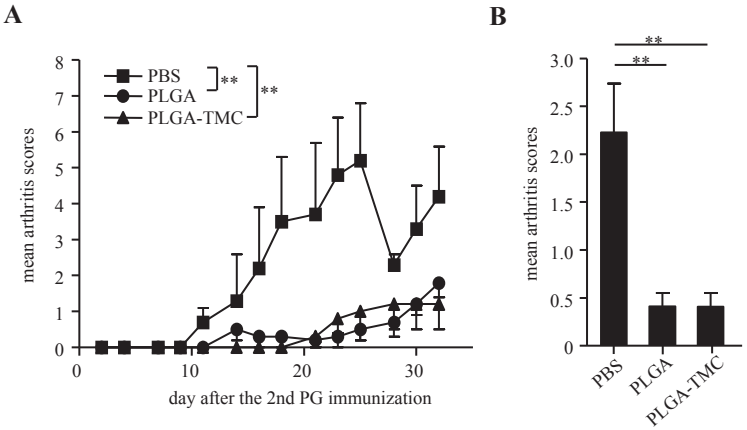

Figure 4 | Nasal application of low-dose mB29a-PLGA containing particles reduces severity of arthritis. A and B. Effect of mB29a-nanoparticles on nasally induced suppression of PG-induced arthritis in BALB/c mice. Mice received 30 µg of mB29a peptide i.n. dissolved in PBS or encapsulated in PLGA or PLGA-TMC nanoparticles prior to arthritis induction. Arthritis scores of mB29a-PBS (black squares), PLGA (black circles) or PLGA-TMC (black triangles) treated mice as assessed by swelling and redness of the paws. Data are shown as the mean arthritis scores ± SEM. of n=3 mice per group. Statistically significant: **, $P< 0.01$.

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PLGA nanoparticles enhance retinaldehyde dehydrogenase enzyme activity in dendritic cells and induce FoxP3$^+$ T-cells *in vitro*

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ABSTRACT

Many autoimmune diseases and other chronic inflammatory disorders are characterized by defective FoxP3$^+$ regulatory T-cell mediated suppression. A potential treatment option for these disorders is to increase the number and activity of regulatory T-cells locally. Both PLGA (poly-lactic-co-glycolic acid) and TMC-TPP (N-trimethyl chitosan tripolyphosphate) nanoparticles have been described to enhance T-cell activation upon nasal application. Since, PLGA and TMC-TPP nanoparticles differentially affect CD4$^+$ T-cell differentiation, we investigated *in vitro* the capacity of both delivery systems to trigger retinoic acid (RA) production in dendritic cells (DCs) as a strategy to enhance the induction of FoxP3$^+$ T-cells. We generated ovalbumin (OVA)-encapsulated PLGA and TMC-TPP nanoparticles that were similar in size (400 nm) but differed in their surface charge and other physico-chemical properties. We demonstrate that OVA-specific T-cells that are activated by CLN-derived DCs treated with PLGA or TMC-TPP nanoparticles show more FoxP3 expression than T-cells that are activated by ILN treated cells. We demonstrate that only OVA-loaded PLGA nanoparticles enhance the induction of FoxP3 in activated T-cells via a TGF-β and RA dependent mechanism by enhancing retinaldehyde dehydrogenase enzyme (RALDH) activity in CLN-derived DCs that is required for RA production. Additionally, detailed analysis of the CD4$^+$ T-cell response reveals that PLGA nanoparticles promote both IL-10 and IFN-γ production, while TMC-TPP nanoparticles enhance IL-4 and IL-17A expression by CD4$^+$ T-cells. Underlining that both APC origin and nanoparticle characteristics determine the expression level of FoxP3 in activated T-cells. In conclusion, our data suggest that PLGA nanoparticles enhance the induction of FoxP3$^+$ T-cells in the CLN through modulation of DC function and might be a very suitable nasal delivery system to treat a wide variety of autoimmune diseases and other chronic inflammatory disorders.

INTRODUCTION

Nasal delivery of vaccines is an attractive route of immunization given the induction of both systemic and mucosal immune responses[1, 2]. In the nose-draining lymphoid tissues including the cervical lymph nodes (CLN), dendritic cells (DCs) are essential regulators of mucosal immune responses, both responses against pathogens and the induction of tolerogenic responses to innocuous antigens[3]. The latter is associated with local accumulation of antigen-specific regulatory T-cells[4]. DCs sample their environment for antigens and are capable of presenting epitopes of these antigens on MHC class II (MHCII) molecules to activate naive $CD4^+$ T-cells[5]. $CD4^+$ T-cells that express T-cell receptors (TCR) that are specific for the presented antigenic peptide will undergo clonal expansion locally in the draining lymph nodes and subsequently differentiate into different subsets such as Th1, Th2, Th17 or regulatory T-cells[6]. $CD4^+$ T-cell differentiation into $FoxP3^+$ regulatory T-cells depends largely on the cytokines present in the local microenvironment and direct interaction with the DCs. For example, in oral tolerance, DCs that produce RA from retinol (vitamin A) in the presence of the cytokine TGF-β promote the induction of immune-suppressive $FoxP3^+$ regulatory T-cells in the perifery, while they inhibit T-cell differentiation into inflammatory Th17 cells[7, 8].

Nanoparticle-based vaccines can increase the uptake of antigens by DCs and thereby enhance the activation of antigen-specific $CD4^+$ T-cells *in vitro* and after nasal vaccine delivery to a greater extent than soluble antigen application. Moreover, nanoparticles not only enhance delivery of protein antigens, but can also skew the subsequently induced T-cell response and thereby at least partially modulate the immunological outcome of vaccination[9, 10]. Previously, we showed that both PLGA (poly-lactic-co-glycolic acid) and TMC-TPP (N-trimethyl chitosan tripolyphosphate) polymer-based antigen delivery systems were non-toxic to nasal epithelium as they did not significantly decrease the ciliary beat frequency[9]. In addition, PLGA and TMC-TPP nanoparticles differentially modulate the outcome of nasal vaccination. The TMC-TPP nanoparticles enhance humoral immunity in mice after nasal administration of influenza antigens[11], whereas PLGA nanoparticles enhance nasal and oral tolerance induction and protection against arthritis[10, 12]. Moreover, we previously showed that intranasal and intramuscular vaccination of mice with PLGA nanoparticles led to enhanced FoxP3 mRNA expression when compared to TMC-TPP vaccination[10].

Limited knowledge is present about the capacity of nanoparticles to modulate the function of DCs and the subsequent effect on $CD4^+$ T-cell differentiation. In this study, we investigate whether PLGA nanoparticles stimulate DCs to enhance the induction of $FoxP3^+$ T-cells irrespective of DC origin (mucosal versus non-mucosal). Hitherto, we generated ovalbumin (OVA)-encapsulated PLGA and TMC-TPP nanoparticles that were similar in size (400 nm), but differed in their surface charge and other physicochemical properties such as chemistry, zeta potential and antigen release profile[9]. Subsequently, we treated total lymph node cells isolated from the CLN and inguinal lymph nodes (ILN) with PLGA or TMC-TPP nanoparticles to address Foxp3 expression as a marker for regulatory T-cell induction

in OVA-specific $CD4^+$ T-cells. Because DCs derived from mucosal draining lymph nodes such as the CLN in general potentiate nasal tolerance induction, we also explored how nanoparticle interaction with CLN-derived $MHCII^+CD11c^+$ DC-like subsets affected the capacity of DCs to modulate expression of FoxP3 in OVA-specific T-cells. The metabolic conversion of retinol to RA in DCs, which drives regulatory T cell differentiation, requires RALDH enzymes[13]. We investigated if PLGA and TMC-TPP nanoparticles differentially induced RALDH expression in DCs and how this affected intracellular cytokine production in activated $CD4^+$ T-cells and their differentiation into $FoxP3^+$ T-cells.
This study was performed to gain insight into the underlying mechanism that drives mucosal tolerance induction after nasal PLGA nanoparticle vaccination. Here, we show that PLGA and TMC-TPP nanoparticles differentially modulate RALDH activity in DCs and how this affects subsequent $CD4^+$ T-cell differentation into $FoxP3^+$ T-cells. The data may assist future rational design of nasal vaccines for the treatment of autoimmune diseases and other chronic inflammatory disorders for which enhanced $FoxP3^+$ regulatory T-cell activation is required to dampen unwanted inflammatory immune responses.

MATERIALS AND METHODS

Mice
Female BALB/c mice (8-16 weeks) were purchased from Charles River Laboratories (Maastricht, The Netherlands). OVA-specific TCR transgenic (Tg) mice on BALB/c background (DO11.10 mice), were bred at the Central Animal Laboratory (GDL), University Utrecht, Utrecht, The Netherlands. Experiments were approved by the Animal Experimental Committee of the University Utrecht, Utrecht, The Netherlands.

Nanoparticle materials
Endotoxin-low ovalbumin protein (OVA) was purchased from Worthington Biochemical Corporation, USA. N-trimethyl chitosan with a degree of quaternization of 17% was obtained from 92% deacetylated (MW 120 kDa) chitosan (Primex, Norway), by NaOH induced methylation as described by Sieval[14]. Poly(lactic-co-glycolic-acid) (PLGA) 50:50 Mw 5000-15000 Da, pentasodium tripolyphosphate (TPP), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), dichloromethane were obtained from Sigma-Aldrich (-Germany) (glycerol was purchased from Sigma and polyvinyl alcohol (PVA) 4-88 (31kDa) was purchased from Fluka.

Nanoparticle preparation, size and zeta potential
PLGA nanoparticles were prepared by a "water-in-oil-in-water" emulsion with solvent evaporation method and TMC nanoparticles were prepared by ionic complexation with TPP as described previously[15]. For PLGA nanoparticle preparation, 50 µl of 10 mg/ml of OVA in 25 mM Hepes pH 7.4 was emulsified with 1 ml of dichloromethane containing 50 mg of PLGA with an ultrasonic processor for 30 sec at 20 W (Branson Instruments, USA).

The secondary emulsion was prepared with 2 ml of 1% (w/v) PVA in water, under sonication (30 sec, 20 W). The double emulsion was then poured into 25 ml of a 0.3% PVA aqueous solution and stirred at 40°C for 1 h. The nanoparticle suspension was then washed twice with water by centrifugation at 8000 g for 10 min. PLGA nanoparticles were freeze-dried and stored at -20°C. For TMC-TPP nanoparticle preparation, 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. A TPP solution (1 mg/ml) was added under continuous stirring to a weight ratio TMC:TPP:OVA of 10:0.9:1. Nanoparticles were collected by centrifugation (15 min at 15000 x g) on a glycerol bed to avoid aggregation. Nanoparticles were dispersed in MiliQ water and stored at 4°C. The hydrodynamic diameter (Z-average and polydispersity index (PDI)) and the zeta potential were determined with a Nanosizer (Malvern Instruments, UK) by dynamic light scattering and laser Doppler electrophoresis, respectively. For that purpose, nanoparticle suspensions were diluted 1:20 in 5 mM Hepes pH 7.4 until a slightly opalescent dispersion was obtained. Encapsulated protein concentration was determined by BCA protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. For that purpose, particles 100 µL nanoparticle suspension (containing 5 mg PLGA or 1 mg TMC) were extracted as described[16]. Encapsulation efficiency (%EE) was calculated according to equation 1 and drug loading (%DL) by equation 2.

$$(1)\quad \%\,EE = \frac{\text{encapsulated protein mass}}{\text{total protein mass}} \times 100$$

$$(2)\quad \%\,DL = \frac{\text{encapsulated protein mass}}{\text{total polymer + protein mass}} \times 100$$

Isolation of lymph node APCs by cell sorting

The deep and superficial CLN and the ILN were isolated from BALB/c mice. Single cell suspensions were prepared by mincing the lymph nodes and straining them through a 70 µm cell strainer. Erythrocytes were depleted from this cell suspension by incubation in ACK lysis buffer (150 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM EDTA, pH 7.4) for 2 min on ice. The cells were resuspended in Iscove's Modified Dulbecco's Medium (IMDM) that contains $5x10^{-5}$ M 2-mercaptoethanol, penicilin (100 units/ml) and streptomycin (100 µg/ml) (Gibco, Germany), 5% FBS (fetal bovine serum, Lonza). Total LN cells (10^5) were used as APC directly or the cells were stained for anti-CD11c-APC (HL3), anti-CD11b-efluor450 (M1/70) and anti-MHCclassII-AF700 (I-A/I-E; M5/114.15.2) on ice in the dark for 30 min (all monoclonal antibodies from Ebioscience), washed and FACS-sorted into $MHCII^+CD11b^-CD11c^+$ or $MHCII^+$ $CD11b^+CD11c^+$ DC-like subsets and used as APC ($5x10^3$) for *in vitro* co-cultures with nanoparticles at final concentrations of 5-10 µg/ml of OVA and $2x10^5$ CFSE-labeled OVA-specific $CD4^+$ T-cells. Cell sorting was performed on a BD Influx cell sorter with a nozzle diameter of 100 µm.

$CD4^+$ T-cell enrichment and CFSE labeling

Spleens were isolated from DO11.10 donor mice and were prepared into single cell suspensions, followed by erythrocyte depletion, as described earlier. $CD4^+$ T-cells were obtained by negative selection with sheep-anti-rat IgG Dynabeads (Dynal, Invitrogen, The Netherlands) using an excess amount of anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-MHC class II (M5/114), anti-CD8 (YTS169). Labeling of cells with carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, The Netherlands) was performed as previously described[4].

Intracellular cytokine and FoxP3 staining

Cells were restimulated with 1 µg/ml of anti-CD3 (145.2C11 hamster-anti-mouse) and 0.5 µg/ml of anti-CD28 (PV1.17-10, home-made) for 45 min at 37°C and brefeldin A (Sigma) was added at a final concentration of 1 µg/ml for 4 h. For intracellular cytokine and FoxP3 staining, the cells were washed twice with FACS buffer and incubated with anti-DO11.10 TCR (KJ1.26; Invitrogen, The Netherlands), anti-CD4 (RM4-5) and anti-CD25 (PC61.5) and incubated in the dark on ice for 30 min (antibodies from Ebioscience). The cells were washed with FACS buffer and each cell pellet was resuspended in 100 µl of fixation/permeabilization buffer at an 1:3 ratio (Ebioscience) and cells were incubated o/n in the dark at 4°C. The cells were washed 3 times with 1x perm wash buffer (Ebioscience) and mouse Fc block (2.4G2) was added. After 15 min of incubation on ice, anti-IL-10-APC (JES5-16), anti-IFN-γ-PE (XMG1), anti-IL-17A-APC (eBio17), anti-FoxP3-PE (antibodies from BD Biosciences) and anti-IL-4-PE (11B11) (Ebioscience), were added and cells were incubated on ice in the dark for 30 min. The cells were washed 4 times with 1x perm wash buffer and analyzed with a FACSCanto II flow cytometer (BD Biosciences) and FlowJo Software V8.8.6.

DC culture

Bone marrow-derived dendritic cells (BMDC) were cultured from BALB/c donor mice as previously described[10]. Briefly, femurs and tibia of adult BALB/c mice were flushed with culture medium. Single cell suspensions were seeded in IMDM supplemented with $5x10^{-5}$ M 2-mercaptoethanol, penicilin (100 units/ml) and streptomycin (100 µg/ml) (Gibco, Germany), 10% FBS (fetal bovine serum, Lonza), 20 ng/ml murine rGM-CSF (Cytogen, The Netherlands). On day 2 and 4, 10 ng/ml murine rGM-CSF was added. On day 8, 70%-80% of the BMDCs expressed both MHCII (anti-MHC class II-PE, M5/114) and CD11c (anti-CD11c-APC, HL3), (antibodies from BD Pharmingen, The Netherlands) and were used for *in vitro* experiments.

