Journal of Controlled Release 138 (2009) 113–121

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

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

Bram Slütter [a,1], Laurence Plapied [b,1], Virgine Fievez [b,c], Maria Alonso Sande [d], Anne des Rieux [b], Yves-Jacques Schneider [c], Elly Van Riet [a], Wim Jiskoot [a], Véronique Préat [b,*]

[a] Leiden/Amsterdam Center for Drug Research, Division of Drug Delivery Technology, Einsteinweg 55, 2333 CC Leiden, The Netherlands
[b] Université Catholique de Louvain, Unité de pharmacie galénique, Avenue Mounier 73 UCL 7320, 1200 Brussels, Belgium
[c] Université catholique de Louvain, Laboratoire de Biochimie cellulaire, toxicologique et nutritionnelle, Institut des Sciences de la Vie, Croix du sud 5, 1348 Louvain-la-Neuve, Belgium
[d] University of Santiago de Compostela, Department of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, 15782 Santiago de Compostela, Spain

ARTICLE INFO

Article history:
Received 19 January 2009
Accepted 2 May 2009
Available online 13 May 2009

Keywords:
Trimethyl-chitosan
Chitosan
Nanoparticles
Oral vaccine
M-cells

ABSTRACT

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

1. Introduction

Whereas most pathogens gain access to their hosts via mucosal surfaces, most human vaccines currently available are licensed for non-mucosal administration e.g. via subcutaneous or intramuscular injections. However, mucosal vaccines have several attractive features compared with parenteral vaccines. Mucosal immune responses are most efficiently induced by administration of vaccines onto mucosal surfaces [1]. Moreover, mucosal immunization is needle-free, patient-friendly and reduces the risk of infection.

Nonetheless, if mucosal vaccination is to become a feasible alternative for parenteral immunization, there are still hurdles to overcome before such an approach can be used widely. Oral vaccine delivery raises particular challenges: the bioavailability of orally delivered antigen is limited by possible degradation in the gastro-intestinal environment. Moreover, most antigens are bulky substances and therefore not easily absorbed into the intestinal epithelium. Finally, the intestine is a fairly immuno-tolerant site, and the default response to an antigen will often be tolerance instead of immunity [2,3].

To facilitate effective mucosal immunization, the antigen must be protected from degradation, its uptake/absorption enhanced and immune cells activated. Therefore, oral vaccines should ideally be multimeric/particulate, adherent to the intestinal surface, effectively target M-cells and efficiently stimulate innate and adaptive immune responses [1]. Polymeric nanoparticles in which the antigen is encapsulated have been designed for oral immunization [4–6]. Various studies have shown increased antibody responses when antigens are orally administered in poly(lactic-co-glycolic acid) (PLGA) particles [7–10]. Particles composed of bioadhesive material like chitosan (CS) and its soluble derivate N-trimethyl chitosan (TMC), characterised by its permanent positive charges irrespective of pH, can prolong the residence time of the antigen in the intestine, increase its permeation and enhance its immunogenicity [11–15]. Although most of the polymers used allow protection of the antigen from degradation, their effects on interaction with intestinal surface and on

* Corresponding author. Tel.: +32 2 764 7320; fax: +32 2 764 7398.
E-mail address: Veronique.preat@uclouvain.be (V. Préat).
[1] Shared coauthorship.

0168-3659/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2009.05.011

antigen uptake is less well recorded and depends on the type of polymer used [4]. The present study addresses the question what happens to an encapsulated antigen once it has survived the harsh gastric conditions and has reached the intestine.

M-cells in the follicle associated epithelium (FAE) of intestinal Peyer's patches and isolated lymphoid follicles are gatekeepers of the mucosal immune system. They sample the gut lumen and transport antigens in the underlying mucosal lymphoid tissue for processing and initiation of an immune response [16]. Given their unique features to transcytose particles, M-cells are an interesting target in oral vaccine delivery. It has been shown that nanoparticles are actively taken up by the FAE through M-cells [5,12,16,17]. Moreover, M-cells have been suggested to transport antigen to underlying dendritic cells (DCs), without any degradation of the antigen, even without a protective carrier [18]. *In vitro* models of human FAE that have been recently developed are useful to study the contribution of M-cells to the transport of nanoparticles [5,17].

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

The aim of this study was to understand the mechanisms by which nanoparticles can enhance immune responses after oral administration apart from protection from degradation. Ovalbumin (OVA) was used as a model antigen and encapsulated with CS and TMC polymers to form nanoparticles. CS particles have been described as potential oral vaccine carrier [12] and transport of these particles by M-cells has been observed [21]. A drawback of CS is its water solubility. With CS' pK_a of 6.2, at physiological pH primary amines groups are protonated and consequently OVA/CS nanoparticles will loose their repulsive surface charge and show colloidal instability. The slightly acid environment of the jejunum will promote the stability of CS nanoparticles, but as soon as these particles are transported to the subepithelial space to interact with immune cells, the physiological pH will be deleterious for its stability. As TMC carries a permanent positive charge, OVA/TMC nanoparticles will not be affected by small pH shifts and may be a more suitable carrier for mucosal vaccination [22].

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

2. Materials and methods

2.1. Materials

N-trimethyl chitosan with a degree of quaternisation of 15% was obtained from 92% deacetylated (MW 120 kDa) chitosan (Primex, Siglufjordur, IC), by NaOH induced methylation as described by Sieval [6]. Poly(lactic-co-glycolic acid) 50:50 (PLGA) (L:G 50:50 average M_w 5000–15,000) pentasodium tripolyphosphate (TPP), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), dichloromethane, dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ovalbumin (OVA) grade V and sodium cholate were obtained from Sigma-Aldrich (Steinheim, DE). FITC-ovalbumin (FITC-OVA) was purchased from Molecular Probes (Invitrogen Breda, NL). All culture media, including penicillin/streptomycin (PEST) and trypsin were obtained from Gibco (Invitrogen, Carlsbad, CA), unless indicated otherwise.

2.2. Nanoparticle preparation

CS and TMC nanoparticles were prepared by ionic complexation with pentasodium tripolyphosphate (TPP) [23]. CS and OVA or OVA-FITC were dissolved in a 0.1 M acetate buffer (pH 5) to a final concentration of respectively 1 mg/ml and 0.1 mg/ml. A TPP solution (1 mg/ml) was added under continuous stirring to weight ratio CS:TPP:OVA of 10:1.2:1. TMC and OVA or FITC-OVA were dissolved in a 5 mM Hepes buffer (pH 7.4) to a final concentration of 1 mg/ml and 0.1 mg/ml, respectively. TPP was added under continuous stirring to a weight ratio TMC:TPP:OVA of 10:1.8:1. Nanoparticles were collected by centrifugation (30 min, 15,000 g) on a glycerol bed, to avoid aggregation.

PLGA particles were prepared by a "water-in-oil-in-water" solvent evaporation method described by Garinot [8]. Briefly, 50 µl of 10 mg/ml OVA or FITC-OVA in 10 mM phosphate buffer saline pH 7.4 (PBS) was emulsified with 1 ml of dichloromethane containing 50 mg of PLGA with an ultrasonic processor for 15 s at 70 W (Branson Instruments, CT, USA). The secondary emulsion was prepared with 2 ml of 1% (w/v) sodium cholate in water. The double emulsion was then poured into 100 ml of a 0.3% sodium cholate aqueous solution, and stirred at 37 °C for 45 min. The nanoparticle suspension was then washed twice in PBS by centrifugation at 22,000 g for 1 h.

2.3. Nanoparticle characterisation

Particle size distribution was determined by means of dynamic light scattering (DLS) using a NanoSizer ZS (Malvern Instruments, Malvern UK). The zeta potential of the particles was measured with the NanoSizer ZS by laser Doppler velocimetry. Before the measurement, samples were diluted in 5 mM Hepes pH 7.4 or 50 mM acetate buffer pH 5.9 until a slight opalescent dispersion was obtained.

The OVA content of the particles was determined with a BCA protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

The encapsulation efficiency was expressed in percentage as the amount of OVA encapsulated compared to the amount of OVA used to form the nanoparticles.

The process yield was expressed in percentage as the weight of nanoparticles compared to the amount of polymers used for the formulation.

2.4. Immunization with ovalbumin-loaded nanoparticles

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

Mice received intramuscular injection of OVA (50 µg OVA/50 µl) as a positive control, or intraduodenal injections of OVA, OVA-loaded CS nanoparticles (OVA/CS) or TMC nanoparticles (OVA/TMC) (100 µl containing 50 µg of OVA in HBSS pH 5.9 for CS and HBSS pH 7.4 for others). A boost was applied in similar fashion, 14 days after the priming. Blood samples were collected by retro-orbital punctures 14, 28, 42 days after priming. Sera isolated by centrifugation were stored at −20 °C before analysis. OVA-specific IgG, IgG1 and IGg2a levels were determined by enzyme-linked immunosorbent assay (ELISA) [8]. Serum dilutions were made in OVA-coated plates (Nunc-Immnuno Plate F96 MAXISORP) and detection of anti-OVA antibodies

was carried out using peroxidase-labelled rat anti-mouse immunoglobulin G, G1 and G2a (LO-IMEX, Brussels, BE). IgG titres were defined as the logarithm of the inverse of the sera dilution corresponding to an absorbance equal to 0.2.

2.5. Visualisation of OVA transport in vivo

Female Balb/c mice were administered 50 μg of free FITC-OVA or FITC-OVA encapsulated in TMC or CS nanoparticles by intraduodenal injection. After 1 h, mice were sacrificed, pieces of jejunum and Peyer's patches were harvested and washed with PBS. Tissues were fixed with formaldehyde for 90 min and incubated with PBS containing 0.2% Triton X-100 and Rhodamine-phalloïdin (1/50 v/v) (Molecular Probes, Oregon, USA) to stain cells membranes. Scanning laser confocal microscopy (Zeiss LSM510 with META detector, mounted on an inverse microscope and equipped for classical confocal microscopy and multiphoton microscopy) was used to visualise the luminal side and to perform *x–z* analysis. Images were edited with Adobe Photoshop (CS4 Extended v.11).

2.6. Cell lines

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

2.7. Effect of polymers on cell viability

Toxicity of the formulations on Caco-2 cells was assessed using the MTT method. Caco-2 cells (10,000/well) were seeded in a 96-well plate (Nunc, Roskilde, DK) and maintained for 2 days at 37 °C and 5% CO_2. After 1 h exposure to 1 mg/ml of the various delivery systems, the cells were washed 3× with Hank's Balanced Salt Solution (HBSS) and incubated for 3 h with 0.5 mg/ml MTT in DMEM. Medium was removed and the purple formazan crystal was dissolved in 100 μl DMSO. Absorbance at 570 nm was measured using a μQuant ELISA plate reader (Biotek, Winooski, VT, USA).

2.8. In vitro human FAE culture

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

2.9. Nanoparticle transport in vitro

For transport experiments mono- and co-culture inserts were reversed to their original orientation in 12-well plates (Corning) and washed with HBSS. After 20 min of equilibration at 37 °C the transepithelial electrical resistance (TEER) was measured using a home made chop stick electrode couple to a MilliCellers® multimeter (Millipore, NL). Nanoparticle formulations containing FITC-OVA were diluted to a final concentration of 1 mg/ml in HBSS pH 5.9 for CS and pH 7.4 for other formulations (corresponding to approximately 0.9×10^8 CS, 1.0×10^8 TMC and 1.0×10^8 PLGA nanoparticles per ml, as determined by flow cytometry) and 400 μl were applied to the apical compartment of the insert. At the basolateral side 1.2 ml HBSS pH 7.4 was used as acceptor compartment. After incubation for 60 min at 37 °C the amount of particles in the acceptor compartment was determined using flow cytometry (FACScalibur, Becton Dickinson) [5,8,17]. The transport of free FITC-OVA was evaluated by fluorimetry with an FS920 fluorimeter (Edinburgh Instruments, Campus Livingston, UK).

The Papp (cm/s) was calculated as

$$\text{Papp} = dQ / dtAC_0$$

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

The apical compartment was harvested and centrifuged (14,000 g, 30 min). The supernatant was used to quantify the release of lactate dehydrogenase (LDH) according to the manufacturer's instructions (Cytotoxicity detection kit LDH, Roche, Woerden, NL).

2.10. Human monocytes derived dendritic cells culture

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

2.11. Interaction of nanoparticles with dendritic cells (DCs)

DCs were incubated for 4 h at 37 °C in RPMI 1640 and 500 U/ml GMCSF with 2 μg/ml FITC-OVA either free or encapsulated in TMC, CS or PLGA nanoparticles (polymer concentration 20, 20 and 100 μg/ml respectively). Cells were washed 3 times with PBS containing 1% w/v bovine serum albumin and 2% v/v FBS before FITC-OVA association with DCs was quantified using flow cytometry (FACScalibur, Becton Dickinson). Live cells were gated based on forward and side scatter. FITC-OVA association was expressed as the mean fluorescence intensity (MFI) in the FL-1 channel. Histogram overlay were created with WinMDI vs 2.9.