BMDC aldefluor assay after nanoparticle treatment

BMDCs (10^5) were cultured in 96-wells flat-bottom plates in the presence of PLGA-OVA, TMC-TPP-OVA, soluble OVA (sOVA) at a final concentration of 5 µg/ml of OVA or in complete IMDM at 37°C. After 48 h, RALDH activity in individual cells was measured using ALDEFLUOR staining kits (StemCell Technologies, France), according to the

manufacturer's protocol with minor modifications. Briefly, cells suspended at 10^6 cells per ml in ALDEFLUOR assay buffer containing activated ALDEFLUOR substrate (365 nM) with or without the RALDH inhibitor diethylaminobenzaldehyde (DEAB, 7.5 μM) were incubated in the dark in a water bath at 37°C for 45 min. After incubation, the cells were washed with ALDEFLUOR assay buffer and resuspended in FACS buffer (PBS and 2% FBS). Cells were analyzed with a FACSCanto II flow cytometer (BD Biosciences) and FlowJo Software V8.8.6.

***In vitro* retinol experiments**

For the retinol supplemented cultures, $2x10^4$ BMDCs, $5x10^3$ sorted $CD11b^-CD11c^+MHCclassII^+$ or $CD11b^+CD11c^+MHCclassII^+$ cells were cultured in a volume of 200 μl of complete IMDM supplemented with 50 nM or 25 nM retinol (Fluka, Sigma-Aldrich, Zwijndrecht, The Netherlands) and 2 ng/ml human rTGF-β1 (PeproTech) and PLGA, TMC-TPP, sOVA at final concentrations of 5, 10 or 100 μg/ml of OVA. After 1 h of culture at 37°C, $2x10^5$ OVA-specific $CD4^+$ T-cells labeled with CFSE were added and cells were cultured for 5 days at 37°C.

Statistics

Statistical analysis was performed with GraphPad Prism software Inc. (version 5.0c). For *in vitro* analysis of RALDH expression one-way ANOVA (two-tailed) with Bonferroni correction was used. Error bars represent the standard error of the mean. Statistical differences for the mean values are indicated as follows: *, $P<0.05$.

RESULTS

Nanoparticle characteristics

OVA was encapsulated into PLGA and TMC-TPP nanoparticles with an average size of about 400 nm (Table 1). TMC-TPP nanoparticles showed a higher tendency to form aggregates than PLGA nanoparticles as is shown by the relatively high PDI of the TMC-TPP nanoparticles. Because aggregate formation might affect the experimental outcome, the TMC-TPP nanoparticles were ultrasonicated for 15 sec before use to remove aggregates. Brief sonication does not affect the OVA encapsulation and native structure of the nanoparticles as shown previously[9]. PLGA nanoparticles carried a negative charge, while TMC-TPP nanoparticles were positively charged at physiological pH. OVA was more efficiently encapsulated into PLGA than into TMC-TPP nanoparticles, though the latter achieved higher drug loading (Table 1).

CLN cells treated with PLGA nanoparticles enhance the expression of FoxP3 in OVA-specific $CD4^+$ T-cells

We previously showed that PLGA and not TMC-TPP nanoparticles enhanced the expression of FoxP3 mRNA in cells isolated from the CLN and the non-mucosal ILN

after nasal or intramuscular immunization, respectively[10]. However, we observed that the expression of FoxP3 mRNA was higher in cells isolated from the CLN compared to ILN-derived cells. To investigate whether the differences in enhanced Foxp3 mRNA induced by PLGA was due to the origin of the APC, we treated total CLN or ILN cells *in vitro* with OVA encapsulated in PLGA and TMC-TPP nanoparticles and studied subsequent OVA-specific $CD4^+$ T-cell differentiation into $FoxP3^+$ T-cells. Total FoxP3 expression was enhanced in the OVA-specific $CD4^+$ T-cell population when these cells

Table 1 | Physical properties of OVA-loaded nanoparticles

Nanoparticles	Size (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency (%)	Drug loading (%)
PLGA	371 ± 17.9	0.2 ± 0.0	-16.8 ± 4.7	66 ± 3.8	0.84 ± 0.16
TMC-TPP	400 ± 9.4	0.5 ± 0.1	17.0 ± 1.6	27.8 ± 5.0	2.43 ± 0.44

Data represent the mean of 3 (PLGA) and 5 (TMC-TPP) independently prepared batches ± standard error of the mean. Measurements were performed in 5mM Hepes pH 7.4

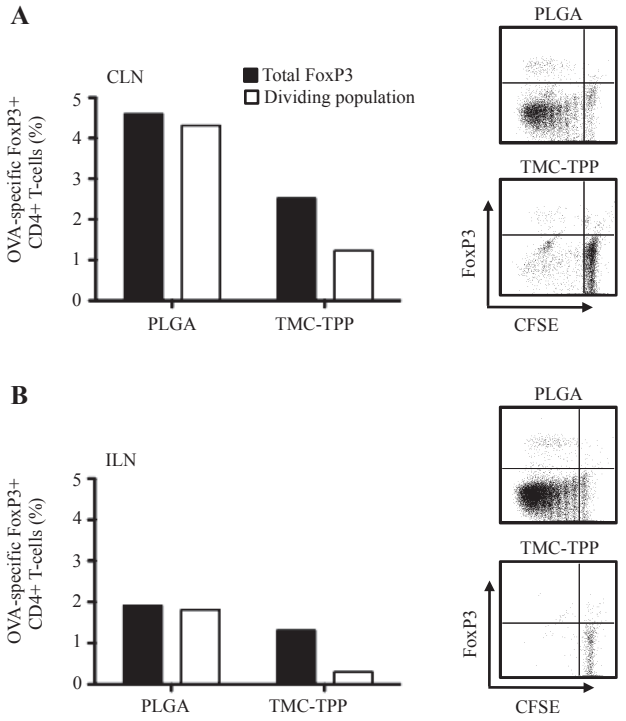

Figure 1 | CLN cells treated with PLGA nanoparticles enhance the expression of FoxP3 by $CD4^+$ T-cells. CLN (A) and ILN (B) cells were cultured in the presence of PLGA-OVA and TMC-TPP-OVA and CFSE-labeled $CD4^+$ T-cells (DO11.10) and after 3 days, FoxP3 expression of OVA-specific T-cells was assessed. Data are representative for 1 experiment with pooled CLN and ILN cells from 13 mice. Data are shown as FACS plots or total $FoxP3^+$ cells or as the dividing OVA-specific $FoxP3^+$ T-cell population.

were activated by CLN (figure 1A) or ILN (figure 1B) cells that were treated with PLGA nanoparticles and much less by cells treated with TMC-TPP. Moreover, PLGA enhanced the expression of FoxP3 in the dividing T-cell population to a greater extent than TMC-TPP nanoparticles. In addition, T-cells activated by CLN cells treated with PLGA or TMC-TPP nanoparticles showed more FoxP3 expression than T-cells that were activated by ILN treated cells (Figure 1). In summary, both APC origin and nanoparticle characteristics determine the expression level of FoxP3 in activated T-cells.

DC subsets treated with PLGA nanoparticles enhance the expression of FoxP3 in activated T-cells

PLGA treated CLN-derived DC subsets enhanced the expression of FoxP3 in antigen specific T-cells (previous section). To verify whether this was DC mediated, we FACS-sorted MHCII$^+$CD11b$^-$CD11c$^+$ and MHCII$^+$CD11b$^+$CD11c$^+$ DC-subsets (from now on referred to as CD11c$^+$ and CD11b$^+$CD11c$^+$, respectively, figure 2A) isolated from CLN and ILN and characterized the DC subsets that enhanced the expression of FoxP3 in T-cells after nanoparticle treatment. The CD11c$^+$ and CD11b$^+$CD11c$^+$ DC subsets isolated from CLN and ILN were treated with OVA-encapsulated PLGA, TMC-TPP nanoparticles or soluble OVA (sOVA) as a control and we measured subsequent OVA-specific FoxP3$^+$ T-cell differentiation. FoxP3 expression was enhanced in the dividing OVA-specific CD4$^+$ T-cell population when these cells were activated by PLGA treated CD11c$^+$ and CD11b$^+$CD11c$^+$ DCs isolated from the CLN (figures 2B-2C) and ILN (figures 2D-2E). TMC-TPP treated CD11c$^+$ and CD11b$^+$CD11c$^+$ DCs isolated from the CLN (figures 2B-2C) and ILN (figures 2D-2E) did not enhance the FoxP3 expression in OVA-specific CD4$^+$ T-cells. In addition, the data also showed that the expression of FoxP3 in T-cells was higher with PLGA treated CD11c$^+$ DCs compared to the CD11b$^+$CD11c$^+$ DCs, which suggests that especially PLGA nanoparticle interaction with the CD11c$^+$ DCs enhanced the expression of FoxP3 in activated OVA-specific T-cells.

PLGA nanoparticles enhance RALDH enzyme activity in BMDCs

Thus far, we showed that DC activation by OVA-loaded PLGA nanoparticles promoted the expression of FoxP3 in activated OVA-specific T-cells. Mucosal DCs can induce FoxP3$^+$ regulatory T-cells via a TGF-β and RA-dependent mechanism. The metabolic conversion of retinol to RA requires the expression of RALDH enzymes in DCs. Therefore, we first investigated *in vitro* if PLGA nanoparticles can modulate the function of DCs by enhancing RALDH activity. MHCII$^+$CD11c$^+$ BMDCs were treated with OVA-encapsulated PLGA and TMC-TPP nanoparticles or sOVA as a control. The cells were subsequently treated with active ALDEFLUOR substrate with or without the RALDH inhibitor DEAB. Representative flow cytometry plots show the gating strategy to identify the cell population with RALDH enzyme activity (figure 3A). In contrast to sOVA and medium controls, BMDC treatment with PLGA nanoparticles showed enhanced RALDH activity, while treatment with TMC-TPP nanoparticles decreased RALDH expression in the BMDCs (figure 3B).

RALDH$^+$ BMDCs induce FoxP3$^+$ T-cells in a TGF-β and RA-dependent mechanism
Next, we explored if BMDCs that express RALDH also induced FoxP3 expression in OVA-specific CD4$^+$ T-cells in a TGF-β and RA-dependent manner. To investigate this, we treated BMDCs with sOVA in medium supplemented with retinol, TGF-β or a combination of TGF-β and retinol. Representative flow cytometry plots are shown for the different culture conditions (figure 4A) and the percentages of FoxP3 expression in the total and dividing OVA-specific CD4$^+$ T-cell population are shown in figure 4B. BMDC treatment with only low TGF-β or 25nM of retinol did not induce FoxP3 in OVA-specific CD4$^+$ T-cells. However, a combination of retinol and low TGF-β treatment enhanced the induction of FoxP3 in OVA-specific CD4$^+$ T-cells compared to medium control (Figure 4A-B). In summary, the data show that RALDH$^+$ BMDCs induce FoxP3$^+$ T-cells through a TGF-β and RA dependent mechanism.

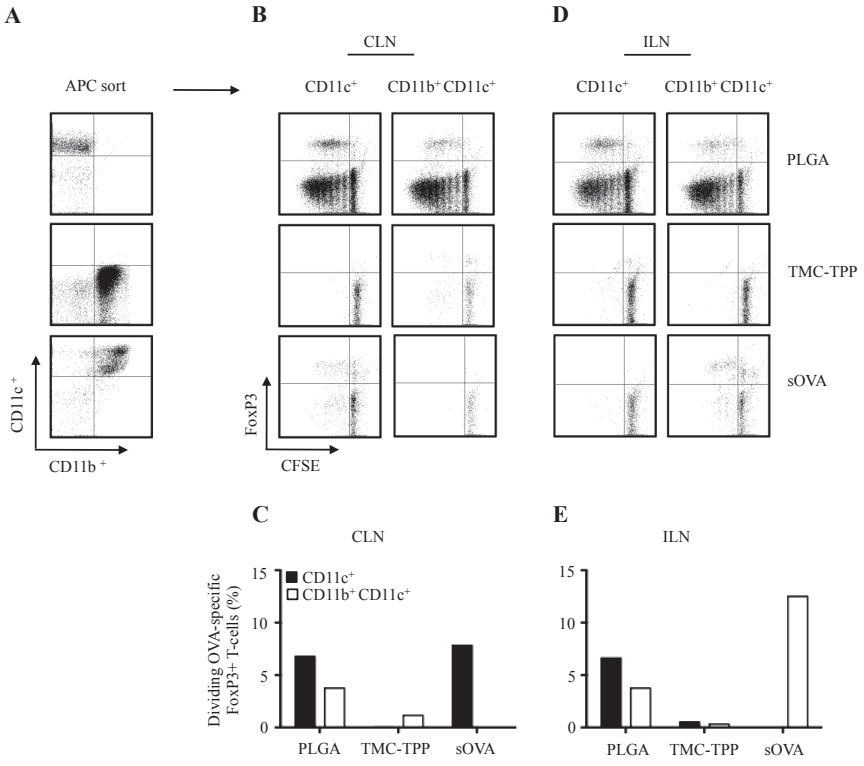

Figure 2 | CD11c$^+$ DC subsets treated with PLGA nanoparticles enhance the expression of FoxP3 by CD4$^+$ T-cells
CD11c$^+$ and CD11b$^+$CD11c$^+$ sorted cells (A) isolated from CLN (B and C) and ILN (D and E) were treated with PLGA-OVA, TMC-TPP-OVA and sOVA and CFSE-labeled CD4$^+$ T-cells (DO11.10) and after 5 days, FoxP3 expression of OVA-specific T-cells was assessed. Representative FACS plots are shown for CLN (B) and ILN (D) cocultures and percentages of FoxP3 positive T-cells of the dividing population with subsets isolated from CLN (C) and ILN (E). Data are representative for one experiment with pooled CLN and ILN cells from 13 mice.

PLGA nanoparticles enhance IFN-γ and IL10 expression, while TMC-TPP nanoparticles promote IL-4 and IL-17A expression by T-cells

Thus far, we showed that PLGA nanoparticles enhanced the expression of RALDH enzymes in BMDCs (figure 3B) and that RALDH$^+$ DCs enhance the induction of FoxP3$^+$ T-cells via a TGF-β and RA dependent mechanism (figure 4). RA in combination with TGF-β is known to promote the induction of immune-suppressive FoxP3$^+$ regulatory T-cells, while a high dose of RA alone can inhibit T-cell differentiation into inflammatory Th17 cells[8]. Our data suggest that PLGA nanoparticles stimulate DCs to produce RA from retinol to induce FoxP3$^+$ T-cells, while TMC-TPP nanoparticles decreased RALDH expression in DCs and therefore most likely also RA production and probably promote T-cell differentiation into Th17 cells. To investigate this, we treated BMDCs with OVA-encapsulated PLGA and TMC-TPP nanoparticles or sOVA as a control in medium alone or supplemented with retinol and TGF-β. Detailed analysis of intracellular cytokine production showed that only BMDCs treated with PLGA nanoparticles enhanced the expression of intracellular IFN-γ in activated OVA-specific CD4$^+$ T-cells (figure 5A). In contrast, BMDCs treated with TMC-TPP enhanced the expression of IL-4 (figure 5B) and IL-17A (figure 5C) cytokines in activated OVA-specific CD4$^+$ T-cells. Both BMDCs treated with PLGA or TMC-TPP slightly induced IL-10 expression (figure 5D). The presence of retinol did not affect the intracellular cytokine production in OVA-specific CD4$^+$ T-cells. In conclusion, PLGA nanoparticles promoted FoxP3$^+$ T-cell differentiation and intracellular IL-10 and IFN-γ cytokine production and inhibited T-cell differentiation into Th17 cells, which was not seen after TMC-TPP nanoparticle treatment. Conversely, we showed that TMC-TPP nanoparticles promoted subsequent T-cell differentiation into Th17 cells, most likely through inhibition of RA production by BMDCs.