2.12. Effect of nanoparticles on DC maturation

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

2.13. Statistics

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

3. Results and discussion

3.1. Nanoparticle formulations

All OVA-loaded nanoparticles showed a mean size distribution between 200 and 300 nm with comparable size distributions and a good process yield (Table 1). With an ionic complexation method using TPP, adapted from Calvo [23], OVA was efficiently encapsulated into fairly monodisperse (PDI<0.25) CS and TMC nanoparticles with a mean hydrodynamic diameter of approximately 300 nm. For comparison, monodisperse (PDI<0.15) PLGA nanoparticles with an average size of 240 nm were prepared by emulsification/solvent extraction [8].

TMC nanoparticles carried a positive surface charge at physiological pH. CS nanoparticles however lost their positive zeta potential at pH 7.4 due to deprotonation of the primary amine groups and showed major colloidal instability. Therefore, the characterisation and *in vitro* experiments with CS nanoparticles were conducted at pH 5.9. PLGA nanoparticles carried a negative surface charged partly due to deprotonated carboxyl groups.

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

3.2. Mucosal immunization with OVA-loaded TMC and CS nanoparticles

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

Two weeks after priming, TMC nanoparticles showed an immune response in 5 out of 8 mice, whereas free OVA, OVA encapsulated in CS nanoparticles or intramuscular injection of OVA induced a low IgG response in 2 out of 8 mice (Fig. 1a). On day 42 (4 weeks after boosting), all mice vaccinated with OVA-loaded nanoparticles showed a strong and significant enhancement (over 1000 fold) in IgG production compared to intraduodenal immunization with free OVA (Fig. 1b). No significant difference in IgG titres was observed between the 2 groups vaccinated with nanoparticles. Compared to intramuscular injection of unadjuvanted OVA, TMC and CS nanoparticles elicited a higher but more scattered immune response.

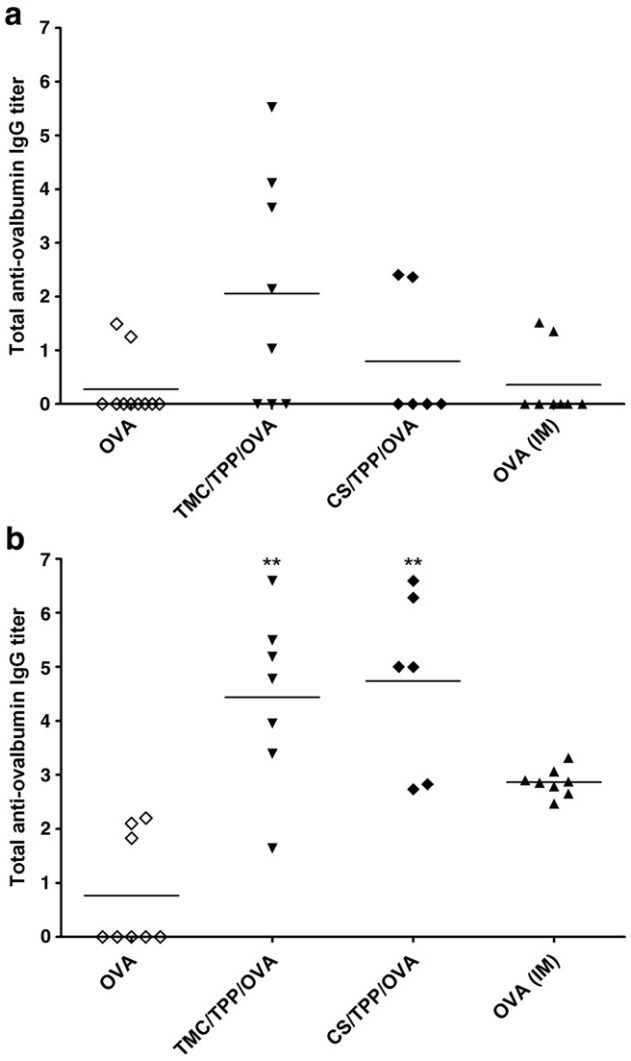

Fig. 1. OVA-specific IgG titres in serum of Balb/c mice intraduodenally fed with OVA solution, OVA-loaded TMC nanoparticles or CS nanoparticles, as compared to intramuscular injection with OVA solution. a) Total IgG titres 14 days after priming. b) Total IgG titres 42 days after priming (28 days after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

Table 1
Characteristics of TMC, CS and PLGA nanoparticles.

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

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

Interestingly, OVA administered by intramuscular injection, while yielding a 100 fold higher IgG titre than free OVA given intraduodenally, resulted in a lower immune response than the intraduodenal delivery of OVA in nanoparticulate formulations. This could indicate that the increased immune response caused by encapsulation of the antigen is not solely due to a more efficient delivery of the antigen to the target cells but also to an intrinsic immunopotentiating capacity of the nanoparticles [19,20].

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

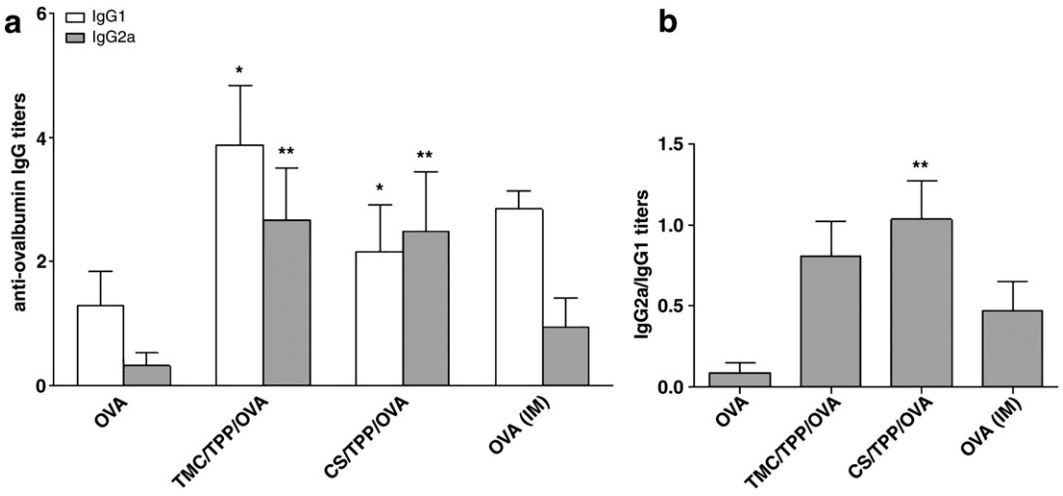

Fig. 2. OVA-specific IgG1 and IgG2a titres in serum of Balb/c mice intraduodenally fed with OVA solution, OVA-loaded TMC and CS nanoparticles. Intramuscular injection was used as a positive control. a) IgG1 and IgG2a 28 days after boost. *IgG1 and **IgG2a induced by TMC/TPP/OVA and CS/TPP/OVA were significantly higher than those induced by free OVA ($p<0.05$). b) Ratio of IgG2a on IgG1 titres 28 days after boost. **Ratio induced by OVA-loaded CS nanoparticles immunization was significantly different from those induced by OVA solution ($p<0.01$).