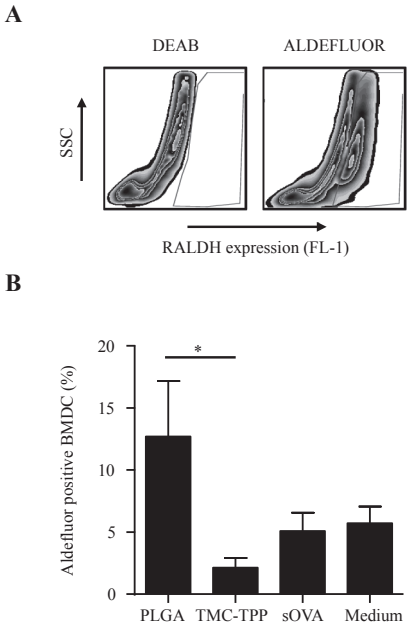

Figure 3 | PLGA nanoparticles enhance the activity of RALDH enzymes in BMDCs. BMDCs were treated with PLGA-OVA, TMC-TPP-OVA and sOVA or medium as a control for 48 h and analyzed for RALDH activity using ALDEFLUOR assay. Representative FACS plots are shown for ALDEFLUOR signal and DEAB inhibitor (A) and percentages of ALDEFLUOR positive cells above DEAB background (B). Data are representative for 5 independent experiments; mean ± s.e.m. Statistically significant *, P<0.05.

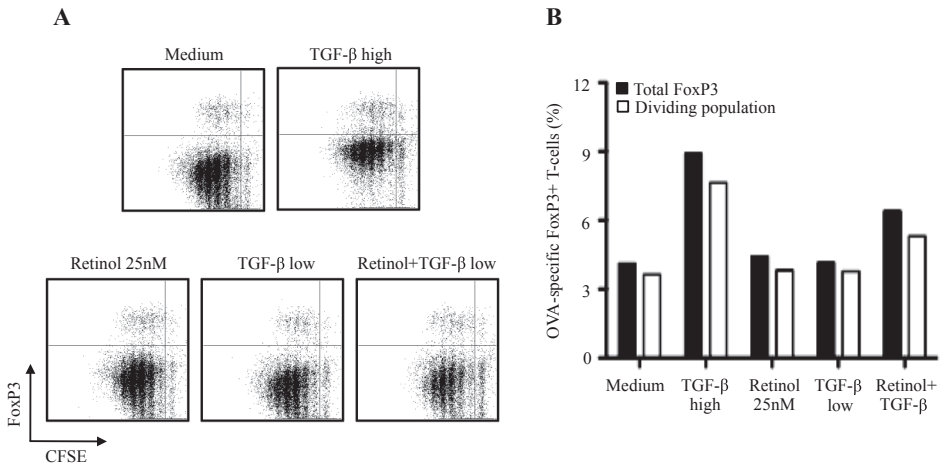

Figure 4 | RALDH$^+$ BMDCs enhance FoxP3 expression in CD4$^+$ T-cells via a TGF-β and RA dependent mechanism. BMDCs were treated with sOVA (100 μg/ml) in medium only or supplemented with human TGF-β low, retinol 25nM or a combination or TGF-β high as a positive control and CFSE-labeled CD4$^+$ T-cells (DO11.10). After 5 days, FoxP3 expression of OVA-specific T-cells was assessed. Representative FACS plots are shown (A) and cell percentages of FoxP3 positive T-cells (B). Data are representative for one *in vitro* experiment.

PLGA nanoparticles enhance the capacity of CLN-derived DCs to induce FoxP3$^+$ T-cells via a TGF-β and RA-dependent mechanism

We demonstrated that both PLGA interaction with CLN-derived CD11c$^+$ DCs and RALDH activity in BMDCs enhance the induction of FoxP3$^+$ T-cells. Therefore, we hypothesized that PLGA nanoparticles enhance the expression of RALDH in CLN-derived CD11c$^+$ DCs, which enables them to produce RA that in combination with TGF-β induces FoxP3 expression in OVA-specfic CD4$^+$ T-cells. To investigate this, CLN-derived CD11c$^+$ and CD11b$^+$CD11c$^+$ DCs were FACS sorted and treated with OVA-encapsulated PLGA, TMC-TPP nanoparticles or sOVA as a control in medium alone or supplemented with retinol and TGF-β. Representative flow cytometry plots are shown for the CD11c$^+$ DCs (figure 6A) and CD11b$^+$CD11c$^+$ DCs (figure 6C) and the percentages of FoxP3 expression in the dividing OVA-specific CD4$^+$ T-cell population are shown for the CD11c$^+$ DCs (figure 6B) and CD11b$^+$CD11c$^+$ DCs (figure 6D) for the different treatments, as indicated. Only PLGA nanoparticles enhanced the capacity of CLN-derived CD11c$^+$ DCs and CD11b$^+$CD11c$^+$ DCs to induce TGF-β and RA dependent FoxP3 expression in OVA-specific CD4$^+$ T-cells (figure 6). Moreover, the CD11c$^+$ DCs were superior to the CD11b$^+$CD11c$^+$ DCs in inducing FoxP3$^+$ T-cells. In summary, we suggest that, PLGA and TMC-TPP nanoparticles differentially induced RALDH expression in CLN-derived CD11c$^+$ DCs and that specifically PLGA nanoparticle interaction with the CLN-derived CD11c$^+$ DCs promoted subsequent FoxP3$^+$ T-cell induction in a TGF-β and RA dependent fashion.

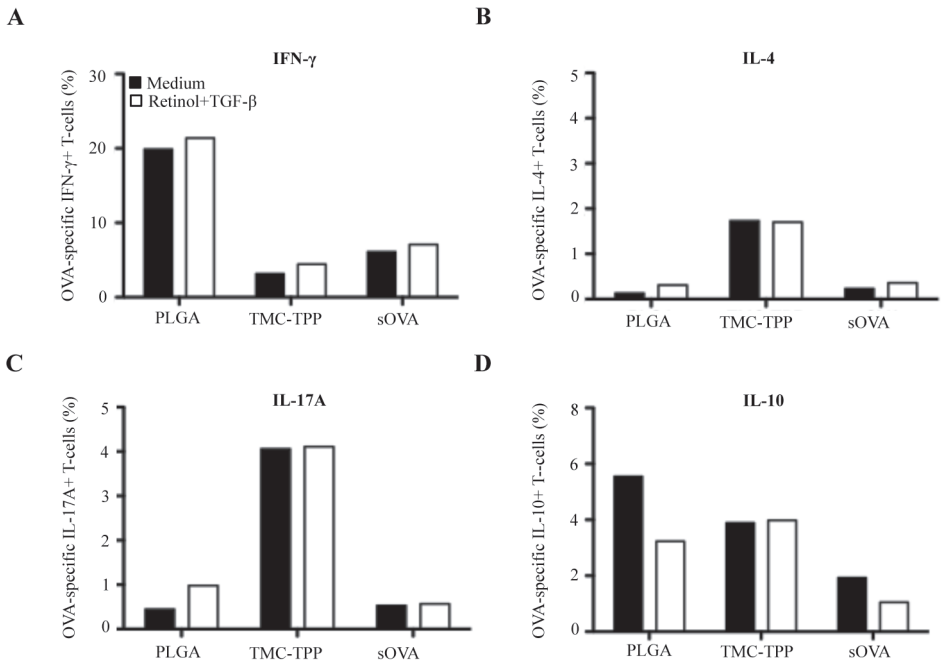

Figure 5 | PLGA nanoparticles induce IFN-γ and IL10 production, while TMC-TPP treatment promotes IL-4 and IL-17 expression. BMDCs were treated with PLGA-OVA, TMC-TPP-OVA and sOVA or medium as a control in the presence of retinol 25nM and human TGF-β low and CFSE-labeled $CD4^+$ T-cells (DO11.10). After 5 days, the cells were restimulated with anti-CD3 and anti-CD28, followed by Brefeldin A treatment for 4 h. Data represent the precentages of T-cells positive for intracellular cytokine signals of IFN-γ (A), IL-4 (B), IL-17A (C) and IL-10 (D). Data are representative for one *in vitro* experiment.

DISCUSSION

Previously, we showed that PLGA and TMC-TPP nanoparticles not only enhance the delivery of antigens to APCs, but also differentially skew the subsequent immune response and modulate the outcome of nasal vaccination[9, 10]. Moreover, PLGA but not TMC-TPP nanoparticles enhanced nasal tolerance induction by accumulation of $FoxP3^+$ T-cells in the local dLNs[10]. Literature describes that the tolerogenic characteristics of the nose-draining CLNs are essential for nasal tolerance induction[17], however, mechanisms are not well known. Because DCs are known to be essential regulators of mucosal immune responses we explored how PLGA and TMC-TPP nanoparticles can differentially modulate DC function to enhance or break immune tolerance.

In figure 1, we showed that both APC origin (CLN vs ILN) and nanoparticle characteristics determine the expression level of FoxP3 in activated T-cells. However, when DC subsets were FACS-sorted from the CLN and ILN, we did not observe a difference in FoxP3 expression when DC subsets were treated with PLGA nanoparticles (figure 2). We suggest

that *in vivo* the ratio of $CD11c^+$ and $CD11b^+CD11c^+$ in the CLN an ILN might be different from the *in vitro* situation. As we used a similar concentration of DCs of a single subset for *in vitro* nanoparticle stimulation this might explain why ILN-derived cells enhance Foxp3 expression in activated T-cells in a similar way as CLN-derived cells do.

There is currently not much known about the interaction of TMC-TPP nanoparticles with DCs. We previously showed that although OVA-encapsulated TMC-TPP nanoparticles are poorly taken up by human DCs (hDCs) they still significantly enhanced MHCII, CD83 and CD86 costimulatory molecule expression on the cell surface of hDCs compared to sOVA alone[9]. Mechanisms of action of PLGA nanoparticles have been studied more extensively both *in vitro* and *in vivo* than those of TMC-TPP nanoparticles. After PLGA nanoparticles have been taken up by DCs, they slowly release encapsulated antigen and this specific PLGA characteristic might favor the induction of nasal/mucosal tolerance,

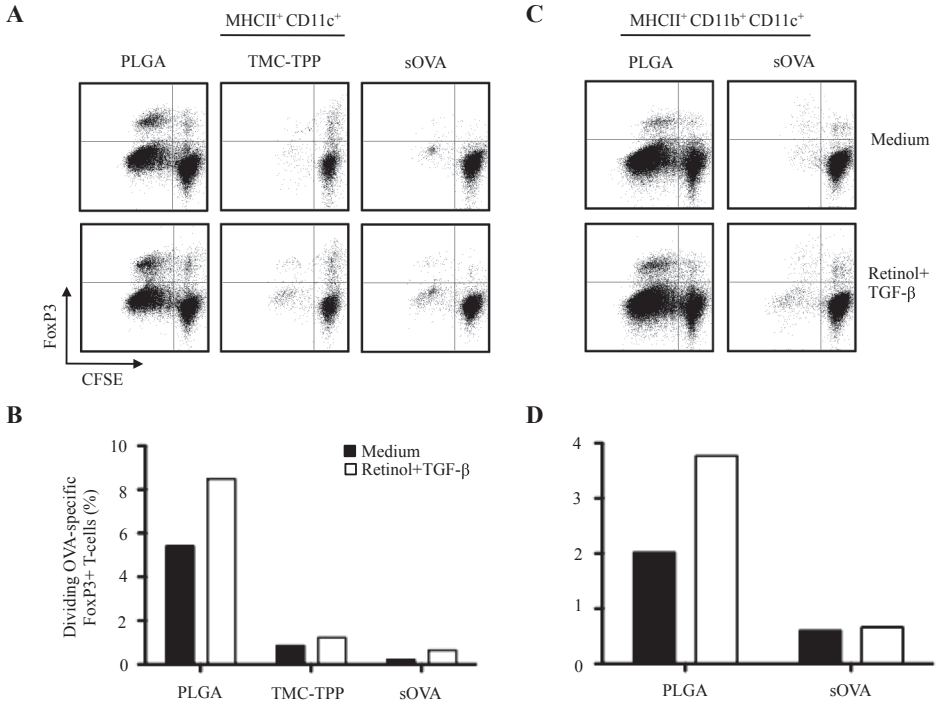

Figure 6 | PLGA nanoparticles enhance the capacity of CLN-derived DCs to induce FoxP3 expression in T-cells via a TGF-β and RA dependent mechanism. BMDCs were treated with PLGA-OVA, TMC-TPP-OVA and sOVA (5μg/ml) or medium as a control in the presence of retinol 50nM and human TGF-β low and CFSE-labeled $CD4^+$ T-cells (DO11.10). After 5 days, FoxP3 induction was assessed by flow cytometry. Representative FACS plots are shown for $CD11c^+$ subsets (A) and $CD11b^+CD11c^+$ subsets (C) and cell percentages of the FoxP3 positive dividing T-cell population cultured with $CD11c^+$ subsets (B) and $CD11b^+CD11c^+$ subsets (D). Data are representative for one *ex vivo* experiment.

as it has been shown that nasal tolerance can be induced by repeated application of low doses of free antigen[18]. TMC-TPP nanoparticles degrade fast under physiological conditions and therefore antigen is released more rapidly compared to PLGA nanoparticles[9, 10]. PLGA nanoparticles can prolong the expression of MHCI and MHCII molecules on the cell surface of DCs, while they do not significantly enhance maturation of DCs[19], describing another characteristic of PLGA nanoparticles that modulates DC phenotype and function to enable them to enhance mucosal tolerance induction.

This study describes a new functional characteristic of PLGA nanoparticles, which may explain their capacity to enhance nasal tolerance. We showed that the major important difference between the effects of PLGA and TMC-TPP nanoparticles appeared to be the induction of RALDH expression in DCs. PLGA nanoparticles significantly enhanced RALDH expression in BMDCs, whereas TMC-TPP nanoparticles decreased RALDH expression in BMDCs (figure 3B). We also showed that this difference in RALDH expression modulated $CD4^+$ T-cell differentiation, as DCs treated with PLGA nanoparticles enhanced not only T-cell activation but also the expression of FoxP3 in OVA-specific $CD4^+$ T-cells via a TGF-β and RA dependent mechanism (figure 6). Moreover, PLGA nanoparticle-induced RA production by DCs inhibited T-cell differentiation into inflammatory Th17 cells, while TMC-TPP-induced downregulation of RA production promoted the differentiation into Th17 cells (figure 5). The reciprocal Th17 and Treg differentiation mediated by RA is in accordance with the literature[8].