the nanoparticulate formulations could be due to proton scavenger properties of CS and TMC, which may facilitate endosomal escape of the antigen and thereby promote antigen presentation by the MHC class I pathway [31].

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

3.3. Localisation of antigen in Peyer's patch

A confocal microscopy of the gut of mice after intraduodenal delivery of free or encapsulated FITC-labelled OVA was performed to check if the TMC and CS particles were specifically taken up by M-cells in Peyer's patch.

Free FITC-OVA was detected in neither regular (Fig. 3a) nor Peyer's patch epithelium (Fig. 3b), which could account for the poor immunogenicity of OVA observed after intraduodenal administration. Consistent with previous studies conducted by van der Lubben [12,21], who found M-cell specific uptake of CS microparticles, uptake of FITC-OVA encapsulated in CS (Fig. 3d) or TMC (Fig. 3f) nanoparticles was detected mainly in the Peyer's patches. In general, regular intestinal epithelium exhibited less intense OVA related fluorescence than the M-cell rich Peyer's patch area (Fig. 3c and e). *x–z* analysis clearly shows that CS and TMC nanoparticles were taken up by cells in the jejunum and Peyer's patches. This indicates that 1 h after of intraduodenal administration a significant part of the antigen has accumulated in the Peyer's patch, which is most probably due to M-cell specific uptake.

The ability of M-cells to transport particulate structures is well established [33] and increasing the M-cell uptake has become an attractive strategy to improve current mucosal vaccines [34–36]. The particulate nature of the encapsulated FITC-OVA therefore seems to be one of the major reasons for the active uptake by the FAE. To support these findings and to assess whether encapsulation in CS or TMC nanoparticles is beneficial for M-cell mediated transport, FITC-OVA transport studies over an *in vitro* FAE was performed.

3.4. In vitro studies with FAE

3.4.1. Cell viability and monolayer integrity

As cell viability and monolayer integrity are vital for the interpretation of the transport experiments, the toxicity of the nanoparticles on Caco-2 cells was assessed with an MTT assay and a LDH release test, and monolayer integrity was evaluated by monitoring the TEER (Table 2).

At 1.0 mg/ml, the same concentration as used in the transport experiments, no significant cytotoxicity of CS and PLGA nanoparticles on Caco-2 cells was observed by the MTT assay. However, TMC nanoparticles caused a 20% reduction of cell survival ($p<0.05$). No LDH release (<1%) was detected. MTT as well as LDH assay performed at pH 5.9 showed similar results (data not shown). The toxicity of TMC could be due to its higher positive charge density leading to a higher interaction with cell membrane and higher uptake [32].

CS and TMC have been reported as enhancers of oral absorption, due to the opening of tight junctions between the epithelial cells [11,12,14,15,32]. The relatively small but significant decrease of electrical resistance across the monolayer of both Caco-2 monocultures and co-cultures induced by CS and TMC particles ($p<0.05$) indicates that nanoparticles composed of these cationic biopolymers modified the tight junctions. In contrast, no changes in TEER were observed after exposure to PLGA nanoparticles. Although tight junction opening has been described as mechanism promoting antigen uptake, it is unlikely that an antigen encapsulated in a 300 nm sphere, will diffuse through these tight junction openings as FITC-dextran (4 kDa and 12 kDa) were not significantly transported by co-cultures [5]. Therefore the immune-enhancing effect of CS and

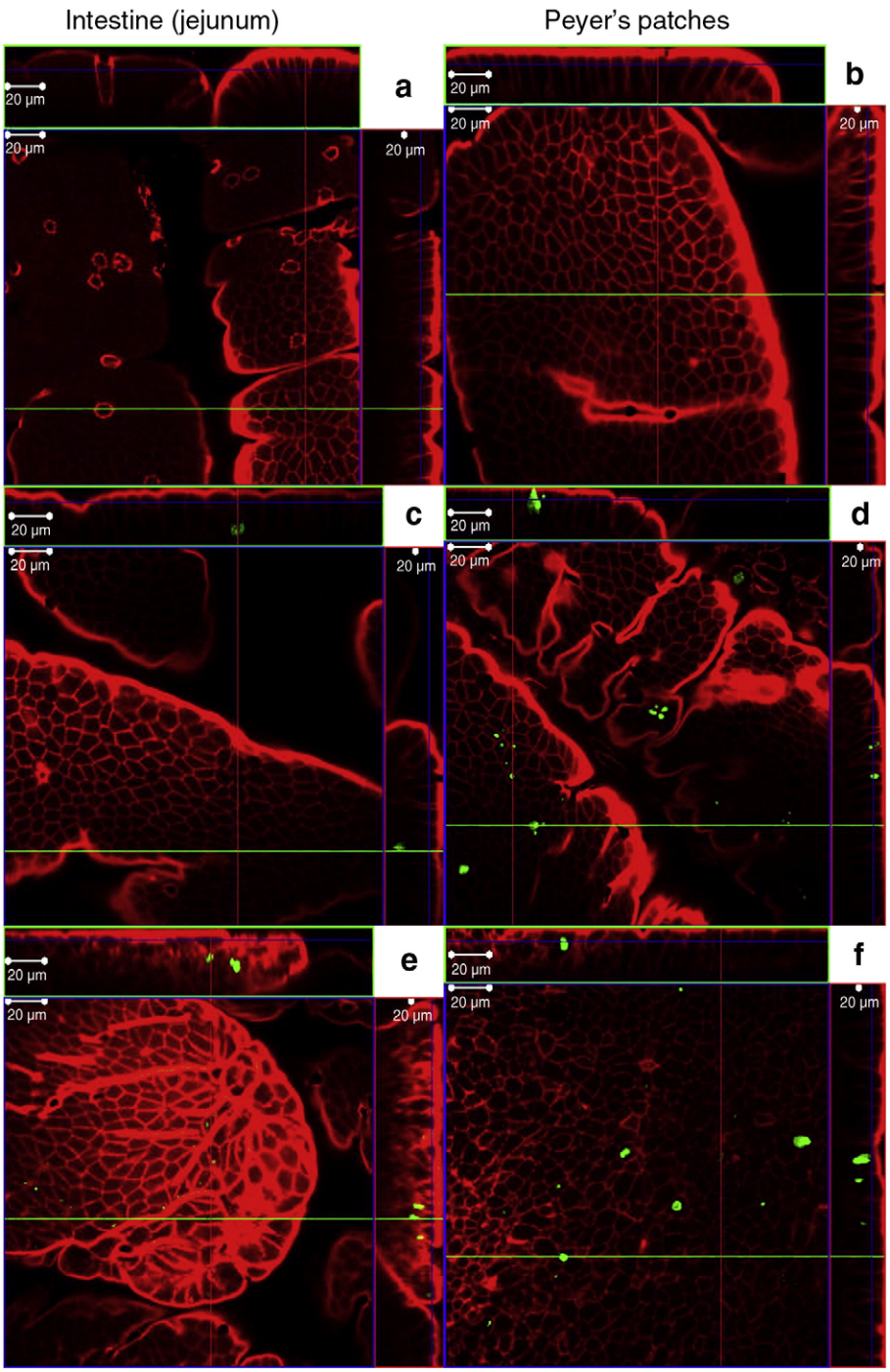

Fig. 3. Representative confocal microscopy images of murine jejunum (a, c, e) and a Peyer's patch (b, d, f) from murine jejunum, isolated 1 h after intraduodenal injection with 50 μg of FITC-OVA alone (a, b), incorporated in CS nanoparticles (c, d) or in TMC nanoparticles (e, f). Tissues were formaldehyde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

TMC nanoparticles should not be attributed to their ability to modify tight junctions.

3.4.2. Nanoparticle transport across human FAE culture

M-cells present in the FAE are known for their transcytotic transport capacity and may be critical for effective antigen transport to subepithelial immune cells [1,16]. Therefore, nanoparticle transport across the intestinal epithelium was investigated *in vitro* by measuring the amount of FITC-OVA-loaded particles in the basolateral compartment of Caco-2 mono-cultures as well as Caco-2/Raji-B co-cultures. The Raji-B cells induce the generation of M-cells within the epithelial cell layer, permitting us to discriminate between M-cell dependent and M-cell independent transport (Fig. 4).

All types of nanoparticles were more transported in the presence of M-cells, although only CS and TMC particle transport increased significantly ($p<0.01$). The increase in CS and TMC particle transport in the co-culture is consistent with the preferential localisation in Peyer's patches *in vivo* (Fig. 3).

TMC particle transport by the mono-culture and co-culture was higher than CS and PLGA particle transport, independent of the presence of M-cells ($p<0.01$). Transport by co-cultures was equivalent for PLGA and CS particles. It could be related to the higher positive

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

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

#$p<0.05$, compared to OVA solution, °HBSS pH 7.4, *HBSS pH 5.9.
[a] Cell viability was assessed by an MTT test. Results are expressed as percentage (mean ± SEM; $n = 14$) of the medium control (100%).
[b] Monolayer integrity was investigated by measuring the TEER before and after experiment. Values represent mean ± SEM ($n = 14$).

charge density which is a critical factor for bioadhesion and penetration enhancement towards intestinal epithelium. It could also be related to the increase of quaternization degree favouring the mucoadhesion or to the small toxic effect of TMC (Table 2), which may have negatively affected the integrity of the monolayer. For the lower transport of CS particles in comparison with TMC particles, a key element could be the instability of the CS particles. The colloidal stability of OVA/CS is fragile and very sensitive to pH shifts, ionic strength and medium composition [37,38]. Thus, it is well possible that many particles exposed to pH>6.5 had aggregated before they reached the basolateral compartment [39,37] or that CS could be less condensed because the amine groups will not be charged and so, be less transported [40,15].

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

3.5. In vitro studies with DCs

3.5.1. Effect of nanoparticles on FITC-OVA association with DC

Immature human DCs were stimulated for 4 h with either FITC-OVA alone or nanoparticles containing FITC-OVA to investigate if encapsulation of OVA increased its association with DCs. Treatment with 2 μg/ml FITC-OVA led to an increase in mean fluorescence intensity compared to unstimulated DCs, indicating association of the antigen with the DCs. The positively charged formulations (OVA/CS and OVA/TMC) showed significantly increased association with DCs (Fig. 5a). Interestingly, OVA incorporated in PLGA nanoparticles was not taken up by DCs more efficiently than free OVA, probably because the DC-PLGA interaction was less pronounced due to the unfavourable electrostatic interactions between the negative surfaces of PLGA nanoparticles and the DC's cell membrane.

3.5.2. Effect of nanoparticles on DC maturation

Uptake of antigen by DC does not necessarily mean that a successful immune response will be started. DCs sample their environment continuously, but only become activated if the antigen is recognized as dangerous. To measure whether nanoparticles supply a 'danger signal' next to increasing the delivery of the antigen, the maturation of human monocyte DC was studied. After 48 h of exposure to the different OVA formulations, clear differences in the maturation status of the DCs could be observed (Fig. 5b). The positive control, LPS-stimulated DCs expressed maturation markers to a very high extent, as 90% of the total DCs population was positive for MHCII as well as CD86. DCs treated with OVA showed no maturation, but OVA/TMC nanoparticles increased the number of MHCII/CD86 double positive DCs ($p<0.01$). The type of biopolymer seems to be an important parameter determining DCs maturation, as for PLGA and CS nanoparticles no significant increase of mature DCs was observed. Induction of DC maturation by CS and PLGA particles has been investigated by other groups with inconclusive results. Increased DC maturation by PLGA and CS biofilms *in vitro* [42] and by CS solutions *in vivo* [30] has been shown, but also studies claiming no stimulatory effect of these biopolymers exist [43,44]. Fisher et al. have recently addressed this discrepancy [45] and explained it by slight differences in preparation of the formulations and possible endotoxin contamination. To rule out LPS contamination in our experiments, TLR-4 transfected HEK cells were exposed to the formulations and we found the LPS content to be below the detection limit (<0.1 ng/ml, data not shown).