The specific physicochemical characteristics of PLGA and TMC-TPP nanoparticles determine the outcome of the immune response[10]. The particle size is one of the parameters that is most adequately described to modulate immune responses[20]. However, previous studies also showed that intranasally applied antigen-loaded PLGA nanoparticles of different sizes (200, 500 and 1000 nm) induced similar immune responses[21]. As the OVA-encapsulated PLGA and TMC-TPP nanoparticles in our study had a similar mean hydrodynamic diameter of approximately 400 nm (table 1), we assumed that size variation between the particles is most likely not the factor that caused the differences between RALDH induction in DCs and subsequent $CD4^+$ T-cell differentiation.
We suggest that the interaction of nanoparticles with CLN-derived DC subsets plays an important role in the outcome of the immune response. We showed that PLGA nanoparticles are more potent in targeting CLN-derived DCs compared to TMC-TPP (figures 2 and 6). Although the positively charged TMC-TPP nanoparticles should enhance the interaction with the negatively charged cell membranes of DCs and subsequent antigen uptake due to favourable electrostatic interactions[22, 23], we suggest that the slow antigen release kinetics of PLGA nanoparticles, together with a lower expression of costimulation and enhanced induction of RALDH expression in DCs ([9] and figure 3) modulates the phenotype and function of DCs, which enhances their capacity to induce antigen-specific $FoxP3^+$ T-cells and tolerogenic immune responses.

CONCLUSION

PLGA nanoparticles significantly increase the expression of RALDH in DCs and enhance the induction of FoxP3 expression in antigen-specific $CD4^+$ T-cells via a TGF-β and RA dependent mechanism. We propose that nasal vaccination with PLGA nanoparticles induces $FoxP3^+$ T-cells in the CLN through the induction of RALDH in CLN-derived DCs. Since the functional impairment of $FoxP3^+$ T-cells is the hallmark of a wide variety of autoimmune diseases, PLGA nanoparticles may turn out to be very suitable as nasal therapeutic vaccine delivery system to treat autoimmune diseases and other chronic inflammatory disorders. TMC-TPP nanoparticles are more potent delivery systems in the field of vaccination against infectious diseases when humoral immune responses and inflammatory Th17 cells are needed.

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Heat shock proteins are therapeutic targets in autoimmune diseases and other chronic inflammatory conditions

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ABSTRACT

Introduction

Exploitation of antigen-specific regulatory T-cells (Tregs) as critical regulators in the control of chronic inflammatory diseases is hampered by the obscure nature of most disease-relevant autoantigens. Heat shock proteins (Hsp) are possible targets for Tregs due to their enhanced expression in inflamed (stressed) tissues and there is evidence that Hsp can induce anti-inflammatory immunoregulatory T-cell responses.

Areas covered

Recent publications showing that exogenous administration of stress proteins has induced immunoregulation in various models of inflammatory disease and has also been shown to be effective in first clinical trials in humans. Now, also in the light of a growing interest in T-cell regulation, it is of interest to further explore the mechanisms through which Hsp can be utilized to trigger immunoregulatory pathways, capable of suppressing such a wide and diversified spectrum of inflammatory diseases.

Expert opinion

Therapeutic approaches via exploitation of antigen specific Tregs will benefit from tailor-made combination therapies. Combining current therapeutic approaches with Hsp specific therapies enhancing natural immune regulation might expedite the entry of antigen-specific Tregs into the therapeutic arsenal of the anti-inflammatory therapeutics.

INTRODUCTION

Breakdown of immunoregulatory mechanisms can lead to loss of self tolerance and autoimmune disease, coinciding with chronic inflammation and tissue damage. The target organ may differ between autoimmune diseases, but basic features of failing regulatory systems and chronic inflammation are shared between the various diseases[1]. Many existing therapies in autoimmune diseases are based on general suppression of inflammation and the side effects observed with these therapies illustrate the pressing need for more specific interventions. Regulatory T-cells (Tregs) are pivotal controllers of autoaggressive immune responses and inflammation, and decreased Treg numbers and/or functioning have been associated with autoimmune disease[1]. Therefore, Treg became frequently studied targets for more specific immunotherapy. Especially antigen-specific targeting of Treg would enable local and tailor made interventions, while obviating the negative side effect of general immuno-suppression.

Self-antigens that participate in inflammation, irrespective of the etiology of the different autoimmune diseases, are held to be candidate antigens for antigen-specific interventions. Rather than tolerance induction to disease inciting self-antigens, which are frequently unknown, general self-antigens expressed at sites of inflammation would allow targeting of disease independent, but inflammatory-site-specific, regulatory mechanisms. Preferably, such self-antigens should be abundantly expressed and up-regulated at the inflammatory site. In this perspective heat shock proteins (Hsp) have several characteristics that can make them highly attractive targets for antigen-specific therapy. Being stress proteins, Hsp expression is increased during inflammation. This and the fact that Hsp are conserved and immunodominant proteins make them ideal targets for the immune regulation.

Several studies have shown that Hsp derived peptides are abundantly present in the clefts of MHC-class II molecules. Not only different length variants, but also peptides derived from members of several Hsp70 families have been eluted, indicating that Hsp70 is abundantly present in MHC class II especially under conditions of stress where intracellular Hsp expression is induced and autophagy enhanced[2]. Autophagy in particular chaperone-mediated autophagy is a mechanism that is activated as part of the cellular response to different stressors and which promotes the MHC class II presentation of peptides that are derived from intracellular source proteins including cytosolic Hsp that are normally presented by the MHC class I molecule[3-5].

The regulatory potential of exogenously administered Hsp has been shown before, both clinically in patients and in experimental models of inflammatory diseases[6]. Decreased Hsp inducibility has been observed in aged individuals and in some immune disorders[7, 8]. We suggest that reduced Hsp levels can result in failure of Hsp-specific regulation of inflammation and that this may contribute to increased disease susceptibility in elderly individuals.

FAILING HSP MEDIATED T-CELL REGULATION IN AGEING AND CHRONIC INFLAMMATORY DISEASES

With ageing susceptibility to inflammatory disease increases. Although many immunological processes, like reduced T-cell specificity and T-cell regulation most likely are involved, reduced stress responsiveness may also contribute. Several studies have shown that stress induced Hsp expression is inversely correlated with age in rodents[9, 10] and in humans[10-13. Also, in aged immune cells, both lymphocytes and monocytes, decreased expression of inducible Hsp70 has been observed[13, 14]. A declined stress response will have multiple consequences at the level of the cell and the immune system. Decreased stress-induced Hsp expression leads to failure of Hsp-specific immunoregulation and thus increases the susceptibility to development of immune disorders. This can happen by multiple means; first, the Hsp-specific Treg response itself is malfunctioning for instance as result of decreased T-cell numbers or reduced regulatory properties. Second, the Hsp-specific Treg population is normal, both in T-cell numbers and phenotype, yet activation of these Treg, during inflammation, is reduced due to decreased expression and presentation of the self-antigen (Hsp). Targeting Hsp-specific Treg, for immunotherapy purposes in inflammatory disease, might be possible by boosting the Hsp-specific Treg via peptide specific therapies or boosting Hsp expression itself.

BOOSTING HSP-MEDIATED T-CELL REGULATION WITH EXOGENOUS HSP

In a variety of rodent models of inflammatory diseases, boosting Hsp-specific Treg has been achieved successfully by application of Hsp-proteins or Hsp-derived peptides of bacterial- and self-origin. Hsp60 and Hsp60 derived peptides have been studied most extensively, but immunoregulatory effects have also been observed for other Hsp family members like Hsp70, BiP and Hsp10, reviewed in[6]. In the model of proteoglycan induced arthritis (PGIA), a model for chronic and relapsing arthritis which shares several characteristics with human RA, Hsp70 application reduced disease both after i.p. immunization and nasal application[15, 16]. PGIA disease development depends not only on $CD4^+$ T-cells, but also on B-cells and PG-specific antibodies[17]. Clinical and histopathological features in the PGIA model resemble human RA[18, 19].

Most autoimmune diseases have unknown causative agents and this forms a major drawback for the development of antigen-specific interventions. However, antigen-specific immunomodulation of T-cell function via altered peptide ligands (APLs) like Hsp peptides is a promising approach for the treatment of autoimmune diseases that are characterized by unwanted activation of T-cells[20].

Experimental evidence has been obtained that microbial Hsp peptides that were homologous to self-peptides, were able to trigger self-Hsp reactive T-cells, with disease suppressive regulatory potential[21, 22]. These cross-reactive Hsp-specific Tregs were suggested to exert their regulatory function in the periphery when exposed to for instance self-Hsp up-

regulated at a site of inflammation[6]. Nasal application of a Mycobacterial Hsp60 peptide that resembled a self-peptide expressed at the site of inflammation prevented adjuvant arthritis upon immunization before disease induction[23, 24].
Recently, we have mapped the immunodominant T-cell epitopes of mycobacterial Hsp70 and found that nasal administration of one of the highly conserved peptides, called B29, or its mouse homologues (mB29a and mB29b) reduced PGIA severity demonstrating the immunoregulatory potential of the B29 peptide. Administration of the B29 peptide via parenteral vaccination induced potent antigen-specific Tregs that were able to suppress inflammation upon adoptive transfer in recipients with established PGIA. Furthermore, selecting the B29-induced Tregs on expression of Lymphocyte Activation Gene-3 (LAG-3) enabled us to isolate highly effective suppressors, because as few as 4.000 LAG-3$^+$ B29-induced Tregs were sufficient to suppress PGIA upon transfer (van Herwijnen et al, submitted).

TRANSLATION FROM EXPERIMENTAL MODELS TO HUMANS

Peptide mediated epitope-specific therapy has proven highly promising in animal models but some fundamental differences between models and humans complicate translation to the clinic. One major difference is the fact that all inbred animals carry the same MHC while the human HLA system is a highly polymorphic system. Candidate peptides for immunotherapy in humans must be recognized by the human immune system and must thus be able to bind molecules of the human HLA system. The identified peptide B29 is located in an extremely conserved region of the Mt Hsp70 molecule, consequently the mouse homologue peptides are completely identical to the homologous sequence of human Hsp70. The potential relevance of Hsp peptide-specific T-cell responses for disease outcome has been demonstrated before by the finding that T-cell recognition of Hsp60 peptides correlated with better disease prognosis in patients suffering from juvenile arthritis[25, 26]. Interestingly, longitudinal observations showed that remission coincided with raised Hsp60 T-cell responses[25-27]. Recently, this has been reproduced in juvenile dermatomyositis (JDM). As observed in the synovium of RA patients, muscle expression of both Hsp60 and Hsp70 was found in biopsies from JDM patients[28]. This indicated that also in JDM, Hsp60 and Hsp70 can be targets for the local immune system.

Interestingly, oral treatment in RA patients, with the dnaJP1 peptide that is derived from dnaJ Hsp, was seen to induce epitope-specific immune modulation in patients with active RA[29], a shift from pro-inflammatory T-cell responses to IL-4 and IL-10 producing and FoxP3 expressing Tregs that coincided with increased expression of molecules that down-regulate adaptive immunity[30, 31].

The Hsp60 based peptide intervention in type I diabetes mellitus has reached now an advanced stage of development. Following the initial experiments using microbial

Hsp60 as a protective antigen in a multitude of experimental autoimmunity models, p277 (present in mammalian Hsp60) was mapped as the critical diabetes protective peptide in the NOD diabetes mouse model. Initial clinical trials indicated the capacity of p277 to cause a Th1 to Th2 shift at the level of $CD4^+$ T-cells. In addition in patients with newly diagnosed type I diabetes, levels of preserved endogenous insulin production was seen[32]. Diapep277 was shown especially potent in these newly diagnosed patients where beta cells reserves are still good. Peptide specific therapy might halt disease but does not induce beta cell regeneration. Later follow-up studies have shown that p277 had the capacity to trigger a regulatory profile in cells of the innate immune system through TLR2, which may have contributed to the success of p277 to drive a Treg response[33]. Although the anti-inflammatory actions of Hsp proteins have been reported widely, also pro-inflammatory effects of Hsp have been described[7, 34]. However, there is controversy concerning the capacity of Hsp to directly activate the innate immune system via TLR signaling as many (recombinantly prepared) Hsp protein batches have been shown to be contaminated by TLR ligands[35]. To develop a safe therapeutic approach that benefits from the induction of Hsp-specific Tregs without activation of the innate immune system via TLR4 (either directly or via contaminants) Hsp-specific peptides have great potential. This has been confirmed by the Diapep277 studies showing that no TLR4 activating pro-inflammatory actions were present. A recent larger randomized controlled phase 3 multicenter trial was successfully completed, showing that the p277 trial had reached its primary and secondary endpoints[36]. Herewith Hsp60 based p277 is the first immunological intervention that met clinical endpoints in type I diabetes trials.

THERAPEUTIC STRATEGIES; INDUCTION OF REGULATORY T-CELL RESPONSES WITH SPECIFIC PEPTIDES

The exposure route, the antigen and the presence or absence of an adjuvant can be critical to specifically induce Treg. In animal models, boosting immunoregulation has proven successful after administration of Hsp60 or Hsp70 via multiple routes: intragastric[37-39], intranasal[24, 40] and parenteral[16]. In the clinical setting, especially the mucosal route will be an attractive way to induce antigen-specific tolerance because it allows easy and non-invasive delivery and carries low risk of toxicity[41, 42]. In addition, mucosal antigen application induces both local mucosal and systemic immune responses, in contrast to a non-mucosal route of vaccination[43-45]. The immune response following mucosal antigen application depends on many factors, such as the nature of the antigen, the formulation (soluble versus particulate), antigen dose, size, frequency of administration and delivery to the mucosal tissues[46]. In addition, the use of an adjuvant might be required to achieve a sufficient mucosal immune response. Although the type of immune response that is induced following mucosal antigen application might depend on the nature of the antigen that is used as seen in cholera-toxin B subunit immunization studies[47, 48], adjuvant

characteristics may also play an important role. A multitude of mucosal delivery systems have been developed including nanoparticles. For tolerance induction to self-antigens used for vaccination in autoimmune diseases one would prefer to combine a self-antigen with a vaccine that favors tolerance induction. Nanoparticles are available as non-toxic delivery systems with promise for nasal vaccination[49-52].

Recently, we have shown that encapsulation of a dominant murine Hsp70 peptide mB29a in PLGA (poly-lactic-co-glycolic acid) containing nanoparticles enhanced the tolerogenic capacity of the peptide and reduced severity of proteoglycan-induced arthritis after nasal administration, whereas, encapsulation of the same antigen into TMC-TPP (N-trimethyl chitosan tripolyphosphate) nanoparticles enhanced humoral immunity but did not suppress disease activity[50, 53].

These data confirm that particle-antigen combinations need to be carefully constructed to design successful vaccines that induce the preferred type of immune response.

BOOSTING ENDOGENOUS HSP70 EXPRESSION WHEN HSP INDUCIBILITY IS DECREASED DURING AGING OR DISEASE

Likely enough, Hsp need to be abundantly expressed during inflammation in order to act as dependable triggers for the activity of Tregs in inflammatory sites. During ageing, but possibly also in certain diseases there exists a reduced capacity to up-regulate Hsp's (Figure 1).

Enhanced expression of inducible Hsp, in response to stress, is predominantly mediated by HSF1, a transcription factor sustained in a monomeric form in the cytosol by binding to Hsp70 and Hsp90. Upon stress, the increased presence of damaged proteins will cause competitive dissociation of the chaperones from HSF1, allowing HSF1 trimerization and translocation of the functional trimer to the nucleus. Subsequently, binding of HSF1 to heat shock binding elements (HSE), in the Hsp promoter regions, and phosphorylation of HSF1 leads to transcription of Hsp[54, 55]. How age affects the stress response is not completely known but decreased HSF1 binding to Hsp promoter regions has been reported[9, 56]. An age related decline in HSF1 protein levels was suggested as a possible explanation[9]. Alternatively, while total HSF1 protein levels stay constant, numbers of misfolded HSF1 monomers will increase under ageing. Consequently, augmented trimerization of HSF1 monomers incorporating such misfolded monomers will decrease the total number of functional HSF1 trimers during ageing[10]. A third explanation might result from inhibitors of HSF1-HSE binding or loss of HSF1 enhancers[57, 58]. Irrespective of the exact mechanism, decreased HSF1 binding to Hsp promoter regions will reduce the expression of stress proteins.