As only the (positively charged) TMC nanoparticles induced DC maturation, but no DC maturation was found after exposure to soluble antigen, (negatively charged) PLGA particles and (positively charged) CS particles, the nanoparticles material rather than the zeta potential, is an important factor in the process of DCs maturation. Effect of TMC on cell viability could also trigger DC maturation. As CS particles were incapable of activating DCs, but were effective in inducing an immune response, CS apparently acts as an adjuvant via a different mechanism. Possibly a depot is formed by OVA/CS particles that aggregate as soon as they are secreted into the subepithelial space and come into contact within a higher pH and medium constituents [39]. Why TMC has immunopotentiating capacity is unclear. To our best knowledge, this is the first time that the direct effect of TMC nanoparticles on DCs was assessed and TMC is a not a known ligand for pathogen recognition receptors. Further investigation into the adjuvant effect of TMC will therefore be necessary.

4. Conclusion

This study shows that if the right nanoparticle material is selected, the transport of the antigen into the FAE, association with APC, maturation of DCs and modulation of the immune response can be accomplished. The particulate nature of the delivery system increases the M-cell specific transport into the FAE and a positive surface charge seems to cause an enhanced association of antigen with DCs, increasing the probability of successful antigen uptake. Moreover, TMC

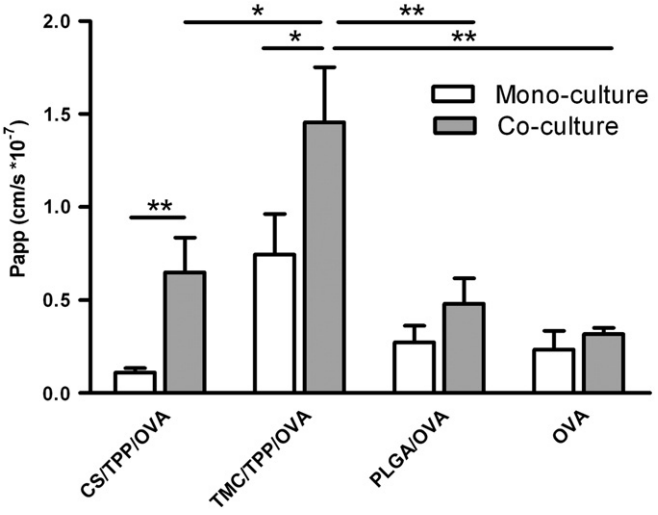

Fig. 4. FITC-OVA-loaded nanoparticle transport over Caco-2 monolayers (white bars) and Caco-2/Raji-B co-cultures (grey bars) quantified by FACS analysis. Transport is expressed as apparent permeability (Papp). Error bars represent standard error of the mean (SEM). M-cell, only present in co-culture significantly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

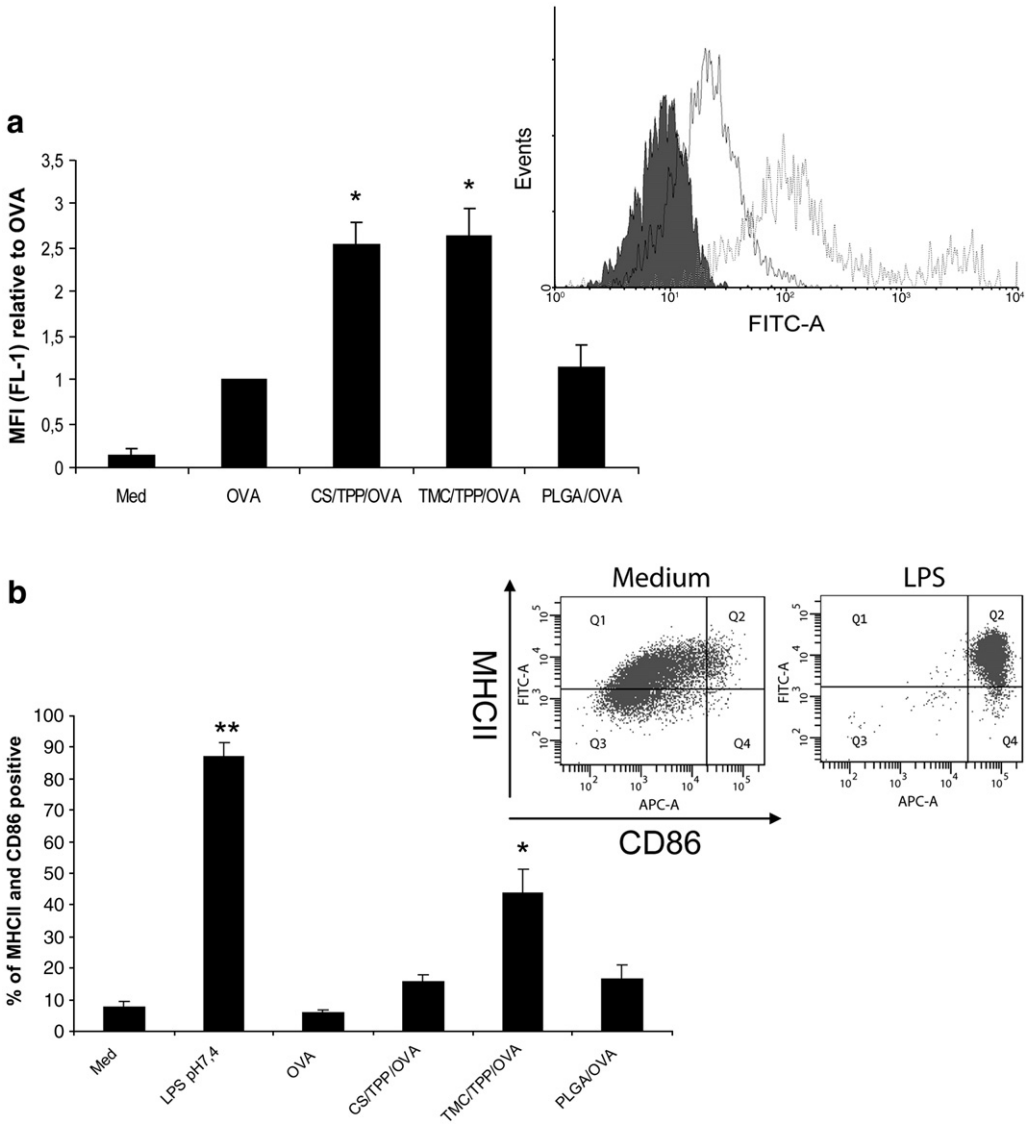

Fig. 5. Interaction of OVA with human monocyte derived DCs. DCs were exposed to 2 μg/ml soluble or encapsulated FITC-OVA for 4 h. a) OVA-FITC association with DCs measured by flow cytometry. Bars represent mean values of 5 experiments from 5 different donors. Insert: Representative overlay histogram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

nanoparticles exerted intrinsic adjuvant properties, as it induces maturation of DCs. These characteristics make TMC nanoparticles a promising delivery system for mucosal vaccination.