Besides ageing, some autoimmune diseases have been associated with an impaired expression of Hsp. For example, Hsp60[59] and members of the Hsp70 family[7, 8] are involved in the pathogenesis of JIA and diabetes mellitus, respectively, and possibly other autoimmune disorders that have not yet been fully explored for this purpose.

Peripheral Hsp expression is important for Hsp-mediated immunoregulation. However, when HSP inducibility declines with age or is deficient in certain disease states, it may be necessary to not only administer exogenous Hsp, but also to amplify endogenous Hsp expression under stress conditions. Although such up-regulation of stress induced Hsp levels would be especially helpful in the aged individual where, due to decreased Hsp, the target antigen of Hsp-specific Treg will be reduced, in individuals with a normal stress response boosting this response could enhance induction and activation of Hsp-specific Treg in the periphery and amplify their regulatory function during inflammation. Various compounds have been found that can enhance Hsp expression and some of these compounds have been described to have anti-inflammatory capacities[60-64]. Recently we have shown that *in vivo* targeting of the stress response with an Hsp co-inducer, called carvacrol, actually leads to T-cell mediated suppression of inflammation[65]. Moreover, preliminary data suggest that carvacrol in combination with thermal stress induced tolerogenic dendritic cells (DCs) that can transfer protection in arthritis models (Spiering et al. submitted).

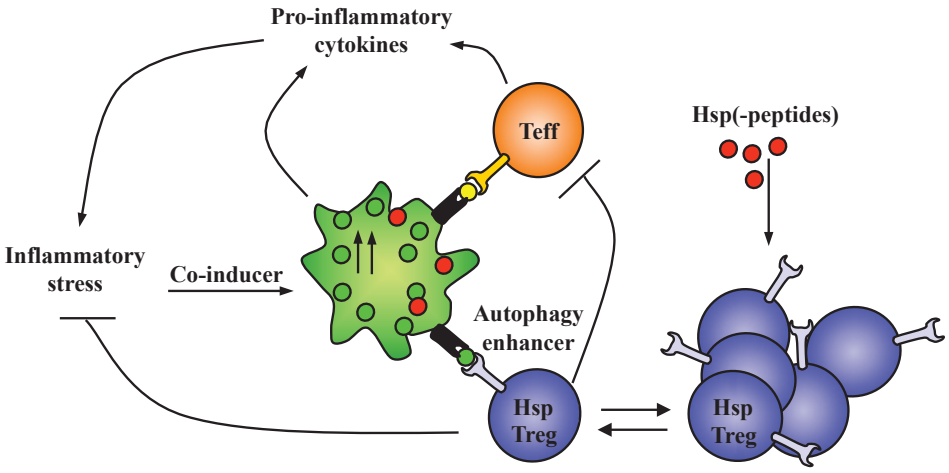

Figure 1 | Hsp-specific T-cell immunity as target for immunotherapy. Expression of inducible Hsp70 is increased in response to various forms of stress and triggers naturally occurring feedback mechanisms (green dots in APC represent endogenous Hsp); During inflammation, enhanced Hsp70 levels will amplify immunoregulation by Hsp70-specific Treg and, upon activation, these T-cells can dampen ongoing inflammation. Antigen-specific immunotherapy with exogenous Hsp or peptides (red dots) will boost the number and function of Hsp-specific Treg. On the other hand, boosting endogenous Hsp expression will increase Hsp-specific Treg numbers, function and expression of the target antigen at the site of inflammation. Autophagy enhancers will augment presentation of Hsp-peptides on the MHC class II-leading to enhanced T-cell activation.

BOOSTING HSP IMMUNOREGULATION WITH CARVACROL

Carvacrol and its isomer thymol are the major components of essential oils, obtained by distillation of (dried) plant matter, from the herbs oregano and thyme[66]. Thus far, carvacrol has been mainly studied as an anti-bacterial agent[67] and from that perspective, carvacrol has been described to increase the expression of Hsp60 but not Hsp70 in prokaryotes[68]. The effect of carvacrol on eukaryotic cells had not been addressed before. We showed that carvacrol did not induce Hsp70 itself, but it is a potent enhancer of stress induced Hsp70 expression in mammalian cells *in vitro*. Oral carvacrol administration ameliorated development of PGIA. Since adoptive transfer of T-cells, from carvacrol treated donor mice, suppressed PGIA effectively, we reasoned that functional Treg were induced.
The carvacrol findings provided proof of principle that the immune system can respond to compound induced Hsp expression. Previously, another compound with Hsp co-inducing capacity, curcumin has been shown to suppress arthritis[69, 70] and to inhibit the immunostimulatory capacity of LPS stimulated DCs via inhibition of LPS-induced NF-κB activation[71]. In this study the production of LPS induced IL-12p70 was decreased in the presence of curcumin whereas the production of IL-10 was unaffected. Combined with our observation that carvacrol can modulate APC function this shows a broad effect of Hsp induction and immune modulation. Since carvacrol is a natural dietary constituent, clinical application of carvacrol seems relatively safe. Furthermore, the finding that carvacrol was effective through the oral exposure route will enable easy administration of the compound, making it an promising candidate for boosting Hsp immunoregulation.

PERSPECTIVES

Hsp-specific T-cell responses have been positively associated with a better disease prognosis[26, 72]. In addition, the immunosuppressive action of Hsp has been demonstrated in multiple rodent disease models. So, it is attractive to speculate that simply enhancing Hsp immunoregulation in either way could be used as therapy. Obviously, this is oversimplified. Depending on multiple factors such as disease etiology and inflammatory status, patient age and genetic background, difficulties will be encountered. In general, defects in for example positive or negative selection in the thymus, IL-2 production by effector T-cells or IL-10 or TGF-β production by Tregs can lead to loss of peripheral tolerance as a result of decreased T-cell numbers or functioning[73]. Some of these defects might also influence Hsp-specific Treg. For example our findings that Hsp70-induced suppression of arthritis failed in the absence of IL-10[16], illustrated that defects in IL-10 production will also influence Hsp-specific Treg. Furthermore, as disease progresses, severe ongoing inflammation has been described to obstruct the effectiveness of antigen-specific Tregs[74, 75]. It is currently not known if Hsp-specific Treg can also be hampered by ongoing inflammation. Recently, it has been reported that natural or thymus derived

Treg but not induced Treg can convert into Th17 cells after exposure to IL-6 and TGF-β[76]. Besides Th1 cells, Th17 cells are major pathogenic effector cells in many autoimmune diseases. Whether Hsp-specific Treg can convert into Th17 cells has not been studied, but if so, timing and route of boosting the Hsp response could be important to avoid exacerbation of disease in stead of induction of regulation.

COMBINATION THERAPY

Autoimmune diseases such as RA are complex diseases where a multiplicity of pathogenic elements have a combined contribution to the disease process. Since several immune functions, rather than individual pathways should perhaps be targeted, combination therapies might offer new opportunities to overcome obstacles seen for single approach therapies. In RA, a combination therapeutic approach with the use of disease modifying antirheumatics (DMARDs) and biologics has become standard practice[77-79]. The main challenge is the maintenance of low disease activity and remission, once achieved. This challenge can probably be met only by an evolving strategy of moving from generalized anti-inflammatory interventions towards a more disease-specific approach.
The concept that the immune circuit we are targeting is not disease but rather inflammation specific is supported by data in other diseases in humans and in animal models of autoimmunity, such as EAE, diabetes and IBD[25, 33, 80]. The epitope-specific approach also has the potential to be combined with current therapeutics. In the animal model of RA (adjuvant-induced arthritis) epitope-specific tolerization was combined with anticytokine therapy, which led to full disease control. Moreover, the dose of anticytokine therapy can be lowered significantly. In rats with AA, the combination of an arthritogenic peptide (Hsp60 p180–188) with one-third of the dose of Etanercept led to significant disease improvement and regulatory immune deviation (81). Thus, combination therapy will probably increase antigen-specific Treg responses and simultaneously allow lower dose usage of the immunosuppressive agent. In addition, alternative combination therapies would be interesting.

We postulated that in individuals with decreased stress-induced Hsp expression, declined Hsp-specific Treg numbers and function can be restored by therapeutic intervention. Combination of boosting T-cell numbers, with exogenous Hsp vaccination, and simultaneously enhancing Hsp expression with co-inducers would be attractive. However, increased expression of Hsp will only have an antigen-specific effect on the Hsp-specific Treg response, if this is accompanied by enhanced presentation of Hsp-epitopes on MHC class-II. Thus, a combination of compounds that co-induce Hsp expression and enhance Hsp-peptide presentation can, at least in theory, act synergistically. Alternatively, agents that promote induction or activation of Treg can be combined with Hsp co-inducers or Hsp peptide application. In this respect, rapamycin or analogues have been described as autophagy inducers, and also the increased activation of Treg upon rapamycin has been

reported[82]. The above mentioned studies illustrate the (potential) synergistic interplay between targeting Hsp immunoregulation through various pathways and therapies currently established or under investigation.

CONCLUSIONS

Hsp are important controllers of cellular and immune homeostasis and are expressed in almost every tissue. Because Hsp expression is directly influenced by both intracellular and extracellular stress signals, Hsp levels reflect the state of the cell and the tissue and can be used to translate stress signals into effector mechanisms. Activation of Hsp-specific Treg is such an effector mechanism and will be important to maintain immune homeostasis. As discussed here various factors influence immune suppressive capacity of the Hsp response. Factors like, the T-cell response itself, expression of the Hsp antigen and presentation of Hsp-peptides on MHC class-II. Targeting these factors, either or not in combination with established therapies, can be an interesting means for antigen-specific immunotherapy to prevent or suppress autoimmune disease. The data presented here enhance basic knowledge on how Hsp-specific T-cells can modulate the immune system, which will contribute to development of new or refinement of existing intervention strategies to treat autoimmune disease.

EXPERT OPINION

In our opinion Treg targeted therapies have great potential for the future. However, especially in the case of antigen-specific Tregs, practical application is hampered by the frequent absence of unique and critical autoimmune disease associated antigens. In addition, it may be difficult to redirect ongoing immune responses under inflammatory conditions. It is clear from the data mentioned that Hsp proteins and peptides have an immunomodulatory role and are well recognized by the immune system. By their nature Hsp are up-regulated at sites of inflammation, which may enable them to function as targets for Hsp/peptide induced Tregs. The Hsp epitope-specific approach has the potential to be combined with current therapeutics, such as the TNF inhibitors in the case of RA[83]. Combinational therapy in a rat model for arthritis showed the potential of such an approach leading to full disease control.

In our opinion it is likely that future therapeutic approaches aiming at antigen-specific Tregs will benefit from tailor-made combination therapies. Combining current therapeutic approaches, such as biologicals that dampen inflammation, with Hsp-specific therapies enhancing natural immune regulation might expedite the entry of antigen-specific Tregs into the therapeutic arsenal of the anti-inflammatory therapeutics.

Declaration of interest
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7

Summarizing Discussion

The research described in this thesis aimed to unravel adaptive immune responses induced after nasal administration of several particle based vaccines that can either enhance protection against invading pathogens or induce a state of peripheral tolerance.

Four main research topics were addressed in this thesis

1. The role of Toll-like receptor 2 (TLR2) in activation of local and systemic virus-specific immune responses was evaluated after nasal administration of bacterium-like particles mixed with influenza split virus (BLP-SV).
2. The characteristics of poly (lactic-co-glycolic acid) (PLGA), PLGA-N-trimethyl chitosan (PLGA-TMC) and TMC-tripolyphosphate (TMC-TPP) nanoparticles were correlated to their capacity to induce antibody production.
3. The potential of PLGA, PLGA-TMC and TMC-TPP nanoparticles to enhance $CD4^+$ T-cell proliferation and their capacity to induce nasal tolerance was addressed.
4. The capacity of PLGA nanoparticles to trigger dendritic cells (DCs) to enhance RALDH activity to induce forkhead box P3 ($FoxP3^+$) T-cells via a TGF-β and retinoic acid (RA) dependent mechanism was evaluated.

Mucosal vaccine development

Although most infections and environmental allergies are acquired through the mucosal membranes, surprisingly, most vaccines are still delivered via the parenteral route because this route of vaccination is associated with effective protective antigen-specific humoral immune responses, the gold standard to measure vaccine efficacy. However, the continuous emergence of new pathogens and growing drug resistance of microorganisms requests for innovative vaccination strategies. Compared to parenteral vaccination one major advantage of the mucosal route of vaccination is the ability to activate both mucosal and systemic immune responses[1]. In addition, vaccination at one mucosal site can induce immunity at peripheral mucosal sites via the common mucosal immune system. The vaccine characteristics required depend largely on the immune responses that are desired and can be divided into two categories, defence responses against invading pathogens[2] and tolerance induction to allergens or autoantigens[3]. There are various routes of mucosal vaccination of which the oral and nasal route are most accepted and easily accessible. The nasal route of vaccination might be preferred over oral vaccination due to a lower proteolytic activity in the nasal mucosa that provides better antigen stability and because it often requires a lower dose of antigen, which reduces the chance of producing negative side effects[4]. However, the efficacy of nasal vaccines is often limited, because vaccine antigens need to find their way through the mucosal layer to ensure their uptake by epithelial cells. In addition, the uptake of antigen needs to occur in a limited time frame as the nasal vaccine antigens are constantly cleared from the nasal cavity by the ciliary movement. Therefore, different kinds of vaccine adjuvants and delivery particles are available and under development to help enhance the mucoadhesiveness, delivery and the immunogenicity of nasal vaccines, including a variety of adjuvants tested for nasal application, BLPs, lipid-based vaccines, virus-like particles (VLPs) and nanoparticles.

Live attenuated vaccines

Live attenuated vaccines are produced by attenuation of an infectious agent either viral or bacterial-derived to alter the pathogen making it less virulent, though still able to infect the host. Unlike subunit vaccines or whole inactivated vaccines, live attenuated vaccines consist of a complete set of antigens used to mimic natural infection to enhance protective immunity against the pathogen. Most live attenuated vaccines are administered via the parenteral route, because this route of vaccination is in general associated with effective protective immune responses against infectious agents. The Food and Drug Administration (FDA) approved the first intranasal (i.n.) live attenuated influenza vaccine (LAIV) called FluMist (Add Immune) for human use in 2003. An advantage of FluMist is that it is developed in such a way that it can be easily adapted to present the surface antigens of seasonal influenza virus[5]. In general, live attenuated vaccines have proven to be very effective in stimulating the immune system to produce pathogen-specific immune responses required to clear infections and induce a longer duration of protection by inducing memory T-cell and B-cell responses. Disadvantages of live attenuated vaccines are that they can not be used in immunocompromized patients, in patients that have egg allergy and these vaccines need to be kept and transported under stringent conditions, all factors that limit its application. Synthetic vaccines are a promising alternative to live attenuated vaccines, because they are composed of a protein, peptide or of antigen-encoding DNA and do not contain any genes encoding virulence factors. However, these vaccines often require adjuvants and/or delivery systems to enhance the immunogenicity of the vaccine antigens. Next, the characteristics and immunostimulatory properties of nasal adjuvants and particle delivery systems will be discussed.