Acknowledgements

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

References

[1] M.R. Neutra, P.A. Kozlowski, Mucosal vaccines: the promise and the challenge, Nat. Rev., Immunol. 6 (2) (2006) 148–158.
[2] B. Dubois, A. Goubier, G. Joubert, D. Kaiserlian, Oral tolerance and regulation of mucosal immunity, Cell Mol. Life Sci. 62 (12) (2005) 1322–1332.
[3] A.M. Mowat, Anatomical basis of tolerance and immunity to intestinal antigens, Nat. Rev., Immunol. 3 (4) (2003) 331–341.
[4] A. des Rieux, V. Fievez, M. Garinot, Y.J. Schneider, V. Preat, Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach, J. Control Release 116 (1) (2006) 1–27.
[5] A. des Rieux, V. Fievez, I. Theate, J. Mast, V. Preat, Y.J. Schneider, An improved *in vitro* model of human intestinal follicle-associated epithelium to study nanoparticle transport by M cells, Eur. J. Pharm. Sci. 30 (5) (2007) 380–391.
[6] A.B. Sieval, M. Thanou, A.F. Kotze, J.E. Verhoef, J. Brussee, H.E. Junginger, Preparation and NMR characterization of highly substituted N-trimethyl chitosan chloride, Carbohydr. Polym. 36 (2–3) (1998) 157–165.
[7] A. Wierzbicki, I. Kiszka, H. Kaneko, D. Kmieciak, T.J. Wasik, J. Gzyl, Y. Kaneko, D. Kozbor, Immunization strategies to augment oral vaccination with DNA and viral vectors expressing HIV envelope glycoprotein, Vaccine 20 (9–10) (2002) 1295–1307.
[8] M. Garinot, V. Fievez, V. Pourcelle, F. Stoffelbach, A. des Rieux, L. Plapied, I. Theate, H. Freichels, C. Jerome, J. Marchand-Brynaert, Y.J. Schneider, V. Preat, PEGylated PLGA-based nanoparticles targeting M cells for oral vaccination, J. Control Release 120 (3) (2007) 195–204.
[9] K. Iaoui-Attarki, E. Fattal, S. Pecquet, S. Trolle, E. Chachaty, P. Couvreur, A. Andremont, Mucosal immunogenicity elicited in mice by oral vaccination with phosphorylcholine