Nasal vaccine adjuvants

Adjuvants that are used for nasal vaccines are known to potentiate immune responses. In addition, the risk of developing antigen- or adjuvant-induced side effects is often reduced, because the nasal route of vaccine delivery not only requires a lower dose of antigen but often also a lower amount of adjuvant to elicit strong immune responses[4]. The type of immune responses that adjuvants elicit depend on their specific physicochemical characteristics. They can enhance the uptake and presentation of the antigen by targeting specific immune cells such as microfold cells (M-cells) and DCs and/or trigger specific pathogen recognition (PRRs) receptors to elicit active inflammatory immune responses. A group of adjuvants that has been studied extensively for use in nasal vaccines is bacteria-derived. These pathogen-associated molecular patterns (PAMPs) are recognized by specific PRRs that are expressed by antigen presenting cells (APCs). These are potent adjuvants in nasal vaccination and include Toll-like receptor (TLR), Nod-like receptor, scavenger receptor and C-type lectin receptor ligands[6].

The enterotoxins cholera toxin (CT) and heat-labile enterotoxin (LT) are potent mucosal adjuvants that can induce systemic and mucosal immune responses. The first commercial nasal virosomal influenza vaccine adjuvanted with LT became available in 1997, but was withdrawn from the market because its use was associated with an increased risk of

developing Bell's palsy[7, 8]. Therefore, to improve safe use of CT and LT, non-toxic mutants have been developed and tested[9] including recombinant CTB (rCTB) that has been described not only to enhance protective immune responses against influenza virus[10, 11], but has also been associated with enhanced mucosal tolerance induction[12].

TLR ligands have been described as potent adjuvants for nasal vaccines against influenza infection. Intranasal pre-treatments with especially TLR2 and TLR4 ligands were found to protect mice against lethal influenza pneumonia in an antigen independent manner[13]. Intranasal vaccination of mice with the TLR3 ligand Poly (I:C) induced protective immunity against influenza virus[14]. Moreover, i.n. immunization with influenza-derived peptides coupled to bacterial-derived lipids induced DC maturation via TLR2 binding and enhanced activation of IFN-γ secreting $CD8^+$ T-cells at the site of infection after i.n. exposure to influenza virus[15].
A recently developed intranasally applied system for vaccination against influenza virus is BLPs. Previous studies showed that non-living BLPs from the food grade bacterium *Lactococcus lactis* are effective stimulators of local and systemic immune responses when administered intranasally[16] and that BLPs supplemented with influenza vaccine resulted in protection against both homologous and heterologous influenza infections[17]. Since BLPs specifically interacted with human TLR2 *in vitro*[18], we investigated the role of TLR2 in the activation of influenza A virus (IAV)-specific adaptive immune responses after nasal administration of BLP-SV using TLR2 knock-out mice.

Bacterium-like particles
In **Chapter 2**, we showed that both local and systemic IAV-specific IFN-γ producing T-cells induced after i.n. BLP-SV vaccination required the activation of TLR2 by BLPs. Although we detected IFN-γ producing T-cells after i.n. BLP-SV administration, the IFN-γ-producing cell type remains unknown. Influenza A is an RNA virus that enters the nucleus for viral protein replication and infects mainly epithelial cells[19] and macrophages[20, 21] of the respiratory tract. Therefore, a combination of a Th1 and cytotoxic T-lymphocyte (CTL) response might be preferred given that CTLs recognize viral peptides presented by infected target cells such as epithelial cells and immediately release a number of anti-viral mediators, including perforin and granzymes, IFN-γ that inhibits viral replication, they induce apoptosis of virus infected cells and contribute to cross-protection against virusses of different subtypes[22]. While, infected macrophages stimulate Th1 cells to secrete IFN-γ that in turn activates macrophages to kill intracellular virus that reside in vesicles[23]. Other immune cells such as natural killer T (NKT)-cells[24], NK-cells[25] and γδ-T-cells[26] can also produce IFN-γ during viral infection and might contribute to anti-viral immunity during influenza infection.
In addition to IFN-γ production, we also observed that i.n. BLP-SV vaccination modestly skewed the response towards Th17 type. Th17 cells have been associated with nasal vaccination[27], however, the role of Th17 and other IL-17 producing cells in influenza infections is still not completely clear[28]. There is evidence that IL-17 producing cells might

be beneficial during severe influenza infections, since enhanced numbers of IL-17 producing influenza specific T cells can protect the host against an otherwise lethal viral infection[29]. Moreover, inhibition of Th17 mediated immunity by the influenza A virus itself, enhances the risk of complicating secondary *S. aureus* infections[30]. To understand the role of Th17 cells in influenza infection further investigation is required.

Activation of B-cells that differentiate into plasmacells and secrete antigen-specific neutralizing antibodies remains the gold standard to determine vaccine efficacy. Besides T-cell activation, we detected systemic IAV-specific IgG production after i.n. BLP-SV vaccination and class switching to IgG2c that correlates with IFN-γ production. Especially at mucosal sites, B-cells produce secretory IgA (SIgA) that forms a first line of protection against invading pathogens[31, 32]. B-cell responses and SIgA switching can be mediated by Th2 cells that secrete IL-5 cytokines required for proliferation and terminal differeniation of $SIgA^+$ B-cells into plasmacells[33]. Based on our data, we could not confirm that SIgA class switching was mediated by IL-5. Therefore, we suggest that other factors might be involved such as direct TLR2 signaling in B-cells[34] or production of RA. TLR2 stimulation by zymosan enhances RALDH2 expression required for RA production in splenic DCs that stimulates $FoxP3^+$ Tregs[35]. However, RA in combination with IL-6 is known to promote class switching to IgA[36].
To summarize, compared to live attenuated vaccines, BLP-SV vaccination is considered to be well tolerated and safe. Since the BLPs are mixed with influenza monovalent split vaccines, there is no risk of reversion of virulence. Although protection against both homologous and heterologous influenza infections has been observed, knowledge of the BLPs mechanism of action after nasal delivery remains unclear and needs further investigation.

Virus-like particles (VLPs)
VLPs are an innovative tool for the development of nasal vaccines. VLPs are composed of specific viral proteins and mimic conformational structures of viruses, but as they do not contain any viral material they are non-infectious and therefore a safe alternative to live attenuated vaccines. VLPs can be generated in a variety of expression systems, including bacteria, virus, plant cells and yeast. In general, VLPs are well tolerated and have been described to induce effective virus-specific humoral and cellular immune responses both after parenteral and nasal administration against a variety of viral pathogens such as the 1918 pandemic H1N1 influenza A virus[37], severe acute respiratory syndrome coronavirus[38], human papilloma virus 16[39] and human immunodeficiency virus (HIV)-1[40].
To summarize, VLPs hold promise as vaccine particles and adjuvants in vaccination strategies to prevent a variety of viral infections that affect especially the mucosal tissues.

Lipid-based systems

Liposomes were first described by Alec D Bangham and are vesicles composed of one or more phospholipid membranes that can deliver a broad range of molecules[41]. Liposomes can be mixed, coated or encapsulated with antigen and can induce both humoral and cellular immune responses. The antigens that are used can vary from protein, peptide, DNA-complexes to whole inactivated virus vaccine. For example, liposomes mixed with an inactivated whole measles virus vaccine activated humoral immune responses by the induction of virus-specific systemic IgG and mucosal SIgA responses after nasal administration[42]. In addition, cationic liposome-DNA complexes showed that not only humoral immunity, but also antigen-specific cellular immune responses can be induced after nasal delivery[43]. Liposomes not only enhance the delivery and immunogenicity of the vaccine candidates, but the adjuvant activity of liposomes can also be adapted by incorporation of PAMPs, such as TLR ligands[44]. Another strategy described is the incorporation of an influenza-derived peptide that is specifically recognized by CTLs and when supplemented with costimulatory molecules results in mucosal CTL responses against influenza[45].

Immune stimulatory complexes (ISCOMs) and ISCOMATRIX both have the size of a virus and a particulate structure and are prepared from cholesterol, lipids, and the saponin Quil A. They can incorporate or associate an antigen and can be used both as parenteral and nasal vaccine adjuvant and have been described to induce systemic and mucosal humoral immune responses and CTL responses[46-48]. Moreover, the component saponin mediates strong immunostimulatory responses and can bind to M-cells to improve antigen uptake and delivery to subepithelial regions. ISCOMS can disrupt the endosomal membrane and therefore enhance the delivery of antigen to the cytosol of APCs and promote efficient antigen-presentation to naive T-cells[49]. Influenza vaccine incorporated into ISCOMATRIX has been shown to induce protective humoral immune responses after a single i.n. immunization[50].

Virosomes are similar to liposomes and mimic viral structures. Virosomal influenza vaccines have been most extensively investigated and are the only virosomal vaccines that have been tested via the nasal route[51-54]. Virosomes can enhance antigen uptake and antigen presentation by DCs[55, 56] that subsequently activate naive $CD4^+$ T-cells and CTLs[57, 58].

Taken together, lipid-based structures are very potent adjuvants for mucosal vaccines designed for therapy to protect against multiple infectious diseases. An alternative to adjuvants and lipid-based structures are the cost-effective biodegradable nanoparticles that can be easily prepared, modified and stored.

Nanoparticles

Nanoparticles are available as non-toxic delivery systems with promise for nasal vaccination. A major advantage of vaccine delivery particles is that the target antigen can be encapsulated by the particle to protect the antigen from enzymatic degradation. Moreover, addition of mucoadhesive substances such as chitosan or its derivate TMC that interact with the negatively charged mucin-like molecules mainly by ionic interactions

can increase the antigen residence time in the nasal cavity. Chitosan has been described to open tight junctions and to increase the uptake probability by the epithelium and the submucosal DCs after nasal administration[59, 60]. Little is known about the physicochemical characteristics of nanoparticles in relation to the immune response they induce following nasal vaccination. Many factors have been described to play a role, including the nature of the antigen (immune suppressive or immunogenic), the formulation (soluble versus particulate), nanoparticle size, antigen dose and frequency of administration[4].

In **Chapter 3**, we introduced live imaging techniques as a novel method to determine the residence time of ovalbumin (OVA) in the nasal cavity. The capacity of PLGA, PLGA-TMC and TMC-TPP nanoparticles to decrease the clearance of OVA protein from the nasal cavity was addressed. Mice that were i.n. vaccinated with TMC-TPP nanoparticles showed a significantly prolonged nasal residence time compared to PLGA and PLGA-TMC nanoparticle treatment. Since the positively charged particles have been described to enhance vaccine efficacy in nasal vaccination[61], we suggested that the positive charge of the TMC-TPP nanoparticles results in a decreased clearance rate of OVA from the nasal cavity after nasal administration. In addition, the ciliary beat frequency used as a measure for nasal cilia toxicity revealed that none of the particles were found to be toxic to the cilia or M-cells[62].

Furthermore, the characteristics of PLGA, PLGA-TMC and TMC-TPP nanoparticles were correlated to their capacity to induce antibody production after nasal administration. Interestingly, only the TMC-TPP nanoparticles induced OVA-specific mucosal SIgA and systemic IgG antibody responses after nasal administration, whereas after intramuscular administration all classes of nanoparticles enhanced the systemic OVA-specific IgG antibody response. However, like TMC-TPP nanoparticles, PLGA-TMC nanoparticles also have a positive zetapotential, suggesting that the positive surface charge is not the only factor that drives humoral immunity after nasal administration. The TMC-TPP nanoparticles were found not to promote the antigen uptake by human DCs and murine DCs (**Chapter 3 and 4**) *in vitro*. Since encapsulated antigen is not easily taken up by B-cells[63], we suggested that TMC-TPP nanoparticles that release their content rapidly after nasal delivery might promote the uptake of antigen by B-cells. Moreover, TMC as an adjuvant has been described to promote skewing towards Th2 cells that are critical for humoral immunity[64] and these observations correlated with enhanced IgG class switching to IgG1.

Based on the observations described in **Chapter 3**, we concluded that TMC-TPP nanoparticles were superior to PLGA-containing nanoparticles in the stimulation of both mucosal and systemic humoral immune response after nasal application.

Since little was known about the role of $CD4^+$ T-cells in nasal vaccination, we investigated how different nanoparticle treatment might influence the activation of $CD4^+$ T-cells. In **Chapter 4**, we described that PLGA, PLGA-TMC and TMC-TPP nanoparticles all enhanced OVA-specific $CD4^+$ T-cell responses after nasal administration, whereas only TMC-TPP nanoparticles were found to enhance the number of OVA-specific B-cells in

the nose draining CLN compared to PLGA-containing nanoparticles and the data correlated with the observations described in **Chapter 3**. Kim Wu *et al* described that, mice that were fed a single dose of 40 μg of type II collagen (CII)-containing PLGA particles, showed reduced severity of arthritis and reduced anti-CII-specific IgG antibody titers and CII-specific T-cell responses[65]. Therefore, we investigated if PLGA nanoparticle characteristics might also enhance nasal tolerance induction. We observed that in contrast to TMC-TPP, i.n. delivery of PLGA nanoparticles not only enhanced the expression of Foxp3 in the nose-draining cervical lymph nodes (CLN), but also reduced the delayed-type hypersensitivity reaction against OVA. Additionally, we investigated whether enhanced nasal tolerance induction mediated by PLGA treatment was sufficient to suppress a chronic inflammatory response. The immunosuppressive Hsp70-peptide mB29a was encapsulated into each of the nanoparticles and we tested the capacity of these nanoparticles to modulate the response towards mucosal tolerance in the proteoglycan-induced arthritis (PGIA) mouse model after nasal administration. PLGA-treated mice showed significantly reduced mean arthritis scores compared to control mice. Confirming our hypothesis, an immunosuppressive function for TMC-TPP was not observed, because the mean arthritis scores observed in these mice were not significantly lower than that of control mice. Furthermore, the lower mean arthritis scores observed after PLGA or PLGA-TMC treatment coincided with a later onset of disease and lower maximum arthritis scores.

Many autoimmune diseases and other chronic inflammatory conditions are characterized by defective $FoxP3^+$ Tregs mediated suppression. A potential treatment option for these disorders is to increase the number and activity of local Tregs[66]. Therefore, we investigated *in vitro* the capacity of both OVA-encapsulated PLGA and TMC-TPP nanoparticles to trigger RA production in DCs as a strategy to enhance the induction of $FoxP3^+$ T-cells (**Chapter 5**). Only PLGA nanoparticles enhanced the capacity of DCs isolated from the nose-draining CLN to induce OVA-specific $FoxP3^+$ T-cells via a TGF-β and RA dependent mechanism, that correlated with significantly enhanced RALDH activity in PLGA-treated DCs compared to TMC-TPP nanoparticle treatment. Moreover, PLGA nanoparticles induced RA production by DCs and inhibited T-cell differentiation into inflammatory Th17 cells, while TMC-TPP mediated down regulation of RA production promoted the differentiation into Th17 cells. The reciprocal Th17 and Treg cell differentiation mediated by RA is in accordance with the literature[67].

In conclusion, we described a new characteristic of PLGA nanoparticles, which might contribute to their capacity to enhance nasal tolerance induction. We suggested that nasal PLGA nanoparticle administration favors the induction of $FoxP3^+$ T-cells in the CLN through modulation of local DC function and might therefore be a suitable delivery system for the treatment of autoimmune diseases and other chronic inflammatory conditions.

TMC-TPP nanoparticles enhanced humoral immune responses and promoted the differentiation of Th17 cells and might therefore be of interest in vaccination strategies to prevent infectious diseases, such as respiratory infections of bacterial and viral origin (Figure 1).

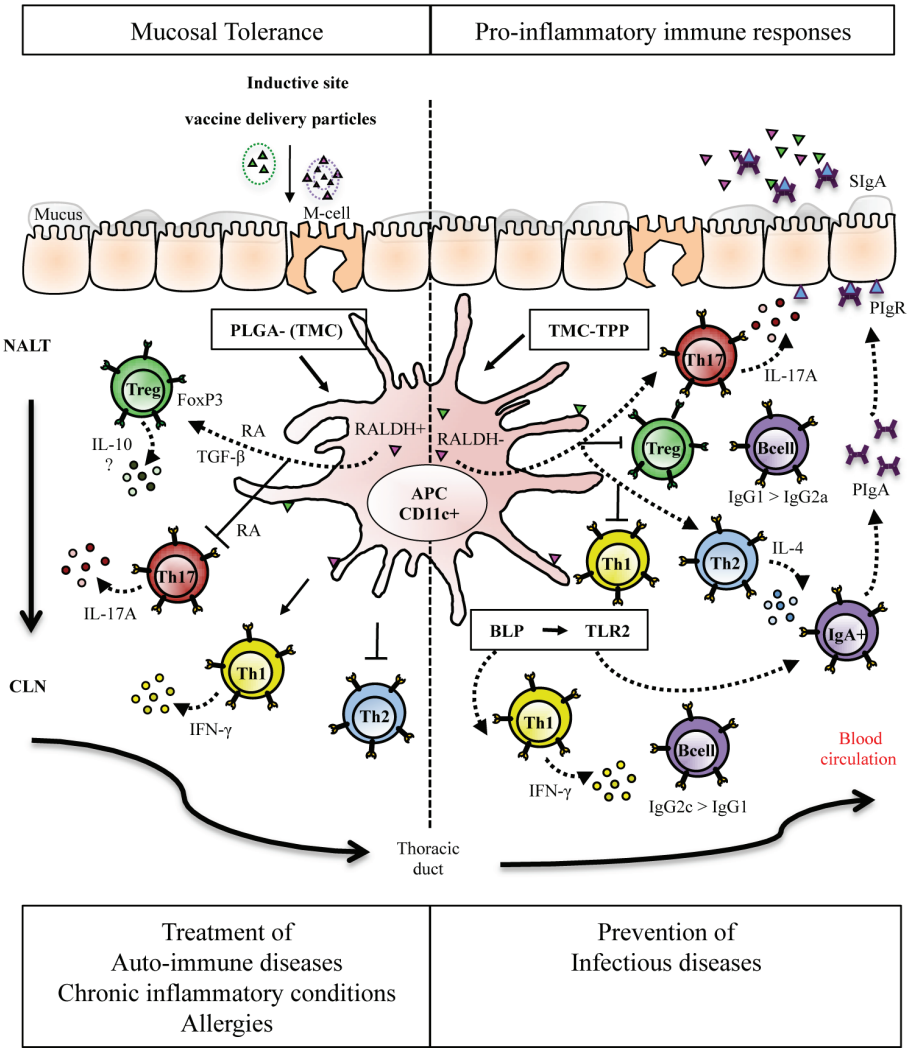

Figure 1 | Adaptive immune responses induced by PLGA-(TMC), TMC-TPP and BLP particles after nasal delivery. PLGA-(TMC) nanoparticles favor the induction of mucosal tolerance after nasal delivery (**Chapter 4**). PLGA nanoparticles can enhance the activity of RALDH in $CD11c^+$ DCs that is required for RA production. RA in the presence of the cytokine TGF-β can induce FoxP3 expression in $CD4^+$ T-cells *in vitro* (**Chapter 5**). PLGA-containing nanoparticles enhance Th1 cell differentiation, whereas TMC-TPP nanoparticles enhance the activation of Th2 cells and humoral immune responses, both systemic IgG and mucosal SIgA responses (**Chapter 3 and 4**). In contrast, TMC-TPP nanoparticles have been associated with an increased production of IL-17A cytokines that are produced by Th17 cells and correlate with the observations that TMC-TPP nanoparticles decrease the RALDH enzyme activity in $CD11c^+$ DCs, which most likely enables them to generate RA that is known to suppress Th17 cell differentiation (**Chapter 5**). Nasal delivery of BLPs results in IFN-γ producing T-cells, systemic IgG2c and mucosal SIgA antibody responses in a TLR2 dependent fashion (**Chapter 2**). Taken together, PLGA containing nanoparticles are suitable nasal vaccine delivery particles for the treatment of autoimmune diseases and allergies, while TMC-TPP nanoparticles and BLPs are more potent delivery particles/ adjuvants for prophylactic vaccination against infectious diseases.

FUTURE PERSPECTIVES

The aim of this thesis was to investigate the potential of innovative nanoparticles to enhance the delivery of nasal vaccines and to monitor protective adaptive immune responses after nasal vaccination. In the next paragraphs, nasal vaccine design and application recommendations are discussed for future vaccine development.

Although the application of live attenuated vaccines via the nasal route can induce protective pathogen-specific pro-inflammatory immune responses, the risk of reversion of virulence remains to be a major drawback for using live attenuated vaccines. As an alternative, whole inactivated virus or subunit vaccines have been developed, but these vaccines often require potent adjuvants and/or vaccine delivery particles of which many have been developed over the past years. For more than a century the adjuvant alum was the only adjuvant approved for human use. However, a variety of adjuvants have been developed over the past years and have shown to be capable of increasing the delivery and immunogenicity of nasal vaccines. Adjuvants that target PRRs expressed by APCs promote pro-inflammatory immune responses after nasal immunization and are potent adjuvant candidates for therapy to induce protective immunity against pathogenic microorganisms. Some adjuvants have been found to be too toxic for human use, such as the TLR4 ligand LPS and enterotoxins, however non-toxic mutants have been developed. In this thesis a recently developed non-toxic TLR2 ligand, the BLPs, were tested as an adjuvant for nasal influenza vaccines.

In **Chapter 2** it is shown that TLR2 strongly contributes to BLPs induced influenza A virus-specific systemic and mucosal immune responses after nasal BLP-SV immunization. However, it is still unclear if TLR2 activation by BLPs is fully responsible for the enhanced activation of the adaptive immune system. Therefore, BLP interaction with TLR2 *in vivo* needs further study. This is not easy to accomplish, because TLR2 is expressed on a diverse repertoir of immune cells including DCs[68], macrophages[69], T-cells[70, 71], B-cells[72] and M-cells[73] capable of differentially regulating the immune response. After i.n. administration, BLPs will first encounter epithelial cells that line the nasopharynx-associated lymphoid tissue (NALT) mucosa[31]. TLR2 signaling on M-cells has been described to enhance the transport of antigens to the subepithelial region and thereby also enhances the possibility for antigen to be recognized and taken up by APCs, most likely DCs[74]. To study this in more detail, it would be interesting to fluorescently label BLPs and study the interaction with the nasal epithelium including M-cells after nasal administration. Moreover, it would also be possible to study the uptake by DCs in the NALT or CLN after nasal delivery as well as direct interaction of BLPs with different lymphocytes. Another important feature of TLR2 is that it can form heterodimers with TLR1 and TLR6. Especially TLR2/TLR1 dimers were shown important in the induction of a protective mucosal Th17 immune response *in vivo*[75, 76], whereas TLR2/TLR6 heterodimers were not[77]. Further research that will focuss on the interaction of BLPs with immune cells after nasal application is needed

to understand its mechanism of action *in vivo* and to ensure efficacy of future BLP-based nasal vaccine development.
Similar to other particle systems including lipid-based structures and VLPs that are used for nasal influenza vaccines, BLPs also induced strong virus-specific humoral and cellular immune responses. An advantage of liposomes is that there are many options to modify liposomes for the design of vaccine candidates and together with their potential to enhance mucosal and systemic immune responses this makes liposomes very attractive as vaccine delivery particles for nasal application. The major drawback for the development of modified liposomes for vaccination are the high cost of manufacturing. ISCOMS are a promising adjuvant that can induce protective immune responses against viral pathogens. Although the nasal route of vaccination in general requires a much lower dose of both antigen and adjuvant, the toxic effects of the component Quil limits the use of ISCOMS as an adjuvant for mucosal vaccines. An advantage of BLPs is that conjugation or incorporation of antigen is not required as the nasal vaccine can be simply mixed with BLPs before use. A disadvantage might be that the antigen is not protected against proteolytic degradation as it is when it is encapsulated into for example biodegradable nanoparticles such as the TMC-TPP nanoparticles. Nasal application of TMC-TPP nanoparticles has been described in the field of influenza vaccination and elicits both antigen-specific systemic IgG and mucosal SIgA humoral immune responses[2]. A major drawback for using TMC-TPP nanoparticles is that these systems are very unstable affecting long-time storage. Also the term particles and the effort value to produce them could be questioned. Since TMC is the active component of TMC-TPP nanoparticles and has shown to be an effective adjuvant by itself, alternative TMC-based formulations have also been developed and tested and showed to be capable of enhancing the delivery and immunogenicity of for example whole inactivated virus (WIV) after nasal vaccination against influenza[64].

In contrast to pro-inflammatory immune responses, nasal antigen application can also enhance tolerance to an innocuous antigen and is often associated with the induction of FoxP3$^+$ Tregs. In **Chapter 4** it was shown that especially PLGA-containing nanoparticles enhanced nasal tolerance induction. PLGA-TMC nanoparticles were designed to enhance the mucoadhesiveness of the PLGA nanoparticles to the mucosal epithelial cells. PLGA-TMC nanoparticles showed intermediate tolerance inducing capacity compared to the PLGA nanoparticles, most likely due to the pro-inflammatory stimulatory characteristics of the TMC content. However, when combined to an immunosuppressive Hsp70 peptide, the PLGA-TMC nanoparticles delayed the onset of arthritis symptoms in mice similar to the PLGA nanoparticles. These observations underline that not only the nature of the antigen, comparable to vaccination studies with CTB as adjuvant[12], but also the adjuvant/particle characteristics affect the immunological outcome after nasal vaccination. This underlines the importance of carefully selecting both candidates to ensure the efficient and safe design of nasal vaccines.

Concluding remarks

Nasal vaccination holds promise for the future as it enables needle-free application, immunization of large populations in a short time-period and induces both systemic and mucosal protective immune responses. The development of vaccine delivery particles for nasal vaccines has emerged over the past years due to a better understanding of the characteristics of the nasal cavity. Research focused on the mechanisms of vaccine delivery particles and the immune responses they elicit after nasal application is required to design effective and safe nasal vaccines in the future.

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List of abbreviations

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LIST OF ABBREVIATIONS

ADH	alcohol dehydrogenases
APC	antigen presenting cell
BCR	B-cell receptor
BLP	bacterium-like particles
BMDC	bone-marrow derived dendritic cell
CFSE	5,6-carboxy-succinimidyl-fluoresceine-ester
CLN	cervical lymph node
CT	cholera toxin
CTL	cytotoxic T-lymphocyte
DC	dendritic cells
dLN	draining lymph node
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
FAE	follicle associated epithelial cells
FCS	fetal calf serum
FDA	food and drug administration
FITC	fluorescein isothiocyanate
FoxP3	forkhead box P3
GATA-3	gata binding protein 3 transcription factor
GM-CSF	granulocyte macrophage colony-stimulating factor
HA	influenza haemagglutinin
Hsp	heat shock protein
IAV	influenza A virus
IFA	incomplete Freund's adjuvant
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
ILN	inguinal lymph node
i.m.	intramuscular
IMDM	iscove's modified dulbecco's media
i.n.	intranasal
i.p.	intraperitoneal
i.v.	intraveneous
KO	knockout
LAIV	live attenuated influenza vaccine
LPS	lipo-polysaccharide
LT	heat-labile enterotoxin
M-cell	microfold cell

MFI	mean fluorescent intensity
MHC	major histocompatibility complex
NALT	nasopharynx-associated lymphoid tissue
NK(T)	natural killer (T-cell)
NP	nanoparticle
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PDI	polydispersity index
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PGIA	proteoglycan induced arthritis
PIgA	polymeric immunoglobulin A
PIgR	polymeric immunoglobulin receptor
PLGA	poly(lactic-co-glycolic acid)
PRR	pathogen recognition receptor
RA	retinoic acid
RALDH	retinaldehyde dehydrogenase
rCTB	recombinant cholera toxin subunit B
RORγt	orphan nuclear receptor RORγt transcription factor
s.c.	subcutaneous
SIgA	secretory immunoglobulin type A
SV	split virus
T-bet	T-box transcription factor
TCR	T cell receptor
TGF-β	transforming growth factor beta
Th	t helper cell
TLR2	Toll-like receptor 2
TMC	N-trimethyl chitosan
TPP	tripolyphosphate
Tregs	regulatory T-cells
VLP	virus-like particles
WIV	whole inactivated virus
ZP	zetapotential

NEDERLANDSE SAMENVATTING

Nasale vaccinatie

De continue toename van nieuwe pathogenen en de groeiende resistentie van micro-organismen voor de huidige therapieën vraagt dringend om innovatieve vaccinatie strategieën. Een alternatief voor toediening van vaccins via injectie therapie is de toediening via de mucosale route. De ontwikkeling van mucosale vaccins is de laatste jaren sterk toegenomen. Van alle verschillende mucosale routes die er zijn (oog, neus, oraal, rectaal, vaginaal) worden over het algemeen de orale en nasale toedieningroutes het best geaccepteerd. De nasale route kan verkozen worden boven de orale route van toediening vanwege de lagere proteolytische activiteit in de neusholte die voorkomt dat het antigeen (vaak een eiwit) vroegtijdig wordt afgebroken. Tegelijkertijd zorgt dit ervoor dat een lagere dosis antigeen vaak afdoende is om een goede immuunrespons te induceren. Daarnaast gaat een lagere dosis vaccin vaak gepaard met een verlaagd risico op het ontwikkelen van bijwerkingen. Net als de overige mucosale toedieningroutes, stimuleert de nasale route van vaccineren zowel de systemische als mucosale adaptieve immuunrespons die ook wel het immunologisch geheugen wordt genoemd. Wanneer een antigeen via de neus wordt toegediend kunnen er twee verschillende immuunresponsen geïnduceerd worden. Antigenen die onder normale omstandigheden niet herkend worden als lichaamsvreemd en waar ons immuunsysteem dus niet op hoort te reageren zijn o.a. allergenen (pollen, huisstofmijt) of voedsel componenten, zoals gluten, maar dit kunnen ook bacteriën zijn die deel uitmaken van de commensale flora. Wanneer we deze antigenen via de nasale route toedienen dan kunnen we een tolerogene immuun respons induceren (mucosale tolerantie), die ervoor zorgt dat ons immuunsysteem deze antigenen niet langer ziet als lichaamsvreemd of schadelijk. In tegenstelling tot mucosale tolerantie inductie tegen onschadelijke of lichaamseigen antigenen wil je dat het immuunsysteem wel reageert met een pro-inflammatoire immuunrespons gericht tegen het pathogeen dat ziekte veroorzaakt.