encapsulated in poly (D,L-lactide-co-glycolide) microspheres, Vaccine 16 (7) (1998) 685–691.
[10] R. Rajkannan, M.D. Dhanaraju, D. Gopinath, D. Selvaraj, R. Jayakumar, Development of hepatitis B oral vaccine using B-cell epitope loaded PLG microparticles, Vaccine 24 (24) (2006) 5149–5157.
[11] S.M. van der Merwe, J.C. Verhoef, J.H.M. Verheijden, A.F. Kotze, H.E. Junginger, Trimethylated chitosan as polymeric absorption enhancer for improved peroral delivery of peptide drugs, Eur. J. Pharm. Biopharm. 58 (2) (2004) 225–235.
[12] I. van der Lubben, J.C. Verhoef, G. Borchard, H.E. Junginger, Chitosan for mucosal vaccination, Adv. Drug Deliv. Rev. 52 (2) (2001) 139–144.
[13] H.Y. Zhou, X.G. Chen, W.F. Zhang, *In vitro* and *in vivo* evaluation of mucoadhesiveness of chitosan/cellulose acetate multimicrospheres, J. Biomed. Mater. Res. A 83 (4) (2007) 1146–1153.
[14] F. Chen, Z.R. Zhang, F. Yuan, X. Qin, M. Wang, Y. Huang, *In vitro* and *in vivo* study of N-trimethyl chitosan nanoparticles for oral protein delivery, Int. J. Pharm. 349 (1–2) (2008) 226–233.
[15] G. Sandri, M.C. Bonferoni, S. Rossi, F. Ferrari, S. Gibin, Y. Zambito, C.G. Di, C. Caramella, Nanoparticles based on N-trimethylchitosan: evaluation of absorption properties using *in vitro* (Caco-2 cells) and *ex vivo* (excised rat jejunum) models, Eur. J. Pharm. Biopharm. 65 (1) (2007) 68–77.
[16] D.J. Brayden, M.A. Jepson, A.W. Baird, Keynote review: intestinal Peyer's patch M cells and oral vaccine targeting, Drug Discov. Today 10 (17) (2005) 1145–1157.
[17] E. Gullberg, M. Leonard, J. Karlsson, A.M. Hopkins, D. Brayden, A.W. Baird, P. Artursson, Expression of specific markers and particle transport in a new human intestinal M-cell model, Biochem. Biophys. Res. Commun. 279 (3) (2000) 808–813.
[18] S. Dahan, F. Roth-Walter, P. Arnaboldi, S. Agarwal, L. Mayer, Epithelia: lymphocyte interactions in the gut, Immunol. Rev. 215 (2007) 243–253.
[19] S.T. Reddy, M.A. Swartz, J.A. Hubbell, Targeting dendritic cells with biomaterials: developing the next generation of vaccines, Trends Immunol. 27 (12) (2006) 573–579.
[20] Y. Zheng, J.R. Kovacs, E.S. Gawalt, H. Shen, W.S. Meng, Characterization of particles fabricated with poly(D, L-lactic-co-glycolic acid) and an ornithine–histidine peptide as carriers of oligodeoxynucleotide for delivery into primary dendritic cells, J. Biomater. Sci., Polym. Ed. 17 (12) (2006) 1389–1403.
[21] I. van der Lubben, F.A. Konings, G. Borchard, J.C. Verhoef, H.E. Junginger, *In vivo* uptake of chitosan microparticles by murine Peyer's patches: visualization studies using confocal laser scanning microscopy and immunohistochemistry, J. Drug Target. 9 (1) (2001) 39–47.
[22] M. Amidi, S.G. Romeijn, G. Borchard, H.E. Junginger, W.E. Hennink, W. Jiskoot, Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system, J. Control Release 111 (1–2) (2006) 107–116.
[23] P. Calvo, C. RemunanLopez, J.L. VilaJato, M.J. Alonso, Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers, J. Appl. Polym. Sci. 63 (1) (1997) 125–132.
[24] M. Rescigno, M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J.P. Kraehenbuhl, P. Ricciardi-Castagnoli, Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria, Nat. Immunol. 2 (4) (2001) 361–367.
[25] E.C. de Jong, P.L. Vieira, P. Kalinski, J.H.N. Schuitemaker, Y. Tanaka, E.A. Wierenga, M. Yazdanbakhsh, M.L. Kapsenberg, Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells *in vitro* with diverse Th cell-polarizing signals, J. Immunol. 168 (4) (2002) 1704–1709.
[26] P.L. Vieira, P. Kalinski, E.A. Wierenga, M.L. Kapsenberg, E.C. de Jong, Glucocorticoids inhibit bioactive IL-12p70 production by *in vitro*-generated human dendritic cells without affecting their T cell stimulatory potential, J. Immunol. 161 (10) (1998) 5245–5251.
[27] I. Gutierro, R.M. Hernandez, M. Igartua, A.R. Gascon, J.L. Pedraz, Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres, Vaccine 21 (1–2) (2002) 67–77.
[28] G.P. Li, Z.G. Liu, B. Liao, N.S. Zhong, Induction of Th1-type immune response by chitosan nanoparticles containing plasmid DNA encoding house dust mite allergen der p 2 for oral vaccination in mice, Cell. Mol. Immunol. 6 (1) (2009) 45–50.
[29] E.A. McNeela, I. Jabbal-Gill, L. Illum, M. Pizza, R. Rappuoli, A. Podda, D.J.M. Lewis, K.H. G. Mills, Intranasal immunization with genetically detoxified diphtheria toxin induces T cell responses in humans: enhancement of Th2 responses and toxin-neutralizing antibodies by formulation with chitosan, Vaccine 22 (8) (2004) 909–914.
[30] C. Porporatto, I.D. Bianco, S.G. Correa, Local and systemic activity of the polysaccharide chitosan at lymphoid tissues after oral administration, J. Leukoc. Biol. 78 (1) (2005) 62–69.
[31] P. Strong, H. Clark, K. Reid, Intranasal application of chitin microparticles down-regulates symptoms of allergic hypersensitivity to *Dermatophagoides pteronyssinus* and *Aspergillus fumigatus* in murine models of allergy, Clin. Exp. Allergy 32 (12) (2002) 1794–1800.
[32] B. Sayin, S. Somavarapu, X.W. Li, M. Thanou, D. Sesardic, H.O. Alpar, S. Senel, Mono-N-carboxymethyl chitosan (MCC) and N-trimethyl chitosan (TMC) nanoparticles for non-invasive vaccine delivery, Int. J. Pharm. 363 (1–2) (2008) 139–148.
[33] L.J. Hathaway, J.P. Kraehenbuhl, The role of M cells in mucosal immunity, Cell Mol. Life Sci. 57 (2) (2000) 323–332.
[34] A.R. Foxwell, A.W. Cripps, J.M. Kyd, Optimization of oral immunization through receptor-mediated targeting of M cells, Hum. Vaccine 3 (5) (2007) 220–223.
[35] F. Roth-Walter, B. Bohle, I. Scholl, E. Untersmayr, O. Scheiner, G. Boltz-Nitulescu, F. Gabor, D.J. Brayden, E. Jensen-Jarolim, Targeting antigens to murine and human M-cells with *Aleuria aurantia* lectin-functionalized microparticles, Immunol. Lett. 100 (2) (2005) 182–188.
[36] B. Slutter, N. Hagenaars, W. Jiskoot, Rational design of nasal vaccines, J. Drug Target. 16 (1) (2008) 1–17.
[37] T. Lopez-Leon, E.L.S. Carvalho, B. Seijo, J.L. Ortega-Vinuesa, D. Bastos-Gonzalez, Physicochemical characterization of chitosan nanoparticles: electrokinetic and stability behavior, J. Colloid Interface Sci. 283 (2) (2005) 344–351.
[38] T. Sato, T. Ishii, Y. Okahata, *In vitro* gene delivery mediated by chitosan. Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency, Biomaterials 22 (15) (2001) 2075–2080.
[39] O. Germershaus, S.R. Mao, J. Sitterberg, U. Bakowsky, T. Kissel, Gene delivery using chitosan, trimethyl chitosan or polyethylenglycol-graft-trimethyl chitosan block copolymers: establishment of structure–activity relationships *in vitro*, J. Controlled Release 125 (2) (2008) 145–154.
[40] A.F. Kotze, H.L. Luessen, B.J. de Leeuw, A.G. de Boer, J.C. Verhoef, H.E. Junginger, Comparison of the effect of different chitosan salts and N-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2), J. Control Release 51 (1) (1998) 35–46.
[41] E.G. Ragnarsson, I. Schoultz, E. Gullberg, A.H. Carlsson, F. Tafazoli, M. Lerm, K.E. Magnusson, J.D. Soderholm, P. Artursson, *Yersinia pseudotuberculosis* induces transcytosis of nanoparticles across human intestinal villus epithelium via invasin-dependent macropinocytosis, Lab. Invest. 88 (11) (2008) 1215–1226.
[42] J.E. Babensee, A. Paranjpe, Differential levels of dendritic cell maturation on different biomaterials used in combination products, J. Biomed. Mater. Res. Part A 74A (4) (2005) 503–510.
[43] A. Luzardo-Alvarez, N. Blarer, K. Peter, J.F. Romero, C. Reymond, G. Corradin, B. Gander, Biodegradable microspheres alone do not stimulate murine macrophages *in vitro*, but prolong antigen presentation by macrophages *in vitro* and stimulate a solid immune response in mice, J. Control. Release 109 (1–3) (2005) 62–76.
[44] C. Wischke, H.H. Borchert, J. Zimmermann, I. Siebenbrodt, D.R. Lorenzen, Stable cationic microparticles for enhanced model antigen delivery to dendritic cells, J. Control. Release 114 (3) (2006) 359–368.
[45] S. Fischer, E. Uetz-von Allmen, Y. Waeckerle-Men, M. Groettrup, H.P. Merkle, B. Gander, The preservation of phenotype and functionality of dendritic cells upon phagocytosis of polyelectrolyte-coated PLGA microparticles, Biomaterials 28 (6) (2007) 994–1004.