Nasale route en activatie van immuuncellen

Antigeen toegediend via de neus wordt in de nasale holte opgenomen door epitheel cellen die het antigeen verder transporteren naar het onderliggende nasopharynx-geassocieerde lymfoïde weefsel ook wel de NALT genoemd. In de NALT bevinden zich antigeen presenterende cellen (APCs), zoals dendritische cellen (DCs) die zich vaak vlak onder de epitheel cellen bevinden en zo gemakkelijk en snel de antigenen kunnen opnemen. DCs verwerken de antigenen tot kleine eiwit stukjes (peptiden) die ze vervolgens presenteren in MHC moleculen op hun celoppervlak aan naïeve T-cellen. Wanneer een T-cel een receptor tot expressie brengt op het celoppervlak die specifiek het peptide en het MHC herkent, dan wordt de T-cel geactiveerd en zal gaan delen en differentiëren.

T-cellen kunnen differentiëren in verschillende subsets, bijvoorbeeld een T helper 1 (Th1), Th2, of Th17 cel welke een pro-inflammatoire immuunrespons induceren die nodig is bij het bestrijden van infectieziekten. Th2 cellen zijn belangrijk voor het activeren van

B-cellen, de cellen die antilichamen aanmaken. B-cellen gelegen in de mucosale lymfoïde weefsels scheiden IgA (SIgA) uit, een antilichaam dat een laag vormt op het oppervlak van het epitheel in de neusholte en als belangrijkste taak heeft de kolonisatie en het binnendringen van pathogenen te beperken en te voorkomen. Op een gegeven moment is het van belang dat deze immuun cellen worden afgeremd. Daarom zijn er ook T-cel subsets die een regulerend effect hebben, de zogehete regulerende T-cellen (Tregs), waarvan de $FoxP3^+$ T-cellen een voorbeeld zijn. Deze Tregs spelen ook een belangrijke rol bij het afremmen van autoreactieve cellen die lichaamseigen antigenen herkennen als schadelijk en kunnen op deze manier chronische ontstekingsreacties afremmen.

Adjuvanten en nanopartikels voor nasale vaccins

De immuunrespons die volgt na nasale antigeen toediening hangt af van verschillende factoren, zoals de oorsprong van het antigeen (immuunsuppressief of immunogeen), de formulering (oplosbaar of partikel), de grootte van het antigeen, de dosis en de frequentie van toediening. Hoewel nasale antigeen toediening een kostenbesparende methode kan zijn van vaccineren, beperkt de fysiologie van de neusholte vaak een goede werking van vaccins, doordat ze vroegtijdig verwijderd worden uit de neusholte en onvoldoende de tijd hebben om te worden opgenomen door het epitheel. Daarom worden er diverse adjuvanten en vaccine delivery systemen ontwikkeld die de bindingstijd in de neusholte kunnen verlengen en de immunogeniteit van nasale vaccins kunnen verhogen.

Adjuvant

Het adjuvant dat geselecteerd wordt hangt vaak af van de gewenste immuunrespons die geïnduceerd dient te worden na vaccinatie. Dit kan een pro-inflammatoire respons zijn voor de preventie van infectieziekten of het induceren van een tolerogene respons die de voorkeur heeft voor de behandeling van een auto-immuunziekten of een allergie. Het is van essentieel belang dat de vaccin formulering zo ontworpen wordt dat ons immuun systeem een optimale immuunrespons induceert met een zo laag mogelijk risico op het ontstaan van bijwerkingen. Adjuvanten die gebruikt worden voor nasale vaccins kunnen de immuunrespons moduleren en versterken en ze kunnen onderverdeeld worden in verschillende categorieën. Het soort immuunrespons dat ze induceren hangt sterk af van hun specifieke fysisch/chemische eigenschappen. Zo kan een adjuvant de opname en presentatie van een antigeen door epitheel cellen in de neusholte en door APCs vergroten. Specifieke receptoren activeren op het oppervlak van APCs en op deze manier de inductie van bijvoorbeeld een pro-inflammatoire immuunrespons vergroten of juist het tegenovergestelde effect hebben door een regulerende respons te induceren.

Nanopartikels

Doordat het eerste contact met pathogene micro-organismen en allergenen uit de omgeving plaats vindt via de mucosale membranen is de interesse in het ontwikkelen van mucosale vaccins de laatste jaren sterk toegenomen. Een breed scala aan vaccin delivery partikels zijn momenteel beschikbaar en in ontwikkeling om de immunogeniteit en de opname van

nasale vaccins te vergroten. Een voordeel van een partikel ten opzichte van een adjuvant is dat het vaccin antigeen kan worden ingebouwd in de partikels, terwijl een adjuvant vaak gemixt wordt met een antigeen en als losse bestanddelen worden toegediend. Door het vaccin in te bouwen in een partikel systeem is het beschermd tegen enzymatische afbraak en dit zorgt er tevens voor dat er vaak minder van het vaccin nodig is om een goede immuun respons op te wekken. Minder vaccin betekent vaak ook een kleinere kans op het ontstaan van bijwerkingen. De toedieningsroute van het vaccin hangt vaak af van de locatie van het aangedane weefsel, terwijl de vaccin eigenschappen vaak bepaald worden door de te induceren immuunrespons, een pro-inflammatoire respons gericht tegen een pathogeen of mucosale tolerantie inductie tegen lichaamseigen of onschadelijke antigenen.

DIT PROEFSCHRIFT

Er zijn diverse mogelijkheden om nasale vaccins te modificeren met als belangrijkste doel de werking van het vaccin te versterken. Dit proefschrift beschrijft welk effect verschillende partikel systemen hebben op ons immuunsysteem na nasale toediening. Er is bestudeerd of een partikel systeem een tolerogene immuunrespons of juist een pro-inflammatoire respons induceert door de differentiatie van T-cellen en B-cellen in detail te bestuderen na nasale toediening om zo te achterhalen voor welke toepassing een partikel systeem het beste gebruikt kan worden.

Bacterium-Like Particles (BLPs)

Het influenza A virus (IAV) beter bekend als het griepvirus is een zeer besmettelijk virus dat via onze ademhalingswegen ons lichaam binnendringt. Wereldwijd sterven er nog zeer veel mensen aan de gevolgen van een influenza infectie. Er wordt veel onderzoek gedaan na het verbeteren van influenza vaccins en het testen van alternatieve routes, waaronder de nasale route, ter vervanging van de huidige injectie therapie. Zoals eerder vermeld heeft een nasaal vaccin vaak een adjuvant nodig. Verschillende Toll-like receptor (TLR) liganden zijn getest als adjuvant voor nasale “influenza” vaccins om de werking van het vaccin versterken.

Uit voorgaande studies blijkt dat BLPs lokale en systemische immuunresponsen induceren na nasale toediening. Verder is *in vitro* aangetoond dat BLPs specifiek binden aan een bepaalde receptor, de TLR2. BLPs zijn afkomstig van de Gram positieve bacterie *Lactococcus lactis* en bestaan uit peptidoglycaan dat specifiek herkent wordt door de TLR2.

In **hoofdstuk 2** is onderzocht in de muis of BLP-geïnduceerde lokale en systemische T-cel en B-cel responsen gericht tegen het IAV afhankelijk zijn van de interactie van BLPs met TLR2 na nasale toediening.

Om de rol van TLR2 aan te kunnen tonen is gebruik gemaakt van TLR2 knockout (TLR2KO) muizen, die geen TLR2 tot expressie brengen. Als de werking van BLPs afhangt van hun interactie met de TLR2, dan zullen de TLR2KO muizen minder sterke

IAV-specifieke T-cel en B-cel responsen induceren. Het influenza split virus (SV) dat virale eiwit componenten bevat zonder infectieus te zijn wordt gemixt met de BLPs (BLP-SV) en dit wordt vervolgens via de neus toegediend in de wild type muizen (hebben wel TLR2 expressie) en de TLR2KO muizen.
In de TLR2KO muizen wordt na nasale vaccinatie met BLP-SV niet alleen een verlaagde lokale en systemische IAV-specifieke-Th1 cel respons, maar ook een gereduceerde B-cel respons gedetecteerd vergeleken met de wild-type muizen. Kort samengevat, de systemische IAV-specifieke IFN-γ Th1-cel en de lokale mucosale IAV-specifieke SIgA B-cel respons geïnduceerd na nasale BLP-SV vaccinatie maken dat BLPs een zeer geschikte kandidaat zijn als adjuvant voor nasale vaccinatie om infecties veroorzaakt door het influenza A virus te voorkomen.

PLGA, PLGA-TMC en TMC-TPP nanopartikels
Naast de BLPs is er ook onderzocht welk effect de specifieke eigenschappen van de synthetische poly-lactic-co-glycolic-acid (PLGA), poly-lactic-co-glycolic-acid N-trimethyl chitosan (PLGA-TMC) and N-trimethyl chitosan tripolyphosphate (TMC-TPP) nanopartikels hebben op ons immuunsysteem na nasale toediening. Er is gebruik gemaakt van een model eiwit ovalbumine (OVA) het hoofdbestanddeel van kippeneiwit dat is ingebouwd in de verschillende nanopartikels waar de muizen nasaal mee gevaccineerd zijn.

In **hoofdstuk 3** is onderzocht welk effect de partikels hebben op de B-cel activiteit na nasale vaccinatie. Na intramusculaire toediening van PLGA, PLGA-TMC en TMC-TPP nanopartikels wordt een systemische OVA-specifieke IgG antilichaam respons waargenomen die hoger is dan de OVA controle (zonder partikel) groep. Alleen na nasale toediening van TMC-TPP nanopartikels wordt er een SIgA respons in de neusspoelingen en een systemische IgG respons in het serum gedetecteerd die even sterk is als na intramusculaire toediening.

In **hoofdstuk 4** is gekeken naar het effect van de PLGA, PLGA-TMC and TMC-TPP nanopartikels op de activatie van OVA-specifieke $CD4^+$ T-cellen na nasale toediening. Drie dagen na nasale partikel vaccinatie wordt een verhoogde OVA-specifieke $CD4^+$ T-cel respons waargenomen in de lokale neus-drainerende cervicale lymfeknopen (CLN) vergeleken met de OVA controle groep. Alleen na nasale PLGA partikel vaccinatie wordt een verhoogde expressie van FoxP3 gedetecteerd in de CLN, een belangrijke marker voor regulerende T-cellen. Antigeen toegediend via de neus kan een pro-inflammatoire immuunrespons induceren, zoals we in **hoofdstuk 2** voor de BLPs en in **hoofdstuk 3** voor TMC-TPP partikels hebben gezien. Naast deze pro-inflammatoire immuunrespons is het ook mogelijk om mucosale tolerantie te induceren. Daarom is onderzocht of de PLGA, PLGA-TMC en TMC-TPP partikel eigenschappen de nasale mucosale immuunrespons kunnen moduleren om zo een meer tolerogene respons te induceren. Voor dit onderzoek is gebruik gemaakt van een delayed-type hypersensitivity (DTH) model. Het idee van dit

model is dat muizen eerst nasaal behandeld worden met de OVA-partikels of OVA zonder partikel als controle groep en dat er vervolgens een overgevoeligheidsreactie (DTH) wordt geïnduceerd gericht tegen OVA door het OVA eiwit via een niet-mucosale route te injecteren in de muis. De overgevoeligheidsreactie wordt gemeten door de oordikte te bepalen voor en na het induceren van de DTH. Muizen die nasaal behandeld waren met PLGA partikels laten een gereduceerde DTH zien dan de muizen die behandeld werden met TMC-TPP nanopartikels of OVA zonder partikel. Alleen de PLGA partikels promoten de inductie van nasale tolerantie.

Heat shock eiwitten (Hsps) kunnen anti-inflammatoire immuunregulerende T-cel responsen induceren. Onderzoek heeft aangetoond dat bepaalde Hsp70 peptiden de symptomen en de inductie van artritis kunnen onderdrukken. Een uitgebreid overzicht van de werking en toepassing van Hsps als therapeutische targets in auto-immuunziekten en overige chronische ontstekingsziekten staat beschreven in **hoofdstuk 6**.
In dit onderzoek is het immuunsuppressieve Hsp70-mB29a-peptide ingebouwd in PLGA en PLGA-TMC nanopartikels en zijn muizen nasaal behandeld om nasale tolerantie te induceren. Vervolgens is er in deze muizen artritis geïnduceerd door de muizen te injecteren met een humaan kraakbeen eiwit proteoglycaan. De resultaten uit het onderzoek tonen aan dat de muizen die behandeld zijn met Hsp70-mB29a-peptide geladen PLGA partikels de inductie en de symptomen van proteoglycaan geïnduceerde artritis onderdrukken.

Hoofdstuk 4 laat zien dat PLGA nanopartikels nasale tolerantie induceren ten opzichte van TMC-TPP nanopartikels. Retinoic acid (RA) een afbraakproduct van vitamine A in combinatie met TGF-β is beschreven als mechanisme om orale tolerantie te induceren door de expressie van FoxP3 in T-cellen te verhogen. Voor de productie van RA zijn bepaalde enzymen nodig die retinaldehyde dehydrogenases (RALDH) worden genoemd.
In **hoofdstuk 5** is *in vitro* aangetoond dat PLGA nanopartikels de expressie van RALDH enzymen in $CD11c^+$ DCs verhogen. $RALDH^+$ DCs kunnen RA produceren dat in aanwezigheid van TGF-β de expressie van FoxP3 in OVA-specifieke T-cellen verhoogd. Omdat het niet goed functioneren van $FoxP3^+$ T-cellen vaak geassocieerd wordt met een breed scala aan auto-immuunziekten, kan er op basis van de data geconcludeerd worden dat de PLGA nanopartikels geschikt zijn als nasaal therapeutisch vaccin partikel systeem voor de behandeling van auto-immuunziekten en overige chronische ontstekingsziekten. TMC-TPP nanopartikels daarentegen zijn meer geschikt bij preventieve vaccinatie tegen infectieziekten, waarbij ook een sterke B-cel respons en antigeen-specifieke antilichaam productie gewenst is.

Algemene conclusie
Nasale vaccinatie is een veelbelovend alternatief voor de huidige vaccins die via injecties worden toegediend. Daarnaast biedt nasaal vaccineren de mogelijkheid om in een korte tijd grote populaties te vaccineren en kan er na nasale toediening niet alleen een systemische,

maar ook een lokale mucosale immuunrespons geïnduceerd worden. Het ontwikkelen van partikels die de werking en opname van nasale vaccins verbeteren is de laatste jaren sterk gegroeid, mede door een beter inzicht in de werking van het nasaal mucosaal immuunsysteem. Onderzoek naar de specifieke mechanismen van vaccin delivery partikels na nasale toediening is nodig om in de toekomst effectieve en veilige nasale vaccins te kunnen ontwikkelen.

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CURRICULUM VITAE

Chantal Keijzer was born on December 4th 1979 in Naarden, The Netherlands. In 2004 she obtained her Bachelor of Applied Science degree at the Institute of Life Sciences and Chemistry, Utrecht, The Netherlands. In 2005 she started the Master Biomedical Sciences at the Vrije University in Amsterdam. Before receiving her Master degree in 2007, she did two research internships; in 2006 at the Royal Tropical Institute in Amsterdam on development of a diagnostic test for early identification of a *Plasmodium falciparum* infection under supervision of dr. Henk Schallig and dr. Petra Mens and in 2007 at the Wilhelmina Children's Hospital, UMC Utrecht on a project concerning inhibitory immune receptors under supervision of prof. dr. Linde Meyaard. In April 2008 she started her PhD project on "nanoparticles for nasal delivery of vaccines: monitoring adaptive immune responses" led by prof. dr. Willem van Eden and dr. Femke Broere at the department of Infectious Diseases and Immunology of the University Utrecht. The research results of this project are described in this thesis.

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